UNIVERSITÀ
DI PAVIA

Dipartimento di Biologia e Biotecnologie “L. Spallanzani”

Seeds in the context of agricultural research: combining traditional and innovative approaches to boost seed quality

Chiara Forti

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXII– A.A. 2016-2019

UNIVERSITÀ
DI PAVIA

Dipartimento di Biologia e Biotecnologie "L. Spallanzani"

Seeds in the context of agricultural research: combining traditional and innovative approaches to boost seed quality

Chiara Forti

Supervised by Prof. Alma Balestrazzi

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXII – A.A. 2016-2019

Ciò che non ti uccide, ti rende più forte,
a Chiara.

Abstract

One of the challenges of the last few years is to provide conditions allowing to feed the overgrowing world population while also taking into consideration land availability and the global climate change. In this PhD thesis, different methods have been developed, useful to promote a sustainable agriculture. The experimental work focuses mainly on aspects related to Seed Technology, particularly the development of improved seed priming protocols, combined with the use of novel molecular hallmarks for seed germination. Besides this, efforts were also dedicated to develop genetic engineering methods useful to improve the complex trait of seed vigor, as a step to support both basic and applied research.

Seed germination is a critical parameter for the successful development of sustainable agricultural practices. While germination is impaired by environmental constraints related to the climate change scenario, priming is a pre-sowing technique that promote germination and seedling robustness by managing a series of crucial parameters during seed imbibition. In particular, it enhances the antioxidant/DNA repair activities triggered during the pre-germinative metabolism. A hydropriming protocol was developed for *Solanum melongena* L. and its crop wild relatives *Solanum torvum* Swartz and *Solanum villosum* Miller, and for the first time the molecular dynamics of pre-germinative metabolism were explored in primed seeds. Molecular hallmarks (expression patterns of antioxidant/DNA repair genes combined with free radical profiles) were used to discriminate between high- and low-quality lots. This study allowed to disclose the plasticity of the pre-germinative metabolism in eggplant, stimulated by priming that imposes a plethora of heterogeneous molecular responses.

In a parallel work, hydropriming and biopriming have been applied to legume model organism *Medicago truncatula* seeds to enhance germination on contaminated soil. Phenotypic parameters were monitored and compared to find the most appropriate treatment combinations. The expression profiles of specific genes were used as molecular indicators of seed quality, to investigate seedling fitness under the imposed treatments. The results show that, while hydropriming significantly enhanced *M. truncatula* seed germination biopriming contributed to improve seedling development. The observed response reflected the up-regulation of antioxidant/DNA repair genes. Overall, the reported data further strengthen the pivotal role of the DNA damage response in plant adaptation to heavy-metal soil contamination. Although genetic transformation has enabled fundamental insights into plant biology, unfortunately for most crops transformation and regeneration remain

arduous. Genome editing techniques are an innovative and powerful strategy to introduce mutations or insert/substitute wide portion of DNA at a targeted site. These approaches have extremely important implications for agriculture and environment. Genome editing provides novel opportunities to enhance crop productivity if accompanied by an efficient transformation protocol. In this work, a novel vector for genome editing able to target the *Tdp1β* gene (tyrosyl-DNA phosphodiesterase 1β) in *M. truncatula* was developed. TDPs are enzymes responsible for the repair of topoisomerase-mediated DNA damage and their involvement in the DNA Damage-Response (DDR) mechanisms triggered during the seed pre-germinative metabolism has been reported. Based on their peculiar expression profiles, the *Tdp1α* and *Tdp1β* genes are highlighted as markers of seed quality. In particular, the TDP1β isoform has been identified only in plants and its role in the DDR context is still not fully understood.

Aside being a cereal model plant, *Oryza sativa* is one of the world's most important crops and a major staple food that feeds more than three billion people worldwide. Rice cultivation in Europe is restricted to a few southern countries, Italy is the major producer in terms of quantity and varieties. Some elite cultivars, such as Vialone Nano, are recalcitrant to genetic transformation. These local varieties (from Lombardy and Veneto) are very appreciated at a culinary level but they suffer severe susceptibility to diseases and/or pathogens. To safeguard this variety, an efficient genetic transformation protocol has been hereby developed, as an essential step for the targeted improvement of relevant agronomic traits.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my advisors Prof. Alma Balestrazzi and Dott. Anca Macovei for guided me during my PhD studies for their patience, knowledge and constant scientific support.
I also want to thank my colleagues at CREA-GB where I spent my first year of PhD.
Many thanks to all my colleagues of Plant Biotechnology Lab, especially Francesca, Valentino, Matteo and Francesco for helping me to conduct all this work.
Thanks to Maraeva, Domenico and Carla for being colleagues but also, and especially, good friends!

Thanks to Laura and Cinzia for always encouraging me even in the worst moments.
Thanks to Cristina, Paola and Piermarco because you are literally saving my life.
Thanks to Chiara and Matteo, the best 'best friends', always with me.

Grazie alla mia famiglia e ai miei piccolini, siete la mia forza nonché ragione di vita.

Et enfin, grâce à toi… unique, différent… spécial!

Abbreviations

2,4-D (2,4-dichlorophenoxyacetic acid)
AA (ascorbic acid)
ABA (abscisic acid)
ACT (actin)
ALC (alcatraz)
AP (apurinic/apyrimidinic)
APRT (adenine phosphoribosyl transferase)
APX (ascorbate peroxidase)
ASH (ascorbate)
ATP (adenosine triphosphate)
BAP (6-Benzylaminopurine)
BER (Base Excision Repair)
Blc (β-lycopene cyclase)
BP (biopriming)
BPHP (biopriming + hydropriming)
BSN (broad spectrum nutrient priming)
CAT (catalase)
CCD (carotenoid cleavage dioxygenase)
CCM (co-cultivation medium)
CIM (callus induction medium)
CPDs (cyclo-butane pyrimidine dimers)
CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)
CTAB (Hexadecyl Trimethyl Ammonium Bromide)
CTRL (control)
CV (coefficient of variation)
CWR (crop wild relative)
CWRPs (cell wall remodeling proteins)
DB (dry back)
DCFH-DA (2',7'-dichlorofluorescein diacetate)
DEP (dense and erect panicle)
DEPC (diethyl pyrocarbonate)
DHA (dehydro ascorbate)
DHAR (dehydroascorbate reductase)
DOG1 (delay of germination 1)
DSB (double strand break)
DW (dry weight)
ECM (embryo conversion medium)

EF1 (elongation factor 1)
EIM (embryo induction medium)
ELF1α (elongation factor 1α)
EMS (ethyl methane sulfonate)
EPM (embryo proliferation medium)
ERCC (excision repair cross-complementing)
ET (ethylene)
FAD (flavin adenin dinucleotide)
FAD2 (fatty acid desaturase 2)
FAO (Food and Agriculture Organization)
FPG (formamidopyrimidine DNA glycosilase/lyase)
FW (fresh weight)
G (germinability)
GA (gibberellin)
GAPDH (glyceraldehyde 3-phosphate dehydrogenase)
GFP (green fluorescent protein)
GG-NER (global genome-NER)
GM (genetically modified)
Gn1a (grain number 1a)
GPOX (guaiacol peroxidase)
GPX (glutathione peroxidase)
GR (glutathione reductase)
GR (glutathione reductase)
GS (grain size)
GSH (reduced glutathione)
GST (glutathione S-transferase)
GUS (β-glucuronidase)
GW (grain weight)
GWAS (Genome Wide Association Studies)
HP (hydropriming)
HPT (hygromycin phosphotransferase)
HR (Homologous Recombination)
IAA (indole-3-acetic acid)
IIA (auxin induced)
IPA (ideal plant architecture)
ISTA (International Seed Testing Associaton)
JA (jasmonate)
LB (left border)
LCY (lycopene ε-cyclase)
Lig (ligase)
LOX3 (lypoxigenase)
LP (long-patch repair)
MAX (more axillary growth)
MDHAR (monodehydroascorbate reductase)
MeJA (methyl jasmonate)

MGT (mean germination time)
miRNA (microRNA)
MLO (mildew resistant locus O)
MMS (methyl methane sulfonate)
MR (mean germination rate)
MRE (meiotic recombination)
MS (Murashige & Skoog)
Mt: *Medicago truncatula*
MT2 (type 2 metallothionein)
MTL (matrilinear)
NAA (1-Naphthaleneacetic Acid)
NADH (nicotinamide adenine dinucleotide)
NADPH (nicotinamide adenine dinucleotide phosphate)
NB (Nipponbare)
NBS (nibrin)
NCED (9-*cis*-epoxycarotenoid dioxygenase)
NER (nucleotide excision repair)
NHEJ (Non-Homologous End Joining)
NOS (nopaline synthase)
NPs (nanoparticles)
OD (optical density)
OGG1 (8-oxoguanine DNA glycosylase)
OSD (omission of second division)
PAIR1 (homologous pairing aberration in rice meiosis 1)
PAM (protospacer adjacent motif)
PARP (poly(ADP-ribose) polymerase)
PCA (principal component analysis)
PCD (programmed cell death)
PCR (polymerase chain reaction)
PEG (polyethylene glycol)
PGPB (plant growth-promoting rhizosphere)
PP2Acs (phosphatase 2A)
PReM (pre-regeneration medium)
PVY (potato virus Y)
*q*RT-PCR (quantitative real-time PCR)
QTL (Quantitative Trait Loci)
RAD (RAD double strand break protein)
RB (right border)
RdDM (RNA-directed DNA methylation)
REC (meiotic recombination protein)
ReM (regeneration medium)
RISC (RNA-induced silencing complex)
RoM (rooting medium)

ROS (Reactive Oxygen Species)
RPA (replication protein A)
SA (salicylic acid)
SBE (starch branching enzyme)
SD (standard deviation)
SDB (seed defence biopriming)
SGR1 (stay green 1)
sgRNA (single guide RNA)
siRNA (small interfering RNA)
Sm: *Solanum melongena*
SMI (selection medium I)
SMII (selection medium II)
SOD (superoxide dismutase)
SP (short-patch repair)
SSB (single strand break)
St: *Solanum torvum*
Sv: *Solanum villosum*
TALEN (Transcription Activator-Like Effector Nuclease)
TC-NER (transcription coupled-NER)
TDP (tyrosyl-DNA phosphodiesterase)
TFIIH (transcription elongation factor IIH)
TFIIS (transcription factor)
TGW (thousand grain weight)
tracrRNA (trans-activating crRNA)
TUB (tubulin)
U (uncertainty)
UBI (ubiquitin)
UVH (DNA repair protein)
UVR (UVB-resistance)
VN (Vialone Nano)
XP (xeroderma pigmentosum)
XRCC4 (X-ray repair cross-complementing)
YEP (yeast extract peptone)
Z (synchronization)
ZFN (Zinc Finger Nuclease)

Contents

1. Introduction

1.1. The seeds in the context of global agricultural challenges

1.1.1. The current state of the agricultural context

The estimated global population increase of 9 billion people by 2050 will have a dramatic influence on the agricultural and food systems, as evidenced by recent FAO (Food and Agriculture Organization) reports (FAO, 2017. *The future of food and agriculture-Trends and challenges*. www.fao.org). Hence, a substantial increase in the global food production is required for the next 40 years, predicted to be equal to the entire production of the last 12.000 years (FAO, 2017). Moreover, this enormous challenge must be achieved in spite of climatic changes and limited amounts of land, water, and fossil fuels (Ray et al., 2013). Fortunately, through plant breeding, the seed industry has managed to overcome many challenges in the past and we can only assume that it will continue to do so, as we need to produce more and better food for a growing population. In addition, the recent discovery of genome editing tools offers a powerful alternative to accelerate crop improvement and address the major challenges in agricultural research (Chen et al., 2019).

In 1960, the production of one hectare was able to feed two people, but by 1995 one hectare fed four people and by 2025 one hectare should produce food for at least five people. This shows that, over a period of 35 years, the agricultural industry has managed to double food production in order to meet the food demand. But now, a 25% increase in food production over a period of 15 years must be achieved with the same amount of land and often under more adverse conditions, such as depleted and degraded soils (FAO, 2017).

The increase in productivity obtained with the use of high-quality seeds of improved varieties can reduce the cost of food by allowing farmers to spend less on labor; this is perhaps the most visible part of what the seed can provide. However, in the context of adverse climatic conditions it has been estimated that the average crop yield losses caused by drought, cold, and salinity, overpass 50% (Mahajan and Tuteja, 2005). At this point, the continuous development of new varieties with improved yields will positively impact land resources (e.g. by decreasing deforestation). Abiotic stresses are also reported to induce notable changes in seed composition and quality (Farooq et al., 2018). Such considerations have relevant outcomes not only on seed survival but also on agricultural production and human nutrition (Forti et al., 2019). Also, in this case, improved varieties can provide benefits in terms of the enhanced nutritional quality. Several classic examples include

Golden Rice, supplemented with vitamin A and iron (Potrykus, 2001), and soybean varieties rich in Omega 3 fatty acids or low saturated oils (Harris et al., 2008; Kinney et al., 1996; Bernal-Santos et al., 2010). Improved seeds can also help minimize storage losses, shown to reach up to 40% (Kumar and Kalita, 2017). Aside their importance as food and feed sources, seeds are also used as raw material for several industries, for the production of oil, biofuels, cosmetic products, and fibers (Powell, 2009).

For all these reasons, the agricultural sector has reached a consensus to prioritize food safety with increased productivity and sustainability. One of the means to satisfy this purpose could be the production of seeds with enhanced, accelerated, and synchronized germination which could help farmers and the seed industry to meet these priorities (Paparella et al., 2015; Macovei et al., 2017; Pagano et al., 2017).

1.1.2. The seed: a brief description of a complex system

Seeds are the main propagating vectors and the most complex and successful mean of sexual reproduction in higher plants. Within the seed there is the zygotic embryo resulting from the double fertilization of the ovule by the pollen (Gossot and Geitmann, 2007; Linkies et al., 2010). Seeds have also a protective coat that shelters the embryo against injuries following the dispersion into the environment. Plants, unlike animals, are sessile organisms and they cannot escape from unfavorable conditions for their survival. Thanks to seeds, plants can disperse their offspring to new locations, thus surviving hostile environmental conditions. During seed maturation the embryo growth is temporarily stopped, storage tissue is produced and reserves are accumulated inside the seed to provide the food source for the developing embryo before germination. At the same time, seeds acquire desiccation tolerance that allows them to survive in the dehydrated state (Dekkers et al., 2015). Desiccation tolerance is the ability to cope with conditions of extreme water loss, avoiding irreversible damage to the cellular macromolecules and structures. Seed enters in a quiescent state called dormancy, during which germination is delayed until the proper conditions occur (Linkies et al., 2010; Sano et al., 2016). Plants species show various degrees of seed dormancy and such variability is also observed within a population of seeds of the same species (Andersson et al., 1998). In this regard, it is necessary to distinguish quiescence from dormancy. Quiescent seeds are mature dried seeds characterized by a low moisture content (5-15%) and a basal metabolic activity (Finch-Savage and Bassel, 2016). An interesting feature of quiescent seeds is that they are able to survive for many years in this state (Long et al., 2015). In many cases, quiescent seeds need only to be hydrated at suitable temperatures in presence of oxygen to resume a normal level of metabolism

(Bewley et al., 2013). By contrast, the dormancy state prevents mature seeds from germination, acting as a suppressive mechanism also under favorable environmental conditions that should support germination (Baskin and Baskin, 2004). To assess the presence of dormancy, seeds can be imbibed and maintained under suitable climate and habitat conditions and the resulting germination profiles can be monitored (Willis et al., 2014; Finch-Savage et al., 2017). From an agricultural point of view, dormancy strongly limits seeds germination causing significant yield and economic losses (Shu et al., 2015).

Plants have evolved different mechanisms that regulate dormancy establishment and release, involving water uptake, balanced hormonal effects and the occurrence of specific environmental conditions. For example, in many species seed dormancy is physiologically induced in the embryo by balanced hormonal signals and by low humidity and temperature. In other species, dormancy maintenance is mainly promoted by the physical resistance opposed by the seed coat to the protruding radicle and the growing embryo. The different strategies of dormancy maintenance are not mutually exclusive and contribute to the complexity of this phenomenon (Penfield, 2017).

Dormancy is regulated by the contrasting effects of various classes of phytohormones and their signaling cascades. Abscisic acid (ABA) is the main hormone that promotes dormancy maintenance since it accumulates during seed maturation (Bewley, 1997). Conversely, gibberellins (GA), ethylene (ET) and other hormones act antagonistically to ABA, promoting dormancy release and germination initiation (Graeber et al., 2012). Moreover, several environmental parameters, including temperature, water, nitrate availability and light, are transduced and integrated into hormonal signaling via specific transcription factors, thus optimizing the regulation of dormancy in response to suitable environments and seasonal cycles (Dekkers et al., 2016).

In synergy with hormones, many molecular players contributing to the regulation of germination timing have been identified. The *DOG1* (*DELAY OF GERMINATION*) gene is one of the most investigated, first discovered in *Arabidopsis* and then identified in other species (*DOG-LIKE* genes). It appears to be a key regulator for the maintenance of dormancy and for various aspects related to seed maturation, including the interaction with the ABA biosynthesis pathway and the accumulation of reservoir metabolites (Dekkers et al., 2016). The *DOG1* gene participates to ABA-GA hormonal regulation by repressing GA biosynthesis and promoting ABA signaling (Graeber et al., 2010). Seed dormancy can be modulated also at an epigenetic level. In *Triticum* spp. and *Hordeum* spp., RNA-directed DNA methylation (RdDM) and the RNA-induced silencing complex (RISC) have been shown to regulate seed maturation and dormancy through small interfering RNA (siRNA) (Singh and Singh, 2012; Singh et al., 2013).

Hormonal control and genetic/epigenetic backgrounds represent some of the many layers of dormancy regulation and their influence on seed germination cumulates with the presence of physical barriers. The structures enveloping the embryo (endosperm, seed coat) offer mechanical resistance for embryo growth and radicle emergence, but these have also been interpreted as a major cause of germination delay independent on the plant species (Finch-Savage and Leubner- Metzger, 2006).

Not all seeds can enter a dormant state. According to their capacity to undergo after-ripening desiccation, seeds are classified into recalcitrant and orthodox. The recalcitrant seeds are unable to survive desiccation and, as a consequence, if the environmental conditions are unsuitable for germination, seeds deteriorate. The orthodox seeds can survive desiccation and enter in a dormant state, remaining viable for variably long periods (Roberts, 1973; Rajjou and Debeaujon, 2008). Dormancy rises survival possibilities of a seed population under variable environmental conditions. Nonetheless, under the temporal and practical constrains of agriculture, a variable delay in seed germination may represent an economical drawback (Baskin and Baskin, 2004; Long et al., 2015). Indeed, many specific techniques have been developed to act on the timing of dormancy release in order to make seed germination more uniform, anticipated, and abundant. These protocols include many physical, chemical, mechanic, and biological treatments and are comprehensively indicated as 'Seed Priming' (Paparella et al., 2015). The production of seeds with enhanced, accelerated, and synchronized germination can ensure successful harvest, a fundamental goal from many points of view (Paparella et al., 2015; Macovei et al., 2017; Pagano et al., 2017). In this regard, the evaluation of seed vigor has important implications for the seed industry.

Seed vigor is defined by ISTA (International Seed Testing Association, www.seedtest.org) as "the sum total of those properties of the seed which determine the level of activity and performance of the seed or seed lot during germination and seedling emergence" (ISTA, 1995). ISTA provides seed operators (e.g. companies, germplasm banks) with rules in order to develop reproducible tests for laboratories able to assess the potential performance of seeds in terms of germination and seedling robustness. By monitoring seed quality through germination tests carried out under unfavorable conditions, it is possible distinguish low-vigor seeds from high-vigor seeds (Rajjou et al., 2012; Ventura et al., 2012; Finch-Savage and Bassel, 2016). Differences in seed vigor are due not only to seed physiology, but also to the various phases of seed production and seed lot processing. Recently, thanks to the advances in the field of plant molecular biology, it is now possible to focus on novel

seed quality markers that associate with high germination levels and can thus be used to help breeding programs (Macovei et al., 2016).

1.2.The seed pre-germinative metabolism

1.2.1. Seed germination: facts and factors

Efficient seed germination is essential for crop productivity. The successful establishment of early seedlings requires rapid and uniform emergence and seedling growth (**Fig. 1**). Germination is a transition phase of the plant life cycle which is fundamental for reproduction (Lutts et al., 2016). It consists of a complex phenomenon of many physiological and biochemical changes leading to the activation of embryo growth (Parihar et al., 2014).

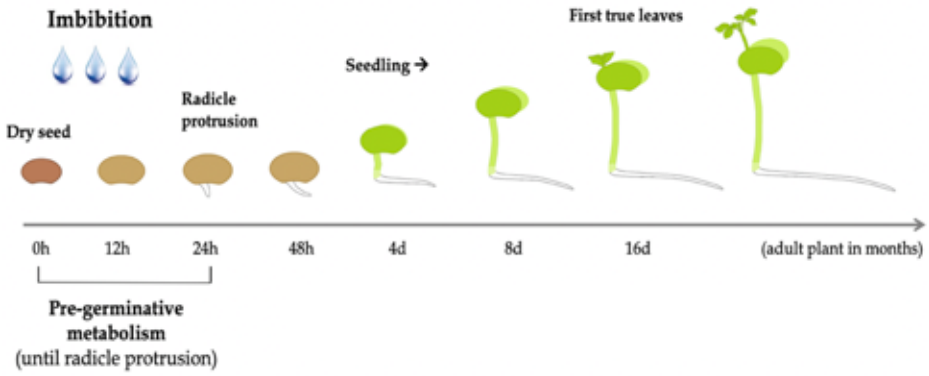


Fig. 1. Schematic representation of seed germination. The timepoints are indicative for legume species. Dry seeds, radicle protrusion, seedling and the appearance of the first true leaves are indicated (Pagano, 2019).

Germination can be described as a progressive temporal process composed of three phases characterized by different degrees of water up-take by the seed. During phase I (seed imbibition), the rapid water up-take is necessary to break the dormant state of the dry seed. The passive imbibition of dry tissues is associated with water movement, first occurring in the apoplastic spaces. During phase II, the imbibed seed continues to absorb water but more slowly. In this crucial phase, the re-establishment of metabolic activities and repairing processes occurs at the cellular level. This permits the activation of the 'pre-germinative metabolism' (**Fig. 1**). Among the main molecular mechanisms featuring pre-germinative metabolism, DNA repair and the antioxidant response represent essential processes that need to be fulfilled before the seed can proceed to the next phase and reach an active

germination state (Macovei et al., 2016). Before the end of phase II, germination remains a reversible process: the seed can be dried back and remains alive during storage, being able to re-initiate germination under favorable conditions (Lutts et al., 2016). The priming techniques are based on this unique feature of the seed. Finally, during phase III, the rapid water uptake is resumed. At this point, the initiation of growth processes associated to cell elongation leads to radicle protrusion.

The vulnerable and complex process of seed germination depends on decisive and specific changes in tissue and cell properties. From a biomechanical perspective, the germination process depends on the balance of two opposing forces: the increasing growth potential of the radicle counteracted by the resistance of the testa and endosperm tissues covering the radicle. After testa rupture, the growth potential of the radicle must overcome the resistance of the endosperm micropylar to protrude, thereby completing germination through endosperm rupture. At the same time, endosperm resistance decreases through tissue softening, a process called endosperm weakening (Bewley, 1997; Müller et al., 2006). Besides its important role as nutrient source, the endosperm is involved also in the regulation of germination timing (Friedman, 1998; Linkies et al., 2010). Endosperm weakening is associated with the action of several cell wall remodeling proteins (CWRPs) (Finch-Savage and Leubner-Metzger, 2006; Nonogaki et al., 2007) and the presence of reactive oxygen species (ROS), e.g. superoxide (O^{2-}), hydroxyl radicals (•OH) and hydrogen peroxide (H_2O_2). ROS can directly (breaking the cell wall polymers leading to cell wall loosening) or indirectly (signaling components) be involved in the regulation of germination timing (Fry et al., 2001; Bailly, 2004).

1.2.2. Molecular aspects of seed pre-germinative metabolism

The pre-germinative metabolism, activated during phase II of germination (**Fig. 2**), is one of the most captivating aspects of seed biology (Macovei et al., 2016). The term 'pre-germinative metabolism' refers to all the processes that lead the transition from the quiescent state of dry seeds toward the active proliferating state of germinating seeds. Considering that industrial seedling production and intensive agricultural production systems require seed stocks with high rate of synchronized germination and low dormancy and that the phases I and II of seed germination are crucial for seed viability, one of the main research topic for scientists is to understand the molecular players and mechanisms involved in the pre-germinative metabolism.

Following seed imbibition, a chain of events is initiated: enzyme activation, DNA damage and repair, *de-novo* synthesis of nucleic acids and protein production, activation of ATP metabolism, breakdown, translocation

and use of reserve storage material, activation of antioxidant pathways (Paparella et al., 2015; Macovei et al., 2016) (**Fig. 2**). In the first two phases of pre-germinative metabolism, seed metabolism and respiration are triggered through the reactivation of the mitochondrial electron transport chain, leading to increased ROS production. This can result in DNA damage and the subsequent activation of DNA repair is essential for a successful seed germination (**Fig. 2**) (Waterworth et al., 2015; Macovei et al., 2016).

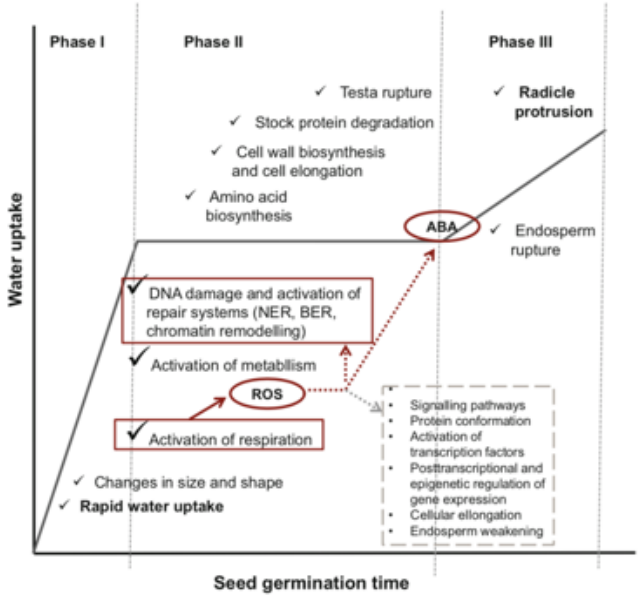


Fig. 2. Schematic representation of the seed imbibition process and mechanisms activated in the seed (Macovei et al., 2016).

1.2.2.1. ROS and phytohormones: crucial players in seed germination

The dual role of ROS, as signaling and damaging molecules, present throughout the entire plant life cycle, is also maintained at seed level (Bailly et al., 2008; Kumar et al., 2015). The modulation of ROS production within the seed is essential for the viability and capability of seed to germinate. In addition, ROS are responsible for the interaction with phytohormones and also act at several levels: protein structure or folding, activation of transcription factors, cellular elongation, post-transcriptional and epigenetic regulation of gene expression, endosperm weakening and pathogen protection (Weitbrecht et al., 2011; Kumar et al., 2015).

A deep connection between ROS, hormones and DNA repair exist at all stages of seed development, from embryogenesis to seed filling (Parreira et al., 2016; Verdier et al., 2008), dormancy and germination (El-Maarouf-

Bouteau et al., 2013). Specific antioxidant systems, both enzymatic and non-enzymatic, have evolved to maintain the ROS balance (Gill and Tuteja, 2010; Balestrazzi et al., 2011a). ROS-related DNA damage during storage can result in loss of telomere sequences, strand breaks and oxidative lesions (Balestrazzi et al., 2011b, 2015).

From a physiological point of view GA, ABA, ET, and jasmonate (JA) signaling and metabolism influence the developmental processes of seed germination (Linkies et al., 2011; Yan et al., 2014). ABA is involved in the induction and maintenance of seed dormancy and inhibits seed germination (Nambara et al., 2010). During seed development, the production of maternal and embryonic ABA is important to prevent precocious germination (Kanno et al., 2010). In contrast to ABA, GA promotes seed germination and negatively regulates dormancy as it releases coat-mediated seed dormancy (Koornneef, 2002; Kucera et al., 2005). Plant hormones control seed germination as internal mediators of developmental phases (**Fig. 3**). Similarly, external environmental factors, such as light, temperature, moisture, oxygen and nutrients, have a role to play during germination in a tight interplay with hormones. It has been shown that endosperm rupture is promoted by GA and inhibited by ABA, which seems to involve the exchange of ABA- and GA-related signals between endosperm and embryo (**Fig. 3**) (Finch-Savage et al., 2006; Kucera et al., 2005). GA and ABA thereby antagonistically regulate downstream mechanisms that mediate the two key processes important for the completion of germination of endospermic seeds, namely embryo elongation and endosperm weakening (Linkies et al., 2011; Pagano et al., 2018).

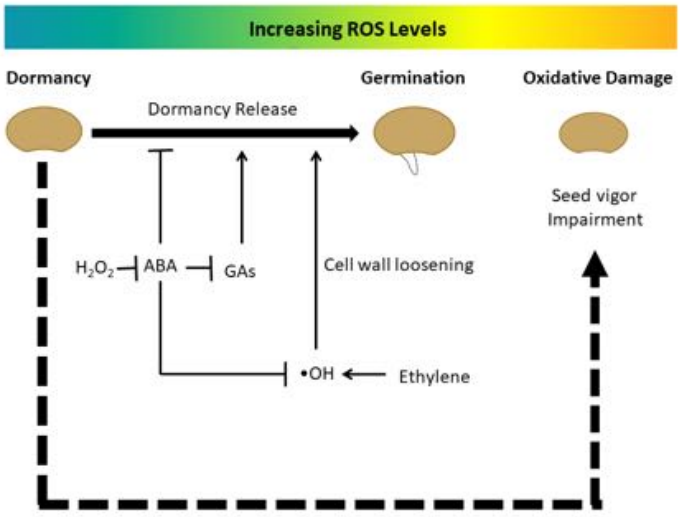


Fig. 3. Connecting ROS and phytohormones in seed physiology (Pagano et al., 2018). ABA inhibits hydroxyl radical (·OH) production and dormancy release, counteracting the effects of GA. Instead, GA and ethylene promote dormancy release. Hydrogen peroxide (H_2O_2) inhibits the biosynthesis of ABA, whereas ·OH promotes dormancy release contributing also to the cell wall loosening required for germination. ABA, abscisic acid; GAs, gibberellins.

While the mechanisms underlying the ABA-GA antagonism are well known (Nambara et al., 2010; Weitbrecht et al., 2011), the molecular mechanisms by which ET promotes and JA inhibits seed germination are not completely explained (Kucera et al., 2005; Matilla et al., 2008). ET has diverse effects on plant growth and development. It is involved in stress responses, in the ripening of climacteric fruits, flowering, aging, seedling growth, seed dormancy release and germination (Klee and Clark, 2004; Matilla, 2000). ET promotes seed germination and antagonism with ABA has been shown in *A. thaliana* (Ghassemian et al., 2000; Linkies et al., 2009) and several other species (Kucera et al., 2005; Matilla et al., 2008). Instead, JA inhibits seed germination and plant growth, regulates defense and it is also involved in pollen and embryo development, senescence, fruit ripening and allelopathy (Delker et al., 2006; Browse et al., 2009). Several studies demonstrated that JA plays a role during seed germination (Dave et al., 2011; Preston et al., 2009). Moreover, it has been shown that JA and methyl jasmonate (MeJA) can inhibit seed germination of *Solanum lycopersicum*, *Brassica napus*, *Linum usitatissimum*, *Lupinus luteus*, and *Zea mays* (Miersch et al., 2008; Oh et al., 2009; Wilen et al., 1991; Zalewski et al., 2010; Norastehnia et al., 2007).

The cross-talk between ROS and phytohormones during seed germination is a fascinating point to investigate (**Fig. 3**). Several studies on hormones-ROS interaction have demonstrated that exogenously applied ABA resulted in reduced ROS accumulation in germinating barley (Ishibashi et al., 2012), rice (Ye et al., 2012), lettuce (Zhang et al., 2014a) and sunflower (El-Maarouf-Bouteau et al., 2015) seeds. GA application, on contrary, was shown to increase ROS production (Lariguet et al., 2013). Other phytohormones, such as ET, JA, salicylic acid (SA), auxin, cytokinin or brassinosteroids are also implicated in the ROS-mediated regulation of seed germination (Miransari and Smith, 2014; El-Maarouf-Bouteau et al., 2015). The mechanisms of phytohormones interactions during seed germination are still poorly understood at the molecular level, specifically at early stages.

1.2.2.2. Omics approaches to decipher the molecular bases of pre-germinative metabolism

Different approaches have been utilized to understand the role of molecular players and factors that influence the pre-germinative metabolism and seed quality. In model species, omics studies are being applied to identify candidate genes and metabolic pathways that are activated during seed germination (Catusse et al., 2008). Transcriptomic studies have highlighted that seed development and environment influence gene expression during seed germination (Ogawa et al., 2003; Finch-Savage et al., 2007). Post-transcriptional regulatory mechanisms have important roles in the modulation of seed germination. Expression analysis of microRNA (miRNA) revealed that the miRNA-mediated regulatory metabolism is rapidly activated during pre-germinative metabolism (He et al., 2015). Proteomic analysis was conducted to analyze seed dormancy related to adverse conditions (Pawlowski and Staszak, 2016; Lu et al., 2016). Genomics, based on Quantitative Trait Loci (QTL) analysis, was a powerful strategy to identify loci and genes involved in the control of important seed traits (Wan et al., 2005; Gu et al., 2008; Ogbonnaya et al., 2008; Sato et al., 2016). Recently, to overcome the limitations of QTL analysis, Genome Wide Association Studies (GWAS) became more popular. This technique allows a quantification of the association between each genotyped marker and a phenotype of interest scored across a large number of individuals (Korte and Farlow, 2013). For example, Hatzig et al. (2015) conducted a GWAS in oilseed rape (*Brassica napus* L.) to identify genomic regions influencing seed germination.

Nutritional quality of seed is an important aspect due to the fact that the consumption of sprouted seeds is becoming more popular in various parts of the world. The nutritional content (e.g. essential amino acids, sugars or phenolic compounds) of the seed is influenced by the species, seed quality and environmental conditions in which germination occurs (Chavan et al., 1989; El-Adawy et al., 2003). Thanks to metabolomic analysis, changes observed in the metabolic pathways related to flavonoids and phenolic acids associated with sprouting were characterized (Tang et al., 2014).

By combining all the different types of omics, systems biology provides the tools for the integration of the different datasets derived from the same biological sample, thus providing an overview of all processes and regulatory reactions (e.g. during seed germination). An interacting cycling between 'wet-lab' (experimental part) and 'dry lab' (computational part) approaches is needed to generate a comprehensive understanding of biological systems (Rohn et al., 2012). Systems biology deliverables includes the identification of complex gene regulatory networks composed of genes, coding and non-coding RNAs, proteins, metabolites and signaling components (Long et al.,

2008) and their relevance for plant development and response to environmental stimuli (Moreno-Risueno et al., 2010). One of the first network models built using publicly available gene expression data from *Arabidopsis thaliana* is SeedNet (www.vseed.nottingham.ac.uk; Bassel et al., 2011). In particular, the SeedNet network describes the global transcriptional interactions that mediate the transition between dormancy and germination.

Understanding the mechanisms related to pre-germinative metabolism is useful for industrial seedling production and seed companies in order to be able to produce high-quality seed stocks in short time, with high levels of germination and low dormancy. At the moment, all the approaches previously mentioned have significantly contributed to expand the knowledge of the pre-germinative metabolism; however, more studies are needed to provide an exhaustive picture of the entire process.

1.3. Seed priming: designing a 'perfect' mix of tradition and innovation to produce high-quality seeds

1.3.1. Definition and historical perspective of seed priming

Seed priming is a pre-sowing treatment which leads to a physiological state that enables seeds to germinate more efficiently (Chen et al., 2011). Priming is a technique that allows controlled seed rehydration to trigger the metabolic processes normally activated during the early phase of germination but preventing the seed transition towards full germination (**Fig. 4**). The priming treatment must be stopped before the loss of desiccation tolerance occurs and this event overlaps with the phenological stage of radicle protrusion (Paparella et al., 2015). During the priming treatment, seeds are soaked in presence or absence of aeration, in water or in solutions with high osmotic potential. The purpose of soaking in the osmotic solution is to prevent the absorption of water amounts sufficient for radicle emergence, thus delaying water up-take and expanding the lag phase of seed metabolism (Mustafa et al., 2017).

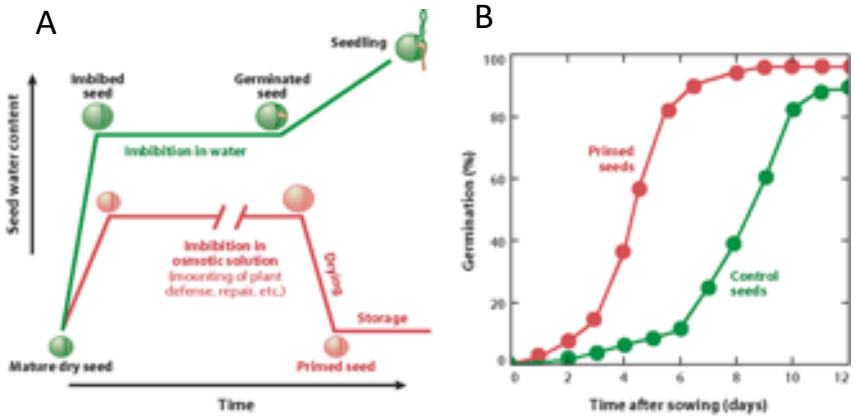


Fig. 3. Schematic representation of the effects of seed priming. **(a)** Seed water relations during priming. The treatment allows the hydration and the advancement of metabolic and repair processes while preventing germination and loss of desiccation tolerance. **(b)** Germination test showing improved vigor of primed seeds (Rajjou et al., 2012).

Priming techniques to improve germination had been used since centuries by farmers and later on by seed companies. Humans established contact with seeds since the dawn of agriculture and they quickly realized that many seeds do not germinate easily and uniformly (Lutts et al., 2016). The Greek Theophrastus (ca. 372-287 BC) already focused on seed physiology and suggested that the germination process may be temporarily interrupted (Everari et al., 1984). Theophrastus investigated the seed response during germination observing that cucumber (*Cucumis sativus*) seeds soaked in water prior to sowing resulted into faster germination (Theophrastus, Enquiry into Plants, Book VII, I.6). The Roman naturalist Gaius Plinius Secundus (A.D. 23-79), collected most of the knowledge on seed physiology in his Encyclopaedia, the Naturalis Historia. In his work, he refers to the relevance of presoaking cucumber seeds in water and honey to improve germination.

Several centuries later, these techniques were still used by farmers for a wide range of species (*Triticum* spp., *Secale* spp., *Ordeum* spp.), according to the French agronomist Olivier de Serres (1539-1619) (Everari et al., 1984). During these treatments, seeds were soaked for 2 days in manure water and then dried in the shade before sowing. In 1664, Evelyn et al. showed that temperature treatments prior sowing may have an impact on germination while one century later, Ingenhousz et al. (1779) analyzed the influence of light on seedling emergence. Charles Darwin (1809-1882) tested osmopriming conditions by submerging cress (*Lepidium sativum*) and lettuce (*Lactuca sativa*) seeds in salty sea water and demonstrated that the treatment was able to enhance germination (Darwin 1855c, 1855d). Furthermore,

during the 19th century, numerous botanists started to describe morphological processes associated with seed germination (Amici et al., 1830, Sachs et al., 1859). Sachs et al. (1887) experimented the impact of various compounds (including tyrosine and asparagine) before and during germination.

The discovery of plant hormones in the 1920s underlined the crucial role of these compounds in seed desiccation tolerance, reserve mobilization, as well as cell division and cell elongation necessary during germination. Later on, these results were confirmed by Ells (1963) who highlighted some key points in seed treatment, paving the way to the modern concept of seed priming. He noticed that tomato seeds treated with a nutrient solution showed increased germination rate while May et al. (1962) observed that seeds dried at different timepoints following imbibition resulted in quicker germination. The possibility to influence final germination as a consequence of pre-sowing treatment has led to a wide range of empirical methods for numerous cultivated plant species during the 1970s (Khan et al., 1992). The pioneer work by Heydecker et al. (1973) showed that seed priming could be successfully used to improve germination and emergence under stressful conditions. Heydecker carried out priming experiments in carrot (*Daucus carota*), red beet (*Beta vulgaris*) and onion (*Allium cepa*). This study demonstrated that the imbibition of onion seeds in an osmotic solution (polyethylene glycol, PEG) for 6 days, followed by drying (dry-back), exhibited higher levels of RNA production during germination than untreated seeds. Moreover, the primed seed displayed an increased rate of radicle emergence depending on all three components of the preparatory treatment: osmotic potential, temperature and duration.

In those early times, an increasing number of reports indicated that 'imbibing and drying' pre-treatments exerted beneficial effects on seed germination in a wide range of horticultural and cereal species, particularly under stress conditions (Austin et al., 1969; Hegarty, 1970; Berrie and Drennan, 1971). Since then, seed priming has been adopted by the seed companies as a routine tool to improve seed vigor. From a molecular point of view, during priming, the pre-germinative metabolism becomes activated and maintained for an extended period in order to enhance the seed repair response, in terms of DNA repair and antioxidant mechanisms (Hussain et al., 2016a, 2016b). DNA repair pathways and the antioxidant response are crucial to preserve genome integrity, improve germination parameters and seedling development. A key step in the protocol of seed priming is the so-called 'dry-back' (**Fig. 4**). Seeds must be re-dried, after priming, until they reach the original water content (approximately 5%) before sowing or storage.

The benefits of seed priming have been widely reported. Priming strategies may grant several economic and agronomic advantages to cultivated plants. Numerous data reported an improvement in the rate and uniformity of germination, seedlings growth and stress resistance. Along with synchronous and fast emergence, primed seeds show reduced photo- and thermo-dormancy, a wider range of germination temperatures and better capacity to compete with weeds and pathogens (Ellis et al., 1988; Hill et al., 2008). The success of seed priming is strongly correlated to plant species/genotype and physiology, seed lot, age and vigor, as well as to the priming method applied, presence/absence of light, osmotic potential, temperature, water potential, presence/absence of aeration, and duration of treatment (Parera and Cantliffe, 1994; Varier et al., 1998; Hussain et al., 2006). The priming process not only impacts seeds but the whole plant system and may be defined as an induced state whereby the plant reacts more rapidly and more efficiently to a stress (Balmer et al., 2015). From this point of view, plants exposed to a primary constraint are able to trigger a temporary metabolic adaptation leading to a stress memory and allowing them to adapt more efficiently to subsequent stress episodes.

Priming has been routinely used to improve seed vigor in the main vegetable crops, e.g. carrot (Pereira, 2009), leek and onion (Nunes et al., 2000; Caseiro et al., 2004), celery, lettuce (Fessel et al., 2001; Ouhibi et al., 2014), endive, pepper (Venkatasubramanian et al., 2010; Song et al., 2017), tomato (Di Girolamo and Barbanti, 2012), eggplant (Fanan and Novembre, 2007; Nascimento and Lima 2008; Zhang et al., 2011; Mahesh et al., 2017), asparagus (Bittencourt et al., 2004), beetroot (Alipor et al., 2019), cauliflower and rape (Kubala et al., 2015; Hassini et al., 2017; Lechowska et al., 2019), fennel (Tahaei et al., 2016), and milk thistle (Migahid et al., 2019). Herbs, like *Rosmarinus officinalis* and *Salvia splendens*, can also benefit from seed priming (Di Girolamo and Barbanti, 2012). Large-scale routine priming of commercial cereal seeds is more difficult to achieve, although advantages are well known (Murungu et al., 2004). Priming provides optimal conditions to accelerate seed germination when requested by the cereal grain and brewing industry (Yaldagard et al., 2008).

1.3.2. Classification of seed priming techniques

Several methods of seed priming have been developed in order to ameliorate seed germination (**Fig. 5**). The most applied priming techniques hereby described are: hydropriming, osmopriming, solid matrix priming, chemo-priming, thermopriming, hormopriming, biopriming, and nutripriming.

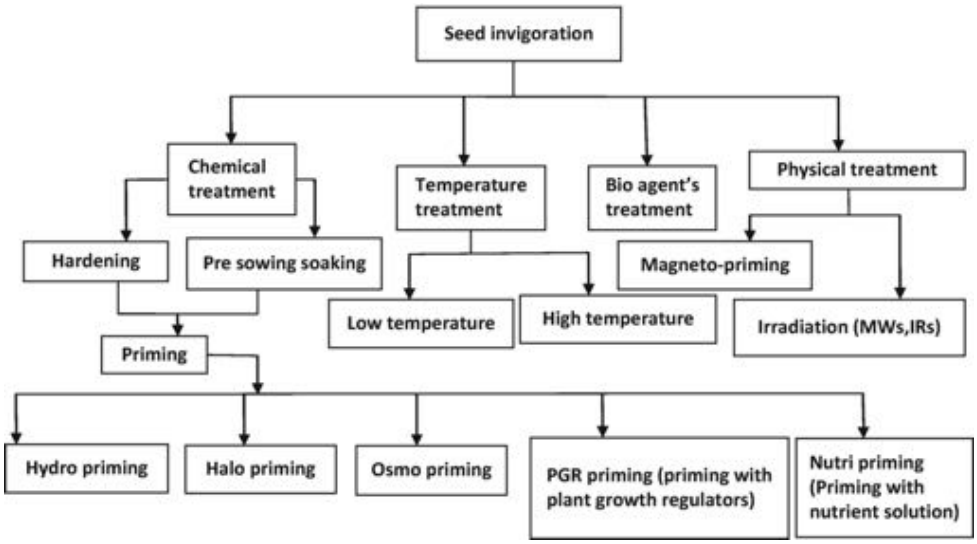


Fig. 5. Schematic representation of seed priming (invigoration) techniques (Bose et al., 2018).

1.3.2.1. Hydropriming

Hydropriming is the simplest method, seeds are soaked, in presence or absence of aeration, in water under optimal temperature conditions and re-dried to original moisture content prior to sowing or storage. This method is simple, low-cost, and environment-friendly (Taylor et al., 1998). The main disadvantage of hydropriming is the uncontrolled water up-take by seeds. This is a consequence of free water availability to seeds during hydropriming, so that the rate of water up-take depends only on the seed tissue affinity to water (Taylor et al., 1998). Moreover, this technique may result in an unequal degree of seed hydration, thus leading to a lack of simultaneous metabolic activation within seeds followed by unsynchronized emergence (McDonald et al., 2000). Due to these limiting factors, it is important to define carefully the treatment duration as well as temperature, and the water volume used in hydropriming to ensure desired level of seed hydration and to prevent radicle protrusion (Taylor et al., 1998). Despite these limitations, several reports highlighted the beneficial effect of hydropriming on seed germination and seedling growth under both optimal and stress conditions in various crop plants, e.g. chickpea, maize (Rahman et al., 2011), wheat (Basra et al., 2002; Fercha et al., 2013), rice (Goswami et al., 2013), Indian mustard (Srivastava et al., 2010), canola (Omidi et al., 2009), sunflower (Kaya et al., 2006), mung bean (Posmyk et al., 2007), and pepper (Patade et al., 2012). Among the

different techniques of hydropriming, 'drum priming' had been patented by Rowse (1991). In this technique, seeds are gently rotating in a drum connected with a boiler that generates vapor, hence gradually hydrated since the vapor condenses into liquid water inside the drum. Drum priming allows seed imbibition in a controlled manner. The machine is connected to a computer that is able to measure the increase in seed relative mass during the treatment. The time and water volume required to complete seed rehydration are strictly controlled to reach gradual seed imbibition (Warren et al., 1997).

1.3.2.2. Osmopriming

Another type of priming protocol is osmopriming, based on the use of osmotic solutions at low water potential, thus facilitating the control of water up-take. In the presence of osmotic agents, water enters the seed slowly and this allows the gradual imbibition of the seed and activation of the early phases of germination but prevents radicle protrusion (Di Girolamo et al., 2012; Khan, 2010). Usually, the water potential of priming agents varies from -1.0 to -2.0 MPa (Kubala et al., 2014). However, both the value of water potential and the duration of the priming treatment need to be adjusted, depending on the species, cultivar, and seed lot. During imbibition, water entry into the seed associates with progressive ROS accumulation and oxidative damage of cellular components (lipid membranes, proteins, nucleic acids). The main goal of osmopriming is to limit the ROS-mediated oxidative injury by delaying water entry. The osmotic agent mainly used is polyethylene glycol (PEG), a polyether compound with several industrial applications, commercially available in a wide range of molecular weights. The chemical features of PEG are recognized as extremely useful in pre-sowing treatments since the inert nature of the molecule avoids cytotoxic effects, and the large molecular size (6000-8000 Daltons) prevents absorption of the molecule into the seed (Michel and Kaufman, 1973; Heydecker and Coolbear, 1977). However, PEG shows some disadvantages such as the high costs and the extremely high viscosity which limits oxygen transfer within the solution. Some of these technical constraints can be overcame by using effective aeration systems (Bujalski and Nienow, 1991). Other compounds used for osmopriming are mannitol, sorbitol, glycerol, and inorganic salts such as NaCl, KCl, KNO_3, K_3PO_4, KH_2PO_4, $MgSO_4$, and $CaCl_2$ (Yacoubi et al., 2013). Most of these agents are less expensive, easier to aerate and to be removed as compared to PEG. Priming carried out with salt solutions is also known as 'halopriming'. Priming with these compounds can either be effective as the PEG-based treatment or it can trigger significantly different responses, depending on seed morphology. Priming effectiveness can be influenced by the presence of a semi-permeable outer layer, a structural

component typical of certain seeds (Pill, 1995). This amorphous tissue, localized between pericarp and seed coat, can reduce or avoid ion/solute exchange, thus controlling seed permeability to water and priming agents (Zhou et al., 2013). The choice of the osmotic agent is a crucial step and it is essential to assess the levels of ion accumulation that could result into cytotoxic effects and nutritional imbalance within the seed (Bradford, 1995; Balestrazzi et al., 2011a; Gomes et al., 2012).

1.3.2.3. Solid matrix priming

Solid matrix priming was developed to overcome the high costs of osmotic agents and technical problems related to aeration and the volume of the priming solution. Seeds are mixed with solid (organic or inorganic) materials able to adjust the moisture content, thus allowing a controlled water up-take (Harman and Taylor, 1988; Khan, 2010). The substrate used as matrix must be carefully selected since it can significantly influence priming performance. The materials utilized as matrices should possess specific physical and chemical features such as low matrix potential, minimal water solubility, high water holding capacity and surface area, no toxicity to seeds and ability to adhere to seed surface (McDonald et al., 2000; Di Girolamo et al., 2012). Many natural substances have been used as matrices, e.g. coal, sawdust, vermiculite, calcined kaolin, charcoal and commercial substrates such as Agro-Lig® (a humic acid-based product used to improve soil features) and Micro-Cel E (a non-soluble, synthetic calcium silicate) (Hacisalihoglu et al., 2018). The treatment is preferentially carried out in a sealed container that permits air circulation, avoiding excessive evaporation. Solid matrix priming simulates the natural imbibition process occurring in soil, leading to the same results obtained with liquid priming but at reduced costs. Furthermore, solid matrix priming can be integrated by adding chemical or biological agents known to improve seed performance (McDonald, 2000; Madsen et al., 2018). Inclusion of GAs, fungicides, or *Bacillus subtilis* to matrix priming leads to improved stand establishment and productivity in some vegetable crops grown under tropical conditions (Adoreoli et al., 2002). The application of GA_3 (gibberellin A3) to matrix priming enhanced the germination quality of hot pepper seeds (*Capsicum annuum*) (Ilyas et al., 2002). Moreover, solid matrix priming with *Trichoderma viride* improved seedling emergence and yield of okra (*Abelmoschus esculentus*) under low temperatures (Pandita et al., 2010).

1.3.2.4. Chemo-priming

The chemo-priming treatment was developed to prevent microbial contamination. Conventional disinfectants, such as sodium hypochlorite (NaOCl) or hydrochloric acid (HCl), natural substances, and agrochemical

(e.g. fungicides and pesticides), are added to the priming solution (Parera and Cantliffe, 1990). Although these treatments limit the losses in germination caused by pathogens, several parameters (disinfectant concentration, treatment duration, solution temperature, and seed age) need to be evaluated with attention (Khan, 1992). To date, the chemo-priming technique relies on the use of innovative materials for seed treatment, e.g. pesticide–collagen hydrolysate mixtures with bioactive properties which allow to reduce pesticide dispersal in the environment. More ecological-friendly tools are contemplated, such as those made of microencapsulated plant extracts with insecticidal and fungicidal properties (Gaidau et al., 2014).

1.3.2.5. Thermopriming

Seed treatment at different temperatures before sowing is defined as thermopriming. This method has been widely demonstrated to improve germination efficiency under adverse environmental conditions, reducing thermo-inhibition of seed germination (Huang et al., 2002). Low temperatures generally provide the best results. Although not so widely applied, thermopriming at high temperatures has been used in some species, resulting in advantages in germination especially for plants adapted to warm climates (Khalil et al., 1983). Thermopriming combined with other treatments resulted in beneficial effects on germination parameters of white spruce (*Picea glauca*), enough to improve nursery practices for commercial seedling production (Liu et al., 2013). Hydro-thermal priming is based on hydropriming treatments carried at different temperatures (Alipor et al., 2019). This method has been tested in sugar beet (*Beta vulgari*s), both under laboratory and field conditions. A 15% increase in seedling emergence was reported in the field. The positive effect of this type of treatment was observed on the final germination percentage, mean germination time, and uniformity of germination (Alipor et al., 2019).

1.3.2.6. Hormopriming

A relevant impact on seed metabolism and germination has been observed with hormopriming treatments when seeds are imbibed in the presence of plant growth regulators. The most frequently used regulators are ABA, GA, ET, SA, auxins, cytokinins (e.g. kinetin), and polyamines. Concomitant priming with GA_3 and PEG improved the photosynthetic properties, antioxidant system, seedling emergence and growth of white clover (*Trifolium repens*) on heavy-metal polluted soil (Galhaut et al., 2014). Priming sprout wheat seeds with GA_3 increased grain yield and salt tolerance (Iqbal et al., 2013). Enhanced salt tolerance, growth, and grain yield of wheat were also observed after kinetin-mediated priming (Iqbal et al., 2006). The

critical role of phytohormones exogenously supplied to seeds for improving the plant response to salinity stress was stated in wheat seeds primed with ascorbic acid and SA as this pretreatment increases the ability of wheat to grow successfully under salt stress, whereas hormonal priming with ABA was not effective in this case (Afzal et al., 2006). In a recent paper, Araujo et al. (2019) highlighted a relevant aspect of the seed response to phytohormones, namely the risk of excess genotoxic damage resulting from the prolonged exposure of seeds to kinetin.

1.3.2.7. Biopriming

Biopriming involves seed imbibition coupled with bacterial inoculation (Callan et al., 1990). This treatment not only increases the rate and uniformity of germination but additionally protects seeds against soil and seed-borne pathogens. Hydration of seeds infected with pathogens during priming can result in a stronger microbial growth and consequently impairment of plant health. However, applying antagonistic microorganisms during priming is an ecological approach to overcome this problem (Reddy et al., 2013). It has been found that biopriming is a much more effective approach to disease management than other techniques such as pelleting and film coating (Müller et al., 2008). Actually, the use of biopriming with plant growth-promoting bacteria (PGPB) as an integral component of agricultural practice shows great promise (Gulick et al., 2012; Timmusk et al., 2014). Seed priming with living PGPR (plant growth-promoting rhizobacteria) increases speed and uniformity of germination, ensures rapid, uniform, and high establishment of crops, and improves harvest quality and yield (Mahmood et al., 2016). During the biopriming treatment, when the pre-germinative metabolism starts, there is a significant expansion of the PGPR population that grows in the spermosphere (the site surrounding the seed where the interaction between soil, microbial communities and the germinating seed takes place), even before sowing (Taylor and Harman, 1990). This proliferation of antagonist PGPR inside the seeds is 10-fold higher than that observed for pathogens, and such feature enables the plant to survive pathogen attack and invasion (Callan et al., 1990). Biopriming procedures ensure the entrance of endophytic bacteria into the seeds along with avoiding the treatment at high temperature (Moeinzadeh et al., 2010). Biopriming with rhizospheric bacteria has been reported in crops such as carrot (Jensen et al., 2002), sweet corn (*Zea mays* var. *saccharata*) (Callan et al., 1990, 1991) and tomato (Legro and Satter, 1995; Warren and Bennett, 1999). Seed biopriming can also be performed using a combination of priming with biologically active compounds (elicitors) that trigger plant immunity (Song et al., 2017). This technology is called ‘seed defence biopriming’ (SDB). Many bacterial heat-stable

metabolites have been so far tested, in particular cyclodipeptides, for their ability to induce SDB in cucumber and pepper seeds, thus triggering plant immunity. Bacterial cyclodipeptides are a class of small molecules that exhibit diverse biological activities as antifungal compounds. As an example, cyclo(L-Phe–D-Pro) and cyclo(L-Leu–L-Pro) act against *Fusarium sporotrichioides* and *Aspergillus parasiticus*, respectively (Ström et al., 2002; Yan et al., 2004), thus confirming the prediction that SDB treatment elicits immunity from the seed to the whole plant and enables plants to maintain effective disease control without growth penalty under greenhouse and field conditions. In pearl millet (*Pennisetum glaucum*), biopriming with *Pseudomonas fluorescens* isolates enhanced plant growth and resistance against downy mildew disease (Ray et al., 2004).

1.3.2.8. Nutripriming

Nutripriming is a more recent technique in which seeds are soaked with solutions containing the limiting nutrient instead of pure water. This was made in order to improve the nutritional requirements by providing key elements and to stimulate the biochemical pathways that influence seed quality, germination parameters, and seedling establishment (Farooq et al., 2013). Seed priming with Zn improved productivity of chickpea and wheat (Arif et al., 2007), germination and early seedling growth of rice (Abbas et al., 2014), and the development and root growth of maize seedling exposed to low temperatures at the root levels (Imran et al., 2013). Potassium (K)-based priming brought favorable effect on the growth and nutrient status of cotton (*Gossypium* spp.) seedling under saline conditions (Shaheen et al., 2015). Some nutripriming techniques are commonly used by seed companies in the process of seed production and preparation for growers. The most used methods, namely 'broad spectrum nutrient' seed priming (BSN), is based on seed imbibition carried with a mixture of minerals, such as zinc, copper, manganese, molybdenum, and phosphorus, which has been proved to fertilize the seed and provide the nutrients for early seedling growth. This positively affects germination, seedling vigor, and root system development (Farooq et al., 2012).

1.3.3. Parameters used to evaluate seed vigor

The overall benefits associated with seed priming can be summarized as an increased level of seed vigor, observed in the treated samples. Seed vigor is a complex trait, difficult to target since it represents the whole set of properties conditioning the performance of seed lots in a wide range of environmental conditions (Zhang et al., 2015). Due to the several components contributing to this trait, a complex array of measurement and analyses

related to the germination process needs to be performed in order to evaluate seed vigor in response to priming treatments. Several mathematical models have been used until now, often with contrasting results and interpretations. Ranal and Garcia de Santana (2006) have provided a comprehensive review covering the measurements required to describe the germination process by taking in consideration the time, rate, homogeneity, and synchrony of germination as most informative parameters. These characteristics are important not only for plant physiologists and seed technologists, but also for ecologists. Indeed, it is possible to predict the degree of success of a species based on the capacity of the harvested seeds to germinate through time, allowing the use of these high-quality seeds in nature. This opens novel perspectives in the study of seed ecology and for the management of germplasm collections maintained in seed banks.

The most descriptive parameters included in this review are germination percentage, mean germination time, coefficient of variation of the germination time, mean germination rate, uncertainty and synchronization index.

Germination percentage or germinability (***G***), is expressed as the mean number of germinated seeds per day calculated in percentage. Mean germination time (***MGT***) is defined as the weighted mean of the germination time and is calculated according to the following formula:

$$\bar{t} = \sum_{i=1}^{k} n_i t_i / \sum_{i=1}^{k} n_i$$

where t_i is time from the start of the experiment to the i^{th} observation (day); n_i: number of seeds germinated in the time i (not the accumulated number, but the number correspondent to the i^{th} observation), and k is the last time of germination.

The coefficient of variation of the germination time (***CVt***) is calculated as:

$$CV_t = (s_t / \bar{t})100$$

where s_t is the standard deviation of the germination time and t the mean germination time.

Labouriau (1970) demonstrated that the coefficient of velocity (CV) of Kotowski (1926) could be used to calculate the mean germination rate (***MR***) as:

$$\bar{v} = CV/100 = 1/\bar{t}$$

where $\bar{t}$: mean germination time and CV: coefficient of velocity. Consequently, the mean germination rate (v) is defined as reciprocal of the

mean germination time, since the mean rate increases and decreases with $1/\bar{t}$, not with $\bar{t}$ (Labouriau 1983b).

Uncertainty (***U***) describes the levels of uncertainty associated to the distribution of the relative frequency of germination and is quantified by means of a measurement named synchronization index (*E*). The uncertainty associated to the distribution of the relative frequency of germination (*U*) was expressed by Labouriau and Valadares (1976) as:

$$\overline{E} = -\sum_{i=1}^{k} f_i \log_2 f_i \text{ , being } f_i = n_i / \sum_{i=1}^{k} n_i$$

where f_i is the relative frequency of germination, ni the number of seeds germinated on the day *i*, and *k* the last day of observation.

The synchronization (***Z***) index has been proposed to evaluate the synchrony of one seed with another included in the same replication of one treatment. It is defined as:

$$Z = \sum C_{n_i,\,2} / N$$

where

$$C_{n_i,\,2} = n_i(n_i - 1)/2$$

$$N = \sum n_i(\sum n_i - 1)/2$$

$C_{n_i,\,2}$: combination of the seeds germinated in the time *i*, two together and n_i: number of seeds germinated in the time *i*. When the germination of all seeds occur at the same time *Z* will be 1 instead it will be 0 when at least two seeds could germinate, one at each time.

All these standardized germination measurements are applied to evaluate seed germination and seedling emergence. Researchers have to carefully choose the measurements and the interval of evaluation that better describe the experiment and the results obtained according to their objectives.

1.3.4. Innovation in seed priming: the use of molecular tools

It is well known that pre-sowing priming treatments induce a peculiar physiological status in seeds by boosting the pre-germinative metabolism, and this feature makes priming a promising strategy to improve seed quality and subsequently plant behavior in the field. There is a strong interest of farmers and seed companies to find suitable and cheap priming treatments but also to precisely identify the agronomical properties improved as a result of priming in cultivated species. Despite the studies so far carried out on the physiological mechanisms associated with seed priming, several open questions still need to be addressed. Within this context, a deeper understanding of the molecular processes that drive the seed response to

priming, at the level of pre-germinative metabolism, will provide molecular hallmarks (genes, proteins, metabolites) useful for predicting the effectiveness of novel pre-sowing treatments.

Thanks to the recent advances in the field of plant molecular biology, it is now possible to focus on novel seed quality markers that associate with high germination levels and thus can be used to help breeding programs, crop productivity, and preserve biodiversity (Macovei et al., 2016). New opportunities to elucidate the molecular bases of the priming process are provided by the use of holistic approaches (e.g. 'omics' tools). Similarly, nondestructive and noninvasive methods such as digital image technology may be used in a more precise way to study the kinetics of imbibition in relation to the modification of the seed ultrastructure (Toorop et al., 1998; Bolte et al., 2011; Kubala et al., 2015).

To date, researchers and seed technologists are focusing on improving some specific weakness points of seed priming. Indeed, despite the wide use and the relevance of these technique, it is still much based on empirical features that limit the work of seed technologists and farmers. A gap of knowledge still exists concerning the molecular aspects of seed priming, mostly at the level of seed pre-germinative metabolism. Further investigations will help to uncover additional molecular hallmarks (genes, proteins, metabolites) necessary to predict the effectiveness of novel pre-sowing treatments. In this context, several reports have already highlighted links between intense DNA repair, antioxidant response, and high-quality seeds in model plants (Balestrazzi et al., 2011a, 2011b, 2015; Donà et al., 2013; Macovei et al., 2011, 2016; Waterworth et al. 2010, 2016; Pagano et al., 2017, 2019).

1.3.4.1. Nanotechnology

In the recent years, nanotechnologies have been developed and applied in agriculture (Marchiol, 2018). Nanotechnology, or the science and technology conducted at the nanoscale, is a rapidly evolving field expected to bring innovations in agriculture and food industry, allowing to increase food production in a sustainable manner as well as to protect and ameliorate crops (Marchiol, 2018). Nanotechnology has developed nanomaterial formed by aggregates of atoms or molecules with a diameter of approximately 1-100 nm (Aslani et al., 2014). These molecules display unique physicochemical properties like catalytic reactivity, high surface area, size and shape. They have the potential to open new paradigms and introduce new strategies in agriculture (Mani and Mondal, 2016). Although the use of nanomaterials for agricultural applications is a relatively new approach as compared to their use in biomedical and industrial sectors, it appears highly promising.

Applications of nanoparticles (NPs) in agriculture need to be low-cost, ecofriendly, biocompatible and non-toxic (Liu et al., 2015; Mahakham et al., 2016). Plant-based materials represent the best candidates for synthesizing biocompatible NPs, due to the biochemical diversity of plant extract, non-toxic phytochemical components, non-pathogenicity, and flexibility in reaction parameters as compared to chemical synthesis methods (Mahakham et al., 2016; Singh et al., 2016b). During the past decade, a number of patents and products incorporating engineered NPs into agricultural practices, e.g. nanopesticides, nanofertilizers, and nanosensors, have been developed with the collective goal to promote the efficiency and sustainability of agricultural practices requiring less input and generating less waste than conventional products and approaches (Servin et al., 2015). The nanoparticles were also used for the improvement of plant traits against environmental stress and diseases (Gogos et al., 2012; Chen et al., 2011), and to treat seeds with low germinability.

These advances in nanotechnology also led to developing a novel type of priming, 'nanopriming'. In this case, seed priming is carried with synthetic nanoparticles (Servin et al., 2015). There have been already some published reports describing the use of metal-based NPs (e.g. AgNPs, AuNPs, CuNPs, FeNPs, FeS_2NPs19, TiO_2NPs, ZnNPs1, ZnONPs) and carbon-based NPs (e.g. fullerene and carbon nanotubes) as priming agents (Aslani et al., 2014; Anderson et al., 2016). The mechanism behind seed nanopriming might be different from that of classic pre-sowing seed treatment carried without drying the seeds. At the moment, a comprehensive study on the physiological and molecular mechanism underlying the effects of nanopriming on seed germination is still missing. However, based on the existing experimental evidence, three main hypotheses have been proposed.

(1) One of the possible mechanisms is based on the hypothesis of Khodakovsakay and collaborators (2011). These authors carried out experiments using carbon nanotubes, which were able to penetrate the seed coat, producing small pores. These pores increased the flow of water inside the seed and caused the overexpression of the aquaporin genes (Villagarcia et al., 2012; Qian et al., 2013; Lahiani et al., 2013). The progressive imbibition stimulates the embryo to produce the phytohormones gibberellins, that in turn trigger the transcription of the gene encoding α-amylase (Man et al., 2013). Once produced, this enzyme is secreted from the aleurone layer inside the endosperm, where it will be able to hydrolyze the starch. In this first hypothesis, the nanopores, produced by nanoparticles, accelerate water absorption and, consequently, the activation of seed metabolism. Once the aquaporin genes are up-regulated, the water channels can facilitate the diffusion of ROS (Cordeiro et al., 2015).

(2) The second hypothesis involves ROS molecules such as H_2O_2, $\bullet O_2^-$ and •OH. This hypothesis has been elaborated based on the observation that when the amount of ROS increased there was also a strong up-regulation of the genes coding for aquaporins (Cordeiro et al., 2015). It is important to mention that ROS, under a certain threshold, perform the function of signal molecules, stimulating germination and plant development (Maurel et al., 2015; Cordeiro et al., 2015). The amount of ROS in seed tissues must be maintained within a certain threshold, the so-called 'oxidative window' to induce germination. Several antioxidant enzymes are responsible for keeping this amount under a tight control (Bailly et al., 2008; Leymarie et al., 2012). In addition to this function, ROS, especially the radical •OH, participate in the loosening of the cell wall. This is a fundamental process that acts through a mechanism of mechanical resistance, limiting cell growth (Müller et al., 2009). Cell wall loosening is found in all stages of plant development. It has been shown that some ROS participate in this process, favoring the breaking of the seed coat and the protrusion of the radicle during the germination (Richards et al., 2015; Liu et al., 2016a). Higher ROS production was observed in germinating seeds treated with nanopriming (Li et al., 2017). These higher ROS levels can act as a signal, consistent with the 'oxidative window' concept, resulting in better germination and faster seedling growth compared with that observed in unprimed seeds. To summarize this hypothesis, Mahakham (2017) suggested that nanopriming reboots ROS and the antioxidant system in seeds.

(3) The third hypothesis proposes the impact of silver nanoparticles as catalysts. These nanoparticles have been shown to increase the speed of the starch hydrolysis reaction, but the exact mechanism by which they operate is not yet clear. An explanation could be that the enzyme α-amylase interacts, via disulfide bridges, with the functional groups bound to the surface of the nanoparticles. In this way, an enzyme-nanoparticle complex is formed as the enzyme α-amylase is immobilized on the surface of the nanoparticle (Ernest et al., 2013; Misson et al., 2015). In the immobilized form, some enzymes tend to be more stable and to interact more easily with the substrate (Ernest et al., 2013). Another explanation could be that, in the form linked to the nanoparticle, the enzyme undergoes a conformational change that makes it more similar to the substrate (Deka et al., 2012). The enzyme α-amylase, therefore, could behave in one of these two ways described and this would explain the increase in enzymatic activity observed in seeds treated with nanopriming. This increase subsequently results in an enhanced amount of soluble sugars in the cell and, consequently, in higher availability of soluble sugars. These are necessary to generate the energy required for seedling growth, thereby leading to increased germination speed. Water will therefore

tend to enter the cell by osmosis, causing the phenomenon of increased water up-take observed by researchers in seeds treated with the nanopriming technique (Hussain et al., 2015; Zheng et al., 2016).

1.3.4.2. Seed priming, biotic and abiotic stresses

Several studies reported an interesting aspect of seed priming: it can boost the seed germination and seedling establishment under non-optimal conditions, e.g. drought or salinity (Kumar et al., 2019). These types of treatments can alleviate stress levels in seedling resulting in better productivity levels under environmental constrains (Macovei et al., 2014; Ibrahim et al., 2016; Li et al., 2017; Bajwa et al., 2018). This is rather important in the context of promoting sustainable agriculture and addressing the big pressure due to the rising global population under drastic changes in the climate conditions. Actually, the situation is aggravated by both abiotic (cold, drought, salinity, water deficit, extreme pH, or pollution) and biotic (pathogenic organisms, weed infestations, pests) stresses.

Considering all these serious issues that the current agriculture has to face and the resulting strong pressure at global level, both researchers and farmers are now focusing their efforts to develop management practices or tools to be used alone or in combination to ensure the production sustainability, cost effectiveness, and healthy ecosystems (Kumar et al., 2012). In this context, the need to improve seed quality is a relevant issue and a priority for researchers. Priming is currently envisaged as a powerful tool that can be easily adopted in different environments and for different crops to improve seed quality in order to obtain plants which can withstand various types of stresses and adverse environmental conditions (Kumar et al., 2019). Galhaut (2013) reported an example of priming applied to a heavy-metal polluted soil. White clover seeds were primed either with GA or PEG. Both priming treatments increased the germination speed and final germination percentage in the presence of heavy-metals (Cd and Zn). Another sustainable application of priming was applied for the bioremediation of petroleum-contaminated soil (Das et al., 2016). Seed biopriming was carried out using PGPR from *Pseudomonas* spp. and applied to Indian ginseng (*Withania somnifera*). The results showed high values for germination, shoot and radicle length, and fresh and dry weight when compared to non-primed seed, underlining the effectiveness of the treatment.

1.4. Antioxidant mechanisms in the context of seed germination

Various biotic and abiotic stress factors (e.g. salinity, UV radiation, heavy-metals, drought, extreme temperatures, nutrient deficiency, air pollution, pathogen attacks) can perturbate the equilibrium between the

production and scavenging of ROS (Foyer et al., 2005; Gill and Tuteja, 2010). This leads to ROS overproduction at the cellular level with consequent deleterious effects. Indeed, free radicals are highly reactive and toxic, causing damage to proteins, lipids, carbohydrates and DNA and ultimately resulting in oxidative stress also at the seed level (**Fig. 5**). ROS accumulation is the major cause of loss of crop productivity worldwide (Mittler, 2002; Gill et al., 2011). Organelles such as chloroplasts, mitochondria, and peroxisomes, carry highly oxidizing metabolic activities or they represent the subcellular compartments where intensive rates of electron flow take place, thus releasing high amounts of free radical species in the plant cells (Rasmusson et al., 2004; Gill and Tuteja et al., 2010). To protect themselves against these toxic oxygen intermediates, plant cells and organelles employ antioxidant defense systems. The cells are equipped with an excellent antioxidant defense machinery able to detoxify the effects of ROS resulting from a wide range of environmental stresses.

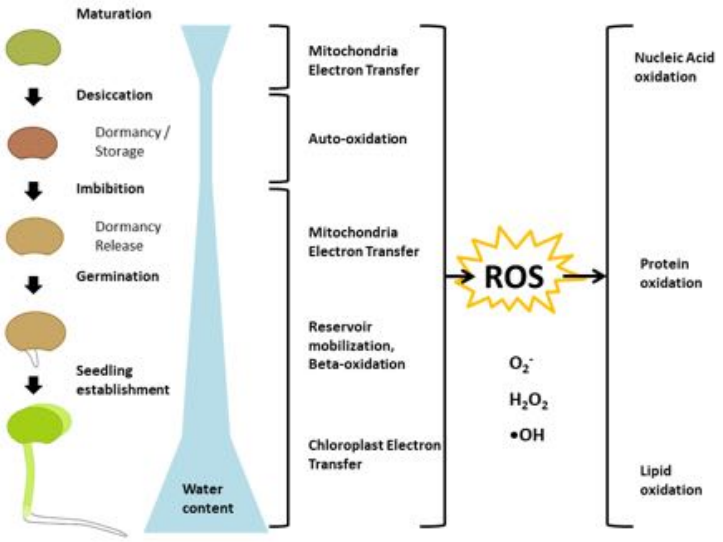


Fig. 6. Timing and mechanisms of ROS production in seeds and their main effects on cell macromolecules (Pagano et al., 2018).

Although ROS play a relevant role as key regulatory molecules essential for cells, they cause severe damage when produced in excess or when the antioxidant machinery does not properly function. Thus, it is of great importance to maintain an equilibrium between ROS production and ROS scavenging to allow appropriate plant development under changing environments.

At the seed level, the antioxidant defense machinery offers protection against damage induced by oxidative stress in order to allow germination. In other words, enzymatic detoxification as well as repair of cell membrane are the main processes required to limit oxidative injury during imbibition and they significantly contribute to delay seed ageing and eventually the loss of seed viability (Tavakol Afshari et al., 2007).

An antioxidant is defined as any substance that delays, prevents or removes oxidative damage to a target molecule. In this definition are included both enzymes and non-enzymatic compounds (Govindaraj et al., 2017). Plant possess very efficient enzymatic and non-enzymatic antioxidant defense systems that can counteract the harmful effects of ROS *in planta* and at the seed level (**Fig. 7**) (Kibinza et al., 2006; Gill et al., 2011). The activity of these antioxidant metabolites and enzymes are responsible for the high levels of seed resistance to oxidative damage as well as for minimizing cell damage (Alscher, 2002; Tabatabaei, 2015). Several studies have established that the induction of the cellular antioxidant machinery is important for protection against various stresses (Tuteja et al., 2007; Khan and Singh, 2008; Gill et al., 2011). The ability of seeds to produce antioxidant compounds considerably differs depending on species and genotype (Mansouri-Far et al., 2015).

Fig. 7. Seed antioxidant defence. Non-enzymatic (ascorbic acid, reduced glutathione, tocopherol, phenolic compounds, alkaloids, carotenoids and non-protein amino acids) and enzymatic components (superoxide dismutase, catalase, ascorbate peroxidase, monodehydroascorbate reductase, glutathione reductase, dehydroascorbate reductase, glutathione peroxidase and glutathione S-transferases) (modified from Hasanuzzaman et al., 2011).

1.4.1. The fundamental role of the antioxidant defense system in seeds

After maturation, orthodox seeds can survive long periods of storage in dehydrated and dormant conditions. During dormancy, and especially at low temperatures, the cytoplasm undergoes changes in terms of physical properties since there is a transition from a metabolically active fluid state to a viscous glassy state in which the mobility of metabolites, macromolecules, and cellular structures is severely restricted by the reduced water content. This reduced metabolic activity limits the ROS production within mitochondria, chloroplasts, and other cellular compartments, thus preventing extensive damage to cellular components before germination occurs (Buitink and Leprince, 2008). Nevertheless, in dry conditions, non-enzymatic sources of oxidative damage are still active, namely lipid peroxidation (Wilson and McDonald, 1986) and the Amadori and Maillard reactions (Murthy and Sun, 2000), thus requiring active detoxification systems to prevent extensive cellular damage and the loss of seed vigor.

The activity of the enzymatic machinery implicated in ROS detoxification is strongly limited during seed dormancy because of the dehydrated glassy state of the cytoplasm in most orthodox seeds (Rajjou et al., 2012). As during the dehydrated state the activity of the antioxidant enzymes is strongly impaired, many orthodox seeds have evolved non-enzymatic systems to scavenge the ROS produced during storage. Indeed, several classes of molecules are accumulated during seed maturation. Polyphenols, like flavonoids, are among the most common and relevant non-enzymatic antioxidants found in the seed coat, endosperm, and embryo, as a consequence of specific developmental signals. Following seed imbibition and consequent metabolism reactivation, the metabolic pathways leading to ROS production, as well as the enzymatic machinery involved in their detoxification, resume their activities (Vranová et al., 2002; Apel et al., 2004). For instance, superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GSR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) are produced in seeds and they act at different levels during germination (Bailly, 2004, Kumar et al., 2015). Protection from stress is expected also during seed maturation and desiccation in order to grant enhanced longevity.

1.4.2. Enzymatic antioxidant defense systems

Enzymatic antioxidants include several types of enzymes generally highly conserved through evolution.

SOD is a metalloprotein found in both prokaryotic and eukaryotic cells (Bandyopadhyay et al.,1999). In conjunction with CAT, SOD acts as an

enzymatic oxidant detoxification system (Mustafa and Lee, 1977). The up-regulation of SOD isoforms is implicated in fighting oxidative stress caused by biotic and abiotic environmental factors and this system plays a critical role in the survival of plants under adverse environmental conditions (Harinasut et al., 2003; Kukreja et al., 2005; Gapińska et al., 2008) Furthermore, it has been reported that priming treatments stimulate the activity of SOD and other ROS scavenging enzymes in cauliflower (Fujikura and Karssen, 1992), sunflower (Bailly et al., 2000) and tomato seeds (Van Pijlenet al., 1995).

CATs are tetrameric eme-containing enzymes, localized in peroxisomes, that promote redox reactions. They catalyze the decomposition of H_2O_2 to water and oxygen and thus protects the cell from oxidative damage caused by H_2O_2 and OH^-(Bandyopadhyay et al., 1999; Garg and Machanda, 2009). CATs contribute to remove lipid peroxidation and protect the mitochondrial components from oxidative damage (Chander and Kapoor, 1990). Decreased CAT activity was associated with ageing, accompanied by an increase in lipid peroxidation and loss of seed vigor and viability in maize (Oliver et al., 1990) and sunflower (Bailly et al., 2002). Stimulation of CAT activity during germination has been reported in seeds of soybean (Gidrol et al., 1994) and maize (Guan and Scandalios, 2002). CAT isozymes have been studied extensively in higher plants, e.g. *Hordeum vulgare* (Azevedo et al., 1998), *Helianthus annuus* cotyledons (Azpilicueta et al., 2007) and *Brassica* spp. (Frugoli et al., 1996).

Peroxidases catalyze the oxidation of a wide range of electron donors, among which H_2O_2 and AA (Ascorbic Acid) in $2H_2O$ and DHA (Dehydro ascorbate) and H_2O_2 and GSH (reduced glutathione) in H_2O and GSSG (two glutathione molecules linked by a disulfide bond) playing an active role in scavenging the endogenous H_2O_2 (Mazumdar et al., 1997). The peroxidase activity was positively associated with the germination percentage in maize (Oliver et al., 1990). In particular, lipid peroxidation is considered as the most damaging process known to occur in every living organism. Lipid peroxidation was shown to produce highly reactive free radical intermediates that resulted in damaged membranes, proteins and nucleic acids, leading to loss of viability in maize (Leprince et al., 1990).

APX is involved in scavenging of H_2O_2 and utilizes ascorbate as the electron donor. The APX family consists of five different isoforms including the thylakoid (tAPX) and glyoxisome membrane (gmAPX) isoforms, as well as chloroplast stromal soluble (sAPX) and cytosolic (cAPX) isoforms (Noctor and Foyer, 1998). APX has a higher affinity for H_2O_2 than CAT and it has a crucial role in the management of ROS under oxidative stress. Indeed,

enhanced expression of APX in plants has been demonstrated during different stress conditions (Zlatev et al., 2006; Khan et al., 2007; Singh et al., 2008). Chen and Arora (2011) demonstrated that osmopriming in spinach (*Spinacia oleracea*) seeds reduces the levels of CAT and SOD activities, increasing the seed germination potential and stress tolerance. Another study revealed the presence of elevated constitutive APX activity in *Brassica juncea* seeds under elevated nichel (Ni) concentrations (Thakur and Sharma, 2015).

Guaiacol peroxidase (GPOX) decomposes indole-3-acetic acid (IAA) in 3-methylene-oxindole and has a role in the biosynthesis of lignin and defense against biotic stresses by removing H_2O_2 (Hinman and Lang, 1965; Ricard and Job, 1974). GPOX preferentially uses aromatic electron donors, such as guaiacol and pyragallol (Asada, 1999). The activity of GPOX varies considerably depending upon plant species and stress conditions (Milone et al., 2003; Koji et al., 2009). GPOX activity was studied in barley seeds irradiated with different doses of γ-ray. It has been shown that both GPOX and CAT increase their activity in the dose range that causes growth stimulation in the seedling (Volkova et al., 2016). Yadu et al., (2017) investigated the role of salicylic acid (SA) and nitric oxide (NO) in improving salinity tolerance in *Pisum sativum* seeds. Both SA and NO serves as signal molecules in plant stress responses and play a crucial role in key regulatory pathways of growth, development and metabolism. SA and NO had significant inducing effects on GPOX, SOD, CAT and APX activities, resulting in a decline of ROS accumulation.

GPXs are a large family of isozymes that use GSH to reduce H_2O_2 and organic and lipid hydroperoxides and help plant cells to fight oxidative stress (Noctor et al., 2002). Some studies were carried out to analyze GPX activity in seeds. The exposure of *Brassica juncea* seeds to high levels of Cu increase the activity of this antioxidant enzyme (Yadav et al., 2018). Mahesh et al., (2017) discovered that eggplant (*Solanum melongena*) seeds primed with SA displayed an increased GPX activity.

Glutathione reductase (GR) is a flavo-protein oxidoreductase involved in the ASH-GSH (ascorbate-glutathione) cycle and playing an essential role in the defense system against ROS by sustaining the reduced status of GSH (Gill and Tuteja, 2010). GR catalyzes the reduction of GSH, a molecule involved in many metabolic regulatory and antioxidant pathways in plants. GR catalyses the NADPH-dependent reduction of the disulphide bond of GSSG (oxidized glutathione), thus maintaining the GSH pool (Reddy and Raghavendra, 2006; Chalapathi and Reddy, 2008). GR and GSH play a crucial role in determining the tolerance of a plant under various stresses (Chalapathi and Reddy, 2008). Sharma and Dubey (2005) noted a significant increase in GR activity in drought-stressed *Oryza sativa* seedlings. Enhanced

activity of GR was observed by Yadav et al. (2018) in *Brassica juncea* seeds treated with high levels of copper (Cu) and exogenous application of the brassinosteroid castasterone. Furthermore, it was found that GR maintained a low level of activity in aged oat (*Avena sativa*) seeds primed with NO. With the application of low NO concentrations to these aged seeds, a protective effect was observed demonstrated by an improvement in seed vigor and increase of H_2O_2 scavenging ability. It is important to underline that aged seeds progressively accumulate H_2O_2, and the resulting oxidative damage causes loss of vigor and an extension of mean germination time (Mao et al., 2018).

Plant glutathione S-transferases (GST) are a large and diverse group of enzymes which catalyze the conjugation of electrophilic xenobiotic substrates with glutathione (GSH). These enzymes are known to act in herbicide detoxification, hormone homeostasis, vacuolar sequestration of anthocyanin, tyrosine metabolism, hydroxyperoxide detoxification, regulation of apoptosis and in plant responses to biotic and abiotic stresses (Dixon et al., 2010). GSTs have the potential to remove cytotoxic or genotoxic compounds, which can react or damage DNA, RNA and proteins (Noctor et al., 2002). In fact, GSTs can reduce peroxides with the help of GSH and produce scavengers of cytotoxic and genotoxic compounds. In *Brassica juncea* seeds, GST displayed high level of activity under Cu stress (Yadav et al., 2018). The same response was found by Yang et al., (2012) in soybean (*Glycine max*) seeds exposed to different Cd concentrations. Furthermore, GST activity showed a pronounced exposure time-dependent response.

MDHAR is a flavin adenin dinucleotide (FAD) enzyme found in plants with chloroplastic and cytosolic isoforms. MDHAR exhibits a high specificity for monodehydroascorbate (MDHA) as the electron acceptor, preferring NADH rather than NADPH as the electron donor. Along with APX, MDHAR is also located in peroxisomes and mitochondria, where it scavenges H_2O_2 (del Río et al., 2002). It was observed that MDHAR enzymatic activity decreases under Cu-mediated stress in *Brassica juncea* seeds (Yadav et al., 2018). Moreover, the level of enzyme activity was found decreased in artificially aged rice seeds (Yin et al., 2014). According to the current hypothesis, an overall depression of the antioxidant machinery composed by CAT, APX and MDHAR could be responsible for ROS accumulation in artificially aged seeds, leading to a loss of vigor (Yin et al., 2014).

DHAR regenerates ASH from the oxidized state and regulates the redox state of the cellular ASH pool. This function is crucial for ensuring tolerance to abiotic stresses and limiting ROS accumulation (Gill and Tuteja, 2010). High copper levels applied to *B. juncea* seeds triggered a high DHAR activity

(Yadav et al., 2018). Garnczarska et al., (2009) investigated the ability of yellow lupine (*Lupinus luteus*) fresh seeds to tolerate desiccation and found that the DHAR activity increases before the seed reaches physiological maturity and decreases thereafter.

1.4.3. Non-enzymatic antioxidant defense systems

Non-enzymatic antioxidants include ascorbic acid (vitamin C), carotenoids, glutathione (GSH), α-tocopherols (vitamin E), flavonoids and proline.

Ascorbic acid is the most abundant, powerful and water-soluble antioxidant able to prevent or minimize the ROS-dependent damage in plants (Smirnoff, 2005). It plays a key role in detoxification of O_2^- radical **(**Foyer et al., 1991) and helps in the regeneration of oxidized vitamin C at the level of membranes, hence it is required to maintain membrane structure. Ascorbic acid content increased progressively during seed development in French bean and desiccation remarkably reduced the levels of this compound in developed and dehydrated seeds (Arrigoni et al., 1992). A positive association between germination and ascorbic acid content has been reported in French bean (Innocenti et al., 1994), wheat (De Gara et al., 1997), pea (Pallanca and Smirnoff, 1999) and maize (De Gara et al., 2000).

Other mechanisms, able to remove excess excitation energy in photosynthetic membranes, involve the isoprenoid compounds. In all photosynthetic organisms, the carotenoids β-carotene, zeaxanthin, and tocopherols, act as photoprotectants, either by dissipating excess excitation energy (e.g. heat) or by scavenging ROS and suppressing lipid peroxidation (Gill and Tuteja, 2010). Carotenoids are pigments found in plants and microorganisms, recognized as efficient quenchers of singlet oxygen. There are over 600 carotenoids occurring in nature. They directly scavenge free radicals and protect cells and tissues from oxidative damage by inhibiting lipid peroxidation. Indeed, low concentrations of β-carotene were reported to effectively protect membrane lipids from oxidative reactions leading to peroxidation (Krinsky, 1992). In *H. vulgare* seedlings, the carotenoids content decreases under Cd-stress. Exposure to cadmium caused a reduction of plant length, biomass, and leaf pigment content (Demirevska-Kepova et al., 2006). The *γ*-irradiated seeds of two red pepper cultivars displayed different response related to the carotenoid activity. The effects of irradiation on the enzymatic activity measured was the opposition between the two cultivars. This behavior has been hypothesized to be cultivar-dependent (Kim et al., 2014).

Glutathione (GSH) is one of the major antioxidants in the soluble fraction of cells involved in the protection of membranes from oxidative attack

(Moron et al., 1979). It occurs abundantly in reduced form in plant tissues within all cell compartments like cytosol, endoplasmic reticulum, vacuole, mitochondria, chloroplasts, peroxisomes as well as in apoplast (Mittler et al., 1992; Jimenez et al., 1998). GSH plays a role in free radical scavenging in aged sunflower (De Paula et al., 1996) and watermelon seeds (Hsu and Sung, 1997). This antioxidant molecule is required in several physiological processes, including regulation of sulfate transport, signal transduction, conjugation of metabolites, detoxification of xenobiotics (Xiang et al., 2001), and the expression of stress-responsive genes (Mullineaux and Rausch, 2005).

Tocopherols are a lipid soluble antioxidant and are considered as potential scavengers of ROS and lipid radicals (Hollander-Czytko et al., 2005). They represent an integral component of biomembranes and helps as effective 'radical chain-breaking' molecules (Marcus et al., 1998). Tocopherols are localized in the thylakoid membrane of chloroplasts. In plants, four isomers (α-, β-, γ-, and δ-tocopherol) were found and α-tocopherol showed the highest antioxidant activity due to the presence of three methyl groups in its molecular structure (Kamal-Eldin and Appelqvist, 1996). Addition of vitamin E has improved the germinability of parsley (*Petroselinum crispum*), onion (*Allium cepa*) and pepper (*Capsicum annuum*) seeds (Woodstock et al., 1983) whereas loss of tocopherol is one of the manifestations of seed deterioration. Depletion of tocopherol in soybean (*Glycine max*) seeds enhanced the loss of desiccation tolerance. Furthermore, the lower tocopherol content detected following ageing suggested that this molecule is utilized for protecting the seed against free radical damage (Seneratna et al., 1988).

Flavonoids are commonly found in leaves, floral parts and pollens. They usually accumulate in the plant vacuole as glycosides, but they also occur as exudates on the surface of leaves and other aerial plant parts (Vierstra et al., 1982). Flavonoids are suggested to have many functions among which flowers, fruits and seed pigmentation, protection against UV light, defense against phytopathogens, plant fertility and pollen germination, or acting as signal molecules in plant-microbe interactions (Olsen et al., 2010).

A peculiar non-enzymatic antioxidant is proline, an amino acid fundamental to counteract the effects of ROS in microbes, animals and plants and a potential inhibitor of programmed cell death (PCD) (Chen and Dickman et al., 2005). A dramatic accumulation of proline occurs in presence of salt, drought and metal stress, and this may be due to increased synthesis or decreased degradation of the amino acid. Free proline has been proposed to act as an osmoprotector, a protein stabilizer, a metal chelator, an inhibitor of LPO and OH• and O_2 scavenger (Ashraf and Foolad, 2007; Trovato et al., 2008; Smirnoff and Cumbes, 1989). In an interesting study, Chen and

Dickman (2005) reported that addition of proline to *DARas* (oncogenic fungal *Ras* gene) mutant cells of *Colletotrichum trifolii*, a fungal pathogen of alfalfa (*Medicago sativa*), effectively quenched ROS levels and prevented cell death. It was suggested that the ability of proline to scavenge ROS and to inhibit ROS-mediated apoptosis can be an important function in response to cellular stress. An accumulation of proline was observed in safflower (*Carthamus tinctorius*) seeds subjected to salt stress which means that high salt tolerance could be attributed also to the proline action (Siddiqui, 2011). Moreover, an increase of proline content may represent a response to heavy-metal toxicity in soybean seeds (Yang et al., 2012).

1.5. DNA damage and DNA repair in plants

Plants are highly vulnerable to abiotic stresses occurring ubiquitously in the environment which results in oxidative injury at the cellular level and, ultimately, in genotoxic damage targeting the double helix structure (Tuteja et al., 2009; Manova and Gruszka, 2015). The various stresses are essentially considered as a critical factor which impairs the plant fitness and productivity by affecting genome stability (Tuteja et al., 2009; Roldan-Arjona and Ariza, 2009; Balestrazzi et al., 2011b). Plants have evolved a complex network of mechanisms for DNA damage detection and repair dedicated to ensure genome stability through removal of DNA lesions and reconstitution of the original genetic information (Bray and West, 2005; Yoshiyama et al., 2013). The ability of plants to maintain genome integrity was shown to decrease with plant age mainly due to a reduction in the efficiency and relative contribution of specific DNA repair pathways (Golubov et al., 2010). The choice of a repair pathway and its mode of action depends primarily on the cell type and its proliferation level/cell cycle stage, as well as on the type of the lesion and its impact in the genomic context (Britt, 1999).

Because of their sessile lifestyle, plants need strictly controlled but flexible DNA repair mechanisms responsive to the changing environment. Common external factors can trigger and modulate the efficiency of specific DNA repair pathways, such as recombination or photo-repair (Li et al., 2002; Boyko et al., 2005; Chang et al., 2008). It is important to underline that a major source of DNA lesions is the intracellular metabolism that contributes to increase significantly the concentration of free radicals in the environment surrounding the DNA (Sharma et al., 2012). In recent years, significant advancements have been made in the molecular characterization of the repair pathways and genes mediating these processes in both dicot and monocot crop plants (Balestrazzi et al., 2011a, 2011b; Yoshiyama et al., 2013; Manova and Gruszka, 2015).

The DNA lesions are divided into two main categories: single- and double-stranded. The first category is comprised of lesions disturbing only one DNA strand, such as oxidized or alkylated base damage, deamination, abasic (apurinic and/or apyrimidinic, AP) sites, DNA adducts, intra-strand cross-links, DNA photoproducts and single-strand DNA breaks (SSBs) (Tuteja et al., 2009). The second category includes lesions affecting both DNA strands, such as inter-strand cross-links and double-strand DNA breaks (DSBs), the latter being the most severe type of DNA damage in the eukaryotic genome. All DNA-associated processes involved in the transmission, expression and maintenance of genetic information have the potential to cause SSBs or DSBs in DNA (Bessho, 2003; Montecucco and Biamonti, 2013). Perception of genotoxic stress and the activation of signal transduction mechanisms lead to cell cycle arrest and this allows the regulated expression of DNA repair genes. Several genotoxic agents can simultaneously cause both DNA damage within the nucleus and oxidative stress outside the nuclear compartment, leading to complex molecular activity which might ultimately result in PCD (Balestrazzi et al., 2012).

The main DNA repair pathways hereby briefly described are Base Excision Repair (BER), Nucleotide Excision Repair (NER), Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR).

1.5.1. Excision repair

The excision repair is divided in base (BER) and nucleotide (NER) excision repair (**Fig. 8**).

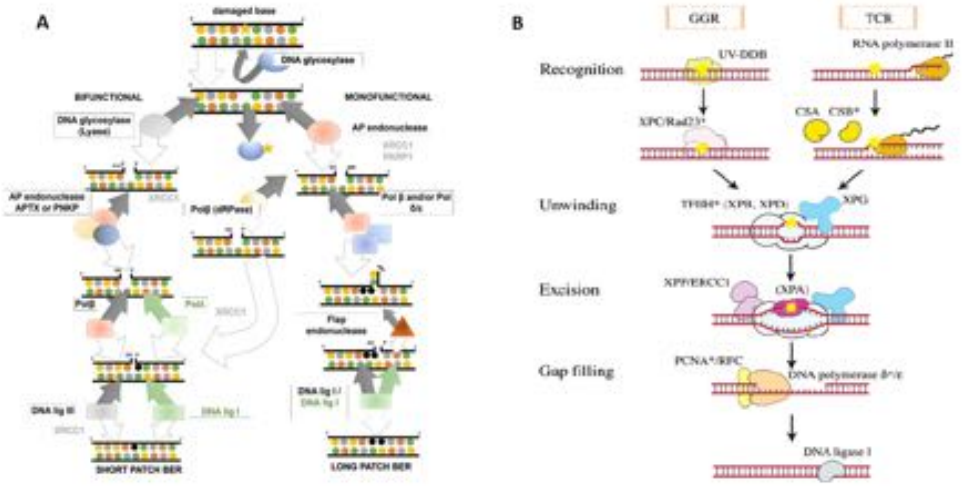


Fig. 8. Excision repair in plants. **(A)** Schematic representation of BER pathway (de Medeiros et al., 2015). **(B)** Schematic representation of NER pathway (Kimura et al., 2006).

BER is involved in the removal of oxidative base damage, alkylated, deaminated, abasic (apurinic and/or apyrimidinic, AP) sites and single-strand breaks (SSBs) (**Fig. 8A**). It requires the activity of DNA glycosylases, enzymes able to catalyze the hydrolysis of the N-glycosidic bond of the damaged deoxynucleoside, causing the excision of the damaged bases (Roldan-Arjona and Ariza, 2009). The resulting AP sites are then processed by AP endonucleases which cleave at the 5' end of the AP site, releasing 3'-OH and 5'- deoxyribose- 5'-phosphate termini. The gap is subsequently filled by inserting a single nucleotide (Short-Patch repair, SP, carried out by the DNA polymerase β) or several nucleotides (Long-Patch repair, LP, performed by replicative DNA polymerases δ or ε) and finally the ends are rejoined by DNA Ligase III (SP-BER) and DNA Ligase I (LP-BER). Poly(ADP-Ribose)Polymerase 1 (PARP-1) is a nuclear protein which interacts with factors involved in the modulation of chromatin architecture and cell recovery from DNA damage, is among the BER components responsive to stresses (Douchet-Chablaud et al., 2001). In *Arabidopsis*, both *AtPARP1* and *AtPARP2* genes are stress responsive and the preferential accumulation of the *AtPARP2* transcript in response to heavy-metal stress suggests for specific roles played in plants by each gene (Douchet-Chablaud et al., 2001). Other key players in BER, the enzymes OGG1 (8-oxoguanine DNA glycosylase), FPG (formamidopyrimidine-DNA glycosylase) and TDPs (tyrosyl-DNA phosphodiesterases) are described in detail in Chapter 1.5.2.1..

The NER mechanism is involved in the enzymatic removal of cyclo-butane pyrimidine dimers (CPDs), phospho-products and other bulky lesions that cause helix distortions (Liu et al., 2010). NER is one of the most versatile DNA repair systems composed of two distinct sub-pathways acting on transcriptionally active or inactive DNA, respectively (Cordoba-Canero et al., 2009) (**Fig. 8B**). The Transcription Coupled-Nucleotide Excision Repair (TC-NER) sub-pathway removes damage from the transcribed strands of active genes and it is triggered upon blockage of RNA polymerase II (RNAPII) at a DNA damaged site while the Global Genome-Nucleotide Excision Repair (GG-NER) sub-pathway repairs lesions throughout the genome. NER is triggered following damage recognition, and the process starts with the incision of the damaged DNA strand carried on each side of the lesion and release of the oligonucleotide that harbors the lesion. Subsequently, repair synthesis occurs and ligation seals the repair patch. TC-

NER is activated by stalling of RNAPII during transcription while in GG-NER, the lesion is detected by the UV-DDB (UV-Damaged DNA-Binding) protein complex and by a complex containing the XPC (Xeroderma Pigmentosum group C), RAD23 and centrin proteins (Cordoba-Canero et al., 2009). Subsequently, both TC-NER and GG-NER require TFIIH (Transcription Elongation Factor-II H) to unwind the DNA helix around the damaged site. Conserved helicase motifs and DNA unwinding activity are associated with the XPD and XPB subunits of the TFIIH complex (Tuteja et al., 2009). XPD and XPB bind and extend the single-stranded DNA around the damaged site. Other proteins stabilize the repair complex and properly orient the structure-specific endonucleases XPF (Xeroderma Pigmentosum group F), XPG (Xeroderma Pigmentosum group G) and ERCC (Excision Repair Cross-Complementing) Protein 1. Following excision of the lesion, the oligonucleotide containing the damaged site is released, polymerase δ or ε-mediated DNA repair synthesis and ligation occur, thus completing the process.

1.5.2. Double strand break repair

The main mechanisms involved in the repair of DSBs in plants are Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR), shown schematically in **Fig. 9**.

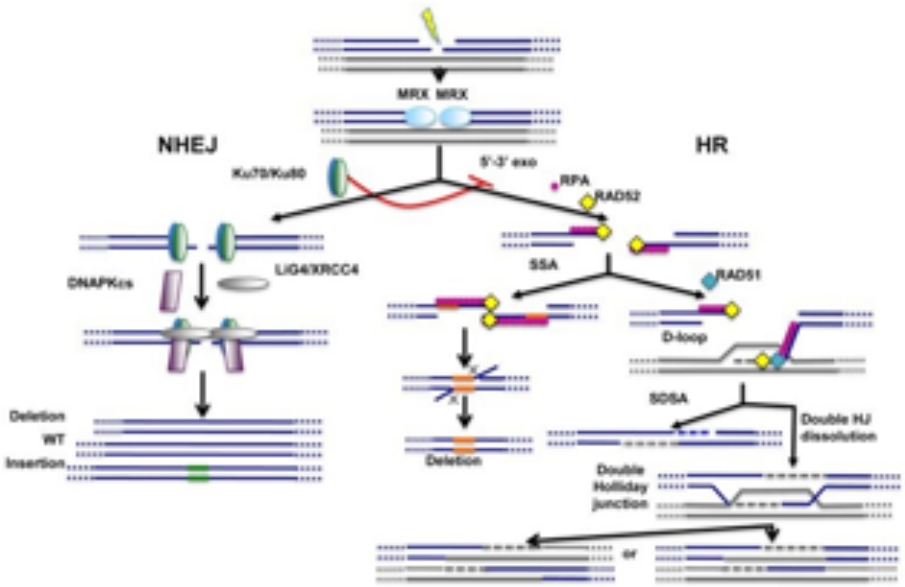


Fig. 9. Main pathways of DNA repair. Non-homologous end-joining (NHEJ) and homologous recombination (HR) pathways act competitively to repair DNA double-strand breaks (DSBs) (Decottignies, 2013). RPA, Replication Protein A. SSA, Single-Strand Annealing. LIG4/XRCC4, Ligase4/X-ray Repair Cross-Complementing protein 4. DNA PKcs, DNA-dependent protein kinase. Ku70/Ku80, Ku heterodimer. SSA, Synthesis-dependent Strand Annealing.

NHEJ represents the major pathway for the repair of two-ended DSBs throughout the cell cycle in most plant tissues (West et al., 2002; Groth et al., 2012). Direct or two-ended DSBs, characterized by the presence of two free ends are considered as the most critical DNA lesions leading to chromosomal rearrangements. In the NHEJ pathway, this type of lesion is repaired by end-to-end-joining, largely independent of DNA sequence. The well-characterized and highly conserved canonical NHEJ pathway is mediated by the Ku70-Ku80 complex, which binds and stabilizes broken DNA ends, whilst ligation is performed by the Lig4-XRCC4 complex (Friesner and Britt, 2003; Gallego et al., 2003). DNA ends arising from damage typically require enzymatic processing to form suitable substrates for end-joining carried by DNA ligases, and processing in plants may involve the MRE11-RAD50-NBS1 complex (Heacock et al., 2004). In recent years, genetic analyses have revealed that higher plants also have the capacity to repair DSBs by a number of alternative Ku-independent NHEJ mechanisms (Charbonnel et al., 2011; Charbonnel et al., 2010).

HR is involved in the repair of more complex and persisting DSBs produced in heterochromatin regions of G2-phase cells (Krejci et al., 2012). HR uses an intact copy of the damaged region as a template, and this allows error-free repair. A homologous template is used for repair in a pathway mediated by the Rad51 recombinase protein in conjunction with accessory proteins (Sancar et al., 2004). Rad51 facilitates the formation of a physical connection between the invading DNA substrate and homologous duplex DNA, leading to the formation of heteroduplex DNA. Although there is predominant requirement for Rad51, DSBs can be repaired by alternative pathways, e.g. the Single-Strand Annealing (SSA) pattern. It has been reported that ssDNA sequences generated during DSBs processing might contain stretches of homology at both sides of the DSB and that these sequences can be annealed and ligated. Modulation of the HR process also requires the interplay between Rad51 and the ssDNA-binding factor RPA complex (Replication Protein A), since the amount of RPA-ssDNA is sensed by checkpoint kinases to elicit cell cycle arrest and allow time for repair (Choi et al., 2010).

1.5.3. Role of DNA repair in the context of seed germination: the crucial balance between genotoxic stress, repair and seed quality

Oxidative stress, resulting from the uncontrolled ROS production, is a major determinant of mutagenesis and cellular ageing and is the principal cause of cellular deterioration in seeds (McDonald, 1999; Bailly, 2004; Kranner et al., 2010). DNA damage accumulates in seeds even in the absence of external stresses because of the inherent instability of DNA in the cellular environment (Lindahl, 1993). Moreover, the germination of seed is more sensitive to adverse environmental conditions which negatively impact on final yields through reduced emergence and slower and weaker seedling establishment (Rajjou et al., 2012).

In seed, DNA damage arises during storage and imbibition, thus reducing seed and seedling vigor (Waterworth et al., 2015). DNA damage must be repaired early during imbibition prior to the initiation of cell division, to maintain germination potential and minimize mutagenesis in subsequent seedling development (Waterworth et al., 2016). The requirement for extended repair of accumulated damage underlies the delay of germination characteristic to low vigor seed (Waterworth et al., 2010). In particular, seed aging is associated with progressive accumulation of DNA damage in the embryo, including increased levels of base loss, generating abasic sites, base modification, SSBs and DSBs (Córdoba-Cañero et al., 2014). DNA is naturally unstable in aqueous solution, although the reduced water content of orthodox seeds provides some protection against water-derived radicles. The lowered moisture content of the desiccated seed reduces the rate of genome damage but in the absence of repair, lesions accumulate over time (Walters et al., 2006). Apurinic sites were detected at a frequency of 3.8×10^{-5} per nucleotide in the quiescent embryo, and levels further increased 4-fold upon imbibition (Dandoy et al., 1987). High levels of oxidative DNA damage accumulated in seeds require effective repair pathways during imbibition.

1.5.3.1. BER is a key player for seed quality

Bailly (2004) proposed a model in which substantial levels of oxidative genome damage are generated upon seed imbibition, further enhancing the damage accumulated during quiescence. Evidence for base damage induced during seed imbibition was provided by an analysis of 7,8-dihydro-8-oxoguanine (8-oxoG), a major oxidative form of base damage. Differently from animals, plants possess both the OGG1 and FPG enzymes which are able to remove oxidized bases (Macovei et al., 2011a; Córdoba-Cañero et al., 2014). The plant OGG1 and FPG enzymes were isolated and characterized for the first time in *A. thaliana*. The *At*OGG1 enzyme predominantly acts on the 8-oxo-dG while the DNA apurinic sites represent the main target of

*At*FPG. Accelerated seed ageing at increased temperature and humidity increased 8-oxoG levels in dry seeds of wild-type Arabidopsis and levels were further increased at 24 h of imbibition (Chen et al., 2012); however, the 8-oxoG levels were greatly reduced in transgenic lines overexpressing *AtOGG1*, most markedly after 24 h imbibition, consistent with increased DNA repair capacity in these plants. Additionally, transgenic *Arabidopsis* seeds overexpressing *AtOGG1* exhibited around 50% lower levels of 8-oxoG in quiescent seeds compared with wild-type lines. Elevated levels of *AtOGG1* expression correlated with a significant increase in seed resilience to controlled deterioration and improvement in germination vigor under abiotic stresses (e.g. heat, salt, and oxidative stress induced by methyl viologen). This study illustrates that base damage can limit germination along with the potential for genetic enhancement of seed vigor through modification of DNA repair enzyme levels or activities (Chen et al., 2012).

The possible roles played by the *MtOGG1* and *MtFPG* genes in *M. truncatula* have been investigated by Macovei et al. (2011a). Expression analysis of these genes was carried out during *M. truncatula* seed imbibition, in presence/absence of an osmotic agent, a process that accumulates consistent levels of 8-oxo-dG (Balestrazzi et al., 2011a). The up-regulation of both genes during seed rehydration was indicative of their involvement in DNA repair mechanisms, under physiological conditions. When imbibition was carried out with an osmotic agent, at conditions that delay water up-take and reduce germination efficiency, a delay in the up-regulation of both *MtOGG1* and *MtFPG* genes was observed. Furthermore, the up-regulation peaked at a time point during rehydration that corresponded to the highest accumulation of 8-oxo-dG (Balestrazzi et al., 2011a).

An intriguing BER component is the enzyme tyrosyl-DNA phosphodiesterase (Tdp1) extensively investigated in animal cells, due to the role of this enzyme in the repair of topoisomerase I-DNA covalent lesions (Yang et al., 1996). Macovei et al. (2010) demonstrated the presence of a small *Tdp1* gene family in plants composed by *Tdp1α* and *Tdp1β* genes encoding the Tdp1α and β isoforms. These genes were significantly up-regulated during *M. truncatula* seed imbibition, although with a temporal shift in transcript accumulation (Macovei et al., 2010). The *MtTdp1α* and *MtTdp1β* genes were up-regulated in seeds under osmopriming conditions, showing a temporal profile that overlapped DNA damage accumulation (Balestrazzi et al., 2011a). The expression profiles of the *MtTdp1* genes were evaluated also in plantlets grown *in vitro* using copper and PEG as stress agents. Both *Tdp1* genes were significantly up-regulated in response to heavy-metal and osmotic stress, suggesting for a requirement of the *Tdp1* function under stress conditions. From this point of view, the response of

MtTdp1 genes to stress seems to be in agreement with the literature available on the animal *Tdp1* gene (Lu et al., 2004). Lebedeva et al., (2011) discovered a novel role played by Tdp1: the enzyme contributes to the removal of AP sites, the key BER intermediates (Barnes and Lindhal, 2004).

1.5.3.2. NER mechanism in seeds

The NER pathway is active in imbibed seeds and it is required for the maintenance of seed viability (Costa et al., 2001). This was demonstrated by a mutation in *Xeroderma pigmentosum* group B protein (XPB1), resulting in reduced germination relative to wild-type seeds after treatment with hypochlorite, an agent that induces oxidative DNA damage. The expression of several NER genes increased at the end of the seed development process in *Phaseolus vulgaris*, as reported by Parreira et al. (2018), a finding consistent with the presence of NER activity in imbibing seeds. The data obtained from Parreira et al. (2018) show a higher number of NER-related genes differentially expressed, compared with the number of genes from other repair mechanisms, throughout seed development. In particular, during the desiccation stage the main NER genes up-regulated were: *RAD23* (*RADIATION SENSITIVE 23*), *UVH3* (*DNA REPAIR PROTEIN UVH3*) and *UVR1* (*UVB-RESISTANCE 1*), *RAD1* and *UVH1* (Restriction Endonuclease type II-like superfamily protein), *XPB1* (*XERODERMA PIGMENTOSUM COMPLEMENTATION GROUP B1*) and *ERCC (DNA EXCISION REPAIR PROTEIN ERCC-1)*, *UVR7* (Parreira et al., 2018).

Interesting clues are provided by the literature on TC-NER, as in the case of TFIIS (Transcription Elongation Factor II-S) which stimulates RNAPII to transcribe DNA regions containing oxidative lesions. Grasser et al. (2009) showed that the *TFIIS* gene from *A. thaliana* is involved in the regulation of seed dormancy. When the expression profiles of the *MtTFIIS* gene were investigated in *M. truncatula* seeds during imbibition, a significant up-regulation was observed, highlighting the possible involvement of *TFIIS* in the seed repair response (Macovei et al., 2011).

1.5.3.3. NHEJ pathway in seeds

Riha et al. (2002) have established an *Arabidopsis* plant model defective in NHEJ, harboring a mutated version of the Ku70 protein. Differently from what observed with seedlings, the mutant *Ku70* seeds were hypersensitive to methyl methanesulfonate (MMS), a monofunctional alkylating agent that produces abasic lesions and single-strand nicks. The mutant *Ku70* seeds were characterized by a dramatic reduction of germination, suggesting the requirement of NHEJ during early plant development. Although germination of Ku70-deficient seeds was unaffected under normal conditions, the

additional damage caused by MMS could not be repaired in the absence of Ku70 (Riha et al., 2002).

DNA ligase enzymes catalyze the final DNA end-joining step in almost all DNA repair pathways and Lig4 functions in the canonical NHEJ pathway. Plants also possess an additional unique DNA ligase termed Lig6, which has an N-terminal motif shared with mammalian Artemis, a protein responsible for processing hairpin structures at DNA ends (Pannicke et al., 2004; Waterworth et al., 2010). *Arabidopsis lig6*, *lig4*, and *lig4 lig6* mutant seeds displayed hypersensitivity to accelerated ageing, establishing a genetic link between DSBs and seed longevity. In particular, the mutant *lig6* seeds showed reduced vigor when germinated under cold stress or oxidative stresses (Waterworth et al., 2010). The additive nature of the *lig4* and *lig6* mutations establishes distinct roles for each ligase in seed longevity and germination. A QTL for seed longevity was subsequently identified to co-localize with a chromosomal region containing *Lig4* (Nguyen et al., 2012).

1.5.3.4. HR implication in seed germination

Like NHEJ, the HR-mediated repair of DSBs is also important in seeds. This was certified by analysing the γ-irradiated maize *rad51* mutants which displayed delayed germination and high seedling mortality compared to wild-type lines (Li et al., 2007). This result is consistent with the studies conducted in *A. thaliana* which demonstrated that Rad51 plays a role in seed physiology and *rad51C* gene is essential for HR; the lack of this specific function compromises seed production (Abe et al., 2005). HR may have only a minor role in early seed imbibition, because cells in the quiescent embryo are typically found in G_1 phase. However, HR may play a more consistent role during DNA replication that occurs later in germination (Barroco et al., 2005; Masubelele et al., 2005).

1.6. Genome Editing: the tool of the future

1.6.1. A general overview on genome editing

Plant biotechnology has entered in a new era in regard to methods that can replace conventional mutagenesis approaches such as EMS (Ethyl Methane Sulfonate) and γ-radiation (Hajiahmadi et al., 2019). In this context, genome editing has become a powerful strategy to accelerate crop improvement. Differently from traditional genetic engineering, where genes are inserted randomly into the genome, this technique leverages the site-specific nucleases (SSNs) combined with DNA recognition domains to insert specific modification at predefined genomic sites (Bogdanove and Voytas, 2011). Genome editing techniques allow to introduce mutations (insertions,

deletions, bases substitution) or insert/substitute wide portions of DNA at a targeted site (**Fig. 10**). These techniques have extremely important implications for human health, agriculture and environment. Consequently, it has an enormous potential given by the high accuracy in recognizing specific DNA sequences. The power of this innovative technique concerns many areas of basic and applied research, ranging from the agri-food sector to the biomedical field (Hsu et al., 2014). Moreover, the discovery of CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) gene editing system (Jinek et al., 2012) has revolutionized the research in both animal and plant biology.

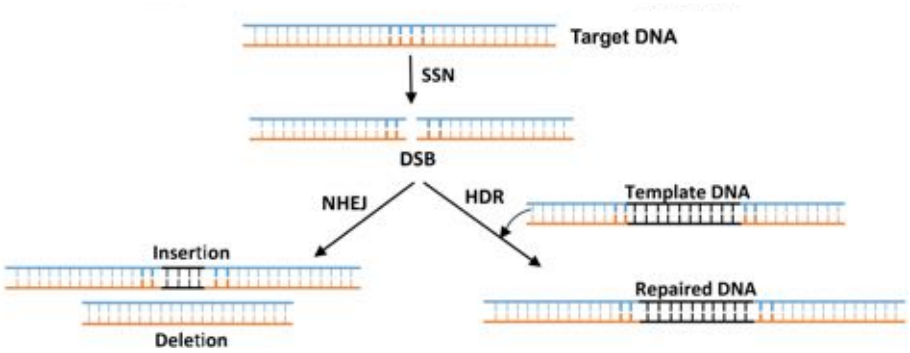


Fig. 10. Schematic representation of site-specific nuclease (SSN)-induced genome editing. The DSBs introduced by SSN complexes stimulates the endogenous NHEJ and HR repair machineries, introducing targeted insertion/deletion (Sedeek et al., 2019).

Thanks to genome editing approaches it is possible to accelerate basic research using model organisms for the study of genes of interest and related mutations. By modifying the genome of an organism through targeted insertions or deletions it is possible to evaluate how these mutations influence the phenotype (Liu and Moschou, 2018). This allows a more in-depth knowledge of gene functions and relative regulatory mechanisms. By modifying targeted cellular metabolic pathways, it is possible to develop new varieties of crops characterized, for instance, by enhanced tolerance to biotic and abiotic stresses (Tebas et al., 2014; Baltes and Voytas, 2016; Shih et al., 2016; Li et al., 2018; Sedeek et al., 2019; Debbarma et al., 2019).

1.6.2. The versatility of the CRISPR/Cas9 tool

Among the genome editing tools, the CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats) system is gaining the highest interest, as the most user-friendly, highly efficient and simple technique (Belhaj et al.,

2013; Barakate and Stepens, 2016; Hajiahmadi et al., 2019). Unlike ZFNs (Zinc Finger Nucleases) and TALENs (Transcription Activator-Like Effector Nucleases) which requires long recognition sequences and difficult cloning procedures, the design of the CRISPR constructs is much more facilitated as it involves the use of a single guide RNA (sgRNA) of about 20 nucleotides complementary to the DNA stretch within the target gene. The acronym CRISPR (Jansen et al., 2002) refers to tandem repeats flanked by non-repetitive DNA stretches that were first observed in the downstream *iap* (isozyme alkaline phosphatase) genes in *E. coli* (Ishino et al., 1987). In 2005, these non-repetitive sequences were found to be homologous to foreign DNA sequences derived from plasmids and phages. Subsequently, the mechanism of homology-dependent cleavage was explored for genome editing and the technology of CRISPR/Cas9 cleavage became a promising genome editing tool (Mojica et al., 2005; Liu et al., 2017).

According to its structure and function, the Cas9 nuclease can be divided into two parts (**Fig. 11**): *(i)* the recognition part interacts with the sgRNA and target DNA; *(ii)* the nuclease part has two nuclease domains (HNH and RuvC) and a PAM (protospacer adjacent motif) interacting domain (Ma et al., 2016). The sgRNA is produced by joining the crRNA and the transRNA with the artificial tetraloop (Jinek et al., 2012). The seed sequence of the sgRNA is essential for the specificity of Cas9. The Cas9 nuclease and the sgRNA form a dual ribonuclease complex capable of binding the complementary strand of the target site introducing a DSB 3-4 bases upstream of the PAM. The Cas9 nuclease is composed of two domains, the RuvC-like (an endonuclease domain named for an *E. coli* protein involved in DNA repair domains and the HNH (an endonuclease domain named for characteristic histidine and asparagine residues) domain, each cutting one DNA strand. Implementing the CRISPR technology requires few relatively simple steps, e.g. *(i)* identification of the PAM sequence in the target gene, *(ii)* synthesis of a sgRNA, *(iii)* cloning the sgRNA into a suitable binary vector, *(iv)* introduction into host cell lines transformation followed by *(v)* screening, and *(vi)* validation of edited lines (Jaganthan et al., 2018).

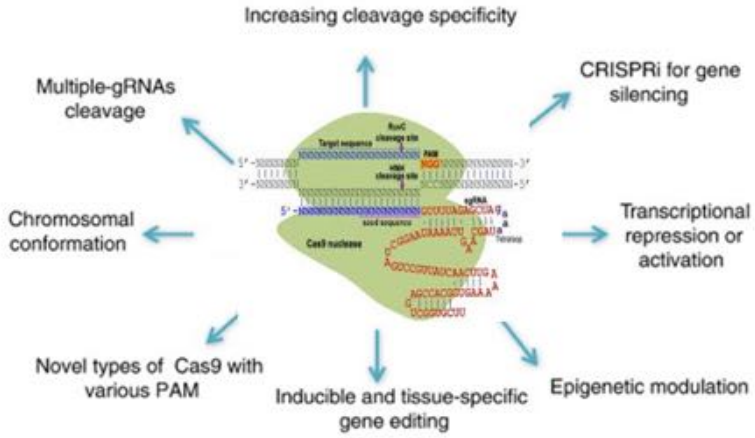


Fig. 11. CRISPR/Cas9 system and its applications (modified from Ma et al., 2016 and Tycko et al., 2017).

The CRISPR/Cas9 system is highly versatile and can be used for different genome editing applications (Tycko et al., 2017) (**Fig. 11**). The standard approach is to perform gene knockouts or DNA sequences substitutions using the existing information on allele sequences. In addition, it can be utilized for transcriptional regulation using a construct consisting of an sgRNA (single guided RNA) linked to a dCas9 (deadCas9 or deactivated Cas9, with inactivated cutting domains). Sequences of transcription regulators can be merged into the dCas9 sequence, such as activators (CRISPRa) or inhibitor (CRISPRi). The sgRNA-dCas9-CRISPRa/i system recognizes and binds to a specific DNA sequence and through activators/inhibitors it can regulate the transcription in a precise and stable way, without inserting cuts into the genome. When dCas9 is fused with a repressor is possible to highly inhibit the transcription of endogenous genes (Gilbert et al., 2013). Furthermore, this system can regulate the transcription of several sequences simultaneously through the use of multiple sgRNAs (Zalatan et al., 2015). Another possible use addresses epigenomic editing. This approach allows to modify histones by modulating the methylation status of the genome. This permits the activation or repression of specific genes in a transient or stable manner (Hilton et al., 2015). Instead, the base editing system allows to insert point mutations at a target site without cutting the DNA sequence and limiting the onset of non-specific mutations (Komor et al., 2016). A further application of the CRISPR/Cas9 technique concerns the visualization of specific loci on chromosomes (imaging) thanks to the fusion of dCas9 with fluorescent molecules. This allows visualization of target sequences in *in vivo* (Chen et al., 2013).

The latest application of the CRISPR/Cas9 system is RNA targeting. RNA is essential to make proteins and being able to target RNA would allow researchers to answer many important questions about RNA biology (Strutt et al., 2018). In the last years, several studies demonstrated that there is the possibility that certain Cas enzymes might have intrinsic RNA-guided RNA cleavage activity. This analysis revealed that certain types (IIA and IIC) of Cas can bind and cleavage single-stranded RNA sequences with no requirement for a PAM sequence (Strutt et al., 2018; Liu et al., 2016b; Batra et al., 2017). Furthermore, they found that this activity can inhibit gene expression. Shmakov et al., (2015) demonstrate the presence of another type of Cas, Cas13, able to recognize and cut RNA sequences instead of DNA sequences. This adaptive defense mechanism is supposed to be used by bacteria to eliminate RNA phages or to eliminate specific phage with DNA sequences. Its use would therefore allow it to act not only on the genome, but also directly on the transcriptome (Abudayyeh et al., 2016). The same authors (Abudayyeh et al., 2017) identified Cas13a from *Leptotrichia wadei* (LwaCas13a) as the endonuclease most effective in mammalian and plants cells that can be heterologously expressed for targeted knockdown of either reporter or endogenous transcript. This CRISPR/Cas application is highly important in agriculture, for instance for plant protection against viruses (Zhang et al., 2018). Zhan et al., (2019) demonstrate that in potato (*Solanum tuberosum*) a CRISPR/Cas13a/sgRNA constructs was able to suppress the potato virus Y (PVY) accumulation and disease symptoms. The sgRNAs were designed against conserved coding regions of three different PVY strains. In plant cells, the action of CRISPR/Cas13-RNA targeting is comparable to RNA interference, while having the advantage of reduced off-target effects (Abudayyeh et al., 2017).

1.6.3. CRISPR/Cas9 in the context of agricultural research

One of the major challenges of research in agriculture concerns the need to increase productivity and quality of cultivated species. Within this context, genome editing represents effective and precise alternative tools to modify the plants genome and improve crop quality. Hence, the use of the CRISPR/Cas system in plants of agricultural and economic interest have already allowed to modify desired characteristics. Although, unlike the U.S.A. where most of these approaches are not subjected to GM regulatory issues, in Europe the situation is more complex as genome edited crops are still considered GM (Wolt and Wolf, 2018; Faure and Napier, 2018).

In only a few years (2013-2019), the number of plants modified through the use of this innovative system has increased dramatically as showed in **Fig. 12**. The figure was developed by performing a PubMed NCBI

(https://www.ncbi.nlm.nih.gov/pubmed/) search using the following key words "CRISPR/Cas and plants". The CRISPR-Cas9 system has been successfully applied to improve many important crops rice (Lin et al., 2018; Begemann et al., 2017; Baysal et al., 2016; Sun et al., 2016; Zong et al., 2017), *Brassica* spp. (Yang et al., 2017; Braatz et al., 2017; Sun et al., 2018 Murovec et al., 2018), maize (Svitashev et al., 2016; Zhu et al., 2016; Zong et al., 2017; Lee et al., 2019), wheat (Upadhyay et al., 2013; Liang et al., 2017; Zong et al., 2017; Howells et al., 2018), tomato (Brooks et al., 2014; Pan et al., 2016; Ueta et al., 2017; Dahan-Meir et al., 2018; Veillet et al., 2019), *Medicago sativa* (Gao et al., 2018), and the model legume *Medicago truncatula* (Meng et al., 2017).

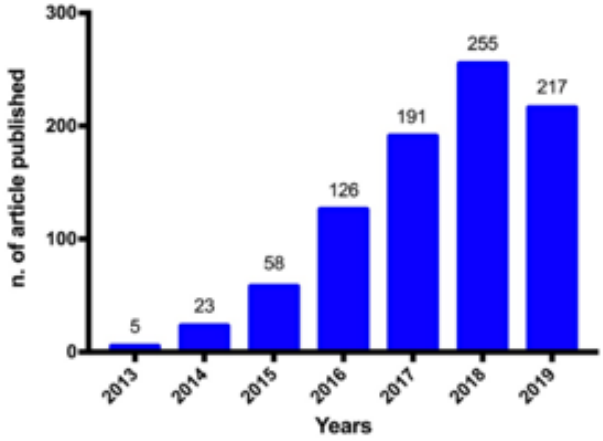


Fig. 12. Number of publications related to the application of CRISPR/Cas9 technique in plants (PubMed-NCBI, 12 August 2019).

To cite a few applications, the CRISPR/Cas system has been used to improve plant fitness by providing immunity to viral infections (Baltes et al., 2015) as well as to fight different other pathogens attacks. For instance, tomato was the subject of studies carried out by Nekrasov et al. (2017) who developed the 'Tomelo' variety. It is considered a non-transgenic tomato variety (at least in the U.S.A.) resistant to the powdery mildew fungal pathogen *Oidium neolycopersici*. The *MLO* (*MILDEW RESISTANT LOCUS O*) gene, that confers susceptibility to fungi causing the powder disease (Acevedo-Garcia et al., 2014), was targeted in this case. Still in tomato, a CRISPR/Cas9-mediated mutagenesis of *CCD8* (*CAROTENOID CLEAVAGE DIOXYGENASE*) gene was used to induce the resistance of plants against the parasitic weed boomrapes (*Phelipanche aegyptiaca*) (Bari et al., 2019). Taking into account the impact of plant parasitic weeds on agriculture and the

difficulty to constitute efficient control methods, the genome editing approach offers novel solutions to fight this problem (Bari et al., 2019).

Other applications targeted nutritional and productivity traits. For instance, the production of seedless fruits was carried out by targeting *IIA9* (*AUXIN-INDUCED 9*), a key gene controlling parthenocarpy, an important trait with agricultural value for industrial purpose and food quality (Gorguet et al., 2005; Ruan et al., 2012). As results, the regenerated mutants exhibited morphological changes in leaf shape and seedless fruit, a characteristic of parthenocarpic tomato (Ueta et al., 2017). Moreover, through the CRISPR/Cas9 mediated editing of *SBEIIb* (*STARCH BRANCHING ENZYME IIb*) gene, a high-amylose rice variety was developed (Sun et al., 2017). This trait was pursued because rice is one of the major staple food crops consumed be nearly half of the world population (FAO, 2004) and cereals with high content in amylose and resistant starch offer potential health benefits (Sun et al., 2017). Zheng et al., (2019) simultaneously knocked-out the two *MAX1* (*MORE AXILLARY GROWTH 1*) homologous genes, which regulates plant height and axillary bud at growth, using the CRISPR/Cas9 system in rapeseed (*Brassica napus*). This targeted mutagenesis resulted in improved plant architecture and increased yield (Zheng et al., 2019). By targeting endogenous genes involved in morphogenesis, it is possible to easily select the mutated plants by visibly observing their phenotypic changes (Qin et al., 2007).

Considering the need to increase productivity and quality of cultivated species under adverse environmental conditions in order to feed the over-growing population of the planet, genome editing has the potential to accelerate crop improvement. Crop performance is strongly dependent on seed quality traits. Moreover, seeds can be considered as common denominators to address both plant productivity and improved human nutrition (Forti et al., 2019).

1.6.4. Genome editing in the context of seed biology

Seeds are considered one of the main vectors conditioning crop yield. Traditional breeding, the original method used to improve yield, relies on the production of various QTL combinations and subsequent selection the most favorable breeds (Zuo and Li, 2014; Shen et al., 2018), but it is quite a time-consuming process. Hence, crop yield improvements need to be accelerated in order to avoid an eventual food insecurity situation. To do so, GM crops represent a solid alternative, already proven to be effective when considering the much-studied examples related to the production of herbicide and insect resistant crops (Brookes and Barfoot, 2017; Paul et al., 2018).

With the advent of genome editing techniques, researchers have further committed themselves in finding faster and easier ways to address this issue. The traits edited through the use of the CRISPR/Cas9 system span from seed size and number to plant productivity and heterosis (Forti et al., 2019). Below are presented some examples of genome editing applications dedicated to improve seeds quality.

In rice, panicle size is controlled by a transcription factor (TF) named DEP1 (Dense and Erect Panicle) (Huang et al., 2009) while another TF, IPA1 (Ideal Plant Architecture) controls tillering (*Oryza sativa* L.) (Miura et al., 2010). CRISPR/Cas9 has been applied to mutate these two TFs and rice plants with phenotypic changes in plant architecture and subsequent increase in plant productivity were obtained (Li et al., 2016).

Seed size and number are important agronomic parameters to evaluate crop yield. A direct approach to increase yield is to knock-out genes that negatively affect this parameter, as reported in rice (Xu et al., 2016; Li et al., 2016). Using CRISPR/Cas9 technology, the *GS3* (Grain Size 3), *GW2* (Grain Weight 2), *GW5* (Grain Weight 5), *TGW6* (Thousand Grain Weight 6) and *Gn1a* (Grain number 1a) genes were silenced as these are negative regulators of grain size, number and weight.

Genome editing tools are also helpful to improve the seed nutrition content. Nutrient deficiency is a major challenge especially for people in third-world countries who do not afford to have a balanced diet and rely on staple food crops with low levels of micronutrient (e.g. minerals, vitamins and amino acids) (Forti et al., 2019). Due to the need better nutritional foods, increasing efforts are now focusing on this complex trait through the use of the genome editing techniques (Yu and Tian, 2018). One of the targeted traits was the seed oil composition. This because vegetable oils are important for both human consumption and industrial application. *FAD2* (Fatty Acid Desaturase 2) gene is one of the main targets to address seed oil content. It is involved in the conversion of oleic to linoleic acid (Okuley et al., 1994). Oleic acid is a valuable monounsaturated fatty acid with high oxidative stability that can help in the prevention of several diseases such as cardio-vascular associated disorders (Lopez-Huertas, 2010). Soybean was the first crop targeted for the modification of oil seed content (Demorest et al., 2016). Subsequently other species, such as peanuts (Wen et al., 2018), rice (Abe et al., 2018) and false fax (*Camelina sativa*) (Jiang et al., 2017), were modified. Li et al. (2018) carried out an interesting work in which the lycopene content was enhanced in tomatoes. The authors used a multiplex genome editing dedicated to boost lycopene production by targeting the carotenoid biosynthetic pathway. These targeted mutations in *SGR1* (*STAY GREEN 1*), *Blc* (ß-lycopene cyclase), *LCY-E* and *LCY-B1* (*LYCOPENE ε-CYCLASE*),

allowed an increasing of 5.1-fold of lycopene content in the fruits. In the last few years, researchers applied the genome editing techniques also to lower the amount of deleterious carbohydrates (Syahariza et al., 2013), antinutritional elements such as phytic acid or acrylamide (Shukla et al., 2009; Liang et al., 2014), to decrease sugar content (Clasen et al., 2016), or to develop low gluten wheat designated for patients suffering from coeliac disease (Sánchez-León et al., 2018).

Other important aspects of genome editing address seed physiology and development. This can be tracked as far as pollen development. For instance, Wang et al. (2019) developed a multiplex CRISPR/Cas9 vector to simultaneously target the *REC8* (*MEIOTIC RECOMBINATION PROTEIN*), *PAIR1* (*HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS 1*)), *OSD1* (*OMISSION OF SECOND DIVISION 1*) and *MTL* (*MATRILINEAR*) genes in rice. These targeted mutations in multiple genes, playing important role in meiosis, resulted in the production of rice hybrid lines able to self-pollinate and produce true-breeding progeny trough seeds. CRISPR/Cas9 methodology was used to target genes involved in pollen development to generate male sterile lines also in two other important cereals, namely maize and bread wheat (Chen et al., 2018; Singh et al., 2018).

Seed preservation during processing and storage is a key trait to promote sustainable agricultural practices without additional economic losses. In *Brassica napus*, a CRISPR/Cas9 construct was designed to target the two *ALC* (*ALCATRAZ*) alleles while in rice, TALEN construct was developed to target the *LOX3* (*LIPOXYGENASE 3*) gene. This approach resulted in reduced seed shattering during harvest and enhanced resistance during storage (Ma et al., 2015; Braatz et al., 2017).

Plant phytohormones play multiple roles in plant growth and development. Among them, ABA and GAs are involved in maintaining the equilibrium between seed dormancy and germination (Rodríguez-Gacio et al., 2009). CRISPR/Cas9 targeting of the *NCED4* (*9-cis-EPOXYCAROTENOID DIOXYGENASE 4*) gene, encoding a key enzyme in ABA biosynthesis in lettuce, resulted in the development of lines with an enhanced range of optimal temperatures for seed germination (Huo et al., 2013). This study showed that more than 70% of the seeds were able to germinate at temperatures up to 37ºC, when the usual germination temperature of lettuce ranges between 15ºC and 25ºC (Bertier et al., 2018).

The cited examples prove that genome editing is being successfully applied to improve seed quality and crop performance. Further advances in both technological development and basic research will allow the use of these tools to also target the highly complex processes involved in the seed pre-germinative metabolism.

2. Aims of the research

In the context of the current scenario associated to the agricultural challenges that need to be addressed to feed the overgrowing population, this work of thesis focuses mainly on aspects related to Seed Technology. In view of this, the development of improved seed priming protocols, defining novel molecular hallmarks of seed germination, and the application of genetic engineering methods to improve a complex agronomic trait such as seed vigor, represent important requisites for both basic and translational research.

To this purpose, the following specific goals were identified and my PhD project was developed accordingly, using different experimental systems:

1. *Design of protocols for seed priming to improve germination and seedling performance in horticultural and legume crops*.

Eggplant (*Solanum melongena*), the third most prominent product from the *Solanum* genus, after potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*), is still difficult to manage in terms of seed quality. In this work, eggplant accessions and wild crop relatives showing contrasting levels of seed vigor and germination profiles were investigated at the physiological and molecular level to assess the response to priming, select reliable molecular hallmarks (gene expression and ROS profiles) and define improved protocols.

Medicago truncatula, a valuable source of animal feed as well as a model legume, was investigated to expand the information so far obtained on the pre-germinative metabolism only under laboratory conditions (Macovei et al., 2010; Balestrazzi et al., 2011; Pagano et al., 2017, 2019; Araujo et al., 2019) to the more complex field-like conditions. In this case, seed hydropriming was evaluated in the context of soil contamination as a mean to putatively enhance seedling establishment under stress conditions. Attention was focused on antioxidant and DNA repair genes, e.g. the *Tdp1* (*TYROSYL-DNA PHOSPHODIESTERASES*) genes.

2. *Development of plant genetic transformation protocols as tools to improve seed quality.*

Plant genetic transformation has enabled fundamental insights into plant biology and revolutionized commercial agriculture. Unfortunately, for most crops, transformation and regeneration remain arduous even after more than 30 years of technological advances. Genome editing techniques are an innovative and powerful strategy that allow to introduce mutations on insert/substitute wide portion of DNA at a targeted site. These techniques have extremely important implications for human health, agriculture and

environment. Genome editing provides novel opportunities to enhance crop productivity if accompanied by an efficient transformation protocol.

In this work, a novel vector for genome editing able to target the *Tdp1β* gene (*TYROSYL-DNA PHOSPHODIESTERASE 1β*) in *M. truncatula* was developed. The TDP1 enzyme is involved in the repair of topoisomerase I-DNA covalent lesions. In particular, the *Tdp1β* gene encoding the β isoform was identified only in plants and its role is not fully understood. The expression of the *Tdp1* genes in plants is correlated with different stress conditions in several experimental systems, including cell suspensions, aerial parts, and seeds of *M. truncatula* (Balestrazzi et al., 2011a; Macovei et al., 2018), suggesting their recurrent involvement in the plant response to genotoxic stress. Its modulation during the seed pre-germinative metabolism is indicative for its use as a marker of seed quality.

Aside being a cereal model plant, *O. sativa* is one of the world's most important crops and a major staple food that feeds more than three billion people worldwide. Rice cultivation in Europe is restricted to a few southern countries and Italy is the major producer. Some cultivars with high commercial importance, are recalcitrant to genetic transformation, as in the case of Vialone Nano, a local rice variety (from Lombardy and Veneto) very appreciated at the culinary level, but highly susceptible to diseases and/or pathogens. To safeguard this variety, it is necessary to develop an efficient genetic transformation protocol in order to improve and preserve it.

3. Material and methods

3.1. Plant material

In this work, 49 different accessions of eggplants and wild relatives derived from the germplasm collection located at CREA-GB (Consiglio per la Ricerca in agricoltura e l'analisi dell'Economia Agraria - centro di Genomica e Bioinformatica) in Montanaso Lombardo (Lodi, Italy) were analyzed.

Seeds from a commercial cultivar of *Medicago truncatula* (Var. Jemalog) were kindly provided by Fertiprado L.d.a. (Portugal).

The rice cultivars Vialone Nano and Nipponbare were provided by Dr. Gianpiero Valè (CREA-SCS, Consiglio per la Ricerca in agricoltura e l'analisi dell'Economia Agraria - centro di Sperimentazione e Certificazione delle Sementi). The rice seeds used in this study were collected in 2016 from the open field at CREA- SCS (Vercelli, Italy).

3.2. Seed germination parameters

Fresh seeds of *Solanum melongena* L. (inbred line '67/3') and its wild relatives *Solanum torvum* Swartz 1788 (turkey berry) and *Solanum villosum* Miller (hairy nightshade) were extracted from physiologically ripe fruits produced by plants cultivated in an open field at CREA-GB (Montanaso Lombardo). In this study, five '67/3' seed lots collected during subsequent years (2014, 2015, 2016, 2017, 2018) were investigated. As for the wild relatives, three *S. tovum* seed lots (collected during 2004, 2017 and 2018), and two *S. villosum* seed lots (collected during 2015 and 2018) were analysed. For germination tests, seeds were transferred to Petri dishes (diameter 90 mm) containing two filter papers moistened with 2.5 ml of H_2O, sealed and kept in a growth chamber at 22°C under light conditions with photon flux density of 150 μmol m^{-2} s^{-1}, photoperiod of 16/8 h and 70-80% relative humidity. Seeds with protrusion of the primary radicle were considered germinated and counted every day after imbibition. Germination parameters, calculated as described by Ranal and Garcia de Santana (2006) are listed in **Table 1.** For each treatment, three independent replications with 20 seeds per replication were analysed. Seeds and seedlings were harvested at the indicated time points, the fresh weight was measured and samples were stored in liquid N_2 for molecular analyses.

Table 1. Germination parameters used in this study as reported by Ranal and Garcia de Santana (2006). For each parameter, definition, formula, limits of measurement and unit are shown.

Parameter	Formula	Limits	Unit
G **mean number of germinated seeds per day expressed in percentage**	G = (100* n. of germinated seeds)/ Total n. of seeds	$0 \leq G \leq 100$	%
MGT **mean germination time (*)**	$\bar{t} = \sum_{i=1}^{k} n_i t_i / \sum_{i=1}^{k} n_i$	$0 < t \leq k$	day
CVG **coefficient of velocity of germination**	$CVG = (\sum_{i=1}^{k} f_i / \sum_{i=1}^{k} f_i x_i) 100$	$0 < CVG \leq 100$	%
MGR **mean germination rate**	$v = CV/100$	$0 < v \leq 1$	day^{-1}
U **uncertainty associated to the distribution of the relative frequency of germination (**)**	$\bar{E} = -\sum_{i=1}^{k} f_i \log_2 f_i$, being $f_i = n_i$	$0 \leq U \leq \log_2 n$	bit
Z **synchronization index (***)**	$Z = \sum C_{n_i, 2} / N$	$0 \leq Z \leq 1$	Unit less

(*) t_i is time from the start of the experiment to the i^{th} observation (day); n_i: number of seeds germinated in the time i (not the accumulated number, but the number correspondent to the i^{th} observation), and k is the last time of germination. (**) f_i is the relative frequency of germination, n_i the number of seeds germinated on the day i, and k the last day of observation. (***) $C_{ni,\ 2}$: combination of the seeds germinated in the time i, two together, and n_i the number of seeds germinated in the time i.

For *M. truncatula*, seed germination was carried out in the soil and seedling emergence was monitored for a two-week period. The seeds grown in trays containing two types of agricultural soils (denominated as Soil_A and Soil_B) collected from the Varanasi agricultural area (namely Karsada). Both soil types were regionally collected *in situ* from the field and no additional treatments were implemented.

The soil properties are given in **Table 2**. The two types of soil mainly differ in their content of total dissolved solids (TDS) based on their vicinity to the power plant at Karsada.

The experiment was performed in October 2018, when day/night temperature ranged between 28-31/20-24°C and the relative humidity was around 83%. Each tray row represented experimental replicates (R1-R5)

containing 21 seeds, and three seeds per square, respectively. Other two independent experiments with the same setup were performed in August 2019 when day/night temperature ranged between 31-36/24-29°C and the relative humidity was around 90%. During this period, seeds were stored at room temperature in a sealed plastic container.

As the first true trifoliate leaves start to appear after the 7th day, the 14th day after sowing was selected to have fully developed seedlings in all treatments.

The total germination percentage (%) was calculated at the end of the experiment while the time required for 50% of seeds to germinate (T_{50}) was calculated according to the following formula (Farooq et al., 2005):

$$T_{50} = t_i + [(N / 2 - n_i) (t_i - t_j)] / n_i – n_j$$

where, N is the final number of germination and n_i, n_j cumulative number of seeds germinated by adjacent counts at times t_i and t_j when $n_i<N/2< n_j$.

Table 2. Soil properties. EC, electrical conductivity. TDS, total dissolved solids. ds, deciSiemens. ppm, parts per million.

type	pH	EC (ds/m)	Salinity (ppm)	TDS (ppm)
Soil_A	8.1	0.15	0.07	82.9
Soil_B	8.23	0.2	0.09	103.0

3.3. Phenotyping parameters

For eggplant experiments, phenotyping analyses were performed as follows; radicle length and fresh weight (FW) were measured in seven-day old *S. melongena*, *S. torvum*, and *S. villosum* seedlings. Seedlings were then placed in oven at 60°C for 24 h, and the dry weight (DW) was measured. Values are expressed as mean ± SD (standard deviation) of three independent replications with 15 seedlings for each replication.

In the case of *M. truncatula* experiments, seedlings were collected at 14 days after sowing. Seedling length, FW and DW were measured as described above. Data are presented as mean ± SD of five biological and five technical replicates.

3.4. Seed priming protocols

3.4.1. Hydropriming

The following hydropriming protocol was applied to the eggplant ‘67/3’ line and its wild relative *S. torvum*. Overall, 45 seeds (15 seeds for each replicate) were soaked in 400 ml of H_2O under aeration produced by a Wave

Air Pump Mouse 2 Beta aerator (De Jong Marinelife B.V., The Netherlands) with the following parameters: 220-240 V, 50 Hz, 2.3 W, output 1.8 l min^{-1}, pressure 0.012 MPa. This treatment was performed for 24 h, 48 h, 72 h, and 96 h, respectively, at 24°C. For dehydration (dry-back process, DB) primed seeds were transferred into glass tubes, placed between two cotton disks, covered with silica beads (disidry® Orange Silica Gel, The Aerodyne, Florence, Italy) with a seed : silica ratio of 1 : 10, and kept at 24-25°C. Seed fresh weight was monitored every 15 min for 2 h until the weight of dry seed was reached (**Table 3**). In the case of *S. villosum*, the seeds were treated for 2, 4, 8, 12 and 24 h, respectively, under the same conditions (**Table 3**). The different priming timepoints are due to the accelerated germination profile observed for this wild relative. Accordingly, the DB step was completed in 0.5 h.

Table 3. Hydropriming treatments applied to *S. melongena* '67/3' line and its wild relatives. DB, dry-back.

Genotype	Time (h)	DB (h)
'67/3'	24, 48, 72, 96	2
S. torvum	24, 48, 72, 96	2
S. villosum	2, 4, 8, 12, 24	0.5

For *M. truncatula*, the hydropriming protocol was same as the one indicated for eggplant, with the specifications that the seed material was collected at 2 h (HP2) and 4 h (HP4) following treatment. A non-primed control (HP0) was also used. Subsequently, seeds were dried using filter paper and immediately used for sowing.

3.4.2. Biopriming

For *M. truncatula* seeds, biopriming was performed with two formulations of bacterial cultures prepared by mixing 1×10^8 cells in 2 g of talc (carrier material) (CDH, India). The seeds were initially treated with jaggery syrup to facilitate the adhesion of bacteria to the seed surface. Subsequently, the treated seeds were incubated for about 16 h at approximately 25-30°C. The bacterial formulations contained two different strains of *Bacillus spp*. isolated from mustard (*Brassica juncea*) rhizosphere (BP1) and linseed (*Linum usitatissimum*) rhizosphere (BP2), respectively. Both strains tested positive for phosphate solubilization, production of indole acetic acid and siderophores (Prasad, personal communication). An additional treatment was performed by combining 4 h of hydropriming and BP1 strain (hereby denominated as BP1HP4).

3.5. Quantification of ROS (reactive oxygen species)

The fluorogenic dye 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, Milan Italy) was used to quantify ROS levels in dry and imbibed seeds of eggplant and wild relatives. Following deacetylation by cellular esterases, the dye is converted to a non-fluorescent molecule which is subsequently oxidized by ROS into the highly fluorescent 2',7'-dichlorofluorescein. DFC can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm, respectively. The assay was carried as described by Macovei et al. (2016), with the following modifications. Seeds were collected at the indicated time points and dried on filter paper. Samples (three seeds per time point) were incubated for 15 min with 50 µl of 10 µM DCFH-DA and subsequently fluorescence at 517 nm was determined using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia), setting the program for one cycle of 30 s at 25°C. As negative control, a sample containing only DCFH-DA was used to subtract the baseline fluorescence. Relative fluorescence was calculated by normalizing samples to controls and expressed as Relative Fluorescence Units (R.F.U.).

3.6. Molecular analysis

3.6.1. RNA extraction and cDNA synthesis

RNA was extracted from treated and untreated seeds of *S. melongena* and wild relatives and from *M. truncatula* seeds and 14-days-old seedlings. RNA isolation was carried out using the TRIZOL® Reagent (Fisher Molecular Biology, Trevose, U.S.A.) according to the supplier's indications. with the following changes. RNAs were extracted from three biological replicates consisting of a pool of seeds/seedlings (approximately 100 mg each). Seeds were grinded to a fine powder in liquid N_2 with a mortar and pestle. Each sample was transferred the sample to a 1.5 ml Eppendorf tube containing 750 µl of TRIZOL® Reagent and incubated at room temperature (r.t.) for 5 min. Subsequently, chloroform (150 µl) was added, samples were incubated at r.t. for 3 min and centrifuged at 11.500 rpm at 4°C for 15 min. The aqueous phase, containing RNA, was transferred into a new 1.5 ml tube. An equal volume of isopropanol was added, the sample was mixed briefly and incubated at r.t. for 10 min. RNA was then pelleted by centrifugation (11.500 rpm, 4°C, 10 min). The supernatant was removed, the pellet was washed twice with 500 µl of 70% ethanol and once with 500 µl of 100% ethanol, then dried at r.t. and finally dissolved in 20 µl of DEPC (diethyl pyrocarbonate)-treated

H_2O. RNA was quantified using a Biochrom™ WPA Biowave DNA UV-Vis Spectrophotometer (Biochrom Ltd, Cambridge, U.K.). RNA integrity was verified by electrophoresis on 1% agarose gel. For each sample, 2 µl were mixed with 3 µl of DEPC-treated H_2O and 1 µl of 5X Green GoTaq® Flexi Buffer (Promega, Italy). The 1Kb DNA Ladder RTU (GeneDireX Inc., U.S.A.) was used as molecular weight marker. RNA samples were treated with DNase I, RNase-free (1 U μl^{-1}) (ThermoFisher Scientific, Milan, Italy) to remove DNA, according to manufacturer's suggestions and RNA was then quantified as previously described. cDNAs were obtained using the RevertAid™ First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Milan, Italy) according to the manufacturer's suggestions.

3.6.2. Design of oligonucleotides for *q*RT-PCR and selection of reference genes

Oligonucleotide primers were designed using the Real-Time PCR Primer Design program Primer3Plus (https://primer3plus.com) from GenScript and further validated through the online software Oligo Analyzer (https://eu.idtdna.com/calc/analyzer). The sequence of *S. melongena* genes used in this study were provided by Dr. Laura Bassolino (CREA-GB), based on the available reference genome. For each oligonucleotide set, the nucleotide sequence is listed in **Table 4**. The following genes were tested: *SmOGG1* (Accession N° SMEL_004g210790.1), *SmFPG* (Accession N° SMEL_003g194660.1), *SmTDP1α* (Accession N° SMEL_003g171200.1), *SmAPX* (Accession N° SMEL_006g245760.1.01), *SmSOD* (Accession N° SMEL_001g139700.1.01).

To identify the most stable reference genes under the tested conditions, a GeNorm (https://genorm.cmgg.be/) analysis was carried out in *S. melongena*, *S. villosum* and *S. torvum* using the following housekeeping genes *SmGADPH* (Accession N° AB110609.1), *SmAPRT* (Accession N° JX448345.1), *SmEF1* (Accession N° X14449.1), *SmTUB* (Accession N° DQ205342.1), *SmPP2Acs* (Accession N° AY325817.1). The *SmAPRT* and *SmGADPH* resulted the most stable in *S. melongena*, *SmPP2Acs* and *SmTUB* in *S. torvum*, and *SmAPRT* and *SmPP2Acs* in *S. villosum* (**Fig. 13A-C**). These were subsequently used as reference genes.

Table 4. List of oligonucleotide sequences designed for *q*RT-PCR analysis in *S. melongena* and wild relatives and *M. truncatula.* FPG, formamidopyrimidine DNA glycosylase. OGG1, 8-oxoguanine glycosylase/lyase. TDP, tyrosyl-DNA phosphodiesetrase. APX, ascorbate peroxidase. SOD, superoxide dismutase. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. PP2A, phosphatase 2A. TUB, tubulin. EF, elongation factor. APRT, adenine phosphoribosyl transferase. MT2, type 2 metallothionein. ACT, actin. UBI, ubiquitin. ELF*α*, elongation factor.

Gene (accession N°)	Forward Primer (5'-3')	Reverse Primer (5'-3')	Efficiency
SmAPX (SMEL_006g245760.1.01)	GCAGTTTCCCATCCTCTCCC	GGTGGTTCTGGCTTGTCCTC	1.73
SmSOD (SMEL_001g139700.1.01)	CTGGAAATGCTGGCGGAAG	GGAGGAATCAACCCTGGAGC	1.81
SmOGG1 (SMEL_004g210790.1)	TTATTGACCAGCAGCCCACA	ATACACCAGCAACACCCCTT	1.70
SmFPG (SMEL_003g194660.1)	CCAAAAGAATACGGGAGGTGA	TTCTGGCTCTTCATCTTGACC	1.73
SmTDP1α (SMEL_003g171200.1)	GCTTCACCAGGCAACAAACA	CCCACGCACTCTCATCAATC	1.70
SmGAPDH (AB110609.1)	GGTGCCAAGAAGGTTGTGAT	GGTTACGATCAACGTGTTGC	1.81
SmPP2Acs (AY325817.1)	GGACTCTCACCATCCCTTGA	ACCAACCCTTATAGTGGAG	1.80
SmTUB (DQ205342.1)	CCAGACAGGATGATGCTCAC	TACCAGGAACTGTTGCTTCG	1.80
SmEF1 (X14449.1)	ACCAAGATTGACAGGCGTTC	GGAAACGACTTATGGGAGGT	1.79
SmAPRT (JX448345.1)	TGGCGCCTCATGATCCGATTCTTA	ACTCCAACACGCTCAAGAAGCCTA	1.75
MtOGG1 (Medtr3g088510)	AAACACCGCACCTTCTCAAT	TGTGGAGATGTTTGAGGGAA	1.73
MtFPG (Medtr2g126800)	TCCTTTCAATTCGGTATGGC	GCTCCAAACCATCGTCTAGC	1.76
MtSOD (Medtr7g114240)	CCTGAGGATGAGACTCGACA	GAACAACAACAGCCCTTCCT	1.79

MtAPX (Medtr4g061140)	AGCTCAGAGGTTTCATCGCT	CGAAAGGACCACCAGTCTTT	1.76
MtMT2 (Medtr8g060850)	CATGTCAAGCTCATGCGGCAAC	TGCCGTAGTTGTTTCCCTTCCC	1.72
MtACT (Medtr3g095530)	TCAATGTGCCTGCCATGTATG	ACTCACACCGTCACCAGAATC	1.70
MtTUB (Medtr7g089120)	TTTGCTCCTCTTACATCCCGTG	GCAGCACACATCATGTTTTTGG	1.82
MtUBI (Medtr3g091400)	GCAGATAGACACGCTGGGA	AACTCTTGGGCAGGCAATAA	1.81
MtGAPDH (Medtr3g085850)	TGCCTACCGTCGATGTTTCAGT	TTGCCCTCTGATTCCTCCTTG	1.75
MtELF1α (GenBank EST317575)	GACAAGCGTGTGATCGAGAGA	TTCACGCTCAGCCTTAAGCT	1.69

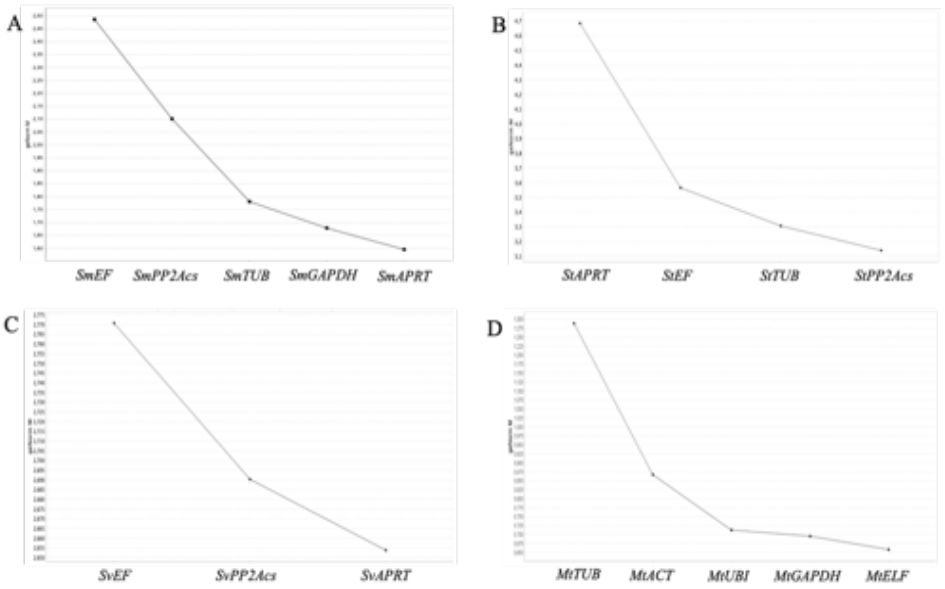


Fig. 13. Selection of reference genes for *q*RT-PCR analysis. (A) *S. melongena*. (B) *S. torvum*. (C) *S. villosum*. (D) *M. truncatula*. Average expression stability values (M), calculated using GeNorm algorithm (https://genorm.cmgg.be), of the putative reference genes analyzed in primed and unprimed seeds throughout the tested time points of the experimental design. PP2A, phosphatase 2A; EF, elongation factor; APRT, adenine phosphoribosyl transferase; TUB, tubulin; ACT, actin; UBI, ubiquitin; GADPH, glyceraldehyde 3-phosphate dehydrogenase.

For *M. truncatula*, the following genes were tested: *MtOGG1* (Accession N° Medtr3g088510), *MtFPG* (Accession N° Medtr2g126800), *MtSOD* (Accession N° Medtr7g114240), *MtAPX* (Accession N° Medtr4g061140), *MtMT2* (*TYPE 2 METALLOTHIONEIN*; Accession N° Medtr8g060850) (**Table 4**). Also, in this case GeNorm was used to identify the most stable reference genes under the tested conditions. The tested genes were: *MtTUB* (Accession N° Medtr7g089120), *MtACT* (Accession N° Medtr3g095530), *MtUBI* (Accession N° Medtr3g091400), *MtGAPDH* (Accession N° Medtr3g085850) and *MtELF1α* (Accession N° GenBank EST317575). The *MtELF1α* and *MtGADPH* genes resulted most stable (**Fig. 13D**) and were used as reference genes.

3.6.3. Quantitative real-time polymerase chain reaction (*q*RT-PCR) analysis

For all used species and treatments, *q*RT-PCR was performed with the Maxima SYBR Green qPCR Master Mix (2X) (Thermo Fisher Scientific) according to supplier's indications, using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics Pty Ltd, Brisbane, Australia). Amplification conditions were as follows: denaturation at 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 30 s and 72°C for 30 s. The raw, background-subtracted fluorescence data provided by the Rotor-Gene 6000 Series Software 1.7 (Corbett Robotics) was used to estimate PCR efficiency (E) and threshold cycle number (C_t) for each transcript quantification. The Pfaffl method (Pfaffl, 2001) was used for relative quantification of transcript accumulation and statistical analysis was performed with REST2009 Software V2.0.13 (Qiagen GmbH, Hilden, Germany). Results are presented in terms of relative expression and given as mean ± SD of three independent replicates.

3.7. Genetic transformation protocols

3.7.1. The CRISPR-Cas9 vector preparation

A bioinformatic analysis was performed on the *M. truncatula Tdp1β* gene to identify the appropriate sgRNA sequence. The *MtTdp1β* nucleotide sequence (Medtr8g095490) was retrieved from Phytozome v.12.1.5

(https://phytozome.jgi.doe.gov/pz/portal.html). The amino acid sequence (ABE85647.1) was obtained from NCBI (https://www.ncbi.nlm.nih.gov/). To identify the sgRNA sequence, the E-CRISP design online tool (http://www.e-crisp.org/E-CRISP/) was used. Based on previous work (Liu et al., 2016b), several factors were evaluated including: 1) the position of the target site in the gene and in the corresponding amino acid sequence, 2) the PAM sequence, 3) the GC percentage, and 5) the type of nucleotides present in positions 3 and 20 in sgRNA. The sgRNA selected for the application of genome editing to target the *Tdp1β* gene is shown in **Table 5.** The choice of the sgRNA was based on its location within the gene/protein sequence. Indeed, the target site is located upstream of the highly conserved catalytic domain HKD1, thus in-frame mutations leading to the formation of premature stop codons would result in the production of a nonfunctional protein.

Table 5. Selected sgRNA sequence for *Tdp1β* gene knockout.

Gene	Target sequence (5'-3')	Target protein	PAM sequence	Cas type	GC%	N3	N20
Tdp1β	GGTAAAGACCACAAGAGACA	GKDHKRH	TGG	spCas9	45%	T	A

Once identified, the sgRNA was synthesized and cloned into a the CRISPR-Cas9 vector by Genewiz (https://www.genewiz.com/en-GB/). The CRISPR-Cas9 vector (pK7WGF2::hCas9), containing the Cas9 from *Streptococcus pyogenes* (spCas9) optimized for human use and fused to the green florescent protein (GFP), was purchased from Addgene (https://www.addgene.org/crispr/). This vector was previously showed to be efficient also for plant transformation (Nekrasov et al., 2013). The construct hereby named CRISPR_Tdp1β, contains the Cas9 cassette, the sgRNA cassette, and the plant selection cassette (**Fig. 14**). The sgRNA cassette contains the U6 promoter from *Arabidopsis thaliana*, the *Tdp1β* sgRNA, a tracrRNA (*trans*-activating crRNA) sequence and the NOS (nopaline synthase) terminator sequence.

Fig. 14. Schematic representation of the vector used for gene knockout. RB, Right Border; LB, Left Border; 35S, cauliflower mosaic virus promoter; spCas9h, Cas9 nuclease; GFP, Green Fluorescent Protein; NOSt, nopaline synthase terminator; AtU6, *Arabidopsis thaliana* promoter; *Tdp1β*, sgRNA for the *Tdp1β* gene; Kan, kanamycin; SpecR, spectinomycin resistance.

The prepared plasmid was inserted into *Escherichia coli* (strain DH5α) by electroporation in order to produce multiple copies by using standard protocols (Maniatis Molecular Cloning), followed by plasmid purification using the AurumTM plasmid Mini Kit (Bio-Rad Laboratories, Segrate MI, Italy), performed as indicated by the manufacturers. Subsequently, the CRISPR_Tdp1β plasmid was transformed into *Agrobacterium tumefaciens* (EHA105 strain) cells by electroporation, following standard protocol (Maniatis Molecular Cloning). To certify the plasmid insertion into the bacteria a series of standard PCR reactions (using PCRBIO DNA Taq Polymerase, PCR Biosystem Ltd, London, as indicated by the suppliers' instructions) was carried out using the following primer sets: FwWhCass (5'-GGAGTGATCAAAAGTCCCACATC-3') and RevWhCass (5'-GTCAAGCAGATCGTTCAAAC-3') which amplify the sgRNA cassette (459 bp), FwhCas9 (5'-AAAGCGTCAAGGAACTGCTG-3') and RevhCas9 (5'-AATGTTTTCTGCCTGCTCCC-3') which amplify a part of the Cas9 cassette (455 bp), FweGFP (5'-GGCTCAAAAGAACAGCACGG-3') and RevGFP (5'-CGAGTGACAGGGCGATAAGA-3') which amplify a part of the *GFP* marker gene (550 bp).

3.7.2. *Agrobacterium*-mediated genetic transformation of *M. truncatula* cell suspension cultures

3.7.2.1 Preparation of plant material

The CRISPR_Tdp1β construct was subsequently used for *Agrobacterium*-mediated transformation of *M. truncatula* cells, obtained as shown in **Fig. 15**.

Fig. 15. Schematic representation of the procedure used to obtain *M. truncatula* cell suspension cultures.

M. truncatula (Jemalong M9-10a genotype) seeds were scarified, sterilized and placed on MS30 medium (Murashige and Skoog, 1962). The scarification and sterilization steps were performed as follows. Seeds were transferred in a glass jar, and absolute sulfuric acid (H_2SO_4) was added with a glass pipette in order to cover all the seeds, the jar was slowly shaken for 5 min until black spots were observed on the surface of the seed. Subsequently, all sulfuric acid was removed and seeds were washed with cold sterile H_2O five times. Commercial bleach (50% dilution) was then added to cover the seeds, and after stirring slowly for 5 min, five consecutive washes with sterile

H_2O were performed. Seeds were incubated in 70% EtOH, for 2 min under slow stirring followed by other five consecutive washes with sterile H_2O. Finally, seeds were maintained in sterile H_2O for 2 min and once rehydration was completed, they were transferred into a Magenta™ vessel containing MS30 (Murashige-Skoog) medium containing 3% (w/v) sucrose, 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D, Micropoli, Milan, Italy), incubated at 4° C in the dark for 2 days, and then transfer to the growth chamber at 23°C for one month under light conditions with a photon flux density of 150 µmol m^{-2} s^{-1}, photoperiod of 16/8 h and 70-80% relative humidity. The resulting *M. truncatula* plantlets were used to develop calli. Leaves excised from plantlets were cultivated *in vitro* on MSOG medium containing 4.4 g/l MS salts/vitamins (Duchefa Biochemie B.V., Netherlands), 30 g/l sucrose (Panreac química s.l.u., Spain), 2 g/l gelrite (Duchefa Biochemie B.V., Netherlands), 0.5 g/l kinetin (Duchefa Biochemie B.V., Netherlands), 0.5 g/l 2.4-D (2,4-dichlorophenoxyacetic acid, Duchefa Biochemie B.V., Netherlands) (pH 5.7), in a growth chamber as previously described. The cut leaves were arranged with the nervure facing downwards in Petri dishes containing the MSOG medium. The plates were incubated at 26°C for 21 days and subsequently transferred in the dark. Finally, calli were kept for another 30 days at 26°C in dark conditions and transferred to fresh medium every month. *M. truncatula* cell suspension cultures were obtained by transferring a single callus propagation in liquid MS30 medium. Cells were transferred to fresh liquid medium every 8 days and kept at 23°C, under shaking (80 rpm) in dark conditions (Iantcheva et al., 2014).

3.7.2.2. Bacterial co-cultivation

In order to allow the co-cultivation step, the engineered *A. tumefaciens* strain containing the CRISPR_Tdp1β plasmid was grown as follows. A pre-inoculum of bacterial cells was performed in 2 ml liquid medium YEP (1% yeast extract, 2% peptone, 2% glucose/dextrose) containing rifampicin (50 µg/ml) and spectinomycin (50 µg/ml), and incubated at 28°C under continuous shaking (180 rpm) for 48 h. The resulting bacterial culture was used to prepare an inoculum of 20 ml in the same medium as previously described. *Agrobacterium* cells were finally collected by centrifuging for 15 min at 3500 rpm, and the pellet was resuspended in an equal volume of liquid MS30 medium. Bacterial cultures with final OD_{600} = 1 were used for the co-cultivation step.

The *M. truncatula* cell suspension was maintained in 250 ml flasks containing 50 ml of MS30 medium. The experimental set-up included 4 flasks of suspension cells to which 4 different transformation protocols were applied:

1. co-cultivation of 1 ml of bacterial culture containing the CRISPR_Tdp1β plasmid for 2 days
2. co-cultivation of 3 ml of bacterial culture containing the CRISPR_Tdp1β plasmid for 2 days
3. co-cultivation of 1 ml of bacterial culture containing the CRISPR_Tdp1β plasmid for 5 days
4. co-cultivation of 3 ml of bacterial culture containing the CRISPR_Tdp1β plasmid for 5 days

At the end of the co-cultivation, carbenicillin (250µg/ml; used as a selection agent to eradicate *A. tumefaciens*) and kanamycin (50µg/ml; used as a selection agent for the plant cells transformed with the plasmid CRISPR_Tdp1β) were added.

3.7.2.3. Screening for the transformation events

Following the co-cultivation of *M. truncatula* suspension cells with the transformed *A. tumefaciens* strain, fluorescence optical microscope analysis was performed to verify the expression of the *GFP* reporter gene. An Olympus BX51 fluorescence microscope (Olympus Italia S.R.L., Milan, Italy) was used with an excitation filter of 485/20 and a barrier filter of 530/25 for the visualization of GFP. The images were obtained using an Olympus MagnaFire camera equipped with Olympus Cell F software (Olympus Italia S.R.L., Milan, Italy). Subsequently, DNA was extracted from *M. truncatula* cell suspension cultures using the CTAB (Hexadecyl Trimethyl Ammonium Bromide)-based protocol as previously described by Dellaporta et al. (1983). Finally, PCR analysis was performed to amplify the *eGFP* and *hCas9* genes, respectively, as described above (see Section 3.7.1.).

3.7.3. *Agrobacterium*-mediated transformation of *M. truncatula* leaf discs and petioles

For the transformation of leaf discs and peduncles, the protocol used was carried out as described by Araùjo et al. (2004). This is an efficient protocol for transformation and regeneration through embryogenesis. Using the *M. truncatula* M9-10a genotype and the methodology described, it is possible to recover transgenic plants within 4 months of *in vitro* culture.

3.7.3.1. Plant material and *A. tumefaciens* transformation

To obtain the plant material, *M. truncatula* seeds were scarified, sterilized and placed on MS30 solid medium as described above (see Section 3.7.2.1.). Subsequently, young, fully expanded leaves and petioles were used as explants for the genetic transformation. In parallel, leaves derived from *in*

vitro re-propagated plants were also utilized. The culture of *A. tumefaciens* EHA105 strain transformed with the CRISPR_Tdp1β plasmid and the *A. tumefaciens* EHA105 strain containing the control plasmid pK7WGF2::hCas9 (no sgRNA cassette) were prepared as mentioned above (see Section 5.1.2.2.). Bacterial cultures with final OD_{600} = 1.5-1.6 and the addition of acetosyringone (100 µM) (Duchefa Biochemie B.V., Netherlands) were kept in the dark for 30 min and then used for genetic transformation. Subsequently, individual leaflets or petioles were placed onto a wet sterile filter paper in a Petri dish to prevent desiccation and wounded perpendicularly to the mid-nervure using a scalpel blade previously dipped into the *Agrobacterium* suspension. Explants were transferred with the abaxial side-down (7-9 leaflets per Petri dish). Co-cultivation was carried out on solid EIM (Embryo Induction Medium) (4.4 g/l MS salts and vitamins, 30 g/l sucrose, 2 g/l gelrite, 0.45 µM 2,4-D, 0.91 µM zeatin) supplemented with 100 µM acetosyringone, in a dark growth chamber at 23°C for 5 days.

3.7.3.2. Growth of embryogenic calli

Inoculated explants were transferred to EIM containing 100 mg/l kanamycin and 500 mg/l carbenicillin to start the selection procedure, and maintained in growth chamber at 23°C, photon flux density of 150 µmol m^{-2} s^{-1}, photoperiod of 16/8 h, and 70-80% relative humidity. To maintain the selective pressure, the developed embryogenic calli were transferred weekly to fresh selective medium. Three weeks after inoculation, embryogenic calli were transferred to growth-regulator-free EPM (Embryo Proliferation Medium) (4.4 g/l MS salts and vitamins, 30 g/l sucrose, 2 g/l gelrite) supplemented with antibiotics as previously described. Calli were cultivated on the same medium (refreshed each week) until somatic embryos reached the late torpedo/dicotyledonar stage (30-60 days). The last step requires the transfer of calli to ECM (Embryo Conversion Medium) medium (4.4 g/l MS salts and vitamins, 30 g/l sucrose, 7 g/l microagar) supplemented with 170 µM kanamycin and 0.6 mM carbenicillin. The experimental setup for this protocol is given in **Table 6**.

Table 6. Schematic representation of the M9-10a transformation procedure. M, medium; A, antibiotic supplementation; EIM, Embryo Inducing Medium; EPM, Embryo Proliferation Medium; ECM, Embryo Conversion Medium. Km, kanamycin. Carb, carbenicillin.

Plant material	Explant infection and co-culture		Somatic embryogenesis induction		Embryo maturation and proliferation		Embryo conversion		Plantlet rooting	
	M	A	M	A	M	A	M	A	M	A
Wounded explants of M9-10a	EIM	100 µM acetosyringone	EIM	100 mg/Km 500 mg/l Carb	EPM	100 mg/Km 500 mg/l Carb	ECM	100 mg/Km 250 mg/l Carb	ECM	50 mg/Km
	5 days		3 sub-cultures (16 d)		4-8 sub-cultures (30-60 d)		2-4 sub-cultures (15-30 d)		2 sub-cultures (15 d)	

3.7.3.3. Selection of transformed calli

Following transformation, *M. truncatula* calli (control and transformed) were used to detect the presence of the GFP signal by using a transilluminator Biorad Chemidoc MP Imaging System and Image Lab Software (Bio-Rad Laboratories, Inc.).

3.7.4. *Agrobacterium*-mediated transformation of *Oryza sativa*

3.7.4.1. Regeneration protocol for *Oryza sativa* var. Vialone Nano

The regeneration protocol is an integration between the methods published by Hiei et al. (2006) and Sahoo et al. (2011). Mature seeds of Vialone Nano (VN) and Nipponbare (NB), used as control, were de-husked and sterilized in three steps using: (1) dH_2O and Teepol (Sigma-Aldrich) for 30 sec, (2) 70% ethanol for 30 sec, (3) commercial bleach diluted 1:3 with sterile dH_2O for 10 min (twice), (4) final rinse in sterile dH_2O under laminar air flow, (5) dry on sterile filter paper. Subsequently, sterile seeds were placed on Callus Induction Medium (CIM) (20 seeds per plate) (**Table 7**) and incubated for 2 weeks under dark at 28°C to induce callus. This time is required for the development of white and nodular embryogenic calli, which are separated by the scutellum and transferred to fresh CIM for another week under dark at 28°C. Calli were transferred on Pre-Regeneration Medium (PReM) (15 calli per plate) (**Table 7**) to start tissue differentiation and further incubated for 2 weeks under dark at 28°C. Then, calli were transferred on Regeneration Medium (ReM) (5-6 calli per plate) (**Table 7**) and incubated for 2-3 weeks under 16 h light/8 h dark conditions at 24°C with a photon flux density of 150 µmol m^{-2} s^{-1} and 70-80% relative humidity, to complete differentiation, proliferate and develop shoots. ReM contains the synthetic cytokinin 6-Benzylaminopurine (BAP, Duchefa Biochemie B.V.) and the auxin 1-Naphthaleneacetic Acid (NAA, Duchefa Biochemie B.V.). Well-developed shoots were transferred in Magenta™ containers (Sigma-Aldrich) containing Rooting Medium (RoM) (**Table 7**) and kept for 2 weeks as

previously described. Regenerated plantlets were initially grown in Magenta^{TM} containers for 2 weeks and later transferred (acclimatized) to soil pots kept in a non-cooled iron-glass greenhouse. At the end of the life cycle, seeds were collected, dried, and conserved at 4°C. The seeds collected from the greenhouse are meant to be used as starting material for following transformation experiments.

Table 7. Media composition for rice regeneration protocol. CIM, callus induction medium. PReM, pre-regeneration medium. ReM, regeneration medium. RoM, rooting medium. LS, Linsmaier and Skoog vitamins.

Stock solution	**ml/l**			
	CIM	**PReM**	**ReM**	**RoM**
N6 major I (10X)	100	100	100	-
N6 major II (10X)	100	100	100	-
MS major (10X)	-	-	-	100
MS minor (100X)	-	-	-	10
FeNaEDTA (1000X)	1	1	1	1
B5 minor (1000X)	1	1	1	-
B5 vitamins (1000X)	1	1	1	-
LS vitamins (1000X)	-	-	-	1
Glycine (1000X)	-	-	-	2
Chemical	**g/l**			
Proline	0,5	0,5	0,5	-
Glutamine	0,5	0,5	0,5	-
Casamino Acids (CEH)	0,3	0,3	0,3	-
MES	-	-	-	0,5
Inositol	-	-	-	0,1
Sucrose	30	30	30	-
Adjust volume to 1 liter				
pH	5.8	5.8	5.8	5.8
Gelrite	6	5	5	-
Plant Agar	-	-	-	4
Growth regulator	**ml/l**			
2,4-D (1000X)	3	3	-	-
6-BAP (1 mg/l)	-	2	3	-
ABA (5 mg/l)	-	5	-	-
NAA (1 mg/l)	-	1	-	-

3.7.4.2. Plant material, plasmid and *Agrobacterium* culture

The transformation procedure was carried out using a standard plasmid kindly provided by Ming-Bo Wang (CSIRO, Commonwealth Scientific and Industrial Research Organization - Agriculture and Food, Canberra, Australia). The plasmid (15.430 bp) contains the *GUS* reporter gene encoding β-glucuronidase, the *HPT* gene conferring hygromycin resistance, and the *SpecR* gene conferring spectinomycin resistance, as selectable marker for bacteria.

Mature seeds of VN and NB varieties were de-husked, sterilized, and placed on CIM as described in the above regeneration protocol (see Section 3.7.4.1). Embryogenic calli of both varieties were sub-cultured for another week on fresh CIM. Two days before transformation, the *Agrobacterium* culture (5 g beef extract, 1 g yeast extract, 5 g sucrose, 5 g peptone, 0.98 g $MgSO_4$, 15 g agar, 50 mg spectinomycin, 25 mg rifampicin, pH 7.5) containing the plasmid was prepared and grown for 48 h at 28°C. Subsequently, the culture was centrifuged for 10 min at 4000 rpm and resuspended to an OD_{600} ~ 1. The resuspended culture was transferred to Petri dishes containing the calli (15 calli per plate) on liquid Co-Cultivation Medium (CCM) (**Table 8**) and kept for 15 min at room temperature under shaking. The calli were dried on sterile filter paper and plated onto Petri dishes containing solid CCM. The calli were co-cultivated for 3 days in a growth chamber at 28±2°C under dark. As controls (CTRL), non-transformed calli of each variety were used and placed on CCM solid medium under the same conditions.

3.7.4.3. Standard *Agrobacterium*-mediated transformation protocol for rice

After co-cultivation, the calli must be thoroughly washed (two times for 1 min and four times for 15 min with sterile dH_2O and cefotaxime 250 mg/l) and dried (on sterile paper) to remove bacteria. Subsequently, 10-15 calli per plate were transferred on Selection Medium I (SMI) supplemented with antibiotics (**Table 8**). The control calli were separated as follows: half on SMI (denominated as CTRL) which serve as a negative control (as non-transformed these will die under antibiotic selection), and another half on SMI without antibiotics (denominated CTRL/CTRL) which serve as control for the efficiency of the regeneration protocol. All material was incubated for two weeks at 26-28°C under dark conditions. A subsequent selection was carried out on Selection Medium II (SMII) supplemented with antibiotics (**Table 8**) whereas the CTRL/CTRL calli were transferred on SMII without antibiotics. The calli were incubated for two weeks at 26-28°C under dark

conditions. After the two selection steps (SMI and SMII), it is expected that the small transgenic hygromycin resistant calli would start to proliferate.

Calli were transferred to PReM supplemented with cefotaxime (100 mg/l) and hygromycin (50 mg/l), while CTRL/CTRL calli were transferred on the same medium without antibiotics and incubated at 26-28°C under dark conditions. After two weeks, or when proliferating calli have increased in size, they were transferred to ReM supplemented with hygromycin (25 mg/l) and incubated for 2-3 weeks at 28°C under light conditions. In about 1-2 weeks calli turned green and shoots started to differentiate. The root induction and acclimatization steps were performed as previously.

Table 8. Media composition for rice transformation protocol. CCM, co-coltivation medium. SMI, selection medium I. SMII, selection medium II.

Stock solution		**ml /l**	
	CCM	**SMI**	**SMII**
R2 major I (10X)	100	100	100
N6 major II (10X)	88.5	88.5	88.5
R2 minor (1000X)	1	1	-
B5 minor (1000X)	-	-	1
FeNaEDTA (10X)	1	1	1
B5 vitamins (1000X)	-	-	1
LS vitamins (400X)	2.5	2.5	-
Chemical		**g /l**	
Proline	-	-	0.5
Glutamine	-	-	0.5
Casamino Acids (CEH)	-	-	0.3
Glucose	10	-	-
Sucrose	-	30	30
Adjust volume to 1 liter			
pH	5.2	6	6
Gelrite	-	5	5
Acetosyringone	150 μM	-	-
Growth regulator		**ml /l**	
2,4-D (1000X)	3	3	3
Antibiotic		**mg /l**	
Cefotaxime	-	400	400
Hygromycin	-	50	100

3.7.4.4. Protocol optimization for the transformation of Vialone Nano cultivar

As the firstly developed protocol was unsuccessful for Vialone Nano, the next combination of techniques implemented was based on the protocols developed by Sah et al. (2014) and Slamet-Loedin et al. (2014). The CCM and RoM media were used also in this protocol. As a first step, it was necessary to assess the optimal concentration of 2,4-D for the efficient development of VN calli.

The experiment was set up using the same components of the previous cited media while changing the 2,4-D concentration as follows: 1, 2, 3, 4, and 5 mg/l. VN and NB seeds were sterilized as described above (see Section 5.1.4.1.) and placed on CIM with different 2,4-D concentration and plates were kept for two weeks at 25± 1°C under dark conditions. After the white and nodular calli were separated by scutellum, these were subjected to the previously described regeneration protocol (see Section 7.3.4.1).

Hence, the implemented experimental design used, in parallel, two different protocols, hereafter denominated as (F: Hiei and Sahoo) and (A: Sah and Slamet-Loedin). Six different types of media combinations were used as follow (**Table 9**):

1) A medium with [1 mg/l] of 2,4-D called A1
2) A medium with [2 mg/l] of 2,4-D called A2
3) A medium with [3 mg/l] of 2,4-D called A3
4) F medium with [1 mg/l] of 2,4-D called F1
5) F medium with [2 mg/l] of 2,4-D called F2
6) F medium with [3 mg/l] of 2,4-D called F3

In this experiment, four different modes of inoculation with *A. tumefaciens* were implemented. Changes consisted in: (1) decreasing the time (mins) of co-cultivation of calli with bacteria, (2) decreasing the bacteria concentration (OD), (3) decreasing the number of days of calli incubation on CCM (**Table 10**). In both protocols (A and F), the type and amount of antibiotics used were the same as in the previous transformation test (see Section 3.7.4.3. **Table 8**). Following the washing steps as previously described earlier (see Section 7.3.4.3.), calli transformed with protocol (A) were placed on SM, whereas calli transformed with protocol (F) were placed on SMI. After 14 days, calli were transferred on fresh SM for protocol (A) or to SMII for protocol (F).

Table 9. Media composition for rice transformation protocol. Comparison between media of protocol (A) and media of protocol (F). CIM, callus induction medium. SM, selection medium. PReM, pre-regeneration medium. ReM, regeneration medium.

Stock solution	**ml/l**							
	CIM		**SM**		**PReM**		**ReM**	
	F	A	F	A	F	A	F	A
N6 major I (10X)	100	-	100	200	100	-	100	-
N6 major II (10X)	100	-	88.5	100	100	-	100	-
FeNaEDTA(10X)	10	10	10	10	10	10	10	10
MS major (10X)	-	100	-	-	-	100	-	100
MS minor (100X)	-	10	-	-	-	10	-	10
B5 minor (1000X)	1	-	-	30	1	-	1	-
B5 vitamins (100X)	1	-	-	5	1	-	1	-
MS vitamins (400X)	-	1	2.5	-	-	5	-	5
R2 minor (1000X)	-	-	1	-	-	-	-	-
Glycine (100X)	-	10	-	10	-	-	-	-
Chemical	**g /l**							
Proline	0.5	0.6	-	0.5	0.5	0.6	0.5	-
Glutamine	0.5	-	-	0.3	0.5	-	0.5	-
CEH	0.3	-	-	0.5	0.3	-	0.3	-
Sucrose	30	-	30	-	30	-	30	30
Maltose	-	40	-	20	-	30	-	-
Mannitol	-	-	-	36	-	-	-	-
Sorbitol	-	-	-	-	-	20	-	-
Inositol	-	0.1	-	-	-	-	-	-
MES	-	0.5	-	-	-	-	-	-
Adjust volume to 1 liter								
pH	5.8	5.8	6	5.8	5.8	5.8	5.8	5.8
Gelrite	5	6	5	5	6	6	6	6
Growth regulator	**ml/l**							
2,4-D (1000X)	1-2-3	1-2-3	1-2-3	1-2-3	-	-	-	-
6-BAP (1 mg/ml)	-	-	-	0.2	2	-	3	-
ABA (5 mg/ml)	-	-	-	-	5	-	-	-
NAA (1 mg/ml)	-	-	-	1	1	1	0.5	1
Kinetin (2 mg/ml)	-	-	-	-	-	2	-	2
Antibiotic	**mg/l**							
Cefotaxime	-	-	400	400	100	100	100	100
Hygromycin	-	-	50	50	50	50	25	25

Table 10. Different combinations utilized for the inoculation of Vialone Nano calli with *Agrobacterium*. Co-Co, co-coltivation.

Combinations	Co-Co time (min)	Co-Co days	OD
1	15	3	1
2	15	3	0.5
3	5	2	1
4	5	2	0.5

3.7.4.5. Screening for the transformation events

Leaves from control and transformed rice plants were sampled to confirm the presence of the transgene. The GUS (β-glucuronidase) assay and PCR analysis were performed. The GUS assay was performed as previously reported by Jefferson and Wilson (1991), by placing the leaves collected from transformed and CTRL plants in a Petri dish containing the GUS assay solution containing 1 mM X-Gluc (5-bromo-4-choloro-3-indolyl) β-D-glucuronic acid, Sigma-Aldrich) in 50 mM Na_2HPO_4, pH 7.0 and 0.1% Triton X-100 (Sigma-Aldrich). Samples were incubated in the assay solution at 37°C overnight. Since the blue color is difficult to visualize against the dark green background of mature leaves, samples were then placed in 70% ethanol and de-stained. Further assessment was carried out using standard PCR (DreamTaq DNA Polymerase, Thermo Scientific™, following the suppliers' instructions) with GUS specific primers (forward- 5'-ATGGTCCGTCCTGTAGAAACCCC-3' and reverse- 5'-CACCACCTGCCAGTCAACAGACG-3').

3.8. Statistical analysis

In eggplant and wild relatives, the effects of priming *versus* unprimed control, in terms of germination percentage, days, and their interaction were analyzed using Two-way ANOVA (Analysis of Variance) ('*' $P < 0.05$; '**' $P < 0.01$, '***' $P < 0.001$, '****' $P < 0.0001$) carried out with the statistical software GraphPad Prism 8 (GraphPad Software Inc., California). Comparison between unprimed control and different priming treatments were carried out as follows. For each treatment, three biological replicates were considered. Means were then compared using the Post-Hoc Tukey's HSD (Honest Significant Difference) test. Means with a significance value lower than 0.05 ($P \leq 0.05$) were considered statistically different. Statistical analysis of phenotyping data and *q*RT-PCR data was performed using the Student's *t*-test. Asterisks indicate statistically significant differences determined using Student's *t*-test ('*' $P < 0.05$; '**' $P < 0.01$, '***' $P < 0.001$). The efficacy of treatment *versus* unprimend control was carried out using Mann- Withney test ('*' $P < 0.05$; '**' $P < 0.01$, '***' $P < 0.001$). Principal Component Analysis (PCA) was performed using all measured

parameters (genotype, hydropriming treatment, germination %, ROS levels, gene expression profiles) and the ClustVis program available at https://biit.cs.ut.ee/clustvis/ (Metsalu and Vilo, 2015).

In the *M. truncatula* experiments, for each variable, significant differences between treatments were determined with Two-way ANOVA (Analysis of Variance) using the statistical tool developed by Assaad et al. (2015). For each treatment, five biological replicates were considered. Means were compared using the Duncan test, where means with a significance value lower than 0.05 were considered statistically different. Letters are used to indicate significant differences among all samples. The Student' *t*-test (*, $p < 0.05$) was used to statistically compare the gene expression profiles in *M. truncatula* seeds, where HP2 and HP4 were reported to the dry seeds (DS) for each gene.

Principal Component Analysis was performed using all measured parameters (germination %, T50, seedling length, FW, DW, gene expression profiles) and the ClustVis program. Pattern search analysis was conducted using the PatternHunter tool available at https://biit.cs.ut.ee/clustvis/ (Metsalu et al., 2015).

4. Results

4.1 Seed priming: from the lab to the field

4.1.1. Screening of germination profiles in an eggplant germplasm seed bank

A screening of the entire germplasm collection composed of 380 genotypes hosted at CREA-GB (Montanaso Lombardo, Lodi, Italy) was carried out in pots to evaluate the germination profile. Following this preliminary test, 49 different accessions, comprising 35 *S. melongena* varieties and 14 wild relatives were selected (**Fig. 16**). For all the 49 genotypes, germination tests were performed in Petri dishes at DBB-UNIPV, in order to monitor the timing of radicle protrusion, and classify those eggplant varieties and wild relatives having contrasting phenotype in terms of dormancy (**Tab. 11**).

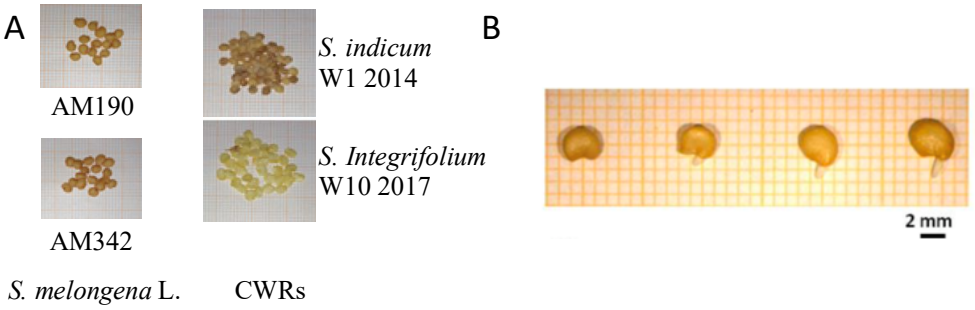


Fig. 16. (A) Seeds of some eggplant varieties and wild relatives of the CREA-GB germplasm collection used for the screening of germination profiles. (**B)** *S. melongena* L. seeds at the phenological stage of radicle protrusion.

Table 11. List of genotypes from the CREA-GB germplasm collection subjected to germination tests and classified, based on the time required to observe radicle protrusion, as Early: 0-6 days, Normal: 7-14 days, Late: >14 days. Values are expressed as mean ± SD of three independent replications with 15 seeds for each replication. CWR, crop wild relative. n.d., not detected due to microbial contamination.

S. melongena **Accession N° (*)**	**Germination (%)**	**Phenotype**
AM11	98.33 ± 2.36	Normal
AM 29	96.67 ± 4.70	Early
AM45	100.00 ± 0.00	Early

AM109	100.00 ± 0.00	Early
AM116	100.00 ± 0.00	Early
AM146	100.00 ± 0.00	Early
AM158	98.33 ± 2.36	Early
AM179	98.33 ± 2.36	Early
AM181	100.00 ± 0.00	Early
AM190	100.00 ± 0.00	Early
AM192	68.33 ± 15.46	Normal
AM201	100.00 ± 0.00	Early
AM210	100.00 ± 0.00	Normal
AM211	20.00 ± 16.33	Early
AM212	90.00 ± 8.16	Normal
AM224	93.33 ± 6.24	Normal
AM226	50.00 ± 24.5	Early
AM238	98.33 ± 2.36	Normal
AM243	n.d.	-
AM245	100.00 ± 0.00	Normal
AM248	98.33 ± 2.36	Early
AM274	75.00 ± 14.72	Early
AM278	100.00 ± 0.00	Early
AM279	100.00 ± 0.00	Normal
AM280	93.33 ± 4.71	Normal
AM286	100.00 ± 0.00	Normal
AM288	100.00 ± 0.00	Early
AM293	100.00 ± 0.00	Early
AM305	100.00 ± 0.00	Early
AM312	100.00 ± 0.00	Early
AM313	73.33 ± 12.47	Early
AM323	93.33 ± 9.43	Early
AM333	96.67 ± 4.71	Early
AM342	90.00 ± 14.14	Early
AM375	98.33 ± 2.36	Early
CWR (year of collection)	**Germination (%)**	**Phenotype**
S. aethiopicum (2011)	98.33 ± 2.36	Early
S. consona (2007)	22.22 ± 15.71	Late
S. indicum (2014)	n.d.	-
S. indicum (2015)	n.d.	-
S. integrifolium (2008)	80.00 ± 16.33	Normal
S. integrifolium (2017)	100.00 ± 0.00	Early
S. mistretta (2005)	n.d.	-
S. mistretta (2007)	33.33 ± 27.22	Late
S. santo stefano (2005)	n.d.	-
S. santo stefano (2007)	44.44 ± 15.71	Late
S. sodomeum (1987)	n.d.	-
S. torvum (2013)	n.d.	-
S. torvum (2017)	100.00 ± 0.00	Normal
S. virginianum (2013)	100.00 ± 0.00	Normal

(*) The *S. melongena* accessions hereby listed were collected in different years from 1987 to 2017.

At the end of the germination tests, based on the time required to observe radicle protrusion, the eggplant and CWR genotypes were classified as "EARLY" if germination took place within 6 days from the beginning of imbibition, "NORMAL" if germination occurred between 7 and 14 days, and "LATE" if germination took place after 14 days (**Tab. 11**). Among the tested samples, 24 eggplant varieties and two CWR genotypes (*S. aethiopicum*, *S. integrifolium*) were classified as "EARLY" whereas 10 eggplant varieties three CWR genotypes (*S. integrifolium, S. torvum, S. virginianum*) were classified as "NORMAL". Finally, three CWR genotypes (*S. consona*, *S. mistretta*, *S. santo stefano*) were classified as "LATE". germinated after 14 days. The seeds of seven accessions showed molds contamination on their surface and they did not germinate. Data from germination tests were further elaborated by calculating specific parameters according to Ranal et al. (2006) and the estimated values are showed in **Tab. 12**.

Table 12. Germination parameters calculated based on results of germination tests carried out in *S. melongena* and wild relatives (CWR). Germinability (*G*). Mean germination time (*MGT*). Coefficient of variation of the time (*CV*). Mean germination rate (*MR*). Uncertainty (*U*). Synchronization index (*Z*). Values are expressed as mean ± SD of three independent replications with 15 seeds for each replication.

S. melongena **Accession N°**	***G*** **(%)**	***MGT*** **(days)**	***CV*** **(%)**	***MR*** **(day^{-1})**	***U*** **(bit)**	***Z***
AM11	98.33 ± 2.36	11.98 ± 0.23	68 ± 2.02	0.015 ± 0.0005	2.181 ± 0.530	0.223 ± 0.1
AM 29	96.67 ± 4.70	9.71 ± 1.47	36.56 ± 10.10	0.029 ± 0.0082	2.173 ± 0.37	0.157 ± 0.08
AM45	100 ± 0.00	10.73 ± 2.21	96.58 ± 45.84	0.012 ± 0.0045	2.33 ± 0.29	0.205 ± 0.052
AM109	100 ± 0.00	4.70 ± 0.38	336.73 ± 35.96	0.003 ± 0.0003	1.705 ± 0.448	0.368 ± 0.139
AM116	100 ± 0.00	4.29 ± 0.07	360.11 ± 20.95	0.003 ± 0.0002	1.25 ± 0.087	0.431 ± 0.029
AM146	100 ± 0.00	5.22 ± 0.51	293.34 ± 36.66	0.004 ± 0.0005	0.70 ± 0.75	0.693 ± 0.328
AM158	98.33 ± 2.36	5.53 ± 1.15	265.44 ± 60.37	0.004 ± 0.001	1.80 ± 0.67	0.334 ± 0.189
AM179	98.33 ± 2.36	6.97 ± 1.08	193.73 ± 53.21	0.005 ± 0.0015	2.178 ± 0.46	0.218 ± 0.088
AM181	100 ± 0.00	3.77 ± 0.32	177.86 ± 23.70	0.006 ± 0.0007	1.271 ± 0.370	0.415 ± 0.110
AM190	100 ± 0.00	5.00 ± 0.98	116.73 ± 44.39	0.009 ± 0.0031	2.015 ± 0.28	0.222 ± 0.059
AM192	68.33 ± 15.46	12.57 ± 0.85	42.80 ± 14.81	0.026 ± 0.0105	2.582 ± 0.74	0.149 ± 0.125
AM201	100 ± 0.00	8.23 ± 0.25	26.55 ± 2.97	0.038 ± 0.0045	1.884 ± 0.221	0.229 ± 0.056
AM210	100 ± 0.00	9.47 ± 0.60	115.62 ± 12.92	0.009 ± 0.001	1.885 ± 0.567	0.345 ± 0.166
AM211	20 ± 16.33	7.00 ± 8.89	45.90 ± 79.50	8.67 ± 15.01	0.333 ± 0.57	0.00 ± 0.00

AM212	90 ± 8.16	12.32 ± 2.07	41.78 ± 6.36	0.024 ± 0.004	2.363 ± 0.171	0.113 ± 0.042
AM224	93.33 ± 6.24	16.02 ± 0.65	25.17 ± 9.74	0.005 ± 0.0206	2.664 ± 0.325	0.133 ± 0.049
AM226	50 ± 24.5	11.09 ± 1.57	81.09 ± 50.89	0.015 ± 0.007	1.693 ± 0.61	0.081 ± 0.073
AM238	98.33 ± 2.36	15.34 ± 0.34	32.90 ± 5.21	0.031 ± 0.0048	2.468 ± 0.252	0.167 ± 0.041
AM245	100 ± 0.00	9.07 ± 0.50	125.22 ± 12.34	0.005 ± 0.0008	19.68 ± 0.282	0.263 ± 0.074
AM248	98.33 ± 2.36	7.88 ± 0.30	151.81 ± 11.58	0.007 ± 0.0005	2.179 ± 0.18	0.217 ± 0.04
AM274	75 ± 14.72	11.59 ± 0.38	59.31 ± 19.04	0.018 ± 0.005	3.146 ± 0.111	0.065 ± 0.035
AM278	100 ± 0.00	11.13 ± 1.74	20.81 ± 6.97	0.052 ± 0.019	2.243 ± 0.281	0.422 ± 0.408
AM279	100 ± 0.00	9.93 ± 0.32	12.50 ± 2.21	0.082 ± 0.0133	1.989 ± 0.206	0.230 ± 0.034
AM280	93.33 ± 4.71	14.76 ± 0.92	42.10 ± 3.55	0.005 ± 0.0021	2.428 ± 0.255	0.115 ± 0.050
AM286	100 ± 0.00	14.07 ± 2.10	74.52 ± 7.66	0.014 ± 0.0014	1.738 ± 0.511	0.167 ± 0.153
AM288	100 ± 0.00	6.20 ± 1.73	42.10 ± 3.55	0.016 ± 0.011	2.193 ± 0.443	0.171 ± 0.09
AM293	100 ± 0.00	5.90 ± 0.35	74.26 ± 9.90	0.014 ± 0.0017	1.121 ± 0.339	0.474 ± 0.102
AM305	100 ± 0.00	4.02 ± 0.29	412.52 ± 36.49	0.002 ± 0.0002	2.329 ± 0.120	0.193 ± 0.016
AM312	100 ± 0.00	6.47 ± 1.42	73.72 ± 25.41	0.015 ± 0.005	2.084 ± 0.558	0.193 ± 0.114
AM313	73.33 ± 12.47	6.36 ± 0.86	50.84 ± 21.27	0.022 ± 0.008	2.015 ± 0.192	0.141 ± 0.05
AM323	93.33 ± 9.43	9.17 ± 1.35	33.36 ± 11.27	0.032 ± 0.0103	2.206 ± 0.143	0.154 ± 0.047
AM333	96.67 ± 4.71	9.13 ± 0.57	22.36 ± 8.48	0.05 ± 0.021	1.331 ± 0.75	0.461 ± 0.297
AM342	90 ± 14.14	9.37 ± 4.84	84.89 ± 22.18	0.012 ± 0.004	2.313 ± 0.170	0.141 ± 0.034
AM375	98.33 ± 2.36	4.79 ± 0.29	319.84 ± 16.83	0.003 ± 0.0002	1.492 ± 0.276	0.406 ± 0.128

CWR	*G* (%)	*MGT* (days)	*CV* (%)	*MR* (day^{-1})	*U* (bit)	*Z*
S. aethiopicum	98.33 ± 2.36	6.74 ± 0.53	200.03 ± 17.38	0.005 ± 0.0004	2.429 ± 0.334	0.173 ± 0.036
S. consona	22.22 ± 15.71	11.67 ± 10.12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.267 ± 0.00
S. integrifolium	80 ± 16.33	12.99 ± 0.64	88.56 ± 16.93	0.005 ± 0.0021	1.669 ± 0.223	0.089 ± 0.084
S. integrifolium	100 ± 0.00	10.00 ± 0.61	22.13 ± 3.98	0.046 ± 0.008	1.859 ± 0.405	0.267 ± 0.102
S. mistretta	33.33 ± 27.22	15.33 ± 13.61	42.43 ± 73.48	0.003 ± 0.005	0.00 ± 0.00	0.267 ± 0.577
S. santo stefano	44.44 ± 15.71	20.83 ± 5.01	43.59 ± 75.50	0.005 ± 0.005	0.333 ± 0.577	0.00 ± 0.00
S. torvum	100 ± 0.00	8.83 ± 0.51	131.63 ± 13.29	0.008 ± 0.0007	1.916 ± 0.486	0.214 ± 0.056
S. virginianum	100 ± 0.00	15.47 ± 1.29	81.99 ± 5.25	0.005 ± 0.001	1.738 ± 0.511	0.167 ± 0.153

Results of these analysis revealed that almost all the wild relatives were characterized by longer germination times compared to the eggplant accessions. Among these 49 genotypes, the following accessions were chosen for further analyses:

i) AM210 also known as the inbred line '67/3' (classified as NORMAL) of high relevance for molecular investigations because both reference genome and transcriptome are available (Barchi et al., 2019, www.eggplantgenome.org)
ii) the CWR *Solanum torvum* Swartz 1788 (turkey berry) (classified as NORMAL) was chosen due to its relevance as a source of genes for the resistance to root-knot nematodes and soil-borne diseases. Indeed, this wild relative is widely used as rootstock for grafting in eggplant cultivation (Gousset et al., 2005). Low seed quality is a recurrent issue in *S. torvum* that often shows strong physiological dormancy and several factors must be taken into account when dealing with such a recalcitrant system (Ranil et al., 2015).
iii) the CWR *Solanum villosum* Miller (hairy nightshade) classified as EARLY was selected for the high germination speed and homogeneity.

Germination profiles of these three varieties are shown in **Fig. 17**.

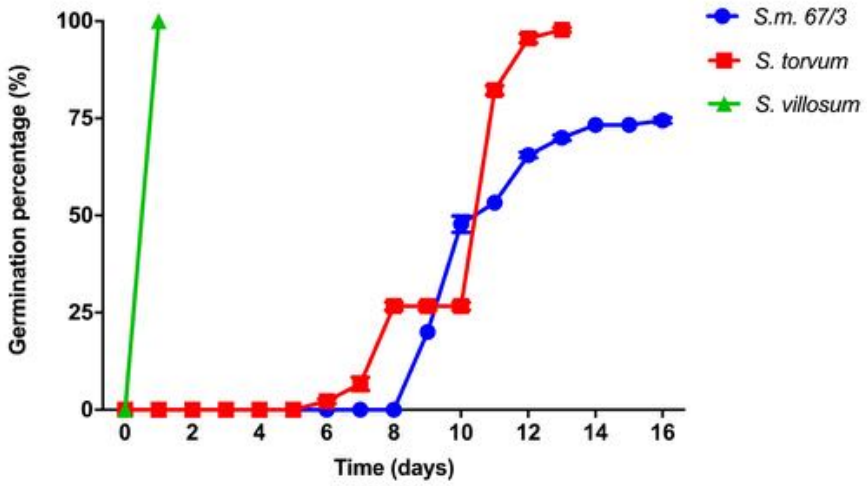


Fig. 17. Germination curves of *S. melongena* '67/3', *S. torvum* and *S.villosum*. Values are expressed as mean ± SD of three independent replications with 20 seeds for each replication.

In the case of '67/3', germination started at the 9th day from the beginning of imbibition with an estimated value of 20±1.41%. Germination percentage

further increased up to 74.44±0.76 % at the 16th day and values did not change until the end of the experiment (20th day). For *S. torvum*, germination started at the 6th day (2.22±0.58%) and stopped at the 13th day reaching the maximum value of 97.78±0.58%. On the contrary, *S. villosum* seeds reached the highest germination percentage (100%) in one day.

The availability of seeds with contrasting germination profiles, and thus different levels of vigor, represents a suitable experimental system for investigating the impact of priming as well as the molecular mechanisms underlying the seed response to these treatments. Understanding how these seeds work at the physiological and molecular level, in the context of the pre-germinative metabolism, is an essential step towards the design of improved priming protocols and the identification of quality hallmarks.

4.1.2. Testing the seed lot-dependent variability of the '67/3' line, *S. torvum* and *S. villosum*

Germination performance can significantly vary among seed lots of the same accession, an undesired feature for commercially relevant crops. Such a variability was observed also in this work, for both the '67/3' line and the CWRs. A seed lot is defined as a group of seeds that share features as year of collection, geographical provenance, environmental conditions in which they were grown, harvest and storage conditions.

For the '67/3' genotype seed lots collected during five different years (2014, 2015, 2016, 2017 and 2018) were analyzed. Results of germination tests and the related parameters are shown in **Fig. 18** and **Tab. 14**.

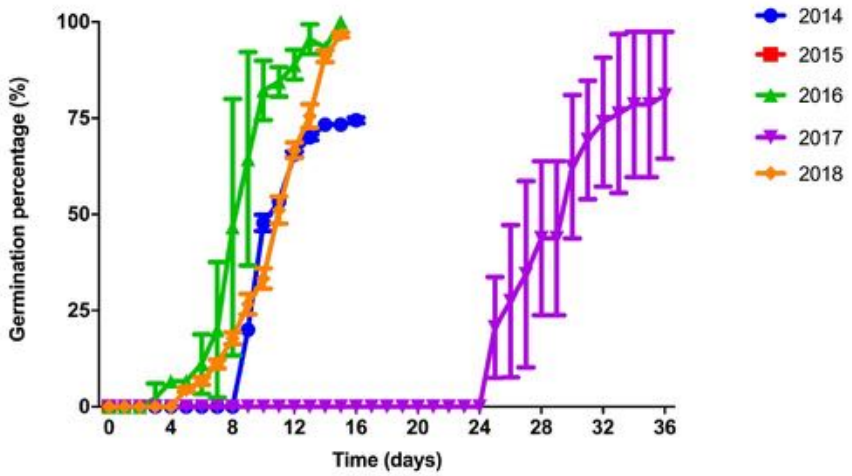


Fig. 18. Germination curves of five '67/3' seed lots collected in different years (2014, 2015, 2016, 2017 and 2018). Values are expressed as mean ± SD of three independent replications with 20 seeds for each replication.

As shown in **Fig. 18**, the seed lot collected in 2014 started germination at the 9th day from the beginning of imbibition and reached the maximum germination percentage (74.44±0.76%) at the 16th day. The seed lot collected in 2015 was unable to germinate after 36 days from the beginning of imbibition. In the case of the year 2016, germination occurred at the 3rd day (6.67±0.00%) from the beginning of imbibition and reached the maximum value (100±0.00 %) at 15 days. The seed lot collected in 2018 started to germinate at the 5th day (4.44±0.58%) and the maximum value (96.67±0.71%) was recorded at 15 days. Results of statistical analysis carried out using Two-way ANOVA (F: 27.79; DF: 140; *P*: 0.0001****) as well as comparison between different seed lots carried out using the Post-Hoc Tukey's HSD test ($P \leq 0.05$) are shown in **Table 13**.

Table 13. Results of statistical analysis carried out using Two-way ANOVA (F: 27.79; DF: 140; *P*: 0.0001****). A comparison between the different seed lots (2014-2015-2016-2017 and 2018) were carried out using the Post-Hoc Tukey's HSD test ($P \leq 0.05$).

Comparison	q	DF	*P* value	Significance
2014-2015	85.15	360	<0.0001	****
2014-2016	34.14	360	<0.0001	****
2014-2017	52.02	360	<0.0001	****
2014-2018	25.62	360	<0.0001	****
2015-2016	114.3	360	<0.0001	****
2015-2017	28.13	360	<0.0001	****
2015-2018	105.8	360	<0.0001	****
2016-2017	86.16	360	<0.0001	****
2016-2018	8.53	360	<0.0001	****
2017-2018	77.64	360	<0.0001	****

In the case of *S.torvum*, seed lots collected during 2004, 2017 and 2018 were tested (**Fig. 19** and **Tab. 14**). Only the seed lot collected in year 2017 was able to germinate, differently from the lots collected in 2004 and 2018. As shown in **Fig. 19**, the seed lot of 2017 started to germinate at the 7th day from the beginning of imbibition (18.33±8.50%), reaching a maximum value at the 15th day (100±0.00%).

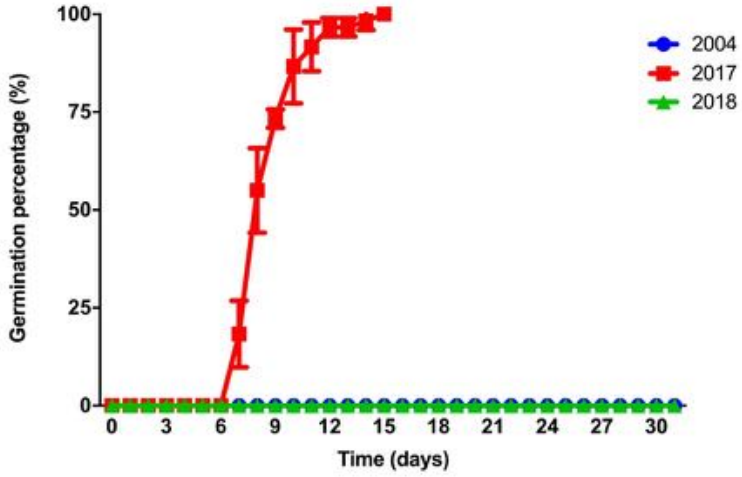


Fig. 19. Germination curve of the *S. torvum* seed lot collected in 2017. Values are expressed as mean ± SD of three independent replications with 20 seeds for each replication.

Two distinct *S. villosum* seed lots, collected in 2015 and 2018, were analyzed. As shown in **Fig. 20**, germination started at the 2nd and 3rd day from the beginning of imbibition, with estimated values of 50±15.01% (2015) and 23.33±23.09% (2018) and reaching the maximum level (100±0.00%) at the 3rd and 4th day, respectively. Additional parameters calculated to evaluate the variability of the germination process are showed in **Table 14**. Results of statistical analysis carried out using Two-way ANOVA (F: 204.6; DF: 4; *P*: 0.0001****) reveal a significant difference between the two seed lots.

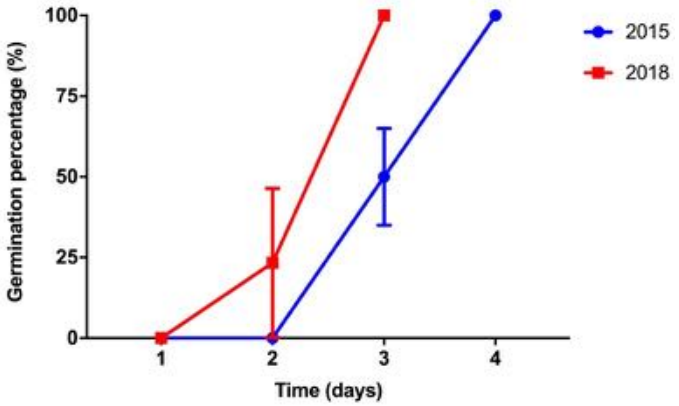


Fig. 20. Germination curve of the *S. villosum* seed lots collected in 2015 and 2018. Values are expressed as mean ± SD of three independent replications with 20 seeds for each replication.

Table 14. Germination parameters calculated based on results of germination tests carried out in different *S. melongena* '67/3' and CWRs seed lots. Germinability (*G*). Mean germination time (*MGT*). Coefficient of variation of the time (*CV*). Mean germination rate (*MR*). Uncertainty (*U*). Synchronization index (*Z*). Values are expressed as mean ± SD of three independent replications with 15 seeds for each replication.

Seed lot (years)	***G* (%)**	***MGT* (days)**	***CV* (%)**	***MR* (day^{-1})**	***U* (bit)**	***Z***
'67/3' (2014)	74.44 ± 1.58	10.59 ± 0.01	220.39 ± 6.56	0.0045 ± 0.00014	2.227 ± 0.018	0.231 ± 0.016
'67/3' (2016)	100 ± 0.00	8.95 ± 0.91	76.34 ± 17.86	0.005 ± 0.0034	2.393 ± 0.052	0.178 ± 0.006
'67/3' (2017)	77.78 ± 19.24	28.56 ± 1.34	62.70 ± 12.05	0.005 ± 0.003	2.525 ± 0.137	0.118 ± 0.013
'67/3' (2018)	97.78 ± 3.85	11.05 ± 1.10	42.03 ± 9.86	0.025 ± 0.006	2.50 ± 0.29	0.15 ± 0.05
CWR	***G* (%)**	***MGT* (days)**	***CV* (%)**	***MR* (day^{-1})**	***U* (bit)**	***Z***
S. torvum (2017)	100 ± 0.00	8.83 ± 0.51	131.63 ± 13.29	0.008 ± 0.0007	1.916 ± 0.486	0.214 ± 0.056
S. villosum (2015)	100 ± 0.00	2.49 ± 0.10	521.42 ± 25.26	0.005 ± 0.00009	0.980 ± 0.015	0.478 ± 0.01
S.villosum (2018)	100 ± 0.00	2.1 ± 0.05	875.01 ± 23.22	0.0012 ± 0.00015	0.46 ± 0.16	0.81 ± 0.01

The parameters taken in consideration describe the seed lots quality and predict the seedling performance. Based on the results obtained from the germination test and relative parameters analysis, it is possible to classify the seed lots as 'low-quality' and 'high-quality'. The '67/3' seed lots collected in 2014, 2016 and 2018 were classified as high-quality while those of 2015 and 2017 were defined as low-quality. In the case of CWRs, the *S. torvum* seeds of 2017 and both *S. villosum* lots were classified as high-quality.

4.1.3. Development of an optimized hydropriming protocol for the *Solanum melongena* '67/3' line

The cultivated eggplant is susceptible to a variety of biotic and abiotic stresses, which may cause great losses in yields and reduce quality of the final products. Along with this unfavorable feature, eggplant frequently shows seed dormancy, low germination rates and uniformity levels. The availability of effective priming protocols is expected to boost seed quality. Thus, the subsequent step in this work was the design of optimized priming protocols to speed up germination of eggplant seeds and the molecular characterization of the pre-germinative metabolism challenged by the treatments. To this purpose, the experimental work was performed using the '67/3' line, whose genome is currently available. Among the different types of priming, hydropriming (HP) was chosen because this is a low cost, simple and reliable treatment, with limited economic impact on seed industry and extensive field application.

HP was first tested on '67/3' seeds collected in 2014. Seeds soaked in H_2O for 24 h, 48 h, 72 h, and 96 h (hereby named treatments HP24, HP48, HP72, and HP96) were subjected to dry-back (DB, 2 h) and then immediately used for germination tests. Results of germination tests carried with treated seeds and unprimed (UP) seeds are shown in **Fig. 21.** UP seeds started germination at 9 days from the beginning of imbibition and reached the maximum germination percentage (74.44±0.71%) at 16 days whereas hydropriming always resulted in anticipated germination compared to untreated seeds. In the case of primed seeds, germination occurred at 5 days (40.00±0.00 %, HP24), 4 days (6.00±0.71 %, HP48), 3 days (2.00±0.71 %, HP72), and 2 days (4.00±0.00 %, HP96) from the beginning of imbibition. Results of statistical analysis carried out using Two-way ANOVA (F: 45.66; DF: 4; *P*: 0.0004***) as well as comparison between UP seeds and primed seeds (HP24, HP48, HP72, and HP96) carried out using the Post-Hoc Tukey's HSD test ($P \leq 0.05$) are shown in **Table 15**.

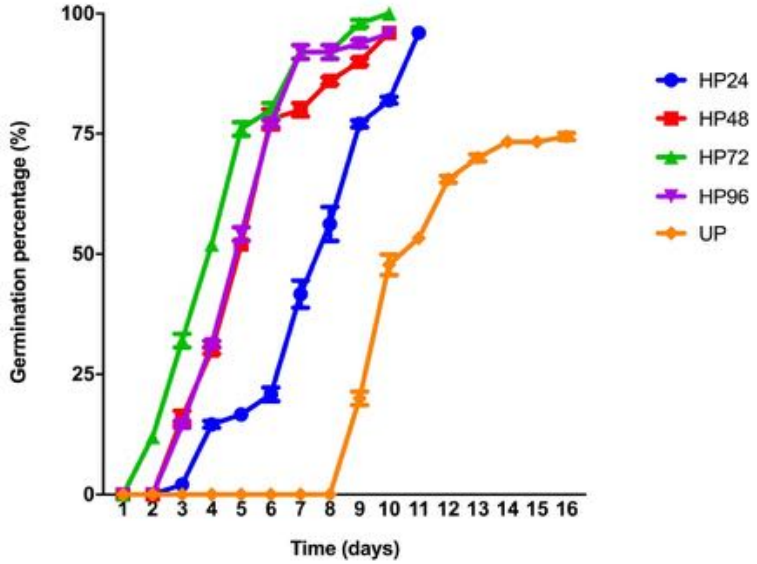


Fig. 21. Results from germination tests carried out with '67/3' seeds treated with hydropriming for 24, 48, 72, and 96 h and unprimed (UP) seeds. Values are expressed as mean ± SD of three independent replications with 15 seeds for each replication. Statistical analysis was carried out using Two-way ANOVA (F: 45.66; DF: 4; *P*: 0.0004***). Comparison between UP and HP24, HP48, HP72, HP96 were carried out using the Post-Hoc Tukey's HSD test ($P \leq 0.05$) (see **Table 15**).

Table 15. Results of statistical analysis carried out using Two- way ANOVA (F: 45.66; DF: 4; *P*: 0.0004***). Comparison between UP and HP24, HP48, HP72, HP96 were carried out using the Post-Hoc Tukey's HSD test ($P \leq 0.05$).

UP *vs* HP	q	DF	*P*	Significance
24 h	4.090	61.70	0.0407	*
48 h	4.674	61.39	0.0133	*
72 h	6.141	62.00	0.0005	***
96 h	6.007	60.94	0.0007	***

Data collected from this experiment were used to calculate the previously described germination parameters. The peak value (*PV*) is used as index of germination speed. As reported in **Fig.22,** UP seeds showed an estimated *PV* of 4.92 % day^{-1} whereas for all the tested HP treatments the *PV* values were always higher. It is worth noting that HP72 resulted into the highest *PV* value, recorded at 5 days. The germination index (*GI*), used to measure the time

requested for each seed to germinate, is shown in **Fig. 23**. Also, in this case, the highest value was observed for HP72.

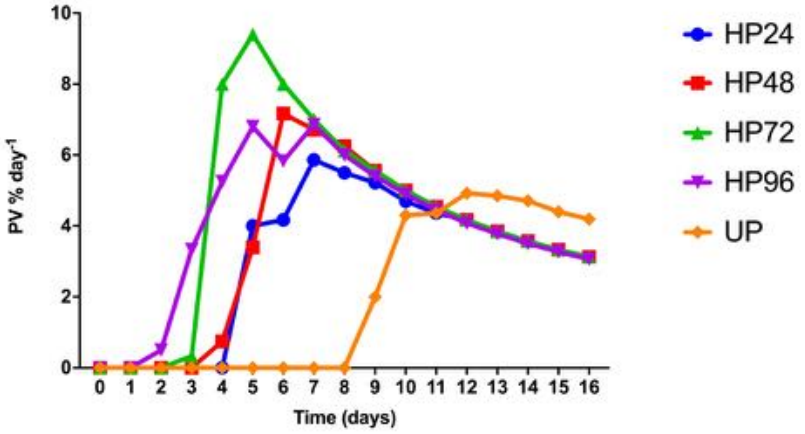


Fig. 22. Peak value (*PV*) as index of germination speed calculated for '67/3' seeds treated with hydropriming (HP) for 24, 48, 72, and 96 h and for unprimed (UP) seeds.

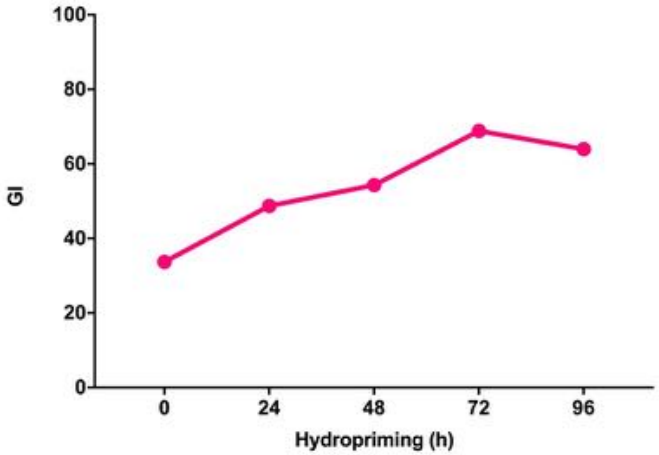


Fig. 23. Germination index (*GI*, time requested for each seed to germinate) calculated for '67/3' seeds treated with hydropriming (HP) for 24, 48, 72, and 96 h and UP seeds.

Additional parameters describing the germination profiles of *S. melongena* seeds treated with hydropriming were calculated according to Ranal et al. (2006) and the estimated values are shown in **Table 16**. Germinability (*G*) was the highest (100 %) for HP24, HP48 and HP72 and slightly lower (98±2.80 %) for HP96. The hydropriming treatment carried out

for 72 h turned out to be the best in terms of mean germination time (*MGT*) since it showed the lowest value (4.48±0.00 days). The coefficient of variation of the time (*CV*) was higher in 72 h compared to other treatment, with a minimal standard deviation (468.00±0.15 %). Mean germination rate (*MGR*) values were similar between the different treatment, but also in this case HP72 was the best one, showing the lowest rate (0.00214±0.00 day^{-1}). Uncertainty (*U*) was significantly lower in HP72 (1.22±0.20 bit), compared to the other treatments, and this was indicative of synchronized germination. *Z* or synchronization index was closer to 1 in the case of HP48 (0.34±0.10) and HP72 (0.31±0.30). For each parameter, a comparison between UP and all the tested HP treatments was carried out using the Student's *t* test ($P<0.05$) (**Tab. 16**).

Table 16. Germination parameters calculated based on results of germination tests carried out on *S. melongena* '67/3'seeds treated with hydropriming (HP) for increasing time (24 h, 48 h, 72 h, and 96 h) and unprimed (UP) seeds. Values are expressed as means ± SD from three independent replicates. Asterisks indicate statistically significant differences determined using Student's t test (P<0.05). *G*, Germinability. *MGT*, Mean germination time. *CVG*, Coefficient of velocity of germination. *MGR*, Mean germination rate. *U*, Uncertainty. *Z*, Synchronization index. h, hours.

Treament	***G* (%)**	***MGT* (days)**	***CVG* (%)**	***MGR* (day^{-1})**	***U* (bit)**	***Z* (unit less)**
HP 24 h	100.0 ± 0.00*	6.56 ± 0.05**	288.00 ± 3.41*	3.4 x 10^{-3} ± 7.07 x 10^{-5} *	2.08 ± 0.00	0.26 ± 0.00
HP 48 h	100.0 ± 0.00*	5.78 ± 0.08**	339.76 ± 6.29**	2.9 x 10^{-3} ± 5.65 x 10^{-5} *	1.70 ± 0.38	0.34 ± 0.10
HP 72 h	100.0 ± 0.00*	4.48 ± 0.00***	468.00 ± 0.15*	2.1 x 10^{-3} ± 0.00*	1.22 ± 0.20	0.31 ± 0.30
HP 96 h	98.0 ± 2.80*	4.77 ± 0.18*	426.02 ± 33.09	2.3 x 10^{-3} ± 1.98 x 10^{-4} **	2.01 ± 0.38	0.25 ± 0.05
UP	74.4 ± 1.57	10.58 ± 0.01	220.38 ± 6.55	4.5 x 10^{-3} ± 1.41 x 10^{-4}	2.22 ± 0.02	0.23 ± 0.01

Seed priming not only improves germination parameters but also exerts beneficial effects at the level of seedling growth and stress tolerance. In order to assess any impact of the HP72 protocol on the growth of *S. melongena* seedlings developed from primed seeds, a phenotyping analysis was carried out. Seven-day old seedlings from primed and unprimed seeds were evaluated in terms of fresh/dry weight and radicle length. Results from these analyses are shown in **Table 17**. Statistical analysis did not highlight significant changes in the root length of seedlings resulting from the HP72 treatment, compared to those developed from unprimed seeds. A significant (P<0.05) increase in fresh weight was recorded only in seedlings developed from seeds primed for 72 h, compared to control. The seedling phenotype is shown in **Fig. 24.**

Table 17. Results of phenotyping analyses performed on seven-day old *S. melongena* seedlings (line '67/3') developed from seeds treated with hydropriming (HP72) and untreated (UP). Values are expressed as mean ± SD of three independent replications with 15 seedlings for each replication. Asterisks indicate statistically significant differences determined using Student's *t*-test. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

Parameter	UP	HP72
fresh weight (mg/15 seedling)	37.40 ± 0.168	42.5 ± 0.028*
dry weight (mg/15 seedling)	4.65 ± 0.029	3.6 ± 0.005
radicle length (mm)	38.50 ± 2.12	47.0 ± 21.21

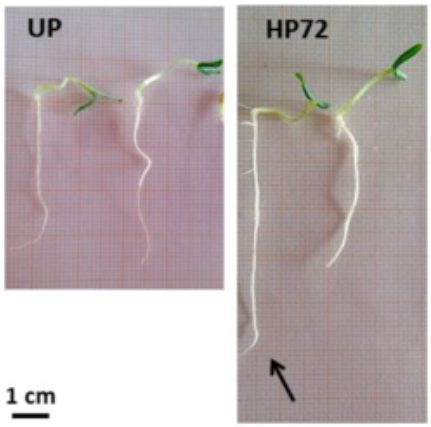


Fig. 24. Phenotype of seven-day old *S. melongena* seedlings. UP, unprimed. HP, hydroprimed.

4.1.4. Experimental design for the molecular profiling of seed priming in *S. melongena*

Based on the seed response to priming, the HP72 protocol was selected for subsequent molecular analyses. As a first step, an experimental design was set up in order to figure out the most representative timepoints during priming (**Fig. 25**). At the selected timepoints, seeds were collected and stored for ROS detection and *q*RT-PCR analyses. Seeds were collected at the dry state (dry seed, DS) and during the treatment at 24, 48 and 72 h. An additional timepoint was fixed at the end of the dehydration (dry-back, DB). In order to monitor the seed response during the germination process, additional timepoints were fixed, as representative of the temporal window overlapping the pre-germinative metabolism. As shown in **Fig. 25,** seeds were collected throughout imbibition at 2, 4, 8, 16, 24, 48 and 72 h and finally at the end of germination (radicle protrusion stage, RD).

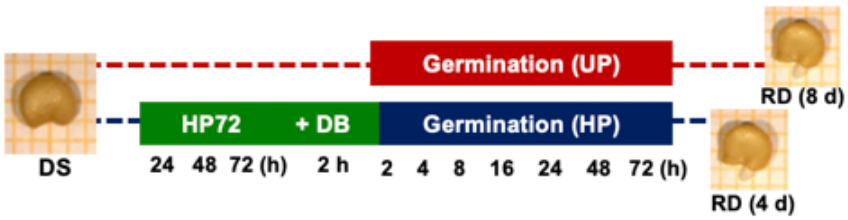


Fig. 25. Experimental design showing the most representative timepoints (24, 48, 72 h) during the HP72 treatment, followed by the end point of the dehydration step (dry-back, DB, 2 h). The germination test carried with unprimed (UP) seeds and primed (HP) seeds was monitored throughout imbibition at 2, 4, 8, 16, 24, 48 and 72 h and finally at the end of germination (radicle protrusion stage, RD).

Due to the limited amount of '67/3' seeds available for each lot/year, molecular analyses were performed using seeds collected during year 2018. As shown in **Fig. 26**, when this seed lot underwent the HP72 treatment, the subsequent germination tests confirmed the expected beneficial effects of hydropriming. UP seeds (2018) started to germinate at the 5th day (4.44±0.58%) and reached the maximum value at the 15th day (97.67±0.71%) while the HP72 seeds anticipated the germination at the 3rd day (4.44±0.58%) and showed the highest germination percentage (100±0.00%) at the 13th day. Statistical analysis carried out using Two-way ANOVA (P<0.05) highlight significant differences between the UP and HP72 seeds. The related germination parameters (Ranal et al., 2006) are illustrated in **Table 18**.

The HP72 treatment anticipated germination, as evidenced by *MGT* (11.05±1.10 and 8.84±0.87 days, for UP and HP72 samples, respectively) but no significant effects on synchronization were observed (*U* values of 2.50±0.29 and 2.57±0.11 bit, for UP and HP72 samples, respectively). Statistical analysis carried out using Student's t test highlight significant difference in *CVG* (42.03±9.86 and 77.61±17.23 %, for UP and HP72 samples) (**Table 18**).

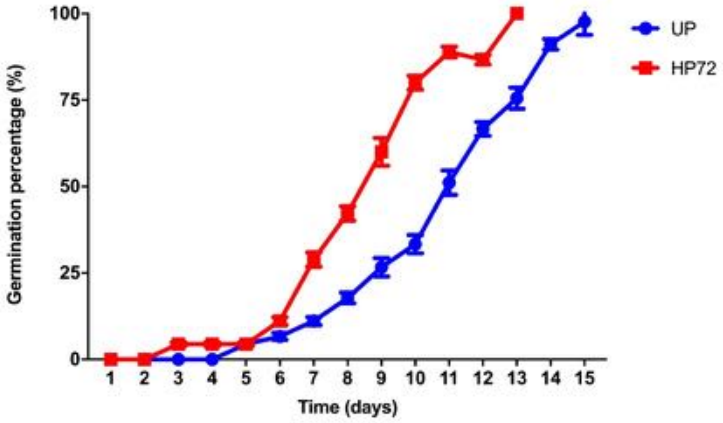


Fig. 26. Results from germination tests carried out with '67/3' seeds collected in year 2018, treated with the HP72 protocol. The germination curve of unprimed (UP) seeds is also shown. Values are expressed as mean ± SD of three independent replications with 15 seeds for each replication. Statistical analysis was carried out using Two-way ANOVA (F: 9.861; DF: 1; *P*: 0.0348*). Comparison between UP and HP72 were carried out using Mann-Whitney test (U: 86.5; *P*: 0.2872 ns).

Table 18. Germination parameters calculated for the '67/3' seed lot collected during 2018 and treated with hydropriming (HP72 protocol). *G*, Germinability. *MGT*, Mean germination time. *CVG*, Coefficient of velocity of germination. *MGR*, Mean germination rate. *U*, Uncertainty. *Z*, Synchronization index. UP, unprimed seeds. Asterisks indicate statistically significant differences determined using Student's *t*-test. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

Parameter	UP	HP72
G (%)	97.78 ± 3.85	100.00 ± 0.00
MGT (days)	11.05 ± 1.10	8.84 ± 0.87
CVG (%)	42.03 ± 9.86	77.61 ± 17.23*
MGR (day^{-1})	0.025 ± 0.006	0.013 ± 0.00
U (bit)	2.50 ± 0.29	2.57 ± 0.11
Z (unit less)	0.15 ± 0.05	0.136 ± 0.005

4.1.4.1. Hydropriming boosts ROS accumulation in *S. melongena* seeds

ROS levels were measured using the DCFH-DA fluorescent dye in dry and imbibed '67/3' seeds at selected time points. The experimental design is shown in **Fig. 25**. Samples were collected at 24 h, 48 h, and 72 h during HP and subsequently after the dry-back step (DB, 2 h of dehydration). During germination tests, unprimed (UP) and primed (HP) seeds were collected at 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, and 72 h from the beginning of imbibition. Results of these experiments performed on '67/3' samples, dry seeds (DS) and seeds collected throughout the HP treatment (24 h, 48 h, 72 h) are shown in **Fig. 27A**. HP triggered a significant (P = 0.04) ROS accumulation at 24 h (27.63±8.82 R.F.U.), 14.62-fold compared to DS (1.89±0.53 R.F.U.). At 48 h and 72 h, the recorded ROS levels were still significantly higher (15.72±3.55 and 8.71±1.70 R.F.U., respectively; P = 0.02 and 0.01) (**Fig. 27A**). Following dry-back, ROS levels further decreased compared to the 72 h timepoint, reaching an estimated value of 1.02±0.25 R.F.U. (P = 0.08), similar to those detected in the dry seeds (**Fig. 27A**, + DB). A different pattern was detected in the UP samples since a peak in ROS levels was observed at 8 h and 16 h of imbibition (8.84±1.83 and 12.50±4.40 R.F.U., respectively; P = 0.02 and 0.04), compared to the DS (1.89±0.53 R.F.U.) (**Fig. 27B**, UP). ROS levels were decreased at the subsequent time points, from 24 to 72 h, although they appeared still significantly higher (P = 0.03 and 0.00008), compared to DS (4.68±1.23 R.F.U., 24 h; 3.50±0.86 R.F.U., 48 h; 4.31±0.23 R.F.U., 72 h) (**Fig. 27B**, UP).

The RD stage, that marks the end of germination and features extensive metabolic changes other than those associated with the pre-germinative metabolism (Pagano et al., 2018), was not considered for ROS measurements. Based on the reported data, the HP72 treatment featured enhanced ROS accumulation within the first 24 h, whereas no consistent changes in ROS pattern were associated with the accelerated germination profile of HP seeds.

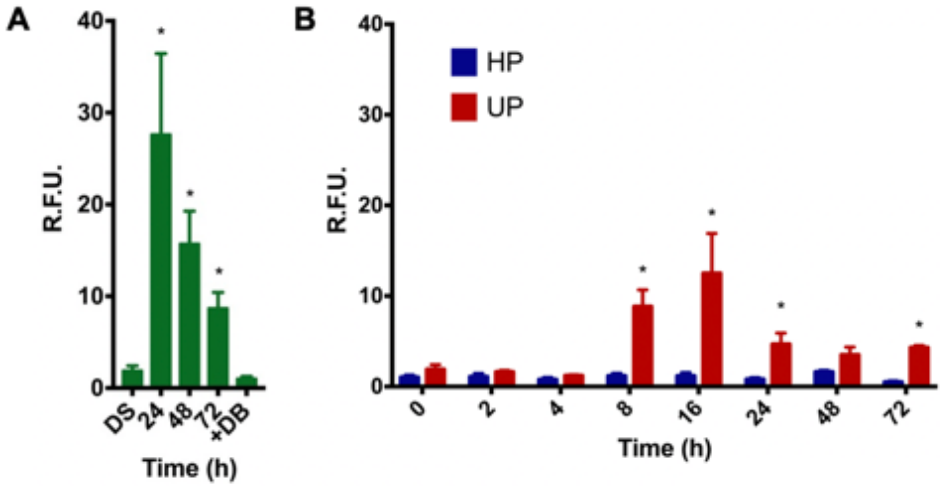


Fig. 27. Result of ROS accumulation assay carried out with '67/3' seeds treated with the HP72 hydropriming protocol and collected at the selected timepoints. Values are expressed as mean ± SD of three independent replications with 3 seeds for each replication. Statistical analysis was carried out using the Student's *t* test ($P<0.05$). DS, dry seed. +DB, hydropriming + dry back. UP, unprimed. HP. Hydroprimed.

4.1.4.2. Identification and validation of candidate genes for the molecular profiling of the seed response to priming in *S. melongena*

The experimental work carried for this thesis is part of a research projects titled *"Seed wake-up with aptamers: a new technology for dormancy release and improved seed priming strategy (WAKE-APT)"* sponsored by CARIPLO FOUNDATION (www.fondazionecariplo.it; www.wake-apt.it). Dr. Laura Bassolino (CREA-GB; CREA-CI) contributed to this work by retrieving from the *S. melongena* '67/3' genome a list of target genes with roles in DNA repair, chromatin remodeling and antioxidant defence already reported to play a role in the seed pre-germinative metabolism. In addition, a set of putative reference genes useful for the *q*RT-PCR-based analysis is shown (**Table 19**). The list includes genes involved in chromatin remodelling, e.g. genes coding for histone deacetylase HDA19/HD1 which participates in the transcriptional repression of the *AtABI3 (ABSCISIC ACID INSENSITIVE)* gene promoter during early seedling development in *Arabidopsis* (Ryu et al., 2014) and HD2B histone deacetylase up-regulated by treatments that break seed dormancy (Yano et al., 2013). The list also includes the *TFIIS* (transcription elongation factor IIS) gene that in *Arabidopsis* has been shown to control the expression of the *DOG1* gene responsible for a dormant phenotype. *DOG1* gene expression is seed-specific and transcript levels drop during seed imbibition (**Table 19**). Thus, *TFIIS* and *DOG1* genes are

expected to be expressed at high levels in dormant seeds. The GA (gibberellin)-mediated pathway is negatively controlled by the so-called DELLA proteins, consisting of five members in *Arabidopsis*: GA-INSENSITIVE (GAI), REPRESSOR OF *ga1-3* (RGA), RGA-LIKE1 (RGL1), RGA-LIKE2 (RGL2), and RGA-LIKE3 (RGL3). *OGG1* and *FPG* genes (**Table 19**), key components of the BER (Base Excision Repair) pathway responsible for the removal of oxidative DNA lesions, were included in the list. Previous reports have highlighted the requirement for both *OGG1* and *FPG* transcripts during seed imbibition carried out both under physiological conditions, osmotic stress (Macovei et al., 2011) and in response to stress agents as the HDAC inhibitors trichostatin A and sodium butyrate (Pagano et al., 2017, 2018).

Table 19. List of target and reference genes retrieved from the *S. melongena* '67/3' genome.

TARGET GENES			
Gene (accession N.°)	**Protein**	**Function**	**Reference**
SmHDA19/HD1	Histone deacetylase	CHROMATIN REMODELING	Pagano et al., 2017
SmHD2A (SMEL_010g355120.1.01)	Histone deacetylase	CHROMATIN REMODELING	
SmHD2B (SMEL_009g320230.1)	Histone deacetylase	CHROMATIN REMODELING	
SmTdp1 α (SMEL_003g171200.1)	Tyrosyl-DNA phosphodiesterase	DNA REPAIR	Macovei et al., 2010
SmTFIIS (SMEL_010g346840.1.)	Transcription elongation factor	DNA REPAIR	Macovei et al., 2011a
SmAPX (SMEL_006g245760.1.01)	Ascorbate peroxidase	ANTIOXIDANT RESPONSE	Balestrazzi et al., 2011
SmSOD (SMEL_001g139700.1.01)	Superoxide dismutase	ANTIOXIDANT RESPONSE	
SmRGL2 (SMEL_011g363840.1.01)	Transcription factor	SEED DORMANCY	Née et al., 2017
SmDOG1A (SMEL_003g170150.1.01)	Transcription factor	SEED DORMANCY	
SmDOG1B (SMEL_012g384970.1)	Transcription factor	SEED DORMANCY	
SmOGG1 (SMEL_004g210790.1)	DNA glycosylase/lyase	DNA REPAIR	Macovei et al., 2011b
SmFPG (SMEL_003g194660.1)	DNA glycosylase/lyase	DNA REPAIR	
REFERENCE GENES			
Gene (accession N.°)	**Protein**	**Function**	**Reference**
SmGAPDH (AB110609.1)	Glyceraldehyde-3-phosphate dehydrogenase	GLYCOLYSIS	Barbierato et al., 2017
SmPP2Acs (AY325817.1)	Catalytic subunit of protein phosphatase 2A	PROTEIN PHOSPHATASE ACTIVATOR	Dekkers et al., 2011
SmTUB (DQ205342.1)	Tubulin	MAJOR BUILDING BLOCK OF MICROTUBULES	Cao et al., 2016
SmEF1 (X14449.1)	Elongation factor 1	PROTEIN BIOSYNTHESIS	Dekkers et al., 2011
SmAPRT (JX448345.1)	Adenine phosphoribosyltransferase	NUCLEOSIDE METABOLISM	Gantasala et al., 2013

Five putative reference genes were also evaluated (**Table 19**). The *SmGAPDH* gene encodes glyceraldehyde 3-phosphate dehydrogenase that catalyses the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate, involved in glycolysis (Barbierato et al., 2017). *SmPP2Acs* gene encodes the catalytic subunit of protein phosphatase 2A, one of the four major Ser/Thr phosphatases implicated in the negative control of cell growth and division (Dekkers et al., 2011). The *SmTUB* gene encodes tubulin, a structural component of microtubules. *SmEF1* gene codes for the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome (Cao et al., 2016). The *SmAPRT* gene encodes adenine phosphoribosyltransferase acting in the mechanisms for the

distribution of CpG dinucleotides, production of AMP and inorganic pyrophosphate from adenine and 5- phosphoribosyl-1-pyrophosphate (PRPP). It also produces adenine as a by-product of the polyamine biosynthesis pathway (Gantasala et al., 2013). For each candidate gene, oligonucleotide primers were designed, validated *in silico* by cross-check with the *S. melongena* database as well as by standard PCR analysis carried out on cDNA synthesized from seed RNA. The reference genes *SmEF*, *SmPp2AC*s, *SmTUB*, *SmGAPDH* and *SmAPRT* encoding proteins involved in basic cellular processes (housekeeping genes) are commonly used as controls for gene expression analyses in plants. As a first step, each candidate gene was tested by *q*RT-PCR using as template cDNA samples representative of each timepoint of the experimental design. The constitutive transcriptional profile of the candidate reference genes was evaluated using the GeNorm tool. Based on the M values provided by the software, *SmGAPDH* and *SmAPRT* resulted as the most suitable reference genes (**Fig. 13, paragraph 3.6.2.**).

4.1.4.3. Hydropriming induces temporally distinct waves of up-regulation of antioxidant and BER genes

In order to assess the effects of the HP72 treatment on the seed antioxidant defence and DNA repair response, the expression profiles of genes involved in the seed pre-germinative metabolism were investigated using *q*RT-PCR, namely *SmSOD*, *SmAPX*, *SmOGG*, *SmFPG* and *SmTDP1*α. Results of *q*RT-PCR analyses are shown in **Fig. 28A** as changes in the expression of antioxidant and BER genes. Values represent fold changes of transcript levels where FC = primed seed (HP)/dry seed (DS) and FC(HP/DS) values < 1 or > 1 correspond to gene down- or up-regulation, respectively (**Fig. 28A**, dashed line). When considering the effects of HP72 treatment along the tested time points, compared to DS, the *SmSOD* gene was down-regulated, as indicated by the observed FC(HP/DS) values (< 1) whereas up-regulation of the *SmAPX* gene was evident after 24 h of treatment. Among the tested BER genes, only *SmOGG1* showed early up-regulation at 24 h (**Fig. 28A**).

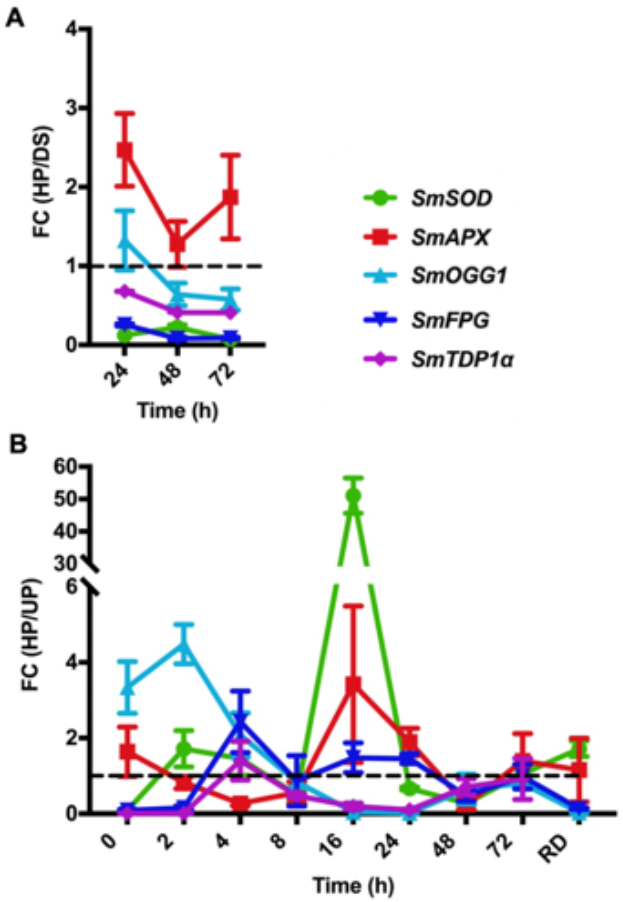


Fig. 28. Impact of hydropriming (HP72) on the expression profiles of antioxidant and DNA repair genes in '67/3' seeds. Results of *q*RT-PCR analyses are reported as expression changes of antioxidant and BER genes. Values are expressed as mean ± SD of three independent replications with 20 seeds for each replication. SOD, superoxide dismutase. APX, ascorbate peroxidase. OGG1, 8-oxoguanine glycosylase/lyase. FPG, formamidopyrimidine-DNA glycosylase. TDP1α, tyrosyl-DNA phosphodiesterase1α. RD, radicle protrusion.

Results of *q*RT-PCR analyses performed on the '67/3' samples, both HP and UP seeds, collected during the germination test (0 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, and 72 h) and at the radicle protrusion stage (RD) are shown in **Fig. 28B** as expression changes of antioxidant and BER genes. Values represent fold changes of transcript levels where FC = primed seed (HP)/unprimed seeds (UP) and FC(HP/UP) values < 1 or > 1 correspond to gene down- or up-regulation, respectively (**Fig. 28B**, dashed line). When comparing HP seeds subjected to dry-back with UP dry seeds (**Fig. 28B**, 0 h),

the observed FC(HP/UP) values indicated down-regulation of the *SmSOD* gene while the HP-dependent up-regulation of *SmAPX* and *SmOGG1* genes was evident. The FC(HP/UP) value of *SmOGG1* significantly increased following dry-back (from 0.80±0.15 at 72 h up to 3.34± 0.70), suggesting for enhanced expression during the dehydration step. Besides the early up-regulation of *SmAPX* and *SmOGG1* genes, the impact of the HP treatment on seed imbibition is evidenced by distinct waves of responses. At 2 h, the *SmOGG1* gene was still up-regulated in HP seeds whereas the recorded increase in FC(HP/UP) for the *SmSOD* gene (1.72±0.48), compared to 0 h, highlights the influence of the treatment on this antioxidant component of the eggplant pre-germinative metabolism (**Fig. 28B**, 2 h). At 4 h of imbibition, the treatment resulted into a distinctive response of BER genes. Indeed, despite the observed decrease in the FC(HP/UP) value, the *SmOGG1* gene was still up-regulated at this time point. HP72 triggered also the expression of *SmFPG* and *SmTDP1*α genes, based on the estimated FC(HP/UP) values of 2.43±0.81 and 1.40±0.52, respectively (**Fig. 28B**, 4 h). At 16 h, a new wave of response was observed featuring up-regulation of the antioxidant gene *SmAPX* with an estimated FC(HP/UP) value of 3.42±2.07 as well as a very strong response of *SmSOD* gene (FC(HP/UP) = 51.07±5.43) (**Fig. 28B**, 16 h). The slight fluctuations detected at the end of the experiment, at 48 h and 72 h, and the changes observed at the radicle protrusion stage (RD) showed that the impact of HP was apparently diminished when approaching the end of germination. To better clarify the dynamics of the seed response resulting from the concomitant changes in the *SmOGG1* mRNA and ROS levels, the ratio between the *SmOGG1* transcript level and the ROS amount (OGG1: ROS) was calculated. A significant increase in the OGG1: ROS ratio occurred in HP seeds, compared to UP, during early imbibition (from 0 to 4 h) (**Fig. 29**, HP). In the absence of treatment, such a response was delayed since in this case the OGG1: ROS ratio peaked at 24 h (**Fig. 29**, UP).

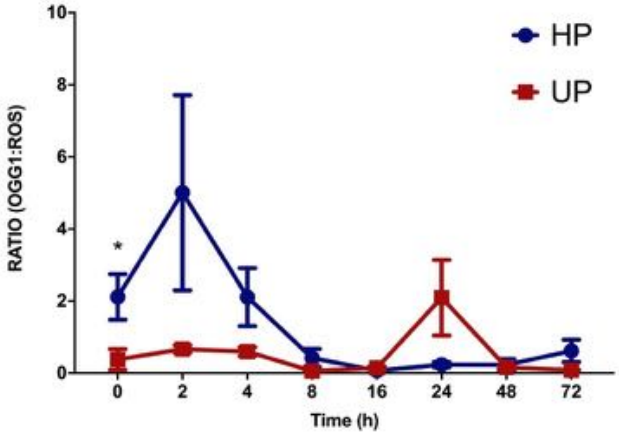


Fig. 29. Ratio between the *SmOGG1* transcript level and the ROS amount (OGG1: ROS) calculated during the germination test (0 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, and 72 h) of '67/3' seeds. Values are expressed as mean ± SD of three independent replications with 15 seeds for each replication. Asterisks indicate statistically significant differences determined using Student's *t*-test ($P < 0.05$). OGG1, 8-oxoguanine glycosylase/lyase. ROS, reactive oxygen species.

The observed differences in gene expression and ROS patterns suggest that the temporal 'window' spanning the first hours of eggplant seed imbibition provide information useful to predict the efficacy of hydropriming. To further explore the potential of this 'window', ROS profiles and gene expression analysis were carried in the *S. melongena* '67/3' seed lots previously characterized, showing variable quality.

4.1.5.1. The HP72 protocol is effective on seed lots showing different quality

The HP72 protocol was applied to '67/3' seed lots collected during subsequent years (2015, 2016, 2017), germination tests were carried out and the resulting data were used to calculate the specific germination parameters. For each year, a comparison between the UP seeds and HP72 seeds was carried out using Two-way ANOVA ($P<0.05$) and Mann Whitney test ($P<0.05$) (**Fig. 30, A, B, and C**). As shown in **Table 20**, the UP seeds of the 2015 lot were unable to germinate, however the HP72 treatment was able to rescue germination. Vitality test based on TTC assay revealed that this seed lots was vital, infact the priming treatment confirm this data. For this lot, the recorded values for the HP72 sample were 55.56±21.42 % (*G*), 6.52±0.86 days (*MGT*), 59±42.48 % (*CVG*), 0.0269±0.022 day^{-1} (*MGR*), 1.61±0.87 bit (*U*) and 0.34±0.28 (*Z*). As for the 2016 lot, a significantly ($P<0.05$) lower *MGT* (6.20±0.00 days) was observed for the primed seeds compared to UP

(8.95±0.91 days) whereas only slight differences were noticed in *U* values (UP, 2.39±0.05 bit; HP72, 2.04±0.16 bit) (**Table 20**). The 2017 lot showed a late germination profile, however the HP72 treatment could anticipate germination as evidenced by *MGT* values (UP, 28.56±1.34 days; HP72, 25.51±0.79 days) whereas no effects on synchronization were observed, based on *U* values (UP, 2.53±0.14 bit; HP72, 2.74±0.18 bit). Considering the overall results and the response to priming, the different eggplant seed lots were still classified as high-quality (2014, 2016, 2018) and low-quality (2015, 2017) lots.

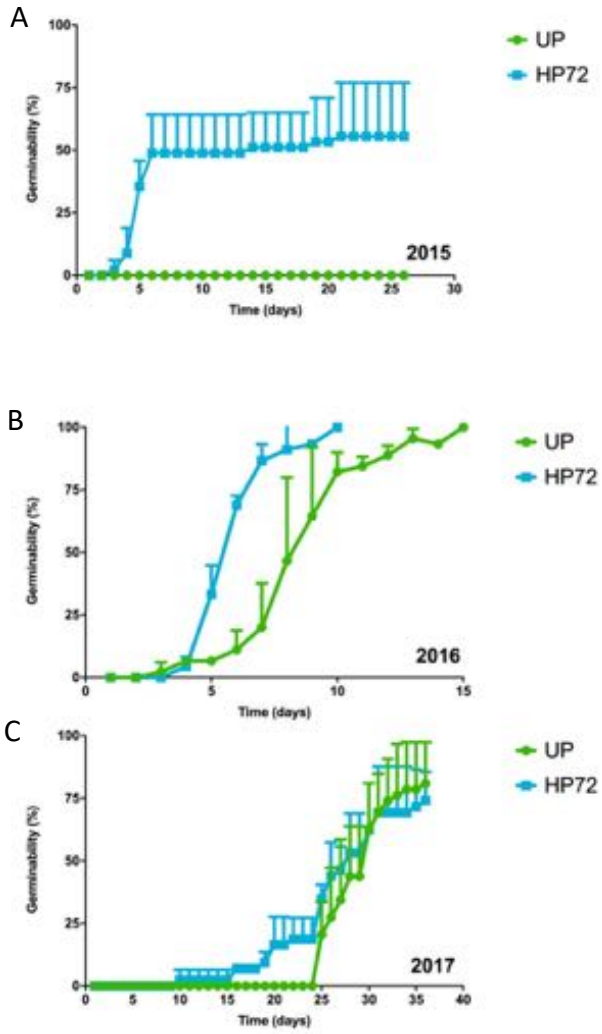


Fig. 30. Results from germination tests carried out with '67/3' seed lots collected during year 2015 (**A**), 2016 (**B**) and 2017 (**C**), and treated with the HP72 protocol. For each year, the germination curve of unprimed (UP) seeds is also shown. Values are expressed as mean ± SD of three independent replications with 15 seeds for each replication. Statistical analysis was carried out using Two-way ANOVA (2015, F: 26.91; DF: 1; *P*: 0.0066**; 2016, F: 27.51; DF: 1; *P*: 0.0063**; 2017, F: 1.111; DF: 1; *P*: 0.3512 ns). Comparison between UP and HP72 were carried out using Mann-Whitney test (2015, U: 42; *P* < 0.0001****; 2016, U: 138; *P*: 0.2045 ns); 2017, U: 470.5; *P*: 0.0352*).

Table 20. Germination parameters calculated for different '67/3' seed lots collected during 2015, 2016, and 2017 and treated with hydropriming (HP72 protocol). *G*, Germinability. *MGT*, Mean germination time. *CVG*, Coefficient of velocity of germination. *MGR*, Mean germination rate. *U*, Uncertainty. *Z*, Synchronization index. UP, unprimed seeds. Asterisks indicate statistically significant differences determined using Student's *t*-test. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

	2015		2016		2017	
Parameter	**UP**	**HP72**	**UP**	**HP72**	**UP**	**HP72**
G (%)	0	55.56 ± 21.42	100.00 ± 0.00	100.00 ± 0.00	77.78 ± 19.24	74.13 ± 11.43
MGT (days)	0	6.52 ± 0.86	8.95 ± 0.91	6.20 ± 0.00*	28.56 ± 1.34	25.51 ± 0.79*
CVG (%)	0	59.00 ± 42.48	76.34 ± 17.85	148.70 ± 0.94*	62.70 ± 12.00	65.00 ± 10.59
MGR (day^{-1})	0	0.030 ± 0.02	0.005 ± 0.003	0.007 ± 0.00	0.005 ± 0.003	0.016 ± 0.00
U (bit)	0	1.61 ± 0.87	2.39 ± 0.05	2.04 ± 0.16*	2.53 ± 0.14	2.74 ± 0.18
Z (unit less)	0	0.340 ± 0.28	0.18 ± 0.00	0.230 ± 0.01*	0.19 ± 0.02	0.077± 0.04

4.1.5.2. Hydropriming treatment boosts ROS accumulation in *S. melongena* seed lots showing different quality

The levels of free radical species were assessed in the different '67/3' seed lots (2014, 2015, 2016 and 2017) previously characterized for their germination profiles and response to hydropriming. The experimental design is shown in **Fig. 31**. For each lot, the unprimed samples included dry seeds (UP) as well as rehydrated seeds collected at 2 h of imbibition (UP2) while the primed samples included seeds that underwent the HP72 treatment followed by dry-back (HPDB) and seeds collected at 2 h of imbibition (HPDB2). The sampling was limited to this early time point with the aim to speed-up the prediction of the seed response and reduce costs, in view of possible future applications.

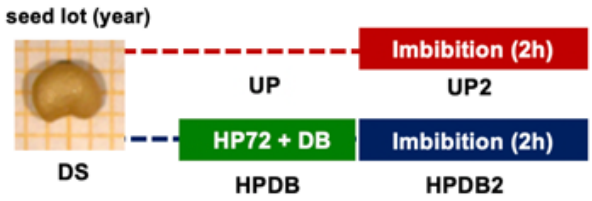


Fig. 31. Experimental design showing the time points selected to carry out the molecular analysis in the different '67/3' seed lots (2014, 2015, 2016, 2017 and 2018). DS, dry seed. UP, unprimed. UP2, unprimed and imbibed for 2h. HPDB, hydroprimed and dried back. HPDB2, hydroprimed, dried back and imbibed for 2h.

As shown in **Fig. 32**, the '67/3' seeds were grouped, based on their germination profiles, as 'high-quality' (2014, 2016, 2018) and 'low-quality' (2015, 2017) lots. Comparable ROS levels were detected in the UP seeds of high-quality' lots (1.54±0.51 R.F.U., 2014; 1.37±0.66 R.F.U., 2016; 1.73±0.85 R.F.U., 2018). At 2 h of imbibition (UP2) ROS levels were decreased in the 2014 and 2016 seed lots (0.68±0.23 and 0.44±0.01 R.F.U., respectively, compared to dry seeds. A similar trend was observed also in the 2018 lot (**Fig. 32**). The impact of HP72 treatment on the high-quality seed lots, in terms of ROS accumulation, was not apparently relevant since ROS levels detected in both HPDB and HPDB2 samples did not exceed the amount measured in the UP seeds whereas the low-quality seed lots showed a significant (P = 0.02, 2015 and P = 0.03, 2017) enhancement in ROS levels following the HP72 treatment (**Fig. 32**). The seed lots collected in 2015 and 2017 did not show high germinability. In one case (2015), no germination at all was observed while the HP72 treatment induced germination, although at a low percentage (55.56±21.42 %) (**Table 20**). In the other case (2017), the

seed lot showed suboptimal germination (77.78±19.24 %) and there was no benefit from hydropriming (**Table 20**). It is possible that the poor-quality germination performance of the 2015 and 2017 seed lots correlates with the presence of high ROS levels. On the other hand, the ROS profiles in the seed lots of years 2014, 2016, and 2018 should reflect their high-quality germination performance (**Table 20**).

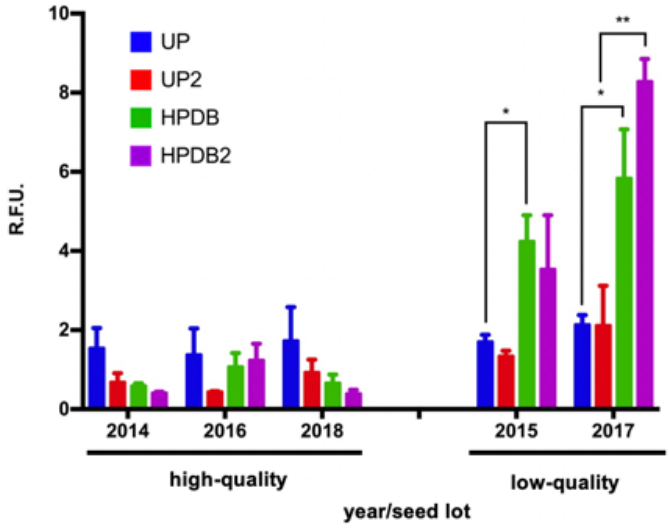


Fig. 32. Result of ROS accumulation assay carried out with different '67/3' seed lots (2014, 2015, 2016, 2017 and 2018) treated with the HP72 protocol and collected at the selected timepoints. Values are expressed as mean ± SD of three independent replications with 3 seeds for each replication. Statistical analysis was carried out using the Student's *t* test ($P<0.05$). UP, unprimed. UP2, unprimed and imbibed for 2 hours. HPDB, hydroprimed and dried back. HPDB2, hydroprimed, dried back and imbibed for 2 hours.

4.1.5.3. Impact of hydropriming on the expression of antioxidant/BER genes in high- and low-quality eggplant seed lots

In order to correlate ROS profiles with gene expression patterns, *q*RT-PCR analyses were carried on the different '67/3' seed lots according to the experimental design shown in **Fig. 31**. Values represent fold changes of transcript levels where FC = primed and dried-back seed (HPDB)/dry seed (DS) and FC(HPDB/DS) values < 1 or > 1 correspond to gene down- or up-regulation, respectively (**Fig. 33**, dashed line). Apparently the HP72 treatment did not impact the expression of *SmSOD* and *SmAPX* genes in the high-quality seed lots. As for the low-quality samples, up-regulation of *SmSOD*

gene was detected in one lot (2015, FC(HPDB/DS) = 3.44 ± 0.35) (**Fig. 33 A**). In the case of DNA repair genes, the HP72 treatment resulted in *SmOGG1* up-regulation only in the 2014 and 2018 seed lots, as shown by the FC(HPDB/DS) values (4.86±0.20 and 4.17±1.29, respectively) whereas no substantial changes were detected the seed lots collected in 2015, 2016 and 2017. The *SmFPG* gene showed a lot-dependent expression profile in the high-quality samples and no significant changes occurred in the low-quality seeds. The effects of HP72 treatment on the *Tdp1α* gene expression was heterogeneous across both the high-quality and low-quality seed lots (**Fig. 33 A**).

Results of the *q*RT-PCR analysis performed on imbibed seeds are shown in **Fig. 33 B**. Values represent fold changes of transcript levels where FC = primed seed (HP_{2h})/unprimed seed (UP_{2h}) and FC(HP_{2h} /UP_{2h}) values < 1 or > 1 correspond to gene down- or up-regulation, respectively (**Fig. 33 B**, dashed line). At 2 h of imbibition the hydropriming did not trigger substantial up-regulation of the *SmSOD* gene in almost all the seed lots (**Fig. 33 B**), however up-regulation of *SmAPX* gene was evident in both high-quality and low-quality seed lots. The estimated FC(HP_{2h}/UP_{2h}) values were 9.23±2.69 (2014), 4.13±1.18 (2015), 1.61±0.47 (2016), 2.66±1.59 (2017), and 0.83±0.16 (2018). Up-regulation of *SmOGG1* gene also occurred in almost all the tested seed lot except for year 2014. As shown in **Fig. 33 B,** the estimated FC(HP_{2h}/UP_{2h}) values were 2.13±0.54 (2015), 2.98±0.66 (2016), 1.55±0.40 (2017), and 4.46±0.82 (2018). Following imbibition, the *SmFPG* gene was down-regulated in all the tested seed lots. The *Tdp1α* gene expression showed fluctuations in all the tested seed lots (**Fig. 33 B**). The emerging picture highlights a complex pattern of gene expression that reflects profiles sometimes unique to a seed-lot as well as responses shared by different seed lots. This is the case of *SmOGG1*, *SmFPG*, and *SmTdp1α* genes, whose patterns suggest that these BER functions might be useful to trace the seed response, particularly in seeds primed and subjected to dry-back.

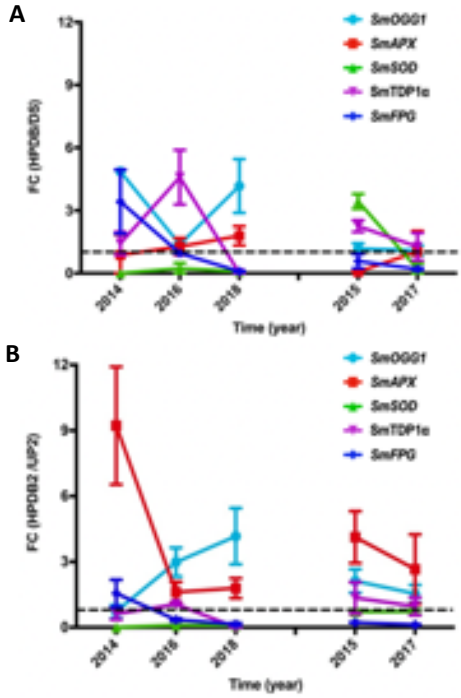


Fig. 33. Impact of hydropriming on the expression profiles of antioxidant and DNA repair genes in different '67/3' seed lots (2014, 2015, 2016, 2017 and 2018). Results of *q*RT-PCR analyses are reported as expression changes of antioxidant and BER genes. Values are expressed as mean ± SD of three independent replications with 20 seeds for each replication. SOD, superoxide dismutase. APX, ascorbate peroxidase. OGG1, 8-oxoguanine glycosylase/lyase. FPG, formamidopyrimidine-DNA glycosylase. TDP1α, tyrosyl-DNA phosphodiesterase 1α.

4.1.6. Impact of hydropriming on eggplant wild relatives

4.1.6.1. Development of an optimized hydropriming protocol for *Solanum torvum*

Hydropriming (HP) was tested on *S. torvum* seeds collected in 2017. Seeds soaked in H_2O for 48 h and 72 h (HP48 and HP72) were subjected to dry-back (DB, 2 h) and then immediately used for germination tests. Results of germination tests carried with primed and unprimed (UP) seeds are shown in **Fig. 34**. UP seeds started germination at 6 days from the beginning of imbibition and reached the maximum germination percentage (97.78±3.85%) at 13 days whereas hydropriming always resulted in anticipated germination compared to untreated seeds. In the case of primed seeds, germination occurred at 4 days (3.70±1.15%, HP48; 2.22±0.58%, HP72) from the beginning of imbibition. Results of statistical analysis carried out using Two-

way ANOVA (F: 8.452; DF: 24; *P*: <0.0001****) as well as comparison between UP and primed seeds performed using the Post-Hoc Tukey's HSD test ($P \leq 0.05$) are shown in **Tab. 21**. Both HP48 and HP72 treatments result in significant differences, compared to UP. However, the differences between HP48 and HP72 occurring at the 6th and 7th day allowed to choose HP72 as the most effective hydropriming treatment (**Table 21** and **Fig. 34**).

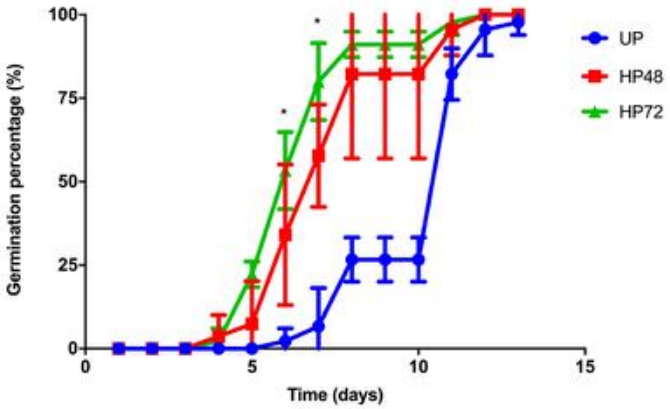


Fig. 34. Results from germination tests carried out with *S. torvum* seeds treated with hydropriming (HP48 and HP72) and with unprimed (UP) seeds. Values are expressed as mean ± SD of three independent replications with 15 seeds for each replication. Statistical analysis was carried out using Two-way ANOVA (F: 8.452; DF: 24; *P*: <0.0001****). Comparison between UP and HP48 and HP72 were carried out using the Post-Hoc Tukey's HSD test ($P \leq 0.05$).

Table 21. Results of the comparison between HP48 and HP72 treatments carried out using the Post-Hoc Tukey's HSD test ($P \leq 0.05$).

Comparison	day	q	DF	*P* value	Significance
HP48 *vs* HP72	6	3.458	78	0.0437	*
HP48 *vs* HP72	7	3.989	78	0.0166	*

Germination parameters were calculated, as reported in **Table 22**. The *G* value increased (from 97.78±3.85 to 100 %) in response to HP48 and HP72. Germination was also accelerated since the *MGT* value was reduced (from 10.26±0.44 to 7.55±1.31 and 6.71±0.30 days) respectively in HP48 and HP72 seeds compared to UP. The effects of hydropriming on *S. torvum* seeds were further evident when considering the *CVG* value, that increased from 47.60±2.97 % (UP) up to 78.24±33.30 (HP48) and 130.82±9.780 % (HP72).

For each parameter, a comparison between UP and HP treatments was carried out using the Student's t test ($P<0.05$) (**Table 22**). Based on the seed response, the HP72 protocol was selected for subsequent molecular analyses.

Table 22. Germination parameters calculated based on results of germination tests carried out on *S. torvum* seeds treated with hydropriming (HP) for increasing time (48 h and 72 h) and unprimed (UP) seeds. Values are expressed as means ± SD from three independent replicates. Asterisks indicate statistically significant differences determined using Student's t test ($P<0.05$). *G*, Germinability. *MGT*, Mean germination time. *CVG*, Coefficient of velocity of germination. *MGR*, Mean germination rate. *U*, Uncertainty. *Z*, Synchronization index. h, hours.

Treatment	***G* (%)**	***MGT* (days)**	***CVG* (%)**	***MGR* (day^{-1})**	***U* (bit)**	***Z* (unit less)**
HP 48 h	100.0 ± 0.00	7.55 ± 1.31	78.24 ± 33.30	0.0145 ± 0.0062	2.046 ± 0.20*	0.198 ± 0.043 ***
HP 72 h	100.0 ± 0.00	6.71 ± 0.03***	130.82 ± 9.80**	0.008 ± 0.0006*	2.14 ± 0.35	0.20 ± 0.10
UP	97.78 ± 3.85	10.26 ± 0.44	47.60 ± 2.97	0.021 ± 0.0015	1.53 ± 0.24	0.36 ± 0.08**

4.1.6.2. Experimental design for the molecular profiling of seed priming in *S. torvum*

Based on results obtained from the molecular analysis carried out in the '67/3' line, an experimental design was set up in order to figure out the most relevant timepoints during priming (**Fig. 35**). At the selected timepoints, seeds were collected and stored for ROS detection and *q*RT-PCR analyses. Seeds were collected at the dry state (dry seed, DS), at the end of hydropriming treatment and subsequent dry-back (DB). In order to monitor the seed response during the early phase of germination process, additional timepoints were fixed at 2h after imbibition and samples were hereby named UP2 (unprimed+2h) and HPDB (hydroprimed, dried back+2h). The sampling was limited to these early timepoints to assess whether it was possible to speed-up the prediction of the seed response, in view of future applications.

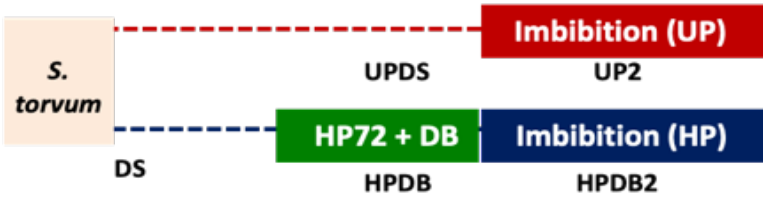


Fig. 35. Experimental design showing the timepoints taken in consideration for molecular analysis (UPDS, UP2, HPDB and HPDB2). The germination test carried with unprimed (UP) seeds and primed (HP) seeds was monitored throughout imbibition at 2 hours. UPDS, unprimed dry seed. UP2, unprimed and imbibed for 2 hours. HPDB, hydroprimed and dried back. HPDB2, hydroprimed, dried back and imbibed for 2 hours.

Molecular analyses were performed using *S. torvum* seeds collected in 2017. As shown in **Fig. 36**, when this seed lot underwent the HP72 treatment, the subsequent germination tests confirmed the expected beneficial effects of hydropriming. UP seeds started to germinate at the 6th day (2.22±0.58%) and reached the maximum value at the 13th day (97.78±3.58%) while the HP72 seeds anticipated the germination at the 4rd day (2.22±0.58%) and showed the highest germination percentage (100±0.00%) at the 12th day. Statistical analysis carried out using Two-way ANOVA (P<0.05) highlighted significant differences between the UP and HP72 seeds (F: 100.5, DF: 12; *P*: <0.0001****). A comparison between UP seeds and HP72 seeds using the Bonferroni test ($P \leq 0.05$) revealed a significant difference from day 5 to day 11 (**Table 23**).

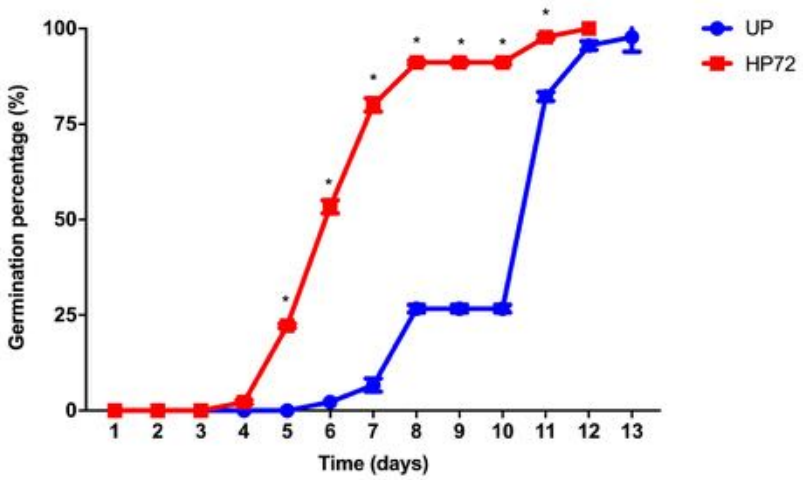


Fig. 36. Results from germination tests carried out with *S. torvum* seeds treated with hydropriming (HP72) and unprimed (UP) seeds. Values are expressed as mean ± SD of three independent replications with 15 seeds for each replication. Statistical analysis was carried out using Two-way ANOVA (F: 100.5; DF: 12; *P*: <0.0001****). Comparison between UP and HP72 were carried out using Bonferroni test ($P \leq 0.05$).

Table 23. Results of statistical analysis carried out using Two-way ANOVA (F: 100.5; DF: 12; *P*: <0.0001****). A comparison between UP and HP72 seeds was carried out using the Bonferroni test ($P \leq 0.05$).

Comparison	day	t	DF	*P* value	Significance
UP *vs* HP72	5	7.361	26	<0.0001	****
UP *vs* HP72	6	16.93	26	<0.0001	****
UP *vs* HP72	7	24.29	26	<0.0001	****
UP *vs* HP72	8	21.34	26	<0.0001	****
UP *vs* HP72	9	21.34	26	<0.0001	****
UP *vs* HP72	10	21.34	26	<0.0001	****
UP *vs* HP72	11	5.153	26	0.0003	***

The related germination parameters were calculated (**Table 24)**. The *G* value increased (from 97.78±3.85 to 100%) in response to the HP72 treatment. Germination was also accelerated since the *MGT* value was

reduced (from 10.26±0.44 to 6.71±0.30 days) in the HP72 seeds compared to UP. The effects of hydropriming on *S. torvum* seeds were further evident when considering the *CVG* value, that increased from 47.60±2.97 % (UP) up to 130.82±9.780 % (HP72). For each parameter, a comparison between UP and HP72 was carried out using the Student's *t* test ($P<0.05$). The results evidenced a significant difference in terms of *MGT*, *CVG*, *MGR*, and *Z* values.

Table 24. Germination parameters calculated based on results of germination tests carried out on *S. torvum* seeds treated with hydropriming (HP) for 72 h and unprimed (UP) seeds. Values are expressed as means ± SD from three independent replicates. Asterisks indicate statistically significant differences determined using Student's *t* test ($P<0.05$). *G*, Germinability. *MGT*, Mean germination time. *CVG*, Coefficient of velocity of germination. *MGR*, Mean germination rate. *U*, Uncertainty. *Z*, Synchronization index. h, hours.

Treament	***G* (%)**	***MGT* (days)**	***CVG* (%)**	***MGR* (day^{-1})**	***U* (bit)**	***Z* (unit less)**
HP 72 h	100.0 ± 0.00	6.71 ± 0.03***	130.82 ± 9.80**	0.008 ± 0.0006*	2.14 ± 0.35	0.20 ± 0.10*
UP	97.78 ± 3.85	10.26 ± 0.44	47.60 ± 2.97	0.021 ± 0.0015	1.53 ± 0.24	0.36 ± 0.08

In order to assess the impact of the HP72 protocol on the growth of *S. torvum* seedlings developed from primed seeds, a phenotyping analysis was carried out. Seven-day old seedlings from primed and unprimed seeds were evaluated in terms of fresh/dry weight and radicle length. Results from these analyses are shown in **Table 25**. Statistical analysis did not highlight significant changes in the dry weight and root length of seedlings resulting from the HP72 treatment, compared to those developed from unprimed seeds. A significant (P<0.05) decrease in fresh weight was recorded only in seedlings developed from seeds primed for 72 h, compared to control.

Table 25. Results of phenotyping analyses performed on seven-day old *S. torvum* seedlings developed from primed (HP72) and unprimed (UP) seeds. Values are expressed as mean ± SD of three independent replications with 15 seedlings for each replication. Asterisks indicate statistically significant differences determined using Student's *t*-test. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

Parameter	UP	HP72
fresh weight (mg/15 seedling)	8.2 ± 0.0002	5.4 ± 0.0006*
dry weight (mg/15 seedling)	1.2 ± 0.0002	0.9 ± 0.0001
radicle length (mm)	12.00 ± 2.65	13.00 ± 4.04

4.1.6.3. ROS accumulation in crop wild relatives: the case study of *S. torvum*

ROS levels were measured using the DCFH-DA fluorescent dye in dry and imbibed *S. torvum* seeds according to the experimental design (**Fig. 35**). The *S. torvum* seed lot collected in 2017 reveal significant (P:0.008**) differences in ROS level in the unprimed seeds compared with hydroprimed seeds (UP and HPDB, 1.10±0.22 and 0.27±0.13 R.F.U., respectively) (**Fig. 37**). It should be noticed that the observed ROS profile is similar to those observed in the 'high-quality' seed lots of the eggplant '67/3' line.

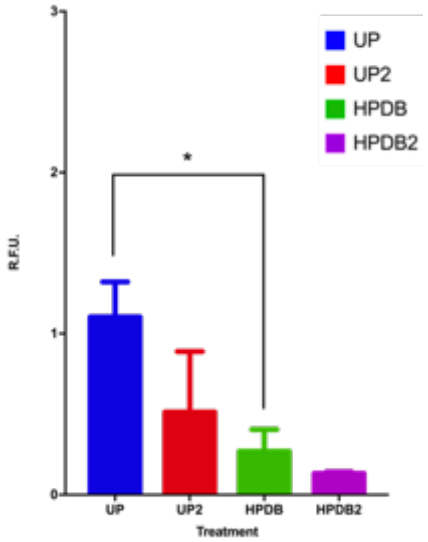


Fig. 37. Result of ROS accumulation assay carried out with *S. torvum* seeds treated with the HP72 hydropriming protocol and collected at the selected timepoints. Values are expressed as mean ± SD of three independent replications with 3 seeds for each replication. Statistical analysis was carried out using the Student's *t* test (P<0.05). UP, unprimed. UP2, unprimed and imbibed for 2 hours. HPDB, hydroprimed and dried back. HPDB2, hydroprimed, dried back and imbibed for 2 hours.

4.1.6.4. Impact of hydropriming on the expression of antioxidant/BER genes in *S. torvum*

The oligonucleotide primers previously used for the eggplant '67/3' line were tested on *S. torvum* cDNA samples. Only those for *APRT, EF, TUB, PP2Acs, SOD*, *APX* and *TDP1α* genes gave specific amplification products and were subsequently used for *q*RT-PCR analyses. The reference genes (*StTUB* and *StPP2Acs*) were selected using the geNorm algorithm (**Fig. 13B**).

Results are shown in **Fig. 38** where values represent fold changes of transcript levels and FC(HP/UP)) and FC(HP_{2h} /UP_{2h}) values < 1 or > 1 correspond to gene down- or up-regulation, respectively (**Fig. 38**, dashed line). Based on the FC values (*StSOD*, FC =0.21±0.11; *StAPX*, FC = 0.018±0.004; *StTdp1α*, FC =0.68±0.26 and *StSOD*, FC =0.024±0.008; *StAPX*, FC =0.009±0.006; *StTdp1α*, FC =0.26±0.089 respectively for HP/UP and HP_{2h} /UP_{2h}), all these genes turned out to be down-regulated in *S. torvum* in response to HP72. No significative differences were detected using the Student's *t* test (P<0.05).

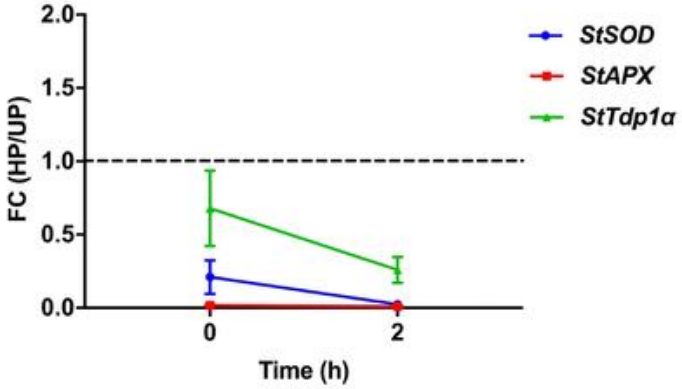


Fig. 38. Impact of hydropriming on the expression profiles of antioxidant and DNA repair genes in *S. torvum* seeds. Results of *q*RT-PCR analyses are reported as expression changes of antioxidant and BER genes. Values are expressed as mean ± SD of three independent replications with 20 seeds for each replication. SOD, superoxide dismutase. APX, ascorbate peroxidase. TDP1α, tyrosyl-DNA phosphodiesterase 1α.

4.1.6.5. Development of an optimized hydropriming protocol for *Solanum villosum*

The wild relative *S. villosum* was selected since it showed a contrasting phenotype, compared to most of the CREA-GB accessions analysed. Indeed, the acceletated germination profile of *S. villosum* provides an interesting model for comparisons at the physiological and molecular levels with eggplant accessions that display problems related to germination.

Hydropriming (HP) was tested on *S. villosum* seeds collected in 2015. Seeds soaked in H_2O for 2, 4, 12 and 24 h (HP2, HP4, HP12 and HP24) were subjected to dry-back (DB, 0.5 h) and then immediately used for germination tests. Results of germination tests carried with HP and UP treated seeds are shown in **Fig. 39.** UP seeds started germination at 3 days from the beginning of imbibition and reached the maximum germination percentage (100.00±0.00%) at 4 days whereas hydropriming always resulted in anticipated germination compared to untreated seeds. In the case of primed seeds, germination occurred at 2 days (51.11±10.18%, HP24) and 3 days (31.11±10.18%, HP2; 26.67±6.67%, HP4; 100.00±0.00%, HP12) from the beginning of imbibition. For HP12 and HP24 seeds, germination was completed at 3 days. Results of statistical analysis carried out using Two-way ANOVA (F: 68.69; DF: 8; *P*: <0.0001****) as well as comparison between UP and primed (HP2, HP4, HP12 and HP24) seeds carried out using the Post-Hoc Tukey's HSD test ($P \leq 0.05$). All types of treatment are significant compared to the unprimed (UP) seeds (**Table 26**).

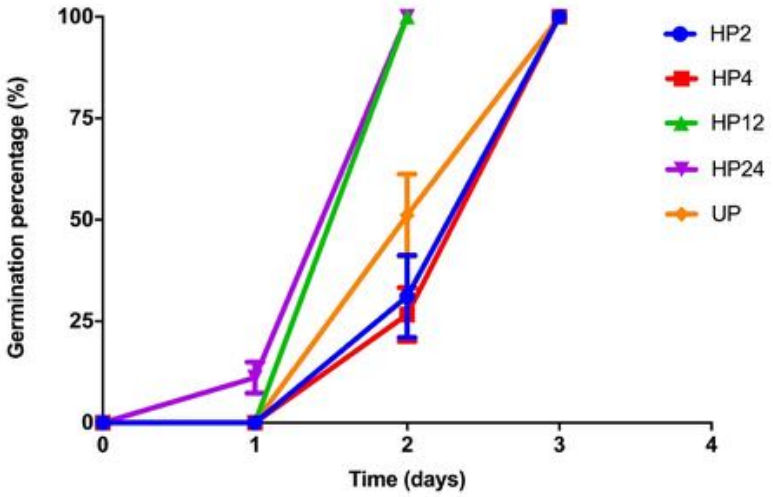


Fig. 39. Results from germination tests carried out with *S. villosum* seeds treated with hydropriming for 2, 4, 12 and 24 h and with unprimed (UP) seeds. Values are expressed as mean ± SD of three independent replications with 15 seeds for each replication. Statistical analysis was carried out using Two-way ANOVA (F: 68.69; DF: 8; *P*: <0.0001****). Comparison between UP and HP2, HP4, HP12 and HP24 were carried out using the Post-Hoc Tukey's HSD test ($P \leq 0.05$).

Table 26. Results of the comparison between UP and the different type of treatment (HP48 and HP72) were carried out using the Post-Hoc Tukey's HSD test ($P \leq 0.05$).

Comparison	q	DF	*P* value	Significance
UP *vs* HP2	4.744	10	0.0453	*
UP *vs* HP4	5.798	10	0.0144	*
UP *vs* HP12	11.6	10	<0.0001	****
UP *vs* HP24	14.23	10	<0.0001	****

The germination parameter were calculated, as reported in **Table 27**, *G* value was already 100 % in UP seeds, however the HP12 and HP24 treatments were able to accelerate germination as indicated by *MGT* values that decreased from 2.49±0.10 days (UP) to 2.00±0.00 days (HP12) and 1.89±0.04 days (HP24). The effects of hydropriming on *S. villosum* seeds were further evident when considering the *CVG* value, that increased from 521.42±25.26 % (UP) up to 672.81±0.00 %(HP12) and 718.91±16.63 %

(HP24). For each parameter, a comparison between UP and all the tested HP treatments was carried out using the Student's *t* test ($P<0.05$) (**Table 27**).

A phenotyping analysis was carried out in order to assess the impact of the different hydropriming protocol on the growth of *S. villosum* seedlings developed from primed seeds. Seven-day old seedlings from primed and unprimed seeds were evaluated in terms of fresh/dry weight and radicle length. Results from these analyses are shown in **Table 28**. Statistical analysis highlighted significant changes in the fresh weight and root length of seedlings resulting from the HP24 treatment, compared to those developed from unprimed seeds. A significant ($P<0.05$) change was also recorded in root length of seedling derived from seeds treated for 12 h. Based on the germination profile and phenotyping analysis, the HP24 protocol was selected for subsequent molecular analyses.

Table 27. Germination parameters calculated based on results of germination tests carried out on *S. villosum* seeds treated with hydropriming (HP) for increasing time (2 h, 4 h, 12 h, and 24 h) and unprimed (UP) seeds. Values are expressed as means ± SD from three independent replicates. Astersks indicate statistically significant differences determined using Student's t test (P<0.05). *G*, Germinability. *MGT*, Mean germination time. *CVG*, Coefficient of velocity of germination. *MGR*, Mean germination rate. *U*, Uncertainty. *Z*, Synchronization index. h, hours.

Treament	*G* (%)	*MGT* (days)	*CVG* (%)	*MGR* (day^{-1})	*U* (bit)	*Z* (unit less)
HP 2 h	100.0 ± 0.00	2.69 ± 0.1	474.79 ± 21.72	0.021± 9.8 x 10^{-5}	0.87 ± 0.13	0.56 ± 0.09
HP 4 h	100.0 ± 0.00	2.73 ± 0.07*	465.06 ± 13.92*	2.2 x 10^{-3} ± 6.4x 10^{-5}	0.83± 0.1	0.58 ± 0.07
HP 12 h	100.0 ± 0.00	2.00 ± 0.00*	672.81 ± 0.00**	1.5 x 10^{-3} ± 0.00*	0.00 ± 0.00***	1.00 ± 0.00***
HP 24 h	100.0 ± 0.00	1.89 ± 0.04**	718.91 ± 16.63*	1.4 x10^{-3} ± 3.38 x 10^{-5}**	0.50 ± 0.12 *	0.78 ± 0.07*
UP	100.0 ± 0.00	2.49 ± 0.10	521.42 ± 25.26	5 x 10^{-3} ± 9.2 x 10^{-5}	0.98 ± 0.02	0.48 ± 0.01

Table 28. Results of phenotyping analyses performed on seven-day old *S. villosum* seedlings developed from seeds treated with hydropriming (HP2, HP4, HP12 and HP24) and untreated (UP). Values are expressed as mean ± SD of three independent replications with 15 seedlings for each replication. Asterisks indicate statistically significant differences determined using Student's *t*-test. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

Parameter	UP	HP2	HP4	HP12	HP24
fresh weight (mg/15 seedling)	13.2 ± 0.002	13.7 ± 0.003	12.2 ± 0.0007	14.0 ± 0.002	9.3 ± 0.002*
dry weight (mg/15 seedling)	0.8 ± 0.003	1.9 ± 0.001	0.9 ± 0.0002	1.1 ± 0.0002	0.9 ± 0.0002
radicle length (mm)	62 ± 2.87	64 ± 4.36	66 ± 2.33	74 ± 1.23*	69 ± 5.87*

4.1.6.6. Experimental design for the molecular profiling of seed priming in *S. villosum*

As a first step, an experimental design was set up in order to figure out the most representative timepoints during priming (**Fig. 40**). At the selected timepoints, seeds were collected and stored for ROS detection and *q*RT-PCR analysis. Seeds were collected at the dry state (dry seed, DS) and during the treatment at 2, 4, 8, 16 and 24 h. An additional timepoint was fixed at the end of the dehydration (dry-back, DB). In order to monitor the seed response during the germination process, additional timepoints were fixed, as representative of the temporal window overlapping the pre-germinative metabolism. As shown in **Fig. 40,** seeds were collected throughout imbibition at 2, 4, 8, 12, 16, 24 h and finally at the end of germination (radicle protrusion stage, RD).

Fig. 40. Experimental design showing the most representative timepoints (2, 4, 8, 16 and 24 h) during the HP24 treatment, followed by the end point of the dehydration step (dry-back, DB, 0.5 h). The germination test carried with unprimed (UP) seeds and primed (HP) seeds was monitored throughout imbibition at 2, 4, 8, 12, 16, 24 and finally at the radicle protrusion stage (RD).

Molecular analyses were performed using *S. villosum* seeds collected in 2018. As shown in **Fig. 41** when this seed lot underwent the HP24 treatment, the subsequent germination tests confirmed the expected beneficial effects of hydropriming. UP seeds started to germinate at the 2th day (23.33±23.09%) and reached the maximum value at the 3th day (100%) while the HP24 seeds anticipated the germination at the 1rd day (26.67±5.77%) and showed the highest germination percentage (100%) at the 2th day. Statistical analysis carried out using Two-way ANOVA ($P<0.05$) highlight significant differences between the UP and HP24 seeds (F: 24.06, DF: 2; P: <0.0004***). A comparison between UP seeds and HP24 seeds using the Bonferroni test ($P \leq 0.05$) revealed a significative difference at day 2 (**Fig. 41 and Table 29**).

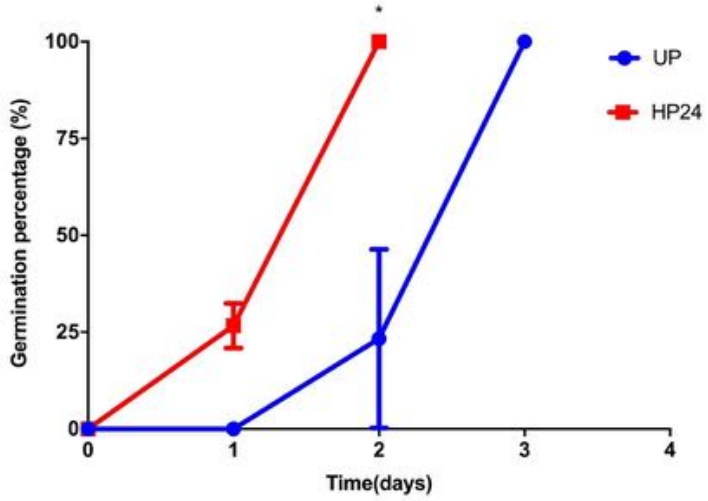


Fig. 41. Results from germination tests carried out with *S. villosum* seeds treated with hydropriming (HP24) and unprimed (UP) seeds. Values are expressed as mean ± SD of three independent replications with 15 seeds for each replication. Statistical analysis was carried out using Two-way ANOVA (F: 24.06; DF: 2; *P*: <0.0004***). Comparison between UP and HP24 were carried out using Bonferroni test ($P \leq 0.05$).

Table 29. Results of the comparison between UP seeds and HP24 seeds carried out using the Bonferroni test ($P \leq 0.05$).

Comparison	day	t	DF	P value	Significance
UP *vs* HP24	1	3.361	12	0.085	ns
UP *vs* HP24	2	9.662	12	<0.0001	****
UP *vs* HP24	3	0	12	>0.9	ns

The germination parameters were calculated (**Table 30)**. The *G* value was 100% in both UP and HP24 seeds. However, germination was significantly accelerated since the *MGT* value was reduced (from 2.77±0.23 to 1.73±0.06 days) in the HP24 seeds compared to UP. The effects of hydropriming on *S. villosum* seeds were further evident when considering the *CVG*, *MGR* and *U* values. A comparison between UP and HP24 treatment, for each parameter was carried out using the Student's *t* test ($P<0.05$). The results displayed a significative difference in terms of mean germination time (*MGT*), speed of germination (*CVG*) and mean germination rate (*MGR*).

Table 30. Germination parameters calculated for the seed lot of *S. villosum* treated with hydropriming (HP24). *G*, Germinability. *MGT*, Mean germination time. *CVG*, Coefficient of velocity of germination. *MGR*, Mean germination rate. *U*, Uncertainty. *Z*, Synchronization index. UP, unprimed seeds. Asterisks indicate statistically significant differences determined using Student's *t*-test. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

Parameter	UP	HP24
G (%)	100.00 ± 0.00	100.00 ± 0.00
MGT (days)	2.77 ± 0.23	1.73 ± 0.06*
CVG (%)	277.84 ± 33.85	503.88 ± 20.01**
MGR (day^{-1})	0.004 ± 0.0004	0.002 ± 8.1 x 10^{-5}*
U (bit)	0.646 ± 0.31	0.83 ± 0.09
Z (unit less)	0.61 ± 0.33	0.57± 0.06

Also in this case, phenotyping characterization was carried out. Seven-day old seedlings from primed and unprimed seeds were evaluated in terms of fresh/dry weight and radicle length. Results from these analyses are shown in **Table 31**. Statistical analysis did not highlight significant changes in the parameters taken in consideration.

Table 31. Results of phenotyping analyses performed on seven-day old *S. villosum* seedlings developed from seeds treated with hydropriming (HP24) and unprimed (UP) seeds. Values are expressed as mean ± SD of three independent replications with 15 seedlings for each replication. Asterisks indicate statistically significant differences determined using Student's *t*-test. (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

Parameter	UP	HP24
fresh weight (mg/15 seedling)	8.2 ± 0.0004	10.5 ± 0.0002
dry weight (mg/15 seedling)	1.1 ± 0.0033	0.8 ± 0.0002
radicle length (mm)	66.00 ± 2.85	64.00 ± 4.98

4.1.6.7. ROS accumulation in crop wild relatives: the case study of *S. villosum*

ROS levels were measured using the DCFH-DA fluorescent dye in dry and imbibed *S. villosum* seeds according to the experimental design (**Fig. 40**). Samples were collected at 2, 4, 8, 16 and 24 h during HP and subsequently after the dry-back step (DB, 0.5 h of dehydration). During germination tests,

unprimed (UP) and primed (HP) seeds were collected at 2, 4, 8, 12, 16, 24 h from the beginning of imbibition. Results of these experiments are shown in **Fig. 42**. Statistical analysis was carried out using the Student's *t* test (*P*<0.05).The results reveal significant (*P*:0.05*) differences in ROS level in the unprimed and imbibed for 16 h seeds compared with hydroprimed and imbibed for 16 h seeds (UP16 and HPDB16, 0.84±0.17 and 2.90±3.74 R.F.U., respectively).

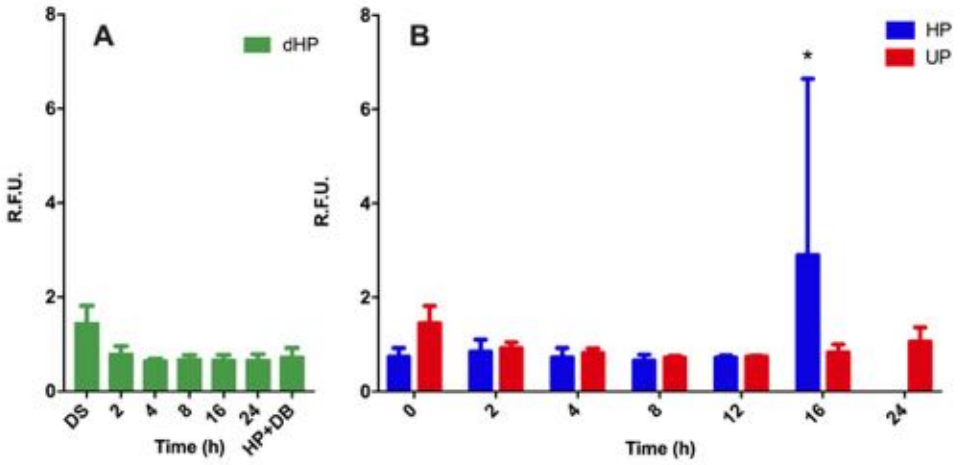


Fig. 42. Result of ROS accumulation assay carried out with *S. villosum* seeds treated with the HP24 hydropriming protocol and collected at the selected timepoints. Values are expressed as mean ± SD of three independent replications with 3 seeds for each replication. Statistical analysis was carried out using the Student's *t* test (*P*<0.05). DS, dry seed. +DB, hydropriming + dry back. UP, unprimed. HP, hydroprimed.

4.1.6.8. Impact of hydropriming on the expression of antioxidant/BER genes in *S. villosum*

The oligonucleotide primers previously used for the eggplant '67/3' line were also tested on *S. villosum* cDNA samples, only those for *APRT, EF, PP2Acs, SOD*, *APX* and *TDP1α* genes gave specific amplification products and were subsequently used for *q*RT-PCR. Reference genes for *q*RT-PCR analysis were selected using geNorm algorithm (**Fig. 13C**).

Results of *q*RT-PCR analyses performed on *S. villosum* samples collected during the HP24 treatment (at 2, 4, 8, 16 and 24 h) and compared to the DS are shown in **Fig. 43A**. Values represent fold changes of transcript levels where FC = primed seed (HP)/dry seed (DS) and FC(HP/DS) values < 1 or > 1 correspond to gene down- or up-regulation, respectively (**Fig. 43A**, dashed line). When considering the effects of HP24 treatment along the tested time

points, compared to DS, the antioxidant genes were down-regulated. As indicated by the observed FC(HP/DS) values (< 1) a borderline up-regulation (1.10±0.2) of the *SvTdp1α* gene at 16 h was revealed.

Results of *q*RT-PCR analyses performed on the *S. villosum* samples, both HP and UP seeds, collected during the germination test (0, 2, 4, 8, 12, 16 and 24) and at the radicle protrusion stage (RD) are shown in **Fig. 43B** as expression changes of antioxidant and BER genes. Values represent fold changes of transcript levels where FC = primed seed (HP)/unprimed seeds (UP) and FC(HP/UP) values < 1 or > 1 correspond to gene down- or up-regulation, respectively. When comparing HP seeds subjected to dry-back with UP dry seeds (**Fig. 43B**, 0 h), the observed FC(HP/UP) values indicated down-regulation of the three genes taken in consideration. The impact of the HP treatment on seed imbibition is evidenced by distinct waves of responses. At 2h, the FC(HP/UP) value of *SvTdp1α* significantly increase (307.94±109.9) displaying a wave of up-regulation, there is also an up-regulation of *SvAPX* gene (2.07±1.13), suggesting for enhanced expression during the first phases of germination. At 4 h of imbibition the *SvTdp1α* and *SvAPX* genes were still up-regulated (17.41±3.04 and 1.74±0.9 respectively). At 8 and 12h, the treatment resulted into a distinctive response of antioxidant and BER genes. Indeed, *SvAPX* (23.35±4.47 and 52.62±26.79), *SvSOD* (28.65±2.35 and 293.17±120.21) and *SvTdp1α* (5.00±1.6 and 16.65±11.69) were up-regulated. In particular, *SvSOD* gene reveal, as before for *SvTdp1α* at 2h, another wave of up-regulation. At 16 h, the expression level of *SvSOD* decrease drastically (0.09±0.04) instead, *SvAPX* gene gets ready for a new wave of up-regulation checked at the radicle protrusion stage (RD) (283.67±207.09). Statistical analysis was carried out using the Student's t test ($P<0.05$). It is worth noting that *SvAPX* gene always showed significant expression changes, except for the 2 h and 4 h timepoints and the RD stage. Also, *SvSOD* gene always showed significant expression changes, except for the 0 h (DS and HP24 + DB) and 16 h timepoints while *SvTdp1α* gene did not displayed significant differences only during the treatment at 16 h.

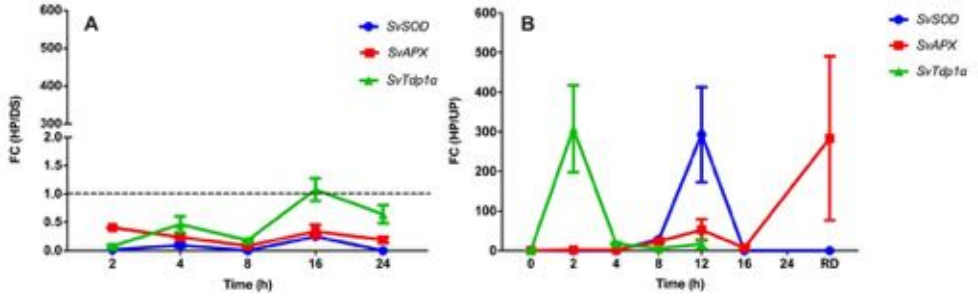


Fig. 43. Impact of hydropriming on the expression profiles of antioxidant and DNA repair genes in *S. villosum* seeds. Results of *q*RT-PCR analysis are reported as expression changes of antioxidant and BER genes. Values are expressed as mean ± SD of three independent replications with 20 seeds for each replication. SOD, superoxide dismutase. APX, ascorbate peroxidase. TDP1α, tyrosyl-DNA phosphodiesterase 1α.

4.1.7. Impact of priming in *Medicato truncatula* seeds grown in agricultural solis

4.1.7.1. Priming improves *Medicago truncatula* seed germination in soil and upregulates specific genes used as indicators of seed quality

Alongside the work carried out in eggplant, where priming has been applied under laboratory conditions, priming treatments have also been performed in the field using *M. truncatula* as a model system. It is the case, the seeds subjected to hydropriming and biopriming were germinated in two types of agricultural soil (Soil_A and Soil_B). The field experiments were carried out at the Institute of Environment and Sustainable Development (IESD), Banaras Hindu University (BHU), Varanasi (India), within the frame of a TECO (Technological Eco-Innovations for the Quality Control and the Decontamination of Polluted Waters and Soils) collaborative project. The experimental design of the study is illustrated in **Fig. 44.**

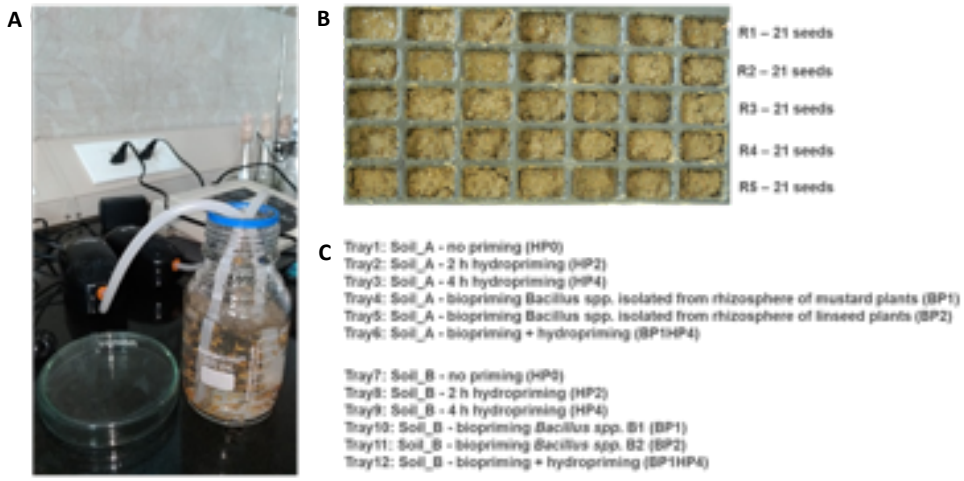


Fig. 44. Experimental design and working system. **(A)** Hydropriming treatment of *M. truncatula* seeds. **(B)** Germination tray, containing five replicates of 21 seeds each. **(C)** treatment labels.

4.1.7.2. Priming treatments enhanced *M. truncatula* seed germination and seedling development

The seeds subjected to hydropriming and biopriming were grown on Soil_A and Soil_B to evaluate seed germination and seedling performance. In non-primed seeds (HP0), the germination percentage on soil_A was 40.2±5.06% while a slight decrease (33.2±5.81%) was observed when seeds were grown on soil_B (**Fig. 45, A**). The relatively low basal germination in soil may be due to the higher than optimal temperatures registered at the growing site, and storage conditions. Hydropriming performed for 4 h had significantly increased seed germination percentage on both types of soil. The highest germination percentage was observed with the HP2 treatment (60±11.72 %) on Soil_B and HP4 treatment (60±9.35 %) on Soil_A, respectively. Conversely, biopriming and the combination between biopriming and hydropriming did not result in any significant differences compared to non-primed samples (HP0) (**Fig. 45, A**). The overall differences between the imposed conditions, from a statistical point of view, are mostly due to the priming treatments ($P < 0.001$) rather than the soil type ($S = 0.468$). The positive effect of hydropriming on seed germination was confirmed also in a second experiment carried out in August 2019 (**Figure 46**). Although in this case, the germination percentage was considerably lower than in the first experiment, hydropriming treatments still resulted in enhanced germination. As the temperatures during this period were higher than the previous conditions, these experiments also confirm that germination is affected by

high temperatures and storage conditions. Garcia et al., 2006 indicated that the optimal temperature for *M. truncatula* seed germination is 18-20˚C while paper envelopes or aired plastic vials are recommended for storage conditions.

When considering the germination speed (T_{50}), it is possible to observe that non-primed seeds germinated in about 3 days, independently of the type of soil (**Fig. 45, B**). In this case, the hydropriming treatments did not result in any significant differences compared to the non-primed control. However, biopriming seemed to negatively affect germination speed as the maximum percentage of germination was reached in up to 8 days in some of these cases (e.g. BP2). The high standard deviations are because the germination of these seeds was not uniform. Also in this case, the differences are mainly attributed to the priming treatments ($P < 0.001$).

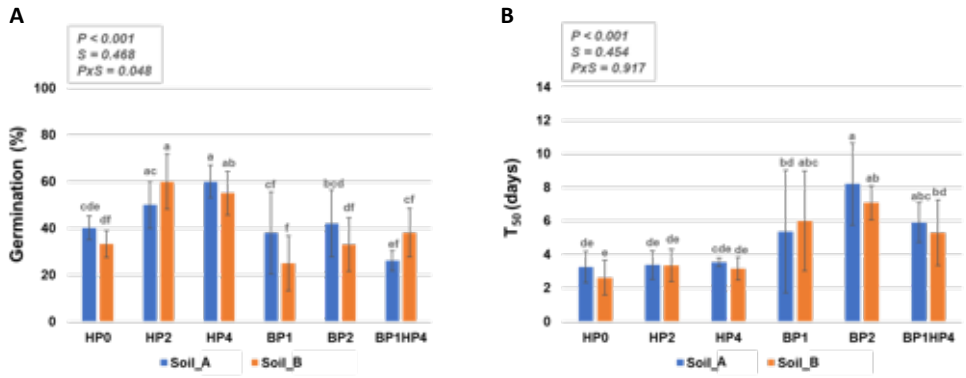


Fig. 45. Germination of primed and non-primed *M. truncatula* seeds grown on two types of agricultural soils (Soil_A and Soil_B) collected from Varanasi. **(A)** Germination percentage (%) calculated after 14 days of seedling monitoring. **(B)** The time required for 50% of seeds to germinate (T_{50}) calculated on everyday basis. Data are represented as means ± SD of five replicates. Significant differences (as per Duncan test) are shown with lowercase letters and the significance (*P*-values) for each type of treatment as well as the interaction between treatments is included in the upper panel of each graphic (P, priming; S, soil, PxS, interaction between priming and soil). HP0, non-primed control; HP2, 2h hydropriming; HP4, 4 h hydropriming; BP1, *Bacillus* strain isolated from mustard rhizosphere; BP2, *Bacillus* strain isolated from and linseed rhizosphere; BP1HP4, 4 h of hydropriming and BP1 strain.

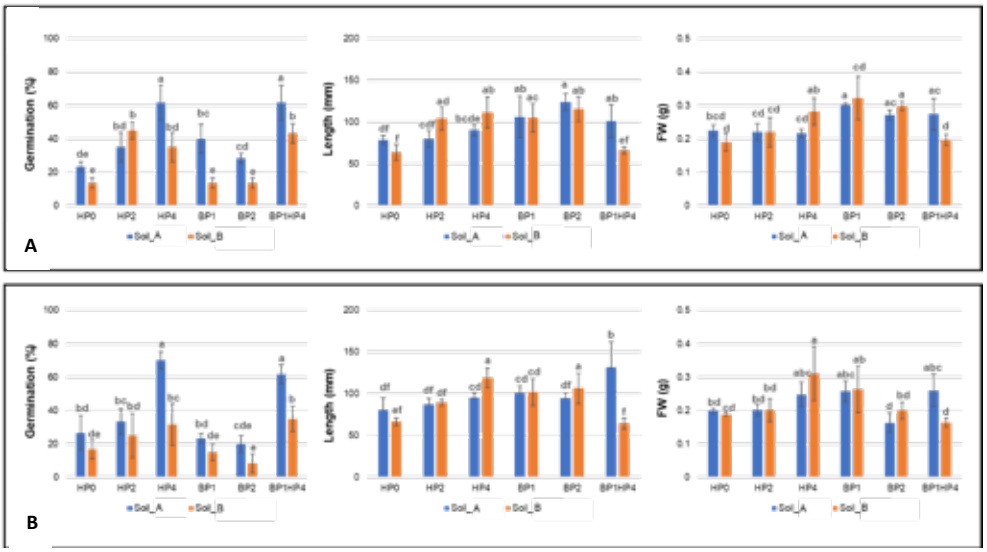


Fig. 46. Phenotipic characterization of primed and non-primed *M. truncatula* seeds grown in two types of agricultural soils (Soil_A and Soil_B) from Varansi region in August 2019. The experiment was performed when day/night temperature ranged between 31-36/24-29°C and the relative humidity was around 90%. Measurements of germination percentage (%), seedling length (mm), and fresh weight (FW, g) were performed in two independent experiments (**A, B**) each consisting of five replicates (21 seeds per replicate). Data are represented as means ± SD of five replicates. Significant differences (as per Duncan test) are shown with lowercase letters. HP0, non-primed control; HP2, 2h hydropriming; HP4, 4 h hydropriming; BP1, *Bacillus* strain isolated from mustard rhizosphere; BP2, *Bacillus* strain isolated from and linseed rhizosphere; BP1HP4, 4 h of hydropriming and BP1 strain.

If the germination percentage is more influenced by the hydropriming treatments, the seedling growth, on the other hand, seems to be more influenced by the biopriming treatments. This is mostly reflected by enhanced biomass rather than seedling length, as no statistically significant differences are observed in this parameter in most of the cases. However, a significant difference was observed when seedlings grown from non-primed seeds (HP0) on Soil_B as compared with those grown in Soil_A; namely, a decrease in seedling length is noticed when seeds are grown in Soil_B (**Fig. 47, A**). Interestingly, this was significantly recovered only by the HP2 treatment. The fact that seedling growth is affected by the type of soil is evident also when measuring seedling biomass (reflected by the FW values) under non-primed conditions (HP0) (**Fig. 47, B**). However, FW and DW were improved by biopriming, especially when seedlings were grown in the Soil_B (**Fig. 47, B,C**). Similar to the germination parameters, from a statistical point of view,

the broad differences between the observed responses are primarily due to the priming treatments. The improved seedling growth is evident also in the second experiment performed in August 2019 (**Fig. 46**). In this case, even enhanced seedling length was observed when considering the HP4 (Soil_B), BP2 (Soil_B), and BP1HP4 (Soil_A) conditions. Higher biomass, in terms of fresh weight, was registered under BP1 (Soil_A) and BP2 (Soil_B) conditions. These experiments show that even if the germination rate is decreased, the priming treatments still provide support for better seedling development in the first two weeks of the plants' life.

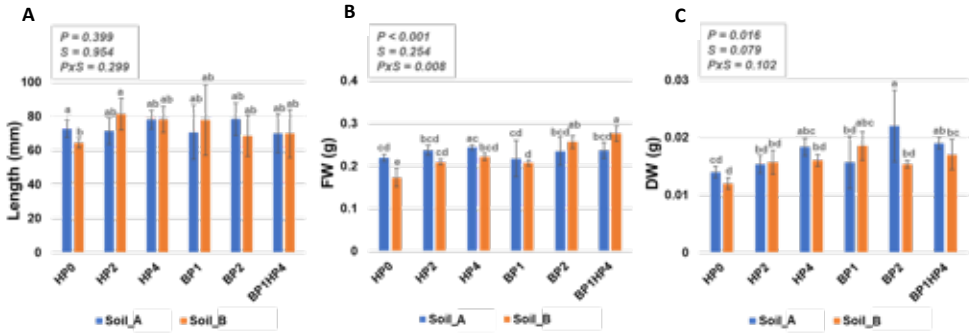


Fig. 47. Evaluation of *M. truncatula* seedling growth on two types of agricultural soils (Soil_A, Soil_B) collected from Varanasi for 14 days. **(A)** Seedling length (mm). **(B)** Measurement of fresh weight (FW, g). **(C)** Measurement of dry weight (DW, g). Data are represented as means ± SD of five replicates. Significant differences are shown with lowercase letters and the *P*-values are included in the upper panel of each graphic (P, priming; S, soil, PxS, interaction between priming and soil). HP0, non-primed control; HP2, 2h hydropriming; HP4, 4 h hydropriming; BP1, *Bacillus* strain isolated from mustard rhizosphere; BP2, *Bacillus* strain isolated from and linseed rhizosphere; BP1HP4, 4 h of hydropriming and BP1 strain.

4.1.7.3. Enhanced expression of genes involved in DNA repair and antioxidant response is characteristic to seedlings grown from primed seeds

In our previous works, we reported that certain genes that play roles in DNA repair and antioxidant responses can be used as indicators of seed quality and seeding growth (Balestrazzi et al., 2011a; Araùjo et al., 2019). Here we focused on the expression profiles of OGG1 (8-Oxoguanine DNA glycosylase) and FPG (Formamidopyrimidine-DNA glycosylase), involved in Base Excision Repair (BER), APX (Ascorbate peroxidase) and SOD (Superoxide dismutase), encoding known ROS scavengers, and MT2 (Metallothionein type 2), proven to act both as scavenger and repair enzyme (Balestrazzi et al., 2011a). The expression profiles of these genes were tested both in dry and hydroprimed seeds as well as in the two-weeks old seedlings

grown in soil. In seeds, it is possible to observe that 2 h and 4 h of hydropriming did not significantly change the relative expression of OGG1 and MT2 genes as compared to dry seeds (DS). Conversely, significant upregulation was registered for the SOD and APX genes in response to both hydropriming timepoints while the FPG gene was upregulated only in response to the HP4 treatment (**Figure 48, A**). When considering the OGG1 gene expression, it is possible to observe that the seedlings grown from bioprimed seeds (BP1, BP2, BP1HP4) in Soil_B presented a significant upregulation of the OGG1 gene (**Figure 48, B**). As for the FPG, downregulation of the gene is observed in non-primed seeds grown in Soil_B (**Figure 48, C**). The hydropriming treatment for 2 h (HP2) resulted in a significant upregulation of the gene in both Soil_A and Soil_B. A similar trend was observed in the case of the MT2 gene, where hydropriming and the combination between hydropriming and biopriming (BP1HP4) resulted in enhanced gene expression (**Figure 48, D**). Differently, the expression of APX and SOD genes was more influenced by the biopriming treatments as well as the BP1HP4 combination. In the case of the APX gene, the highest expression was induced by the BP2 treatment on Soil_B (**Figure 48, E**) whereas the SOD gene peaked when the BP1 treatment was implemented on the same soil type (**Figure 48, F**).

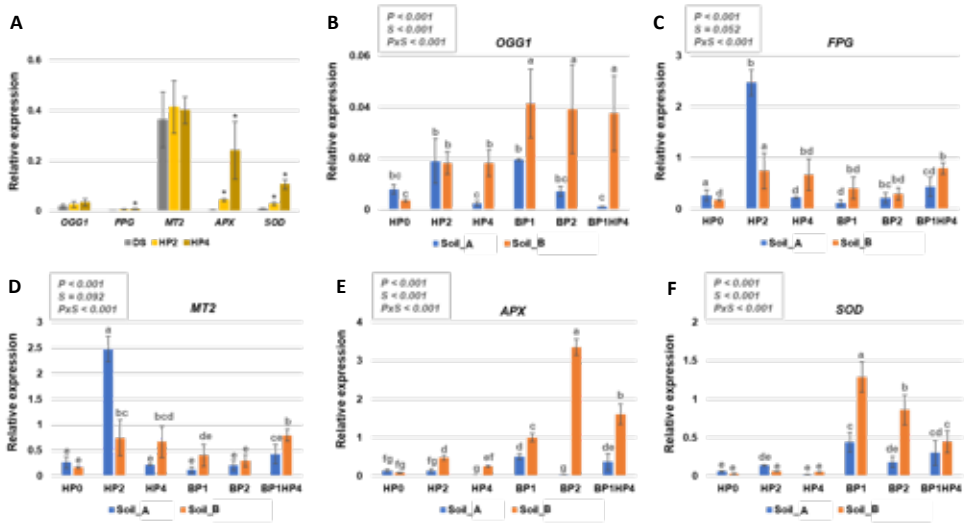


Figure 48. Gene expression profiles in *M. truncatula* seeds and 14-days-old seedlings grown from primed and non-primed seeds on two types of agricultural soils (Soil_A, Soil_B) collected from Varanasi. **(A)** Relative expression of *OGG1*, *FPG*, *MT2*, *APX*, and *SOD* genes in dry seeds (DS) and seeds treated with hydropriming for 2 h (HP2) and 4 h (HP4). Asterisks '*' show statistical significance ($p < 0.05$)

compared to DS. **(B)** Relative expression of *OGG1* gene in seedlings. **(C)** Relative expression of *MT2* gene in seedlings. **(D)** Relative expression of *FPG* gene in seedlings. **(E)** Relative expression of *APX* gene in seedlings. **(F)** Relative expression of *SOD* gene in seedlings. Data are represented as means ± SD of five replicates. Significant differences are shown with lowercase letters and the *P*-values are included in the upper panel of each graphic (P, priming; S, soil, PxS, interaction between priming and soil). HP0, non-primed control; HP2, 2h hydropriming; HP4, 4 h hydropriming; BP1, *Bacillus* strain isolated from mustard rhizosphere; BP2, *Bacillus* strain isolated from and linseed rhizosphere; BP1HP4, 4 h of hydropriming and BP1 strain.

4.1.7.4. Integrative data analyses

To better understand how the samples behave comparatively, a PCA analysis was performed taking into consideration the imposed conditions (soil type and priming treatments) and measured variables (germination %, T_{50}, seedling length, FW, DW, and gene expression profiles). The two main principal components extracted accounted for 65.7% of the variance (**Figure 49**). Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. X and Y axis show principal component 1 (PC1) and principal component 2 (PC2) that explain 44.8% and 20.9% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. Variables OGG1 (0.41), APX (0.40), SOD (0.45), and MT2 (0.45) seem to be mostly correlated with PC1, while variables DW (0.66), seedling length (0.55), and T_{50} (0.32) are correlated with PC2. It is however interesting to observe how the treatments are grouped based on the type of soil and priming treatments. Mainly, the Soil_A samples are mostly clustered together (in red); although, samples Soil_B_HP2 and Soil_B_HP4 (in blue) are an integral part of this cluster (**Figure 49**). This is indicative of the fact that the hydropriming treatments seem to be able to rescue the seedlings grown on Soil_B, having a higher TDS concentration than Soil_A.

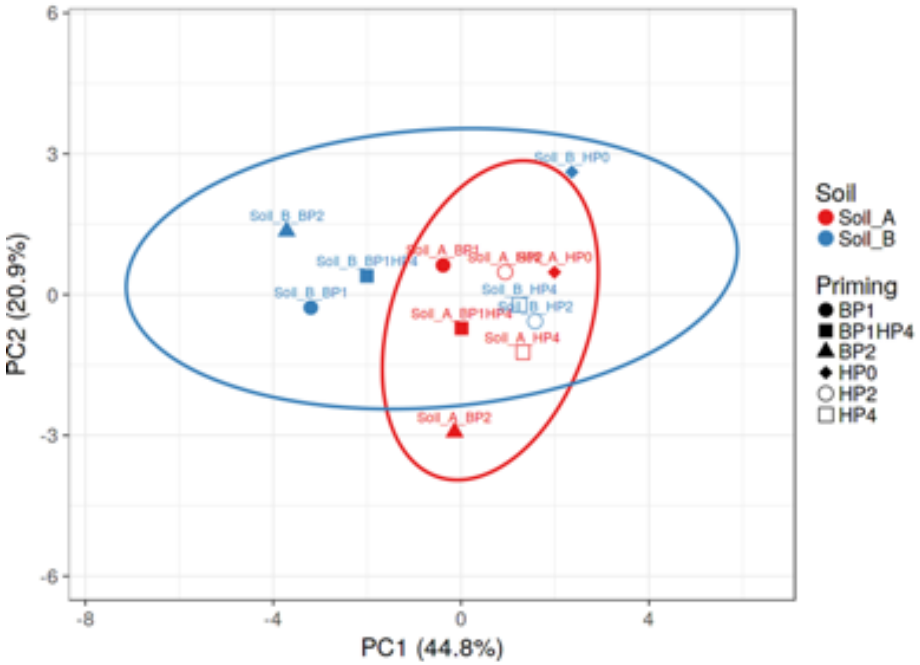


Fig. 49. Integrative analyses of primed and non-primed seeds grown on control (Soil_CTRL) and heavy-metal (Soil_HM) contaminated soils. **(A)** Principal Component Analysis loading plot explaining the distribution of samples based on the imposed conditions and measured variables. **(B)** Pattern search distribution of samples taking into consideration germination % and gene expression profiles. HP0, non-primed control; HP2, 2h hydropriming; HP4, 4 h hydropriming; BP1, *Bacillus* strain isolated from mustard rhizosphere; BP2, *Bacillus* strain isolated from and linseed rhizosphere; BP1HP4, 4 h of hydropriming and BP1 strain.

4.2. Plant genetic transformation as a premise to improve seed quality: development of optimized protocols in monocots and dicots model species

4.2.1. Genome editing applied to mutate the *MtTdp1β* gene in *M. truncatula*

The enzyme tyrosyl-DNA phosphodiesterase (Tdp1) is a BER component extensively investigated in animal cells due to its role in the repair of topoisomerase I-DNA covalent lesions. In plants, the small *Tdp1* gene family is composed by *Tdp1α* and *Tdp1β* genes encoding the Tdp1α and β isoforms. The expression of the *Tdp1* genes is correlated with different stress conditions in several experimental systems, including cell suspensions, aerial parts, and seeds of *M. truncatula* (Balestrazzi et al., 2011a; Sabatini et al., 2017;

Macovei et al., 2018), suggesting their recurrent involvement in the plant response to genotoxic stress. The modulation of *Tdp1α* gene during the seed pre-germinative metabolism is indicative for its use as a marker of seed quality, as exemplified in *S. melongena*. In *M. truncatula*, the *MtTdp1α* gene has been well characterized (Donà et al., 2013) while the role of *MtTdp1β* gene is not fully understood.

Hence, to better characterize the putative functions of *MtTdp1β*, a CRISPR/Cas9 approach was pursued to induce targeted mutations to knockdown or silence the gene. The selected sgRNA (see **Table 5, paragraph 3.7.1.**) targets the site located upstream of the catalytic HKD1, essential for the proper functioning of the protein; if mutated, it could lead to the formation of a non-functional protein. The CRISPR_Tdp1β construct (synthesized by Genewiz), described in Materials & Methods (see **Fig. 14** section 3.7.1.), was introduced into *A. tumefaciens* EHA105 strain by electroporation. The *Agrobacterium*-mediated transformation of *M. truncatula* was carried out using three different types of plant material: cell suspension, petioles, and leaf discs.

4.2.1.1. *Agrobacterium*-mediated genetic transformation of *M. truncatula* cell suspension

The *A. tumefaciens* culture containing the CRISPR_Tdp1β construct was used for the genetic transformation of cell suspension of *M. truncatula*. Differences in cell growth were also observed in relation to the concentration of *A. tumefaciens* culture (1 or 3 ml of culture) and duration of co-cultivation (2 or 5 days). The best result was obtained when the suspension cells were co-cultivated for 2 days with 1 ml of bacterial suspension.

After co-cultivation, fluorescence optical microscope analysis was performed to verify the expression of the *GFP* reporter gene present in the construct. The cells emitting fluorescence are indicated by red arrows. In **Fig. 50, A**, it is possible to observe a transformed microcallus in the fluorescent field while **Fig. 50 B** shown the same microcallus under the bright, black and white, field. **Fig. 50 C** and **D** show the same analysis performed on a wild-type microcallus, that did not contain the GFP protein.

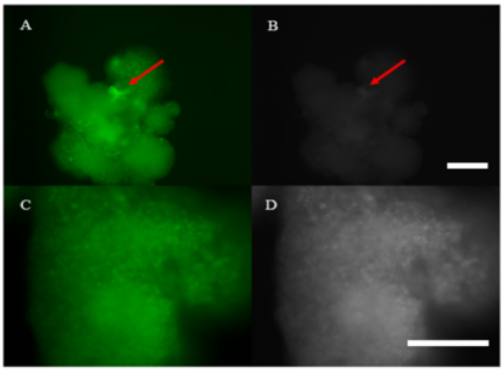


Fig. 50. Fluorescence microscopy observation of the *M. truncatula* cells transformed with the CRISPR_Tdp1β construct. **(A)** transformed cells visible in the fluorescent field; **(B)** transformed cells visible in the bright field (black and white); **(C)** wild-type non-transformed cells visible in the fluorescent field; **(D)** wild-type cells visible in the bright field (black and white). A and B, bar = 200 µm. C and D, bar = 20 µm

In addition to microscopy analyses, PCR was performed to amplify the *GFP* (552 bp) and *hCas9* (455 bp) genes (**Fig. 51**).

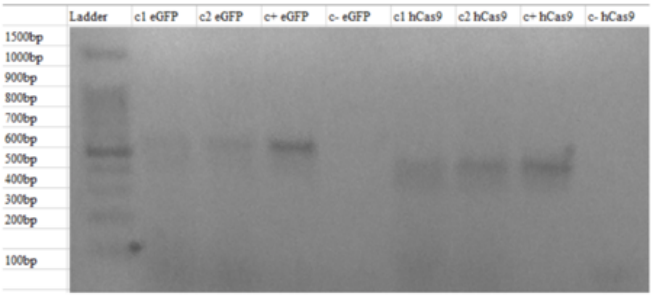


Fig. 51. Electrophoresis on agarose gel showing the results of PCR amplification for GFP and hCas9 sequences present in the DNA extracted from control and transformed suspension cells.

4.2.1.2. *Agrobacterium*-mediated genetic transformation of *M. truncatula* petioles

The petioles utilized were obtained either from *in vitro* continuosly repropagated *M. truncatula* plants or from newly *in vitro* plants derived from scarified seeds. **Table 32** shows the number of explants utilized to carry out the experiment and the number of calli developed from transformed petioles. The use of petioles derived from newly germinated seeds or repropagated plants was intended as a test to understand if the origin of the petioles could influence transformation efficiency. No differences were highlighted between

the petioles derived from different origins. Petioles used as control and those transformed only with Cas9, derived from *in vitro* repropagated plants, were eliminated after the 5-day co-cultivation phase because *Agrobacterium* was not removed by antibiotics. In petioles derived from scarified seed, there was a significant reduction of number of petioles between the day 26 and day 33, probably due to selection with antibiotics. Interestingly, the calli developed from petioles transformed with Cas9 vector were healthier than the calli derived from petioles transformed with the CRISPR_Tdp1β construct.

Table 32. Experimental time-lapse. The number of petioles used for *Agrobacterium*-mediated transformation and resulting calli derived from petioles obtained either from seed or from repropagated material are indicated. C, control, non-transformed petioles/calli. Cas9, petioles/calli transformed with *A. tumefaciens* containing the plasmid with only the Cas9 cassette. Tdp1β, petioles/calli transformed with *A. tumefaciens* containing the CRISPR_Tdp1β vector.

Day	Explant						Calli					
	Seed			Repropagated			Seed			Repropagated		
	C	***Cas9***	***Tdp1β***	***C***	***Cas9***	***Tdp1β***	***C***	***Cas9***	***Tdp1β***	***C***	***Cas9***	***Tdp1β***
0	14	15	50	-	12	13	-	-	-	-	-	-
5	14	15	50	-	-	7	-	-	-	-	-	-
12	13	15	39	-	-	7	1	14	24	-	-	6
19	13	15	39	-	-	7	1	13	20	-	-	8
26	13	15	39	-	-	7	1	16	19	-	-	9
33	7	14	16	-	-	5	1	15	16	-	-	7
40	7	14	16	-	-	5	1	15	16	-	-	7
47	7	14	16	-	-	5	1	15	16	-	-	7
54	-	13	14	-	-	5	1	14	15	-	-	7
61	-	13	14	-	-	5	1	13	12	-	-	5
68	-	13	14	-	-	5	1	13	15	-	-	5
75	-	13	14	-	-	5	1	13	15	-	-	5

After 40 days, the developed calli were tested for the presence of the GFP signal by using a transilluminator. In **Fig. 52, A** and **C**, it is possible to observe the calli derived from petioles transformed with the construct containing only the Cas9 under normal and UV light, respectively, while **Fig. 52, B** and **D** shows the calli derived from petioles transformed with the CRISPR_Tdp1β construct. Red arrows indicate the presence of GFP fluorescence.

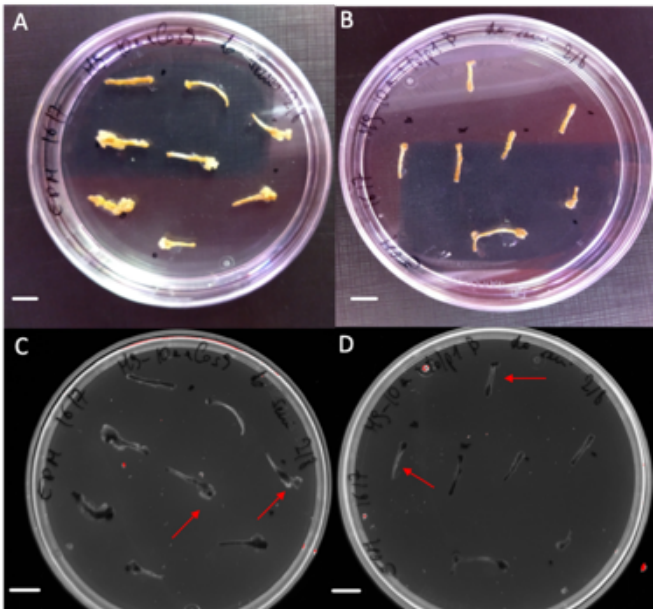


Fig. 52. Images of *M. truncatula* calli derived from *Agrobacterium*-mediated transformation of petioles. (**A)** calli transformed with Cas9 vector visualized at normal light (**B**) calli transformed with Tdp1β vector visualized at normal light (**C**) calli transformed with Cas9 vector visualized at the transilluminator. (**D**) calli transformed with Tdp1β vector visualized at the transilluminator. Red arrows indicate the GFP fluorescence. Bar = 1 cm

The selected calli are currently maintained on embryo maturation and proliferation medium (EPM). Then, the embryos will be isolated from the callus and transferred to the embryo conversion medium (ECM) to permit the growth of plantlets. Once developed, the plantlets will be transferred on rooting medium to allow the development of the root system. The fact that CRISPR-Tdp1β calli are less developed than control calli (obtained from material transformed with the construct containing only the Cas9 without the sgRNA) suggests that the gene is successfully targeted and may have important roles in cell differentiation and proliferation.

4.2.1.3. *Agrobacterium*-mediated genetic transformation of *M. truncatula* leaf discs

Genetic transformation was finally carried out using *M. truncatula* leaf discs derived from *in vitro* repropagated plant or from *in vitro* plants grown from scarified seeds. **Table 33** illustrates the number of explants utilized to

carried out the experiment and the number of calli developed from transformed leaf discs. The use of leaf discs derived from newly germinated seeds or repropagated plants was intended as a test to understand if the origin of the leaf discs could influence the efficiency of transformation. Comparing the two types of leaf discs, those derived from *in vitro* repropagated plants generated a greater number of calli compared to those produced by plants derived from scarified seeds. Another observation relates to the leaf color after the 5 five days of co-culture: leaf discs from repropagated plants were green and a little bit light yellow while the other ones were yellow. Similar to the experiments performed with petioles, the calli developed from leaf discs transformed with Cas9 vector were healthier than the calli derived from leaf discs transformed with the CRISPR_Tdp1β construct.

Table 33. Experimental time-lapse. The number of leaf discs used for *Agrobacterium*-mediated transformation and obtained calli derived from leaf discs obtained either from seed or from repropagated material are indicated. C, control, non-transformed leaf discs/calli. Cas9, leaf discs/calli transformed with *A. tumefaciens* containing the plasmid with only the Cas9 cassette. Tdp1β, leaf discs/calli transformed with *A. tumefaciens* containing the CRISPR_Tdp1β vector.

Day	Explant						Calli					
	Seed			Repropagated			Seed			Repropagated		
	C	***Cas9***	***Tdp1β***	***C***	***Cas9***	***Tdp1β***	***C***	***Cas9***	***Tdp1β***	***C***	***Cas9***	***Tdp1β***
0	14	22	63	-	25	19	-	-	-	-	-	-
5	14	21	63	-	25	7	-	-	-	-	-	-
12	14	21	62	-	25	7	-	-	-	-	-	-
19	14	21	62	-	25	7	-	-	4	-	6	8
26	14	21	60	-	25	7	-	8	23	-	12	11
33	14	18	20	-	23	5	-	10	14	-	11	4
40	14	18	20	-	23	5	-	11	14	-	11	4
47	5	18	18	-	22	5	-	11	12	-	11	-
54	5	13	15	-	12	5	-	12	12	-	10	-
61	5	13	14	-	11	5	-	11	14	-	6	-
68	5	13	14	-	11	5	-	11	14	-	6	-
75	5	13	14	-	11	5	-	8	9	-	3	-

After 40 days, the developed calli were tested for the presence of the GFP signal by using a transilluminator. In **Fig. 53, A** and **C**, it is possible observe

the calli obtained from leaf discs transformed with the construct containing only the Cas9 under normal and UV light, respectively, while **Fig. 53, B** and **D** show the calli obtained from leaf discs transformed with the CRISPR_Tdp1β construct. Red arrows indicates the presence of GFP fluorescence.

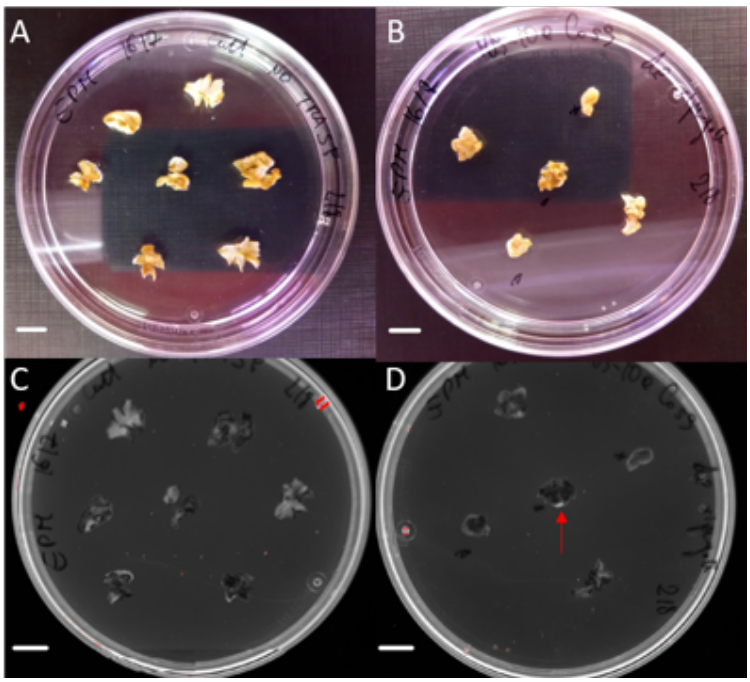


Fig. 53. Images of *M. truncatula* calli derived from *Agrobacterium*-mediated transformation of leaf discs. (**A)** Control calli visualized in normal light (**B**) Transformed calli visualized in normal light (**C)** Control calli visualized under the transilluminator (**D**) Control calli visualized at the transilluminator. Red arrow indicates the GFP fluorescence. Bar = 1 cm

Genetic transformation of *M. truncatula* petioles and leaf discs was carried out in the same time, hence also the calli derived from leaf discs are currently on ECM. The putative involvement of the targeted gene in cell proliferation is confirmed once again since also in this case the CRISPR-Tdp1β calli were less developed than the control calli. As future work, it will be interesting to calculate the transformation efficiency of each type of explant used and establish the transformation and mutation rate.

4.2.2. Development of a protocol for transformation of local rice variety as a tool for future genome editing application

This part of the work contributes to expand the knowledge on rice genetic engineering since it aims to establish a regeneration and transformation protocol for a valuable local Italian rice variety, Vialone Nano. Italy is the largest rice producer in Europe with 1.5 Million Metric Tons produced in 2015 (USDA, 2015). Lombardy and Piedmont regions are at the top of the country's ranking (FAO, 2004). Vialone Nano is a medium-grain japonica variety, traditionally grown in Italy since 1937 (Fabris, 2014), developed at the CREA-RIS - Unità di ricerca per la risicoltura (Vercelli) by crossing the Vialone variety with a dwarf (Nano) variety. Vialone Nano, although it has good sensorial qualities, it has a relatively low productivity and is highly susceptible to blast (*Magnaporthe oryzae*) disease (Urso et al., 2016). Hence, genetic engineering could be of use to develop improved lines, but this variety has never been attempted to regenerate and transform *in vitro*. This part of the work was performed at CREA-GB in Montanaso Lombardo.

Production of embryogenic calli with high regeneration capacity is a prerequisite for highly efficient transformation of rice. Callus induction and regeneration frequency are conditioned by a plethora of factors like genotype, *in vitro* culture conditions, nutrients, hormone composition, antibiotic concentration, temperature, *Agrobacterium* strain, and methods used to wash out the bacteria after co-cultivation (Sahoo et al., 2011, Sah et al., 2013). It is generally thought that the genotype remains a major limiting factor restricting successful transformation in *japonica* rice (Hiei et al., 1997, Hiei and Komari, 2008).

4.2.2.1. Development of a regeneration protocol for Vialone Nano cultivar

The protocol used for this experiment is an integration between Hiei et al. (2006) and Sahoo et al. (2011) published methodologies. The sterility of the materials used for regeneration is a key step for a successful procedure. Apart sterilization, media composition and temperature, other important factors conditioning the regeneration potential of rice seeds are the number and proximity of the material in each plate. For this reason, it is suggested to start with more material per plate and reduce the number after each re-plating. For instance, we started with 20 seeds per Petri dish (plate) on CIM, continued with 15 calli per plate when using PReM and only 5-6 calli on the same container when using ReM. This allows availability to more nutrients and hence, better calli/plantlet development.

The purpose of this experiment was to test the regeneration potential of Vialone Nano (VN), compared with Nipponbare (NB) reference, as well as to obtain seeds uncontaminated from external biotic and abiotic factors present in the field. The protocol hereby developed led to different responses between the two cultivars. VN calli developed one week later and were smaller than NB calli. VN calli differentiation is slower, but under greenhouse conditions plants grew healthier. **Fig. 54** shows the entire cycle of regeneration of *O. sativa* starting from the seeds to the maturation of seeds in greenhouse.

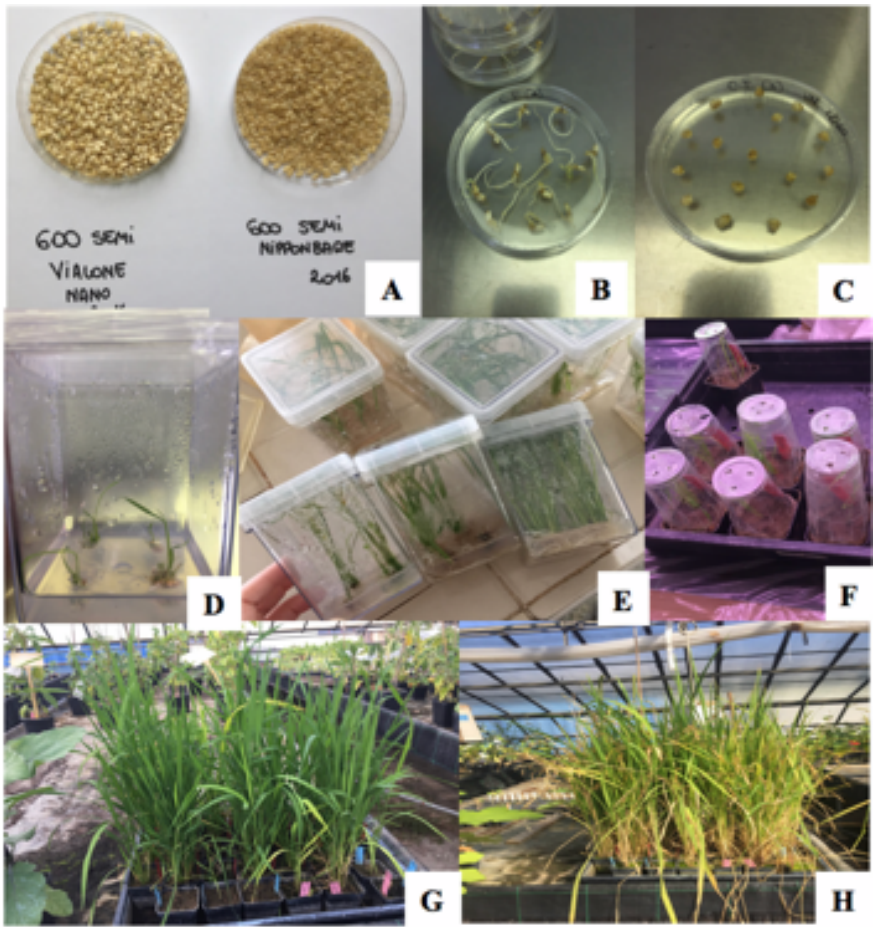


Fig. 54. Rice *in vitro* regeneration procedure. (**A**) Seed selection; (**B**) Germinated seeds and calli formation on CIM; (**C**) Embryogenic calli; (**D**) Regenerated shoots; (**E**) Regenerated plantlets with roots; (**F**) Plantlets transferred into pots for acclimatization; (**G**) Rice plants in greenhouse; (**H**) Mature plants producing seeds.

4.2.2.2. *Agrobacterium*-mediated rice transformation

The transformation procedure was carried out using a standard plasmid donated by Ming-Bo Wang (CSIRO, Commonwealth Scientific and Industrial Research Organization - Agriculture and Food, Canberra, Australia). The plasmid is 15430 base pairs long and contains β-glucuronidase (GUS) as reporter gene, hygromycin (HPT) selection for plants and spectinomycin (SpecR) as selectable marker for bacteria (**Fig. 55**).

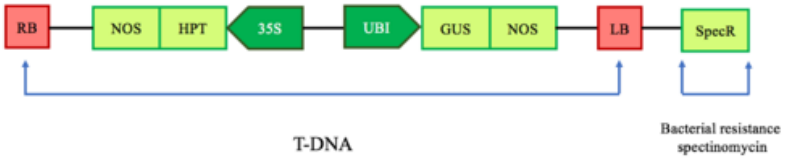


Fig. 55. Schematic representation of the plasmid used for transformation. RB, Right Border; LB, Left Border; NOS, nopaline synthase terminator; HPT, hygromycin; 35S, cauliflower mosaic virus promoter; UBI1-P, ubiquitin promoter; GUS, β-glucuronidase; SpecR, spectinomycin resistance.

Both VN and NB variety were subjected to genetic transformation mediated by *A. tumefaciens*. After the co-cultivation step, the calli were incubated for two weeks at 26-28°C under dark conditions. After the two selection steps (SMI and SMII), it is expected that the small transgenic hygromycin resistant calli would start to proliferate. Healthy proliferating calli of creamy-white colour were observed only in the case of NB reference, while the VN calli were brown and dying (**Fig. 56, B**). This indicates that protocols suitable for NB transformation might not be as efficient for VN local variety, even though both cultivars are *japonica* rice, usually more prone to transformation when compared to *indica* rice. At this point, the transformants regeneration protocol was carried out only for NB. These plants were acclimatized to greenhouse (**Fig. 56, F**) and seeds were collected.

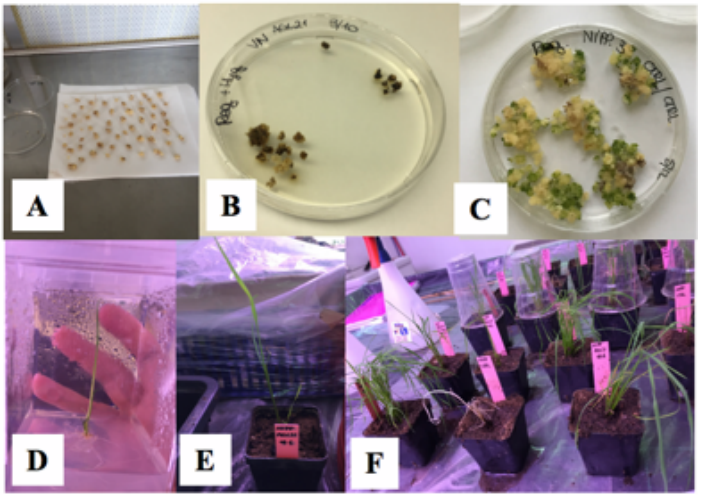


Fig. 56. *Agrobacterium*-mediated transformation of rice calli. (**A**) Calli after co-cultivation with *Agrobacterium.* (**B**) VN calli on ReM. (**C**) NB calli on ReM (**D)** Regenerated NB plantlet transferred on RoM (**E-F**) NB plants transferred in soil and kept in acclimatization room.

4.2.2.3. Protocol optimization for the transformation of Vialone Nano cultivar

One of the problems encountered during the first transformation test of VN variety was the calli formation. By using different concentrations of 2,4-D, it is expected to improve the formation of calli upon transformation. This is because 2,4-D is a synthetic auxin, the hormone responsible for plant growth (Gamborg et al., 1968). Low concentrations of 2,4-D can stimulate RNA, DNA, and protein synthesis, leading to uncontrolled cell division (calli formation) (Raghavan, 2004). On the other hand, high concentrations of 2,4-D can inhibit cell division and growth (Tu et al., 2001). With this experiment, the optimal concentration of 2,4-D for the VN callus development is to be tested.

A first experiment was set up to use the same components of the previous media while altering only the concentration of 2,4-D as follows: 1, 2, 3, 4, and 5 mg/L. The results showed that 4 and 5 mg/L of 2,4-D lead to a brown-black colour and death of the calli (**Fig. 57**). This was confirmed by the data shown in the **Table 34.** The calli grown on CIM with 1, 2 and 3 mg/L of 2,4-D generated many healthy plantlets. Overall, the best 2,4-D concentrations for VN calli were 1 and 3 mg/L. This is confirmed by the percentage of induction of the callus (respectively 95% and 78.3%) and also by the simple observation of the VN calli. Calli grown on 1, 2 and 3 mg/L of 2,4-D were white and larger than those grown on higher concentrations.

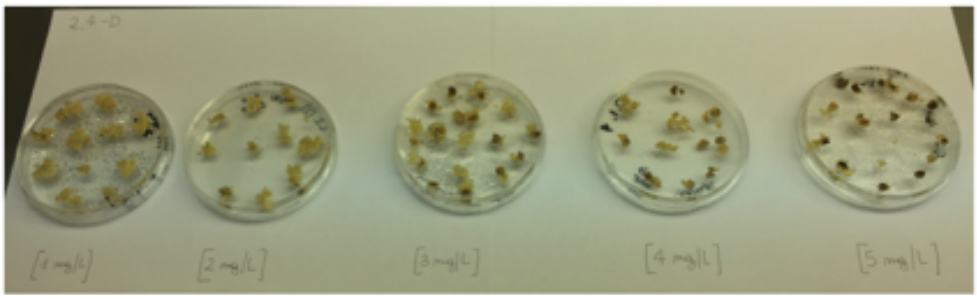


Fig. 57. Development of Vialone Nano calli on CIM with increasing (1, 2, 3, 4, and 5 mg/L) 2,4-D concentrations.

Table 34. Percentage of Vialone Nano callus induction in response to different 2,4-D concentrations.

2,4-D [mg/l]	VN seeds	VN calli	% callus induction
1	60	57	95
2	60	31	51.6
3	60	47	78.3
4	60	22	36.6
5	60	12	20

The newly developed protocol in this study was based on combining two previously published methodologies (Sah et al., 2014; Slamet-Loedin et al., 2014). The CCM and RoM media were the same in both protocols. Optimizations of our protocol retained only one type of Selection Medium (SM). Hence, the new experimental design implemented for rice transformation used the two different protocols, hereafter denominated as (F) and (A). During the regeneration phase, the only transformed calli that have succeeded in generating plantlets were the A3VN with the combination n. 3 (A3VN3, see **Table 10**, paragraph **3.7.4.4.**). It is important to specify that this protocol was unsuccessful for the NB reference genotype. The controls behaved as expected, with CTRL combination (non-transformed on antibiotics) being dead, while the CTRL/CTRL (non-transformed without antibiotics) calli were vital and have generated a large number of plantlets. Subsequently, the resulted plantlets were transferred on RoM, followed by potting and greenhouse acclimatization **(Fig. 58)**. **Table 35** resumes the outcome of each combination carried out during this transformation procedure and relative efficiency of transformation was calculated.

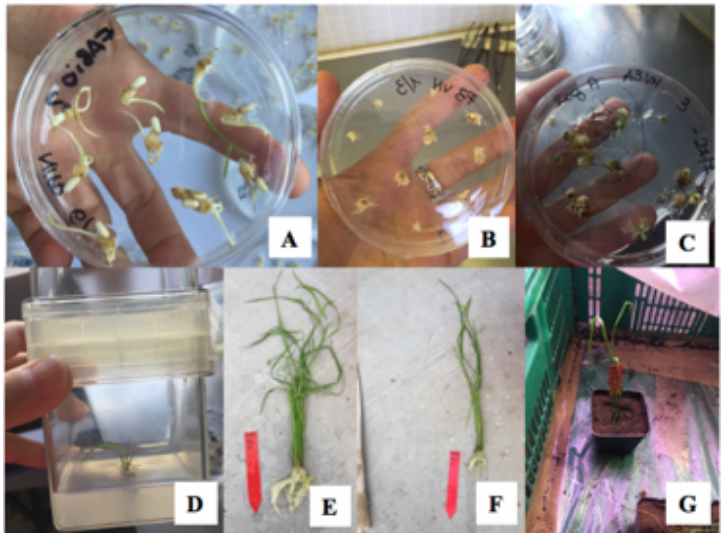


Fig. 58. Vialone Nano transformation protocol. (**A**) Seeds on CIM after two weeks of *in vitro* maintenance. (**B**) VN calli on F3 medium immediately after *Agrobacterium* inoculation. (**C**) Regenerated shoots on ReM. (**D**) Plantlets on RoM. (**E-F**) Regenerated plants with roots before being transfer into pots. (**G**) Transformed Vialone Nano plant in greenhouse.

Table 35. Comparison of different protocols for Vialone Nano transformation.

Combinations	Medium type and rice variety	No. of inoculated calli on CIM	No. of calli on ReM	No. of regenerated plantlets in greenhouse	Transformation efficiency (%)*
1	A1 NB	11	0	-	-
	A1 VN	12	3	-	-
	F1 NB	7	3	-	-
2	A2 NB	11	0	-	-
	A2 VN	11	2	-	-
	F2 NB	23	15	-	-
	F2 VN	17	2	-	-
3	A3 NB	11	0	-	-
	A3 VN	10	4	5	50
	F3 NB	30	0	-	-
	F3 VN	14	0	-	-
4	A3 NB	10	0	-	-
	A3 VN	10	0	-	-
	F3 NB	13	3	-	-
	F3 VN	2	2	-	-

*: Transformation efficiency (%) = $\frac{number\ of\ transgenic\ plants}{number\ of calli\ inoculated\ with\ Agrobacterium} \times 100$

Following acclimatization to greenhouse, leaves from five transformed plants were sampled to proceed with molecular analysis and confirm the presence of the transgene. To do this, the GUS (β-glucuronidase) assay and PCR analysis were performed (**Fig. 59**). The results showed that the five samples were positive for GUS histochemical staining. Further confirmation was carried out using PCR with GUS specific primers and an example of positive bands of expected size (900 bp) is shown in **Fig. 16E**.

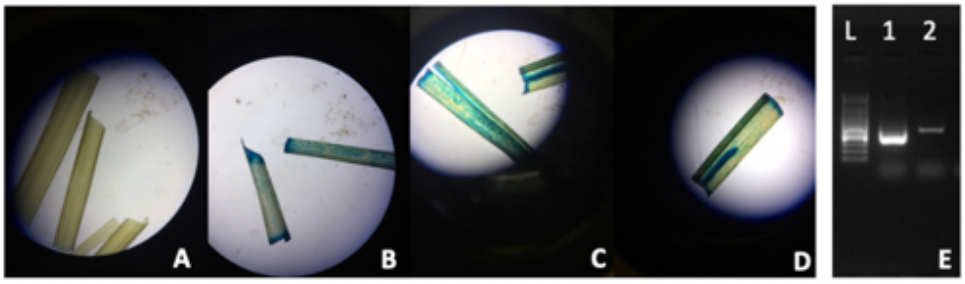


Fig. 59 Histochemical and molecular analysis of Nipponbare transgenic plants. **(A)** GUS staining of non-transformed, negative control plants; (**B**-**D**) GUS staining of positive transgenic VN plants showing blue staining. **(E)** PCR analysis using GUS specific primers; L, ladder 100 bp (Sigma-Aldrich); 1-2, DNA extracted from transformed VN leaves.

These experiments have demonstrated how important are factors like the temperature and the 2,4-D concentration for the development of healthy calli, fundamental to carry out genetic transformation in rice. By comparing the different combinations used to carry out the genetic transformation, it is possible to observe how the culture medium or the amount of *A. tumefaciens* used to do the co-cultivation are decisive for the success of the experiment. The protocol developed is specific for the Vialone Nano variety as it was unsuccessful for the Nipponbare control variety. This is an indication for the need to develop variety-specific protocols for genetic transformation.

The regeneration and transformation protocol hereby developed for the Vialone Nano local variety represent the starting point for genome editing applications to improve this variety. Genome editing techniques can offer useful tools for the sustainable management of biodiversity of local rice varieties such as the Vialone Nano. For instance, it is possible to develop VN plants with higher production performance in unfavorable conditions by modifying the architecture of the plant. Furthermore, it is possible to improve adaptability to cultivation environments thanks to a more efficient use of water and nitrogen for example by modifying the root system.

5. Discussion and conclusions

To cope the actual agricultural challenges, this work of thesis focused mainly on the development of innovative and sustainable methodologies useful to improve the seed quality. In view of this, seed priming protocols were designed and the impact on the pre-germinative metabolism evaluated using molecular tools. Identification of molecular hallmarks useful to predict priming efficacy was also a priority. Gene-transfer methods, such as the development of genetic transformation protocols and the use of genome editing techniques were carried out in two species (*M. truncatula* and rice) that represent well established models for legumes and monocots but also agronomically relevant crops.

The first part of the experimental work focused on the development and application of priming protocols applied to seeds both under laboratory and field conditions. The effects of these treatments on the germination performance, in presence/absence of stress conditions, and their impact on the pre-germinative metabolism were explored in different systems to figure out shared or divergent molecular hallmarks.

Although previous work has evidenced the role of specific DNA repair genes as *OGG1*, *FPG* and *Tdp1*α in the context of seed imbibition with water as well osmotic agents (Macovei et al., 2010, 2011; Balestrazzi et al., 2011b) and other chemicals as sodium butyrate and trichostatin A (Pagano et al., 2017, 2019), this is the first time that a molecular landscape of the pre-germinative metabolism is provided in seeds treated with a standard priming protocol (controlled rehydration plus dry-back).

Indeed, the eggplant seed response to priming was monitored through the expression patterns of antioxidant/DNA repair genes that play pivotal roles during imbibition integrated with free radical profiles.

The HP72 treatment hereby developed accelerates germination in the *S. melongena* inbred line '67/3' resulting in beneficial effects as reported for other priming agents (e.g. salts, growth regulators, vinegard) (Zhang et al 2011; Marvin 2015; Ali et al. 2019). Although these findings provide useful technological solutions to improve seed production, a better understanding of their molecular implications in the context of the pre-germinative metabolism will lead to more consistent and rationale advancements not only in eggplant but also in other valuable horticultural crops.

The biochemical events triggered by water up-take during seed imbibition are associated with ROS accumulation, e.g. the respiratory burst within mitochondria generates the superoxide anion that is dismutated to hydrogen

peroxide as well as the extracellular peroxidases that produce superoxide radicals then converted to hydrogen peroxide (Wojtyla et al., 2016). ROS are source of oxidative damage that is counteracted by the seed through the activation of the antioxidant defence and DNA repair (Waterworth et al., 2010, 2016; Macovei et al., 2010; Pagano et al., 2017; Wojtyla et al., 2016), however these radical species also contribute to germination, e.g. specific oxidative modifications of storage proteins act as signals to trigger reserves mobilization (Job et al., 2005). Being so crucial in seed germination, ROS undergo changes also during seed priming. The eggplant seeds revealed a boost in ROS levels at 24 h of hydropriming (up to 14.62-fold, compared to dry seeds), followed by a progressive decrease throughout the treatment and the levels detected after dry-back were even lower compared to the dry seed.

Accordingly, Kubala et al. (2015) found enhanced ROS levels in rape (*Brassica napus* L.) seeds subjected to osmopriming and a decrease during the subsequent drying. However, high ROS amounts were measured during the post-priming germination in both unprimed and primed *Brassica* seeds, differently from what observed in eggplant. Primed seeds are developmentally more advanced (Chen and Arora, 2013) and it is possible that the ROS peak detected during hydropriming simply anticipates the physiological boost that takes place at 8 h of imbibition in the unprimed seeds.

Changes in free radicals during hydropriming, dry-back and post-priming germination are determined by the ROS-scavenging activity of antioxidant enzymes, among which ascorbate peroxidase and superoxide dismutase. Species-specific dynamics in ROS production and activity of scavenging enzymes during germination have been described, suggesting that the overall interplay between these two players can be tightly rearranged (Bailly et al 2008). The up-regulation of *SmAPX* gene provides evidence that eggplant seeds are dealing with the rise in ROS during hydropriming. On the other hand, it is possible that the down-regulation of *SmSOD* gene reflects a crucial step in the pre-germinative metabolism, as highlighted by Oracz et al. (2009).

These authors showed that inhibition of SOD activity coupled with the stimulation of ROS-producing enzymes was necessary in sunflower (*Helianthus annuus* L.) seeds to promote ROS-mediated carbonylation of proteins, and then trigger germination. A peak in *SmSOD* gene expression occurred during post-priming germination of the eggplant '67/3' seeds, between the 16 h and 24 h time points and this is in agreement with the documented increase in SOD activity of osmoprimed seeds from different crop plants (Bailly et al. 2000; Rouhi et al. 2012).

Maintenance of genome integrity is another big challenge for the seed (Bray and West, 2005; Waterworth et al., 2010, 2016, 2019) and the controlled hydration carried through priming promotes the cellular repair

activities including the removal of DNA lesions (Ashraf and Bray 1993, Kubala et al. 2015; Sharma and Maheshwari 2015). As for eggplant, among the DNA repair genes hereby tested only *SmOGG1* showed up-regulation during the HP72 treatment, suggesting that repair of oxidative DNA damage was requested particularly within the first 24 h of hydropriming. Later on, a peak in *SmOGG1* gene expression occurred in the primed seeds during the dry-back step, supporting for an active response to genotoxic stress and in agreement with the previous study by Chen et al. (2012) who showed that overexpression of *AtOGG1* gene in *Arabidopsis* resulted in lower DNA damage accumulation during the late phase of seed maturation. More recently, Parreira et al. (2018) also demonstrated that activation of DNA repair at the onset of the desiccation phase is a molecular signature of seed maturation in common bean (*Phaseolus vulgaris* L.). When considering the response of imbibed seeds, hydropriming resulted in the early up-regulation of *SmOGG1* gene (2 h) as well as in the up-regulation of the *SmFPG* gene was noticed at 4 h and 16 h of imbibition, and there were no evident peaks in *Tdp1*α gene expression.

A major challenge of seed priming is to design protocols that can work independently of the seed lot quality. Thus, the investigation was extended to eggplant seed lots showing variable germination profiles to test the reproducibility of the HP72 treatment. Furthermore, since the early up-regulation of *SmOGG1* gene in primed seeds at 2 h of imbibition appeared as a promising hallmark, the subsequent investigation on eggplant and wild relatives was restricted within this time point, to verify whether the observed molecular landscape was reproducible in other eggplant seed lots. Indeed, shortcut protocols would be an ideal solution to speed-up the routine assessment of seed quality.

A striking difference between the high-quality and low-quality eggplant seed lots was that the primed, dried-back, seeds with low germinability contained enhanced ROS levels, compared to the unprimed, and this profile was maintained also at 2 h of imbibition. This excessive ROS content might be detrimental and impair eggplant germination in the seed lots collected during 2015 and 2017. At the molecular level, the primed and unprimed dry seeds did not share common expression profiles of *SmSOD* and *SmAPX* genes, thus providing lot-specific responses. The up-regulation of *SmAPX* gene at 2 h of imbibition was a common hallmark of both 2015 and 2017 seed lots, symptomatic of an early antioxidant response triggered by priming that was not present in the high-quality lots. Despite this, the low-quality eggplant seeds were not able to restore an optimal germination performance. Although lot-specific profiles were observed, the level of *SmOGG1* transcript was in most cases higher in the high-quality seeds, compared to the low quality ones.

Overall, the reported data highlight the complexity of the molecular landscape depicted by the expression profiles of *SmOGG1*, *SmFPG*, and *SmTDP1α* genes in each specific seed lot. Looking at the cumulative effect of the gene expression profiles during hydropriming and post-priming germination as well as across lots, it seems that the repair machinery of the eggplant seed is endowed with a remarkable capacity to adapt to the changes induced by priming in the cell metabolism and physiology.

Another intriguing question would be to what extent the knowledge gained in a domesticated crop, as eggplant, can be translated to its wild relatives to speed up their use in breeding programs or other agronomical applications. One of the two wild relatives hereby investigated, the turkey berry (*S. torvum* Swartz 1788, Leptostemonum clade) is supposed to have split from *S. melongena* approximately 6.66 million years ago (Yang et al., 2014). *S. torvum* was chosen due to its relevance as a source of genes for the resistance to root-knot nematodes and soil-borne diseases. Indeed, this wild relative is widely used as rootstock for grafting in eggplant cultivation (Gousset et al. 2005). Low seed quality is a recurrent issue in *S. torvum* that often shows strong physiological dormancy and several factors must be taken into account when dealing with such a recalcitrant system (Ranil et al. 2015).

The other wild relative analyzed, *S. villosum* Miller (red nightshade), is part of the so called *Solanum nigrum* complex that includes African indigenous vegetables characterized by high nutrient content, medicinal properties and secondary metabolites with potential insecticidal activity (Yuan et al. 2018). The germination profile of *S. villosum* showed accelerated germination, compared to eggplant and turkey berry, and the process was further shortened in response to hydropriming.

The seed lots hereby tested provided a molecular picture that further underlines how difficult can be the task of bridging domesticated crops and wild relatives when using the pre-germinative metabolism as a source of 'markers'. One of the goals of the present work was to explore to what extent the knowledge gained in eggplant on the molecular responses induced by priming, in the context of pre-germinative metabolism, could be successfully translated to the wild relatives. Although the analyses carried in eggplant allowed to discriminate between high- and low-quality seed lots, based on ROS profiles, the response observed in the wild relatives was not so straightforward.

To date, the genomic resources of wild relatives are still limited (Yang et al. 2014; Gramazio et al., 2016) and the cloning of specific genes useful to address seed quality issues might be time consuming. Thus, taking advantage of the high degree of conservation among coding sequences, we decided to check whether the oligonucleotide primers designed on the '67/3'

antioxidant/DNA repair gene coding sequences could be used to speed up the investigation in the wild relatives. The available '67/3' oligonucleotide primers were successfully applied to *S. torvum* and *S. villosum*, however the oligonucleotide sets specific for the *OGG1* and *FPG* genes failed to amplify the expected products. The resulting gene expression patterns provide an interesting picture of the dynamics of the pre-germinative metabolism induced by hydropriming in the two wild relatives, and future studies will help clarifying the role of Tdp1α gene.

The priming treatments were also applied to seeds then left to germinate under field conditions, in the Varanasi region (India). This work was part of the TECO challenge project (2018), a collaborative research between the Plant Biotechnology Laboratory (Department of Biology and Biotechnology, University of Pavia) and the Institute of Environment and Sustainable Development (Banaras Hindu University, Varanasi, India).

This was also a unique opportunity to test the expression profiles of genes with crucial roles in the pre-germinative metabolism, in the context of 'on-farm' seed priming and further assess their reliability as hallmarks of seed quality. Agriculture represents a major stamina of the economical and socio-political stability in India and employs the largest work force. About 60% of the geographical area of the country is occupied by agricultural land, most of which is facing one or more types of soil contaminations (Bhattacharyya et al., 2015). Legumes can help restore soil organic matter when used in rotation with non-leguminous crops. Thus, improving legume seed germination using easy and economically accessible methods will upgrade the livelihood of many small farmers.

Within this context, the work carried out focused on the evaluation of hydropriming and biopriming treatments as a mean to improve seed germination and seedling establishment on agricultural soils collected from two different sites in the Varanasi region. The collected soils originate from the province of Karsada, where a power plant is located allowing to convert the municipal solid waste to electric power in an attempt to dispose waste through a sustainable and environmentally friendly process. However, the farmers in the region report loss of agricultural productivity due to the accumulation of solid waste in the soil. This is also revealed when measuring the content of total dissolved solids (TDS), shown to be higher in the soils collected closer to the power plant (Soil_B, **Table 2**).

Hydropriming was chosen mainly because it is very easy to implement, and hence highly accessible also to farmers from underdeveloped regions. The biopriming formulations were selected from local varieties of rhizospheric microorganisms having plant growth-promoting traits under stressful conditions and studied in relation to improved phosphorus nutrition

(Singh et al., 2016; Prasad et al., 2018). Moreover, biopriming can be associated with the elicitation of plant immunity, starting from the seedling stage (Song et al., 2017). The experiment was setup under the natural weather conditions of Varanasi to evaluate the efficiency of the tested protocols in the region.

Our results showed that while hydropriming enhanced seed germination percentage on both types of soils (**Fig. 45, A**), biopriming resulted in improved seedling development on Soil_B, mainly reflected by enhanced biomass (**Fig. 47 B, C**). This is coherent with the fact that hydropriming mainly improves germination a result of the enhanced water uptake and more favourable water relations in primed seeds (Lechowska et al., 2019) whereas biopriming effects come into action later on when the radicle protrudes and it becomes directly exposed to bacteria that can aid in soil nutrient uptake (Yadav et al., 2018; Prasad et al., 2018). Importantly, by repeating the experimental setup under different climatic conditions in August 2019, the observed trend of improved germination and seedling development was once again confirmed (**Fig. 46**). An important finding is the fact that HP2 and HP4 treatments were able to exert a more positive effect on Soil_B (having a higher TDS concentration than Soil_A), as evidenced by the PCA integrative analyses (**Figure 49**). Another recent work shows how hydropriming was able to mitigate the negative effects of cold temperatures on the germination and seedling development in narrow-leaf lupine (*Lupine angustifolius*) (Płazek et al., 2018).

The next step was to examine the expression profiles of genes that play roles in DNA repair and antioxidant defence, previously used as indicators of seed quality during the early stages of germination (namely seed imbibition) (Balestrazzi et al., 2011a, 2011b; Pagano et al. 2017, 2019). This is a rather important aspect because such molecular hallmarks allow the prediction of seed quality in a very short time, thus providing assistance to Seed Companies to design customized priming protocols (Paparella et al., 2015; Macovei et al., 2017, Waterworth et al., 2019; Araùjo et al., 2016). The expression of the chosen genes, initially tested in dry and hydroprimed seeds, showed that while the *FPG, APX,* and *SOD* were upregulated by the treatment, the *OGG1* and *MT2* genes were not responsive at the tested time points (**Figure 48, A**). Nonetheless, in the 14-days-old seedlings, all the tested genes were differentially expressed in response to both priming and soil conditions. The *OGG1* and *FPG* genes, playing similar roles within the BER pathway involved in the removal of oxidized bases like 7,8-dihydro-8-oxoguanine (8-oxo-dG) and formamidopyrimidine (FAPy) lesions, had been previously reported to be upregulated during seed imbibition under physiological conditions or in the presence of osmotic stress (PEG) in *M. truncatula*

(Macovei et al., 2011). However, their expression was mostly induced after 8 h of imbibition with water and 12 h of imbibition in the presence of PEG. More recently, this was further validated in several other conditions, like in the case of treatments with histone deacetylase inhibitors trichostatin A (TSA) and sodium butyrate (NaB) (Pagano et al., 2019; Araùjo et al., 2019). Here, we show that, while the *OGG1* and *FPG* gene expression is downregulated in seedlings grown in Soil_B in the absence of priming, both hydropriming and biopriming resulted in gene upregulation (**Figure 48, B,C**). This indicates that primed seedlings are better equipped to repair the DNA damage that can be induced by the presence of contaminants in the soil. Similarly, the *APX* and *SOD* genes, chosen as molecular hallmarks of the antioxidant response, were upregulated in hydroprimed seeds. In *M. truncatula*, upregulation of *SOD* and *APX* genes has been previously reported starting from the 4th h of seed imbibition with water (Balestrazzi et al., 2011a). In seedlings, the genes were mostly upregulated in response to biopriming (**Figure 48, E, F**). SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide (H_2O_2), while APX plays a key role catalyzing the conversion of H_2O_2 into water, using ascorbate as a specific electron donor (Alscher et al., 2002; Caverzan et al., 2012). Enhanced accumulation of antioxidant enzymes following different seed priming methods (e.g. osmopriming, halopriming, hormopriming) has been previously reported in a number of plant species (*Vigna radiata*, *Moringa oleifera*, *Triticum aestivum, Oryza sativa, Sorghum bicolor, Brassica napus*) (Nouman et al., 2014; Jisha and Puthur, 2015; Islam et al., 2015; Zhang et al., 2015; Salah et al., 2015; Kubala et al., 2015), and these were even correlated with changes in gene expression patterns (Kubala et al., 2015; Salah et al., 2015). Another group of interesting ROS scavengers includes metallothioneins (MTs), small cysteine-rich proteins that accumulate in response to toxic levels of HMs (Cobbet et al., 2002). Besides their role in HM detoxification, MTs are part of the signaling pathway activated by nitric oxide (Balestrazzi et al., 2011a, Balestrazzi et al., 2011b) and have been associated with protection against oxidative injuries also at the nuclear level (Balestrazzi et al., 2009; Donà et al., 2013). In human cells, *MTs* overexpression was associated with the suppression of 8-oxo-dG accumulation in γ-irradiated cells (Yeong et al. 2004), thus suggesting an interplay between MTs and BER components. Under our experimental conditions, *MT2* seems to be mostly upregulated in hydroprimed seedlings (**Figure 48, D**) while no changes in gene expression level were observed in the hydroprimed seeds (**Figure 48, A**). As in the case of *OGG1*, also the *MT2* gene was previously shown to be upregulated only later on during seed imbibition in *M. truncatula* (Pagano et al., 2019). In a different work, the *MT* gene expression was monitored in artificially aged

seeds of two *Silene* species that differ in seed longevity and grow in contrasting habitats (Mondoni et al., 2011). Based on several measured parameters (e.g. ROS accumulation, antioxidant potential, telomere length) and the differential expression of *MT* and *SOD* genes in aged seeds it was possible to distinguish between short- and long-lived seeds under seed bank storage conditions (Donà et al., 2013). In a context where seed researchers are continuously facing the complexity of efficient seed germination and healthy seedling establishment, the genes hereby investigated (*OGG1, FPG, APX, SOD,* and *MT2*) are important molecular hallmarks to test and monitor seed quality and seedling development.

In conclusion, the present study shows that seed priming is an efficient method to enhance seed germination and seedling establishment in agricultural soils collected from contaminated areas of the Varanasi province. While hydropriming improved seed germination percentage, biopriming resulted in improved seedling development. At a molecular level, this is reflected by upregulation of specific genes used as molecular indicators of seed quality.

In the second part of work we focused on the development of genetic transformation protocols tailored for model/crop plants as the legume *M. truncatula* and the monocot *Oryza sativa* as tool to specifically address the issue of seed quality.

Plant genetic transformation has enabled fundamental insights into plant biology and revolutionized commercial agriculture. Unfortunately, for most crops, transformation and regeneration remain arduous even after more than 30 years of technological advances. On the other side, genome editing techniques are an innovative and powerful strategy that allow to introduce mutations on insert/substitute wide portion of DNA at a targeted site. Genome editing provides novel opportunities to enhance crop productivity if accompanied by an efficient transformation protocol.

First of all, in this work was developed a novel vector for genome editing able to target the *Tdp1β* gene in *M. truncatula*. The expression of the different *TDP* genes identified in plants (*TDP1α*, *TDP1β* and *TDP2*) is responsive to different stress conditions in different experimental systems, including cell suspensions, aerial parts and seeds of *M. truncatula* (Balestrazzi et al., 2011a; Macovei et al., 2018) suggesting their recurrent involvement in the plant response to genotoxic stress. The relevance of *TDP1* gene is due to the role of this enzyme in the repair of topoisomerase I-DNA covalent lesions and its involvement in the activation of seed pre-germinative metabolism, for these reasons it can be used as a marker of seed quality. In particular, the *TDP1β* isoform was identified only in plants and its role is not fully understood. For

this reason, our work was carried out to start to characterize the function of the isoform Tdp1β of the gene. The Tdp1β vector, inserted in *A. tumefaciens* were utilized to genetically transform three different explants of *M. truncatula*: cell suspension, petioles and leaf discs. Three different types of explants have been used and then, after the experiment has been completed, a comparison can be made to establish which method is the best, especially in terms of efficiency and time spent. The results show that the transformation took place successfully, as demonstrated by the presence of a fluorescence signal emitted by the GFP protein from petioles and leaf discs as well as cell suspension cultures.

The microcalli developed following transformation of cell suspension cultures were also analyzed at the molecular level by standard PCR, confirming the presence of the construct within the genome of the transformed cells. These experiments are still in progress, as genetic transformation and *in vitro* regeneration is a process that requires, in the case of *M. truncatula*, about four months to be completed.

Although an efficient protocol for the genetic transformation of *M. truncatula* genotype Jemalong M9-10a (Araùjo et al., 2004) was used, only calli developed following co-cultivation. No embryos appeared. These results are difficult to explain. Chabaud et al. (2007) reported that *M. truncatula* Jemalong genotype is highly embryogenic. These authors estimated that up to 97-100% of the leaflet explants cultivated *in vitro* were able to produce embryogenic calli, even under selection. More in general, the estimated efficiency of embryogenesis-mediated regeneration was about 15-50% (explants regenerating transgenic plants). In the attempt to further optimize this protocol, petioles were tested as a new type of explant. Indeed, *M. truncatula* petioles were able to develop calli faster than leaf discs, in accordance with Zhao et al. (2017) who reported that segments of petioles excised from plantlets exhibit better capacity to differentiate calli than leaf discs. This optimization was not sufficient to reach the expected results. Future work will be necessary to further improve other aspects of the protocol, e.g. considerably increasing the number of explants to be used during the co-cultivation step.

Another relevant point relates to the target *Tdp1β* gene, investigated in this study. The presence of multiple *Tdp1* genes in plants (Macovei et al., 2010) expands the needs to design dedicated studies to investigate their implication in plant development and stress response. In human cells, *Tdp1* is strongly linked with complex functions in DNA repair (Pommier et al., 2014), whereas mutations in its catalytic sites are associated with serious diseases (e.g., spinocerebellar ataxia) (Takashima et al., 2002). Studies conducted so far in plants have proven the involvement of the *Tdp1α* gene in DNA damage repair

and stress response (Macovei et al., 2010; Donà et al., 2013; Lee et al., 2010). On the other side, the *Tdp1β* gene is far less characterized. Previous works demonstrate its involvement in chromatin remodeling and DNA repair. The two isoforms encoded by *MtTdp1α* and *MtTdp1β* genes were up-regulated in *M. truncatula* plants in response to heavy metal and osmotic stresses as well as during seed imbibition when DNA repair is required to preserve genome integrity and improve seed vigor (Macovei et al., 2010; Balestrazzi et al., 2011a, 2011b). The reported data from Sabatini et al. (2017) suggest that *Tdp1β* function plays a role in the early response to abiotic stresses. To unfold the reasons of *Tdp1β* early stress response, Sabatini et al. (2017) investigated the presence and distribution of the HIRAN domain (conserved in many DNA-binding proteins) in plants.
This analysis showed that the HIRAN-HKD domain, specific to the Tdp1β protein is ubiquitously found in plants. It has been hypothesized that the HIRAN motif might act in the recruitment of repair/remodeling enzymes to specific DNA sites, playing a role in cell cycle-checkpoints arising from stalled replication forks and post-replication damage (Iyer et al., 2006). The HIRAN domain was investigated in relation to AtRAD5A, a DNA translocase that catalyzes the fork regression and was shown to be able to bind to branched DNA structures and promote DNA repair (Kobbe et al., 2016).

However, all the cited literature refers to the HIRAN-SMARCA type of architecture, while no information on the HIRAN-HKD is available in plants. Based on the assumption that the HIRAN domain could act as a sensor to initiate the repair processes at damaged DNA checkpoints Iyer et al. (2006) hypothesized that the presence of this motif in the Tdp1β protein might sustain the early response of the gene to abiotic stress conditions.
An RNA-Seq analysis carried out in *MtTdp1α*-depleted *M. truncatula* plants revealed that the *MtTdp1β* gene was not able compensate for the lack of *MtTdp1α* gene in planta (Donà et al., 2013). Even though, the *MtTdp1β* gene was up-regulated in the depleted lines.

At the moment, these working hypothesis about the function of the *MtTdp1β* isoform cannot be discarded because the HIRAN domain is present in numerous proteins involved in the processes mentioned above. For this reason, a strategy was design to carry the knock-out of the *MtTdp1β* isoform in the HIRAN domain, to fully understand the function. The targeting of the *MtTdp1β* gene was at high risk because this gene performs an essential function within the cell. In all our working hypothesis the *MtTdp1β* gene is involved in essential processes for cellular viability and genome integrity maintenance. The transformed cell may not survive and not proliferate if the Tdp1β construct manages to inactivate the gene. Indeed, a high mortality rate was recorded among the transformed explants during the *in vitro* culture and

most of the transgenic lines did not survive. This result was not unexpected and it could be an indirect confirmation of the essential role played by the *Tdp1β* gene. The reduced calli viability has drastically compromised the probability of identifying transformation events. In conclusion, the lack of embryo development and the consequent development of plantlets transformed with the Tdp1β construct could be the result of a failed tuning of the transformation protocol or, alternatively, the *Tdp1β* gene has been correctly inactivated. These results highlight the difficulty of working on target genes that play an essential role for the cell.

Finally, the last part of work contributes to expand the knowledge on rice genetic engineering trying to establish a regeneration and transformation protocol for a valuable local Italian rice variety, Vialone Nano. Rice is among the most diverse crop species, accounting for more than 100,000 cultivated varieties. Nonetheless, only few varieties, selected for high disease resistance and productivity, are being extensively cultivated (IRRI, 2007). Major factors limiting rice production are related to sensitivity to different abiotic stress factors, in particular drought, submergence and salinity (Grover and Minhas, 2000). To maximize rice production, it is necessary to use all tools available, including traditional breeding, genetic and genome engineering. In the past, conventional breeding attempted to improve the quality and quantity of rice production but since these are complex traits, this aim is difficult to pursue using only a narrow array of approaches (Grover et al., 2000). With the recent advances in biotechnology and the development of genome editing tools (Voytas and Gao, 2014), it is more feasible to tackle the complex challenges related to rice production. Rice has emerged as a model cereal system for molecular studies, as the complete genome has been sequenced, several tools for functional genomics are available, and genetic transformation protocols have been developed for some varieties (Cantos et al., 2014). Though, many genotypes are recalcitrant to *in vitro* cultures, which makes the development of genetically engineered rice a considerable challenge (Slamet-Loedin and Macovei, 2016). As genetic engineering is strongly dependent on genotype, the availability of efficient *in vitro* plant regeneration method constitutes a major prerequisite (Sah et al., 2014). Problems raise from poor regeneration ability and recalcitrance to various biotechnological techniques (Kumar et al., 2008). Nowadays, a number of transformation strategies are available, the most widely used being particle bombardment and *Agrobacterium*-mediated transformation. Regardless of the chosen methodology, rice transformation has a low efficiency (Slamet-Loedin and Macovei, 2016). *Agrobacterium*-mediated transformation is probably the best method for genetic modification in many plant species because it allows efficient insertion of stable, unrearranged, single-copy sequences into plant genomes (Nishimura et al.,

2006). To successfully carry out genetic transformation in rice, it is essential to establish efficient *in vitro* regeneration methodologies (Raemakers et al., 1997; Sanyal et al., 2005; Dabul, 2009). Rice has a complex *in vitro* tissue culture system, with many varieties being recalcitrant to tissue culture and genetic transformation (Datta et al., 2000). Over time, several protocols have been developed with specific improvements such as altering the type and the age of inoculation material, improved delivery methods, choice of expression vectors and selectable markers, as well as adjustments to tissue culture media (Hiei and Komari, 2006).

Vialone Nano is a medium-grain japonica variety, it has good sensorial qualities, it has a relatively low productivity and is highly susceptible to blast (*Magnaporthe oryzae*) disease (Urso et al., 2016). To develop a transformation and regeneration protocol for Vialone Nano rice, first it was evaluated the *in vitro* regeneration potential using known protocols for *O. sativa* var. *japonica* reference cultivar Nipponbare, the most studied so far. Results obtained from this experiment reveal that the regeneration and transformation protocol utilized was not efficient for Vialone Nano variety. Infact, VN seeds were able to generate calli but with a low frequency and not healthy compared with the control Nipponbare cultivar. The VN calli were more susceptible at temperature than Nipponbare calli and also was important the concentration of hormone 2,4-D, a synthetic auxin responsible for plant growth (Gamborg et al., 1968), utilized during the callus induction stage. As results, the first genetic transformation of Vialone Nano calli was unsuccessful. For these reasons, a new regeneration protocol was developed for the Vialone Nano variety by testing callus development on different 2,4-D concentrations: 1, 2, 3, 4 and 5 mg/l respectively. The best results were obtained using 1 and 3 mg/l of 2,4-D. This was confirmed by the percentage of callus induction (respectively 95% and 78.3%). Calli grown on 1 and 3 mg/L of 2,4-D were white and larger than those grown on higher concentrations. Subsequently, transformation and regeneration protocols for Vialone Nano were developed in parallel with Nipponbare, always used as a reference variety. This has been achieved by modifying the following parameters: **(1)** *Agrobacterium* culture (decreasing OD and exposure time), **(2)** growth media (sugar types and hormone concentrations), **(3)** temperature (decreasing from 28° C to 25°C for callus induction, selection and regeneration steps). From all tested protocols (A1VN, A2VN, F2VN, A3VN and F3VN), only the A3VN combination resulted into the generation of transgenic plants with a transformation efficiency of 50%. Importantly, this is a genotype specific protocol for Vialone Nano as Nipponbare reference did not produce any transformants.

These preliminary results represent the starting point for genome editing applications to improve this variety. Cloning protocols could be carried out to design CRISPR/Cas9 vectors for transformation in Vialone Nano by targeting endogenous genes, that, when silenced/disturbed, can result in improved plants useful to cope the actual agricultural challenges.

In the present work we explore for the first time the dynamics of the pre-germinative metabolism in eggplant *(S. melongena* L.), a valuable horticultural crop, comparing the impact of hydropriming *versus* physiological conditions and looking for useful molecular hallmarks within a set of antioxidant and DNA repair genes that play a pivotal role during early seed imbibition. The investigation also covers the issues of seed quality in lots with variable germinability and it is then further expanded to eggplant wild relatives (*Solanum torvum* and *Solanum villosum*). The plasticity of the seed pre-germinative metabolism, stimulated by priming, imposes a plethora of heterogeneous molecular responses in eggplant and underlined in other plants, that provide a valuable contribution to fill the current knowledge gap on the molecular bases of seed priming. On the other hand, this heterogeneity stands up as an hindrance to the search for molecular markers allowing a quick diagnostics of seed quality.

Then, our studies show that seed priming is an efficient method to overcome the adverse effects of heavy metal soil contamination. Hydropriming improve seed germination percentage, biopriming improve seedling development. At molecular level, this is reflected by up-regulation of genes involved in DNA damage repair and antioxidant defense, allowing a better adaptation to environmental constraints.

Finally, in the last part of our work we considered the importance of developing efficient genetic transformation protocol tailored for *Medicago truncatula*, a model legume and for *Oryza sativa*, Vialone Nano variety, an important local crop plant. This was a starting point for future genome editing application as tool to specifically address the issue of seed quality and preservation enhancing the variety. Overall, this work of thesis provide innovative and sustainable methodologies useful to cope the current agricultural challenges. Knowledge hereby gathered will be possibly translated to other relevant crop plants.

References

- Abbas, S. Q., Hassan, M. U., Hussain, B., Rasool, T. & Ali, Q. (2014). Optimization of Zinc seed priming treatments for improving the germination and early seedling growth of Oryza sativa. *Advancements in Life Sciences*, 2(1), 31-37.
- Abe, K., Araki, E., Suzuki, Y., Toki, S. & Saika, H. (2018) Production of high oleic/low linoleic rice by genome editing. *Plant Physiol Biochem*, 131, 58-62.
- Abe, K., Osakabe, K., Nakayama, S., Endo, M., Tagiri, A., Todoriki, S., Ichikawa, H. & Toki, S. (2005) Arabidopsis RAD51C gene is important for homologous recombination in meiosis and mitosis. *Plant physiology*, 139(2), 896-908.
- Abudayyeh, O. O., Gootenberg, J. S., Essletzbichler, P., Han, S., Joung, J., Belanto, J. J., Verdine, V., Cox, D. B. T., Kellner, M. J., Regev, A., Lander, E. S., Voytas, D. F., Ting, A. Y. & Zhang, F. (2017) RNA targeting with CRISPR-Cas13. *Nature*, 550(7675), 280-284.
- Abudayyeh, O. O., Gootenberg, J. S., Konermann, S., Joung, J., Slaymaker, I. M., Cox, D. B., Shmakov, S., Makarova, K. S., Semenova, E., Minakhin, L., Severinov, K., Regev, A., Lander, E. S., Koonin, E. V. & Zhang, F. (2016) C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science*, 353(6299), aaf5573.
- Acevedo-Garcia, J., Kusch, S. & Panstruga, R. (2014) Magical mystery tour: MLO proteins in plant immunity and beyond. *New Phytol*, 204(2), 273-81.
- Afzal, I., Basra, S. M., Farooq, M. & Nawaz, A. (2006) Alleviation of salinity stress in spring wheat by hormonal priming with ABA, salicylic acid and ascorbic acid. *Int. J. Agric. Biol*, 8(1), 23-28.
- Ali, M., Hayat, S., Ahmad, H., Ghani, M. I., Amin, B., Atif, M. J. & Cheng, Z. (2019) Priming of *Solanum melongena* L. Seeds Enhances Germination, Alters Antioxidant Enzymes, Modulates ROS, and Improves Early Seedling Growth: Indicating Aqueous Garlic Extract as Seed-Priming Bio-Stimulant for Eggplant Production. *Applied Sciences*, 9(11), 2203.
- Alipor, S., Taghvaei, M., Jalilian, A., Kazemeini, A. & Razi, H. (2019) Hydro-thermal priming enhance seed germination capacity and seedling growth in sugar beet. *Cell Mol Biol (Noisy-le-grand)*, 65(4), 90-96.
- Alscher, R. G., Erturk, N. & Heath, L. S. (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J Exp Bot*, 53(372), 1331-41.

- Anderson, J. A., Gipmans, M., Hurst, S., Layton, R., Nehra, N., Pickett, J., Shah, D. M., Souza, T. L. P. & Tripathi, L. (2016) Emerging agricultural biotechnologies for sustainable agriculture and food security. *Journal of agricultural and food chemistry*, 64(2), 383-393.
- Andersson, L. & Milberg, P. (1998) Variation in seed dormancy among mother plants, populations and years of seed collection. *Seed science research*, 8(1), 29-38.
- Andreoli, C. & de Andrade, R. (2002) Integrating matriconditioning with chemical and biological seed treatments to improve vegetable crop stand establishment and yield under tropical conditions. *Seed Technology*, 89-99.
- Apel, K. & Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.*, 55, 373-399.
- Araújo, S., Pagano, A., Dondi, D., Lazzaroni, S., Pinela, E., Macovei, A. & Balestrazzi, A. (2019) Metabolic signatures of germination triggered by kinetin in *Medicago truncatula*. *Scientific Reports*, 9(1), 10466.
- Araújo, S., Paparella, S., Dondi, D., Bentivoglio, A., Carbonera, D., Balestrazzi, A. (2016) Physical methods for seed invigoration: advantages and challenges in seed technology. *Front Plant Sci. 7*, 646.
- Araújo, S., Duque, A. S. R. L. A., Santos, D. M. M. F. d. & Fevereiro, M. P. S. (2004) An Efficient Transformation Method to Regenerate a High Number of Transgenic Plants Using a New Embryogenic Line of *Medicago truncatula* cv. Jemalong. *Plant Cell, Tissue and Organ Culture*, 78(2), 123-131.
- Arif, M., Waqas, M., Nawab, K. & Shahid, M. (2007) Effect of seed priming in Zn solutions on chickpea and wheat. *Afri. Crop Sci. Pro*, 8, 237-240.
- Arrigoni, O., De Gara, L., Tommasi, F. & Liso, R. (1992) Changes in the Ascorbate System during Seed Development of Vicia faba L. *Plant physiology*, 99(1), 235-238.
- Asada, K. (1999) The water-water cycle in chloroplasts: Scavenging of Active Oxygens and Dissipation of Excess Photons. *Annu Rev Plant Physiol Plant Mol Biol*, 50, 601-639.
- Ashraf, M. & Bray, C. (1993) DNA synthesis in osmoprimed leek (*Allium porrum* L.) seeds and evidence for repair and replication. *Seed Science Research*, 3(1), 15-23.
- Ashraf, M. & Foolad, M. R. (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany*, 59(2), 206-216.

- Aslani, F., Bagheri, S., Muhd Julkapli, N., Juraimi, A. S., Hashemi, F. S. G. & Baghdadi, A. (2014) Effects of engineered nanomaterials on plants growth: an overview. *The Scientific World Journal*, 2014.
- Austin, R. B., Longden, P. C. & Hutchinson, J. (1969) Some Effects of 'Hardening' Carrot Seed. *Annals of Botany*, 33(133), 883-895.
- Azevedo, R. A., Alas, R. M., Smith, R. J. & Lea, P. J. (1998) Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. *Physiologia Plantarum*, 104(2), 280-292.
- Azpilicueta, C. E., Benavides, M. P., Tomaro, M. L. & Gallego, S. M. (2007) Mechanism of CATA3 induction by cadmium in sunflower leaves. *Plant Physiol Biochem*, 45(8), 589-95.
- Bailly, C. (2004) Active oxygen species and antioxidants in seed biology. *Seed Science Research*, 14(2), 93-107.
- Bailly, C., Benamar, A., Corbineau, F. & Côme, D. (2000) Antioxidant systems in sunflower (*Helianthus annuus* L.) seeds as affected by priming. *Seed Science Research*, 10, 35-42.
- Bailly, C., Bogatek-Leszczynska, R., Côme, D. & Corbineau, F. (2002) Changes in activities of antioxidant enzymes and lipoxygenase during growth of sunflower seedlings from seeds of different vigour. *Seed Science Research*, 12(1), 47-55.
- Bailly, C., El-Maarouf-Bouteau, H. & Corbineau, F. (2008) From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. *C R Biol*, 331(10), 806-14.
- Bajwa, A. A., Farooq, M. & Nawaz, A. (2018) Seed priming with sorghum extracts and benzyl aminopurine improves the tolerance against salt stress in wheat (*Triticum aestivum* L.). *Physiology and molecular biology of plants*, 24(2), 239-249.
- Balestrazzi, A., Botti, S., Zelasco, S., Biondi, S., Franchin, C., Calligari, P., Racchi, M., Turchi, A., Lingua, G., Berta, G. & Carbonera, D. (2009) Expression of the PsMTA1gene in white poplar engineered with the MAT system is associated with heavy metal tolerance and protection against 8-hydroxy-2′-deoxyguanosine mediated-DNA damage. *Plant Cell Reports*, 28(8), 1179-1192.
- Balestrazzi, A., Confalonieri, M., Macovei, A. & Carbonera, D. (2011a) Seed imbibition in Medicago truncatula Gaertn.: Expression profiles of DNA repair genes in relation to PEG-mediated stress. *J Plant Physiol*, 168(7), 706-13.

- Balestrazzi, A., Confalonieri, M., Macovei, A., Donà, M. & Carbonera, D. (2011b) Genotoxic stress and DNA repair in plants: emerging functions and tools for improving crop productivity. *Plant Cell Rep*, 30(3), 287-95.
- Balestrazzi, A., Donà, M., Macovei, A., Sabatini, M. E., Pagano, A. & Carbonera, D. (2015) DNA repair and telomere maintenance during seed imbibition: correlation of transcriptional patterns. *Telomere and Telomerase*, 2.
- Balmer, A., Pastor, V., Gamir, J., Flors, V. & Mauch-Mani, B. (2015) The 'prime-ome': towards a holistic approach to priming. *Trends Plant Sci*, 20(7), 443-52.
- Baltes, N., Hummel, A., Konecna, E., Cegan, R., Bruns, A., Bisaro, D. & Voytas, D. (2015) Conferring resistance to geminiviruses with the CRISPR–Cas prokaryotic immune system. *Nature Plants*, 1, 15145.
- Baltes, N. J. & Voytas, D. F. (2015) Enabling plant synthetic biology through genome engineering. *Trends Biotechnol*, 33(2), 120-31.
- Bandyopadhyay, U., Das, D. & Banerjee, R.K. (1999) Reactive Oxygen Species: Oxidative Damage and Pathogenesis. *Current Science*, 77, 658-666.
- Barakate, A. & Stephens, J. (2016) An Overview of CRISPR-Based Tools and Their Improvements: New Opportunities in Understanding Plant-Pathogen Interactions for Better Crop Protection. *Frontiers in plant science*, 7, 765-765.
- Bari, V. K., Nassar, J. A., Kheredin, S. M., Gal-On, A., Ron, M., Britt, A., Steele, D., Yoder, J. & Aly, R. (2019) CRISPR/Cas9-mediated mutagenesis of Carotenoid Cleavage Dioxygenase 8 in tomato provides resistance against the parasitic weed *Phelipanche aegyptiaca*. *Sci Rep*, 9(1), 11438.
- Barnes, D. E. & Lindahl, T. (2004) Repair and genetic consequences of endogenous DNA base damage in mammalian cells. *Annu. Rev. Genet.*, 38, 445-476.
- Barrôco, R. M., Van Poucke, K., Bergervoet, J. H., De Veylder, L., Groot, S. P., Inzé, D. & Engler, G. (2005) The role of the cell cycle machinery in resumption of postembryonic development. *Plant Physiology*, 137(1), 127-140.
- Baskin, J. M. & Baskin, C. C. (2004) A classification system for seed dormancy. *Seed Science Research*, 14(1), 1-16.
- Basra, S., Zia, M., Mehmood, T., Afzal, I. & Khaliq, A. (2002) Comparison of different invigoration techniques in wheat (Triticum aestivum L.) seeds. *Pakistan Journal of Arid Agriculture (Pakistan)*.
- Bassel, G. W., Lan, H., Glaab, E., Gibbs, D. J., Gerjets, T., Krasnogor, N., Bonner, A. J., Holdsworth, M. J. & Provart, N. J. (2011) Genome-wide

network model capturing seed germination reveals coordinated regulation of plant cellular phase transitions. *Proceedings of the National Academy of Sciences*, 108(23), 9709.

- Batra, R., Nelles, D. A., Pirie, E., Blue, S. M., Marina, R. J., Wang, H., Chaim, I. A., Thomas, J. D., Zhang, N., Nguyen, V., Aigner, S., Markmiller, S., Xia, G., Corbett, K. D., Swanson, M. S. & Yeo, G. W. (2017) Elimination of Toxic Microsatellite Repeat Expansion RNA by RNA-Targeting Cas9. *Cell*, 170(5), 899-912.e10.
- Baysal, C., Bortesi, L., Zhu, C., Farré, G., Schillberg, S. & Christou, P. (2016) CRISPR/Cas9 activity in the rice OsBEIIb gene does not induce off-target effects in the closely related paralog OsBEIIa. *Molecular Breeding*, 36(8), 108.
- Begemann, M. B., Gray, B. N., January, E., Gordon, G. C., He, Y., Liu, H., Wu, X., Brutnell, T. P., Mockler, T. C. & Oufattole, M. (2017) Precise insertion and guided editing of higher plant genomes using Cpf1 CRISPR nucleases. *Sci Rep*, 7(1), 11606.
- Belhaj, K., Chaparro-Garcia, A., Kamoun, S. & Nekrasov, V. (2013) Plant genome editing made easy: targeted mutagenesis in model and crop plants using the CRISPR/Cas system. *Plant Methods*, 9(1), 39.
- Bernal-Santos, G., O'Donnell, A. M., Vicini, J. L., Hartnell, G. F. & Bauman, D. E. (2010) Hot topic: Enhancing omega-3 fatty acids in milk fat of dairy cows by using stearidonic acid-enriched soybean oil from genetically modified soybeans. *Journal of Dairy Science*, 93(1), 32-37.
- Berrie, A. M. M. & Drennan, D. S. H. (1971) The effect of hydration-dehydration on seed germination. *New Phytologist*, 70(1), 135-142.
- Bertier, L. D., Ron, M., Huo, H., Bradford, K. J., Britt, A. B. & Michelmore, R. W. (2018) High-Resolution Analysis of the Efficiency, Heritability, and Editing Outcomes of CRISPR/Cas9-Induced Modifications of NCED4 in Lettuce (*Lactuca sativa*). *G3 (Bethesda)*, 8(5), 1513-1521.
- Bessho, T. (2003) Induction of DNA replication-mediated double strand breaks by psoralen DNA interstrand cross-links. *J Biol Chem*, 278(7), 5250-4.
- Bewley, J., Bradford, K., Hilhorst, H. & Nonogaki, H. (2013) *Seeds: Physiology of Development, Germination and Dormancy*, third edition. NY: Springer.
- Bewley, J. D. (1997) Seed Germination and Dormancy. *The Plant Cell*, 9(7), 1055.
- Bhattacharyya, R., Ghosh, B. N., Mishra, P. K., Mandal, B., Rao, C. S., Sarkar, D., Das, K., Anil, K. S., Lalitha, M., Hati, K. M. & Franzluebbers, A.

J. (2015) Soil Degradation in India: Challenges and Potential Solutions. *Sustainability*, 7(4), 3528-3570.

- Bittencourt, M. L. C., Dias, D. C. F. S., Dias, L. A. S. & Araújo, E. F. (2004) Effects of priming on asparagus seed germination and vigour under water and temperature stress. *Seed Science and Technology*, 32(2), 607-616.
- Bogdanove, A. J. & Voytas, D. F. (2011) TAL effectors: customizable proteins for DNA targeting. *Science*, 333(6051), 1843-6.
- Bolte, S., Lanquar, V., Soler, M.-N., Beebo, A., Satiat-Jeunemaître, B., Bouhidel, K. & Thomine, S. (2011) Distinct lytic vacuolar compartments are embedded inside the protein storage vacuole of dry and germinating Arabidopsis thaliana seeds. *Plant and Cell Physiology*, 52(7), 1142-1152.
- Bose, B., Kumar, M., Singhal, R. K. & Mondal, S. (2018) Impact of seed priming on the modulation of physico-chemical and molecular processes during germination, growth, and development of crops, in Rakshit, A. & Singh, H. B. (eds), *Advances in Seed Priming*. Singapore: Springer Singapore, 23-40.
- Boyko, A., Filkowski, J. & Kovalchuk, I. (2005) Homologous recombination in plants is temperature and day-length dependent. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 572(1), 73-83.
- Braatz, J., Harloff, H.-J., Mascher, M., Stein, N., Himmelbach, A. & Jung, C. (2017) CRISPR-Cas9 Targeted Mutagenesis Leads to Simultaneous Modification of Different Homoeologous Gene Copies in Polyploid Oilseed Rape (*Brassica napus*). *Plant Physiology*, 174(2), 935.
- Bradford, K. J. (1995) Water relations in seed germination. *Seed development and germination*, 1(13), 351-396.
- Bray, C. M. & West, C. E. (2005) DNA repair mechanisms in plants: crucial sensors and effectors for the maintenance of genome integrity. *New Phytologist*, 168(3), 511-528.
- Britt, A. B. (1999) Molecular genetics of DNA repair in higher plants. *Trends in plant science*, 4(1), 20-25.
- Brookes, G. & Barfoot, P. (2017) Farm income and production impacts of using GM crop technology 1996–2015. *GM Crops & Food*, 8(3), 156-193.
- Brooks, C., Nekrasov, V., Lippman, Z. B. & Van Eck, J. (2014) Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. *Plant Physiol*, 166(3), 1292-7.
- Browse, J. (2009) Jasmonate passes muster: a receptor and targets for the defense hormone. *Annu Rev Plant Biol*, 60, 183-205.

- Buitink, J. & Leprince, O. (2008) Intracellular glasses and seed survival in the dry state. *C R Biol*, 331(10), 788-95.
- Bujalski W & AW, N. (1991) Large-scale osmotic priming of onion seeds: a comparison of different strategies for oxygenation. Sci Hort
- Callan, N. W., Mathre, D. & Miller, J. B. (1990) Bio-priming seed treatment for biological control of Pythium ultimum preemergence damping-off in sh-2 sweet corn. *Plant Disease*, 74, 368-372.
- Cantos, C., Francisco, P., Trijatmiko, K. R., Slamet-Loedin, I. & Chadha-Mohanty, P. K. (2014) Identification of "safe harbor" loci in indica rice genome by harnessing the property of zinc-finger nucleases to induce DNA damage and repair. *Front Plant Sci*, 5, 302.
- Caseiro, R., Bennett, M. A. & Marcos-Filho, J. (2004) Comparison of three priming techniques for onion seed lots differing in initial seed quality. *Seed Science and Technology*, 32(2), 365-375.
- Catusse, J., Job, C. & Job, D. (2008) Transcriptome- and proteome-wide analyses of seed germination. *Comptes Rendus Biologies*, 331(10), 815-822.
- Caverzan, A., Passaia, G., Rosa, S. B., Ribeiro, C. W., Lazzarotto, F. & Margis-Pinheiro, M. (2012) Plant responses to stresses: Role of ascorbate peroxidase in the antioxidant protection. *Genet Mol Biol*, 35(4 (suppl)), 1011-9.
- Chabaud, M., Ratet, P., Araújo, S., Duque, S. & D.G, H. (2007) Agrobacterium tumefaciens-mediated transformation and in vitro plant regeneration of *M. truncatula*.
- Chalapathi Rao, A. S. V. & Reddy, A. R. (2008) Glutathione reductase: a putative redox regulatory system in plant cells. *In:* N.A. Khan, S. S., S. Umar (ed.). Sulfur Assimilation and Abiotic Stresses in Plants. Springer. 111-147.
- Chander, R. & Kapoor, N. K. (1990) High density lipoprotein is a scavenger of superoxide anions. *Biochem Pharmacol*, 40(7), 1663-5.
- Chang, W.-C., Wang, Y.-K., Liu, P.-F., Tsai, Y.-F., Kong, L.-R., Lin, C.-K., Yang, C.-H. & Pan, R.-L. (2008) Regulation of Ku gene promoters in Arabidopsis by hormones and stress. *Functional Plant Biology*, 35(4), 265-280.
- Charbonnel, C., Allain, E., Gallego, M. E. & White, C. I. (2011) Kinetic analysis of DNA double-strand break repair pathways in Arabidopsis. *DNA repair*, 10(6), 611-619.
- Charbonnel, C., Gallego, M. E. & White, C. I. (2010) Xrcc1-dependent and Ku-dependent DNA double-strand break repair kinetics in Arabidopsis plants. *The Plant Journal*, 64(2), 280-290.

- Chavan, J. K., Kadam, S. S. & Beuchat, L. R. (1989) Nutritional improvement of cereals by sprouting. *Critical Reviews in Food Science and Nutrition*, 28(5), 401-437.
- Chen, B., Gilbert, L. A., Cimini, B. A., Schnitzbauer, J., Zhang, W., Li, G.-W., Park, J., Blackburn, E. H., Weissman, J. S., Qi, L. S. & Huang, B. (2013) Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell*, 155(7), 1479-1491.
- Chen, C. & Dickman, M. B. (2005) Proline suppresses apoptosis in the fungal pathogen Colletotrichum trifolii. *Proceedings of the National Academy of Sciences*, 102(9), 3459-3464.
- Chen, H., Chu, P., Zhou, Y., Li, Y., Liu, J., Ding, Y., Tsang, E. W., Jiang, L., Wu, K. & Huang, S. (2012) Overexpression of AtOGG1, a DNA glycosylase/AP lyase, enhances seed longevity and abiotic stress tolerance in Arabidopsis. *Journal of experimental botany*, 63(11), 4107-4121.
- Chen, K. & Arora, R. (2011) Dynamics of the antioxidant system during seed osmopriming, post-priming germination, and seedling establishment in Spinach (*Spinacia oleracea*). *Plant Sci*, 180(2), 212-20.
- Chen, K. & Arora, R. (2013) Priming memory invokes seed stress-tolerance. *Environmental and Experimental Botany*, 94, 33-45.
- Chen, K., Wang, Y., Zhang, R., Zhang, H. & Gao, C. (2019) CRISPR/Cas Genome Editing and Precision Plant Breeding in Agriculture. *Annual Review of Plant Biology*, 70(1), 667-697.
- Chen, R., Xu, Q., Liu, Y., Zhang, J., Ren, D. & Wang, G. (2018) Generation of Transgene-Free Maize Male Sterile Lines Using the CRISPR/Cas9 System. *Front Plant Sci*, 9, 1180.
- Chen, Z. & Gallie, D. R. (2005) Increasing tolerance to ozone by elevating foliar ascorbic acid confers greater protection against ozone than increasing avoidance. *Plant Physiol*, 138(3), 1673-89.
- Choi, J.-H., Lindsey-Boltz, L. A., Kemp, M., Mason, A. C., Wold, M. S. & Sancar, A. (2010) Reconstitution of RPA-covered single-stranded DNA-activated ATR-Chk1 signaling. *Proceedings of the National Academy of Sciences*, 107(31), 13660-13665.
- Clasen, B. M., Stoddard, T. J., Luo, S., Demorest, Z. L., Li, J., Cedrone, F., Tibebu, R., Davison, S., Ray, E. E., Daulhac, A., Coffman, A., Yabandith, A., Retterath, A., Haun, W., Baltes, N. J., Mathis, L., Voytas, D. F. & Zhang, F. (2016) Improving cold storage and processing traits in potato through targeted gene knockout. *Plant Biotechnol J*, 14(1), 169-76.
- Cobbett, C. & Goldsbrough, P. (2002) Phychelatins and metallothioneins: Roles in Heavy Metal Detoxification and Homeostasis. *Annual Review of Plant Biology*, 53(1), 159-182.

- Cordeiro, R. M. (2015) Molecular dynamics simulations of the transport of reactive oxygen species by mammalian and plant aquaporins. *Biochim Biophys Acta*, 1850(9), 1786-94.
- Cordoba-Canero, D., Morales-Ruiz, T., Roldan-Arjona, T. & Ariza, R. R. (2009) Single-nucleotide and long-patch base excision repair of DNA damage in plants. *Plant J*, 60(4), 716-28.
- Costa, R. M., Morgante, P. G., Berra, C. M., Nakabashi, M., Bruneau, D., Bouchez, D., Sweder, K. S., Van Sluys, M. A. & Menck, C. F. (2001) The participation of AtXPB1, the XPB/RAD25 homologue gene from *Arabidopsis thaliana*, in DNA repair and plant development. *The Plant Journal*, 28(4), 385-395.
- Dabul, A. (2009) Screening of a broad range of rice (Oryza sativa L.) germplasm for *in vitro* rapid plant regeneration and development of an early prediction system. *In* vitro *Cell Dev Biol- Plant*. 4(45), 414-420.
- Dahan-Meir, T., Filler-Hayut, S., Melamed-Bessudo, C., Bocobza, S., Czosnek, H., Aharoni, A. & Levy, A. A. (2018) Efficient in planta gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system. *The Plant Journal*, 95(1), 5-16.
- Dandoy, E., Schnys, R., Deltour, R. & Verly, W. G. (1987) Appearance and repair of apurinic/apyrimidinic sites in DNA during early germination of *Zea mays*. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 181(1), 57-60.
- Das, A. J. & Kumar, R. (2016) Bioremediation of petroleum contaminated soil to combat toxicity on *Withania somnifera* through seed priming with biosurfactant producing plant growth promoting rhizobacteria. *Journal of environmental management*, 174, 79-86.
- Datta, K., Koukolíková-Nicola, Z., Baisakh, N., Oliva, N. & Datta, S. K. (2000) *Agrobacterium*-mediated engineering for sheath blight resistance of indica rice cultivars from different ecosystems. *Theoretical and Applied Genetics*, 100(6), 832-839.
- Dave, A., Hernandez, M. L., He, Z., Andriotis, V. M., Vaistij, F. E., Larson, T. R. & Graham, I. A. (2011) 12-oxo-phytodienoic acid accumulation during seed development represses seed germination in *Arabidopsis*. *Plant Cell*, 23(2), 583-99.
- De Gara, L., de Pinto, M. C. & Arrigoni, O. (1997) Ascorbate synthesis and ascorbate peroxidase activity during the early stage of wheat germination. *Physiologia Plantarum*, 100(4), 894-900.
- De Gara, L., Paciolla, C., De Tullio, M. C., Motto, M. & Arrigoni, O. (2000) Ascorbate-dependent hydrogen peroxide detoxification and ascorbate regeneration during germination of a highly productive maize hybrid:

Evidence of an improved detoxification mechanism against reactive oxygen species. *Physiologia Plantarum*, 109(1), 7-13.

- de Medeiros, N. M. C., Marques de Medeiros, A. L. (2015) Recent Advances in Plant DNA Repair, in Chen, C. C. (ed), *Advances in DNA Repair,*IntechOpen.
- De Paula, M., Perez-Otaola, M., Darder, M., Torres, M., Frutos, G. & Martinezi-Honduvilla, C. J. (1996) Function of the ascorbate- glutathione cycle in aged sunflower seeds. Physiol plant.
- Debbarma, J., Sarki, Y. N., Saikia, B., Boruah, H. P. D., Singha, D. L. & Chikkaputtaiah, C. (2019) Ethylene Response Factor (ERF) Family Proteins in Abiotic Stresses and CRISPR-Cas9 Genome Editing of ERFs for Multiple Abiotic Stress Tolerance in Crop Plants: A Review. *Mol Biotechnol*, 61(2), 153-172.
- Decottignies, A. (2013) Alternative end-joining mechanisms: A historical perspective. *Frontiers in genetics*, 4, 48.
- Deka, J., Paul, A. & Chattopadhyay, A. (2012) Modulating enzymatic activity in the presence of gold nanoparticles. *RSC Advances*, 2(11), 4736-4745.
- Dekkers, B. J., Costa, M. C., Maia, J., Bentsink, L., Ligterink, W. & Hilhorst, H. W. (2015) Acquisition and loss of desiccation tolerance in seeds: from experimental model to biological relevance. *Planta*, 241(3), 563-77.
- Dekkers, B. J., He, H., Hanson, J., Willems, L. A., Jamar, D. C., Cueff, G., Rajjou, L., Hilhorst, H. W. & Bentsink, L. (2016) The *Arabidopsis* DELAY OF GERMINATION 1 gene affects ABSCISIC ACID INSENSITIVE 5 (ABI5) expression and genetically interacts with ABI3 during *Arabidopsis* seed development. *Plant J*, 85(4), 451-65.
- Del Río, L. A., Corpas, F. J., Sandalio, L. M., Palma, J. M., Gómez, M. & Barroso, J. B. (2002) Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. *Journal of experimental botany*, 53(372), 1255-1272.
- Delker, C., Stenzel, I., Hause, B., Miersch, O., Feussner, I. & Wasternack, C. (2006) Jasmonate biosynthesis in *Arabidopsis thaliana*--enzymes, products, regulation. *Plant Biol (Stuttg)*, 8(3), 297-306.
- Dellaporta, S. L., Wood, J. & Hicks, J. B. (1983) A plant DNA minipreparation: version II. *Plant molecular biology reporter*, 1(4), 19-21.
- Demirevska-Kepova, K., Simova-Stoilova, L., Stoyanova, Z. P. & Feller, U. (2006) Cadmium Stress in Barley: Growth, Leaf Pigment, and Protein Composition and Detoxification of Reactive Oxygen Species. *Journal of Plant Nutrition*, 29(3), 451-468.

- Demorest, Z. L., Coffman, A., Baltes, N. J., Stoddard, T. J., Clasen, B. M., Luo, S., Retterath, A., Yabandith, A., Gamo, M. E., Bissen, J., Mathis, L., Voytas, D. F. & Zhang, F. (2016) Direct stacking of sequence-specific nuclease-induced mutations to produce high oleic and low linolenic soybean oil. *BMC Plant Biol*, 16(1), 225.
- Di Girolamo, G. & Barbanti, L. (2012) Treatment conditions and biochemical processes influencing seed priming effectiveness. I*talian Journal of Agronomy*.
- Dixon, D. P., Skipsey, M. & Edwards, R. (2010) Roles for glutathione transferases in plant secondary metabolism. *Phytochemistry*, 71(4), 338-50.
- Donà, M., Confalonieri, M., Minio, A., Biggiogera, M., Buttafava, A., Raimondi, E., Delledonne, M., Ventura, L., Sabatini, M. E. & Macovei, A. (2013) RNA-Seq analysis discloses early senescence and nucleolar dysfunction triggered by Tdp1α depletion in Medicago truncatula. *Journal of experimental botany*, 64(7), 1941-1951.
- Doucet-Chabeaud, G., Godon, C., Brutesco, C., De Murcia, G. & Kazmaier, M. (2001) Ionising radiation induces the expression of PARP-1 and PARP-2 genes in Arabidopsis. *Molecular Genetics and Genomics*, 265(6), 954-963.
- El-Adawy, T. A., Rahma, E. H., El-Bedawey, A. A. & El-Beltagy, A. E. (2003) Nutritional potential and functional properties of germinated mung bean, pea and lentil seeds. *Plant Foods for Human Nutrition*, 58(3), 1-13.
- El-maarouf-bouteau, H., Meimoun, P., Job, C., Job, D. & Bailly, C. (2013) Role of protein and mRNA oxidation in seed dormancy and germination. *Frontiers in Plant Science*, 4(77).
- El-maarouf-bouteau, H., Sajjad, Y., Bazin, J., Langlade, N., Cristescu, S. M., Balzergue, S., Baudouin, E. & Bailly, C. (2015) Reactive oxygen species, abscisic acid and ethylene interact to regulate sunflower seed germination. *Plant, Cell & Environment*, 38(2), 364-374.
- Ellis, R. H. & Butcher, P. D. (1988) The effects of priming and 'natural' differences in quality amongst onion seed lots on the response of the rate of germination to temperature and the identification of the characteristics under genotypic control. *Journal of Experimental Botany*, 39(7), 935-950.
- Ells, J. E. (1963) The influence of treating tomato seed with nutrient solution on emergence rate and seedling growth. *Proc Am Soc Hort Sci,* 83, 684-687.
- Ernest, V., Shiny, P., Mukherjee, A. & Chandrasekaran, N. (2012) Silver nanoparticles: a potential nanocatalyst for the rapid degradation of starch hydrolysis by α-amylase. *Carbohydrate research*, 352, 60-64.

- Evenari, M. (1984) Seed physiology: its history from antiquity to the beginning of the 20th century.: Bot Rev.
- Fanan, S. & Novembre, A. D. (2007) Priming of eggplant seeds. *Bragantia*, 66, 675-683.
- FAO *www.fao.org*
- Farooq, M., Basra, S. M. A., Ahmad, N. & Hafeez, K. (2005) Thermal Hardening: A New Seed Vigor Enhancement Tool in Rice.: Journal of Integrative Plant Biology.
- Farooq, M., Hussain, M., Usman, M., Farooq, S., Alghamdi, S. S. & Siddique, K. H. M. (2018) Impact of Abiotic Stresses on Grain Composition and Quality in Food Legumes. *J Agric Food Chem*, 66(34), 8887-8897.
- Farooq, M., Irfan, M., Aziz, T., Ahmad, I. & Cheema, S. (2013) Seed priming with ascorbic acid improves drought resistance of wheat. *Journal of Agronomy and Crop Science*, 199(1), 12-22.
- Farooq, M., Wahid, A. & Siddique, K. H. (2012) Micronutrient application through seed treatments: a review. *Journal of soil science and plant nutrition*, 12(1), 125-142.
- Faure, J.-D. & Napier, J. A. (2018) Europe's first and last field trial of gene-edited plants? *eLife*, 7, e42379.
- Fercha, A., Capriotti, A. L., Caruso, G., Cavaliere, C., Gherroucha, H., Samperi, R., Stampachiacchiere, S. & Lagana, A. (2013) Gel-free proteomics reveal potential biomarkers of priming-induced salt tolerance in durum wheat. *Journal of proteomics*, 91, 486-499.
- Fessel, S. A., Vieira, R., Rodrigues, T. J. D., Fagioli, M. & Paula, R. (2001) Eficiência do condicionamento osmótico em sementes de alface. *Revista Brasileira de Sementes*, 23, 128-133.
- Finch-Savage, W. E. & Bassel, G. W. (2016) Seed vigour and crop establishment: extending performance beyond adaptation. *J Exp Bot*, 67(3), 567-91.
- Finch-Savage, W. E., Cadman, C. S. C., Toorop, P. E., Lynn, J. R. & Hilhorst, H. W. M. (2007) Seed dormancy release in Arabidopsis Cvi by dry after-ripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directed by environmentally specific sensing. *The Plant Journal*, 51(1), 60-78.
- Finch-Savage, W. E. & Footitt, S. (2017) Seed dormancy cycling and the regulation of dormancy mechanisms to time germination in variable field environments. *J Exp Bot*, 68(4), 843-856.
- Finch-Savage, W. E. & Leubner-Metzger, G. (2006) Seed dormancy and the control of germination. *New Phytol*, 171(3), 501-23.

- Forti, C., Gualtieri, C., Pagano, A., Araùjo de Sousa, S., Balestrazzi, A. & Macovei, A. (2019) Genome Editing in the context of Seed research: How these novel biotechnology tools can change the future face of agricultural crop development?, in Tutela, T., Passricha and Saiti (ed), *Advancement in crop improvement techniques* Elsevier Inc. In press
- Foyer, C., Lelandais, M., Galap, C. & Kunert, K. J. (1991) Effects of Elevated Cytosolic Glutathione Reductase Activity on the Cellular Glutathione Pool and Photosynthesis in Leaves under Normal and Stress Conditions. *Plant Physiol*, 97(3), 863-72.
- Foyer, C. H. & Noctor, G. (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell*, 17(7), 1866-75.
- Friedman, W. E. (1998) The evolution of double fertilization and endosperm: an "historical" perspective. *Sexual Plant Reproduction*, 11(1), 6-16.
- Friesner, J. & Britt, A. B. (2003) Ku80-and DNA ligase IV-deficient plants are sensitive to ionizing radiation and defective in T-DNA integration. *The Plant Journal*, 34(4), 427-440.
- Frugoli, J. A., Zhong, H. H., Nuccio, M. L., McCourt, P., McPeek, M. A., Thomas, T. L. & McClung, C. R. (1996) Catalase Is Encoded by a Multigene Family in Arabidopsis thaliana (L.) Heynh. *Plant Physiology*, 112(1), 327.
- Fry, S. C., Dumville, J. C. & Miller, J. G. (2001) Fingerprinting of polysaccharides attacked by hydroxyl radicals in vitro and in the cell walls of ripening pear fruit. *Biochem J*, 357(Pt 3), 729-37.
- Fujikura, Y., & Karssen, C. M. (1992) Effects of controlled deterioration and osmopriming on protein synthesis of cauliflower seeds during early germination. Seed Sci Res. 2, 23-31.
- Gaidau, C., Niculescu, M., Stepan, E., Epure, D.-G. & Gidea, M. (2013) New mixes based on collagen extracts with bioactive properties, for treatment of seeds in sustainable agriculture. *Current pharmaceutical biotechnology*, 14(9), 792-801.
- Galhaut, L., de Lespinay, A., Walker, D. J., Bernal, M. P., Correal, E. & Lutts, S. (2014) Seed priming of Trifolium repens L. improved germination and early seedling growth on heavy metal-contaminated soil. *Water, Air, & Soil Pollution*, 225(4), 1905.
- Gallego, M. E., Jalut, N. & White, C. I. (2003) Telomerase dependence of telomere lengthening in Ku80 mutant Arabidopsis. *The Plant Cell*, 15(3), 782-789.

- Gamborg, O. L., Miller, R. A. & Ojima, K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, 50(1), 151-158.
- Gao, R., Feyissa, B. A., Croft, M. & Hannoufa, A. (2018) Gene editing by CRISPR/Cas9 in the obligatory outcrossing *Medicago sativa*. *Planta*, 247(4), 1043-1050.
- Gapińska, M., Skłodowska, M. & Gabara, B. (2007) Effect of short- and long-term salinity on the activities of antioxidative enzymes and lipid peroxidation in tomato roots. *Acta Physiologiae Plantarum*, 30(1), 11.
- Garg, N. & Manchanda, G. (2009) ROS generation in plants: Boon or bane? *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology*, 143(1), 81-96.
- Garnczarska, M., Bednarski, W. & Jancelewicz, M. (2009) Ability of lupine seeds to germinate and to tolerate desiccation as related to changes in free radical level and antioxidants in freshly harvested seeds. *Plant Physiol Biochem*, 47(1), 56-62.
- GB, A. (1830) Note on pollen action mode on stigmata. Annales Sciences Naturelles Botanique.
- Ghassemian, M., Nambara, E., Cutler, S., Kawaide, H., Kamiya, Y. & McCourt, P. (2000) Regulation of abscisic acid signaling by the ethylene response pathway in *Arabidopsis*. *The Plant Cell*, 12(7), 1117.
- Gidrol, X., Lin, W. S., Degousee, N., Yip, S. F. & Kush, A. (1994) Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. *Eur J Biochem*, 224(1), 21-8.
- Gilbert, L. A., Larson, M. H., Morsut, L., Liu, Z., Brar, G. A., Torres, S. E., Stern-Ginossar, N., Brandman, O., Whitehead, E. H., Doudna, J. A., Lim, W. A., Weissman, J. S. & Qi, L. S. (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell*, 154(2), 442-51.
- Gill, S. S., Khan, N. A., Anjum, N. A. & Tuteja, N. (2010) Amelioration of cadmium stress in crop plants by nutrients management: morphological, physiological and biochemical aspects. *Plant stress*, 5(1), 1-23.
- Gill, S. S. & Tuteja, N. (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem*, 48(12), 909-30.
- Gill, S. S. & Tuteja, N. (2011) Cadmium stress tolerance in crop plants: probing the role of sulfur. *Plant Signal Behav*, 6(2), 215-22.
- Glick, B. R. (2012) Plant growth-promoting bacteria: mechanisms and applications. *Scientifica*, 2012.

- Gogos, A., Knauer, K. & Bucheli, T. D. (2012) Nanomaterials in plant protection and fertilization: current state, foreseen applications, and research priorities. *Journal of agricultural and food chemistry*, 60(39), 9781-9792.
- Golubov, A., Yao, Y., Maheshwari, P., Bilichak, A., Boyko, A., Belzile, F. & Kovalchuk, I. (2010) Microsatellite instability in Arabidopsis increases with plant development. *Plant physiology*, 154(3), 1415-1427.
- Gomes, D. P., da Silva, A. F., Dias, D. C. F., Alvarenga, E. M., da Silva, L. J. & Panozzo, L. E. (2012) Priming and drying on the physiological quality of eggplant seeds. *Horticultura Brasileira*, 30(3), 484-488.
- Gonzales, L. M. (2015) Germination Response of Eggplant (Solanum melongena L.) Seeds to Different Vinegar Concentration as Seed Priming Agents. *International Journal of Scientific and Research Publications*, 5, 1-2250.
- Gorguet, B., van Heusden, A. W. & Lindhout, P. (2005) Parthenocarpic fruit development in tomato. *Plant Biol (Stuttg)*, 7(2), 131-9.
- Gossot, O. & Geitmann, A. (2007) Pollen tube growth: coping with mechanical obstacles involves the cytoskeleton. *Planta*, 226(2), 405-16.
- Goswami, A., Banerjee, R. & Raha, S. (2013) Drought resistance in rice seedlings conferred by seed priming. *Protoplasma*, 250(5), 1115-1129.
- Gousset, C., Collonnier, C., Mulya, K., Mariska, I., Rotino, G. L., Besse, P., Servaes, A. & Sihachakr, D. (2005) Solanum torvum, as a useful source of resistance against bacterial and fungal diseases for improvement of eggplant (*S. melongena* L.). *Plant Science*, 168(2), 319-327.
- Govindaraj, M., Masilamani, P., Alex Albert, V. & Bhaskaran, M. (2017) Role of antioxidant in seed quality- A review. *Agricultural Reviews*, 38(3), 180-190.
- Graeber, K., Linkies, A., Muller, K., Wunchova, A., Rott, A. & Leubner-Metzger, G. (2010) Cross-species approaches to seed dormancy and germination: conservation and biodiversity of ABA-regulated mechanisms and the Brassicaceae DOG1 genes. *Plant Mol Biol*, 73(1-2), 67-87.
- Graeber, K., Nakabayashi, K., Miatton, E., Leubner-Metzger, G. & Soppe, W. J. (2012) Molecular mechanisms of seed dormancy. *Plant Cell Environ*, 35(10), 1769-86.
- Grasser, M., Kane, C. M., Merkle, T., Melzer, M., Emmersen, J. & Grasser, K. D. (2009) Transcript elongation factor TFIIS is involved in *Arabidopsis* seed dormancy. *Journal of molecular biology*, 386(3), 598-611.
- Grover, A. & Minhas, D. (2000) Towards production of abiotic stress tolerant transgenic rice plants: issues, progress and future research needs. Proc indian Natn Acad Sci. B66(1), 13-32.

- Gu, X.-Y., Turnipseed, E. B. & Foley, M. E. (2008) The qSD12 locus controls offspring tissue-imposed seed dormancy in rice. *Genetics*, 179(4), 2263.
- Guan, L. M. & Scandalios, J. G. (2002) Catalase gene expression in response to auxin-mediated developmental signals. *Physiol Plant*, 114(2), 288-295.
- H., H.-C., J., G., I., S., K., W. & E.W., W. (2005) Tocopherol content and activities of tyrosine aminotransferase and cystine lyase in A*rabidopsis u*nder stress conditions. J. Plant Physiol.
- Hacisalihoglu, G., Kantanka, S., Miller, N., Gustin, J. L. & Settles, A. M. (2018) Modulation of early maize seedling performance via priming under sub-optimal temperatures. *PloS one*, 13(11), e0206861.
- Hajiahmadi, Z., Movahedi, A., Wei, H., Li, D., Orooji, Y., Ruan, H. & Zhuge, Q. (2019) Strategies to increase on-target and reduce off-target effects of the CRISPR/Cas9 system in plants. *Int J Mol Sci*, 20(15).
- Harinasut, P., Poonsopa, D., Roengmongkoe, K. & Charoensataporn R (2003) salinity effects on antioxidant enzymes in mulberry cultivar. *Sci Asia*, 29, 109-113.
- Harman, G. E. (1991) Seed treatments for biological control of plant disease. *Crop Protection*, 10(3), 166-171.
- Harris, W. S., Lemke, S. L., Hansen, S. N., Goldstein, D. A., DiRienzo, M. A., Su, H., Nemeth, M. A., Taylor, M. L., Ahmed, G. & George, C. (2008) Stearidonic Acid-Enriched Soybean Oil Increased the Omega-3 Index, an Emerging Cardiovascular Risk Marker. *Lipids*, 43(9), 805-811.
- Hasanuzzaman, M., Hossain, M. A., da Silva, J. A. T. & Fujita, M. (2012) Plant Response and Tolerance to Abiotic Oxidative Stress: Antioxidant Defense Is a Key Factor, in Venkateswarlu, B., Shanker, A. K., Shanker, C. & Maheswari, M. (eds), *Crop Stress and its Management: Perspectives and Strategies*. Dordrecht: Springer Netherlands, 261-315.
- Hassini, I., Baenas, N., Moreno, D. A., Carvajal, M., Boughanmi, N. & Martinez Ballesta, M. D. C. (2017) Effects of seed priming, salinity and methyl jasmonate treatment on bioactive composition of *Brassica oleracea* var. capitata (white and red varieties) sprouts. *J Sci Food Agric*, 97(8), 2291-2299.
- Hatzig, S. V., Frisch, M., Breuer, F., Nesi, N., Ducournau, S., Wagner, M.-H., Leckband, G., Abbadi, A. & Snowdon, R. J. (2015) Genome-wide association mapping unravels the genetic control of seed germination and vigor in *Brassica napus*. *Frontiers in Plant Science*, 6(221).

- He, D., Wang, Q., Wang, K. & Yang, P. (2015) Genome-Wide Dissection of the MicroRNA Expression Profile in Rice Embryo during Early Stages of Seed Germination. *PLOS ONE*, 10(12), e0145424.
- Heacock, M., Spangler, E., Riha, K., Puizina, J. & Shippen, D. E. (2004) Molecular analysis of telomere fusions in Arabidopsis: multiple pathways for chromosome end-joining. *The EMBO journal*, 23(11), 2304-2313.
- Hegarty, T. W. (1970) The possibility of increasing field establishment by seed hardening. *Hon. Res.*, 10, 59-64.
- Heydecker, W. & Coolbear, P. (1977) Seed treatments for improved performance survey and attempted prognosis. *Seed science and technology*.
- Heydecker, W., Higgins, J. & Gulliver, R. L. (1973) Accelerated Germination by Osmotic Seed Treatment. *Nature*, 246(5427), 42-44.
- Hiei, Y. & Komari, T. (2006) Improved protocols for transformation of indica rice mediated by *Agrobacterium tumefaciens*. *Plant cell, tissue, and organ culture*, 2006 v.85 no.3(no. 3), pp. 271-283.
- Hill, H., Bradford, K. J., Cunningham, J. & Taylor, A. G. (2008) Primed lettuce seeds exhibit increased activity to moisture during aging. International Society for Horticultural Science (ISHS), Leuven, Belgium.
- Hilton, I. B., D'Ippolito, A. M., Vockley, C. M., Thakore, P. I., Crawford, G. E., Reddy, T. E. & Gersbach, C. A. (2015) Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat Biotechnol*, 33(5), 510-7.
- Hinman, R. & Lang, J. (1965) Peroxidase-catalyzed oxidation of indole-3-acetic acid. *Biochemistry*, 4(1), 144-158.
- Howells, R. M., Craze, M., Bowden, S. & Wallington, E. J. (2018) Efficient generation of stable, heritable gene edits in wheat using CRISPR/Cas9. *BMC Plant Biol*, 18(1), 215.
- Hsu, J. L. & Sung, J. M. (1997) Antioxidant role of glutathione associated with accelerated aging and hydration of triploid watermelon seeds. *Physiologia Plantarum*, 100(4), 967-974.
- Hsu, P. D., Lander, E. S. & Zhang, F. (2014) Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, 157(6), 1262-78.
- Huang, X., Qian, Q., Liu, Z., Sun, H., He, S., Luo, D., Xia, G., Chu, C., Li, J. & Fu, X. (2009) Natural variation at the DEP1 locus enhances grain yield in rice. *Nat Genet*, 41(4), 494-7.
- Huang, Y., Wang, H. & Chen, K. (2002) Application of seed priming treatments in spinach (*Spinacia oleracea* L.) production. *J. Chinese Soc. Hort Sci*, 48, 117-123.

- Huo, H., Dahal, P., Kunusoth, K., McCallum, C. M. & Bradford, K. J. (2013) Expression of 9-cis-Epoxycarotenoid dioxygenase 4 is essential for thermo-inhibition of lettuce seed germination but not for seed development or stress tolerance. *Plant Cell*, 25(3), 884-900.
- Hussain M, Farooq M, Basra SMA & N., A. (2006) Influence of seed priming techniques on the seedling establishment, yield and quality of hybrid sunflower. International Journal of Agriculture & Biology.
- Hussain, S., Khan, F., Cao, W., Wu, L. & Geng, M. (2016a) Seed Priming Alters the Production and Detoxification of Reactive Oxygen Intermediates in Rice Seedlings Grown under Sub-optimal Temperature and Nutrient Supply. *Front Plant Sci*, 7, 439.
- Hussain, S., Khan, F., Hussain, H. A. & Nie, L. (2016b) Physiological and Biochemical Mechanisms of Seed Priming-Induced Chilling Tolerance in Rice Cultivars. *Front Plant Sci*, 7, 116.
- Hussain, S., Zheng, M., Khan, F., Khaliq, A., Fahad, S., Peng, S., Huang, J., Cui, K. & Nie, L. (2015) Benefits of rice seed priming are offset permanently by prolonged storage and the storage conditions. *Scientific reports*, 5, 8101.
- Iantcheva, A., Revalska, M., Zehirov, G. & Vassileva, V. (2014) *Agrobacterium*-mediated transformation of *Medicago truncatula* cell suspension culture provides a system for functional analysis. *In Vitro Cellular and Developmental Biology - Plant*, 50.
- Ibrahim, E. A. (2016) Seed priming to alleviate salinity stress in germinating seeds. *Journal of Plant Physiology*, 192, 38-46.
- Ilyas, S., Sutariati, G., Suwarno, F. & Sudarsono (2002) Matriconditioning improves the quality and protein level of medium vigor hot pepper seed. *Seed Technology*, 65-75.
- Imran, M., Mahmood, A., Römheld, V. & Neumann, G. (2013) Nutrient seed priming improves seedling development of maize exposed to low root zone temperatures during early growth. *European Journal of Agronomy*, 49, 141-148.
- Innocenti, A. M., Mazzuca, S., Bitonti, M. B., De Gara, L., Liso, R. & Arrigoni, O. (1994) Endogenous rhythm of ascorbic acid in seedling roots of broad bean. Plant Physiol Biochem.
- Iqbal, M. & Ashraf, M. (2013) Gibberellic acid mediated induction of salt tolerance in wheat plants: Growth, ionic partitioning, photosynthesis, yield and hormonal homeostasis. *Environmental and Experimental Botany*, 86, 76-85.

- Iqbal, M., Ashraf, M. & Jamil, A. (2006) Seed enhancement with cytokinins: changes in growth and grain yield in salt stressed wheat plants. *Plant growth regulation*, 50(1), 29-39.
- Ishibashi, Y., Tawaratsumida, T., Kondo, K., Kasa, S., Sakamoto, M., Aoki, N., Zheng, S.-H., Yuasa, T. & Iwaya-Inoue, M. (2012) Reactive Oxygen Species Are Involved in Gibberellin/Abscisic Acid Signaling in Barley Aleurone Cells. *Plant Physiology*, 158(4), 1705.
- Ishino, Y., Shinagawa, H., Makino, K., Amemura, M. & Nakata, A. (1987) Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *J Bacteriol*, 169(12), 5429-33.
- Islam, F., Yasmeen, T., Ali, S., Ali, B., Farooq, M. A. & Gill, R. A. (2015a) Priming-induced antioxidative responses in two wheat cultivars under saline stress. *Acta Physiologiae Plantarum*, 37(8), 153.
- ISTA *www.seedtest.org*
- Iyer, L. M., Babu, M. M. & Aravind, L. (2006) The HIRAN domain and recruitment of chromatin remodeling and repair activities to damaged DNA. *Cell Cycle*, 5(7), 775-82.
- Jaganathan, D., Ramasamy, K., Sellamuthu, G., Jayabalan, S. & Venkataraman, G. (2018) CRISPR for Crop Improvement: An Update Review. *Frontiers in Plant Science*, 9(985).
- Jansen, R., Embden, J. D. A. v., Gaastra, W. & Schouls, L. M. (2002) Identification of genes that are associated with DNA repeats in prokaryotes. *Molecular Microbiology*, 43(6), 1565-1575.
- Jeevan Kumar, S. P., Rajendra Prasad, S., Banerjee, R. & Thammineni, C. (2015) Seed birth to death: dual functions of reactive oxygen species in seed physiology. *Annals of Botany*, 116(4), 663-668.
- Jensen, B., Povlsen, F. V., Knudsen, I. & Jensen, S. D. F. (2001) Combining microbial seed treatment with priming of carrot seeds for control of seed borne *Alternaria* spp, Biocontrol agents: mode of action and interaction with other means of control: proceedings of the phytopathogens WG meeting, 2001.
- Jiang, W. Z., Henry, I. M., Lynagh, P. G., Comai, L., Cahoon, E. B. & Weeks, D. P. (2017) Significant enhancement of fatty acid composition in seeds of the allohexaploid, Camelina sativa, using CRISPR/Cas9 gene editing. *Plant Biotechnol J*, 15(5), 648-657.
- Jimenez, A., Hernandez, J. A., Pastori, G., del Rio, L. A. & Sevilla, F. (1998) Role of the ascorbate-glutathione cycle of mitochondria and peroxisomes in the senescence of pea leaves. *Plant physiology*, 118(4), 1327-1335.

- Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A. & Charpentier, E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, 337(6096), 816-21.
- Jisha, K. C. & Puthur, J. T. (2016) Seed priming with BABA (beta-amino butyric acid): a cost-effective method of abiotic stress tolerance in *Vigna radiata* (L.) Wilczek. *Protoplasma*, 253(2), 277-89.
- Job, C., Rajjou, L., Lovigny, Y., Belghazi, M. & Job, D. (2005) Patterns of protein oxidation in *Arabidopsis* seeds and during germination. *Plant Physiology*, 138(2), 790-802.
- Kamal-Eldin, A. & Appelqvist, L. A. (1996) The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids*, 31(7), 671-701.
- Kanno, Y., Jikumaru, Y., Hanada, A., Nambara, E., Abrams, S. R., Kamiya, Y. & Seo, M. (2010) Comprehensive hormone profiling in developing Arabidopsis seeds: examination of the site of ABA biosynthesis, ABA transport and hormone interactions. *Plant and Cell Physiology*, 51(12), 1988-2001.
- Karcher, S. (2002) Blue plants: Transgenic plants with the GUS reporter gene. *Tested studies for laboratory teaching*. O'Donnell eds, 23, 29-42.
- Kaya, M. D., Okçu, G., Atak, M., Cıkılı, Y. & Kolsarıcı, Ö. (2006) Seed treatments to overcome salt and drought stress during germination in sunflower (*Helianthus annuus* L.). *European journal of agronomy*, 24(4), 291-295.
- Khalil S, Moursy HA & SA, S. (1983) Wheat plant reactions to pre–sowing heat hardening of grains. II. Changes in photosynthetic pigments, nitrogen and carbohydrate metabolism. Bull Egyptian Soc Physiol Sci.
- Khan, A. A. (1992) Preplant physiological seed conditioning. *Horticultural reviews*, 13(1), 131-181.
- Khan, M. & Tripathi, R. (2010) Effects of seed weight and microsite characteristics on germination and seedling fitness in two species of Quercus in a subtropical wet hill forest.
- Khan, N. A. & Singh, S. (2008) Abiotic Stress and Plant Responses. IK international.
- Khan, N. A., Singh, S. & Nazar, R. (2007) Activities of Antioxidative Enzymes, Sulphur Assimilation, Photosynthetic Activity and Growth of Wheat (*Triticum aestivum*) Cultivars Differing in Yield Potential Under Cadmium Stress. *Journal of Agronomy and Crop Science*, 193(6), 435-444.
- Khodakovskaya, M. V., de Silva, K., Nedosekin, D. A., Dervishi, E., Biris, A. S., Shashkov, E. V., Galanzha, E. I. & Zharov, V. P. (2011) Complex genetic, photothermal, and photoacoustic analysis of nanoparticle-plant

interactions. *Proceedings of the National Academy of Sciences*, 108(3), 1028-1033.

- Kibinza, S., Vinel, D., Côme, D., Bailly, C. & Corbineau, F. (2006) Sunflower seed deterioration as related to moisture content during ageing, energy metabolism and active oxygen species scavenging. *Physiologia Plantarum*, 128(3), 496-506.
- Kim, J.-H., Baek, M.-H., Chung, B. Y., Wi, S. G. & Kim, J.-S. (2004) Alterations in the photosynthetic pigments and antioxidant machineries of red pepper (*Capsicum annuum* L.) seedlings from gamma-irradiated seeds. *Journal of Plant Biology*, 47(4), 314-321.
- Kimura, S. & Sakaguchi, K. (2006) DNA repair in plants. *Chemical reviews*, 106, 753-66.
- Kinney, A. J. (1996) Development of genetically engineered soybean oils for food applications. *Journal of Food Lipids*, 3(4), 273-292.
- Klee, H. J. (2004) Ethylene signal transduction. Moving beyond *Arabidopsis. Plant physiology*, 135(2), 660-667.
- Kobbe, D., Kahles, A., Walter, M., Klemm, T., Mannuss, A., Knoll, A., Focke, M. & Puchta, H. (2016) AtRAD5A is a DNA translocase harboring a HIRAN domain which confers binding to branched DNA structures and is required for DNA repair in vivo. *Plant J*, 88(4), 521-530.
- Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A. & Liu, D. R. (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature*, 533(7603), 420-4.
- Koornneef, M., Bentsink, L. & Hilhorst, H. (2002) Seed dormancy and germination. *Curr Opin Plant Biol*, 5(1), 33-6.
- Korte, A. & Farlow, A. (2013) The advantages and limitations of trait analysis with GWAS: a review. *Plant Methods*, 9(1), 29.
- Kotowski, F. (1926) Temperature relations to germination of vegetable seed. *Proceedings of American Society of Horticultural Science*, 23, 176-177.
- Kranner, I., Minibayeva, F. V., Beckett, R. P. & Seal, C. E. (2010) What is stress? Concepts, definitions and applications in seed science. *New Phytologist*, 188(3), 655-673.
- Krejci, L., Altmannova, V., Spirek, M. & Zhao, X. (2012) Homologous recombination and its regulation. *Nucleic Acids Res*, 40(13), 5795-818.
- Krinsky, N. I. (1992) Mechanism of action of biological antioxidants. *Proc Soc Exp Biol Med*, 200(2), 248-54.
- Kubala, S., Wojtyla, L., Quinet, M., Lechowska, K., Lutts, S. & Garnczarska, M. (2015) Enhanced expression of the proline synthesis gene P5CSA in relation to seed osmopriming improvement of *Brassica napus* germination under salinity stress. *J Plant Physiol*, 183, 1-12.

- Kucera, B., Cohn, M. A. & Leubner-Metzger, G. (2005) Plant hormone interactions during seed dormancy release and germination. *Seed Science Research*, 15(4), 281-307.
- Kukreja, S., Nandwal, A., Kumar, N., Sharma, S., Unvi, V. & Sharma, P. (2005) Plant water status, H 2 O 2 scavenging enzymes, ethylene evolution and membrane integrity of *Cicer arietinum* roots as affected by salinity. *Biologia Plantarum*, 49(2), 305-308.
- Kumar, D. & Kalita, P. (2017) Reducing postharvest losses during storage of grain crops to strengthen food security in developing countries. *Foods (Basel, Switzerland)*, 6(1), 8.
- Kumar, P. (2012) The economics of ecosystems and biodiversity: ecological and economic foundations. *Routledge*.
- Kumar, V., Sharma, A., Kaur, P., Sidhu, G. P. S., Bali, A. S., Bhardwaj, R., Thukral, A. K. & Cerda, A. (2018) Pollution assessment of heavy metals in soils of India and ecological risk assessment: A state-of-the-art. *Chemosphere*.
- Kumar, V., Shriram, V., Tukaram, b., Nikam, D., Polavarapu, b., Kishor, B., Jawali, N., Mahadeo, b. & Shitole, G. (2008) Assessment of Tissue Culture and Antibiotic Selection Parameters Useful for Transformation of an Important Indica Rice Genotype Karjat-3. *The Asian and Australasian Journal of Plant Science and Biotechnology*, 2, 84.
- Labouriau, L. (1970) On the physiology of seed germination in *Vicia graminea* Sm. 1. *Anais Acad. bras. Cienc.*, 42(2), 235-62.
- Labouriau, L. (1983) A germinação das sementes. Série de Biologia. *Monografia*, 24.
- Labouriau, L. & Viladares, M. (1976) On the germination of seeds of *Calotropis procera* (Ait.) Ait. f. *Anais*. 2013, 263-284.
- Lahiani, M. H., Dervishi, E., Chen, J., Nima, Z., Gaume, A., Biris, A. S. & Khodakovskaya, M. V. (2013) Impact of carbon nanotube exposure to seeds of valuable crops. *ACS Applied Materials & Interfaces*, 5(16), 7965-7973.
- Lariguet, P., Ranocha, P., De Meyer, M., Barbier, O., Penel, C. & Dunand, C. (2013) Identification of a hydrogen peroxide signalling pathway in the control of light-dependent germination in *Arabidopsis*. *Planta*, 238(2), 381-395.
- Lebedeva, N. A., Rechkunova, N. I. & Lavrik, O. I. (2011) AP-site cleavage activity of tyrosyl-DNA phosphodiesterase 1. *FEBS letters*, 585(4), 683-686.
- Lechowska, K., Kubala, S., Wojtyla, L., Nowaczyk, G., Quinet, M., Lutts, S. & Garnczarska, M. (2019) New insight on water status in germinating

Brassica napus seeds in relation to priming-improved germination. *Int J Mol Sci*, 20(3).

- Lee, K., Zhang, Y., Kleinstiver, B. P., Guo, J. A., Aryee, M. J., Miller, J., Malzahn, A., Zarecor, S., Lawrence-Dill, C. J., Joung, J. K., Qi, Y. & Wang, K. (2019) Activities and specificities of CRISPR/Cas9 and Cas12a nucleases for targeted mutagenesis in maize. *Plant Biotechnology Journal*, 17(2), 362-372.
- Lee, S. Y., Kim, H., Hwang, H. J., Jeong, Y. M., Na, S. H., Woo, J. C. & Kim, S. G. (2010) Identification of tyrosyl-DNA phosphodiesterase as a novel DNA damage repair enzyme in *Arabidopsis*. *Plant Physiol*, 154(3), 1460-9.
- Legro B & H., S. (1995) Biological control of *Pythium* through seed coating and seed priming with *Trichoderma*. in Bradford K, H. T. (ed), *Monterey Proceedings of the 4th National Sympo- sium on Stand Establishment of Horticultural Crops*. Monterey, California, 235–7.
- Leprince, O., Deltour, R., Thorpe, P. C., Atherton, N. M. & Hendry, G. A. F. (1990) The role of free radicals and radical processing systems in loss of desiccation tolerance in germinating maize (*Zea mays* L.). *New Phytologist*, 116(4), 573-580.
- Leymarie, J., Vitkauskaité, G., Hoang, H. H., Gendreau, E., Chazoule, V., Meimoun, P., Corbineau, F., El-Maarouf-Bouteau, H. & Bailly, C. (2011) Role of reactive oxygen species in the regulation of *Arabidopsis* seed dormancy. *Plant and Cell Physiology*, 53(1), 96-106.
- Li, J., Harper, L. C., Golubovskaya, I., Wang, C. R., Weber, D., Meeley, R. B., McElver, J., Bowen, B., Cande, W. Z. & Schnable, P. S. (2007) Functional analysis of maize RAD51 in meiosis and double-strand break repair. *Genetics*, 176(3), 1469-1482.
- Li, M., Li, X., Zhou, Z., Wu, P., Fang, M., Pan, X., Lin, Q., Luo, W., Wu, G. & Li, H. (2016) Reassessment of the four yield-related genes Gn1a, DEP1, GS3, and IPA1 in rice using a CRISPR/Cas9 system. *Front Plant Sci*, 7, 377.
- Li, S., Paulsson, M. & Bjorn, L. O. (2002) Temperature-dependent formation and photorepair of DNA damage induced by UV-B radiation in suspension-cultured tobacco cells. *J Photochem Photobiol B*, 66(1), 67-72.
- Li, X., Wang, Y., Chen, S., Tian, H., Fu, D., Zhu, B., Luo, Y. & Zhu, H. (2018) Lycopene is enriched in tomato fruit by CRISPR/Cas9-mediated multiplex genome editing. *Front Plant Sci*, 9, 559.
- Li, Y., Qin, T., Ingle, T., Yan, J., He, W., Yin, J.-J. & Chen, T. (2017a) Differential genotoxicity mechanisms of silver nanoparticles and silver ions. *Archives of toxicology*, 91(1), 509-519.
- Li, Z., Xu, J., Gao, Y., Wang, C., Guo, G., Luo, Y., Huang, Y., Hu, W., Sheteiwy, M. S. & Guan, Y. (2017b) The synergistic priming effect of

exogenous salicylic acid and H2O2 on chilling tolerance enhancement during maize (*Zea mays* L.) seed germination. *Frontiers in plant science*, 8, 1153.

- Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q., Liu, J., Zhang, H., Liu, C., Ran, Y. & Gao, C. (2017) Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nat Commun*, 8, 14261.
- Liang, Z., Zhang, K., Chen, K. & Gao, C. (2014) Targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system. *J Genet Genomics*, 41(2), 63-8.
- Lin, C.-S., Hsu, C.-T., Yang, L.-H., Lee, L.-Y., Fu, J.-Y., Cheng, Q.-W., Wu, F.-H., Hsiao, H. C.-W., Zhang, Y., Zhang, R., Chang, W.-J., Yu, C.-T., Wang, W., Liao, L.-J., Gelvin, S. B. & Shih, M.-C. (2018) Application of protoplast technology to CRISPR/Cas9 mutagenesis: from single-cell mutation detection to mutant plant regeneration. *Plant Biotechnology Journal*, 16(7), 1295-1310.
- Lindahl, T. (1993) Instability and decay of the primary structure of DNA. *nature*, 362(6422), 709-715.
- Linkies, A., Graeber, K., Knight, C. & Leubner-Metzger, G. (2010) The evolution of seeds. *New Phytol*, 186(4), 817-31.
- Linkies, A. & Leubner-Metzger, G. (2012) Beyond gibberellins and abscisic acid: how ethylene and jasmonates control seed germination. *Plant cell reports*, 31(2), 253-270.
- Linkies, A., Muller, K., Morris, K., Tureckova, V., Wenk, M., Cadman, C. S., Corbineau, F., Strnad, M., Lynn, J. R., Finch-Savage, W. E. & Leubner-Metzger, G. (2009) Ethylene interacts with abscisic acid to regulate endosperm rupture during germination: a comparative approach using *Lepidium sativum* and *Arabidopsis thaliana*. *Plant Cell*, 21(12), 3803-22.
- Liu, C. & Moschou, P. N. (2018) Phenotypic novelty by CRISPR in plants. *Dev Biol*, 435(2), 170-175.
- Liu, L., Lee, J. & Zhou, P. (2010) Navigating the nucleotide excision repair threshold. *Journal of cellular physiology*, 224(3), 585-589.
- Liu, R., Zhang, H. & Lal, R. (2016a) Effects of stabilized nanoparticles of copper, zinc, manganese, and iron oxides in low concentrations on lettuce (*Lactuca sativa*) seed germination: nanotoxicants or nanonutrients? *Water, Air, & Soil Pollution*, 227(1), 42.
- Liu, X., Homma, A., Sayadi, J., Yang, S., Ohashi, J. & Takumi, T. (2016b) Sequence features associated with the cleavage efficiency of CRISPR/Cas9 system. *Scientific Reports*, 6, 19675.
- Liu, X., Wu, S., Xu, J., Sui, C. & Wei, J. (2017) Application of CRISPR/Cas9 in plant biology. *Acta pharmaceutica Sinica. B*, 7(3), 292-302.

- Liu, Y., Kermode, A. & El-Kassaby, Y. (2013) The role of moist-chilling and thermo-priming on the germination characteristics of white spruce (*Picea glauca*) seed. *Seed Science and Technology*, 41(3), 321-335.
- Long, R. L., Gorecki, M. J., Renton, M., Scott, J. K., Colville, L., Goggin, D. E., Commander, L. E., Westcott, D. A., Cherry, H. & Finch-Savage, W. E. (2015) The ecophysiology of seed persistence: a mechanistic view of the journey to germination or demise. *Biol Rev Camb Philos Soc*, 90(1), 31-59.
- Long, T. A., Brady, S. M. & Benfey, P. N. (2008) Systems approaches to identifying gene regulatory networks in plants. *Annual Review of Cell and Developmental Biology*, 24(1), 81-103.
- Lopez-Huertas, E. (2010) Health effects of oleic acid and long chain omega-3 fatty acids (EPA and DHA) enriched milks. A review of intervention studies. *Pharmacol Res*, 61(3), 200-7.
- Lu, T., Pan, Y., Kao, S.-Y., Li, C., Kohane, I., Chan, J. & Yankner, B. A. (2004) Gene regulation and DNA damage in the ageing human brain. *Nature*, 429(6994), 883.
- Lu, X.-j., Zhang, X.-l., Mei, M., Liu, G.-l. & Ma, B.-b. (2016) Proteomic analysis of *Magnolia sieboldii* K. Koch seed germination. *Journal of Proteomics*, 133, 76-85.
- Lutts, S., Benincasa, P., Wojtyla, Ł., Kubala, S., Pace, R., Lechowska, K., Quinet, M. & Garnczarska, M. (2016) Seed Priming: new comprehensive approaches for an old empirical technique. Balestrazzi and Araùjo eds. New challenges in seed biology- Basic and translational research driving seed technology,1-46.
- Ma, L., Zhu, F., Li, Z., Zhang, J., Li, X., Dong, J. & Wang, T. (2015) TALEN-Based Mutagenesis of Lipoxygenase LOX3 Enhances the Storage Tolerance of Rice (*Oryza sativa*) Seeds. *PLoS One*, 10(12), e0143877.
- Ma, Y., Zhang, J., Yin, W., Zhang, Z., Song, Y. & Chang, X. (2016) Targeted AID-mediated mutagenesis (TAM) enables efficient genomic diversification in mammalian cells. *Nat Methods*, 13(12), 1029-1035.
- Macovei, A., Balestrazzi, A., Confalonieri, M. & Carbonera, D. (2010) The tyrosyl-DNA phosphodiesterase gene family in Medicago truncatula Gaertn.: bioinformatic investigation and expression profiles in response to copper-and PEG-mediated stress. *Planta*, 232(2), 393-407.
- Macovei, A., Balestrazzi, A., Confalonieri, M., Faé, M. & Carbonera, D. (2011) New insights on the barrel medic MtOGG1 and MtFPG functions in relation to oxidative stress response in planta and during seed imbibition. *Plant Physiol Biochem*, 49(9), 1040-50.
- Macovei, A., Caser, M., Donà, M., Valassi, A., Giovannini, A., Carbonera, D., Scariot, V. & Balestrazzi, A. (2016) Prolonged Cold Storage

Affects Pollen Viability and Germination along with Hydrogen Peroxide and Nitric Oxide Content in *Rosa hybrida*. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 44(1).

- Macovei, A., Garg, B., Raikwar, S., Balestrazzi, A., Carbonera, D., Buttafava, A., Bremont, J. F., Gill, S. S. & Tuteja, N. (2014) Synergistic exposure of rice seeds to different doses of γ-ray and salinity stress resulted in increased antioxidant enzyme activities and gene-specific modulation of TC-NER pathway. *Biomed Res Int*, 2014, 676934.
- Macovei, A., Pagano, A., Leonetti, P., Carbonera, D., Balestrazzi, A. & Araújo, S. S. (2017) Systems biology and genome-wide approaches to unveil the molecular players involved in the pre-germinative metabolism: implications on seed technology traits. *Plant Cell Rep*, 36(5), 669-688.
- Macovei, A., Pagano, A., Sabatini, M. E., Grandi, S. & Balestrazzi, A. (2018) The human Tyrosyl-DNA phosphodiesterase 1 (hTdp1) inhibitor NSC120686 as an exploratory tool to investigate plant Tdp1 genes. *Genes*, 9(4), 186.
- Madsen, M. D., Svejcar, L., Radke, J. & Hulet, A. (2018) Inducing rapid seed germination of native cool season grasses with solid matrix priming and seed extrusion technology. *PloS one*, 13(10), e0204380.
- Mahajan, S. & Tuteja, N. (2005) Cold, salinity and drought stresses: an overview. *Arch Biochem Biophys*, 444(2), 139-58.
- Mahakham, W., Sarmah, A. K., Maensiri, S. & Theerakulpisut, P. (2017) Nanopriming technology for enhancing germination and starch metabolism of aged rice seeds using phytosynthesized silver nanoparticles. *Scientific reports*, 7(1), 8263-8263.
- Mahakham, W., Theerakulpisut, P., Maensiri, S., Phumying, S. & Sarmah, A. K. (2016) Environmentally benign synthesis of phytochemicals-capped gold nanoparticles as nanopriming agent for promoting maize seed germination. *Science of the Total Environment*, 573, 1089-1102.
- Mahesh, H. M., Murali, M., Anup Chandra Pal, M., Melvin, P. & Sharada, M. S. (2017) Salicylic acid seed priming instigates defense mechanism by inducing PR-Proteins in *Solanum melongena* L. upon infection with *Verticillium dahliae* Kleb. *Plant Physiol Biochem*, 117, 12-23.
- Mahmood, A., Turgay, O., Farooq, M. & Hayat, R. (2016) Seed biopriming with plant growth promoting rhizobacteria: A review. *FEMS Microbiology Ecology*, 92.
- Man, J., Yang, Y., Zhang, C., Zhang, F., Wang, Y., Gu, M., Liu, Q. & Wei, C. (2013) Morphology and structural characterization of high-amylose rice starch residues hydrolyzed by porcine pancreatic α-amylase. *Food hydrocolloids*, 31(2), 195-203.

- Mani, P. K. & Mondal, S. (2016) Agri-nanotechniques for plant availability of nutrients, *Plant Nanotechnology*Springer, 263-303.
- Manova, V. & Gruszka, D. (2015) DNA damage and repair in plants–from models to crops. *Frontiers in plant science*, 6, 885.
- Mansouri-Far, C., Goodarzian-Ghahfarokhi, M., Saeidi, M. & Abdoli, M. (2015) Antioxidant enzyme activity and germination characteristics of different maize hybrid seeds during ageing. *Environmental and Experimental Biology*, 13, 177-182.
- Mao, C., Zhu, Y., Cheng, H., Yan, H., Zhao, L., Tang, J., Ma, X. & Mao, P. (2018) Nitric oxide regulates seedling growth and mitochondrial responses in aged oat seeds. *Int J Mol Sci*, 19(4).
- Marchiol, L. (2018) Nanotechnology in Agriculture: New Opportunities and Perspectives, 121-141.
- Marcus, D. L., Thomas, C., Rodriguez, C., Simberkoff, K., Tsai, J. S., Strafaci, J. A. & Freedman, M. L. (1998) Increased peroxidation and reduced antioxidant enzyme activity in Alzheimer's disease. *Exp Neurol*, 150(1), 40-4.
- Masubelele, N. H., Dewitte, W., Menges, M., Maughan, S., Collins, C., Huntley, R., Nieuwland, J., Scofield, S. & Murray, J. A. (2005) D-type cyclins activate division in the root apex to promote seed germination in Arabidopsis. *Proceedings of the National Academy of Sciences*, 102(43), 15694-15699.
- Matilla, A. & Matilla, M. (2008) Involvement of ethylene in seed physiology. *Plant Science*, 175, 87-97.
- Matilla, A. J. (2000) Ethylene in seed formation and germination. *Seed Science Research*, 10(2), 111-126.
- Maurel, C., Boursiac, Y., Luu, D. T., Santoni, V., Shahzad, Z. & Verdoucq, L. (2015) Aquaporins in Plants. *Physiol Rev*, 95(4), 1321-58.
- May LHE, Milthorpe EJ & FL, M. (1962) Pre-sowing hardening of plants to drought. An appraisal of the contributions by P. A. Gekel. Field Crop Abst
- Mazumdar, A., Adak, S., Chatterjee, R. & Banerjee, R. K. (1997) Mechanism-based inactivation of lacrimal-gland peroxidase by phenylhydrazine: a suicidal substrate to probe the active site. *Biochem J*, 324 (Pt 3), 713-9.
- MB., M. (2000) Seed priming, in Black M, B. J. (ed), *Seed Technology and its Biological Basis.*Sheffield, Sheffield Academic Press, 287-325.
- McDonald, M. (1999) Seed deterioration: physiology, repair and assessment. *Seed Sci. Technol.*, 27, 177-237.

- Meng, Y., Hou, Y., Wang, H., Ji, R., Liu, B., Wen, J., Niu, L. & Lin, H. (2017) Targeted mutagenesis by CRISPR/Cas9 system in the model legume *Medicago truncatula*. *Plant Cell Rep*, 36(2), 371-374.
- Metsalu, T. & Vilo, J. (2015) ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res*, 43(W1), W566-70.
- Michel, B. E. & Kaufmann, M. R. (1973) The osmotic potential of polyethylene glycol 6000. *Plant Physiology*, 51(5), 914.
- Miersch, O., Neumerkel, J., Dippe, M., Stenzel, I. & Wasternack, C. (2008) Hydroxylated jasmonates are commonly occurring metabolites of jasmonic acid and contribute to a partial switch-off in jasmonate signaling. *New Phytol*, 177(1), 114-27.
- Migahid, M. M., Elghobashy, R. M., Bidak, L. M. & Amin, A. W. (2019) Priming of Silybum marianum (L.) Gaertn seeds with H2O2 and magnetic field ameliorates seawater stress. *Heliyon*, 5(6), e01886.
- Milone, M. T., Sgherri, C., Clijsters, H. & Navari-Izzo F. (2003) Antioxidative responses of wheat treated with realistic concentrations of cadmium. Env. Exp. Bot, 50, 265-276.
- Miransari, M. & Smith, D. L. (2014) Plant hormones and seed germination. *Environmental and Experimental Botany*, 99, 110-121.
- Misson, M., Zhang, H. & Jin, B. (2015) Nanobiocatalyst advancements and bioprocessing applications. *Journal of the Royal Society Interface*, 12(102), 20140891.
- Mittler, R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci*, 7(9), 405-10.
- Mittler, R. & Zilinskas, B. A. (1992) Molecular cloning and characterization of a gene encoding pea cytosolic ascorbate peroxidase. *J Biol Chem*, 267(30), 21802-7.
- Miura, K., Ikeda, M., Matsubara, A., Song, X. J., Ito, M., Asano, K., Matsuoka, M., Kitano, H. & Ashikari, M. (2010) *OsSPL14* promotes panicle branching and higher grain productivity in rice. *Nat Genet*, 42(6), 545-9.
- Moeinzadeh, A., Sharif-Zadeh, F., Ahmadzadeh, M. & Tajabadi, F. (2010) Biopriming of sunflower (*'Helianthus annuus'* L.) seed with *'Pseudomonas fluorescens'* for improvement of seed invigoration and seedling growth. *Australian Journal of Crop Science*, 4(7), 564.
- Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J. & Soria, E. (2005) Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol*, 60(2), 174-82.

- Mondoni, A., Probert, R.J., Rossi, G., Vegini, E., Hay, F.R. (2011) Seeds of alpine plants are short-lived: implications for long-term conservation. *Ann Bot. 107*, 171–179.
- Montecucco, A. & Biamonti, G. (2013) Pre-mRNA processing factors meet the DNA damage response. *Front Genet*, 4, 102.
- Moreno-Risueno, M. A., Busch, W. & Benfey, P. N. (2010) Omics meet networks—using systems approaches to infer regulatory networks in plants. *Current Opinion in Plant Biology*, 13(2), 126-131.
- Moron, M. S., Depierre, J. W. & Mannervik, B. (1979) Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochim Biophys Acta*, 582(1), 67-78.
- Mullineaux, P. M. & Rausch, T. (2005) Glutathione, photosynthesis and the redox regulation of stress-responsive gene expression. *Photosynth Res*, 86(3), 459-74.
- Murashige, T. & Skoog, F. (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum*, 15(3), 473-497.
- Murovec, J., Gucek, K., Bohanec, B., Avbelj, M. & Jerala, R. (2018) DNA-free genome editing of *Brassica oleracea* and *B. rapa* protoplasts using CRISPR-Cas9 ribonucleoprotein complexes. *Front Plant Sci*, 9, 1594.
- Mustafa, H. S. (2017) Role of seed priming to enhance growth and development of crop plants against biotic and abiotic stresses. *Role of seed priming to enhance growth and development of crop plants against biotic and abiotic stresses*, 2, 1-11.
- Müller, H. & Berg, G. (2008) Impact of formulation procedures on the effect of the biocontrol agent *Serratia plymuthica* HRO-C48 on *Verticillium* wilt in oilseed rape. *BioControl*, 53(6), 905-916.
- Müller, K., Linkies, A., Vreeburg, R. A., Fry, S. C., Krieger-Liszkay, A. & Leubner-Metzger, G. (2009) In vivo cell wall loosening by hydroxyl radicals during cress seed germination and elongation growth. *Plant Physiology*, 150(4), 1855-1865.
- Müller, K., Tintelnot, S. & Leubner-Metzger, G. (2006) Endosperm-limited Brassicaceae seed germination: abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana. Plant and Cell Physiology*, 47(7), 864-877.
- Murungu, F. S., Chiduza, C., Nyamugafata, P. & Whalley, W. (2004) Effect of on-farm seed priming on emergence, growth and yield of cotton and maize in a semi-arid area of Zimbabwe. *Experimental Agriculture*, 40, 23-36.
- Mustafa, M. & Lee, S. (1977) Biological effects of environmental pollutants: Methods for assessing biochemical changes. *Assessing Toxic*

Effects of Environmental Pollutants. Ann Arbor: Ann Arbor Science Publishers.

- Nambara, E., Okamoto, M., Tatematsu, K., Yano, R., Seo, M. & Kamiya, Y. (2010) Abscisic acid and the control of seed dormancy and germination. *Seed Science Research*, 20(2), 55-67.
- Narayana Murthy, U. & Sun, W. Q. (2000) Protein modification by Amadori and Maillard reactions during seed storage: roles of sugar hydrolysis and lipid peroxidation. *Journal of experimental botany*, 51(348), 1221-1228.
- Nascimento, W. & Baptista de Lima, L. (2008) Condicionamento osmótico de sementes de berinjela visando a germinação sob temperaturas baixas. *Revista Brasileira De Sementes*, 30.
- Nekrasov, V., Staskawicz, B., Weigel, D., Jones, J. D. & Kamoun, S. (2013) Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease, *Nat Biotechnol*. United States, 691-3.
- Nekrasov, V., Wang, C., Win, J., Lanz, C., Weigel, D. & Kamoun, S. (2017) Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. *Scientific Reports*, 7(1), 482.
- Nguyen, T.-P., Keizer, P., van Eeuwijk, F., Smeekens, S. & Bentsink, L. (2012) Natural variation for seed longevity and seed dormancy are negatively correlated in *Arabidopsis*. *Plant Physiology*, 160(4), 2083-2092.
- Nishimura, A., Aichi, I. & Matsuoka, M. (2006) A protocol for *Agrobacterium*-mediated transformation in rice. *Nat Protoc*, 1(6), 2796-802.
- Noctor, G. & Foyer C. H. (1998) A re-evaluation of the ATP: NADPH budget during C3 photosynthesis. A contribution from nitrate assimilation and its associated respiratory activity? J. *Exp. Bot,* 329, 1895-1908.
- Noctor, G., Gomez, L., Vanacker, H. & Foyer, C. H. (2002) Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. *J Exp Bot*, 53(372), 1283-304.
- Nonogaki H, Chen F & Bradford K. J. (2007) Mechanisms and genes involved in germination sensu stricto. Annual plant reviews: seed development, dormancy and germination: Blackwell Publishing Ltd.
- Norastehnia, A., Sajedi, R. & Nojavan-Asghari, M. (2007) Inhibitory effects of methyl jasmonate on seed germination in maize (*Zea Mays*): effect on α-amylase activity and ethylene production. *Genetic and Applied Plant Physiology*, 13-23.
- Nouman, W. (2014) Seed priming improves the emergence potential, growth and antioxidant system of *Moringa oleifera* under saline conditions. *Plant growth regulation*, v. 73(no. 3), pp. 267-278-2014 v.73 no.3.

- Nunes, U. R., Santos, M. d., Alvarenga, E. M. & Dias, D. (2000) Efeito do condicionamento osmótico e do tratamento com fungicida na qualidade fisiológica e sanitária de sementes de cebola (*Allium cepa* L.). *Revista Brasileira de Sementes*, 22(1), 239-246.
- Ogawa, M., Hanada, A., Yamauchi, Y., Kuwahara, A., Kamiya, Y. & Yamaguchi, S. (2003) Gibberellin biosynthesis and response during *Arabidopsis* seed germination. *Plant Cell*, 15(7), 1591-604.
- Ogbonnaya, F. C., Imtiaz, M., Ye, G., Hearnden, P. R., Hernandez, E., Eastwood, R. F., van Ginkel, M., Shorter, S. C. & Winchester, J. M. (2008) Genetic and QTL analyses of seed dormancy and preharvest sprouting resistance in the wheat germplasm CN10955. *Theoretical and Applied Genetics*, 116(7), 891-902.
- Oh, E., Kang, H., Yamaguchi, S., Park, J., Lee, D., Kamiya, Y. & Choi, G. (2009) Genome-wide analysis of genes targeted by Phytochrome interacting factor3-LIKE5 during seed germination in Arabidopsis. *Plant Cell*, 21(2), 403-19.
- Okuley, J., Lightner, J., Feldmann, K., Yadav, N., Lark, E. & Browse, J. (1994) Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. *Plant Cell*, 6(1), 147-58.
- Oliver, C. N., Starke-Reed, P., R Stadtman, E., J Liu, G., M Carney, J. & Floyd, R. (1990) Oliver C.N., Starke-Reed PE, Stadtman E.R., Liu G.J., Carney J.M. & Floyd R.A. Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion-induced injury to gerbil brain. Proc Natl Acad Sci USA 87: 5144−5147. *Proceedings of the National Academy of Sciences of the United States of America*, 87, 5144-7.
- Olsen, K. M., Hehn, A., Jugdé, H., Slimestad, R., Larbat, R., Bourgaud, F. & Lillo, C. (2010) Identification and characterization of CYP75A31, a new flavonoid 3'5'-hydroxylase, isolated from *Solanum lycopersicum*. *BMC plant biology*, 10, 21-21.
- Omidi, H., Khazaei, F., Hamzi, A. S. & Heidari, S. H. (2009) Improvement of seed germination traits in canola (*Brassica napus* L.) as affected by saline and drought stresses. *Plant ecophysiol*. 3.
- Oracz, K., El-Maarouf-Bouteau, H., Kranner, I., Bogatek, R., Corbineau, F. & Bailly, C. (2009) The mechanisms involved in seed dormancy alleviation by hydrogen cyanide unravel the role of reactive oxygen species as key factors of cellular signaling during germination. *Plant Physiology*, 150(1), 494-505.
- Ouhibi, C., Attia, H., Rebah, F., Msilini, N., Chebbi, M., Aarrouf, J., Urban, L. & Lachaal, M. (2014) Salt stress mitigation by seed priming with

UV-C in lettuce plants: growth, antioxidant activity and phenolic compounds. *Plant Physiol Biochem*, 83, 126-33.

- Pagano, A. (2019) *Molecular and metabolic hallmarks of DNA damage response in the context of the seed pre-germinative metabolism and seedling establishment*. PhD thesis University of Study of Pavia.
- Pagano, A., Araújo, S. S., Macovei, A., Leonetti, P. & Balestrazzi, A. (2017) The Seed Repair Response during Germination: Disclosing Correlations between DNA Repair, Antioxidant Response, and Chromatin Remodeling in. *Front Plant Sci*, 8, 1972.
- Pagano, A., de Sousa Araujo, S., Macovei, A., Dondi, D., Lazzaroni, S. & Balestrazzi, A. (2019a) Metabolic and gene expression hallmarks of seed germination uncovered by sodium butyrate in Medicago truncatula. *Plant Cell Environ*, 42(1), 259-269.
- Pagano, A., Forti, C., Gualtieri, C., Balestrazzi, A. & Macovei, A. (2019) Oxidative Stress and Antioxidant Defense in Germinating Seeds: A Q&A Session, 267-289.
- Pallanca, J. E. & Smirnoff, N. (1999) Ascorbic acid metabolism in pea seedlings. A comparison of D-glucosone, L-sorbosone, and L-galactono-1,4-lactone as ascorbate precursors. *Plant physiology*, 120(2), 453-462.
- Pan, C., Ye, L., Qin, L., Liu, X., He, Y., Wang, J., Chen, L. & Lu, G. (2016) CRISPR/Cas9-mediated efficient and heritable targeted mutagenesis in tomato plants in the first and later generations. *Sci Rep*, 6, 24765.
- Pandita, V., Anand, A., Nagarajan, S., Seth, R. & Sinha, S. (2010) Solid matrix priming improves seed emergence and crop performance in okra. *Seed Science and Technology*, 38(3), 665-674.
- Pannicke, U., Ma, Y., Hopfner, K. P., Niewolik, D., Lieber, M. R. & Schwarz, K. (2004) Functional and biochemical dissection of the structure-specific nuclease ARTEMIS. *The EMBO journal*, 23(9), 1987-1997.
- Paparella, S., Araujo, S. S., Rossi, G., Wijayasinghe, M., Carbonera, D. & Balestrazzi, A. (2015) Seed priming: state of the art and new perspectives. *Plant Cell Rep*, 34(8), 1281-93.
- Parera CA & DJ, Cantcliffe. (1994) Pre-sowing seed priming. Hort Rev
- Parihar, P., Singh, S., Singh, R., Singh, V. P. & Prasad, S. M. (2015) Effect of salinity stress on plants and its tolerance strategies: a review. *Environ Sci Pollut Res Int*, 22(6), 4056-75.
- Parreira, J., Balestrazzi, A., Fevereiro, P. & Araújo, S. (2018) Maintaining genome integrity during seed development in *Phaseolus vulgaris* L.: evidence from a transcriptomic profiling study. *Genes*, 9(10), 463.
- Parreira, J. R., Bouraada, J., Fitzpatrick, M. A., Silvestre, S., Bernardes da Silva, A., Marques da Silva, J., Almeida, A. M., Fevereiro, P., Altelaar, A.

F. M. & Araújo, S. S. (2016) Differential proteomics reveals the hallmarks of seed development in common bean (*Phaseolus vulgaris* L.). *Journal of Proteomics*, 143, 188-198.

- Patade, V. Y., Khatri, D., Manoj, K., Kumari, M. & Ahmed, Z. (2012) Cold tolerance in thiourea primed capsicum seedlings is associated with transcript regulation of stress responsive genes. *Molecular biology reports*, 39(12), 10603-10613.
- Paul, M. J., Nuccio, M. L. & Basu, S. S. (2018) Are GM crops for yield and resilience possible? *Trends Plant Sci*, 23(1), 10-16.
- Pawłowski, T. A. & Staszak, A. M. (2016) Analysis of the embryo proteome of sycamore (*Acer pseudoplatanus* L.) seeds reveals a distinct class of proteins regulating dormancy release. *Journal of Plant Physiology*, 195, 9-22.
- Penfield, S. (2017) Seed dormancy and germination. *Curr Biol*, 27(17), R874-r878.
- Pereira, M. D., Dias, D. C. F. d. S., Dias, L. A. d. S. & Araújo, E. F. (2009) Primed carrot seeds performance under water and temperature stress. *Scientia Agricola*, 66, 174-179.
- Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*, 29(9), e45.
- Pill, W. G. (1995) Low water potential and presowing germination treatments to improve seed quality. *Seed quality*, 319-359.
- Pommier, Y., Huang, S. Y., Gao, R., Das, B. B., Murai, J. & Marchand, C. (2014) Tyrosyl-DNA-phosphodiesterases (TDP1 and TDP2). *DNA Repair (Amst)*, 19, 114-29.
- Posmyk, M. & Janas, K. (2007) Effects of seed hydropriming in presence of exogenous proline on chilling injury limitation in *Vigna radiata* L. seedlings. *Acta Physiologiae Plantarum*, 29(6), 509-517.
- Potrykus, I. (2001) Golden rice and beyond. *Plant Physiol*, 125(3), 1157-61.
- Powell, R. G. (2009) Plant seeds as sources of potential industrial chemicals, pharmaceuticals, and pest control agents. *Journal of Natural Products*, 72(3), 516-523.
- Prasad, V., Chaudhary, S. & Singh, A. (2019) Improving Phosphorus Fertility in Soil through Microbial Mediators. *International journal of plant and environment*, 4(2).
- Preston, J., Tatematsu, K., Kanno, Y., Hobo, T., Kimura, M., Jikumaru, Y., Yano, R., Kamiya, Y. & Nambara, E. (2009) Temporal expression patterns of hormone metabolism genes during imbibition of *Arabidopsis*

thaliana seeds: a comparative study on dormant and non-dormant accessions. *Plant Cell Physiol*, 50(10), 1786-800.

- Płażek, A., Dubert, F., Kopeć, P., Dziurka, M., Kalandyk, A., Pastuszak, J., Waligórski, P. & Wolko, B. (2018) Long-term effects of cold on growth, development and yield of narrow-leaf lupine may be alleviated by seed hydropriming or butenolide. *International journal of molecular sciences*, 19(8), 2416.
- Qian, H., Peng, X., Han, X., Ren, J., Sun, L. & Fu, Z. (2013) Comparison of the toxicity of silver nanoparticles and silver ions on the growth of terrestrial plant model *Arabidopsis thaliana*. *Journal of Environmental Sciences*, 25(9), 1947-1956.
- Qin, F., Kakimoto, M., Sakuma, Y., Maruyama, K., Osakabe, Y., Tran, L. S., Shinozaki, K. & Yamaguchi-Shinozaki, K. (2007) Regulation and functional analysis of *ZmDREB2A* in response to drought and heat stresses in *Zea mays* L. *Plant J*, 50(1), 54-69.
- Rahman, M. M., Ahammad, K. U. & Alam, M. M. (2011) Effect of soaking condition and temperature on imbibition rate of maize and chickpea seeds. *Research Journal of Seed Science*, 4(2), 117-124.
- Raj, S., Shetty, N. & Shetty, H. (2004) Seed bio-priming with Pseudomonas fluorescens isolates enhances growth of pearl millet plants and induces resistance against downy mildew. *International Journal of Pest Management - Int J Pest Manage*, 50, 41-48.
- Rajjou, L. & Debeaujon, I. (2008) Seed longevity: survival and maintenance of high germination ability of dry seeds. *C R Biol*, 331(10), 796-805.
- Rajjou, L., Duval, M., Gallardo, K., Catusse, J., Bally, J., Job, C. & Job, D. (2012) Seed germination and vigor. *Annu Rev Plant Biol*, 63, 507-33.
- Ranal, M. A. & Santana, D. G. d. (2006) How and why to measure the germination process? *Brazilian Journal of Botany*, 29, 1-11.
- Ranil, R. H. G., Niran, H. M. L., Plazas, M., Fonseka, R. M., Fonseka, H. H., Vilanova, S., Andújar, I., Gramazio, P., Fita, A. & Prohens, J. (2015) Improving seed germination of the eggplant rootstock *Solanum torvum* by testing multiple factors using an orthogonal array design. *Scientia Horticulturae*, 193, 174-181.
- Rasmusson, A. G., Soole, K. L. & Elthon, T. E. (2004) Alternative NAD(P)H dehydrogenases of plant mitochondria. *Annu Rev Plant Biol*, 55, 23-39.
- Ray, D. K., Mueller, N. D., West, P. C. & Foley, J. A. (2013) Yield trends are insufficient to double global crop production by 2050. *PLOS ONE*, 8(6), e66428.

- Reddy, P.P. (2013) Bio-priming of seeds, in PP, R. (ed), *Recent Advances in Crop Protection.* Springer, 83-90.
- Reddy, A. R. & Raghavendra, A. S. (2006) Photooxidative stress. Springer. *Physiology and molecular biology of stress tolerance in plants*. 157-186.
- Ricard, J. & Job, D. (1974) Reaction mechanisms of indole-3-acetate degradation by peroxidases: a stopped-flow and low-temperature spectroscopic study. *European Journal of Biochemistry*, 44(2), 359-374.
- Richards, S. L., Wilkins, K. A., Swarbreck, S. M., Anderson, A. A., Habib, N., Smith, A. G., McAinsh, M. & Davies, J. M. (2015) The hydroxyl radical in plants: from seed to seed. *J Exp Bot*, 66(1), 37-46.
- Riha, K., Watson, J. M., Parkey, J. & Shippen, D. E. (2002) Telomere length deregulation and enhanced sensitivity to genotoxic stress in *Arabidopsis* mutants deficient in Ku70. *The EMBO Journal*, 21(11), 2819-2826.
- Roberts, E. H. (1973) Predicting the storage life of seeds. *Seed Science and technology*, 1, 499-514.
- Rodriguez-Gacio Mdel, C., Matilla-Vazquez, M. A. & Matilla, A. J. (2009) Seed dormancy and ABA signaling: the breakthrough goes on. *Plant Signal Behav*, 4(11), 1035 - 49.
- Rohn, H., Junker, A., Hartmann, A., Grafahrend-Belau, E., Treutler, H., Klapperstück, M., Czauderna, T., Klukas, C. & Schreiber, F. (2012) VANTED v2: a framework for systems biology applications. *BMC Systems Biology*, 6(1), 139.
- Roldán-Arjona, T. & Ariza, R. R. (2009) Repair and tolerance of oxidative DNA damage in plants. *Mutation Research/Reviews in Mutation Research*, 681(2-3), 169-179.
- Rouhi, H., Aboutalebian, M., Moosavi, S., Karimi, F., Karimi, F., Saman, M. & Samadi, M. (2012) Change in several antioxidant enzymes activity of Berseem clover (*Trifolium alexandrinum* L.) by priming. *International Journal of AgriScience*, 2(3), 237-243.
- Rowse, H. (1991) Methods of priming seeds. *UK Patent*, 2(192), 781.
- Ruan, Y. L., Patrick, J. W., Bouzayen, M., Osorio, S. & Fernie, A. R. (2012) Molecular regulation of seed and fruit set. *Trends Plant Sci*, 17(11), 656-65.
- Sabatini, M. E., Pagano, A., Araùjo, S., Balestrazzi, A. & Macovei, A. (2017) The tyrosyl-DNA phosphodiesterase 1β (Tdp1β) gene discloses an early response to abiotic stresses. *Genes*, 8(11), 305.
- Sachs, J. (1859) About treatments modifying germination strength in seeds. *Botanical Zeitung*, 17, 177-188.

- Sachs, J. (1887) Vorlesungen Lectures on Plant Physiology.
- Sah, S., Kaur, A. & Sandhu, J. (2014) High frequency embryogenic callus induction and whole plant regeneration in Japonica rice Cv. Kitaake. *Rice Research: Open Access*, 2, 1000125.
- Sahoo, K. K., Tripathi, A. K., Pareek, A., Sopory, S. K. & Singla-Pareek, S. L. (2011) An improved protocol for efficient transformation and regeneration of diverse indica rice cultivars. *Plant Methods*, 7(1), 49.
- Sancar, A., Lindsey-Boltz, L. A., Ünsal-Kaçmaz, K. & Linn, S. (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annual review of biochemistry*, 73(1), 39-85.
- Sanchez-Leon, S., Gil-Humanes, J., Ozuna, C. V., Gimenez, M. J., Sousa, C., Voytas, D. F. & Barro, F. (2018) Low-gluten, non-transgenic wheat engineered with CRISPR/Cas9. *Plant Biotechnol J*, 16(4), 902-910.
- Sano, N., Rajjou, L., North, H. M., Debeaujon, I., Marion-Poll, A. & Seo, M. (2016) Staying alive: molecular aspects of seed longevity. *Plant Cell Physiol*, 57(4), 660-74.
- Sanyal I, Singh AK, Kaushik M & D, A. (2005) *Agrobacterium* -mediated transformation of chickpea (*Cicer arietinum* L.) with *Bacillus thuringiensis* cry1Ac gene for resistance against pod borer insect *Helicoverpa armigera*. Plant Sci.
- Sato, K., Yamane, M., Yamaji, N., Kanamori, H., Tagiri, A., Schwerdt, J. G., Fincher, G. B., Matsumoto, T., Takeda, K. & Komatsuda, T. (2016) Alanine aminotransferase controls seed dormancy in barley. *Nature Communications*, 7, 11625.
- Sedeek, K. E. M., Mahas, A. & Mahfouz, M. (2019) Plant genome engineering for targeted improvement of crop traits. *Front Plant Sci*, 10, 114.
- Seneratna, T., Gusse, J. F. & McKersie, B. D. (1988) Age-induced changes in cellular membranes of imbibed soybean axes.: Physiol Plant.
- Servin, A., Elmer, W., Mukherjee, A., De la Torre-Roche, R., Hamdi, H., White, J. C., Bindraban, P. & Dimkpa, C. (2015) A review of the use of engineered nanomaterials to suppress plant disease and enhance crop yield. *Journal of Nanoparticle Research*, 17(2), 92.
- Shaheen, H. L., Iqbal, M., Azeem, M., Shahbaz, M. & Shehzadi, M. (2016) K-priming positively modulates growth and nutrient status of salt-stressed cotton (*Gossypium hirsutum*) seedlings. *Archives of Agronomy and Soil Science*, 62(6), 759-768.
- Sharma, P. & Dubey, R. S. (2005) Modulation of nitrate reductase activity in rice seedlings under aluminium toxicity and water stress: role of osmolytes as enzyme protectant. *J Plant Physiol*, 162(8), 854-64.

- Sharma, P., Jha, A. B., Dubey, R. S. & Pessarakli, M. (2012) Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *Journal of Botany*, 2012, 26.
- Sharma, S. N. & Maheshwari, A. Expression patterns of DNA repair genes associated with priming small and large chickpea (*Cicer arietinum*) seeds.
- Shen, L., Wang, C., Fu, Y., Wang, J., Liu, Q., Zhang, X., Yan, C., Qian, Q. & Wang, K. (2018) QTL editing confers opposing yield performance in different rice varieties. *Journal of Integrative Plant Biology*, 60(2), 89-93.
- Shih, P. M., Liang, Y. & Loque, D. (2016) Biotechnology and synthetic biology approaches for metabolic engineering of bioenergy crops. *Plant J*, 87(1), 103-17.
- Shmakov, S., Abudayyeh, O. O., Makarova, K. S., Wolf, Y. I., Gootenberg, J. S., Semenova, E., Minakhin, L., Joung, J., Konermann, S., Severinov, K., Zhang, F. & Koonin, E. V. (2015) Discovery and functional characterization of diverse class 2 CRISPR-Cas systems. *Mol Cell*, 60(3), 385-97.
- Shu, K., Meng, Y. J., Shuai, H. W., Liu, W. G., Du, J. B., Liu, J. & Yang, W. Y. (2015) Dormancy and germination: How does the crop seed decide? *Plant Biol (Stuttg)*, 17(6), 1104-12.
- Shukla, V. K., Doyon, Y., Miller, J. C., DeKelver, R. C., Moehle, E. A., Worden, S. E., Mitchell, J. C., Arnold, N. L., Gopalan, S., Meng, X., Choi, V. M., Rock, J. M., Wu, Y. Y., Katibah, G. E., Zhifang, G., McCaskill, D., Simpson, M. A., Blakeslee, B., Greenwalt, S. A., Butler, H. J., Hinkley, S. J., Zhang, L., Rebar, E. J., Gregory, P. D. & Urnov, F. D. (2009) Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. *Nature*, 459(7245), 437-41.
- Siddiqi, E. H., Ashraf, M., Al-Qurainy, F. & Akram, N. A. (2011a) Salt-induced modulation in inorganic nutrients, antioxidant enzymes, proline content and seed oil composition in safflower (Carthamus tinctorius L.). *J Sci Food Agric*, 91(15), 2785-93.
- Singh, A., Shankar, A., Gupta, V. K. & Prasad, V. (2016a) *Rhizobacteria*: tools for the management of plant abiotic stresses., in P., S. (ed), *Microbial Biotechnology, 1st Edition.*
- Singh, M., Kumar, M., Albertsen, M. C., Young, J. K. & Cigan, A. M. (2018) Concurrent modifications in the three homeologs of Ms45 gene with CRISPR-Cas9 lead to rapid generation of male sterile bread wheat (*Triticum aestivum* L.). *Plant Mol Biol*, 97(4-5), 371-383.

- Singh, M. & Singh, J. (2012) Seed development-related expression of ARGONAUTE4_9 class of genes in barley: possible role in seed dormancy. *Euphytica*, 188(1), 123-129.
- Singh, M., Singh, S., Randhawa, H. & Singh, J. (2013) Polymorphic homoeolog of key gene of RdDM pathway, ARGONAUTE4_9 class is associated with pre-harvest sprouting in wheat (*Triticum aestivum* L.). *PLoS One*, 8(10), e77009.
- Singh, P., Kim, Y.-J., Zhang, D. & Yang, D.-C. (2016b) Biological synthesis of nanoparticles from plants and microorganisms. *Trends in biotechnology*, 34(7), 588-599.
- Singh, S., Khan, N. A., Nazar, R. & Anjum, N. A. (2008) Photosynthetic traits and activities of antioxidant enzymes in blackgram (*Vigna mungo* L. Hepper) under cadmium stress. *Am J Plant Physiol*, 3, 25-32.
- Slamet-Loedin IH & A, M. (2016) The long and winding road of rice genetic modification technology and its potential. , in Eds. Jones HD, C. (ed), *Biotechnology of Major Cereals*.
- Slamet-Loedin, I. H., Chadha-Mohanty, P. & Torrizo, L. (2014) *Agrobacterium*-mediated transformation: rice transformation. *Methods Mol Biol*, 1099, 261-71.
- Smirnoff, N. (2005) Ascorbate, tocopherol and carotenoids: metabolism, pathway engineering and functions. Antioxidants and Reactive Oxygen Species in Plants: Blackwell Publishing Ltd., Oxford, UK.
- Smirnoff, N. & Cumbes, Q. J. (1989) Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry*, 28(4), 1057-1060.
- Song, G. C., Choi, H. K., Kim, Y. S., Choi, J. S. & Ryu, C.-M. (2017) Seed defense biopriming with bacterial cyclodipeptides triggers immunity in cucumber and pepper. *Scientific Reports*, 7(1), 14209.
- Srivastava, A. K., Lokhande, V. H., Patade, V. Y., Suprasanna, P., Sjahril, R. & D'Souza, S. F. (2010) Comparative evaluation of hydro-, chemo-, and hormonal-priming methods for imparting salt and PEG stress tolerance in Indian mustard (*Brassica juncea* L.). *Acta Physiologiae Plantarum*, 32(6), 1135-1144.
- Strutt, S. C., Torrez, R. M., Kaya, E., Negrete, O. A. & Doudna, J. A. (2018) RNA-dependent RNA targeting by CRISPR-Cas9. *eLife*, 7, e32724.
- Ström, K., Sjögren, J., Broberg, A. & Schnürer, J. (2002) *Lactobacillus plantarum* MiLAB 393 produces the antifungal cyclic dipeptides cyclo (L-Phe-L-Pro) and cyclo (L-Phe-trans-4-OH-L-Pro) and 3-phenyllactic acid. *Appl. Environ. Microbiol.*, 68(9), 4322-4327.

- Sun, Q., Lin, L., Liu, D., Wu, D., Fang, Y., Wu, J. & Wang, Y. (2018) CRISPR/Cas9-mediated multiplex genome editing of the BnWRKY11 and BnWRKY70 genes in *Brassica napus* L. *Int J Mol Sci*, 19(9).
- Sun, Y., Jiao, G., Liu, Z., Zhang, X., Li, J., Guo, X., Du, W., Du, J., Francis, F., Zhao, Y. & Xia, L. (2017) Generation of high-amylose rice through CRISPR/Cas9-mediated targeted mutagenesis of starch branching enzymes. *Front Plant Sci*, 8, 298.
- Sun, Y., Zhang, X., Wu, C., He, Y., Ma, Y., Hou, H., Guo, X., Du, W., Zhao, Y. & Xia, L. (2016) Engineering herbicide-resistant rice plants through CRISPR/Cas9-mediated homologous recombination of acetolactate synthase. *Mol Plant*, 9(4), 628-31.
- Svitashev, S., Schwartz, C., Lenderts, B., Young, J. K. & Mark Cigan, A. (2016) Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. *Nat Commun*, 7, 13274.
- Syahariza, Z. A., Sar, S., Hasjim, J., Tizzotti, M. J. & Gilbert, R. G. (2013) The importance of amylose and amylopectin fine structures for starch digestibility in cooked rice grains. *Food Chem*, 136(2), 742-9.
- Tahaei, A., Soleymani, A. & Shams, M. (2016) Seed germination of medicinal plant, fennel (*Foeniculum vulgare* Mill), as affected by different priming techniques. *Appl Biochem Biotechnol*, 180(1), 26-40.
- Takashima, H., Boerkoel, C. F., John, J., Saifi, G. M., Salih, M. A., Armstrong, D., Mao, Y., Quiocho, F. A., Roa, B. B., Nakagawa, M., Stockton, D. W. & Lupski, J. R. (2002) Mutation of TDP1, encoding a topoisomerase I-dependent DNA damage repair enzyme, in spinocerebellar ataxia with axonal neuropathy. *Nat Genet*, 32(2), 267-72.
- Tang, D., Dong, Y., Guo, N., Li, L. & Ren, H. (2014) Metabolomic analysis of the polyphenols in germinating mung beans (*Vigna radiata*) seeds and sprouts. *Journal of the Science of Food and Agriculture*, 94(8), 1639-1647.
- Tavakol, E. & Pakniyat, H. (2007) Evaluation of some drought resistance criteria at seedling stage in wheat (*Triticum aestivum* L.) cultivars. *Pak J Biol Sci*, 10(7), 1113-7.
- Taylor, A. & Harman, G. (1990) Concepts and technologies of selected seed treatments. *Annual review of phytopathology*, 28(1), 321-339.
- Tebas, P., Stein, D., Tang, W. W., Frank, I., Wang, S. Q., Lee, G., Spratt, S. K., Surosky, R. T., Giedlin, M. A., Nichol, G., Holmes, M. C., Gregory, P. D., Ando, D. G., Kalos, M., Collman, R. G., Binder-Scholl, G., Plesa, G., Hwang, W. T., Levine, B. L. & June, C. H. (2014) Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N Engl J Med*, 370(10), 901-10.

- Thakur, S. & Sharma, S. S. (2015) Characterization of seed germination, seedling growth, and associated metabolic responses of *Brassica juncea* L. cultivars to elevated nickel concentrations. *Protoplasma*, 253(2), 571-580.
- Timmusk, S., El-Daim, I. A. A., Copolovici, L., Tanilas, T., Kännaste, A., Behers, L., Nevo, E., Seisenbaeva, G., Stenström, E. & Niinemets, Ü. (2014) Drought-tolerance of wheat improved by rhizosphere bacteria from harsh environments: enhanced biomass production and reduced emissions of stress volatiles. *PloS one*, 9(5), e96086.
- Toorop, P. E., van Aelst, A. C. & Hilhorst, H. W. (1998) Endosperm cap weakening and endo-β-mannanase activity during priming of tomato (*Lycopersicon esculentum* cv. Moneymaker) seeds are initiated upon crossing a threshold water potential. *Seed Science Research*, 8(4), 483-492.
- Trovato, M., Mattioli, R. & Costantino, P. (2008) Multiple roles of proline in plant stress tolerance and development. *Rendiconti Lincei*, 19(4), 325-346.
- Tuteja, N. (2007) Mechanisms of high salinity tolerance in plants. *Methods Enzymol*, 428, 419-38.
- Tuteja, N., Ahmad, P., Panda, B. B. & Tuteja, R. (2009) Genotoxic stress in plants: shedding light on DNA damage, repair and DNA repair helicases. *Mutation Research/Reviews in Mutation Research*, 681(2-3), 134-149.
- Tycko, J., Hess, G., Jeng, E., Dubreuil, M. & Bassik, M. (2017) The expanding CRISPR toolbox. *Nature Methods.*
- Ueta, R., Abe, C., Watanabe, T., Sugano, S. S., Ishihara, R., Ezura, H., Osakabe, Y. & Osakabe, K. (2017) Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. *Scientific Reports*, 7(1), 507.
- Upadhyay, S. K., Kumar, J., Alok, A. & Tuli, R. (2013) RNA-guided genome editing for target gene mutations in wheat. *G3: Genes|Genomes|Genetics*, 3(12), 2233.
- Urso, S., Desiderio, F., Biselli, C., Bagnaresi, P., Crispino, L., Piffanelli, P., Abbruscato, P., Assenza, F., Guarnieri, G., Cattivelli, L. & Vale, G. (2016) Genetic analysis of durable resistance to *Magnaporthe oryzae* in the rice accession Gigante Vercelli identified two blast resistance loci. *Mol Genet Genomics*, 291(1), 17-32.
- Van Pijlen, J., Kraak, H., Bino, R. & De Vos, C. (1995) Effects of aging and osmopriming on germination characteristics and chromosome aberrations of tomato (*Lycopersicon esculentum* Mill.) seeds. *Seed Science and Technology (Switzerland)*.
- Veillet, F., Perrot, L., Chauvin, L., Kermarrec, M.-P., Guyon-Debast, A., Chauvin, J.-E., Nogué, F. & Mazier, M. (2019) Transgene-free genome editing in tomato and potato plants using *Agrobacterium*-mediated delivery

of a CRISPR/Cas9 cytidine base editor. *International Journal of Molecular Sciences*, 20(2), 402.

- Venkatasubramanian, A. & Umarani, R. (2010) Storability of primed seeds of tomato (*Lycopersicon esculentum*), eggplant (*Solanum melongena*) and chilli (*Capsicum annum*). *Madras Agricultural Journal*, 97(4/6), 117-124.
- Ventura, L., Dona, M., Macovei, A., Carbonera, D., Buttafava, A., Mondoni, A., Rossi, G. & Balestrazzi, A. (2012) Understanding the molecular pathways associated with seed vigor. *Plant Physiol Biochem*, 60, 196-206.
- Verdier, J., Kakar, K., Gallardo, K., Le Signor, C., Aubert, G., Schlereth, A., Town, C. D., Udvardi, M. K. & Thompson, R. D. (2008) Gene expression profiling of *M. truncatula* transcription factors identifies putative regulators of grain legume seed filling. *Plant Molecular Biology*, 67(6), 567.
- Vierstra, R. D., John, T. R. & Poff, K. L. (1982) Kaempferol 3-O-galactoside, 7-O-rhamnoside is the major green fluorescing compound in the epidermis of *Vicia faba*. *Plant Physiol*, 69(2), 522-5.
- Villagarcia, H., Dervishi, E., de Silva, K., Biris, A. S. & Khodakovskaya, M. V. (2012) Surface chemistry of carbon nanotubes impacts the growth and expression of water channel protein in tomato plants. *Small*, 8(15), 2328-2334.
- Volkova, P. Y., Churyukin, R. S. & Geras'kin, S. A. (2016) Influence of γ-irradiated seeds on the enzyme activity in barley seedlings. *Radiats Biol Radioecol*, 56(2), 190-6.
- Voytas, D. F. & Gao, C. (2014) Precision genome engineering and agriculture: opportunities and regulatory challenges. *PLoS Biol*, 12(6), e1001877.
- Vranová, E., Inzé, D. & Van Breusegem, F. (2002) Signal transduction during oxidative stress. *Journal of experimental botany*, 53(372), 1227-1236.
- Walters, C., Reilley, A. A., Reeves, P. A., Baszczak, J. & Richards, C. M. (2006) The utility of aged seeds in DNA banks. *Seed Science Research*, 16(3), 169-178.
- Wan, J. M., Cao, Y. J., Wang, C. M. & Ikehashi, H. (2005) Quantitative trait loci associated with seed dormancy in rice. *Crop Science*, 45(2), 712-716.
- Wang, C., Liu, Q., Shen, Y., Hua, Y., Wang, J., Lin, J., Wu, M., Sun, T., Cheng, Z., Mercier, R. & Wang, K. (2019) Clonal seeds from hybrid rice by simultaneous genome engineering of meiosis and fertilization genes. *Nat Biotechnol*, 37(3), 283-286.
- Warren, J. & Bennett, M. (2000) Bio-osmopriming tomato (*Lycopersicon esculentum* Mill.) seeds for improved seedling establishment. *Bio-*

osmopriming tomato (Lycopersicon esculentum Mill.) seeds for improved seedling establishment., 477-487.

- Warren, J. E. & Bennett, M. A. (1997) Seed hydration using the drum priming system. *HortScience*, 32(7), 1220-1221.
- Waterworth, W. M., Bray, C. M. & West, C. E. (2015) The importance of safeguarding genome integrity in germination and seed longevity. *J Exp Bot*, 66(12), 3549-58.
- Waterworth, W. M., Bray, C. M. & West, C. E. (2019) Seeds and the art of genome maintenance. *Frontiers in Plant Science*, 10(706).
- Waterworth, W. M., Footitt, S., Bray, C. M., Finch-Savage, W. E. & West, C. E. (2016) DNA damage checkpoint kinase ATM regulates germination and maintains genome stability in seeds. *Proceedings of the National Academy of Sciences*, 113(34), 9647-9652.
- Waterworth, W. M., Masnavi, G., Bhardwaj, R. M., Jiang, Q., Bray, C. M. & West, C. E. (2010) A plant DNA ligase is an important determinant of seed longevity. *The Plant Journal*, 63(5), 848-860.
- Weitbrecht, K., Muller, K. & Leubner-Metzger, G. (2011) First off the mark: early seed germination. *J Exp Bot*, 62(10), 3289-309.
- Wen, S., Liu, H., Li, X., Chen, X., Hong, Y., Li, H., Lu, Q. & Liang, X. (2018) TALEN-mediated targeted mutagenesis of fatty acid desaturase 2 (FAD2) in peanut (*Arachis hypogaea* L.) promotes the accumulation of oleic acid. *Plant Mol Biol*, 97(1-2), 177-185.
- West, C. E., Waterworth, W. M., Story, G. W., Sunderland, P. A., Jiang, Q. & Bray, C. M. (2002) Disruption of the Arabidopsis AtKu80 gene demonstrates an essential role for AtKu80 protein in efficient repair of DNA double-strand breaks *in vivo*. *Plant J*, 31(4), 517-28.
- Wilen, R. W., van Rooijen, G. J., Pearce, D. W., Pharis, R. P., Holbrook, L. A. & Moloney, M. M. (1991) Effects of jasmonic acid on embryo-specific processes in *Brassica* and *Linum* oilseeds. *Plant Physiology*, 95(2), 399-405.
- Willis, C. G., Baskin, C. C., Baskin, J. M., Auld, J. R., Venable, D. L., Cavender-Bares, J., Donohue, K. & Rubio de Casas, R. (2014) The evolution of seed dormancy: environmental cues, evolutionary hubs, and diversification of the seed plants. *New Phytol*, 203(1), 300-9.
- Wilson, D. (1986) The lipid peroxidation model of seed aging. *Seed Sci. Technol.*, 14, 269-300.
- Wojtyla, Ł., Lechowska, K., Kubala, S. & Garnczarska, M. (2016) Different modes of hydrogen peroxide action during seed germination. *Frontiers in plant science*, 7, 66.

- Wolt, J. D. & Wolf, C. (2018) Policy and governance perspectives for regulation of genome edited crops in the United States. *Frontiers in Plant Science*, 9(1606).
- Woodstock, L. W., Maxon, S., Faul, K. & Bass, L. (1983) Use of freeze-drying and acetone impregnation with natural and synthetic antioxidants to improve storability of onion, pepper and parsley seeds. J Am Soc Hortic Sci.
- Xiang, C., Werner, B. L., Christensen, E. M. & Oliver, D. J. (2001) The biological functions of glutathione revisited in *Arabidopsis* transgenic plants with altered glutathione levels. *Plant Physiol*, 126(2), 564-74.
- Xu, R., Yang, Y., Qin, R., Li, H., Qiu, C., Li, L., Wei, P. & Yang, J. (2016) Rapid improvement of grain weight via highly efficient CRISPR/Cas9-mediated multiplex genome editing in rice. *J Genet Genomics*, 43(8), 529-32.
- Yacoubi, R., Job, C., Belghazi, M., Chaibi, W. & Job, D. (2013) Proteomic analysis of the enhancement of seed vigour in osmoprimed alfalfa seeds germinated under salinity stress. *Seed Science Research*, 23(2), 99-110.
- Yadav, P., Kaur, R., Kanwar, M. K., Sharma, A., Verma, V., Sirhindi, G. & Bhardwaj, R. (2018) Castasterone confers copper stress tolerance by regulating antioxidant enzyme responses, antioxidants, and amino acid balance in *B. juncea* seedlings. *Ecotoxicol Environ Saf*, 147, 725-734.
- Yadu, S., Dewangan, T. L., Chandrakar, V. & Keshavkant, S. (2017) Imperative roles of salicylic acid and nitric oxide in improving salinity tolerance in *Pisum sativum* L. *Physiol Mol Biol Plants*, 23(1), 43-58.
- Yaldagard, M., Mortazavi, S. A. & Tabatabaie, F. (2008) Application of ultrasonic waves as a priming technique for accelerating and enhancing the germination of barley seed: optimization of method by the taguchi approach. *Journal of the Institute of Brewing*, 114(1), 14-21.
- Yamane, K., Mitsuya, S., Kawasaki, M., Taniguchi, M. & Miyake, H. (2009) Antioxidant capacity and damages caused by salinity stress in apical and basal regions of rice leaf. *Plant Production Science*, 12(3), 319-326.
- Yan, D., Duermeyer, L., Leoveanu, C. & Nambara, E. (2014) The functions of the endosperm during seed germination. *Plant and Cell Physiology*, 55(9), 1521-1533.
- Yan, P.-S., Song, Y., Sakuno, E., Nakajima, H., Nakagawa, H. & Yabe, K. (2004) Cyclo (L-leucyl-L-prolyl) produced by *Achromobacter xylosoxidans* inhibits aflatoxin production by *Aspergillus parasiticus*. *Appl. Environ. Microbiol.*, 70(12), 7466-7473.
- Yang, H., Wu, J.-J., Tang, T., Liu, K.-D. & Dai, C. (2017) CRISPR/Cas9-mediated genome editing efficiently creates specific mutations at multiple loci using one sgRNA in *Brassica napus*. *Scientific Reports*, 7(1), 7489.

- Yang, S., Xie, J. & Li, Q. (2012) Oxidative response and antioxidative mechanism in germinating soybean seeds exposed to cadmium. *Int J Environ Res Public Health*, 9(8), 2827-38.
- Yang, S.-w., Burgin, A. B., Huizenga, B. N., Robertson, C. A., Yao, K. C. & Nash, H. A. (1996) A eukaryotic enzyme that can disjoin dead-end covalent complexes between DNA and type I topoisomerases. *Proceedings of the National Academy of Sciences*, 93(21), 11534-11539.
- Yang, X., Cheng, Y.-F., Deng, C., Ma, Y., Wang, Z.-W., Chen, X.-H. & Xue, L.-B. (2014) Comparative transcriptome analysis of eggplant (*Solanum melongena* L.) and turkey berry (*Solanum torvum* Sw.): phylogenomics and disease resistance analysis. *BMC Genomics*, 15(1), 412.
- Ye, N., Zhu, G., Liu, Y., Zhang, A., Li, Y., Liu, R., Shi, L., Jia, L. & Zhang, J. (2011) Ascorbic acid and reactive oxygen species are involved in the inhibition of seed germination by abscisic acid in rice seeds. *Journal of Experimental Botany*, 63(5), 1809-1822.
- Yeong, H. G., Youn, C.-K., Cho, H.-J., Kim, S.-H., Kim, M.-H., Kim, H.-B., Chang, I.-Y., Lee, Y.-S., Chung, M.-H., You, H.-J. (2004) Metallothionein-III prevents γ-ray induced 8-oxoguanine accumulation in normal and hOGG1-depleted cells. . *J Biol Chem*.
- Yin, G., Xin, X., Song, C., Chen, X., Zhang, J., Wu, S., Li, R., Liu, X. & Lu, X. (2014) Activity levels and expression of antioxidant enzymes in the ascorbate-glutathione cycle in artificially aged rice seed. *Plant Physiol Biochem*, 80, 1-9.
- Yoshiyama, K. O., Sakaguchi, K. & Kimura, S. (2013) DNA damage response in plants: conserved and variable response compared to animals. *Biology (Basel)*, 2(4), 1338-56.
- Yu, S. & Tian, L. (2018) Breeding major cereal grains through the lens of nutrition sensitivity. *Mol Plant*, 11(1), 23-30.
- Yuan, B., Byrnes, D., Giurleo, D., Villani, T., Simon, J. E. & Wu, Q. (2018) Rapid screening of toxic glycoalkaloids and micronutrients in edible nightshades (*Solanum* spp.). *Journal of Food and Drug Analysis*, 26(2), 751-760.
- Zalatan, J. G., Lee, M. E., Almeida, R., Gilbert, L. A., Whitehead, E. H., La Russa, M., Tsai, J. C., Weissman, J. S., Dueber, J. E., Qi, L. S. & Lim, W. A. (2015) Engineering complex synthetic transcriptional programs with CRISPR RNA scaffolds. *Cell*, 160(1-2), 339-50.
- Zalewski, K., Nitkiewicz, B., Lahuta, L. B., Glowacka, K., Socha, A. & Amarowicz, R. (2010) Effect of jasmonic acid-methyl ester on the composition of carbohydrates and germination of yellow lupine (*Lupinus luteus* L.) seeds. *J Plant Physiol*, 167(12), 967-73.

- Zhan, X., Zhang, F., Zhong, Z., Chen, R., Wang, Y., Chang, L., Bock, R., Nie, B. & Zhang, J. (2019) Generation of virus-resistant potato plants by RNA genome targeting. *Plant Biotechnol J*, 17(9), 1814-1822.
- Zhang, F., Yu, J., Johnston, C. R., Wang, Y., Zhu, K., Lu, F., Zhang, Z. & Zou, J. (2015) Seed priming with polyethylene glycol induces physiological changes in sorghum (*Sorghum bicolor* L. Moench) seedlings under suboptimal soil moisture environments. *PLoS One*, 10(10), e0140620.
- Zhang, T., Zheng, Q., Yi, X., An, H., Zhao, Y., Ma, S. & Zhou, G. (2018) Establishing RNA virus resistance in plants by harnessing CRISPR immune system. *Plant Biotechnol J*, 16(8), 1415-1423.
- Zhang, Y., Chen, B., Xu, Z., Shi, Z., Chen, S., Huang, X., Chen, J. & Wang, X. (2014) Involvement of reactive oxygen species in endosperm cap weakening and embryo elongation growth during lettuce seed germination. *Journal of Experimental Botany*, 65(12), 3189-3200.
- Zhang, Y., Liu, H., Shen, S. & Zhang, X. (2011) Improvement of eggplant seed germination and seedling emergence at low temperature by seed priming with incorporation SA into KNO3 solution. *Frontiers of Agriculture in China*, 5(4), 534-537.
- Zhao, T., Wang, Z., Su, L., Sun, X., Cheng, J., Zhang, L., Karungo, S. K., Han, Y., Li, S. & Xin, H. (2017) An efficient method for transgenic callus induction from *Vitis amurensis* petiole. *PloS one*, 12(6), e0179730-e0179730.
- Zheng, M., Tao, Y., Hussain, S., Jiang, Q., Peng, S., Huang, J., Cui, K. & Nie, L. (2016) Seed priming in dry direct-seeded rice: consequences for emergence, seedling growth and associated metabolic events under drought stress. *Plant growth regulation*, 78(2), 167-178.
- Zheng, M., Zhang, L., Tang, M., Liu, J., Liu, H., Yang, H., Fan, S., Terzaghi, W., Wang, H. & Hua, W. (2019) Knockout of two *BnaMAX1* homologs by CRISPR/Cas9-targeted mutagenesis improves plant architecture and increases yield in rapeseed (*Brassica napus* L.). *Plant Biotechnol J*.
- Zhou, J., Wang, Y. & Jahufer, Z. (2013) Location and chemical composition of semi-permeable layer of forage seeds. *Bangladesh Journal of Botany*, 42(1), 23-30.
- Zhu, J., Song, N., Sun, S., Yang, W., Zhao, H., Song, W. & Lai, J. (2016) Efficiency and inheritance of targeted mutagenesis in maize using CRISPR-Cas9. *Journal of Genetics and Genomics*, 43(1), 25-36.
- Zlatev, Z., Lidon, F., Ramalho, J. & Yordanov, I. (2006) Comparison of resistance to drought of three bean cultivars. *Biologia Plantarum*, 50(3), 389-394.

- Zong, Y., Wang, Y., Li, C., Zhang, R., Chen, K., Ran, Y., Qiu, J. L., Wang, D. & Gao, C. (2017) Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nat Biotechnol*, 35(5), 438-440.
- Zuo, J. & Li, J. (2014) Molecular genetic dissection of quantitative trait loci regulating rice grain size. *Annu Rev Genet*, 48, 99-118.

List of original manuscripts

Dear Dr. Balestrazzi,

On 20th Oct 19, I received your manuscript entitled "Molecular dynamics of pre-germinative metabolism in primed eggplant (Solanum melongena L.) seeds" by authors Alma Balestrazzi.

Your manuscript has been assigned the Paper #: HORTRES-02233.

I have asked Zong-Ming (Max) Cheng to assist me with the peer review of your manuscript.

I will contact you as soon as this process is complete.

In the interim, you may check on the status of this manuscript by selecting the "Check Manuscript Status" link under the following URL:

https://mts-hortres.nature.com/cgi-bin/main.plex?el=A7Ca2BBY1A2EEf7F3A9ftdcjEW9rqaiNfYUAuKaHhe8wZ

(Press/Click on the above link to be automatically sent to the web page.)

Thank you for submitting your best work to Horticulture Research.

Sincerely,

Dr. Max Cheng
Editor-in-Chief
Horticulture Research

Dear Editor,

Enclosed, please find the manuscript titled: "Molecular dynamics of pre-germinative metabolism in primed eggplant *(Solanum melongena* L.) seeds" co-authored by Chiara Forti, Valentino Ottobrino, Laura Bassolino, Laura Toppino, Giuseppe Leonardo Rotino, Andrea Pagano, Anca Macovei, and Alma Balestrazzi, to be evaluated for publication on Horticulture Research.

In this study, we explore for the first time the molecular dynamics of the pre-germinative metabolism in primed eggplant *(Solanum melongena* L.) seeds in order to identify hallmarks (expression patterns of antioxidant/DNA repair genes combined with free radical profiles) useful to discriminate between high- and low-quality lots. The hydropriming protocol hereby developed anticipated, or even rescued, germination when applied to lots with variable quality. ROS (reactive oxygen species) raised during hydropriming and dropped after dry-back. Up-regulation of antioxidant/DNA repair genes was observed during hydropriming and the subsequent imbibition. Up-regulation of *SmOGG1* (8-oxoguanine glycosylase/lyase) gene detected in primed seeds at 2 h of imbibition appeared as a promising hallmark. Based on these results, the investigation was restricted within the 2 hours of imbibition, to verify whether the molecular landscape was reproducible in different lots. A complex pattern of antioxidant/DNA repair gene expression emerged, reflecting the preponderance of seed-lot specific profiles. Only the low-quality eggplant seeds subjected to hydropriming showed enhanced ROS levels, both in the dry and imbibed state, and this might be a useful signature to discriminate among lots. The plasticity of the pre-germinative metabolism in eggplant stimulated by priming imposes a plethora of heterogeneous molecular responses that might delay the search for quality hallmarks. However, the information hereby gained could be translated to eggplant wild relatives to speed up their use in breeding programmes or other agronomical applications.

Authors declare that the manuscript represents original work and has not been submitted to an alternative journal.

Looking forward to hearing from you, I send my best regards.

Sincerely yours

Alma Balestrazzi

Dipartimento di Biologia e Biotecnologie "L. Spallanzani"
via Ferrata 1, 27100 Pavia, Italy
E mail: alma.balestrazzi@unipv.it
Tel 00390382985435
Fax 00390382528496

Molecular dynamics of pre-germinative metabolism in primed eggplant (*Solanum melongena* L.) seeds

Chiara Forti[1], Valentino Ottobrino[1], Laura Bassolino[2,3], Laura Toppino[2], Giuseppe Leonardo Rotino[2], Andrea Pagano[1,§], Anca Macovei[1], Alma Balestrazzi[1,*]

[1]Department of Biology and Biotechnology 'Lazzaro Spallanzani', University of Pavia, Italy.
[2] CREA-GB, Research Centre for Genomics and Bioinformatics, Montanaso Lombardo (LO), Italy.
[3] CREA-CI, Research Centre for Cereal and Industrial Crops, Bologna, Italy
[§] Present address: Institute of Plant Genetics, Polish Academy of Sciences, Poznan, Poland

Chiara Forti. E-mail address: chiara.forti01@universitadipavia.it
Valentino Ottobrino. E-mail address: valentino.ottobrino01@universitadipavia.it
Laura Bassolino. E-mail address: laura.bassolino@crea.gov.it
Laura Toppino. E-mail address: laura.toppino@crea.gov.it
Giuseppe Leonardo Rotino. E-mail address: giuseppeleonardo.rotino@crea.gov.it
Andrea Pagano. E-mail address: andrea.pagano01@universitadipavia.it
Anca Macovei. E-mail address: anca.macovei@unipv.it

* Correspondence: Alma Balestrazzi, Department of Biology and Biotechnology 'Lazzaro Spallanzani', University of Pavia, Italy. Tel. 00390382985435. E-mail address: alma.balestrazzi@unipv.it

Abstract

Seed priming, a pre-sowing technique that enhances the antioxidant/DNA repair activities during the pre-germinative metabolism, still retains empirical features. We explore for the first time the molecular dynamics of pre-germinative metabolism in primed eggplant (*Solanum melongena* L.) seeds in order to identify hallmarks (expression patterns of antioxidant/DNA repair genes combined with free radical profiles) useful to discriminate between high- and low-quality lots. The hydropriming protocol hereby developed anticipated, or even rescued, germination when applied to lots with variable quality. ROS (reactive oxygen species) raised during hydropriming and dropped after dry-back. Up-regulation of antioxidant/DNA repair genes was observed during hydropriming and the subsequent imbibition. Up-regulation of *SmOGG1* (8-oxoguanine glycosylase/lyase) gene detected in primed seeds at 2 h of imbibition appeared as a promising hallmark. Based on these results, the investigation was restricted within the first 2 hours of imbibition, to verify whether the molecular landscape was reproducible in different lots. A complex pattern of antioxidant/DNA repair gene expression emerged, reflecting the preponderance of seed-lot specific profiles. Only the low-quality eggplant seeds subjected to hydropriming showed enhanced ROS levels, both in the dry and imbibed state, and this might be a useful signature to discriminate among lots. The plasticity of eggplant pre-germinative metabolism stimulated by priming imposes a plethora of heterogeneous molecular responses that might delay the search for quality hallmarks. However, the information hereby gained could be translated to eggplant wild relatives to speed up their use in breeding programmes or other agronomical applications.

Introduction

Seed priming is a low-cost, pre-sowing technique based on controlled imbibition that boosts the antioxidant and DNA repair activities triggered when the pre-germinative metabolism starts, while avoiding radicle protrusion and loss of desiccation tolerance [1-4]. At the end of the treatment, primed seeds undergo dehydration (dry-back) and finally they are stored and/or commercialised. Enhanced crop yields resulting from primed seeds are generally due to increased tolerance to biotic and abiotic stresses that improves population density as well as individual plant performance. Due to these benefits, the priming technology is gaining relevance as a strategy to address the current and future issues of sustainable crop production on degraded soils [5-7]. Despite the wide use of priming to improve seed quality, this

technique still retains empirical features that delays the work of seed technologists, breeders, and seed bank operators. A deeper understanding of the molecular processes that drive the seed response to priming, at the level of pre-germinative metabolism, will provide molecular hallmarks (genes, proteins, metabolites) useful for predicting the effectiveness of novel pre-sowing treatments [8,9]. Several reports have already highlighted the positive correlation between the antioxidant response and increased seed vigour [10,11] as well as the impact of effective DNA repair on seed quality [12,13]. Seed imbibition features increased oxidative DNA damage, namely enhanced levels of 7,8-dihydro-8-oxoguanine (8-oxodG), as reported in *Medicago truncatula* [9] as well as the up-regulation of key BER (base excision repair) genes as *OGG1* (8-oxoguanine glycosylase/lyase), and *FPG* (formamidopyrimidine DNA glycosylase)[14]. OGG1 (EC: 3.2.2-4.2.99.18) is a bifunctional DNA glycosylase/lyase, typically found in eukaryotes that catalyzes the release of 8-oxo-dG and the cleavage of DNA at the resulting abasic site. The role of OGG1 in counteracting oxidative DNA damage accumulated during seed desiccation has been documented [15,16]. FPG (EC: 3.2.2.23) is responsible for the excision of 8-oxo-dG as well as FAPy (2,6-diamino-4-hydroxy-5-formamidopyrimidine) lesions. Another DNA repair gene, linked to BER, encodes tyrosyl-DNA phosphodiesterase 1 (Tdp1; EC: 3.1.4) that removes the stalled topoisomerase I-DNA complexes through the cleavage of a 3'-phosphotyrosyl bond. In animal cells, the Tdp1 enzyme also interacts with BER proteins for the repair of oxidized DNA lesions [17]. The plant *Tdp1α* gene coding for the most conserved TDP1 isoform is up-regulated during seed imbibition under physiological and oxidative stress conditions [18,9,19,20], and thus it might be involved in the seed response to priming. These studies show that the BER pathway can be source of hallmarks that might assist in developing improved priming treatments.

Eggplant (*Solanum melongena* L.), a member of spiny *Solanum* species native to the Old World and subsequently independently domesticated in India and Cina, is cultivated worldwide for its high nutritional value that correlates with beneficial effects as disease prevention [21,22]. *Solanum* species are mainly propagated by seed. However, dormancy, poor seed germination rate, and low uniformity have been documented in several accessions of *S. melongena* and related wild species [23-27]. Low uniformity as well as scarce germination percentage are crucial factors affecting the use of wild relatives as rootstocks for eggplant and other compatible crop species [28,29]. These issues severely impact breeding programs, with a huge economic impact on seed industries that need to increase product performance while optimizing the use of economic resources.

In this context, seed priming is used to enhance germination and provide faster emergence, uniform stands, and stress tolerant plants [4]. Several reports are currently available, describing the effects of different types of priming protocols on eggplant seed germination and seedling emergence [30-35], however the molecular charaterization of the seed response to priming is still lacking. In the present work, we investigated these issues using the *S. melongena* inbred line '67/3', for which both the reference genome and transcriptome are available [36].

In this study, the role of BER/antioxidant genes as tools to monitor seed priming and its impact on germination was assessed in the '67/3' line by comparing the response of seed lots of different quality. Furthermore, the dynamics of ROS accumulation in primed *versus* unprimed seeds were evaluated as a possible mark of quality.

Results

Hydropriming promotes germination of '67/3' seeds

Hydropriming (HP) was first tested on '67/3' seeds collected in 2014, hereby named Seed Lot 1 (SL1). Seeds soaked in H_2O for 24 h, 48 h, 72 h, and 96 h (treatments HP24, HP48, HP72, and HP96) were subjected to dry-back (DB, 2 h) and then immediately used for germination tests. As shown in Fig. 1, UP seeds started germination at 9 days from the beginning of imbibition and reached the maximum germination percentage (74.44 ± 0.71 %) at 16 days whereas hydropriming always resulted in anticipated germination compared to UP seeds. In the case of primed seeds, germination occurred at 5 days (40.00 ± 0.00 %, HP24), 4 days (6.00 ± 0.71 %, HP48), 3 days (2.00 ± 0.71 %, HP72), and 2 days (4.00 ± 0.00 %, HP96) from the beginning of imbibition.

A more detailed analysis of the germination profiles was performed as described by Ranal and Garcia de Santana [37] (Table 1). *G* (Germinability) was the highest (100 %) for HP24, HP48 and HP72 while a slight reduction (98.00 ± 2.80 %) was observed for HP96. Overall, HP72 turned out to be the best treatment in terms of *MGT* (Mean Germination Time) (4.48 ± 0.00 days) and *CVG* (Coefficient of variation of Velocity of Germination) (468.00 ± 0.15 %) parameters. Both the lowest *MGR* (Mean Germination Rate) and *U* (Uncertainty) values, recorded for HP72 ($2.14 \times 10^{-3} \pm 0.00$ day^{-1} and 1.22 ± 0.20 bit, respectively) were indicative of synchronized germination (Table 1). Biometric parameters measured on seven-day old '67/3' seedlings developed from HP72 seeds highlighted significant changes only in seedling

fresh weight (Table S1). Based on this results, the HP72 treatment was selected for further investigations.

Hydropriming rescues germination in low-quality '67/3' seed lots

The HP72 protocol was applied to '67/3' seed lots collected during the subsequent years (2015, 2016, 2017, 2018, named SL2, SL3, SL4 and SL5). Germination tests were carried out and the resulting data were used to calculate the specific germination parameters. As shown in Table 2, in the case of SL2, UP seeds were not able to germinate whereas the HP72 treatment rescued germination. The recorded values for the HP72 sample were 55.56 ± 21.42 % (*G*), 6.52 ± 0.86 days (*MGT*), 59 ± 42.48 % (*CVG*), 0.0269 ± 0.022 day^{-1} (*MGR*), 1.61 ± 0.87 bit (*U*) and 0.34 ± 0.28 (*Z*). As for SL3, a significantly lower *MGT* (6.20 ± 0.00 days) was observed for the primed seeds compared to UP (8.95 ± 0.91 days). Except for *MGR*, all the other recoded traits in HP72 seeds were significantly different from UP seeds whereas only slight differences were noticed in *U* values (UP, 2.39 ± 0.05 and HP72, 2.04 ± 0.16 bit, respectively) (Table 2). SL4 showed a late germination profile, however the HP72 treatment could anticipate germination as evidenced by *MGT* values (UP, 28.56 ± 1.34 days; HP72, 25.51 ± 0.79 days) whereas no effects were evidenced in other traits, including synchronization (recorded *U* values: UP, 2.53 ± 0.14 bit; HP72, 2.74 ± 0.18 bit). As for SL5, although no significant effects on synchronization were detected, a similar response was reported, with anticipated germination (*MGT* values of 11.05 ± 1.10 and 8.84 ± 0.87 days, for UP and HP72 samples, respectively). By considering the overall data on germination profiles, the '67/3' seeds were grouped as 'high-quality' (SL1, SL3, SL5) and low-quality (SL2, SL4) lots.

Hydropriming boosts ROS accumulation

The experimental design set up for the molecular analyses is shown in Fig. 2A. Samples were collected at 24 h, 48 h, and 72 h during HP and subsequently after the dry-back step (DB). During germination tests, UP and HP seeds were collected at 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, and 72 h from the beginning of imbibition and at the radicle protrusion stage (RD) (Fig. 2A). Based on the germination parameters, the sampling procedure for the radicle protrusion stage was conducted earlier in HP seeds (at 4 days from the beginning of imbibition) than for UP seeds (at 8 days from the beginning of imbibition) (Fig. 2A).

ROS (reactive oxygen species) levels were measured using the DCFH-DA fluorescent dye in dry and imbibed '67/3' seeds at selected time points. These experiments were performed using SL5, both dry seeds (DS) and seeds collected throughout the HP treatment (24 h, 48 h, 72 h) (Fig. 2B). HP triggered a significant ($P = 0.04$) ROS accumulation at 24 h (27.63 ± 8.82 R.F.U.), 14.62-fold compared to DS (1.89 ± 0.53 R.F.U.). At 48 h and 72 h, the recorded ROS levels were still significantly higher (15.72 ± 3.55 and 8.71 ± 1.70 R.F.U., respectively; $P = 0.02$ and 0.01) (Fig. 2B). Following dry-back, ROS levels further decreased compared to the 72 h timepoint, reaching an estimated value of 1.02 ± 0.25 R.F.U. ($P = 0.08$), similar to those detected in the dry seeds (Fig. 1C, + DB). A different pattern was detected in the UP samples since a peak in ROS levels was observed at 8 h and 16 h of imbibition (8.84 ± 1.83 and 12.50 ± 4.40 R.F.U., respectively; $P = 0.02$ and 0.04), compared to the DS (1.89 ± 0.53 R.F.U.) (Fig. 1C, UP). ROS levels were decreased at the subsequent time points, from 24 to 72 h, although they appeared still significantly higher ($P = 0.03$ and 0.00008), compared to dry seeds (4.68 ± 1.23 R.F.U., 24 h; 3.50 ± 0.86 R.F.U., 48 h; 4.31 ± 0.23 R.F.U., 72 h) (Fig. 1C, UP). The RD stage marks the end of germination and features extensive metabolic changes other than those associated with the pre-germinative metabolism [20]. For this reason, this stage was not considered for ROS measurements. Based on the reported data, the HP72 treatment featured enhanced ROS accumulation within the first 24 h, whereas no consistent changes in ROS pattern were associated with the accelerated germination profile of HP seeds.

Hydropriming induces temporally distinct waves of up-regulation of antioxidant and BER genes

In order to evaluate the effects of the HP72 treatment on the seed antioxidant defense and DNA repair response, the expression profiles of selected genes, known to be involved in the seed pre-germinative metabolism [9,19,20] were investigated using qRT-PCR. Putative orthologs corresponding to *S. melongena* (*Sm*) *SmSOD* (superoxide dismutase), *SmAPX* (ascorbate peroxidase), *SmOGG1* (8-oxoguanine glycosylase/lyase), *SmFPG* (formamidopyrimidine-DNA-glycosylase) and *SmTDP1α* (tyrosyl-DNA phosphodiesterase) were identified as best-hits for *A. thaliana* and *M. truncatula* proteins in the Eggplant Genome Browser [36]. Results of qRT-PCR analyses performed on '67/3' samples (harvested in 2018) collected during the HP72 treatment, and compared to the DS, are shown in Fig. 3A as expression changes of antioxidant and BER genes. Values represent fold changes of transcript levels where FC = primed seed (HP)/dry seed (DS) and FC(HP/DS) values < 1 or > 1 correspond to gene down-

or up-regulation, respectively (Fig. 3A, dashed line). Relative gene expression values are shown in Table S2. When considering the effects of HP72 treatment along the tested time points, compared to DS, the *SmSOD* gene was down-regulated, as indicated by the observed FC(HP/DS) values (< 1) whereas up-regulation of the *SmAPX* gene was evident after 24 h of treatment. Among the tested BER genes, only *SmOGG1* showed early up-regulation at 24 h (Fig. 3A).

Results of *q*RT-PCR analyses performed on the '67/3' samples, both HP and UP seeds, collected during the germination test are shown in Fig. 3B, as expression changes of antioxidant and BER genes. Values represent fold changes of transcript levels where FC = primed seed (HP)/unprimed seeds (UP) and FC(HP/UP) values < 1 or > 1 correspond to gene down- or up-regulation, respectively (Fig. 3B, dashed line). Relative gene expression values are shown in Table S3. When comparing HP seeds subjected to dry-back with UP dry seeds (Fig. 3B, 0 h), the observed FC(HP/UP) values indicated down-regulation of the *SmSOD* gene as well as the HP-dependent up-regulation of *SmAPX* and *SmOGG1* genes. The FC(HP/UP) value of *SmOGG1* significantly increased following dry-back (from 0.80 ± 0.15 at 72 h up to 3.34 ± 0.70), suggesting for enhanced expression during the dehydration step. Besides the early up-regulation of *SmAPX* and *SmOGG1* genes, the impact of the HP treatment on seed imbibition is evidenced by distinct waves of responses. At 2 h, the *SmOGG1* gene was still up-regulated in HP seeds whereas the recorded increase in FC(HP/UP) for the *SmSOD* gene (1.72 ± 0.48), compared to 0 h, highlights the influence of the treatment on this antioxidant component of the eggplant pre-germinative metabolism (Fig. 3B, 2 h). At 4 h, the treatment resulted into a distinctive response of BER genes. Indeed, despite the observed decrease in the FC(HP/UP) value, the *SmOGG1* gene was still up-regulated at this time point. HP72 triggered also the expression of *SmFPG* and *SmTDP1α* genes, based on the estimated FC(HP/UP) values of 2.43 ± 0.81 and 1.40 ± 0.52 , respectively (Fig. 3B, 4 h). At 16 h, a new wave of response was observed featuring up-regulation of the antioxidant gene *SmAPX* with an estimated FC(HP/UP) value of 3.42 ± 2.07 as well as a very strong response of *SmSOD* gene (FC(HP/UP) = 51.07 ± 5.43) (Fig. 3B, 16 h). The slight fluctuations detected at the end of the experiment, at 48 h and 72 h, and the changes observed at the radicle protrusion stage (RD) showed that the impact of HP was apparently diminished when approaching the end of germination. To better clarify the dynamics of the seed response resulting from the concomitant changes in the *SmOGG1* mRNA and ROS levels, the ratio between the *SmOGG1* transcript level and the ROS amount (OGG1:ROS) was calculated. A significant increase in

the OGG1:ROS ratio occurred in HP seeds, compared to UP, during early imbibition (from 0 to 4 h) (Fig. S1, HP). The observed differences in gene expression and ROS patterns suggest that the temporal 'window' spanning the first hours of imbibition provides information useful to predict the efficacy of hydropriming. To further explore the potential of this 'window', ROS profiles and gene expression analyses were carried in the '67/3' seed lots previously characterised, showing variable quality.

Correlation between seed quality and ROS profiles

ROS levels were assessed in high- and low-quality '67/3' seed lots. The experimental design is shown in Fig. 4A. For each lot, the unprimed samples included dry seeds (UP) as well as rehydrated seeds collected at 2 h of imbibition (UP2) while the primed samples included seeds that underwent the HP72 treatment followed by dry-back (HPDB) and seeds collected at 2 h of imbibition (HPDB2). The sampling was limited to this early time point with the aim to speed-up the prediction of the seed response and reduce costs, in view of possible future applications. Comparable ROS levels were detected in the UP seeds of high-quality' lots (1.54 ± 0.51 R.F.U., SL1; 1.37 ± 0.66 R.F.U., SL3; 1.73 ± 0.85 R.F.U., SL5). At 2 h of imbibition (UP2) ROS levels were decreased in SL1 and SL3 (0.68 ± 0.23 and 0.44 ± 0.01 R.F.U., respectively) compared to dry seeds. A similar trend was observed also in SL5 (Fig. 4B). The impact of HP72 treatment on the high-quality seed lots was not apparently relevant since ROS levels detected in both HPDB and HPDB2 samples did not exceed the amount measured in the UP seeds whereas the low-quality seed lots showed a significant (P = 0.02, 2015 and P = 0.03, 2017) enhancement following the HP72 treatment (Fig. 4B). SL2 and SL4 did not show high germinability. In the case of SL2, no germination at all was observed while the HP72 treatment induced germination, although at a low percentage (55.56 ± 21.42 %) (Table 2). In the other case (SL4), the seed lot showed suboptimal germination (77.78 ± 19.24 %) and there was no benefit from hydropriming (Table 2). It is possible that the poor-quality germination performance of SL2 and SL4 correlates with high ROS levels. On the other hand, the ROS profiles in SL1, SL3, and SL5 should reflect their high-quality germination performance.

Impact of hydropriming on the expression of antioxidant/BER genes in high- and low-quality eggplant seed lots

In order to correlate ROS profiles with gene expression patterns, *q*RT-PCR analyses were carried on the different '67/3' seed lots according to the experimental design (Fig. 2A) and results are shown in Fig. 5A . Values represent fold changes of transcript levels where FC = primed and dried-back seed (HPDB)/dry seed (DS) and FC(HPDB/DS) values < 1 or > 1 correspond to gene down- or up-regulation, respectively (Fig. 5A, dashed line). Relative gene expression values are reported in Table S4. Apparently the HP72 treatment did not impact the expression profile of *SmSOD* and *SmAPX* genes in the high-quality seed lots. As for the low-quality samples, up-regulation of *SmSOD* gene was detected in one lot (SL2, FC(HPDB/DS) = 3.44 ± 0.35) (Fig. 5A). In the case of DNA repair genes, the HP72 treatment resulted in *SmOGG1* up-regulation only in SL1 and SL5, as shown by the FC(HPDB/DS) values (4.86 ± 0.20 and 4.17 ± 1.29, respectively) whereas no substantial changes were detected in SL2, SL3 and SL4. The *SmFPG* gene showed a seed lot-dependent expression profile in the high-quality samples and no significant changes occurred in the low-quality seeds. The effects of HP72 treatment on the *Tdp1α* gene expression were heterogeneous across both the high-quality and low-quality seed lots (Fig. 5A). Results of the *q*RT-PCR analysis performed on imbibed seeds are shown in Fig. 5B. Values represent fold changes of transcript levels where FC = primed seed (HP_{2h})/unprimed seed (UP_{2h}) and FC(HP_{2h} /UP_{2h}) values < 1 or > 1 correspond to gene down- or up-regulation, respectively (Fig. 5B, dashed line). Relative gene expression values are reported in Table S5. At 2 h of imbibition the hydropriming did not trigger substantial up-regulation of the *SmSOD* gene in almost all the seed lots (Fig. 5B), however up-regulation of *SmAPX* gene was evident in both high-quality and low-quality seed lots. The estimated FC(HP_{2h}/UP_{2h}) values were 9.23 ± 2.69 (SL1), 4.13 ± 1.18 (SL2), 1.61 ± 0.47 (SL3), 2.66 ± 1.59 (SL4), and 0.83 ± 0.16 (SL5). Up-regulation of *SmOGG1* gene also occurred in almost all the tested seed lot except for SL1. As shown in Fig. 5B, the estimated FC(HP_{2h}/UP_{2h}) values were 2.13 ± 0.54 (SL2), 2.98 ± 0.66 (SL3), 1.55 ± 0.40 (SL4), and 4.46 ± 0.82 (SL5). Following imbibition, the *SmFPG* gene was down-regulated in all the tested seed lots. The *Tdp1α* gene expression showed fluctuations in all the tested seed lots (Fig. 5B).

Discussion

In this work, we provide for the first time a molecular landscape of the pre-germinative metabolism in primed eggplant seeds, by integrating the expression patterns of antioxidant/DNA repair genes that play pivotal roles during imbibition with free radical profiles. The first aim of the investigation was the identification of hallmarks useful to

monitor the seed response to priming and discriminate between high- and low-quality lots. The HP72 treatment hereby developed accelerates germination in the *S. melongena* inbred line '67/3' resulting in beneficial effects. Although these findings provide useful technological solutions to improve seed production, a better understanding of their molecular implications in the context of the pre-germinative metabolism will lead to more consistent and rationale advancements not only in eggplant but also in other valuable horticultural crops [4,7].

The biochemical events triggered by water up-take during seed imbibition are associated with ROS accumulation, e.g. the respiratory burst within mitochondria generates the superoxide anion that is dismutated to hydrogen peroxide as well as the extracellular peroxidases that produce superoxide radicals then converted to hydrogen peroxide [38]. ROS are source of oxidative damage that is counteracted by the seed through the activation of the antioxidant defence and DNA repair, however these radical species also contribute to germination since specific oxidative modifications of storage proteins act as signals to trigger reserves mobilization[39]. The eggplant seeds revealed a boost in ROS levels at 24 h of hydropriming, followed by a progressive decrease throughout the treatment and the levels detected after dry-back were even lower compared to the dry seed. Accordingly, Kubala et al.[40] found enhanced ROS levels in rape (*Brassica napus* L.) seeds subjected to osmopriming and a decrease during the subsequent drying. However, high ROS amounts were measured during the germination tests in both unprimed and primed *Brassica* seeds, differently from what observed in eggplant. Primed seeds are considered as an advanced developmental stage[41] and it is possible that the ROS peak detected during hydropriming simply anticipates the physiological boost that takes place at 8 h of imbibition in the unprimed seeds.

Changes in free radicals during hydropriming, dry-back and subsequent germination are determined by the ROS-scavenging activity of antioxidant enzymes, among which ascorbate peroxidase and superoxide dismutase. Species-specific dynamics in ROS production and activity of scavenging enzymes during germination have been described, suggesting that the overall interplay between these two players can be tightly rearranged [38]. The up-regulation of *SmAPX* gene provides evidence that eggplant seeds are dealing with the rise in ROS during hydropriming. On the other hand, it is possible that the down-regulation of *SmSOD* gene reflects a crucial step in the pre-germinative metabolism, as highlighted by Oracz et al.[42]. These authors showed that inhibition of SOD activity coupled with the stimulation of ROS-producing enzymes was necessary in sunflower (*Helianthus annuus* L.) seeds to promote ROS-mediated carbonylation of proteins, and then trigger germination. A peak in *SmSOD*

gene expression occurred during post-priming germination of '67/3' seeds, between the 16 h and 24 h time points and this is in agreement with the documented increase in SOD activity of osmoprimed seeds from different crop plants [43,44].

Maintenance of genome integrity is another big challenge for the seed [13] and the controlled rehydration carried through priming promotes the cellular repair activities including the removal of DNA lesions [40,45]. As for eggplant, among the DNA repair genes hereby tested only *SmOGG1* showed up-regulation during the HP72 treatment, suggesting that repair of oxidative DNA damage was requested particularly within the first 24 h of hydropriming. Later on, a peak in *SmOGG1* gene expression occurred in the primed seeds during the dry-back step, supporting for an active response to genotoxic stress in agreement with Chen et al. [15] who showed that overexpression of *AtOGG1* gene in *Arabidopsis* resulted in lower DNA damage accumulation during the late phase of seed maturation. More recently, Parreira et al.[46] also demonstrated that activation of DNA repair at the onset of the desiccation phase is a molecular signature of seed maturation in common bean (*Phaseolus vulgaris* L.). When considering the response of imbibed seeds, hydropriming resulted in the early up-regulation of *SmOGG1* gene (2 h) as well as in the up-regulation of the *SmFPG* gene was noticed at 4 h and 16 h of imbibition, and there were no evident peaks in *SmTdp1α* gene expression.

A major challenge of seed priming is to design protocols that can work independently of the seed lot quality. Thus, the investigation was extended to eggplant seed lots showing variable germination profiles to test the reproducibility of the HP72 treatment. Furthermore, since the early up-regulation of *SmOGG1* gene in primed seeds at 2 h of imbibition appeared as a promising hallmark, the subsequent investigation on eggplant and wild relatives was restricted within this time point, to verify whether the observed molecular landscape was reproducible both in other eggplant genotypes and seed lots. Indeed, shortcut protocols would be an ideal solution to speed-up the routine assessment of seed quality. A striking difference between the high-quality and low-quality eggplant seed lots was that the primed, dried-back, seeds with low germinability contained enhanced ROS levels, compared to the unprimed, and this profile was maintained also at 2 h of imbibition. This excessive ROS content might be detrimental and impair eggplant germination in the low-quality seed lots. At the level of dry seed, either in presence or absence of hydropriming, the *SmSOD* and *SmAPX* genes showed differences in the expression profiles, resulting in lot-specific responses. The up-regulation of *SmAPX* gene at 2 h of imbibition was a common hallmark of low-quality seed lots, symptomatic of an early antioxidant response triggered by priming that was not present in the

high-quality lots. Despite this, the low-quality eggplant seeds were not able to restore an optimal germination performance. Although lot-specific profiles were observed, the level of *SmOGG1* transcript was in most cases higher in the high-quality seeds, compared to the low quality ones. Overall, the reported data highlight the complexity of the molecular landscape depicted by the expression profiles of *SmOGG1*, *SmFPG*, and *SmTDP1α* genes in each specific seed lot. Looking at the cumulative effect of the gene expression profiles during hydropriming and subsequent germination as well as across lots, it seems that the repair machinery of the eggplant seed is endowed with a remarkable capacity to adapt to the changes induced by priming in the cell metabolism and physiology.

Another intriguing question would be to what extent the knowledge gained in a domesticated crop, as eggplant, can be translated to its wild relatives to speed up their use in breeding programmes or other agronomical applications.

Conclusion

This study explored for the first time the dynamics of the pre-germinative metabolism in eggplant, a valuable horticultural crop, comparing the impact of hydropriming *versus* physiological conditions, and looking for useful molecular hallmarks within a set of antioxidant and DNA repair genes that play a pivotal role during early seed imbibition. The investigation also faced the issues of seed quality in lots with variable germinability. The plasticity of the seed pre-germinative metabolism, stimulated by priming, imposes a plethora of heterogeneous molecular responses, such as those disclosed in the present work, providing a valuable contribution to fill the current knowledge gap on the molecular bases of seed priming. On the other hand, this heterogeneity stands up as an hindrance to the search for molecular markers allowing a quick diagnostics of seed quality. As a complex trait, the response of seeds to priming is difficult to dissect. The temporal sequence of molecular events influenced by environmental and genetic factors that come along with the treatment has to be elucidated. Even more difficult it will be to clarify how these events are coordinated to ensure the seed quality. The information hereby gathered will help to establish future research work dealing with other basic aspects of the DNA damage response in horticultural crops, especially Solanaceae, and their wild relatives that face serious seed quality issues. As for the specific case of eggplants, a larger screening, at the level of seed lots and plant genotypes, will be necessary to accomplish the several open questions that still prevent priming to become a reproducible and reliable technique and support effective agricultural strategies.

Materials and methods

Seeds, germination tests and priming

Fresh seeds of *Solanum melongena* L. (inbred line '67/3' developed by Dr. Giuseppe Leonardo Rotino at CREA-GB, Montanaso Lombardo) were extracted from physiologically ripe fruits produced by plants cultivated in an open field at CREA-GB in Montanaso Lombardo (LO, Italy). For germination tests, seeds were transferred to Petri dishes (diameter 90 mm) containing two filter papers moistened with 2.5 mL of H_2O, sealed and kept in a growth chamber at 22°C under light conditions with photon flux density of 150 µmol m^{-2} s^{-1}, photoperiod of 16/8 h and 70-80% relative humidity. Seeds with protrusion of the primary radicle were considered germinated and counted every day after imbibition. Germination parameters, calculated as described by Ranal and Garcia de Santana[37] are listed in Table S6. For priming, 45 seeds (15 seeds for each replicate) were soaked at 24°C for the indicated time (24 h, 48 h, 72 h, and 96 h) in 400 ml H_2O under aeration produced by a Wave Air Pump Mouse 2 Beta aerator (De Jong Marinelife B.V., The Netherlands) with the following parameters: 220-240 V, 50 Hz, 2.3 W, out put 1.8 l min^{-1}, pressure 0.012 MPa. For dehydration (Dry-Back, DB) primed seeds were transferred into glass tubes, placed between two cotton disks, covered with silica beads (disidry® Orange Silica Gel, The Aerodyne, Florence, Italy) with a seed: silica ratio of 1 : 10, and kept at 24-25°C. Seed fresh weight was monitored every 15 min until the weight of dry seed was reached. Germination tests were carried out with UnPrimed (UP) and primed seeds as follows. For each treatment, three independent replications with 20 seeds per replication were analysed. Seeds and seedlings were harvested at the indicated time points, the fresh weight was measured and samples were frozen using liquid N_2 and stored at -80°C for subsequent molecular analyses.

ROS detection

The fluorogenic dye 2',7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, Milan Italy) was used to quantify ROS (reactive oxygen species) levels in dry and imbibed seeds. Following deacetylation by cellular esterases, the dye is converted to a non-fluorescent molecule which is subsequently oxidized by ROS into the highly fluorescent 2',7'-dichlorofluorescein. DFC can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm, respectively. The assay was carried as described by Macovei et al.[47], with the following modifications. *S. melongena* seeds were

collected at the indicated time points and dried on filter paper. Samples (three seeds per time point) were incubated for 15 min with 50 µl of 10 µM DCFH-DA and subsequently fluorescence at 517 nm was determined using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia), setting the program for one cycle of 30 s at 25°C. As negative control, a sample containing only DCFH-DA was used to subtract the baseline fluorescence. Relative fluorescence was calculated by normalizing samples to controls and expressed as Relative Fluorescence Units (R.F.U.).

RNA extraction, cDNA synthesis and quantitative real-time polymerase chain reaction

RNA isolation was carried out using the TRIZOL® Reagent (Fisher Molecular Biology, Trevose, U.S.A.) according to the supplier's indications. cDNAs were obtained using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Milan, Italy) according to the manufacturer's suggestions. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with the Maxima SYBR Green qPCR Master Mix (2X) (Thermo Fisher Scientific) according to supplier's indications, using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics Pty Ltd). Amplification conditions were as follows: denaturation at 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 30 s and 72°C for 30 s. Oligonucleotide primers were designed using the Real-Time PCR Primer Design program Primer3Plus (https://primer3plus.com) from GenScript and further validated through the online software Oligo Analyzer (https://eu.idtdna.com/calc/analyzer) (Table S7). Quantification was carried out using *SmGADPH* (glyceraldehyde 3-phosphate dehydrogenase, Accession N° AB110609.1) and *SmAPRT* (adenine phosphoribosyl transferase, Accession N° JX448345.1) [48,49] as reference genes for the experimental conditions (treated *versus* untreated) used in this work. Selection was performed using the GeNorm algorithm (https://genorm.cmgg.be) (Supplementary Information, Fig. S2). The raw, background-subtracted fluorescence data provided by the Rotor-Gene 6000 Series Software 1.7 (Corbett Robotics) was used to estimate PCR efficiency (E) and threshold cycle number (C_t) for each transcript quantification. The Pfaffl method[50], was used for relative quantification of transcript accumulation and statistical analysis was performed with REST2009 Software V2.0.13 (Qiagen GmbH, Hilden, Germany). The following genes were tested. *SmOGG1*(8-oxoguanine glycosylase/lyase, SMEL_004g210790.1), *SmFPG* (formamidopyrimidine DNA glycosylase, SMEL_003g194660.1), *SmTDP1α* (tyrosyl-DNA phosphodiesterase 1α,

SMEL_003g171200.1), *SmAPX* (ascorbate peroxidase, SMEL_006g245760.1.01), *SmSOD* (superoxide dismutase, SMEL_001g139700.1.01).

Statistical analysis

The effects of priming *versus* unprimed control in terms of germination percentage, days, and their interaction were analysed using Two-way ANOVA (Analysis of Variance) ('*' P < 0.05; '**' P < 0.01, '***' P < 0.001, '****' P < 0.0001) carried out with the statistical software GraphPad Prism 8 (GraphPad Software Inc., California). Comparison between unprimed control and different priming treatments were carried out as follows. For each treatment, three biological replicates were considered. Means were compared using the Post-Hoc Tukey´s HSD (Honest Significant Difference) test. Means with a significance value lower than 0.05 ($P \leq 0.05$) were considered statistically different. Statistical analysis of phenotyping data and qRT-PCR data was performed using the Student´s *t*-test. Asterisks indicate statistically significant differences determined using Student's *t*-test ('*' $P < 0.05$; '**' $P < 0.01$, '***' $P < 0.001$). The efficacy of treatment *versus* unprimend control was carried out using Mann-Withney test ('*' $P < 0.05$; '**' $P < 0.01$, '***' $P < 0.001$).

Conflict of interest

Authors declare no conflict of interest.

Author contributions

AB, CF, LB, LT, GLR conceived and designed the study. CF, LB, LT, VO, AP performed the experiments. AB, CF, LB wrote the manuscript. LT, GLR, AM, reviewed the manuscript. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by by CARIPLO Foundation in the frame of the WAKE-APT project (Code 2016-0723) ('Seed Wake-up with Aptamers: a New Technology for Dormancy Release and Improved Seed Priming') and by the Italian Ministry of Education, University and Research (MIUR): Dipartimenti di Eccellenza Program (2018–2022) - Dept. of Biology and Biotechnology "L. Spallanzani", University of Pavia (to C. F., A.P., A.M., A.B.). C. F., L.B., and A.P. have been awarded by a Research Fellowship, respectively, from CARIPLO

Foundation in the frame of the WAKE-APT project (Code 2016-0723). Authors thanks Dr. Susana De Sousa Araújo for the helpful discussion.

References

1. Heydecker, W., Higgins, J., Gulliver, R.L. Accelerated germination by osmotic seed treatment. Nature 246, 42-46 (1973).
2. Bailly, C., Benamar, A., Corbineau, F., Côme, D. Free radical scavenging as affected by accelerated ageing and subsequent priming in sunflower seeds. Physiol. Plant. 104, 646-52 (1998).
3. Ventura, L., Donà, M., Macovei, A., Carbonera, D., Buttafava, A., et al. Understanding the molecular pathways associated with seed vigor. Plant Physiol. Biochem. 60, 196-206 (2012).
4. Paparella, S., Araujo, S.S., Rossi, G., Wijayasinghe, M., Carbonera, D. et al. Seed priming: state of the art and new perspectives. Plant Cell Rep. 34, 1281-1293 (2015).
5. Wojtyla, L., Lechowska, K., Kubala, S., Garnczarska, M. Different modes of hydrogen peroxide action during seed germination. Front. Plant Sci. 7, 66 (2016).
6. Farooq, M., Gogoi, N., Hussain, M., Barthakur, S., Paul, S., et al. Effects, tolerance mechanisms and management of salt stress in grain legumes. Plant Physiol. Biochem. 118,199-217 (2017).
7. Macovei, A., Pagano, A., Leonetti, P., Carbonera, D., Balestrazzi, A., et al. Systems biology and genome-wide approaches to unveil the molecular players involved in the pre-germinative metabolism: implications on seed technology traits. Plant Cell Rep. 36, 669-688 (2017).
8. Gallardo, K., Job, C., Groot, S.P., Puype, M., Demol, H., et al. Proteomic analysis of *Arabidopsis* seed germination and priming. Plant Physiol. 126, 835-848 (2001).
9. Balestrazzi, A., Confalonieri, M., Macovei, A., Carbonera, D. Seed imbibition in *Medicago truncatula* Gaertn.: expression profiles of DNA repair genes in relation to PEG-mediated stress. J. Plant Physiol. 168, 706-713 (2011).
10. Bailly, C. Active oxygen species and antioxidants in seed biology. Seed Sci. Res. 14, 93-107 (2004).
11. Kranner, I., Minibayeva, F.V., Beckett, R.P., Seal, C.E. What is stress? Concepts, definitions and applications in seed science. New Phytol. 188, 655-673 (2010).

12. Bray, C.M., West, C.E. DNA repair mechanisms in plants: crucial sensors and effectors for the maintenance of genome integrity. New Phytol. 168, 511-528 (2005).
13. Waterworth, W.M., Bray, C.M., West, C.E. Seeds and the art of genome maintenance. Front. Plant Sci. 10, 706 (2019).
14. Macovei, A., Balestrazzi, A., Confalonieri, M., Faè, M., Carbonera, D. New insights on the barrel medic *MtOGG1* and *MtFPG* functions in relation to oxidative stress response *in planta* and during seed imbibition. Plant Physiol. Biochem. 49, 1040-1050 (2011).
15. Chen, H., Chu, P., Zhou, Y., Li, Y., Liu, J., et al. Overexpression of AtOGG1, a DNA glycosylase/ AP lyase, enhances seed longevity and abiotic stress tolerance in Arabidopsis. J. Exp. Bot. 63, 4107-4121 (2012).
16. Cordoba-Canero, D., Roldan-Arjona, T., Ariza, R.R. Arabidopsis ZDP DNA 30-phosphatase and ARP endonuclease function in 8-oxoG repair initiated by FPG and OGG1 DNA glycosylases. Plant J. 79, 824-834 (2014).
17. Lebedeva, N.A., Rechkunova, N.I., Lavrik, O.I. AP-site cleavage activity of tyrosyl-DNA phosphodiesterase 1. FEBS Lett. 585, 683-686 (2011).
18. Macovei, A., Balestrazzi, A., Confalonieri, M., Carbonera, D. The *Tdp1* (Tyrosyl-DNA phosphodiesterase) gene family in barrel medic (*Medicago truncatula* Gaertn.): bioinformatic investigation and expression profiles in response to copper- and PEG-mediated stress. Planta 232, 393-407 (2010).
19. Pagano, A., Araújo, S., Macovei, A., Leonetti, P., Balestrazzi, A. The seed repair response during germination: disclosing correlations between DNA repair, antioxidant response, and chromatin remodeling in *Medicago truncatula*. Front. Plant Sci. 8,1972 (2017).
20. Pagano, A., De Sousa Araújo, S., Macovei, A., Dondi, D., Lazzaroni, S., et al. Metabolic and gene expression hallmarks of seed germination uncovered by sodium butyrate in *Medicago truncatula*. Plant Cell Environ. 42, 259-269 (2019).
21. Mennella, G., Lo Scalzo, R., Fibiani, M., D'Alessandro, A., Francese, G., et al. Chemical and bioactive quality traits during fruit ripening in eggplant (*S. melongena* L.) and allied species. J. Agr. Food Chem. 60, 11821-1131 (2012).
22. Gürbüz, N., Uluisik, S., Frary, A., Doganlar, S. Health benefits and bioactive compounds of eggplant. Food Chem. 268, 602-610 (2018).

23. Joshua, A. Seed germination of Solanum incanum: an example of germination problems of tropical vegetable crops. Acta Hortic. 83, 155-162 (1978).
24. Ibrahim, M., Munira, M.K., Kabir, M.S., Islam, A.K.M.S., Miah, M.M.U. Seed germination and graft compatibility of wild Solanum as rootstock of tomato. J. Biol. Sci. 1, 701-703 (2001).
25. Demir, L., Ennis, S., Okyu, G., Matthews, S. Vigour tests for predicting seedling emergence of aubergine (*Solanum melongena* L.) seed lots. Seed Sci. Tech. 33, 481-484 (2005).
26. Adebola, P.O., Afolayan, A.J. Germination responses of *Solanum aculeastrum*, a medicinal species of the Eastern Cape, South Africa. Seed Sci. Technol. 34, 735-740 (2006).
27. Taab, A., Andersson, L. Seasonal changes in seed dormancy of *Solanum nigrum* and *Solanum physalifolium*. Weed Res. 49, 90-97 (2009).
28. Ranil, R.H., Niran, H.M.L., Plazas Ávila, M.D.L.O., Fonseka, R., Hemal Fonseka, H., et al. Improving seed germination of the eggplant rootstock *Solanum torvum* by testing multiple factors using an orthogonal array design. Sci. Horticult. 193, 174-181 (2015).
29. Miceli, A., Sabatino, L., Moncada, A., Vetrano, F., D'Anna, F. Nursery and field evaluation of eggplant grafted onto unrooted cuttings of *Solanum torvum* Sw. Sci. Hortic. 178, 203-210 (2015).
30. Zhang, Y., Liu, H., Shen, S., Zhang, X. Improvement of eggplant seed germination and seedling emergence at low temperature by seed priming with incorporation SA into KNO_3 solution. Front. Agric. China 5, 534-537 (2011).
31. Gomes, D.P., Silva, A.F., Dias, D.C.F.S., Alvarenga, E.M., Silva, L.J., et al. Priming and drying on the physiological quality of eggplant seeds. Horticultura Brasileira 30, 484-488 (2012).
32. Gonzalez, L.M.R. Germination response of eggplant (*Solanum melongena* L.) seeds to different vinegar concentration as seed priming agents. Int. J. Sci. Res. Pub. 5, 1-4 (2015).
33. Mahesh, H.M., Murali, M., Anup Chandra Pal, M., Melvin, P., Sharada, M.S. Salycilic acid seed priming instigates defence mechanisms by inducing PR-proteins in *Solanum melongena* L. upon infection with *Verticillum dahlie* Kleb. Plant Physiol. Biochem. 117, 12-23 (2012).

34. Neto, F.J.D., Dalanhol, S.J., Machey, M., Pimentel, A., Rodrigues, J.D., et al. Effects of plant growth regulators on eggplant seed germination and seedling growth. Australian J. Crop Sci. 11, 1277-1282 (2017).
35. Ali, M., Hayat, S., Ahmad, H., Ghani, M.I., Amin, B., et al. Priming of *Solanum melongena* L. seeds enhances germination, alters antioxidant enzymes, modulates ROS, and improves early seedling growth: indicating aqueous garlic extract as seed-priming bio-stimulant for eggplant production. Appl Sci. 9, 2203 (2019).
36. Barchi, L., Pietrella, M., Venturini, L., Minio, A., Toppino, L., et al. A chromosome-anchored eggplant genome sequence reveals key events in Solanaceae evolution. Sci. Rep. 9, 11769 (2019).
37. Ranal, M.A., Garcia de Santana, D. How and why to measure the germination process? Brazilian J. Bot. 29, 1-11 (2006).
38. Bailly, C., El-Maarouf-Bouteau, H., Corbineau, F. From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. C. R. Biol. 331, 806-814 (2008).
39. Job, C., Rajjou, L., Lovigny, Y., Belghazi, M., Job, D. Patterns of protein oxidation in *Arabidopsis* seeds and during germination. Plant Physiol. 138, 790-802 (2005).
40. Kubala, S., Wojtyla, L., Quinet, M., Lechowska, K., Lutts, S., et al. Enhanced expression of the proline synthesis gene *P5CSA* in relations to seed osmopriming improvement of *Brassica napus* germination under salinity stress. J. Plant Physiol. 183, 1-12 (2015).
41. Chen, K., Arora, R. Priming memory invokes seed stress-tolerance. Environ. Exp. Bot. 94, 33-45 (2013).
42. Oracz, K., El-Maarouf-Bouteau, H., Kranner, I., Bogatek, R., Corbineau, F., et al. The mechanisms involved in seed dormancy alleviation by hydrogen cyanide unravel the role of reactive oxygen species as key factors of cellular signaling during germination. Plant Physiol. 150, 494-505 (2009).
43. Bailly, C., Benamar, A., Corbineau, F., Come, D. Antioxidant systems in sunflower (*Helianthus annuus* L.) seeds as affected by priming. Seed Sci. Res. 10, 35-42 (2000).
44. Rouhi, H.R., Aboutalebian, M.A., Moosavi, S.A., Karimi, F.A., Karimi, F., Saman et al. Change in several antioxidant enzymes activity of Berseem clover (*Trifolium alexandrinum* L.) by priming. Int. J. AgriSci. 2, 273-243 (2012).

45. Sharma, S.N., Maheshwari, A. Expression patterns of DNA repair genes associated with priming small and large chickpea (*Cicer arietinum*) seeds. Seed Sci. Tech. 43, 250-261 (2015).
46. Parreira, J.R., Balestrazzi, A., Fevereiro, P., De Sousa Araujo, S. Maintaining genome integrity during seed development in *Phaseolus vulgaris* L.: evidence from a transcriptomic profiling study. Genes 9, E463 (2018).
47. Macovei, A., Caser, M., Donà, M., Valassi, A., Giovannini, A., et al. Prolonged cold storage affects pollen viability and germination along with hydrogen peroxide and nitric oxide content in *Rosa hybrida*. *Not.* Bot. Horti. Agro. 44, 6-10 (2016).
48. Gantasala, N.P., Papolu, P.K., Thakur, P.K., Kamaraju, D., Sreevathsa, R., et al. Selection and validation of reference genes for quantitative gene expression studies by real-time PCR in eggplant (*Solanum melongena* L). BMC Res. Notes 6, 312 (2013).
49. Barbierato, V., Sala, T., Rinaldi, P., Bassolino, L., Barchi, et al. A spiking strategy facilitates housekeeping selection for RT-qPCR analysis under different biotic stresses in eggplant. Protoplasma 254, 2215-2223 (2017).
50. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45 (2001).

Figure legends

Fig. 1 Hydropriming accelerates germination of '67-3' seeds. For priming, seeds were soaked in H_2O for 24 h, 48 h, 72 h, and 96 h under aeration and subsequently re-dried (dry-back) until the weight of the dry seed was reached. Unprimed (UP) and primed (HP) dry seeds were then immediately used for germination tests. Values are expressed as mean ± SD of three independent replications with 20 seeds for each replication. Statistical analysis was carried out using Two-way ANOVA (F = 45.66; DF = 4; $P = 0.0004$***). Comparison between UP and HP24, HP48, HP72, HP96 were carried out using the Post-Hoc Tukey's HSD test ($P \leq 0.05$) (see Supplementary Information, Table S8)

Fig. 2 Impact of hydropriming on ROS levels in '67/3' seeds. **A**. Experimental design showing the selected time points for molecular analyses. Seeds were collected during priming (HP72 protocol) at 24 h, 48 h, and 72 h, as well as at the end of dry-back (DB, 2 h).

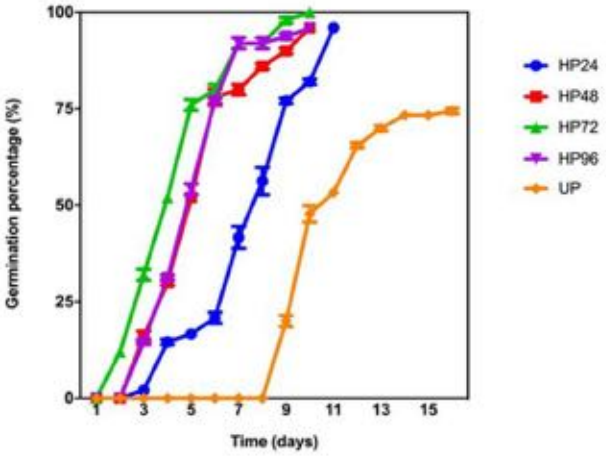
Germination of unprimed (UP) and primed (HP) seeds was monitored by collecting samples throughout imbibition at 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, and 72 h. The last time point, corresponding to the phenological stage of radicle protrusion (RD), was anticipated at 4 days for the primed seeds, compared to unprimed (8 days). ROS levels were measured during hydropriming (**B**) and subsequent germination (**C**) at the selected time points using the DCFH-DA fluorescent dye. Values are expressed as mean ± SD of three independent replications with 20 seeds for each replication. Asterisks indicate statistically significant differences determined using Student's *t*-test ($P < 0.05$). DS, dry seed. R.F.U., relative fluorescence unit. ROS, reactive oxygen species. DCFH-DA, dye 2',7'-dichlorofluorescein diacetate

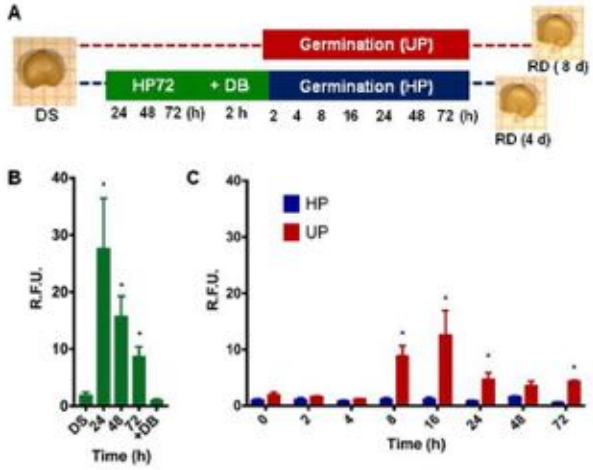
Fig. 3 Impact of hydropriming on the expression profiles of antioxidant and DNA repair genes in '67/3' seeds. **A**. Results of *q*RT-PCR analyses performed on seeds collected during the HP72 treatment (at 24 h, 48 h, and 72 h) and compared to the DS are shown as expression changes of antioxidant and BER genes. Values represent fold changes of transcript levels where FC = primed seed (HP)/dry seed (DS) and FC(HP/DS) values < 1 or > 1 correspond to gene down- or up-regulation, respectively (dashed line). Relative gene expression values are shown in Table S5. **B**. Results of *q*RT-PCR analyses performed on HP and UP seeds collected during the germination test (0 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h, and 72 h) and at the radicle protrusion stage (RD) are shown as expression changes of antioxidant and BER genes. Values represent fold changes of transcript levels where FC = primed seed (HP)/unprimed seeds (UP) and FC(HP/UP) values < 1 or > 1 correspond to gene down- or up-regulation, respectively (dashed line). Relative gene expression values are shown in Table S6. Values are expressed as mean ± SD of three independent replications with 20 seeds for each replication. Asterisks indicate statistically significant differences determined using Student's *t*-test ($P < 0.05$). SOD, superoxide dismutase. APX, ascorbate peroxidase. OGG1, 8-oxoguanine glycosylase/lyase. FPG, formamidopyrimidine DNA glycosylase. TDP1α, tyrosyl-DNA phosphodiesterase 1α

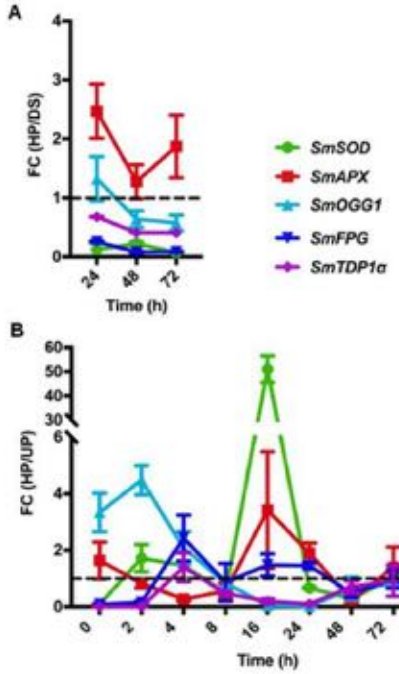
Fig. 4 ROS levels were assessed in the different '67/3' seed lots previously characterised for their germination profiles (high-quality and low-quality lots) and response to hydropriming. **A**. The experimental design is shown: for each lot, the unprimed samples included dry seeds (UP) as well as rehydrated seeds collected at 2 h of imbibition (UP2) while the primed

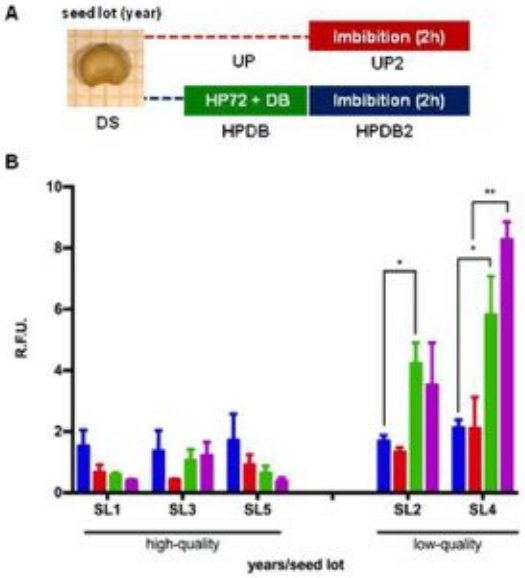
samples included seeds that underwent the HP72 treatment followed by dry-back (HPDB) and seeds collected at 2 h of imbibition (HPDB2). **B.** ROS levels were measured at the selected time points using the DCFH-DA fluorescent dye. Values are expressed as mean ± SD of three independent replications with 20 seeds for each replication. Asterisks indicate statistically significant differences determined using Student's *t*-test ($P < 0.05$). DS, dry seed. R.F.U., relative fluorescence unit. ROS, reactive oxygen species. DCFH-DA, dye 2',7'-dichlorofluorescein diacetate. SL, seed lot

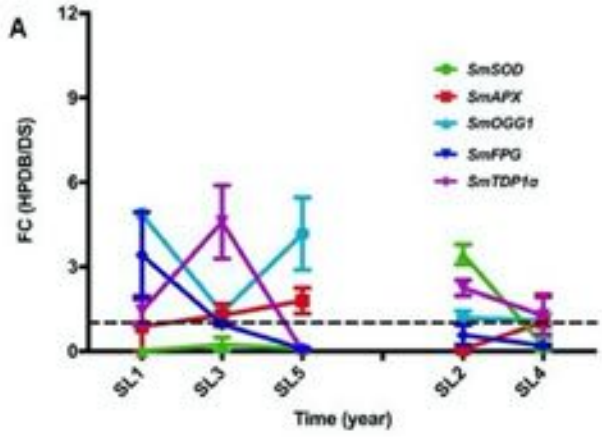
Fig. 5 Impact of hydropriming on the expression profiles of antioxidant and DNA repair genes in different '67/3' seed lots. **A.** Results of *q*RT-PCR analyses performed on seeds collected according to the the experimental design shown in Fig. 4A are reported as expression changes of antioxidant and BER genes. Values represent fold changes of transcript levels where FC = primed and dried-back seed (HPDB)/dry seed (DS) and FC(HPDB/DS) values < 1 or > 1 correspond to gene down- or up-regulation, respectively (dashed line). Relative gene expression values are reported in Table S5. **B.** Results of the *q*RT-PCR analysis performed on imbibed seeds are shown as expression changes of antioxidant and BER genes. Values represent fold changes of transcript levels where FC = primed seed (HP_{2h})/unprimed seed (UP_{2h}) and FC(HP_{2h} /UP_{2h}) values < 1 or > 1 correspond to gene down- or up-regulation, respectively (dashed line). Relative gene expression values are reported in Table S6. Values are expressed as mean ± SD of three independent replications with 20 seeds for each replication. Asterisks indicate statistically significant differences determined using Student's *t*-test ($P < 0.05$). SOD, superoxide dismutase. APX, ascorbate peroxidase. OGG1, 8-oxoguanine glycosylase/lyase. FPG, formamidopyrimidine DNA glycosylase. TDP1α, tyrosyl-DNA phosphodiesterase 1α


Germination percentage (%)
Time (days)
HP24
HP48
HP72
HP96
UP


A
Germination (UP)
RD (8 d)
HP72
+ DB
Germination (HP)
DS
24 48 72 (h)
2 h
2 4 8 16 24 48 72 (h)
RD (4 d)
B
R.F.U.
Time (h)
C
HP
UP
R.F.U.
Time (h)


A
FC (HP/DS)
Time (h)
SmSOD
SmAPX
SmOGG1
SmFPG
SmTDP1α
B
FC (HP/UP)
Time (h)


A
seed lot (year)
Imbibition (2h)
UP
UP2
HP72 + DB
Imbibition (2h)
DS
HPDB
HPDB2
B
R.F.U.
SL1
SL3
SL5
SL2
SL4
high-quality
low-quality
years/seed lot


A
12
9
6
3
0
FC (HPDB/DS)
SmSOD
SmAPX
SmOGG1
SmFPG
SmTDP1a
SL1
SL3
SL5
SL2
SL4
Time (year)

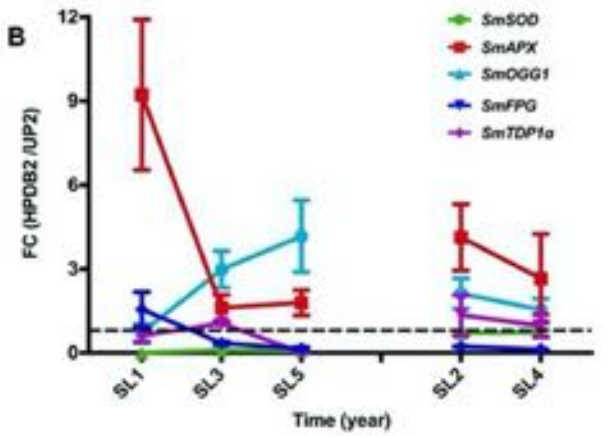

B
12
9
6
3
0
FC (HPDB2 /UP2)
SmSOD
SmAPX
SmOGG1
SmFPG
SmTDP1a
SL1
SL3
SL5
SL2
SL4
Time (year)

Table 1 Germination parameters calculated based on results of germination tests carried out on '67/3'seeds treated with hydropriming (HP) for increasing time (24 h, 48 h, 72 h, and 96 h) and unprimed (UP) seeds. Asterisks indicate statistically significant differences determined using Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$). *G*, Germinability. *MGT*, Mean germination time. *CVG*, Coefficient of velocity of germination. *MGR*, Mean germination rate. *U*, Uncertainty. *Z*, Synchronization index. h, hours

Treatment	*G* (%)	*MGT* (days)	*CVG* (%)	*MGR* (day^{-1})	*U* (bit)	*Z* (unit less)
HP 24 h	100.0 ± 0.00*	6.56 ± 0.05**	288.00 ± 3.41*	$3.4 \times 10^{-3} \pm 7.07 \times 10^{-5}$*	2.08 ± 0.00	0.26 ± 0.00
HP 48 h	100.0 ± 0.00*	5.78 ± 0.08**	339.76 ± 6.29**	$2.9 \times 10^{-3} \pm 5.65 \times 10^{-5}$*	1.70 ± 0.38	0.34 ± 0.10
HP 72 h	100.0 ± 0.00*	4.48 ± 0.00***	468.00 ± 0.15*	$2.1 \times 10^{-3} \pm 0.00$*	1.22 ± 0.20	0.31 ± 0.30
HP 96 h	98.0 ± 2.80*	4.77 ± 0.18*	426.02 ± 33.09	$2.3 \times 10^{-3} \pm 1.98 \times 10^{-4}$**	2.01 ± 0.38	0.25 ± 0.05
UP	74.4 ± 1.57	10.58 ± 0.01	220.38 ± 6.55	$4.5 \times 10^{-3} \pm 1.41 \times 10^{-5}$	2.22 ± 0.02	0.23 ± 0.01

Table 2 Germination parameters calculated for different '67/3' seed lots collected during 2015 (SL2), 2016 (SL3), 2017 (SL4), 2018 (SL5) and treated with hydropriming (HP72 protocol). Asterisks indicate statistically significant differences determined using Student's *t*-test (*, $P < 0.05$). *G*, Germinability. *MGT*, Mean germination time. *CVG*, Coefficient of velocity of germination. *MGR*, Mean germination rate. *U*, Uncertainty. *Z*, Synchronization index. UP, unprimed seeds. SL, seed lot

Seed lot	*G* (%)		*MGT* (days)		*CVG* (%)		*MGR* (day^{-1})		*U* (bit)		*Z* (unit less)	
	UP	HP72	UP	HP72	UP	HP72	UP	HP	UP	HP72	UP	HP72
SL2	0	55.56 ± 21.42	0	6.52 ± 0.86	0	59.00 ± 42.48	0	0.030 ± 0.02	0	1.61 ± 0.87	0	0.340 ± 0.28
SL3	100.00 ± 0.00	100.00 ± 0.00	8.95 ± 0.91	6.20 ± 0.00*	76.34 ± 17.85	148.70 ± 0.94*	0.005 ± 0.003	0.007 ± 0.00	2.39 ± 0.05	2.04 ± 0.16*	0.18 ± 0.00	0.230 ± 0.01*
SL4	77.78 ± 19.24	74.13 ± 11.43	28.56 ± 1.34	25.31 ± 0.79*	62.70 ± 12.00	65.00 ± 10.59	0.005 ± 0.003	0.016 ± 0.00	2.53 ± 0.14	2.74 ± 0.18	0.19 ± 0.02	0.077 ± 0.04
SL5	97.78 ± 3.85	100.00 ± 0.00	11.05 ± 1.10	8.84 ± 0.87	42.03 ± 9.86	77.61 ± 17.23*	0.025 ± 0.006	0.013 ± 0.00	2.50 ± 0.29	2.57 ± 0.11	0.15 ± 0.05	0.136 ± 0.008

Editorial Office <genes@mdpi.com>
a Anca, me, Ajay, Anjali, Alma, Vishal

inglese > italiano Traduci messaggio

Dear Dr. Macovei,

Thank you very much for uploading the following manuscript to the MDPI submission system. One of our editors will be in touch with you soon.

Journal name: Genes
Manuscript ID: genes-658676
Type of manuscript: Communication
Title: Priming improves Medicago truncatula seed germination in soil and upregulates specific genes used as indicators of seed quality
Authors: Chiara Forti, Ajay Shankar, Anjali Singh, Alma Balestrazzi, Vishal Prasad, Anca Macovei *
Received: 18 November 2019
E-mails: chiara.forti01@universitadipavia.it, shankarajay17@gmail.com, singh.anjali241@gmail.com, alma.balestrazzi@unipv.it, vp.iesd@bhu.ac.in, anca.macovei@unipv.it
Submitted to section: Plant Genetics and Genomics, https://www.mdpi.com/journal/genes/sections/p_g_g
Abiotic Stress in Plants: Current Challenges and Perspectives https://www.mdpi.com/journal/genes/special_issues/abiot_stress_plants

You can follow progress of your manuscript at the following link (login required):
https://susy.mdpi.com/user/manuscripts/review_info/316379a895dab379c337c379fa8b1f90

genes

MDPI

Communication

Priming improves *Medicago truncatula* seed germination in soil and upregulates specific genes used as indicators of seed quality

Chiara Forti[1], Ajay Shankar[2], Anjali Singh[2], Alma Balestrazzi[1], Vishal Prasad[2], Anca Macovei[1,*]

[1] Department of Biology and Biotechnology 'L. Spallanzani', University of Pavia, via Ferrata 9, 27100-Pavia, Italy; chiara.forti01@universitadipavia.it, alma.balestrazzi@unipv.it

[2] Institute of Environment and Sustainable Development, Banaras Hindu University, Varanasi 221005, India; shankarajay17@gmail.com, singh.anjali241@gmail.com, vp.jesd@bhu.ac.in

* Correspondence: anca.macovei@unipv.it; Tel.: +39-0382-986580

Academic Editor: name

Received: date; Accepted: date; Published: date

Abstract: Seed germination is a critical parameter for the successful development of sustainable agricultural practices. While seed germination is impaired by environmental constraints emerging from the climate change scenario, several types of simple procedures, known as priming, can be used to enhance it. Seed priming is defined as the process of regulating seed germination by managing a series of parameters during the initial stages of germination. Hydropriming is a highly accessible and economic technique that involves soaking of seeds in water followed by drying, whilst biopriming refers to the inoculation with beneficial microorganism. The present study aims to investigate whether hydropriming and biopriming could enhance seed germination. The germination of *Medicago truncatula* seeds exposed to different time points of hydropriming (2 h, 4 h) and/or bacterial isolates (*Bacillus* spp.) has been followed in trays containing two types of *in situ* agricultural soils collected from the Varanasi region, for a two-week period. This region is believed to be contaminated by solid waste from a nearby power plant. Phenotypic parameters had been monitored and compared to find the most appropriate combination of treatments. Additionally, qRT-PCR was used to evaluate the expression levels of specific genes used as molecular indicators of seed quality. The results show that, while hydropriming significantly enhanced seed germination percentage, biopriming resulted in improved seedling development, represented by increased biomass rather than seedling length. At a molecular level, this is reflected by the upregulation of genes involved in DNA damage repair and antioxidant defence.

Keywords: Gene expression; Germination; *Medicago truncatula*; Seed priming; Soil contamination

1. Introduction

Seed germination is a critical parameter for the successful development of both cultivated crops and wild species. While seed germination is impaired by certain conditions (soil contamination, extreme temperatures, pathogens), several types of simple procedures known as priming can be used to enhance it. Seed priming is defined as the process of regulating seed germination by managing parameters like temperature or seed moisture content during the initial stages of germination [1]. By doing so, the priming techniques bring the seed closer to the point of germination. During priming, seeds are advanced to an equal stage of the germination process to enable fast and uniform emergence when planted. Depending on plant species, seed morphology and physiology, different priming treatments can be applied, triggering the so-called 'pre-germinative metabolism' [2]. This is characterized by rapid water uptake, which in turn activates a series of dynamic biochemical events such as the use of reserve storage compounds (lipids,

carbohydrates, proteins), production of reactive oxygen species (ROS) and subsequent activation of antioxidant enzymes, DNA damage sensing and repair [3]. Indeed, intense DNA repair, essential for *de novo* DNA synthesis in embryo cells [4], and enhanced antioxidant functions are the prerequisites of a successful germination process, and can be used as molecular markers of seed vigour [5-9].

Priming techniques are usually applied during the first phase of germination and are dedicated to accelerate and uniformize the progression of seed through the germination phases. Several seed priming techniques are being currently used; these include treatments with physical (irradiation, ultrasound, magnetic fields), chemical (salts, phytohormones, aptamers), or biological (bacteria, fungi) agents [2, 10]. Among these, hydropriming is a technique for initiating germination (without the emergence of the radicle) that involves soaking of seeds in water followed by drying. Hydropriming allows the seeds to quickly reach a high level of moisture with a constant supply of oxygen, thus increasing the level of metabolites associated with the germination process. This form of hydration followed by seed drying is highly accessible and economical. Seed hydration treatments have proven to be successful and effective in germinating seeds under a variety of stress conditions [11-15]. Biopriming, on the other hand, involves the use of beneficial soil microorganisms that can act as biofertilizers and promote seedling growth by synthesizing plant hormones or increasing nutrients uptake from the soil [16-19]. Hence, an interesting aspect of seed priming relates to the boost of seed germination and seedling establishment under non-optimal conditions. Several studies reported that seed priming can alleviate stress levels in seedlings and this can result in better productivity levels under environmental constraints [13, 19-22].

Soil pollution poses a worrisome threat to agricultural productivity, food safety, and human health. It refers to the presence of chemical substances at higher than normal concentrations that have adverse effects on living organisms. The Status of the World's Soil Resources Report (SWSR) identified soil pollution as one of the main threats affecting global and agricultural ecosystems [23]. This is also the particular case of India as a developing country where industrialization has become heavily intensified and the contamination of agricultural soils increased substantially [24-26]. Contamination of agricultural lands is a major concern for agriculture since certain contaminants can be easily taken up by plant roots and can be translocated into aerial parts, resulting in inhibition of plant growth and, in extreme cases even causes plant death [27, 28].

To evaluate the efficiency of hydropriming and biopriming techniques to enhance seed germination, we used the model legume *Medicago truncatula*, belonging to the *Fabaceae* family. Economically, legumes represent the second most important family of crop plants after *Poaceae* (grass family), accounting for approximately 27% of the world's crop production [29]. The choice of plant species is based on both the economic relevance of legumes in agriculture, as *Medicago* species are mainly used for forage crops, as well as on the fact that the unique property of legumes to develop symbiosis with nitrogen-fixing bacteria could further aid in soil remediation. Primed and non-primed seeds were grown in trays containing two different agricultural soils collected from the Varanasi region and the efficiency on the treatments was evaluated based on phenotypic (germination parameters and seedling biometric measurements) and molecular (expression profiles of genes involved in DNA repair and antioxidant response) aspects. Investigating the expression profiles of specific genes that can be used as indicators of seed quality and seeding growth [5-8] is intended to speed up the selection of high-quality seed lots. Improving seed germination on local agricultural soils using easy and economically accessible methods will upgrade the livelihood of many small farmers.

2. Materials and Methods

2.1. Plant Material and Treatments

The *Medicago truncatula* (Jemalong commercial variety) seeds used in this study were kindly supplied by Fertipeado L.d.a (Portugal). The seeds were subjected to hydropriming and biopriming and grown in trays containing two types of agricultural soils (herein denominated as Soil_A and

Soil_B) collected from the Varanasi agricultural area (namely Karsada), for a time period of two weeks. Both soil types were regionally collected *in situ* from the field and no additional treatments were implemented. The soil characteristics are presented in **Table 1**. The two types of soil mainly differ in their content of total dissolved solids (TDS) based on their vicinity to the power plant at Karsada. As the first true trifoliate leaves start to appear after the 7th day, the 14th day after sowing was selected to have fully developed seedlings in all treatments.

Table 1. Characteristics of used soils. EC, electric conductivity; TDS, content of total dissolved solids.

Sample	pH	EC (ds/m)	Salinity (ppt)	TDS (ppm)
Soil_A	8.1	0.15	0.07	82.9
Soil_B	8.23	0.2	0.09	103

Hydropriming was carried out in a bottle containing purified water under continuous aeration for 2 h (HP2) and 4 h (HP4) (**Figure 1a**). The aeration system was constituted by a *Wave* Air Pump Mouse 2 Beta aerator (De Jong Marinelife B.V., The Netherlands) setup with the following parameters: 220-240 V, 50 Hz, 2.3 W, output 1.8 L min^{-1}, pressure 0.012 MPa. A non-primed control (HP0) was also used.

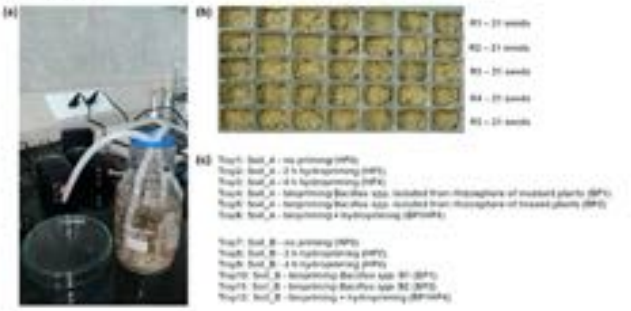


Figure 1. Experimental design and working system. **(a)** Hydropriming treatment of *M. truncatula* seeds. **(b)** Germination tray, containing five replicates of 21 seeds each. **(c)** Treatment labels.

Biopriming was performed with two formulations of bacterial cultures prepared by mixing 1×10^8 cells in 2 g of talc (carrier material) (CDH, India). The seeds were initially treated with jaggery syrup as a standard procedure that helps the adhesion of bacteria to the seed surface. Subsequently, the treated seeds were incubated for about 16 h at approximately 25–30°C. The bacterial formulations contained two different strains of *Bacillus* spp. isolated from mustard (*Brassica juncea*) rhizosphere (BP1) and linseed (*Linum usitatissimum*) rhizosphere (BP2), respectively. Both strains tested positive for phosphate solubilization, production of indoleacetic acid and siderophores (Prasad, personal communication). An additional treatment was performed by combining 4 h of hydropriming and the BP1 strain (hereby denominated as BP1HP4).

Control and primed seeds were sown in germination trays (**Figure 1b**) kept outside in the open area of IESD, BHU, Varanasi, to simulate local climatic conditions. The experiment was performed in October 2018, when day/night temperature ranged between 28-31/20-24°C and the relative

humidity was around 83%. Each tray row represented experimental replicates (R1-R5) containing 21 seeds, and three seeds per square, respectively. Other two independent experiments with the same setup were performed in August 2019 when day/night temperature ranged between 31-36/24-29°C and the relative humidity was around 90%. During this period, seeds were stored at room temperature in a sealed plastic container.

2.2. Phenotypic Analyses

Germination tests were carried out in germination trays containing 21 seeds/row; all experiments were carried out in five replicates. The germinated seeds were counted every 24 h for 14 days. A seed was considered as germinated when the cotyledons were visible above ground. The total percentage (%) of germination was calculated at the end of the experiment, while the time required for 50% of seeds to germinate (T_{50}) was calculated according to the formula (1) [30]:

$$(1)\ T_{50} = t_i + [(N/2 - n_i)(t_j - t_i)] / n_j - n_i$$

where N is the final number of germination and n_i, n_j cumulative number of seeds germinated by adjacent counts at times t_i and t_j when $n_i<N/2< n_j$.

The seedlings length (mm) was measured on millimetric paper at the 14th day. For biomass analysis, seedlings were collected at 14 days after sowing and their fresh weight (FW) was measured (g). Seedlings were then placed in the oven at 60°C for 24 h, and subsequently, the dry weight (DW) was measured (g). Data are presented as mean ± standard deviation (SD) of five biological and five technical replicates.

2.3. RNA extraction, cDNA synthesis, and qRT-PCR analysis

RNA was isolated from primed and non-primed *M. truncatula* seeds and seedlings grown in the two types of soils by using the Trizol (Thermo Fisher, India) reagent, as indicated by the supplier. Total RNA was quantified by agarose gel electrophoresis and spectrophotometric analysis using a WPA Biowave DNA (Biochrom, Cambridge, UK). One microgram of RNA was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, Italy), while qRT-PCR was carried out using the Maxima SYBR Green qPCR Master Mix (Thermo Fisher, Italy) following the suppliers' instructions. C_t values and qRT-PCR efficiency, obtained from the Rotor-Gene 6000 Series Software 1.7 (Corbett Robotics, Australia), were analysed using the REST2009 Software V2.0.13 (Qiagen GmbH, Germany). The qRT-PCR was carried out in a final volume of 12 µL using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Australia). The amplification conditions were: denaturation at 95°C for 10 min (one cycle), followed by 45 cycles of 95°C for 15 s, 60°C for 60 s, each. For each oligonucleotide set, a no-template control was used. The *ELF1α* and *GADPH* genes were used as references, as they resulted the most stable under the tested conditions following the geNorm (https://genorm.cmgg.be/) [31] analysis (**Supplementary Figure S1**). The gene-specific oligonucleotide primers, designed using Primer3 (http://primer3.ut.ee/), are listed in **Supplementary Table S1**. The Pfaffl method [32] was used for the relative quantification of transcript accumulation. Data is represented as relative expression, obtained by dividing (T/R) the expression value of the respective target genes (T) to the expression value of the reference gene (R), obtained as geometrical mean of *ELF1α* and *GADPH* values. All reactions were performed in triplicate and results represented as mean ± SD of each sample.

2.4. Statistical Analyses

For each variable, significant differences between treatments were determined with Two-way ANOVA (Analysis of Variance) using the statistical tool developed by Assaad et al. [33]. For each treatment, five biological replicates were considered. Means were compared using the Duncan test, where means with a significance value lower than 0.05 were considered statistically different. Letters are used to indicate significant differences among all samples. The Student' *t*-test (*, $p < 0.05$)

was used to statistically compare the gene expression profiles in *M. truncatula* seeds, where HP2 and HP4 were reported to the dry seeds (DS) for each gene.

Principal Component Analysis (PCA) was performed using all measured parameters (germination %, T_{50}, seedling length, FW, DW, gene expression profiles) and the ClustVis program available at https://biit.cs.ut.ee/clustvis/ [34].

3. Results

3.1. Hydropriming enhances seed germination

In non-primed seeds (HP0), the germination percentage on control soil was 40.2 ± 5.06% while a slight decrease (33.2 ± 5.81 %) was observed when seeds were grown in contaminated soil (**Figure 2a**). The relatively low basal germination in soil may be due to the higher than optimal temperatures registered at the growing site, and storage conditions [35]. Hydropriming performed for 4 h had significantly increased seed germination percentage on both types of soil. The highest germination percentage was observed with the HP2 treatment (60 ± 11.72 %) on Soil_B and HP4 treatment (60 ± 9.35 %) on Soil_A, respectively. Conversely, biopriming and the combination between biopriming and hydropriming did not result in any significant differences compared to non-primed samples (HP0) (**Figure 2a**). The overall differences between the imposed conditions, from a statistical point of view, are mostly due to the priming treatments ($P < 0.001$) rather than the soil type ($S = 0.468$). The positive effect of hydropriming on seed germination was confirmed also in a second experiment carried out in August 2019 (**Supplementary Figure S2**). Although in this case, the germination percentage was considerably lower than in the first experiment, hydropriming treatments still resulted in enhanced germination. As the temperatures during this period were higher than the previous conditions, these experiments also confirm that germination is affected by high temperatures and storage conditions. Garcia et al. [35] indicated that the optimal temperature for *M. truncatula* seed germination is 18-20°C while paper envelopes or aired plastic vials are recommended for storage conditions.

When considering the germination speed (T_{50}), it is possible to observe that non-primed seeds germinated in about 3 days, independently of the type of soil (**Figure 2b**). In this case, the hydropriming treatments did not result in any significant differences compared to the non-primed control. However, biopriming seemed to negatively affect germination speed as the maximum percentage of germination was reached in up to 8 days in some of these cases (e.g. BP2). The high standard deviations are because the germination of these seeds was not uniform. Also in this case, the differences are mainly attributed to the priming treatments ($P < 0.001$).

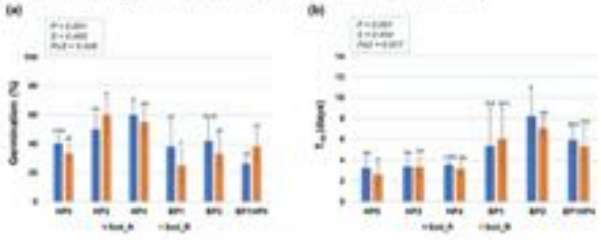


Figure 2. Germination of primed and non-primed *M. truncatula* seeds grown on two types of agricultural soils (Soil_A, Soil_B) collected from Vacaruse. (**a**) Germination percentage (%). (**b**) The time required for 50% of seeds to germinate (T_{50}). Data are represented as means ± SD of five replicates. Significant differences are shown with lowercase letters and the *P*-values are included in the upper panel of each graphic (P, priming; S, soil, PxS, interaction between priming and soil). HP0, non-primed control; HP2, 2h hydropriming; HP4, 4 h hydropriming; BP1, *Bacillus* strain isolated from mustard rhizosphere; BP2, *Bacillus* strain isolated from and linseed rhizosphere; BP1HP4, 4 h of hydropriming and BP1 strain.

3.2. Biopriming treatments improve seedling growth

If the germination percentage is more influenced by the hydropriming treatments, the seedling growth, on the other hand, seems to be more influenced by the biopriming treatments. This is mostly reflected by enhanced biomass rather than seedling length, as no statistically significant differences are observed in this parameter in most of the cases. However, a significant difference was observed when seedlings grown from non-primed seeds (HP0) on Soil_B as compared with those grown in Soil_A; namely, a decrease in seedling length is noticed when seeds are grown in Soil_B (**Figure 3a**). Interestingly, this was significantly recovered only by the HP2 treatment. The fact that seedling growth is affected by the type of soil is evident also when measuring seedling biomass (reflected by the FW values) under non-primed conditions (HP0) (**Figure 3b**). However, FW and DW were improved by biopriming, especially when seedlings were grown in the Soil_B (**Figure 3b,c**). Similar to the germination parameters, from a statistical point of view, the broad differences between the observed responses are primarily due to the priming treatments. The improved seedling growth is evident also in the second experiment performed in August 2019 (**Supplementary Figure S2**). In this case, even enhanced seedling length was observed when considering the HP4 (Soil_B), BP2 (Soil_B), and BP1HP4 (Soil_A) conditions. Higher biomass, in terms of fresh weight, was registered under BP1 (Soil_A) and BP2 (Soil_B) conditions. These experiments show that even if the germination rate is decreased, the priming treatments still provide support for better seedling development in the first two weeks of the plants' life.

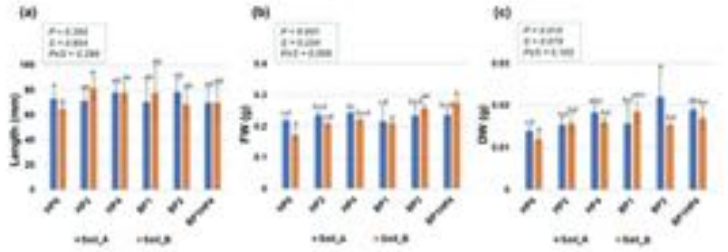


Figure 3. Evaluation of *M. truncatula* seedling growth on two types of agricultural soils (Soil_A, Soil_B) collected from Varunaxi for 14 days. **(a)** Seedling length (mm). **(b)** Measurement of fresh weight (FW, g). **(c)** Measurement of dry weight (DW, g). Data are represented as means ± SD of five replicates. Significant differences are shown with lowercase letters and the *P*-values are included in the upper panel of each graphic (P, priming; S, soil, PxS, interaction between priming and soil). HP0, non-primed control; HP2, 2h hydropriming; HP4, 4 h hydropriming; BP1, *Bacillus* strain isolated from mustard rhizosphere; BP2, *Bacillus* strain isolated from and linseed rhizosphere; BP1HP4, 4 h of hydropriming and BP1 strain.

3.3. Enhanced expression of genes involved in DNA repair and antioxidant response is characteristic to seedlings grown from primed seeds

In our previous works, we reported that certain genes that play roles in DNA repair and antioxidant responses can be used as indicators of seed quality and seeding growth [5-8]. Here we focused on the expression profiles of *OGG1* (8-Oxoguanine DNA glycosylase) and *FPG* (Formamidopyrimidine-DNA glycosylase), involved in Base Excision Repair (BER), *APX* (Ascorbate peroxidase) and *SOD* (Superoxide dismutase), encoding known ROS scavengers, and *MT2* (Metallothionein type 2), proven to act both as scavenger and repair enzyme [36]. The expression profiles of these genes were tested both in dry and hydroprimed seeds as well as in the two-weeks old seedlings grown in soil. In seeds, it is possible to observe that 2 h and 4 h of hydropriming did not significantly change the relative expression of *OGG1* and *MT2* genes as

compared to dry seeds (DS). Conversely, significant upregulation was registered for the *SOD* and *APX* genes in response to both hydropriming timepoints while the *FPG* gene was upregulated only in response to the HP4 treatment (**Figure 4a**). When considering the *OGG1* gene expression, it is possible to observe that the seedlings grown from biopriming seeds (BP1, BP2, BP1HP4) in Soil_B presented a significant upregulation of the *OGG1* gene (**Figure 4b**). As for the *FPG*, downregulation of the gene is observed in non-primed seeds grown in Soil_B (**Figure 4c**). The hydropriming treatment for 2 h (HP2) resulted in a significant upregulation of the gene in both Soil_A and Soil_B. A similar trend was observed in the case of the *MT2* gene, where hydropriming and the combination between hydropriming and biopriming (BP1HP4) resulted in enhanced gene expression (**Figure 4d**). Differently, the expression of *APX* and *SOD* genes was more influenced by the biopriming treatments as well as the BP1HP4 combination. In the case of the *APX* gene, the highest expression was induced by the BP2 treatment on Soil_B (**Figure 4e**) whereas the *SOD* gene peaked when the BP1 treatment was implemented on the same soil type (**Figure 4f**).

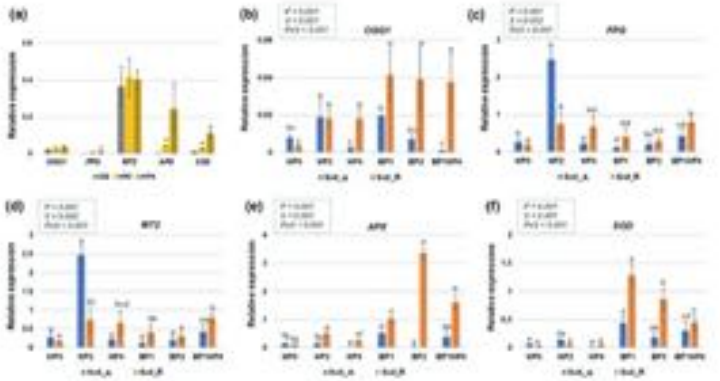
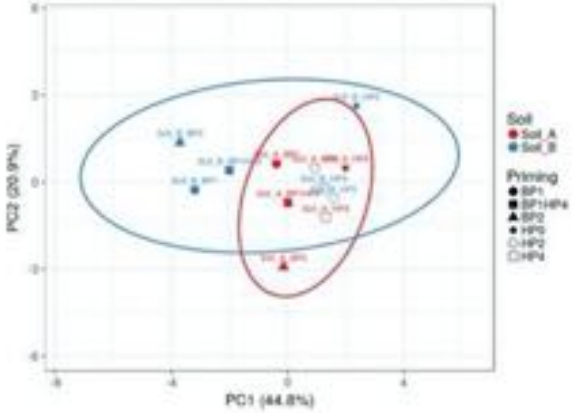


Figure 4. Gene expression profiles in *M. truncatula* seeds and 14-days-old seedlings grown from primed and non-primed seeds on two types of agricultural soils (Soil_A, Soil_B) collected from Varanasi. **(a)** Relative expression of *OGG1*, *FPG*, *MT2*, *APX*, and *SOD* genes in dry seeds (DS) and seeds treated with hydropriming for 2 h (HP2) and 4 h (HP4). Asterisks '*' show statistical significance ($p < 0.05$) compared to DS. **(b)** Relative expression of *OGG1* gene in seedlings. **(c)** Relative expression of *MT2* gene in seedlings. **(d)** Relative expression of *FPG* gene in seedlings. **(e)** Relative expression of *APX* gene in seedlings. **(e)** Relative expression of *SOD* gene in seedlings. Data are represented as means ± SD of five replicates. Significant differences are shown with lowercase letters and the *P* values are included in the upper panel of each graphic (P, priming; S, soil, PxS, interaction between priming and soil). HP0, non-primed control; HP2, 2h hydropriming; HP4, 4 h hydropriming; BP1, *Bacillus* strain isolated from mustard rhizosphere; BP2, *Bacillus* strain isolated from and linseed rhizosphere; BP1HP4, 4 h of hydropriming and BP1 strain.

3.4. Integrative data analysis

To better understand how the samples behave comparatively, a PCA analysis was performed taking into consideration the imposed conditions (soil type and priming treatments) and measured variables (germination %, T_{50}, seedling length, FW, DW, and gene expression profiles). The two main principal components extracted accounted for 65.7% of the variance (**Figure 5**). Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. X and Y axis show principal component 1 (PC1) and principal component 2 (PC2) that explain 44.8% and 20.9% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. Variables OGG1 (0.41), APX (0.40),

287 SOD (0.45), and MT2 (0.45) seem to be mostly correlated with PC1, while variables DW (0.66),
288 seedling length (0.55), and T= (0.32) are correlated with PC2 (**Supplementary Table S2**). It is
289 however interesting to observe how the treatments are grouped based on the type of soil and
290 priming treatments. Mainly, the Soil_A samples are mostly clustered together (in red); although,
291 samples Soil_B_HP2 and Soil_B_HP4 (in blue) are an integral part of this cluster (**Figure 5**). This is
292 indicative of the fact that the hydropriming treatments seem to be able to rescue the seedlings grown
293 on Soil_B, having a higher TDS concentration than Soil_A.



294

295 **Figure 5.** Principal Component Analysis (PCA) loading plot explaining the distribution of samples
296 based on the imposed conditions and measured variables. Soil_A, TDS (content of total dissolved
297 solids) 82.9 ppm; Soil_B, TDS 103 ppm; HP0, non-primed control; HP2, 2h hydropriming; HP4, 4 h
298 hydropriming; BP1, *Bacillus* strain isolated from mustard rhizosphere; BP2, *Bacillus* strain isolated
299 from and linseed rhizosphere; BP1HP4, 4 h of hydropriming and BP1 strain.

300 **4. Discussion**

301 Agriculture represents a major stamina of the economic and socio-political stability of India
302 and employs the largest workforce. About 60% of the geographical area of the country is occupied
303 by agricultural land, most of which are facing one or more types of soil contaminations [37].
304 Legumes can help restore soil organic matter when used in rotation with non-leguminous crops.
305 Thus, improving legume seed germination using easy and economically accessible methods will
306 upgrade the livelihood of many small farmers.

307 Within this context, the present study focused on the evaluation of hydropriming and
308 biopriming treatments as a means to improve seed germination and seedling establishment on
309 agricultural soils collected from two different sites in the Varanasi region. The collected soils
310 originate from the province of Kansada, where a power plant is located allowing to convert the
311 municipal solid waste to electric power in an attempt to dispose waste through a sustainable and
312 environmentally friendly process. However, the farmers in the region report loss of agricultural
313 productivity due to the accumulation of solid waste in the soil. This is also revealed when measuring
314 the content of total dissolved solids (TDS), shown to be higher in the soils collected closer to the
315 power plant (Soil_B, **Table 1**).

316 Hydropriming was chosen mainly because it is very easy to implement, and hence highly
317 accessible also to farmers from underdeveloped regions. The biopriming formulations were selected
318 from local varieties of rhizospheric microorganisms having plant growth-promoting traits under

stressful conditions and studied in relation to improved phosphorus nutrition [38, 39]. Moreover, biopriming can be associated with the elicitation of plant immunity, starting from the seedling stage [40]. The experiment was setup under the natural weather conditions of Varanasi to evaluate the efficiency of the tested protocols in the region.

Our results showed that while hydropriming enhanced seed germination percentage on both types of soils (**Figure 2a**), biopriming resulted in improved seedling development on Soil_B, mainly reflected by enhanced biomass (**Figure 3b,c**). This is coherent with the fact that hydropriming mainly improves germination a result of the enhanced water uptake and more favourable water relations in primed seeds [41] whereas biopriming effects come into action later on when the radicle protrudes and it becomes directly exposed to bacteria that can aid in soil nutrient uptake [17, 39]. Importantly, by repeating the experimental setup under different climatic conditions in August 2019, the observed trend of improved germination and seedling development was once again confirmed (**Supplementary Figure S2**). An important finding is the fact that HP2 and HP4 treatments were able to exert a more positive effect on Soil_B (having a higher TDS concentration than Soil_A), as evidenced by the PCA integrative analyses (**Figure 5**). Another recent work shows how hydropriming was able to mitigate the negative effects of cold temperatures on the germination and seedling development in narrow-leaf lupine (*Lupine angustifolius*) [15].

The next step was to examine the expression profiles of genes that play roles in DNA repair and antioxidant defence, previously used as indicators of seed quality during the early stages of germination (namely seed imbibition) [5-8]. This is a rather important aspect because such molecular hallmarks allow the prediction of seed quality in a very short time, thus providing assistance to Seed Companies to design customized priming protocols [2, 3, 9, 10]. The expression of the chosen genes, initially tested in dry and hydroprimed seeds, showed that while the *FPG*, *APX*, and *SOD* were upregulated by the treatment, the *OGG1* and *MT2* genes were not responsive at the tested time points (**Figure 4a**). Nonetheless, in the 14-days-old seedlings, all the tested genes were differentially expressed in response to both priming and soil conditions. The *OGG1* and *FPG* genes, playing similar roles within the BER pathway involved in the removal of oxidized bases like 7,8-dihydro-8-oxoguanine (8-oxo-dG) and formamidopyrimidine (FAPy) lesions, had been previously reported to be upregulated during seed imbibition under physiological conditions or in the presence of osmotic stress (polyethylene glycol, PEG) in *M. truncatula* [42]. However, their expression was mostly induced after 8 h of imbibition with water and 12 h of imbibition in the presence of PEG. More recently, this was further validated in several other conditions, like in the case of treatments with histone deacetylase inhibitors trichostatin A (TSA) and sodium butyrate (NaB) [6, 7]. Here, we show that, while the *OGG1* and *FPG* gene expression is downregulated in seedlings grown in Soil_B in the absence of priming, both hydropriming and biopriming resulted in gene upregulation (**Figure 4b,c**). This indicates that primed seedlings are better equipped to repair the DNA damage that can be induced by the presence of contaminants in the soil. Similarly, the *APX* and *SOD* genes, chosen as molecular hallmarks of the antioxidant response, were upregulated in hydroprimed seeds. In *M. truncatula*, upregulation of *SOD* and *APX* genes has been previously reported starting from the 4th h of seed imbibition with water [5]. In seedlings, the genes were mostly upregulated in response to biopriming (**Figure 4e,f**). SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide (H_2O_2), while APX plays a key role catalyzing the conversion of H_2O_2 into water, using ascorbate as a specific electron donor [43-46]. Enhanced accumulation of antioxidant enzymes following different seed priming methods (e.g. osmopriming, halopriming, hormopriming) has been previously reported in a number of plant species (*Vigna radiata*, *Moringa oleifera*, *Triticum aestivum*, *Oryza sativa*, *Sorghum bicolor*, *Brassica napus*) [45-50], and these were even correlated with changes in gene expression patterns [49, 50]. Another group of interesting ROS scavengers includes metallothioneins (MTs), small cysteine-rich proteins that accumulate in response to toxic levels of HMs [51]. Besides their role in HM detoxification, MTs are part of the signaling pathway activated by nitric oxide [36, 52] and have been associated with protection against oxidative injuries also at the nuclear level [53, 54]. In human cells, *MTs* overexpression was associated with the suppression of 8-oxo-dG accumulation in γ-irradiated cells [55], thus suggesting an interplay between MTs and

BER components. Under our experimental conditions, *MT2* seems to be mostly upregulated in hydroprimed seedlings (**Figure 4d**) while no changes in gene expression level were observed in the hydroprimed seeds (**Figure 4a**). As in the case of *OGG1*, also the *MT2* gene was previously shown to be upregulated only later on during seed imbibition in *M. truncatula* [6]. In a different work, the *MT* gene expression was monitored in artificially aged seeds of two *Silene* species that differ in seed longevity and grow in contrasting habitats [56]. Based on several measured parameters (e.g. ROS accumulation, antioxidant potential, telomere length) and the differential expression of *MT* and *SOD* genes in aged seeds it was possible to distinguish between short- and long-lived seeds under seed bank storage conditions [57]. In a context where seed researchers are continuously facing the complexity of efficient seed germination and healthy seedling establishment, the genes hereby investigated (*OGG1*, *FPG*, *APX*, *SOD*, and *MT2*) are important molecular hallmarks to test and monitor seed quality and seedling development.

In conclusion, the present study shows that seed priming is an efficient method to enhance seed germination and seedling establishment in agricultural soils collected from contaminated areas of the Varanasi province. While hydropriming improved seed germination percentage, biopriming resulted in improved seedling development. At a molecular level, this is reflected by upregulation of specific genes used as molecular indicators of seed quality.

Supplementary Materials: The following are available online at www.mdpi.com/link: **Figure S1**. geNorm analysis of reference genes. geNorm is a popular algorithm (https://genorm.cmgg.be/) used to determine the most stable reference genes from a set of tested candidate reference. Here, we used the tubulin (TUB), actin (ACT), ubiquitin (UBI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and elongation factor 1α (ELF) genes. The gene expression normalization factor (geNorm M) was calculated for each sample based on the geometric mean of the reference genes. The cDNA extracted from seedlings grown from primed and non-primed seeds was used for this analysis. The most stable genes, presenting the lowest geNorm M, were identified as GADPH and ELF, and were subsequently used for qRT-PCR data normalization, as indicated in Materials & Methods. **Figure S2**. Phenotypic characterization of primed and non-primed *M. truncatula* seeds grown in two types of agricultural soils (Soil_A and Soil_B) from Varanasi region in August 2019. The experiment was performed when day/night temperature ranged between 31-36/24-29°C and the relative humidity was around 90%. Measurements of germination percentage (%), seedling length (mm), and fresh weight (FW, g) were performed in two independent experiments (a, b) each consisting of five replicates (21 seeds per replicate). Data are represented as means ± SD of five replicates. Significant differences (as per Duncan test) are shown with lowercase letters. HP0, non-primed control; HP2, 2h hydropriming; HP4, 4 h hydropriming; BP1, Bacillus strain isolated from *mustard rhizosphere*; BP2, Bacillus strain isolated from and linseed rhizosphere; BP1HP4, 4 h of hydropriming and BP1 strain. **Table S1**. List of oligonucleotides used for the qRT-PCR analysis. **Table S2**. Principal Component Analysis (PCA) loading scores corresponding to each principal component (PC) based on the used variables (T50, seedling length, fresh weight, dry weight, and gene expression profiles). The data were retrieved from ClustVis program available at https://biit.cs.ut.ee/clustvis/.

Acknowledgments: Authors would like to thank Dr. Ana Barradas from Fertiprado L.d.a. (Vairamonte-Monforte, Portugal) for kindly providing the seed material used in this study. A.M. acknowledges the TECO (Technological Eco-Innovations for the Quality Control and the Decontamination of Polluted Waters and Soils) mobility grant awarded by the *Consiglio Nazionale delle Ricerche* (CNR), Italy. V.P. acknowledges the support extended by Institute of Environment and Sustainable Development, Banaras Hindu University, Varanasi, India. A.M., C.F., and A.B. acknowledge the Italian Ministry of Education, University and Research (MIUR): *Dipartimenti di Eccellenza Program* (2018–2022) - Dept. of Biology and Biotechnology "L. Spallanzani", University of Pavia.

Author Contributions: A.M. and V.P. conceptualized the study. C.F. performed the qRT-PCR analyses. Aj.S. and An.S. performed the phenotypic analyses. A.M. and A.B. analyzed the data and wrote the manuscript. All authors read and approved the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. ISTA (International Seed Testing Association). International rules for seed testing. Basserdorf, Switzerland: **2018**.
2. Paparella, S.; Araújo, S.S.; Rossi, G.; Wijayasinghe, M.; Carbonera, D.; Balestrazzi, A. Seed priming: state of the art and new perspectives. *Plant Cell Rep.* **2015**, *34*, 1281-1293. DOI: 10.1007/s00299-015-1784-y.
3. Macovei, A.; Pagano, A.; Leonetti, P.; Carbonera, D.; Balestrazzi, A.; Araújo, S.S. Systems biology and genome-wide approaches to unveil the molecular players involved in the pre-germinative metabolism: implications on seed technology traits. *Plant Cell Rep.* **2017**, *36*, 669-688. DOI: 10.1007/s00299-016-2060-5.
4. Ashraf, M.; Bray, C.M. DNA synthesis in osmoprimed leek (*Allium porrum* L.) seeds and evidence for repair and replication. *Seed Sci Res.* **1993**, *3*, 15–23. DOI: 10.1017/S0960258500001525.
5. Balestrazzi, A.; Confalonieri, M.; Macovei, A.; Carbonera, D. Seed imbibition in *Medicago truncatula* Gaertn.: Expression profiles of DNA repair genes in relation to PEG-mediated stress. *J Plant Physiol.* **2011**, *168*, 706-713. DOI: 10.1016/j.jplph.2010.10.008.
6. Pagano, A.; Araújo, S.S.; Macovei, A.; Leonetti, P.; Balestrazzi, A. The seed repair response during germination: Disclosing correlations between DNA repair, antioxidant response, and chromatin remodeling in *Medicago truncatula*. *Front Plant Sci.* **2017**, *8*, 1972. DOI: 10.3389/fpls.2017.01972.
7. Pagano, A.; de Sousa Araújo, S.; Macovei, A.; Dondi, D.; Lazzaroni, S.; Balestrazzi, A. Metabolic and gene expression hallmarks of seed germination uncovered by sodium butyrate in *Medicago truncatula*. *Plant Cell Environ.* **2019**, *42*, 259-269. DOI: 10.1111/pce.13342.
8. Araújo, S.S.; Pagano, A.; Dondi, D.; Lazzaroni, S.; Pinela, E.; Macovei, A.; Balestrazzi, A. Metabolic signatures of germination triggered by kinetin in *Medicago truncatula*. *Sci Rep.* **2019**, *9*, 10466. DOI: 10.1038/s41598-019-46866-6.
9. Waterworth, W.M.; Bray, C.M.; West, C.E. Seeds and the art of genome maintenance. *Front Plant Sci.* **2019**, *10*, 706. DOI: 10.3389/fpls.2019.00706.
10. Araújo, S.S.; Paparella, S.; Dondi, D.; Bentivoglio, A.; Carbonera, D.; Balestrazzi, A. Physical methods for seed invigoration: advantages and challenges in seed technology. *Front Plant Sci.* **2016**, *7*, 646. DOI: 10.3389/fpls.2016.00646.
11. Casenave, E.C.; Toselli, M.E. Hydropriming as a pre-treatment for cotton germination under thermal and water stress conditions. *Seed Sci Technol.* **2007**, *35*, 88-98. DOI: 10.15258/sst.2007.35.1.08.
12. Yan, M. Hydro-priming increases seed germination and early seedling growth in two cultivars of Napa cabbage (*Brassica rapa subsp. pekinensis*) grown under salt stress. *J Hortic Sci Biotech.* **2016**, *91*, 421-426. DOI: 10.1080/14620316.2016.1162031.
13. Kaya, E.; Seloviđ, A. The effect of hydro and proline seed priming on growth, proline and sugar content, and antioxidant activity of maize under cadmium stress. *Environ Sci Pollut Res Int.* **2018**, *25*, 33270-33280. DOI: 10.1007/s11356-018-3220-7.
14. Plaźek, A.; Dubert, F.; Kopeć, P.; Dziarka, M.; Kalandyk, A.; Pastuszak, J.; Wolko, B. Seed hydropriming and smoke water significantly improve low-temperature germination of *Lupinus angustifolius* L. *Int J Mol Sci.* **2018**, *19*, pii: E992. DOI: 10.3390/ijms19040992.
15. Plaźek, A.; Dubert, F.; Kopeć, P.; Dziarka, M.; Kalandyk, A.; Pastuszak, J.; Wiślgórski, P.; Wolko, B. Long-term effects of cold on growth, development and yield of narrow-leaf lupine may be alleviated by seed hydropriming or butenolide. *Int J Mol Sci.* **2018**, *19*, pii: E2416. DOI: 10.3390/ijms19082416.
16. Biswas, J.C.; Ladha, J.K.; Dazzo, F.B. Rhizobia inoculation improves nutrient uptake and growth of lowland rice. *Soil Sci Soc Am J.* **2000**, *64*, 1644-1650. DOI: 10.2136/sssaj2000.6451644x.
17. Yadav, R.S.; Singh, V.; Pal, S.; Meena, S.K.; Meena, V.S.; Sarma, B.K.; Singh, H.B.; Rakshit, A. Seed bio-priming of baby corn emerged as a viable strategy for reducing mineral fertilizer use and increasing productivity. *Sci Hort.* **2018**, *241*, 93-99. DOI: 10.1016/j.scienta.2018.06.096.
18. Rocier, C.; Gerin, F.; Czarnes, S.; Legendre, L. Biopriming of maize germination by the plant growth-promoting rhizobacterium *Azospirillum lipoferum* CRT1. *J Plant Physiol.* **2019**, *237*, 111-119. DOI: 10.1016/j.jplph.2019.04.011.
19. Macovei, A.; Garg, B.; Raikwar, S.; Balestrazzi, A.; Carbonera, D.; Buttafava, A.; Bremont, J.F.; Gill, S.S.; Tuteja, N. Synergistic exposure of rice seeds to different doses of γ-ray and salinity stress

resulted in increased antioxidant enzyme activities and gene-specific modulation of TC-NER pathway. *Biomed Res Int.* **2014**, *2014*, 676934. DOI: 10.1155/2014/676934.

20. Ibrahim, E.A. Seed priming to alleviate salinity stress in germinating seeds. *J Plant Physiol.* **2016**, *192*, 38-46. DOI: 10.1016/j.jplph.2015.12.011.
21. Li, Z.; Xu, J.; Gao, Y.; Wang, C.; Guo, G.; Luo, Y.; Huang, Y.; Hu, W.; Sheteiwy, M.S.; Guan, Y.; Hu, J. The synergistic priming effect of exogenous salicylic acid and H_2O_2 on chilling tolerance enhancement during maize (*Zea mays* L.) seed germination. *Front Plant Sci.* **2017**, *8*, 1153. DOI: 10.3389/fpls.2017.01153.
22. Bajwa, A.A.; Farooq, M.; Nawaz, A. Seed priming with sorghum extracts and benzyl aminopurine improves the tolerance against salt stress in wheat (*Triticum aestivum* L.). *Physiol Mol Biol Plants.* **2018**, *24*, 239-249. DOI: 10.1007/s12298-018-0512-9.
23. FAO - Food and Agriculture Organization of the United States. Status of the World's Soil Resources Report (SWSR); **2015**. http://www.fao.org/3/a-i5199e.pdf.
24. Machender, G.; Dhakate, R.; Tamma Rao, G.; Loukya, G.; Reddy, M.N. Assessment of trace element contamination in soils around Chirnaeru River Basin, Nalgonda District, India. *Environ Earth Sci.* **2013**, *70*, 1021–1037. DOI: 10.1007/s12665-012-2192-z.
25. Paul, D.; Choudhary, B.; Gupta, T.; Jose, M.T. Spatial distribution and the extent of heavy metal and hexavalent chromium pollution in agricultural soils from Jajmau, India. *Environ Earth Sci.* **2015**, *73*, 3565–3577. DOI 10.1007/s12665-014-3642-6.
26. Kumar, V.; Sharma, A.; Kaur, P.; Singh Sidhu, G.P.; Bali, A.S.; Bhardwaj, R.; Thukral, A.K.; Cerda, A. Pollution assessment of heavy metals in soils of India and ecological risk assessment: a state-of-the-art. *Chemosphere.* **2019**, *216*, 449–462. DOI: 10.1016/j.chemosphere.2018.10.066.
27. Gill, S.S.; Khan, N.A.; Tuteja, N. Cadmium at high dose perturbs growth, photosynthesis and nitrogen metabolism while at low dose it up regulates sulfur assimilation and antioxidant machinery in garden cress (*Lepidium sativum* L.). *Plant Sci.* **2012**, *182*, 112-120. DOI: 10.1016/j.plantsci.2011.04.018.
28. Gill, S.S.; Hasanuzzaman, M.; Nahar, K.; Macovei, A.; Tuteja, N. Importance of nitric oxide in cadmium stress tolerance in crop plants. *Plant Physiol Biochem.* **2013**, *63*, 254-261. DOI: 10.1016/j.plaphy.2012.12.001.
29. Graham, P.H.; Vance, C.P. Legumes: importance and constraints to greater use. *Plant Physiol.* **2003**, *131*, 872-877. DOI: 10.1104/pp.017004.
30. Farooq, M.; Hussain, M.; Usman, M.; Farooq, S.; Alghamdi, S.S.; Siddique, K.H.M. Impact of abiotic stresses on grain composition and quality in food legumes. *J Agric Food Chem.* **2018**, *66*, 8887-8897. DOI: 10.1021/acs.jafc.8b02924.
31. Mestdagh, P.; Van Vlierberghe, P.; De Weer, A.; Muth, D.; Westermann, F.; Speleman, F.; Vandesompele, J. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol.* **2009**, *10*, R64. DOI: 10.1186/gb-2009-10-6-r64.
32. Pfaffl, M.W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, e45. DOI: 10.1093/nar/29.9.e45.
33. Assand, H.I.; Hou, Y.; Zhou, L.; Carroll, R.J.; Wu, G. Rapid publication-ready MS-Word tables for two-way ANOVA. *SpringerPlus.* **2015**, *4*, 33. DOI: 10.1186/s40064-015-0795-z.
34. Metsalu, T.; Vilo, J. Clustvis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res.* **2015**, *43*, W566-W570. DOI: 10.1093/nar/gkv468.
35. Garcia, J.; Barker, D.G.; Journet, E.-P. Seed storage and germination. *In: Medicago truncatula* Handbook, 2006, pp. 1-9.
36. Balestrazzi, A.; Macovei, A.; Tava, A.; Avato, P.; Raimondi, E.; Carbonera, D. Unraveling the response of plant cells to cytotoxic saponins: role of metallothioneins and nitric oxide. *Plant Signal Behav.* **2011**, *6*, 506-519. DOI: 10.4161/psb.6.4.14746.
37. Bhattacharyya, R.; Ghosh, B.N.; Mishra, P.K.; Mandal, B.; Rao, C.S.; Sarkar, D.; Das, K.; Anil, K.S.; Lalitha, M.; Hati, K.M.; Franzluebbers, A.J. Soil degradation in India: Challenges and potential solutions. Sustainability. **2015**, *7*, 3528-3570. DOI:10.3390/su7043528.
38. Singh, A.; Shankar, A.; Gupta, V.K.; Prasad, V. Rhizobacteria: tools for the management of plant abiotic stresses. *In: Microbial Biotechnology. 1st Edition.* Eds. Shukla P. CRC Press. **2016**. https://doi.org/10.1201/9781315367880

39. Prasad, V.; Chaudhary, S.; Singh, A. Improving phosphorus fertility in soil through microbial mediators. *Int J Plant Environ.* **2018**, *4*, 2.
40. Song, G.C.; Choi, H.K.; Kim, Y.S.; Choi, J.S.; Ryu, C.M. Seed defense biopriming with bacterial cyclodipeptides triggers immunity in cucumber and pepper. *Sci Rep.* **2017**, *7*, 14209. DOI: 10.1038/s41598-017-14155-9.
41. Lechowska, K.; Kubala, S.; Wojtyla, Ł.; Nowaczyk, G.; Quinet, M.; Lutts, S.; Garnczarska, M. New insight on water status in germinating *Brassica napus* seeds in relation to priming-improved germination. *Int J Mol Sci.* **2019**, *20*, E540. DOI: 10.3390/ijms20030540.
42. Macovei, A.; Balestrazzi, A.; Confalonieri, M.; Faè, M.; Carbonera, D. New insights on the barrel medic MtOGG1 and MtFPG functions in relation to oxidative stress response in planta and during seed imbibition. *Plant Physiol Biochem.* **2015**, *89*, 1040-1050. DOI: 10.1016/j.plaphy.2015.05.007.
43. Alscher, R.G.; Erturk, N.; Heath, L.S. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *J Exp Bot.* **2002**, *53*, 1331-1341.
44. Caverzan, A.; Passaia, G.; Rosa, S.B.; Ribeiro, C.W.; Lazzarotto, F.; Margis-Pinheiro, M. Plant responses to stresses: Role of ascorbate peroxidase in the antioxidant protection. *Genet Mol Biol.* **2012**, *35*, 1011-1019.
45. Nouman, W.; Basra, S.M.A.; Yasmeen, A.; Gull, T.; Hussain, S.B.; Zubair, M.; Gul, R. Seed priming improves the emergence potential, growth and antioxidant system of *Moringa oleifera* under saline conditions. *Plant Growth Regul.* **2014**, *73*, 267-278. DOI: 10.1007/s10725-014-9887-y.
46. Jisha, K.C.; Puthur, J.T. Seed priming with BABA (β-amino butyric acid): a cost-effective method of abiotic stress tolerance in *Vigna radiate* (L.) Wilczek. *Protoplasma.* **2016**, *253*, 277-289. DOI: 10.1007/s00709-015-0804-7.
47. Islam, F.; Yasmeen, T.; Ali, S.; Ali, B.; Farooq, M.A.; Gill, R.A. Priming-induced antioxidative responses in two wheat cultivars under saline stress. *Acta Physiol Plant.* **2015**, *37*, 153. DOI: 10.1007/s11738-015-1897-5.
48. Zhang, F.; Yu, J.; Johnston, C.R.; Wang, Y.; Zhu, K.; Lu, F.; Zhang, Z.; Zou, J. Seed priming with polyethylene glycol induces physiological changes in sorghum (*Sorghum bicolor* L. Moench) seedlings under suboptimal soil moisture environments. *PLoS One.* **2015**, *10*, e0140620. DOI: 10.1371/journal.pone.0140620.
49. Salah, S.M.; Yajing, G.; Dongdong, C.; Jie, L.; Aamir, N.; Qiuan, H.; Weimin, H.; Mingyu, N.; Jin, H. Seed priming with polyethylene glycol regulating the physiological and molecular mechanism in rice (*Oryza sativa* L.) under nano-ZnO stress. *Sci Rep.* **2015**, *5*, 14278. DOI: 10.1038/srep14278.
50. Kubala, S.; Garnczarska, M.; Wojtyla, Ł.; Clippe, A.; Kosmala, A.; Żmieńko, A.; Lutts, S.; Quinet, M. Deciphering priming-induced improvement of rapeseed (*Brassica napus* L.) germination through an integrated transcriptomic and proteomic approach. *Plant Sci.* **2015**, *231*, 94-113. DOI: 10.1016/j.plantsci.2014.11.008.
51. Cobbet, C.; Goldsbrough, P. Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. *Annu Rev Plant Biol.* **2002**, *53*, 159-182. DOI: 10.1146/annurev.arplant.53.100301.135154.
52. Balestrazzi, A.; Agnoi, V.; Tava, A.; Avato, P.; Biazzi, E.; Raimondi, E.; Macovei, A.; Carbonera, D. Cell death induction and nitric oxide biosynthesis in white poplar (*Populus alba* L.) suspension cultures exposed to alfalfa saponins. *Plant Physiol.* **2011**, *141*, 227-238. DOI: 10.1111/j.1399-3054.2010.01436.x.
53. Balestrazzi, A.; Botti, S.; Zelasco, S.; Biondi, S.; Franchin, C.; Calligari, P.; Racchi, M.; Turchi, A.; Lingua, G.; Berta, G.; Carbonera, D. Expression of the PsMTA1 gene in white poplar engineered with the MAT system is associated to heavy metal tolerance and protection against 8-hydroxy-2′-deoxyguanosine mediated-DNA damage. *Plant Cell Rep.* **2009**, *28*, 1179-1192. DOI: 10.1007/s00299-009-0719-x.
54. Donà, M.; Ventura, L.; Macovei, A.; Confalonieri, M.; Savio, M.; Giovannini, A.; Carbonera, D.; Balestrazzi, A. γ irradiation with different dose rates induces different DNA damage responses in *Petunia x hybrida* cells. *J Plant Physiol.* **2013**, *170*, 780-7. DOI: 10.1016/j.jplph.2013.01.010.
55. Yeong, H.G.; Youn, C.-K.; Cho, H.-J.; Kim, S.-H.; Kim, M.-H.; Kim, H.-B.; Chang, I.-Y.; Lee, Y.-S.; Chung, M.-H.; You, H.-J. Metallothionein-III prevents γ-ray induced 8-oxoguanine accumulation in normal and hOGG1-depleted cells. *J Biol Chem.* **2004**, *297*, 34138-34149. DOI: 10.1074/jbc.M402530200.

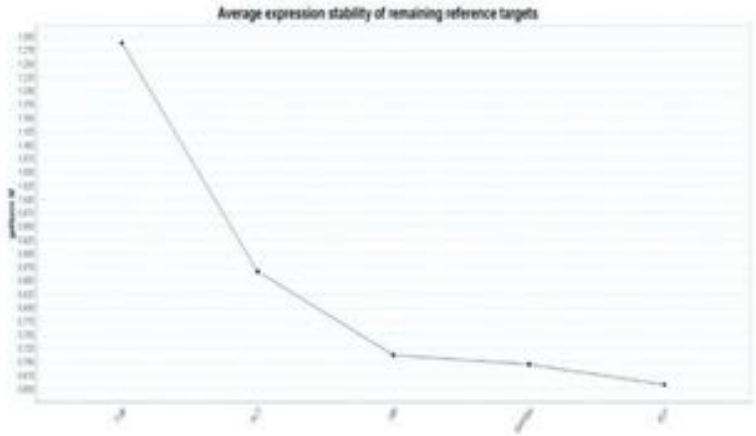
586 56. Mondoni, A.; Probert, R.J.; Rossi, G.; Vegini, E.; Hay, F.R. Seeds of alpine plants are short-lived: implications for long-term conservation. *Ann Bot.* **2011**, *107*, 171–179. DOI: 10.1093/aob/mcq222.
588 57. Dondi, M.; Balestrazzi, A.; Mondoni, A.; Rossi, G.; Ventura, L.; Buttafava, A.; Macovei, A.; Sabatini, M.E.; Valassi, A.; Carbonera, D. DNA profiling, telomere analysis and antioxidant properties as tools for monitoring ex situ seed longevity. *Ann Bot.* **2013**, *111*, 987–998. DOI: 10.1093/aob/mct058.

SUPPLEMENTARY MATERIAL

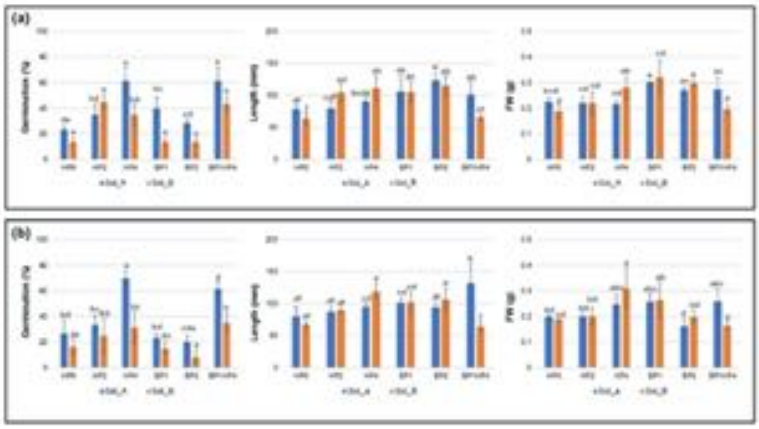
Priming improves *Medicago truncatula* seed germination in soil and upregulates specific genes used as indicators of seed quality

Chiara Forti[1], Ajay Shankar[2], Anjali Singh[2], Alma Balestrazzi[1], Vishal Prasad[2], Anca Macovei[1,*]

[1] Department of Biology and Biotechnology 'L. Spallanzani', University of Pavia, via Ferrata 9, 27100-Pavia, Italy;
[2] Institute of Environment and Sustainable Development, Banaras Hindu University, Varanasi 221005, India;
* Correspondence: anca.macovei@unipv.it; Tel.: +39-0382-985583



Supplementary Figure S1. geNorm analysis of reference genes. geNorm is a popular algorithm (https://genorm.cmgg.be/) used to determine the most stable reference genes from a set of tested candidate reference. Here, we used the tubulin (*TUB*), actin (*ACT*), ubiquitin (*UBI*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and elongation factor 1α (*ELF*) genes. The gene expression normalization factor (geNorm M) was calculated for each sample based on the geometric mean of the reference genes. The cDNA extracted from seedlings grown from primed and non-primed seeds was used for this analysis. The most stable genes, presenting the lowest geNorm M, were identified as *GADPH* and *ELF*, and were subsequently used for qRT-PCR data normalization, as indicated in Materials & Methods.



Supplementary Figure S2. Phenotipic characterization of primed and non-primed *M. truncatula* seeds grown in two types of agricultural soils (Soil_A and Soil_B) from Varanasi region in August 2019. The experiment was performed when day/night temperature ranged between 31-36/24-29°C and the relative humidity was around 90%. Measurements of germination percentage (%), seedling length (mm), and fresh weight (FW, g) were performed in two independent experiments (**a, b**) each consisting of five replicates (21 seeds per replicate). Data are represented as means ± SD of five replicates. Significant differences (as per Duncan test) are shown with lowercase letters. HP0, non-primed control; HP2, 2h hydropriming; HP4, 4 h hydropriming; BP1, *Bacillus* strain isolated from mustard rhizosphere; BP2, *Bacillus* strain isolated from and linseed rhizosphere; BP1HP4, 4 h of hydropriming and BP1 strain.

Supplementary Table S1. List of oligonucleotides used for the qRT-PCR analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Efficiency
OGG1	AAACACCGCACCTTCTCAAT	TGTGGAGATGTTTGAGGGAA	1.73
FPG	TCCTTTCAATTCGGTATGGC	GCTCCAAACCATCGTCTAGC	1.76
SOD	CCTGAGGATGAGACTCGACA	GAACAACAACAGCCCTTCCT	1.79
APX	AGCTCAGAGGTTTCATCGCT	CGAAAGGACCACCAGTCTTT	1.76
MT2	CATGTCAAGCTCATGCGGCAAC	TGCCGTAGTTGTTTCCCTTCCC	1.72
ACT	TCAATGTGCCTGCCATGTATG	ACTCACACCGTCACCAGAATC	1.70
TUB	TTTGCTCCTCTTACATCCCGTG	GCAGCACACATCATGTTTTTGG	1.82
UBI	GCAGATAGACACGCTGGGA	AACTCTTGGGCAGGCAATAA	1.81
GADPH	TGCCTACCGTCGATGTTTCAGT	TTGCCCTCTGATTCCTCCTTG	1.75
ELF1α	GACAAGCGTGTGATCGAGAGA	TTTCACGCTCAGCCTTAAGCT	1.69

Supplementary Table S2. Principal Component Analysis (PCA) loading scores corresponding to each principal component (PC) based on the used variables (TS0, seedling length, fresh weight, dry weight, and gene expression profiles). The data were retrieved from ClustVis program available at https://biit.cs.ut.ee/clustvis/.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
TS0	0.370485	0.317228	0.191904	-0.11232	0.587175	-0.20684	-0.38453	-0.42101	0
Length	-0.04948	0.553522	-0.46642	0.022897	-0.41938	-0.41574	0.116981	-0.33269	-5.65E-17
FW	0.245926	0.218739	0.335223	0.698268	-0.19048	0.385374	0.180849	-0.27243	9.37E-17
DW	0.169995	0.657046	0.175219	-0.14446	-0.00172	0.105279	0.020598	0.690153	1.24E-17
OGG1	0.4099	-0.17129	-0.30431	0.183269	-0.34401	0.068824	-0.71978	0.187853	3.79E-17
FPG	-0.16627	0.118103	-0.65372	0.360568	0.546003	0.29181	0.045082	0.125461	5.85E-17
APX	0.404864	-0.24312	-0.01561	0.371173	0.158847	-0.64267	0.330957	0.301945	-1.06E-16
SOD	0.453506	-0.07425	-0.20291	-0.29753	-0.0261	0.246845	0.296373	-0.09834	-0.70711
MT	0.453506	-0.07425	-0.20291	-0.29753	-0.0261	0.246845	0.296373	-0.09834	0.707107

TWST933-c3-1-2 TWST933-de Bruijn June 14, 2019 15:46 Printer Name: Trim: 279mm x 216mm

CHAPTER 3.1.2

Medicago truncatula, an informative model to investigate the DNA damage response during seed germination

Anca Macovei,[1] Andrea Pagano,[1] Chiara Forti,[1] Susana Araújo,[1,2] and Alma Balestrazzi[1]
[1]Department of Biology and Biotechnology "Lazzaro Spallanzani", University of Pavia, Pavia 27100, Italy
[2]Instituto De Tecnologia Química e Biológica António Xavier (ITQB-UNL), Oeiras 2780-157, Portugal

3.1.2.1 Introduction

Seed germination is triggered by water uptake during imbibition (phase I) with the consequent reactivation of biochemical pathways ("pre-germinative metabolism," phase II). These phases are fundamental to ensure the proper physiological conditions for germination. They also represent the basis to study adequate systems for enhanced seed vigor and seedling ability to withstand environmental stresses. The emergence of radicle (phase III) corresponds to the end of germination. As a consequence of imbibition, the *de novo* synthesis of nucleic acids, proteins, and lipids, as well as ATP production takes place (Bewley et al. 2013). The pre-germinative metabolism includes molecular processes that contribute to the "seed repair response" or the seed ability to withstand oxidative and genotoxic injury (Rajjou and Debeaujon 2008; Balestrazzi et al. 2011a). The DNA damage response (DDR), an essential component of the seed repair response, is still poorly explored. DDR is a highly conserved and intricate network of multiple pathways able to sense and signal DNA damage accumulation (Yoshiyama et al. 2013). As a follow-up, the cell is driven toward either starting efficient DNA repair or induced programmed cell death (Manova and Gruszka 2015). Another relevant component of the seed repair response is the antioxidant system. Seeds face environmental stresses during maturation on the mother plant, post-dispersal storage, as well as during the early phases of germination. All these conditions lead to ROS (reactive oxygen species)-mediated oxidative damage at the level of lipids, proteins, and nucleic acids (Ventura et al. 2012). A schematic representation of our proposed *M. truncatula* seed working system, combining the redox potential and DDR components (sensing, transduction, chromatin remodeling, and DNA repair), is given in Figure 3.1.2.1. In addition, a list of genes (Table 3.1.2.1) with potential direct or indirect roles in DDR during the early phases of seed germination is provided and discussed throughout the text. The present chapter provides a detailed depiction of the current advances related to DNA damage profiles and DDR-mediated mechanisms associated with early seed germination in *M. truncatula*. Within this context, the pivotal role of *M. truncatula* as a working model is clearly underlined.

3.1.2.2 Oxidative DNA damage profiling during early seed germination in *Medicago truncatula*

Under physiological conditions, phase I (imbibition) of germination is accompanied by considerable accumulation of oxidative DNA damage. Using an ELISA (enzyme-linked immunosorbent assay), Macovei et al. (2010) measured the amount of 7,8-dihydro-8-oxoguanine (8-oxo-dG), one of the most common oxidized bases, during *M. truncatula* seed imbibition. A significant increase in the level of 8-oxo-dG was detected after eight hours of imbibition, possibly as a consequence of enhanced ROS production during early imbibition (El-Maarouf-Bouteau and Bailly 2008). The close correlation between water uptake, ROS levels, and genotoxicity became more evident when *M. truncatula* seed imbibition was performed under stress conditions. In seeds imbibed with the osmotic agent polyethylene glycol (PEG), water uptake was delayed whereas prolonged treatment resulted in increased levels of ROS in the radicle (Balestrazzi et al. 2011b). The slow water uptake correlated with a delay in 8-oxo-dG accumulation since the first significant peak occurred after 12 hours of imbibition. Overall, the oxidative DNA damage profile showed significant changes in terms of temporal accumulation and amount, when compared to seeds

The Model Legume Medicago truncatula, First Edition. Edited by Frans J. de Bruijn.

IWST935-c3-1-2 IWST935-de Bruijn June 14, 2019 15:44 Printer Name Trim: 279mm x 216mm

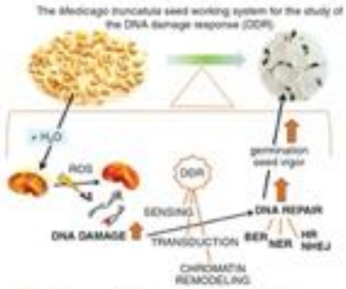


Figure 3.1.2.1 Schematic representation of the molecular events characterizing the DNA damage response (DDR) during *M. truncatula* seed germination. Water uptake during imbibition results in ROS (reactive oxygen species) accumulation. DNA damage triggers DDR-sensing and transduction pathways, resulting in chromatin modulation to allow DNA repair. This leads to enhanced seed germination. BER, base excision repair; NER, nucleotide excision repair; HR, homologous recombination; NHEJ, non-homologous end joining.

imbibed with water (Balestrazzi et al. 2011b). In PEG-treated seeds, the dynamics of water uptake and the profile of oxidative DNA damage were clearly overlapped, highlighting an interrelation between the two parameters. Another reliable and versatile tool to measure both the qualitative and quantitative profiles of DNA damage under physiological and/or oxidative stress conditions is the SCGE (single cell gel electrophoresis) or comet assay. This allows the investigation of nuclei isolated from single cells and discloses size-dependent distribution of intact vs. fragmented DNA molecules when subjected to electrophoresis (Ventura et al. 2013). In the presence of an electric field, the fragmented DNA migrates forming a comet tail while the intact DNA localizes within the comet head. Comets are visualized with fluorescent dyes and scored by visual or automated image analysis (Gichner et al. 2009; Braafladt et al. 2016). The SCGE protocol has been recently optimized by our group for applications during seed germination, particularly in radicles collected at phase III. (Macovei and Pagano, personal communication). Despite the recent advances and the current knowledge available in model species, the dynamics of DNA damage accumulation are still poorly explored, especially in the context of seed imbibition. A deeper understanding of the possible effects that osmotic agents or other chemicals have on DNA integrity is required to develop innovative seed priming protocols and boost seed vigor. This will support the work of operators from seed companies and seed germplasm banks, constantly facing problems with low seed vigor and reduced longevity (Paparella et al. 2015; Araújo et al. 2016a; Macovei et al. 2016).

Table 3.1.2.1 Upregulated genes with direct or indirect roles in DDR during *M. truncatula* seed germination

ID	Gene	Phytozome Acc. No.	Function	References
APX	Ascorbate peroxidase	Medtr4g061140	Catalyzes the H_2O_2-dependent oxidation of organic substrates	Balestrazzi et al. 2011a
SOD	Superoxide dismutase	Medtr7g114240	Catalyzes the dismutation of O^{2-} radicals into O_2 or H_2O_2	Balestrazzi et al. 2011a
MT2	Metallothionein2	[illegible]	Cysteine-rich, low molecular weight proteins, with metal binding properties	Balestrazzi et al. 2016
OGG1	8-oxoguanine DNA glycosylase/lyase	[illegible]	BER enzyme involved in the excision of 8-oxoG that occurs as a result of exposure to ROS	Macovei et al. 2011a
FPG	Formamidopyrimidine-DNA-glycosylase	Medtr2g[illegible]	BER enzyme involved in the recognition and removal of a wide range of oxidized purines from damaged DNA	Macovei et al. 2011a
TFIIS	Transcription elongation factor IIS	Medtr3g[illegible]	NER enzyme involved in assisting RNA polymerase II to bypass oxidative DNA lesions	Macovei et al. 2011b
TFIIS-like	Transcription elongation factor IIS-like	Medtr5g[illegible]	Putative NER enzyme with roles in abiotic stress tolerance	Macovei et al. 2011b
TDP1α	Tyrosyl-DNA phosphodiesterase1α	Medtr7g[illegible]	Involved in the repair of stalled topoisomerase I-DNA complexes	Macovei et al. 2010; Balestrazzi et al. 2011a
TDP1β	Tyrosyl-DNA phosphodiesterase1β	Medtr8g[illegible]	Involved in the repair of stalled topoisomerase I-DNA complexes	Macovei et al. 2010; Balestrazzi et al. 2011a
TDP2α	Tyrosyl-DNA phosphodiesterase2α	Medtr4g[illegible]	Involved in the repair of stalled topoisomerase II-DNA complexes	Confalonieri et al. 2014; Faè et al. 2014
TRRAP	Transformation/transactivation domain-associated protein	Medtr5g[illegible]	Adapter protein found in multiprotein chromatin remodeling complexes with histone acetyltransferase activity	Balestrazzi et al. 2016

TWIST955-c3-1.2 TWIST955-de Bruijn June 14, 2019 15:46 Printer Name: Trim: 279mm x 216mm

3.1.2.3 The seed antioxidant response during early seed germination in *Medicago truncatula*

The free radical scavenging activity of seeds, resulting from the combined action of non-enzymatic and enzymatic components, represents the first barrier against the ROS-mediated genotoxic effects (Bailly et al. 2000; Hsu et al. 2003; Macovei et al. 2016). The seed ROS scavenging activity, expressed as the seed antioxidant potential, is analyzed using the DPPH (1,1-diphenyl-2-picrylhydrazyl) test which exploits the reactivity of the DPPH radical with antioxidant compounds (Braca et al. 2001; Macovei et al. 2011a). The DPPH free radical scavenging activity of *M. truncatula* seeds was significantly higher at four and eight hours following rehydration, both in the presence and absence of PEG (Macovei et al. 2011a). The expression profiles of antioxidant genes have been used as molecular hallmarks of the seed antioxidant response. This is the case of *APX* and *SOD* genes, encoding cytosolic ascorbate peroxidase and superoxide dismutase, respectively (Balestrazzi et al. 2011a,b; Macovei et al. 2010, 2011b). Another player is metallothionein (MT), a ROS scavenger and signal molecule which acts outside and inside the nucleus, with possible interactions with the DNA repair machinery (Wang et al. 2010; Balestrazzi et al. 2009, 2011c). *MT* genes were upregulated during seed imbibition in different plant species (Zhou et al. 2012; Donà et al. 2013a), evidencing that these can be used as hallmarks of the antioxidant response at early germination phases.

3.1.2.4 BER and NER players contribute to oxidative DNA damage repair in *Medicago truncatula* seeds

Base-excision repair (BER) mechanisms play a crucial role in preventing mutations caused by oxidative DNA damage (David et al. 2007). The guanine base is particularly susceptible to oxidation, due to its low redox potential (Neeley and Essigmann 2006). The best-known guanine oxidation product, *8-oxo-dG*, is excised by the OGG1 enzyme (8-oxoguanine DNA glycosylase/lyase). Macovei et al. (2011a) reported on the involvement of the *MtOGG1* gene in the seed repair response. The MtOGG1 protein has all the conserved motifs found in eukaryotic DNA glycosylase/lyases, bifunctional enzymes that catalyze the scission of the N-terminal glycosylic bond which links a damaged base to the sugar moiety of the phosphate backbone and cleave the abasic site by a lyase reaction (David et al. 2007). Another BER player, the *MtFPG* (formamidopyrimidine-DNA-glycosylase) gene, was characterized in the same study (Macovei et al. 2011a). The MtFPG protein contains most of the conserved domains of the FPG superfamily, except for the zinc-finger domain which is missing in plants. In *Escherichia coli* the FPG enzyme catalyzes the removal of *8-oxo-dG* and Fapy lesions at similar rates (Kreval et al. 2004). A comparative study of the OGG1 and FPG enzyme activity in *Arabidopsis thaliana* revealed that the OGG1 enzyme acts predominantly on 8-oxo-dG DNA lesions, while apurinic sites in DNA represent the main target of FPG (Murphy and George 2005). In *M. truncatula*, the *MtOGG1* and *MtFPG* genes were upregulated during seed imbibition, while a delay in upregulation was observed in response to PEG (Macovei et al. 2011a). The gene expression profiles peaked at the timepoint of rehydration in which the highest levels of oxidative DNA damage, measured in terms of *8-oxo-dG* accumulation, were recorded (Balestrazzi et al. 2011b). The contribution of NER (nucleotide excision repair) pathway to genome maintenance during *M. truncatula* seed imbibition has been highlighted as well (Macovei et al. 2010, 2011b). We identified and characterized the *MtTFIIS-like* (transcription elongation factor IIF) gene encoding a product that shares common features with the canonical TFIIS protein, as well as with elongin A and CRSP70 (cofactor required for SP1 activation) transcription factors (Booth et al. 2000). TFIIS not only associates with RNA polymerase II, stimulating transcription of long RNAs but it also enables the enzyme to bypass those regions containing oxidative DNA lesions (Kuraoka et al. 2007). Considering the possible role hypothesized for the animal TFIIS in TC(transcription-coupled)-NER, the expression profiles of *MtTFIIS-like* and *MtTFIIS* genes were investigated during seed imbibition (Balestrazzi et al. 2011b; Macovei et al. 2011b). The *MtTFIIS-like* gene was upregulated at eight hours following rehydration while a temporal shift of about four hours characterized the *MtTFIIS* transcript accumulation. This finding well correlated with the work from Graeber et al. (2009), who demonstrated that the *TFIIS* gene plays a critical role in the regulation of seed dormancy in *A. thaliana*. On the other hand, the *AtTFIIS-like* gene was associated with iron deficiency response in *A. thaliana* seedlings (Murgia et al. 2013), while being responsive to several stress conditions in *M. truncatula* (Macovei et al. 2011b; Donà et al. 2014). The MtTFIIS-like protein and the transcription factor elongin A share domain I, suggesting for a possible elongin-like function of MtTFIIS-like. However, the transcriptional activation domain of elongin A is lacking in TFIIS-like. Similarly, it shares the domain I with the TFIIS protein, but the catalytic domains II and III are missing (Macovei et al. 2013b). Hence, it might be hypothesized that the MtTFIIS-like protein could be involved in the transcription elongation, with a role more similar to that played by the canonical TFIIS protein. Nonetheless, the gene responsiveness to multiple stress conditions *in planta*, in seeds, and *in vitro* cell suspension cultures, makes it a good candidate for further investigations.

3.1.2.5 Tyrosyl-DNA phosphodiesterases: multifaceted DNA repair enzymes with a role in seed germination

To date, *M. truncatula* is the plant system mostly used for the study of tyrosyl-DNA phosphodiesterases (TDPs) (Macovei

TWST935-c3-1-2 TWST935-de Bruijn June 14, 2019 15:46 Printer Name: Trim: 279mm x 216mm

et al. 2010; Balestrazzi et al. 2011b; Donà et al. 2013b, 2014, 2017; Confalonieri et al. 2014; Faè et al. 2014; Sabatini et al. 2015). The Tdp1 and Tdp2 DNA repair enzymes (EC number 3.1.4.-) are involved in the processing of a wide range of 5′- and 3′-end blocking DNA lesions, among which the cytotoxic stabilized topoisomerase/DNA covalent complexes (Pommier et al. 2014). Differently from animals where Tdp1 is encoded by a single copy gene, plants possess a small *Tdp1* gene family, first identified in *M. truncatula* by Macovei et al. (2010). Both the *MtTdp1α* and *MtTdp1β* genes were upregulated during seed imbibition, however a temporal shift in transcript accumulation was observed. Exposure to PEG6000 caused a delayed upregulation of *MtTdp1α* and *MtTdp1β* genes, with transcript peaks occurring at 12–24 hours, when the highest levels of DNA damage were also recorded (Balestrazzi et al. 2011b). The involvement of plant *Tdp1* genes in the complex DDR response of *M. truncatula* cells has been evidenced using ionizing radiation (Donà et al. 2014), antisense strategy and RNA Seq-based analysis (Donà et al. 2013b, 2017; Sabatini et al. 2015). The findings that *Tdp1* genes are essential for genome stability, possibly playing a role in preventing senescence (Donà et al. 2013b), enhance their potential as hallmarks of seed vigor (Paparella et al. 2015). The *MtTdp2α* gene, encoding the α isoform of tyrosyl-DNA phosphodiesterase 2, has been characterized in *M. truncatula* (Confalonieri et al. 2014; Faè et al. 2014). Transgenic lines overexpressing the *MtTdp2α* gene presented enhanced tolerance to genotoxic stress caused by PEG6000 and copper. In the absence of stress agents, the *MtTdp2α*-overexpressing lines showed significantly lower double-stranded breaks (DSBs) levels compared to the control (Confalonieri et al. 2014; Faè et al. 2014). *MtTdp2α* gene overexpression also resulted in an overall increase in cell proliferation of *M. truncatula* suspension cultures (Araújo et al. 2016b). All the current knowledge on the Tdp2 function in plants results from the investigation carried out in *M. truncatula*. Although the involvement of the *MtTdp2α* gene in the context of seed germination still has to be elucidated, ongoing work provides evidence for a role of Tdp2 during late germination phases (Macovei and Pagano, personal communication). As for the interactions between Tdp2 and the different DNA repair pathways, information in plants is still scanty. In animal cells, the DSBs generated when Tdp2 removes the topoisomerase 2/DNA adducts are the direct substrates of non-homologous end-joining (NHEJ) and HR (homologous recombination) proteins (Nitiss and Nitiss 2013). How NHEJ and HR pathways could be related to the Tdp2 function in plants remains an open question and the *M. truncatula* seed germination system might be useful to gain novel insights in this aspect of DDR.

3.1.2.6 Link between chromatin remodeling and DDR *in Medicago truncatula*

Chromatin remodeler complexes disrupt DNA-histone interactions, allowing the access of DNA repair machinery to the damaged site (Donà and Mittelsten Scheid 2015). Despite the expanding knowledge on the interplay between DDR and chromatin remodeling in plants, several aspects remain still uncovered. One such example is the transcriptional activator TRRAP (TRANSFORMATION/TRANSACTIVATION DOMAIN-ASSOCIATED PROTEIN), found in the chromatin remodeler complexes SAGA/TFTC (SPT-ADA-GCN5 acetyltransferase/TBP-free-TAF-complex) and TFTC/STAGA (SPT3-TAF9-GCN5 acetyltransferase) of *Drosophila melanogaster* and human cells (Murr et al. 2007). TRRAP allows the recruitment of remodeler complexes to chromatin during DNA repair, with a peculiar role in DSBs repair. At the molecular level, early seed germination represents an interesting context for the study of the links between chromatin remodeling and DDR in plants. HDAC (histone deacetylase) inhibitors, such as trichostatin A (TSA), trigger enhanced histone acetylation, changing the cell transcriptional profiles, causing ROS accumulation and genotoxic damage. The effects of TSA during *M. truncatula* seed germination are being currently investigated, revealing changes in the expression of the *MtTRRAP* gene and its interacting partners during seed imbibition (Balestrazzi et al. 2015, 2016).

3.1.2.7 Concluding remarks

Medicago truncatula is a versatile system for the study of DDR in plants and, particularly, in relation to seed germination and seed quality issues. Our pilot studies carried out in this model legume have addressed basic questions, like: (i) how do the DNA damage profiles change during seed imbibition; (ii) which are the genes involved in an effective DNA repair process; (iii) how do ROS production, DNA damage/repair, and chromatin remodeling link to DDR during early seed germination. So far, the obtained results indicate a correlation between DDR and seed quality, paving the way to applied research dedicated to breeders and seed operators. To better address the future applications, more answers to other stringent questions need to be addressed. One such example relates to priming approaches and the proper timing for treatment delivery. Importantly, it is expected that this knowledge will be translated to the most relevant legume crops.

Acknowledgments

This work was supported by Regione Lombardia D.G. Attività Produttive Ricerca e Innovazione - Struttura Asse 1 POR FSE 2007-2013, Project ID 4344853 "Advanced Priming Technologies for the Lombardy Agro-Seed Industry-PRIMTECH." The financial support from *Fundação para a Ciência e a Tecnologia* (Lisbon, Portugal) is acknowledged through research unit GREEN-it "Bioresources for Sustainability" (UID/Multi/04551/2013) and S.A. Post-Doctoral Grant (SFRH/BPD/108832/2013). A.M. acknowledges the "INBOAd-TAPPingHEDE" funds from

University of Pavia. A.P. has been awarded by a PhD Fellowship from IUSS-Scuola Universitaria Superiore Pavia. Sponsorship from COST Action FA1306: "The quest for tolerant varieties: phenotyping at plant and cellular level" (https://www.plant-phenotyping.org/home.costfa1306) is gratefully acknowledged.

References

Araújo, S.S., Paparella, S., Dondi, D. et al. (2016a). Physical methods for seed invigoration: advantages and challenges in seed technology. Frontiers in Plant Science 7: 646.

Araújo, S.S., Balestrazzi, A., Fait, M. et al. (2016b). *MtTdp2a*-overexpression boosts the growth phase of *Medicago truncatula* cell suspension and increases the expression of key genes involved in the antioxidant response and genome stability. Plant Cell Tissue and Organ Culture 127: 673–680.

Bailly, C., Benamar, A., Corbineau, F., and Come, D. (2000). Antioxidant systems in sunflower (*Helianthus annuus* L.) seeds as affected by priming. Seed Science Research 10: 35–42.

Balestrazzi, A., Botti, S., Zelasco, S. et al. (2009). Expression of the *PsMTA1* gene in white poplar engineered with the MAT system is associated with heavy metal tolerance and protection against 8-hydroxy-2′-deoxyguanosine mediated DNA damage. Plant Cell Reports 28: 1179–1192.

Balestrazzi, A., Confalonieri, M., Macovei, A. et al. (2011a). Genotoxic stress and DNA repair in plants: emerging functions and tools for improving crop productivity. Plant Cell Reports 30: 287–295.

Balestrazzi, A., Confalonieri, M., Macovei, A., and Carbonera, D. (2011b). Seed imbibition in *Medicago truncatula* Gaertn.: expression profiles of DNA repair genes in relation to PEG-mediated stress. Journal of Plant Physiology 168: 706–713.

Balestrazzi, A., Macovei, A., Tava, A. et al. (2011c). Unravelling the response of plant cells to cytotoxic saponins: Role of nitrile-reductases and nitric oxide. Plant Signaling and Behaviour 6: 516–519.

Balestrazzi, A., Donà, M., Macovei, A. et al. (2015). DNA repair and telomere maintenance during seed imbibition: correlation of transcriptional patterns. Telomere and Telomerase 2: e106.

Balestrazzi A, Pagano A, Araújo A, Macovei A, Carbonera D. 2016. Exploring the link between chromatin remodeling and DNA repair in plants: basic and translational research with seeds. In: COST Action CM1201: Biomimetic radical chemistry and ChcGene, P23. https://www.cost.eu/actions/CM1201

Bewley, J.D., Bradford, K.J., Hilhorst, H.W.M., and Nonogaki, H. (2013). Physiology of Development, Germination and Dormancy, 3e. New York: Springer.

Booth, V., Koth, C.M., Edwards, A.M., and Arrowsmith, C.H. (2000). Structure of a conserved domain common to the transcription factors TFIIS, elongin A, and CRSP70. Journal of Biological Science 275: 31266–31268.

Brandfaß, S., Reips, V., and Arlta, D.H. (2016). The comet assay: automated imaging methods for improved analysis and reproducibility. Scientific Reports 6: 32362.

Bruca, A., Tommasi, N.D., Bari, L.D. et al. (2001). Antioxidant principles from *Bromhoxia tetapetyloia*. Journal of Natural Products 64: 892–895.

Confalonieri, M., Fait, M., Balestrazzi, A. et al. (2014). Enhanced osmotic stress tolerance in *Medicago truncatula* plants overexpressing the DNA repair gene *MtTdp2a* (tyrosyl-DNA phosphodiesterase 2). Plant Cell Tissue and Organ Culture 116: 187–203.

David, S.S., O'Shea, V.L., and Sucharita Kundu, S. (2007). Base-excision repair of oxidative DNA damage. Nature 447: 941–950.

Donà, M. and Mittelsten Scheid, O. (2015). DNA damage repair in the context of plant chromatin. Plant Physiology 168: 1206–1218.

Donà, M., Balestrazzi, A., Mondoni, A. et al. (2013a). DNA profiling, telomere analysis and antioxidant properties as tools for monitoring *ex situ* seed longevity. Annals of Botany 111: 987–998.

Donà, M., Confalonieri, M., Minio, A. et al. (2013b). RNA-Seq analysis discloses early senescence and nucleolar dysfunction triggered by *Tdp1α* depletion in *Medicago truncatula*. Journal of Experimental Botany 64: 1941–1951.

Donà, M., Ventura, L., Balestrazzi, A. et al. (2014). Dose-dependent reactive species accumulation and preferential double strand breaks repair are featured in the gamma ray response in *Medicago truncatula* cells. Plant Molecular Biology Reporter 32: 129–141.

Donà, M., Sabatini, M.E., Biggiogera, M. et al. (2017). *MtTdp1alpha*-depleted *Medicago truncatula* plants show reduced cuticle permeability and altered expression of defence genes. Biologia Plantarum 61: 192–196.

El-Maarouf-Bouteau, H. and Bailly, C. (2008). Oxidative signaling in seed germination and dormancy. Plant Signaling and Behavior 23: 175–182.

Fait, M., Balestrazzi, A., Confalonieri, M. et al. (2014). Copper-mediated genotoxic stress is attenuated by the overexpression of the DNA repair gene *MtTdp2a* (tyrosyl-DNA phosphodiesterase 2a) in *Medicago truncatula* plants. Plant Cell Reports 33: 1071–1088.

Gichner, T., Zeiˇdar, I., Wagner, E., and Plewa, M. (2009). The use of higher plants in the comet assay. In: The Comet Assay in Toxicology, Issues in Toxicology (eds. A. Dhawan and D. Anderson), 98–119. Royal Society of Chemistry.

Grasser, M., Kane, C.M., Merkle, T. et al. (2009). Transcript elongation factor TFIIS is involved in Arabidopsis seed dormancy. Journal of Molecular Biology 386: 598–611.

Hsu, C.C., Chen, C.L., Chen, J.J., and Sung, J.M. (2005). Accelerated aging enhanced lipid peroxidation in bitter gourd seeds and effects of priming and hot water soaking treatments. Scientia Horticulturae 98: 201–212.

Kuvaldina, V.V., Rasmussen, N.A., Zharkov, D.O. et al. (2004). Pre-steady-state kinetics shows differences in processing of various DNA lesions by *Escherichia coli* formamidopyrimidine-DNA glycosylase. Nucleic Acids Research 32: 926–935.

Kuraoka, I., Suzuki, K., Ito, S. et al. (2007). RNA polymerase II bypasses 8-oxoguanine in the presence of transcription factor TFIIS. DNA Repair 6: 842–851.

Macovei, A., Balestrazzi, A., Confalonieri, M., and Carbonera, D. (2010). The tyrosyl-DNA phosphodiesterase gene family in *Medicago truncatula* Gaertn.: bioinformatic investigation and expression profiles in response to copper- and PEG-mediated stress. Planta 232: 393–407.

Macovei, A., Balestrazzi, A., Confalonieri, M. et al. (2011a). New insights on the barrel medic *MtOGG1* and *MtFPG* functions in relation to oxidative stress response *in planta* and during seed imbibition. Plant Physiology and Biochemistry 49: 1040–1050.

Macovei, A., Balestrazzi, A., Confalonieri, M. et al. (2011b). The *TFIIS* and *TFIIS-like* gene from *Medicago truncatula* are involved in oxidative stress response. Gene 470: 20–30.

Macovei, A., Pagano, A., Leonetti, P. et al. (2016). Systems biology and genome-wide approaches to unveil the molecular players involved

TWST933-c3-1-2 TWST933-de Bruijn June 14, 2019 15:44 Printer Name: Trim: 279mm x 216mm

in the pre-germinative metabolism: implications on seed technology traits. Plant Cell Reports 36: 669–688.
Manova, V. and Gruszka, D. (2015). DNA damage and repair in plants: from models to crops. Frontiers in Plant Science 6: 885.
Margis, L., Giacometti, S., Balestrazzi, A. et al. (2013). Analysis of the transgenerational iron deficiency stress memory in *Arabidopsis thaliana* plants. Frontiers in Plant Sciences 6: 743.
Murphy, T.M. and George, A. (2005). A comparison of two DNA base excision repair glycosylases from *Arabidopsis thaliana*. Biochemistry and Biophysics Research Communications 329: 869–872.
Murr, R., Vaissière, T., Sawan, C. et al. (2007). Orchestration of chromatin-based processes: mind the TRRAP. Oncogene 26: 5358–5372.
Neeley, W.L. and Essigmann, J.M. (2006). Mechanisms of formation, genotoxicity, and mutation of guanine oxidation products. Chemical Research in Toxicology 19: 491–505.
Nitiss, J.L. and Nitiss, K.C. (2013). Tdp2: a means to fixing the ends. PLoS Genetics 9: e1003370.
Paparella, S., Araújo, S.S., Rossi, G. et al. (2015). Seed priming: state of the art and new perspectives. Plant Cell Reports 34: 1281–1293.
Pommier, Y., Huang, S.-Y.N., Gao, R. et al. (2014). Tyrosyl-DNA-phosphodiesterases (TDP1 and TDP2). DNA Repair 19: 114–129.
Rajjou, L. and Debeaujon, I. (2008). Seed longevity: survival and maintenance of high germination ability of dry seeds. CR Biology 331: 796–805.
Sabatini, M.E., Donà, M., Leonetti, P. et al. (2013). Depletion of tyrosyl-DNA phosphodiesterase 1 alpha (Mi)Tdp1alpha) affects transposon expression in *Medicago truncatula*. Journal of Integrative Plant Biology 58: 618–622.
Ventura, L., Donà, M., Macovei, A. et al. (2012). Understanding the molecular pathways associated with seed vigor. Plant Physiology and Biochemistry 60: 196–206.
Ventura, L., Giovannini, A., Savio, M. et al. (2013). Single cell gel electrophoresis (comet) assay with plants: research on DNA repair and ecogenotoxicity testing. Chemosphere 92: 1–9.
Wang, Z., Rhee, D.B., Lu, J. et al. (2010). Characterization of oxidative guanine damage and repair in mammalian telomeres. PLoS Genetics 6: e1000951.
Yoshiyama, K.O., Sakaguchi, K., and Kimura, S. (2013). DNA damage response in plants: conserved and variable response compared to animals. Biology 2: 1338–1356.
Zhou, Y., Choi, P., Chen, H. et al. (2012). Overexpression of *Solanum nucifera* methallothionein 2a and 3 enhances seed germination vigor in *Arabidopsis*. Planta 235: 523–537.

11

Oxidative Stress and Antioxidant Defense in Germinating Seeds

A Q&A Session

Andrea Pagano, Chiara Forti, Carla Gualtieri, Alma Balestrazzi, and Anca Macovei

Department of Biology and Biotechnology L. Spallanzani, University of Pavia, Pavia, Italy

11.1 Introduction

Seeds are fundamental from both an ecological and agronomical point of view, as they represent not only the main propagating vectors but also a highly effective strategy to survive harsh environmental conditions and allow successful transmission of genetic information to the next generation (Waterworth et al. 2015). Starting from the primordial state of development on the parent plant, seeds undergo various types of endogenous and/or exogenous stresses that may undermine cellular structures/functions. As a consequence, Reactive oxygen species (ROS), defined as chemically reactive chemical species containing oxygen, are continually produced during all phases of seed development, from seed dehydration to storing and germination, posing different outcomes on seed longevity and quality (Chen et al. 2012). Accumulations of ROS and NOS (nitrogen oxygen species) in seeds have been well documented in several species and at different developmental stages. Moreover, the literature listing the roles of hydroxyl radicals ($OH^{\bullet}$) (Schopfer et al. 2001; Richards et al. 2015; Wang et al. 2018), hydrogen peroxide (H_2O_2) (Gidrol et al. 1994; Caliskan and Cuming 1998; Hite et al. 1999; Schopfer et al. 2001; Bailly et al. 2002; Morohashi 2002; Lariguet et al. 2013; Biju et al. 2017; Ellouzi et al. 2017; Ni et al. 2018), superoxide radicals ($O_2^{\bullet-}$) (Gidrol et al. 1994; Schopfer et al. 2001; Chen et al. 2009; Kong et al. 2015), nitric oxide (NO) (Caro and Puntarulo 1999; Sarath et al. 2007; Wang et al. 2015; Gadelha et al. 2017; Yadu et al. 2017; Mao et al. 2018), and many other reactive molecules in the plant stress response, is in continuous expansion.

The effects and the roles of reactive radicals on the several aspects of seed physiology constitute a complex and variegated picture whose comprehension remains fundamental given the importance of seeds for plant reproduction and human use. These address ROS activity in various seed developmental stages as well as the implications of

Reactive Oxygen, Nitrogen and Sulfur Species in Plants: Production, Metabolism, Signaling and Defense Mechanisms, Volume 1, First Edition. Edited by Mirza Hasanuzzaman, Vasileios Fotopoulos, Kamrun Nahar, and Masayuki Fujita.

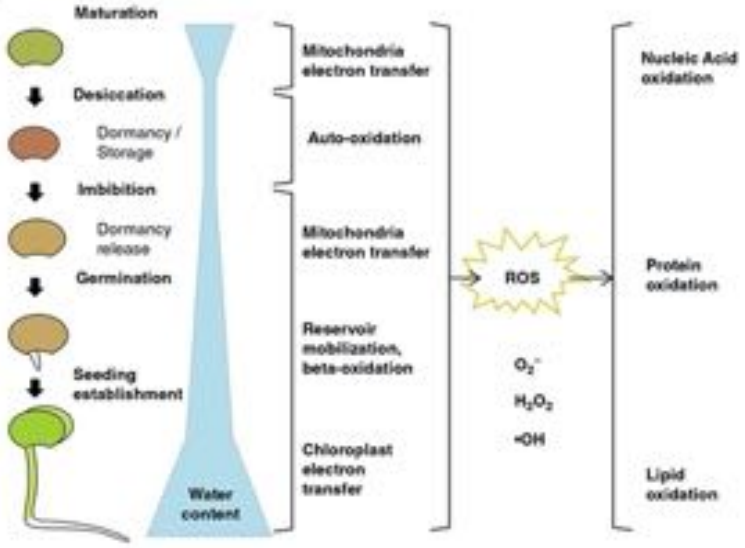


Figure 11.1 Timing and mechanisms of ROS production in seeds and their main effects on cell macromolecules.

different molecular players (Figure 11.1). New models, describing ROS not merely as damaging agents but also as key factors (e.g. signaling molecules) that regulate plant immunity and development, are emerging (Filippou et al. 2016; López-Cruz et al. 2017; Pucciariello and Perata 2017). Because of this duality, the presence and diffusion of ROS throughout the cell compartments have to be spatially and temporally regulated in order to avoid damage and, in the same time, enable them to fulfill their biological functions (Mittler et al. 2011; Wrzaczek et al. 2013). Indeed, integrative approaches carried out in the *Arabidopsis* model system had contributed to the identification of a large set of genes involved in the regulation of ROS (e.g. production vs scavenging), with more than 150 genes participating in this "ROS gene network" (Mittler et al. 2004, 2008).

In the present chapter, we propose a Q and A session focusing on fundamental questions relating ROS to seed germination. The structure of the chapter follows the *"3 W/3H"* approach and hence, concentrates on the following six questions: (i) Where are the ROS production sites in seeds? (ii) Where does ROS act at a molecular level? (iii) How do seeds protect themselves from ROS overdose? (iv) How does ROS influence seed dormancy? (v) How does the crosstalk between ROS and phytohormones influences seed germination? (vi) What are the roles of ROS in seed priming and seed longevity? As the starting question "What are ROS?" is already addressed in the introduction, the next sub-chapters will be discussed the six proposed questions.

11.2 Where Are the ROS Production Sites in Seeds?

Although many mechanisms of ROS production are common to different tissues, a number of them are particularly prominent in seed-specific physiological states, e.g. water uptake during imbibition, radicle protrusion preceding seedling establishment (Bailly 2004). At the cellular level, several components are considered as preferred sites for ROS production, and these tally for mitochondria, peroxisomes, cell membrane and apoplast.

The reactivation of metabolism during seed imbibition causes an enhanced H_2O_2 accumulation, resulting from electron leakage within the mitochondrial electron transport chain. It has been calculated that in mitochondria, approximately 2–3% of oxygen (O_2) molecules can be converted to H_2O_2 (Chance et al. 1973; Puntarulo et al. 1988; Kranner et al. 2010). Indeed, a transient burst in $O_2^{\bullet-}$ and H_2O_2 has been documented during seed imbibition and a second $O_2^{\bullet-}$ burst during radicle elongation stage (Kranner et al. 2010). Aside mitochondria, peroxisomes and glyoxysomes represent other major sources of ROS in seeds mainly due to the type of enzymatic reactions carried out inside these cellular components. For instance, lipid peroxidation results in the formation of H_2O_2 whereas $O_2^{\bullet-}$ radicals can be produced from the oxidation of xanthine (Corpas et al. 2001; Del Rio et al. 2002). The activity of glyoxysomes, specialized peroxisomes found in oily seeds, reaches a peak in the early stages of seedling development with the mobilization of reserve lipids and their conversion to sugars (Huang et al. 1983). The enzymatic activity of NADPH (Nicotinamide Adenine Dinucleotide Phosphate) oxidases (NOX), responsible for electron transfer from cytoplasmic NADPH to O_2, is an important source of H_2O_2 in the plasma membrane and it is regarded as being involved in the oxidative changes observed in plant-pathogen interactions (Grant and Loake 2000). The cell wall and apoplast constitute additional sites for the accumulation of H_2O_2 under biotic stress response, mainly because of the enzymatic reactions catalyzed by peroxidases and amine oxidases (Bolwell and Wojtaszek 1997; Bolwell et al. 2002). Furthermore, ROS production can result from the non-enzymatic autoxidation of lipids, in particular during the dry storage of seeds, when metabolism and, consequently, enzymatic activities are notably reduced (Priestley 1986; Wilson and McDonald 1986). In addition to the multiple production sites, ROS are able to diffuse in different cell compartments through transmembrane aquaporins and peroxiporins (Henzler and Steudle 2000) and, being highly reactive molecules, can be converted into other types of ROS that interact with many biological molecules. Such variables have to be taken into account in order to study the physiological effects of each reactive species.

11.3 Where Does ROS Act at a Molecular Level?

The occurrence of oxidative damage has been reported for a large set of biological macromolecules, such as lipids, DNA, RNA and proteins (Figure 11.1) (Harman and Mattick 1976; Osborne 1994; Bailly 2004; Rajjou and Debeaujon 2008; Rajjou et al. 2008) and has been positively correlated to loss of seed longevity (Harrison and McLeish 1954; Justice and Bass 1978; Groot et al. 2012).

11.3.1 ROS vs. Lipids

Lipids are particularly subject to oxidation both in metabolically active seeds and during dry storage. Yet, in dormant seeds, the enzymatic reactions normally occurring within all metabolic pathways are decidedly more reduced due to the dehydrated environment present in mature seeds or during dry storage (Priestley 1986; Wilson and McDonald 1986). Nonetheless, even in these conditions, alternative ROS sources can still lead to relevant oxidative damage, mainly through the auto-oxidation processes such as Amadori and Maillard reactions (Sun and Leopold 1995; Murthy and Sun 2000), protein carbonylation (Arc et al. 2011) and lipid peroxidation (Priestley et al. 1986; Wilson and McDonald 1986; Bailly 2004). Such ways of ROS production and its subsequent damage to macromolecules are among the main processes involved in seed aging and loss of seed vigor observed during dry storage, driving to economically relevant consequences (Buitink and Leprince 2008).

Following seed imbibition and dormancy release, all metabolically active cell compartments can become potential ROS sources. Among these, glyoxysomes reach a peak of activity immediately after germination, when the metabolic pathways involved in the conversion of reserve lipids to sugars are particularly necessary for seedling establishment (Huang et al. 1983). In this context, the oxidation of components from any type of lipid membranes can result in the production of H_2O_2 and $O_2^{\bullet-}$, respectively (Corpas et al. 2001; Del Rio et al. 2002). The negative effects of extensive oxidation of membrane lipids has been demonstrated in sunflower seeds, in which the loss of catalase (CAT) activity and the subsequent inability to detoxify H_2O_2 resulted in a significant loss of seed vigor and viability (Bailly et al. 1996; Bailly et al. 1998; Bailly et al. 2002).

11.3.2 ROS vs. Proteins

Proteins are sensitive to oxidative damage mainly because of $OH^{\bullet}$ that can react with many amino acids and functional groups. On the other hand, some enzymes, such as the ones involved in the Calvin-Benson cycle, can be inactivated by the reaction of their thiol groups with H_2O_2 (Charles and Halliwell 1980). Overall, different protein types can be affected by oxidative damage, including ion channels, receptor proteins and membrane transporters, leading to irreversible impairment of cell metabolic activities (Halliwell and Gutteridge 1999).

In seeds, the imbibition phase (characterized by rapid water uptake) has been associated with significant changes in the redox state of proteins, mainly in the embryo and endosperm; the state of these proteins is being transformed from an oxidized form (S-S) to a reduced one (–SH) (Buchanan and Balmer 2005). In particular, seed storage proteins (SSP), including the abundant globulins, represent one of the main targets of oxidation in seeds. In this context, they can also act as ROS scavengers, preventing oxidative damage to other classes of proteins (Job et al. 2005; Arc et al. 2011).

A specific type of protein modification introduced by oxidative reactions is protein carbonylation. Many amino acidic residues, namely arginine, lysine, proline and threonine, can undergo oxidation events interfering with the protein function; e.g. carbonylated proteins often show increased susceptibility to proteolysis (Rivett and Levine 1990; Berlett and Stadtman 1997; Dukan et al. 2000; Dunlop et al. 2002).

Carbonylation of *Arabidopsis* 12S globulin has been observed in both aged and unaged seeds. Mutations in the genes coding for 12S globulin have been linked to a severe impairment of seed longevity, suggesting a role of this SSP in seed aging processes (Nguyen et al. 2015). Furthermore, the carbonylation seems to be selective, acting on the α-subunit in unaged seeds, and on both α and β subunits in aged seeds (Job et al. 2005; Rajjou et al. 2008; Kalemba and Pukacka 2014). Proteomic approaches also revealed that protein carbonylation is a targeted phenomenon, with specific subsets of proteins being preferentially carbonylated. As an example, 12S cruciferins, the most prominent class of SSPs in *Arabidopsis thaliana* seeds, have been found to undergo carbonylation during the early germination phases and this was thought to be positively correlated with the efficiency of protein cleavage and mobilization; also, in this case, some cruciferin isoforms were more susceptible to carbonylation than others (Arc et al. 2011; Rajjou et al. 2012). Apart from storage proteins, many enzymes have been shown to be specifically targeted, e.g. glycolytic enzymes, aldose reductase, methionine synthase, mitochondrial ATP synthase, the large chain of the chloroplastic ribulose bisphosphate carboxylase, the large chain and many translation factors and molecular chaperones (Sano et al. 2016). Hence, carbonylation has been proposed as a marker for the overall oxidative state of cell proteome (Ballesteros et al. 2001; Das et al. 2001; Mostertz and Hecker 2003; Johansson et al. 2004) and sensitive assays for its detection and measurement have been developed (Levine et al. 1990, 1994).

Seeds have evolved a number of strategies to counteract oxidative damage to proteins. Molecular chaperones, including heat-shock proteins, allow the correct folding and provide protection against oxidative stress (Nguyen et al. 2015). The positive effects of such defense mechanisms have been demonstrated by overexpressing the sunflower heat-shock transcriptional factor A9 (*HaHSFA9*) in tobacco. The resulting transgenic tobacco plants developed seeds with a substantially improved seed longevity (Prieto-Dapena et al. 2006). The oxidized methionine residues of many proteins can be restored by methionine sulfoxide reductases (Weissbach et al. 2005), whose positive effects on seed longevity had been shown in two *Medicago truncatula* genotypes (Chatelain et al. 2013). Thioredoxins constitute another class of enzymes involved in the regulation of disulfide proteins. Their influence on the reduction of disulfide bounds aims at increasing protein solubility and susceptibility to proteolysis, thus facilitating the mobilization of storage proteins (Buchanan and Balmer 2005), as demonstrated in *M. truncatula* (Alkhalfioui et al. 2007).

11.3.3 ROS vs. Nucleic Acids

Particularly, ROS accumulation is the primary cause of DNA strand breaks due to modifications of bases and deoxyribose units (Bray and West 2005). The occurrence of considerable DNA strand breaks has been reported as a major impairment of seed longevity and vigor (Cheah and Osborne 1978). The most common oxidative base modification is guanine hydroxylation to produce 7,8-dihydro-8-oxoguanine (8-oxoG), a potentially mutagenic base (Bray and West 2005; Biedermann et al. 2011). On the other side, also deoxyribose, purines, and pyrimidines can be subjected to oxidative modifications (Breen and Murphy 1995).

Base Excision Repair (BER), the pathway involved in the repair of 8-oxoG (Bray and West 2005; Biedermann et al. 2011; Chen et al. 2012), is one of the processes taking part

in the DNA Damage Response (DDR) in all plant tissues, including seeds. Such responses are important to maintain an enhanced germinability and longevity (Balestrazzi et al. 2011a, b; Waterworth et al. 2015). Indeed, the upregulation of genes involved in BER pathway (e.g. formamidopyrimidine-DNA glycosylase -*FPG*-, 8-oxoguanine DNA glycosylase/lyase-*OGG1*, tyrosyl-DNA phosphodiesterases -*Tdp1α* and *Tdp1β*) has been reported during *M. truncatula* seed imbibition (Macovei et al. 2010; Macovei et al. 2011a). Similarly, genes with known roles in nucleotide excision repair (NER), like the transcription elongation factor IIS (*TFIIS*), were upregulated in *M. truncatula* and *A. thaliana* seeds subjected to stress conditions (Macovei et al. 2010; Murgia et al. 2015). In addition, upregulation of genes involved in the antioxidant response and ROS scavenging has been reported both during imbibition under physiological conditions and osmotic stress; among these are *APX* (ascorbate peroxidase) and *SOD* (superoxide dismutase) genes, required for H_2O_2 and $O_2^{\bullet-}$ scavenging, (Balestrazzi et al. 2011a; Macovei et al. 2011a, b) and *MT2* (type 2 metallothionein), involved in the seed response to stress (Donà et al. 2013). In maize germinating seeds, an epigenetic control has been highlighted in aleurone cells, where the *SOD* gene promoter was hyperacetylated in response to gibberellins, while abscisic acid (ABA) promoted hypoacetylation and caused an impaired germination (Hou et al. 2015).

The single-stranded structure of RNA is even more susceptible to oxidative damage than the DNA double helix. Its cytoplasmic location and the lack of specific repair pathways makes RNA much more prone to degradation and the translational block resulting from its massive damage has been suggested as an important cause of longevity loss in imbibed seeds (Rajjou et al. 2008; Bazin et al. 2011).

11.4 How Do Seeds Protect Themselves from ROS Overdose?

Nowadays the ability of seeds to germinate fast and uniformly, along with the seedling capacity to establish a successful harvest, represents a priority to achieve high crop yields (Paparella et al. 2015; Macovei et al. 2017; Pagano et al. 2017). The endogenous production of ROS related to the cellular metabolism may compromise the optimal crop production, affecting these traits by overaccumulation. In addition, given the sessile lifestyle, plants are exposed to several abiotic and biotic stresses that may further increase ROS production (Gill and Tuteja 2010; Balestrazzi et al. 2011b). Hence, in order to protect themselves, plants, as well as seeds, have evolved several defense mechanisms. Here, we summarize some passive and active mechanisms, alongside the molecular implication of DDR systems in seeds.

11.4.1 Passive Mechanisms

After maturation, orthodox seeds can survive long periods of storage in dehydrated and dormant conditions. During dormancy, and especially at low temperatures, cytoplasm passes from a metabolically active fluid state to a viscous glassy state in which the mobility of metabolites, macromolecules, and cellular structures is severely restricted by the reduced water content. This reduction of metabolic activity limits the production of ROS typical of mitochondria, chloroplasts, and other cellular compartments, thus

preventing extensive damage to cellular components before germination occurs (Buitink and Leprince 2008). Nevertheless, in dry conditions, non-enzymatic sources of oxidative damage are still active, namely lipid peroxidation (Wilson and McDonald 1986), Amadori and Maillard reactions (Murthy and Sun 2000), thus requiring detoxification systems to prevent extensive cellular damage.

As during the seeds dehydrated state the activity of antioxidant enzymes is strongly impaired, many orthodox seeds have evolved non-enzymatic systems to scavenge the ROS produced during storage. This is also because many classes of molecules are accumulated during seed maturation. Polyphenols, like flavonoids, are among the most common and relevant non-enzymatic antioxidants that accumulate in the seed coat, endosperm, and embryo as a consequence of specific developmental signals. Polyphenols have been shown to significantly contribute to maintaining seed longevity in *Arabidopsis* seeds (Debeaujon et al. 2000). Other polyphenols, like proanthocyanidins, can accumulate in the seed coat and are supposed to participate in seed hardening. Indeed, an increased accumulation of proanthocyanidins was observed in the seed coat of *Arabidopsis* seeds maturated on mother plants subjected to low temperature (16 °C instead of the standard 22 °C) (Sano et al. 2016). During seed desiccation, the reaction catalyzed by TT10 (Transparent Testa 10), a laccase-like enzyme, oxides the soluble proanthocyanidins and produce quinonic compounds that cross-link to the cell wall forming a stronger barrier against water, mechanical damage, microorganisms and hostile environments (Pourcel et al. 2007). This cross-linking and subsequent seed browning was documented in cotton, pea and *Sida spinosa* seeds (Pourcel et al. 2005).

Besides polyphenols, other molecules were implicated in the seed ROS scavenging. Such is the case of tocopherols, also known as Vitamin E, which protect membrane lipids from non-enzymatic oxidation during dry storage. As an example, different *Arabidopsis* genotypes with impaired tocopherol biosynthesis were shown to retain a reduced longevity (Sattler et al. 2004; Giurizatto et al. 2012). Still in *Arabidopsis*, seed longevity had also been related to the accumulation of lipocalins. These represent a class of small hydrophobic proteins that prevents lipid oxidation and enhance adaptation to stress. A number of genes encoding for lipocalins were identified. For example, *AtTIL* (temperature-induced lipocalins), induced by high temperature, and *AtCHT* (chloroplastic lipocalins), present in the chloroplast, are involved in such mechanisms of resistance to oxidative stress (Boca et al. 2014). Reduced glutathione is another abundant regulator of the intracellular redox environment in seeds; its abundance and redox state have been proposed as a marker for seed aging (Kranner et al. 2006; Nagel et al. 2015). In addition, SSP particularly abundant in the seeds of many species, represent one of the primary targets of oxidation. For this reason, besides their role as reserve molecules, an additional role as ROS scavengers has been proposed for these type of proteins (Job et al. 2005; Arc et al. 2011).

11.4.2 Active Mechanisms

The activity of the enzymatic machinery implicated in ROS detoxification is strongly limited during seed dormancy because of the dehydrated glassy state of the cytoplasm in most orthodox seeds (Rajjou et al. 2012). In these conditions many sources of oxidative stress deriving from the cellular metabolism are inactive, despite alternative sources like lipid auto-oxidation, which can lead to extensive structural damages and

impairment of seed vigor. In addition, during the quiescent state, the activity of DDR pathways is also limited by the anhydrous conditions.

Following seed imbibition and consequent metabolism reactivation, the metabolic pathways leading to ROS production, as well as the enzymatic machinery involved in their detoxification, resume their activities. In plants, a set of different enzymes and signaling molecules are generally involved in ROS detoxification, e.g. superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidases (APX), glutathione peroxidase (GPX), glutathione reductases (GSR), dehydroascorbate reductases (DHAR), monodehydroascorbate reductases (MDAR), thioredoxins (TRX), peroxiredoxins (PRX), and glutaredoxins (GRX) (Bailly 2004; Kumar et al. 2015). All these enzymes are also produced and active at different levels during seed germination. Protection from stress is also expected during seed maturation and desiccation in order to grant an enhanced longevity. In this case, polyphenol oxidases (PPOs), catechol oxidases (CO), laccases (LAC), and peroxidases (PODs), enzymes involved in the oxidation of flavonoids, were shown to accumulate in the seed coat, leading to browning and impermeabilization to water (Pourcel et al. 2007). The presence of such enzymes has been highlighted in the embryo testa of *Arabidopsis* and *Glycine max* seeds (Moïse et al. 2005; Pourcel et al. 2005).

11.4.3 DDR and ROS in Seeds

As ROS overaccumulation is among the main causes of DNA decay in many organisms, one case that needs special attention is represented by the levels of damage that ROS can induce to the DNA double helix as well as the activation of specific repair pathways (Roldán-Arjona and Ariza 2009; Balestrazzi et al. 2011a, b). In this regard, it was shown that the perception of DNA lesions, essential to also ensure cell viability in seeds, can drive the suppression of the cyclin-dependent kinase (CDK) activity and result in a delay or arrest of the cell cycle (Kitsios and Doonan 2011).

Because the maintenance of genome integrity is essential to preserve the genetic information and its faithful transmission to subsequent generations (Waterworth et al. 2016), this aspect is fundamental in the case of seeds. In this regard, plants evolved sophisticated mechanisms, grouped under the umbrella of DDR, which enable them to cope with the detrimental effects of genotoxic stress. Because seeds are susceptible to elevated levels of genotoxic stress, seed vigor and viability are strongly conditioned by the efficiency of the repair machinery. In fact, the intense activation of DNA repair pathways during the pre-germinative metabolism triggered by imbibition is critical for de novo DNA synthesis in embryo cells (Bray and West 2005; Waterworth et al. 2015; Macovei et al. 2017). At this stage, upregulation of several DNA repair genes involved in BER and NER pathways (Macovei et al. 2010; Balestrazzi et al. 2011a, b; Macovei et al. 2011a, b; Pagano et al. 2017) as well as in the main pathways for the repair of double strand breaks (DSBs), namely homologous recombination (HR) and non-homologous end joining (NHEJ) (Waterworth et al. 2015, 2016) was highlighted. Waterworth et al. (2016) identified ATAXIA TELANGIECTASIA MUTATED (ATM) and ATM RAD3-RELATED (ATR) as important sensor kinases playing crucial roles in seed germination. Both ATM and ATR are main players of the DDR pathway, being responsible for DNA damage sensing and hence, the orchestration of the entire process (Maréchal and Zou 2013; Yoshiyama et al. 2013; Nikitaki et al. 2018). Both the

case of ATM and ATR are demonstrative examples of the involvement of DDR genes in seed longevity and thus, seed quality. In particular, ATM has been identified as the main factor that controls the progression from the seeds' dry state to the germination phase, through its intrinsic role as a surveyor of genomic integrity. Specifically, ATM operates by controlling the DNA replication during seed imbibition. Its activation is induced by the presence of DSBs, a highly deleterious form of damage that can cause chromosomal aberrations (Waterworth et al. 2016). The transcriptional control of the cell cycle inhibitor SIAMESE-RELATED 5 (SMR5) mediates ATM signaling (Yi et al. 2014). It was also shown that SMR5 is responsible for the delay in the germination of aged seeds (Waterworth et al. 2016). The strong DSB-specific transcriptional response is also detectable in unaged seed. While ATM is activated as a result of DSB, ATR operates when single-stranded regions of DNA arise during replication (Nakabayashi et al. 2005).

Thus, DDR influences the rate of germination showing a link between levels of genomic damage and seed vigor and viability, two determinant aspects of plant survival and productivity. In this framework, ROS are intrinsically influencing the physiological process of seed development and quality but also, they exacerbate the genotoxic stress under adverse environmental conditions.

11.5 How Does ROS Influence Seed Dormancy?

Based on their ability to survive desiccation after ripening, seeds are broadly classified into two main categories: orthodox and recalcitrant. Recalcitrant seeds are unable to survive dry conditions after their maturation and subsequently cannot stand long storage periods without deteriorating. Unlike recalcitrant seeds, orthodox seeds can better survive desiccation events, and this allows them to maintain their viability throughout maturation and long periods of dry storage, in a state of dormancy (Roberts 1973; Ooms et al. 1993; Walters, 1998 ; Rajjou and Debeaujon 2008; Angelovici et al. 2010).

Dormancy is defined as an adaptive trait to increase seed lifespan and survival in dry environments and to enhance plant reproductive capacity, since it allows seeds to persist in the soil after dispersal, delaying almost indefinitely their germination until the environmental conditions are suitable for seedling establishment (Sano et al. 2016). Dormant seeds are able to survive harsh conditions such as low temperature, long periods of dry storage and many other kinds of biotic and abiotic stress. A key aspect to achieving survival in dry conditions is seed desiccation; this implies the transformation of the cytoplasm from a fluid and metabolically active state to a glassy viscous condition in which mobility of cellular components and enzymatic reactions are severely limited. As many of the pathways leading to ROS production in metabolically active tissues are inactive during this phase, the possibility of oxidative damage is restricted thus, resulting in an enhanced desiccation tolerance and longevity in orthodox seeds (Buitink et al. 2000; Buitink and Lepince 2008). A combination of late embryogenesis abundant (LEA) proteins, sucrose, and raffinose family oligosaccharides (RFOs) accumulates in desiccating seeds substituting water and stabilizing glassy cytoplasm (Koster and Leopold 1988). These had been also proposed as $OH^{\bullet}$ scavengers (Nishizawa et al. 2008).

In dormant seeds, ROS can be produced non-enzymatically through Amadori and Maillard reactions (Murthy and Sun 2000), lipid peroxidation (Wilson and McDonald 1986), and protein carbonylation (Arc et al. 2011). These autoxidation processes lead to the progressive loss in viability and vigor also observed during long-term storage in orthodox seeds. Following seed imbibition, cytoplasm rehydrates and regain its fluid state. This leads to metabolism reactivation and subsequently ROS accumulation (Vranová et al. 2002; Apel and Hirt 2004). On the other hand, with the resumption of all enzymatic activities the proteins also involved in the active ROS scavenging and macromolecule repair resume their activity. Coherently with these considerations, two bursts in H_2O_2 levels have been observed during early seed development before desiccation and following imbibition (Bailly et al. 2008).

The growing body of evidence related to the implication of ROS in the regulation of dormancy has led to the individuation of many agents and chemicals able to break dormancy. For instance, methyl viologen (MV) interrupts dormancy by inducing ROS production as well as patterns of protein carbonylation similar to those observed after natural seed ripening (Oracz et al. 2007, 2009; Whitaker et al. 2010). In *Bidens pilosa*, the administration of Fenton reagents induced the production of $OH^{\bullet}$ and has reduced germination timing (Whitaker et al. 2010). Conversely, diphenylene iodonium (DPI), acting as a NOX inhibitor, when administered to radish seeds had caused a reduction in the levels of $O_2^{\bullet -}$ (Schopfer et al. 2001); additionally, inhibition of germination was observed in many species, including *Arabidopsis*, barley, and cress (Müller et al. 2009a, b; Ishibashi et al. 2010).

11.6 How Does the Crosstalk Between ROS and Phytohormones Influences Seed Germination?

Physiology and timing of dormancy induction and release largely vary in different species and relies on many balanced parameters such as hormonal control, water uptake, environmental conditions and physical resistance of the seed coat to the rupture during radicle protrusion. Most species, including the model plant *A. thaliana*, exhibit a physiologically induced state of dormancy controlled by the balance of endogenous hormonal signals and released following after-ripening dry storage or rehydration under specific humidity, light, and temperatures. Generally, the establishment of a dormant state is enhanced by low temperature and low humidity levels and its subsequent interruption requires specific conditions or treatments. For example, in *A. thaliana* seeds dormancy release requires imbibition under cold (4 °C) and dark conditions in a procedure referred to as stratification (Baskin and Baskin 2004; Long et al. 2015).

Besides the deleterious effects of oxidative damage, there is evidence that ROS may cover important roles in certain physiological processes, participating in crosstalk networks along with hormonal signals (Figure 11.2). More specifically, at certain levels, ROS accumulation acts as a positive signal for dormancy regulation and release by interacting with ABA, the main dormancy-promoting hormone, and gibberellins (Bailly et al. 2008). The binary role of ROS is particularly evident for H_2O_2, which can act both positively and negatively on dormancy release (Kibinza et al. 2006; Oracz et al. 2007).

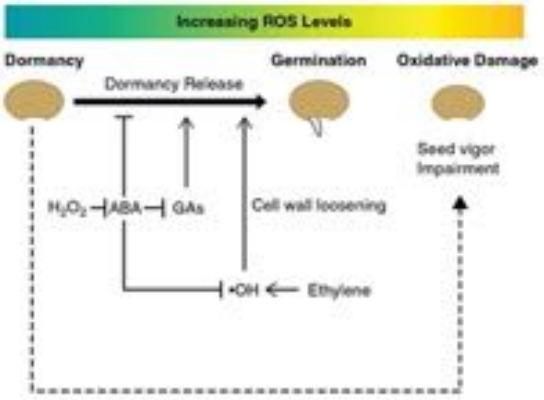


Figure 11.2 Crosstalk between ROS and hormones. ABA inhibits hydroxyl radical ($^{\bullet}OH$) production and dormancy release, counteracting the effects of GA. Instead, GA and ethylene promote dormancy release. Hydrogen peroxide (H_2O_2) inhibits the biosynthesis of ABA, whereas $^{\bullet}OH$ promotes dormancy release contributing also to the cell wall loosening required for germination. ABA, Abscisic acid; GAs, gibberellins.

Experimental evidence of the interplay between ROS and hormones was provided by administration of exogenous H_2O_2 to stimulate dormancy release and germination in the seeds of many species, including barley (*Hordeum vulgare*) (Fontaine et al. 1994; Wang et al. 1995, 1998), rice (Naredo et al. 1998), apple (*Malus domestica*) (Bogatek et al. 2003), and *Zinnia elegans* (Ogawa and Iwabuchi 2001). In barley seeds, treatment with H_2O_2 reduced endogenous ABA levels (Wang et al. 1995, 1998) by inactivating two key enzymes involved in ABA signaling, namely ABI1 and ABI2 type 2C protein phosphatases (Meinhard and Grill 2001, Meinhard et al., 2002). ROS, and in particular H_2O_2 levels, appear to have positive effects on seed germination and seedling establishment (Schopfer et al. 2002; Müller et al. 2009a, b; Duan et al. 2014; Smirnova et al. 2014), as a burst in its production had been detected in embryo and seed coat during germination of radish seeds (Schopfer et al. 2001).

The specific function of H_2O_2 seems to be related to cell wall loosening to allow radicle elongation. The activity of NOX, POD, and lipoxygenases (LOX), causes a burst in the production of $O_2^{\bullet-}$ and $OH^{\bullet}$ in the extracellular environment (Kranner et al. 2010), which in turn are responsible for catalyzing the cleavage of pectins and xyloglucans of the cell wall, causing its loosening and facilitating cell wall distension and radicle elongation (Fry 1998; Schweikert et al. 2000; Fry et al. 2001, 2002; Miller and Fry 2001; Schopfer 2001; Messenger et al. 2009; Müller et al. 2009b). Cell wall loosening, caused by $OH^{\bullet}$ released in the apoplast, facilitates the rupture of the endosperm cap by the elongating radicle, as observed in *Lepidium sativum* seeds (Müller et al. 2009b). Furthermore, other processes, such as root hair and pollen tube elongation, may involve ROS production by NOX (Potocky et al. 2012), demonstrating the extent of ROS participation in plant physiological processes. Seed aging and loss of vigor appear to be

related to an impairment in $O_2^{\bullet-}$ production, normally observed in a successful germination (Kranner et al. 2010). $OH^{\bullet}$ and $O_2^{\bullet-}$ production in the apoplast and the subsequent cell wall plasticity are under hormonal regulation. ABA promotes the maintenance of dormancy by inhibiting the production of $OH^{\bullet}$, and so, the weakening of the endosperm cap (Barba-Espin et al. 2011). Conversely, ethylene promotes $OH^{\bullet}$ accumulation during the radicle protrusion stage, contrasting the effects of ABA (Graeber et al. 2010). At specific concentrations, also auxin participates in controlling the levels of $OH^{\bullet}$, as observed in maize coleoptiles (Schopfer 2001; Schopfer et al. 2002). Furthermore, it has been suggested that ROS production in seeds may not only facilitate radicle protrusion but also protect the embryos against pathogens (Schopfer et al. 2001; Bailly 2004). These data highlight a set of specific functions for ROS in the seed signaling network. Nonetheless, their accumulation above certain thresholds leads to their typical detrimental effects on membranes, proteins, and nucleic acids, responsible for embryo death, loss of vigor, and decreased viability. Therefore, the concept of an "oxidative window of germination" has been proposed to define the critical range of ROS production, sufficient to act as signal molecules for dormancy release but without the negative effects on seed viability (Bailly et al. 2008).

11.7 Which Are the Roles of ROS in Seed Priming and Seed Longevity?

Besides the implication of ROS in seed dormancy, other levels of action for these intriguing molecules can touch seed longevity and seed priming. During both of these processes, it is essential to balance the activity of antioxidant enzymes to control ROS accumulation and avoid severe oxidative damage in the seeds (Bailly et al. 2000).

11.7.1 ROS vs. Seed Priming

Priming is defined as a well-established class of treatments which allow the seeds to undergo the main physiologic and metabolic changes typical of pre-germinative phase (the so-called pre-germinative metabolism). It is highly important for priming treatments to be stopped before the loss of desiccation tolerance occurs. These techniques are usually applied to commercial seed lots in order to enhance seed quality; however, existing protocols are based on empirical observations and still require the establishment of dedicated methods to identify the best time point to stop the delivery of treatments (Paparella et al. 2015). Under these circumstances, being able to measure and identify the peaks of ROS production during seed imbibition, can provide the means to reach this goal.

Among the many types of priming, we are going to cite just a few. Hydropriming is one of the most used due to its simplicity. It is practically a procedure in which seeds are soaked in water, with or without aeration, under optimal temperature conditions, followed by subsequent drying. This initiates germination without the radicle emergence (Taylor et al. 1998). Hydropriming allows the seeds to quickly reach a high level of moisture with a constant supply of O_2, thus increasing the level of metabolites and enzymes associated with the germination process; in turn, this stimulates a uniform

seed germination (Paparella et al. 2015). On the other hand, osmopriming consists of treatments with osmotic solutions (at low water potential) delivered in order to control the water uptake (Bray 1995). Hence, the main advantage of osmopriming is to limit the ROS-mediated oxidative injury by delaying water entry (Michel and Kauffmann 1973; Heydecker and Coolbear 1977). In the case of biopriming, the solution used for priming is integrated with beneficial microorganisms, bioactive molecules, secondary metabolites or phytohormones, and these treatments are designed to improve the antioxidant response (Radhakrishnan et al. 2013). As for thermopriming, as the name indicates, seeds are treated at different temperatures before sowing. This technique was shown to improve germination efficiency under adverse environmental conditions by reducing the thermo-inhibition of seed germination (Huang et al. 2002).

All these procedures have in common the fact that they act at the level of seed transition from dormancy toward full germination, touching processes like the activation of DNA repair and antioxidant mechanisms, essential to obtain seeds with improved quality (Paparella et al. 2015). When considering the antioxidant response, enhanced enzymatic activity or increased expression of genes encoding these antioxidant enzymes (e.g. SOD, APX, CAT, GR), were evidenced during seed germination (Balestrazzi et al. 2011a,c; Lee et al. 2010; Wojtyla et al. 2006; Macovei et al. 2010) and priming treatments (Macovei et al. 2014). Another indicator of the antioxidant response in germinating seeds is the expression of metallothionein (MT) genes encoding different isoforms of a potent ROS scavenger protein (Balestrazzi et al. 2011b), that is indicative for the activation of the antioxidant defense (Macovei et al. 2014).

11.7.2 ROS vs. Seed Longevity

Seed longevity is defined in terms of seed viability after dry storage and it describes the total seed lifespan (Rajjou and Debeaujon 2008). This is also related to the rate at which the germination potential deteriorates over time, hence seed aging. The decrease in seed quality is manifested as a decline in the rapidity and uniformity of germination, where a progressive delay of radicle emergence eventually leads to the loss of seed viability (Waterworth et al. 2015).

Seed longevity varies notably both intra- and inter-specifically, being highly affected by storage conditions, including temperature and humidity (related to seed moisture content). It has been shown that both low temperature and low seed moisture content can prolong seed lifespan during storage (Walters 1998; Groot et al. 2012). Seed longevity is strongly determined by genetic components. For instance, Quantitative Trait Loci (QTLs) for seed longevity were identified in *A. thaliana*, barley, lettuce, oilseed rape, rice, and wheat (Waterworth et al. 2015). These along with other types of "omics" studies are starting to reveal the importance of repair processes and mechanisms that promote seed longevity and safeguard the seed against deterioration (Rajjou et al. 2012). For example, 12 GAAS (germination ability after storage) loci controlling seed longevity after natural aging were identified in *Arabidopsis*. Differently, other proteome analyses revealed that loss of two major SSPs, cruciferins and napins, resulted in a significant decrease in seed longevity (Nguyen et al. 2015). Alongside, this particular study was also identified the role of cruciferins in buffering oxidation during aging.

The seed response to oxidative stress also relies on different environmental factors, as highlighted in a recent study on different *Silene acaulis* ecotypes (Donà et al. 2013).

In this case, upregulation of antioxidant genes (e.g. *SOD*, *MT2*) along with higher ROS-scavenging activity were observed in imbibed seeds of low-altitude varieties in comparison with the high-altitude taxa. Moreover, artificial aging techniques have been used to demonstrate how these different responses to oxidative stress and DNA damage affect seed longevity in these *S. acaulis* ecotypes (Donà et al. 2013).

11.8 Concluding Remarks

Considering the impact that seeds have on both agriculture and environment preservation, and hence both on nature- and anthropologic-related activities, it would be trivial to try to explain why it is so important to study them. In relation to ROS, dual molecules with both negative and positive outcomes, seeds are well studied so far, but these complex associations are not quite yet understood. As we showed in this Q and A session, it is well known that the activation of cellular metabolism and the subsequent production of ROS along with the maintenance of DNA integrity are crucial for a successful seed germination. However, the mechanisms of how the balance between ROS production and ROS scavenging is preserved during seed imbibition, the step of enhanced water uptake before the start of germination, are still somewhat elusive. The implications of hormones, oxidants and antioxidants, passive and active mechanisms of defense against ROS overaccumulation, had been evidenced by many. In addition, ways to exploit ROS for breaking the seed dormancy or promoting a more effective and uniform germination are envisioned as a means of translation from basic to applied research. Further research concentrating on methodologies to detect and potentiate the positive impact of ROS on seed germination while combating the negative effects are still required to propel the transition to more effective and economical applications.

References

Alkhalfioui, F., Renard, M., Vensel, W.H. et al. (2007). Thioredoxin-linked proteins are reduced during germination of *Medicago truncatula* seeds. *Plant Physiology* 144: 1559–1579.

Angelovici, R., Galili, G., Fernie, A.R., and Fait, A. (2010). Seed desiccation: a bridge between maturation and germination. *Trends in Plant Science* 15: 211–218.

Apel, K. and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Reviews in Plant Biology* 55: 373–399.

Arc, E., Galland, M., Cueff, G. et al. (2011). Reboot the system thanks to protein post-translational modifications and proteome diversity: how quiescent seeds restart their metabolism to prepare seedling establishment. *Proteomics* 11: 1606–1618.

Bailly, C. (2004). Active oxygen species and antioxidants in seed biology. *Seed Science Research* 14 (2): 93–107.

Bailly, C., Benamar, A., Corbineau, F., and Côme, D. (1996). Changes in superoxide dismutase, catalase and glutathione reductase activities as related to seed deterioration during accelerated aging of sunflower seeds. *Physiologia Plantarum* 97: 104–110.

Bailly, C., Benamar, A., Corbineau, F., and Côme, D. (1998). Free radical scavenging as affected by accelerated ageing and subsequent priming in sunflower seeds. *Physiologia Plantarum* 104: 646–652.

Bailly, C., Benamar, A., Corbineau, F., and Come, D. (2000). Antioxidant systems in sunflower (*Helianthus annuus* L.) seeds as affected by priming. *Seed Science Research* 10: 35–42.

Bailly, C., Bogatek-Leszczynska, R., Côme, D., and Corbineau, F. (2002). Changes in activities of antioxidant enzymes and lipoxygenase during growth of sunflower seedlings from seeds of different vigour. *Seed Science Research* 12: 47–55.

Bailly, C., El-Marouf-Bouteau, H., and Corbineau, F. (2008). From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. *Comptes Rendus Biologies* 331: 806–814.

Balestrazzi, A., Confalonieri, M., Macovei, A., and Carbonera, D. (2011a). Seed imbibition in *Medicago truncatula* Gaertn.: expression profiles of DNA repair genes in relation to PEG-mediated stress. *Journal of Plant Physiology* 168: 706–713.

Balestrazzi, A., Confalonieri, M., Macovei, A. et al. (2011b). Genotoxic stress and DNA repair in plants: emerging functions and tools for improving crop productivity. *Plant Cell Reports* 30 (3): 287–295.

Balestrazzi, A., Macovei, A., Tava, A. et al. (2011c). Unraveling the response of plant cells to cytotoxic saponins: role of metallothionein and nitric oxide. *Plant Signaling & Behavior* 6: 1–4.

Ballesteros, M., Fredriksson, A., Henriksson, J., and Nyström, T. (2001). Bacterial senescence: protein oxidation in non-proliferating cells is dictated by the accuracy of the ribosomes. *EMBO Journal* 20: 5280–5289.

Barba-Espin, G., Diaz-Vivancos, P., Job, D. et al. (2011). Understanding the role of H_2O_2 during pea seed germination: a combined proteomic and hormone profiling approach. *Plant, Cell and Environment* 34: 1907–1919.

Baskin, J.M. and Baskin, C.C. (2004). A classification system for seed dormancy. *Seed Science Research* 14: 1–16.

Bazin, J., Langlade, N., Vincourt, P. et al. (2011). Targeted mRNA oxidation regulates sunflower seed dormancy alleviation during dry after-ripening. *Plant Cell* 23: 2196–2208.

Berlett, B.S. and Stadtman, E.R. (1997). Protein oxidation in aging, disease and oxidative stress. *Journal of Biological Chemistry* 272: 20313–20316.

Biedermann, S., Mooney, S., and Hellmann, H. (2011). Recognition and repair pathways of damaged DNA in higher plants. In: Selected Topics in DNA Repair (ed. C.C. Chen), 201–236. New York: InTech Publishing.

Biju, S., Fuentes, S., and Gupta, D. (2017). Silicon improves seed germination and alleviates drought stress in lentil crops by regulating osmolytes, hydrolytic enzymes and antioxidant defense system. *Plant Physiology and Biochemistry* 119: 250–264.

Boca, S., Koestler, F., Ksas, B. et al. (2014). *Arabidopsis* lipocalins AtCHL and AtTIL have distinct but overlapping functions essential for lipid protection and seed longevity. *Plant, Cell and Environment* 37: 368–381.

Bogatek, R., Gawronska, H., and Oracz, K. (2003). Involvement of oxidative stress and ABA in CN-mediated elimination of embryonic dormancy in apple. In: The Biology of Seeds: Recent Research Advances (ed. G. Nicolas, K.J. Bradford, D. Côme and H.W. Pritchard), 211–216. Wallingford: CABI Publishing.

Bolwell, G.P. and Wojtaszek, P. (1997). Mechanisms for the generation of reactive oxygen species in plant defence – a broad perspective. *Physiological and Molecular Plant Pathology* 51: 347–366.

Bolwell, G.P., Bindschedler, L.V., Blee, K.A. et al. (2002). The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *Journal of Experimental Botany* 53: 1367–1376.

Bray, C.M. (1995). Biochemical processes during the osmopriming of seeds. In: Seed Development and Germination (ed. J. Kigel and G. Galili), 767–789. New York: Marcel Dekker Inc.

Bray, C.M. and West, C.E. (2005). DNA repair mechanisms in plants: crucial sensors and effectors for the maintenance of genome integrity. *New Phytologist* 168: 511–528.

Breen, A.P. and Murphy, J.A. (1995). Reactions of oxyl radicals with DNA. *Free Radical Biology and Medicine* 18: 1033–1077.

Buchanan, B.B. and Balmer, Y. (2005). Redox regulation: a broadening horizon. *Annual Reviews in Plant Biology* 56: 187–220.

Buitink, J. and Leprince, O. (2008). Intracellular glasses and seed survival in the dry state. *Comptes Rendus Biologies* 331: 788–795.

Buitink, J., Leprince, O., Hemminga, M.A., and Hoekstra, F.A. (2000). Molecular mobility in the cytoplasm: an approach to describe and predict lifespan of dry germplasm. *Proceedings of the National Academy of Sciences of the United States of America* 97: 2385–2390.

Caliskan, M. and Cuming, A.C. (1998). Spatial specificity of H_2O_2-generating oxalate oxidase gene expression during wheat embryo germination. *Plant Journal* 15 (2): 165–171.

Caro, A. and Puntarulo, S. (1999). Nitric oxide generation by soybean embryonic axes. Possible effect on mitochondrial function. *Free Radical Research* (Suppl): S205–S212.

Chance, B., Boveris, A., Oshino, N., and Loschen, G. (1973). The nature of catalase intermediate and its biological function. In: Oxidases and Related Redox Systems (ed. T.E. King, H.S. Mason and M. Morrison), 350–353. Baltimore: University Park Press.

Charles, S.A. and Halliwell, B. (1980). Effect of hydrogen peroxide on spinach (*Spinacia oleracea*) chloroplast fructose bisphosphatase. *Biochemistry Journal* 189: 373–376.

Châtelain, E., Satour, P., Laugier, E. et al. (2013). Evidence for participation of the methionine sulfoxide reductase repair system in plant seed longevity. *Proceedings of the National Academy of Sciences of the United States of America* 110: 3633–3638.

Cheah, K.S.E. and Osborne, D.J. (1978). DNA lesions occur with loss of viability in embryos of ageing rye seed. *Nature* 272: 593–599.

Chen, H., Chu, P., Zhou, Y. et al. (2012). Overexpression of AtOGG1, a DNA glycosylase/AP lyase, enhances seed longevity and abiotic stress tolerance in *Arabidopsis*. *Journal of Experimental Botany* 63: 4107–4121.

Chen, Y.P., Jia, J.F., and Han, X.L. (2009). Weak microwave can alleviate water deficit induced by osmotic stress in wheat seedlings. *Planta* 229 (2): 291–298.

Corpas, F.J., Barroso, J.B., and Del Rio, L.A. (2001). Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. *Trends in Plant Science* 6: 145–150.

Das, N., Levine, R.L., Orr, W.C., and Sohal, R.S. (2001). Selectivity of protein oxidative damage during aging in *Drosophila melanogaster*. *Biochemistry Journal* 360: 209–216.

Debeaujon, I., Leon-Kloosterziel, K.M., and Koornneef, M. (2000). Influence of the testa on seed dormancy, germination, and longevity in Arabidopsis. *Plant Physiology* 122: 403–414.

Del Rio, L.A., Corpas, F.J., Sandalio, L.M. et al. (2002). Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. *Journal of Experimental Botany* 53 (372): 1255–1272.

Donà, M., Balestrazzi, A., Mondoni, A. et al. (2013). DNA profiling, telomere analysis and antioxidant properties as tools for monitoring ex situ seed longevity. *Annals of Botany* 111: 987–998.

Duan, Q., Kita, D., Johnson, E.A. et al. (2014). Reactive oxygen species mediate pollen tube rupture to release sperm for fertilization in *Arabidopsis*. *Nature Communications* 5: 3129. https://doi.org/10.1038/ncomms4129.

Dukan, S., Farewell, A., Ballesteros, M. et al. (2000). Protein oxidation in response to increased transcriptional or translational errors. *Proceedings of the National Academy of Sciences of the United States of America* 97: 5746–5749.

Dunlop, R.A., Rodgers, K.J., and Dean, R.T. (2002). Recent development in the intracellular degradation of oxidized proteins. *Free Radical Biology and Medicine* 33: 894–906.

Ellouzi, H., Sghayar, S., and Abdelly, C. (2017). H_2O_2 seed priming improves tolerance to salinity, drought and their combined effect more than mannitol in *Cakile maritima* when compared to *Eutrema salsugineum*. *Journal of Plant Physiology* 210: 38–50.

Filippou, P., Antoniou, C., Obata, T. et al. (2016). Kresoxim-methyl primes *Medicago truncatula* plants against abiotic stress factors via altered reactive oxygen and nitrogen species signalling leading to downstream transcriptional and metabolic readjustment. *Journal of Experimental Botany* 67 (5): 1259–1274.

Fontaine, O., Huault, C., Pavis, N., and Billard, J.P. (1994). Dormancy breakage of *Hordeum vulgare* seeds: effects of hydrogen peroxide and scarification on glutathione level and glutathione reductase activity. *Plant Physiology and Biochemistry* 32: 677–683.

Fry, S.C. (1998). Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. *Biochemistry Journal* 332: 507–515.

Fry, S.C., Dumville, J.C., and Miller, J.G. (2001). Fingerprinting of polysaccharides attacked by hydroxyl radicals *in vitro* and in the cell walls of ripening pear fruit. *Biochemistry Journal* 357: 729–735.

Fry, S.C., Miller, J.G., and Dumville, J.C. (2002). A proposed role for copper ions in cell wall loosening. *Plant and Soil* 247: 57–67.

Gadelha, C.G., Miranda, R.S., Alencar, N.L.M. et al. (2017). Exogenous nitric oxide improves salt tolerance during establishment of *Jatropha curcas* seedlings by ameliorating oxidative damage and toxic ion accumulation. *Journal of Plant Physiology* 212: 69–79.

Gidrol, X., Lin, W.S., Dégousée, N. et al. (1994). Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. *European Journal of Biochemistry* 224 (1): 21–28.

Gill, S.S. and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* 48 (12): 909–930.

Giurizatto, M.I.K., Ferrarese-Filho, O., Ferrarese, M.D.L.L. et al. (2012). α-Tocopherol levels in natural and artificial aging of soybean seeds. *Acta Scientiarum Agronomy* 34: 339–343.

Graeber, K., Linkies, A., Müller, K. et al. (2010). Cross-species approaches to seed dormancy and germination: conservation and biodiversity of ABA-regulated mechanisms and the Brassicaceae *DOG1* genes. *Plant Molecular Biology* 73: 67–87.

Grant, J.J. and Loake, G.J. (2000). Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiology* 124: 21–29.

Groot, S.P.C., Surki, A.A., de Vos, R.C.H., and Kodde, J. (2012). Seed storage at elevated partial pressure of oxygen, a fast method for analysing seed ageing under dry conditions. *Annals of Botany* 110: 1149–1159.

Halliwell, B. and Gutteridge, J.M.C. (1999). Free Radicals in Biology and Medicine. Oxford: Oxford University Press.
Harman, G.E. and Mattick, L.R. (1976). Association of lipid oxidation with seed ageing and death. *Nature* 260: 323–324.
Harrison, B.J. and McLeish, J. (1954). Abnormalities of stored seeds. *Nature* 173: 593–594.
Henzler, T. and Steudle, E. (2000). Transport and metabolic degradation of hydrogen peroxide in *Chara corallina*: model calculations and measurements with the pressure probe suggest transport of H_2O_2 across water channels. *Journal of Experimental Botany* 51 (353): 2053–2066.
Heydecker, W. and Coolbear, P. (1977). Seed treatments for improved performance-survey and attempted prognosis. *Seed Science and Technology* 5: 353–425.
Hite, D.R., Auh, C., and Scandalios, J.G. (1999). Catalase activity and hydrogen peroxide levels are inversely correlated in maize scutella during seed germination. *Redox Report: Communications in Free Radical Research* 4 (1–2): 29–34.
Hou, H., Wang, P., Zhang, H. et al. (2015). Histone acetylation is involved in gibberellin-regulated sodCp gene expression in maize aleurone layers. *Plant Cell Physiology* 56: 2139–2149.
Huang, A.H.C., Trelease, R.N., and Moore, T.S. (1983). Plant Peroxisomes. New York: Academic Press.
Huang, Y.M., Wang, H.H., and Chen, K.H. (2002). Application of seed priming treatments in spinach (*Spinacia oleracea* L.) production. *Journal of the Chinese Society for Horticultural Science* 48: 117–123.
Ishibashi, Y., Tawaratsumida, T., Zheng, S.-H. et al. (2010). NADPH oxidases act as key enzyme on germination and seedling growth in barley (*Hordeum vulgare* L.). *Plant Protection Science* 13: 45–52.
Job, C., Rajjou, L., Lovigny, Y. et al. (2005). Patterns of protein oxidation in *Arabidopsis* seeds and during germination. *Plant Physiology* 138: 790–802.
Johansson, E., Olsson, O., and Nyström, T. (2004). Progression and specificity of protein oxidation in the life cycle of *Arabidopsis thaliana*. *Journal of Biological Chemistry* 279: 22204–22208.
Justice, O.L. and Bass, L.N. (1978). Principles and Practices of Seed Storage. USDA Agricultural Handbook No. 506, Washington, DC.
Kalemba, E.M. and Pukacka, S. (2014). Carbonylated proteins accumulated as vitality decreases during long-term storage of beech (*Fagi sylvatica L.*) seeds. *Trees* 28: 503–515.
Kibinza, S., Vinel, D., Côme, D. et al. (2006). Sunflower seed deterioration as related to moisture content during ageing, energy metabolism and active oxygen species scavenging. *Physiologia Plantarum* 128: 496–506.
Kitsios, G. and Doonan, J.H. (2011). Cyclin dependent protein kinases and stress responses in plants. *Plant Signaling & Behavior* 6 (2): 204–209.
Kong, L., Huo, H., and Mao, P. (2015). Antioxidant response and related gene expression in aged oat seed. *Frontiers in Plant Science* 19 (6): 158.
Koster, K.L. and Leopold, A.C. (1988). Sugars and desiccation tolerance in seeds. *Plant Physiology* 88: 829–832.
Kranner, I., Birtic, S., Anderson, K.M., and Pritchard, H.W. (2006). Glutathione half-cell reduction potential: a universal stress marker and modulator of programmed cell death? *Free Radical Biology and Medicine* 40: 2155–2165.

Kranner, I., Roach, T., Beckett, R.P. et al. (2010). Extracellular production of reactive oxygen species during seed germination and early seedling growth in *Pisum sativum*. *Journal of Plant Physiology* 167: 805–811.

Kumar, S.J., Prasad, S.R., Banerjee, R., and Thammineni, C. (2015). Seed birth to death: dual functions of reactive oxygen species in seed physiology. *Annals of Botany* 116: 663–668.

Lariguet, P., Ranocha, P., De Meyer, M. et al. (2013). Identification of a hydrogen peroxide signalling pathway in the control of light-dependent germination in Arabidopsis. *Planta* 238 (2): 381–395.

Lee, Y.P., Baek, K.-H., Lee, H.-S. et al. (2010). Tobacco seeds simultaneously over-expressing Cu/Zn-superoxide dismutase and ascorbate peroxidase display enhanced seed longevity and germination rates under stress conditions. *Journal of Experimental Botany* 61 (9): 2499–2506.

Levine, L.R., Garland, D., Oliver, C.N. et al. (1990). Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology* 186: 464–478.

Levine, R.L., Williams, J.A., Stadtman, E.R., and Shacter, E. (1994). Carbonyl assays for determination of oxidatively modified proteins. *Methods in Enzymology* 233: 346–357.

Long, R.L., Gorecki, M.J., Renton, M. et al. (2015). The ecophysiology of seed persistence: a mechanistic view of the journey to germination or demise. *Biological Reviews of the Cambridge Philosophical Society* 90: 31–59.

López-Cruz, J., Óscar, C.S., Emma, F.C. et al. (2017). Absence of Cu-Zn superoxide dismutase BCSOD1 reduces *Botrytis cinerea* virulence in Arabidopsis and tomato plants, revealing interplay among reactive oxygen species, callose and signalling pathways. *Molecular Plant Pathology* 18 (1): 16–31.

Macovei, A., Balestrazzi, A., Confalonieri, M., and Carbonera, D. (2010). The *Tdp1* (Tyrosyl-DNA phosphodiesterase) gene family in barrel medic (*Medicago truncatula* Gaertn.): bioinformatic investigation and expression profiles in response to copper- and PEG-mediated stress. *Planta* 232: 393–407.

Macovei, A., Balestrazzi, A., Confalonieri, M. et al. (2011a). New insights on the barrel medic MtOGG1 and MtFPG functions in relation to oxidative stress response in planta and during seed imbibition. *Plant Physiology and Biochemistry* 49: 1040–1050.

Macovei, A., Balestrazzi, A., Confalonieri, M. et al. (2011b). The TFIIS and TFIIS-like genes from *Medicago truncatula* are involved in oxidative stress response. *Gene* 470 (1–2): 20–30.

Macovei, A., Garg, B., Raikwar, S. et al. (2014). Synergistic exposure of rice seeds to different doses of gamma-ray and salinity stress resulted in increased antioxidant enzyme activities and gene-specific modulation of TC-NER pathway. *BioMed Research International* 2014: 676934.

Macovei, A., Pagano, A., Leonetti, P. et al. (2017). Systems biology and genome-wide approaches to unveil the molecular players involved in the pre-germinative metabolism: implications on seed technology traits. *Plant Cell Reports* 36 (5): 669–688.

Mao, C., Zhu, Y., Cheng, H. et al. (2018). Nitric oxide regulates seedling growth and mitochondrial responses in aged oat seeds. *International Journal of Molecular Sciences* 19 (4): E1052. https://doi.org/10.3390/ijms19041052.

Maréchal, A. and Zou, L. (2013). DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harbor Perspectives in Biology* 5 (9): a012716. https://doi.org/10.1101/cshperspect.a012716.

Meinhard, M. and Grill, E. (2001). Hydrogen peroxide is a regulator of ABI1, a protein phosphatase 2C from *Arabidopsis*. *FEBS Letters* 508: 443–446.

Meinhard, M., Rodriguez, P.L., and Grill, E. (2002). The sensitivity of ABI2 to hydrogen peroxide links the abscisic acid-response regulator to redox signaling. *Planta* 214: 775–782.

Messenger, D.J., McLeod, A.R., and Fry, S.C. (2009). The role of ultraviolet radiation, photosensitizers, reactive oxygen species and ester groups in mechanisms of methane formation from pectin. *Plant, Cell and Environment* 32: 1–9.

Michel, B.E. and Kauffmann, M.R. (1973). The osmotic potential of polyethylene glycol 6000. *Plant Physiology* 51: 914–916.

Miller, G., Shulaev, V., and Mittler, R. (2008). Reactive oxygen signaling and abiotic stress. *Physiologia Plantarum* 133 (3): 481–489.

Miller, J.G. and Fry, S.C. (2001). Characteristics of xyloglucan after attack by hydroxyl radicals. *Carbohydrate Research* 332: 389–403.

Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. (2004). Reactive oxygen gene network of plants. *Trends in Plant Science* 9 (10): 490–498.

Mittler, R., Vanderauwera, S., Suzuki, N. et al. (2011). ROS signaling: the new wave? *Trends in Plant Science* 16: 300–309.

Moïse, J.A., Han, S., Gudynaite-Savitch, L. et al. (2005). Seed coats: structure, development, composition, and biotechnology. *In Vitro Cellular & Developmental Biology - Plant* 41: 620–644.

Morohashi, Y. (2002). Peroxidase activity develops in the micropylar endosperm of tomato seeds prior to radicle protrusion. *Journal of Experimental Botany* 53 (374): 1643–1650.

Mostertz, J. and Hecker, M. (2003). Patterns of protein carbonylation following oxidative stress in wild-type and sigB *Bacillus subtillis* cells. *Molecular Genetics and Genomics* 269: 640–648.

Müller, K., Carstens, A.C., Linkies, A. et al. (2009a). The NADPH-oxidase AtrbohB plays a role in *Arabidopsis* seed after-ripening. *New Phytologist* 184: 885–897.

Müller, K., Linkies, A., Vreeburg, R.A.M. et al. (2009b). In vivo cell wall loosening by hydroxyl radicals during cress seed germination and elongation growth. *Plant Physiology* 150: 1855–1865.

Murgia, I., Giacometti, S., Balestrazzi, A. et al. (2015). Analysis of the transgenerational iron deficiency stress memory in *Arabidopsis thaliana* plants. *Frontiers in Plant Science* 6: 745. https://doi.org/10.3389/fpls.2015.00745.

Murthy, U.N. and Sun, W.Q. (2000). Protein modification by Amadori and Maillard reactions during seed storage: roles of sugar hydrolysis and lipid peroxidation. *Journal of Experimental Botany* 51: 1221–1228.

Nagel, M., Kranner, I., Neumann, K. et al. (2015). Genome-wide association mapping and biochemical markers reveal that seed ageing and longevity are intricately affected by genetic background and developmental and environmental conditions in barley. *Plant, Cell and Environment* 38: 1011–1022.

Nakabayashi, K., Okamoto, M., Koshiba, T. et al. (2005). Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: epigenetic and genetic regulation of transcription in seed. *Plant Journal* 41: 697–709.

Naredo, M.E.B., Juliano, A.B., Lu, B.R. et al. (1998). Responses to seed dormancy-breaking treatments in rice species (*Oryza* L.). *Seed Science and Technology* 26: 675–689.

Nguyen, T.P., Cueff, G., Hegedus, D.D. et al. (2015). A role for seed storage proteins in *Arabidopsis* seed longevity. *Journal of Experimental Botany* 66: 6399–6413.

Ni, J., Wang, Q., Shah, F.A. et al. (2018). Exogenous melatonin confers cadmium tolerance by counterbalancing the hydrogen peroxide homeostasis in wheat seedlings. *Molecules* 23 (4): E799. https://doi.org/10.3390/molecules23040799.

Nikitaki, Z., Holác, M., Donà, M. et al. (2018). Integrating plant and animal biology for the search of novel DNA damage biomarkers. *Mutation Research, Reviews in Mutation Research* 775: 21–38.

Nishizawa, A., Yabuta, Y., and Shigeoka, S. (2008). Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant Physiology* 147: 1251–1263.

Ogawa, K. and Iwabuchi, M. (2001). A mechanism for promoting the germination of *Zinnia elegans* seeds by hydrogen peroxide. *Plant and Cell Physiology* 42: 286–291.

Ooms, J.J.J., Leon-Kloosterziel, K.M., Bartels, D. et al. (1993). Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana*: a comparative study using abscisic acid-insensitive abi3 mutants. *Plant Physiology* 102: 1185–1191.

Oracz, K., El-Maarouf Bouteau, H., Farrant, J.M. et al. (2007). ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation. *Plant Journal* 50 (3): 452–465.

Oracz, K., El-Maarouf-Bouteau, H., Kranner, I. et al. (2009). The mechanisms involved in seed dormancy alleviation by hydrogen cyanide unravel the role of reactive oxygen species as key factors of cellular signaling during germination. *Plant Physiology* 150: 494–505.

Osborne, D.J. (1994). DNA and desiccation tolerance. *Seed Science Research* 4: 175–185.

Pagano, A., Araújo, S., Macovei, A. et al. (2017). The seed repair response during germination: disclosing correlations between DNA repair, antioxidant response, and chromatin remodeling in *Medicago truncatula*. *Frontiers in Plant Science* 8: 1972. https://doi.org/10.3389/fpls.2017.01972.

Paparella, S., Araújo, S.S., Rossi, G. et al. (2015). Seed priming: state of the art and new perspectives. *Plant Cell Reports* 34: 1281–1293.

Potocky, M., Pejchar, P., Gutkowska, M. et al. (2012). NADPH oxidase activity in pollen tubes is affected by calcium ions, signaling phospholipids and Rac/Rop GTPases. *Journal of Plant Physiology* 169: 1654–1663.

Pourcel, L., Routaboul, J.M., Cheynier, V. et al. (2007). Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends in Plant Science* 12: 29–36.

Pourcel, L., Routaboul, J.M., Kerhoas, L. et al. (2005). TRANSPARENT TESTA10 encodes a laccase-like enzyme involved in oxidative polymerization of flavonoids in *Arabidopsis* seed coat. *Plant Cell* 17: 2966–2980.

Priestley, D.A. (1986). Seed Aging, Implications for Seed Storage and Persistence in the Soil. ISBN: 0-8014-1865-8, Cornell University Press, Ithaca (New York).

Prieto-Dapena, P., Castan˜o, R., Almoguera, C., and Jordano, J. (2006). Improved resistance to controlled deterioration in transgenic seeds. *Plant Physiology* 142: 1102–1112.

Pucciariello, C. and Perata, P. (2017). New insights into reactive oxygen species and nitric oxide signalling under low oxygen in plants. *Plant, Cell and Environment* 40 (4): 473–482.

Puntarulo, S., Sanchez, R.A., and Boveris, A. (1988). Hydrogen peroxide metabolism in soybean embryonic axes at the onset of germination. *Plant Physiology* 86: 626–630.

Radhakrishnan, R., Khan, A.L., and Lee, I.-J. (2013). Endophytic fungal pre- treatments of seeds alleviate salinity stress effects in soybean plants. *Journal of Microbiology* 51: 850–857.

Rajjou, L. and Debeaujon, I. (2008). Seed longevity: survival and maintenance of high germination ability of dry seeds. *Comptes Rendus Biologies* 331: 796–805.

Rajjou, L., Duval, M., Gallardo, K. et al. (2012). Seed germination and vigor. *Annual Reviews in Plant Biology* 63: 507–533.

Rajjou, L., Lovigny, Y., Groot, S.P.C. et al. (2008). Proteome-wide characterization of seed aging in *Arabidopsis*: a comparison between artificial and natural aging protocols. *Plant Physiology* 148: 620–641.

Richards, S.L., Wilkins, K.A., Swarbreck, S.M. et al. (2015). The hydroxyl radical in plants: from seed to seed. *Journal of Experimental Botany* 66 (1): 37–46.

Rivett, A.J. and Levine, R.L. (1990). Metal-catalyzed oxidation of *Escherichia coli* glutamine synthetase: correlation of structural and functional changes. *Archives of Biochemistry and Biophysics* 278: 26–34.

Roberts, E.H. (1973). Predicting the storage life of seeds. *Seed Science and Technology* 1: 499–514.

Roldán-Arjona, T. and Ariza, R.R. (2009). Repair and tolerance of oxidative DNA damage in plants. *Mutation Research* 681: 169–179.

Sano, N., Rajjou, L., North, H.M. et al. (2016). Staying alive: molecular aspects of seed longevity. *Plant Cell Physiology* 57 (4): 660–674.

Sarath, G., Hou, G., Baird, L.M., and Mitchell, R.B. (2007). Reactive oxygen species, ABA and nitric oxide interactions on the germination of warm-season C4-grasses. *Planta* 226 (3): 697–708.

Sattler, S.E., Gilliland, L.U., Magallanes-Lundback, M. et al. (2004). Vitamin E is essential for seed longevity and for preventing lipid peroxidation during germination. *Plant Cell* 16: 1419–1432.

Schopfer, P. (2001). Hydroxyl radical-induced cell-wall loosening in vitro and in vivo: implications for the control of elongation growth. *Plant Journal* 28: 679–688.

Schopfer, P., Liszkay, A., Bechtold, M. et al. (2002). Evidence that hydroxyl radicals mediate auxin-induced extension growth. *Planta* 214: 821–828.

Schopfer, P., Plachy, C., and Frahry, G. (2001). Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. *Plant Physiology* 125 (4): 1591–1602.

Schweikert, C., Liszkay, A., and Schopfer, P. (2000). Scission of polysaccharides by peroxidase-generated hydroxyl radicals. *Phytochemistry* 53: 565–570.

Smirnova, A.V., Matveyeva, N.P., and Yermakov, I.P. (2014). Reactive oxygen species are involved in regulation of pollen wall cytomechanics. *Plant Biology* 16: 252–257.

Sun, W.Q. and Leopold, A.C. (1995). The Maillard reaction and oxidative stress during aging of soybean seeds. *Physiologia Plantarum* 94: 94–104.

Taylor, A.G., Allen, P.S., Bennet, M.A. et al. (1998). Seed enhancements. *Seed Science Research* 8: 245–256.

Vranová, E., Inzé, D., and Van Breusegem, F. (2002). Signal transduction during oxidative stress. *Journal of Experimental Botany* 53: 1227–1236.

Walters, C. (1998). Understanding the mechanisms and kinetics of seed aging. *Seed Science Research* 8: 223–244.

Wang, H., Shabala, L., Zhou, M., and Shabala, S. (2018). Hydrogen peroxide-induced root Ca^{2+} and K^+ fluxes correlate with salt tolerance in cereals: towards the cell-based phenotyping. *International Journal of Molecular Sciences* 19 (3): E702. https://doi.org/10.3390/ijms19030702.

Wang, P., Zhu, J.K., and Lang, Z. (2015). Nitric oxide suppresses the inhibitory effect of abscisic acid on seed germination by S-nitrosylation of SnRK2 proteins. *Plant Signaling & Behavior* 10 (6): e1031939.

Wang, M., Heimovaara-Dijkstra, S., and Van Duijn, B. (1995). Modulation of germination of embryos isolated from dormant and non-dormant barley grains by manipulation of endogenous abscisic levels. *Planta* 195: 586–592.

Wang, M., van der Meulen, R.M., Visser, K. et al. (1998). Effects of dormancy-breaking chemicals on ABA levels in barley grain embryos. *Seed Science Research* 8: 129–137.

Waterworth, W.M., Bray, C.M., and West, C.E. (2015). The importance of safeguarding genome integrity in germination and seed longevity. *Journal of Experimental Botany* 66 (12): 3549–3558.

Waterworth, W.M., Footitt, S., Bray, C.M. et al. (2016). DNA damage checkpoint kinase ATM regulates germination and maintains genome stability in seeds. *Proceedings of the National Academy of Sciences of the United States of America* 113: 9647–9652.

Weissbach, H., Resnick, L., and Brot, N. (2005). Methionine sulfoxide reductases: history and cellular role in protecting against oxidative damage. *Biochimica et Biophysica Acta* 1703: 203–212.

Whitaker, C., Beckett, R.P., Minibayeva, F.V., and Kranner, I. (2010). Alleviation of dormancy by reactive oxygen species in *Bidens pilosa* L. seeds. *South African Journal of Botany* 76: 601–605.

Wilson, D.O. and McDonald, M.B. (1986). The lipid peroxidation model of seed aging. *Seed Science and Technology* 14: 269–300.

Wojtyla, L., Garnczarska, M., Zalewski, T. et al. (2006). A comparative study of water distribution, free radical production and activation of antioxidative metabolism in germinating pea seeds. *Journal of Plant Physiology* 163 (12): 1207–1220.

Wrzaczek, M., Brosché, M., and Kangasjärvi, J. (2013). ROS signaling loops – production, perception, regulation. *Current Opinion in Plant Biology* 16: 575–582.

Yadu, S., Dewangan, T.L., Chandrakar, V., and Keshavkant, S. (2017). Imperative roles of salicylic acid and nitric oxide in improving salinity tolerance in *Pisum sativum* L. *Physiology and Molecular Biology of Plants* 23 (1): 43–58.

Yi, D., Alvim Kamei, C.L., Cools, T. et al. (2014). The Arabidopsis SIAMESE-RELATED cyclin-dependent kinase inhibitors SMR5 and SMR7 regulate the DNA damage checkpoint in response to reactive oxygen species. *Plant Cell* 26: 296–309.

Yoshiyama, K.O., Sakaguchi, K., and Kimura, S. (2013). DNA damage response in plants: conserved and variable response compared to animals. *Biology* 2 (4): 1338–1356.

Thank you for agreeing to contribute to **ADVANCEMENT IN CROP IMPROVEMENT TECHNIQUES** (the "**Work**") edited by **Narendra Tuteja, Renu Tuteja, Nishat Passricha** and **Shabnam K. Saifi** (the "**Editor**"). This Agreement between you (the "**Contributor**") and **Elsevier Inc.** (the "**Publisher**") regarding your contribution (the "**Contribution**") outlines the Contributor's and the Publisher's respective obligations and rights.

[ChNum]

Genome Editing in the context of Seed research: How these novel biotechnology tools can change the future face of agricultural crop development?

Chiara Forti[1], Carla Gualtieri[1], Andrea Pagano[1], Susana de Sousa Araújo[2], Alma Balestrazzi[1], Anca Macovei[1,*]

[1] Department of Biology and Biotechnology "L. Spallanzani", University of Pavia, via Ferrata 9, Pavia 27100, Italy

[2] Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa (ITQB-NOVA), Avenida da República, Estação Agronómica Nacional, Oeiras 2780-157, Portugal

* Corresponding author's email address: anca.macovei@unipv.it

[NON PRINT ITEMS]

Abstract: Considering the major challenges in agricultural research, *e.g.* the need to increase productivity and quality of cultivated species under environmental constraints in order to feed the ever-growing population of the Planet, genome editing has the powerful potential to accelerate crop improvement. Within this context, genome editing is perceived as an effective and precise tool to modify plant genomes and improve their quality. Crop performance is strongly dependent on seed quality traits. Moreover, seeds can be considered as common denominators to address both plant productivity and

improved human nutrition. In this chapter, we intend to take-into-account the application of genome editing techniques focusing on seed quality traits, including aspects related to seed vigor, dormancy, germination, as well as features covering seed nutritional/biochemical composition, to address current and future crop improvements.

Key Words: Agriculture, Biotechnology, Genome editing, Nutrition, Seeds

Introduction

Genome editing is a powerful technique that must be used to accelerate crop improvement. Differently from traditional genetic engineering, where genes are inserted randomly into the genome, this set of techniques harness site-specific nucleases (SSNs) combined with DNA/RNA recognition domains to insert designated modifications at predefined sites in the genome (Bogdanove and Voytas, 2011; Chen and Gao, 2014; Sun et al., 2016). This has an implicit significance for human health, agriculture, and the environment. Thanks to genome editing approach it is now possible to accelerate basic research using model organisms for the study of genes of interest and related mutations. By modifying the plant genome through targeted insertions or deletions it is possible to evaluate how these mutations influence the phenotype (Liu and Moschou, 2018). This allows a more in-depth knowledge of gene functions and relative regulatory mechanisms. Moreover, genome editing makes it possible to modify targeted cellular metabolic pathways that allow the development of new varieties of plants of agricultural interest characterized, for instance, by greater tolerance to biotic and abiotic stresses (Baltes and

Voytas, 2016; Shih et al., 2016; Li et al., 2018; Sedeek et al., 2019; Debbarma et al., 2019).

So far, the main tools available for genome editing encompass meganucleases (MNs), zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat-associated proteins (CRISPR-Cas). Among these, the CRISPR-Cas system is most used due to its facility for target design (20 nucleotides long single guide RNAs), high efficiency, and relatively low-costs (Ran et al., 2013; Khatodia et al., 2016; Jaganathan et al., 2018; Chen et al., 2019). This is also reflected by the high number of articles published since the discovery of this technique by Jennifer Doudna and Emmanuelle Carpentier (Jinek al., 2012). For instance, a search carried out on PubMed at the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/pubmed, on 8th March 2019) using the keywords "((CRISPR) AND plant) NOT review" identified more than 1100 published papers so far. All of the available genome editing tools work in a similar manner by generating double-strand breaks (or nicks) at targeted sites whereas the repair of the break is being performed by the endogenous DNA repair systems (Bogdanove and Voytas, 2011; Zhang et al., 2017; Puchta, 2017; Shan and Voytas, 2018). As many comprehensive reviews were dedicated to explaining the structure, functionality, and use of these tools in plants (Chen and Gao, 2014; Baltes and Voytas, 2015; Khatodia et al., 2016; Malzhan et al., 2017; Baltes et al., 2017; Shan and Voytas, 2018; Chen et al., 2019; Kausch et al., 2019), the present chapter mainly focuses on their relevance for Seed research. Hence, this work convenes the application of genome editing techniques with regards to seed production (intended as plant productivity or yield), the nutritional and

biochemical composition of seed, as well as other quality traits that include aspects related to seed vigor, dormancy, and germination.

1. Seeds: the protagonists of agricultural productivity

Seeds are the main propagating vectors in higher plants, as they guarantee plant reproduction. From an evolutionary point of view, seeds represent the fundamental step in the transition from non-vascular plants to seed plants (Spermatophyta). The first fossil evidence of seed-like morphological structures refers to the Progymnosperm, a monophyletic taxon dated in the late Devonian period, about 370 million years ago. This correlates a taxon of extinct 'seed ferns' to the first Gymnosperms and to the ancestors of all the modern Spermatophyta (Niklas, 1997; Linkies et al., 2010). Whereas a monophyletic origin for Angiosperms and Gymnosperms was ascribed, the precise evolutionary connections between the two clades have still to be defined (Linkies et al., 2010).

From seed to seed, across their entire lifecycle, plants are unable to physically escape the deleterious fluctuations present in variable environments. Consequently, evolution has endowed plants with a variegated set of mechanisms and strategies that allows them to face and survive stress conditions (Haak et al, 2017). Since adaptation for survival is a major driving force in evolution, the seed itself is regarded as an adaptive trait to enhance plant survival and diffusion under the fluctuating environmental conditions thought the production of a quiescent and diffusible form able to 'wait' the optimal parameters for germination to occur. In strict interconnection with their role in plant reproduction, seeds encapsulate plant versatility and adaptability to different kinds of biotic (e.g. pathogens)

and abiotic (e.g. drought, flooding, extreme temperatures) stresses (Haak et al, 2017). Among these, salinity stress and soil contaminants were shown to decrease protein content due to nitrogen depletion and altered metabolism, drought stress alters oleic/linoleic acid ratios in legume seeds, whereas heat stress induces protein depletion and oil content increase along with alterations in fatty acid composition (Farooq et al, 2018). It has been estimated that the average crop yield losses caused by drought, cold, and salinity overpass 50% (Mahajan and Tuteja, 2005). Moreover, abiotic stresses are reported to induce notable changes in seed content composition and quality (Farooq et al, 2018). Such considerations have relevant outcomes not only on seed survival but also on agricultural production and human nutrition.

From an anthropological perspective, seeds are essential for several aspects (*e.g.*, nutrition, biodiversity, maintenance of ecosystem niches, landscape, *etc*.). Mainly, the nutritional property of seeds is linked to major alimentary resources for all the population across different cultures, with cereals, fruits, vegetables, and oilseed crops representing the big portions of the seed market. The relevance of the seeds is reflected by the increase in the global seed market (from 30 billion USD in 1996 to 36 billion USD in 2007), and this trend was mainly observed in emerging economies and developing countries (FAO). Moreover, while facing the food demand of a growing population, the global seed industry has been valued at more than 54 billion USD in 2016 and constant growth is predicted for the next years (2017-2023) (Allied Market Research).

In this context, seed industries, breeders, and biotechnologists have identified the main features of interest to optimize the quality of a seed lot at many steps of the production process, including storage, transporting, sowing, plant development and seed maturation.

Thus, seed quality is defined and quantified by taking into account a set of measurable parameters such as germination speed and rates, long-term and after-storage viability, homogeneity and purity of the seed lot, resistance to mechanical damage and the absence of biological and chemical contaminants (Finch-Savage and Bassel, 2016). Many of these characteristics are defined and can be improved according to the requirements of the seed industry and market. Nonetheless, plant and human interests converge in regard to the capacity of seed to produce a healthy and resistant seedling able to withstand biotic and abiotic stresses, thus ensuring plant reproduction along with abundant harvests even under variable environmental parameters, seasonal cycles, and climate changes. Many traits in the genetic background of each seed lot contribute to these properties that are collectively referred to as 'seed vigor' (Finch-Savage and Bassel, 2016), broadly defined as "the sum of those properties that determine the activity and performance of seed lots of acceptable germination in a wide range of environments" (ISTA, 2018).

Seed quality and vigor are variable not only intrinsically, among different crop species, cultivars and seed lots, but also in response to the different cultivation conditions and schedules. Maturation and germination timings are important limiting factors. Indeed, most crop seeds reach their optimal vigor at their physiological maturity which can be difficult to synchronize, while strict temporal windows are often required for harvesting, sowing, germination and growing. Furthermore, as a general tendency, field cultivation leads to poorer and more variable germination performances than laboratory-controlled conditions, making standardization even more difficult (Finch-Savage and Bassel, 2016).

The improvement of seed quality and vigor is a challenge that started to be addressed since ancient times. The notion entails that seed germination can be enhanced through

specific treatments and the first attempts in this sense are historically attested throughout time, from Theophrastus (371–287 B.C.), Gaius Plinius Secundus (A.D. 23–79), Oliver de Serres (1539–1619), up to Charles Darwin (1809–1882). Since the first effective approaches to be discovered, a common element was a water-soaking step before sowing, while more recent protocols implemented also solutions containing salts or osmotic agents (Everari, 1984; Paparella et al, 2015). The development of techniques to improve germination rates, speed and consistency, as well as viability under stress conditions evolved in the modern concept of 'seed priming', broadly defined as 'any treatment that improves seed quality' (Osburn and Schroth, 1989; Paparella et al, 2015). Although different priming protocols have been devised according to the characteristics and the requirements of various species of interest, certain common elements can be identified.

2. Crop yield: a complex trait that can be undertaken with the use of genome editing

Crop yield and quality are quantitative traits generally controlled by multiple genes localized in specific Quantitative Trait Loci (QTLs). Within this context, seeds are considered among the main vectors conditioning crop yield. Traditional breeding, the original method used to improve yield, relies on the production of various QTL combinations and subsequent selection the most favorable breeds (Zuo and Li, 2014; Shen et al., 2018), but it is quite a time-consuming process. Hence, crop yield improvements need to be quickened in order to avoid an eventual food insecurity situation. To do so, genetically modified (GM) crops represent a solid alternative, already proven to be effective when considering the much-studied examples related to the

production of herbicide and insect resistant crops (Brookes and Barfoot, 2017; Paul et al., 2018).

With the advent of genome editing techniques, researchers had further plunged into finding more rapid and easier ways to address these complex traits. Some examples of crops with improved yields developed mainly through the use of the CRISPR/Cas9 system are given in **Table 2.1** and discussed here. The considered traits span from seed size and number, to plant productivity and heterosis. Starting from the last, heterosis (also known as hybrid vigor) has been extensively applied in agriculture to improve plant productivity, although hybrid seed production is quite difficult and expensive for many crops (Schnable and Springer, 2013). Manipulating heterosis was achieved by synthetic apomixes, defined as an asexual reproductive strategy in which offspring are generated through seeds in the absence of meiosis or fertilization (Spillane et al., 2004). A study published this year reported that targeted mutations in multiple genes playing roles in meiosis to produce hybrid rice (*Oryza sativa*) lines with increased heterosis (Wang et al., 2019). Here, a multiplex CRISPR/Cas9 vector was developed to simultaneously target the *REC8* (Meiotic Recombination Protein), *PAIR1* (HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS1), *OSD1* (OMISSION OF SECOND DIVISION1), and *MTL* (MATRILINEAL) genes, obtaining rice hybrids able to self-pollinate and produce true-breeding progeny through seeds. Targeting other well-known transcription factors (TFs) was also associated with phenotypic changes in plant architecture and subsequent increase in plant productivity in rice. For instance, DEP1 (DENSE AND ERECT PANICLE) was shown to control panicle size (Huang et al., 2009) whereas IPA1 (IDEAL PLANT ARCHITECTURE1) is related to tillering in rice (Miura et al., 2010).

By CRISPR/Cas9-mediated mutagenesis of these two TFs, Li et al. (2016) obtained rice plants form the Zhonghua 11 japonica cultivar with improved productivity. For *DEP1*, mutation rates up to 67% were obtained, and some of the mutants grown under filed conditions exhibited decreased plant height, short panicles, and importantly, increased number of flowers per panicle. Similarly, targeting the *IPA1* gene close to the region containing the miR156 target site, base deletions causing protein inactivation, resulted in mutant plants with a dwarf phenotype and increased number of tillers, although at lower mutation rates (27%). Aside directly targeting TFs, another used approach was to insert small changes in the promoter regions of the CLAVATA-WUSCHEL stem cell circuit (CLV-WUS, controlling the meristem size), SELF PRUNING (SP, controlling the flowering time), and SINGLE FLOWER TRUSS (SFT, a flowering repressor that counteracts the florigen hormone) in tomato (*Solanum lycopersicum*), obtaining plants with improved fruit shape and plant architecture
(Rodríguez-Leal et al., 2017). CLAVATA genes were also targeted in *Brassica napus*, an allotetraploid species considered among the most important oil crops worldwide, where mutations induced by CRISPR/Cas9 in all the *CLV3* alleles resulted into the development of plants with more leaves and multilocular siliques, thus also having higher number of seeds (Yang et al., 2018).

Seed size and number are important agronomic parameters to evaluate crop yield. A direct approach to increase yield is to knock out genes that negatively affect it. By doing so with the use of CRISPR/Cas9, rice yield has been improved by knocking out the *GS3* (GRAIN SIZE 3), *GW2* (GRAIN WEIGHT 2), *GW5*, *TGW6* (THOUSAND GRAIN WEIGHT 6), and *Gn1a* (GRAIN NUMBER 1a) genes, well-known negative regulators

of grain size, number, and weight (Xu et al., 2016; Li et a., 2016). More recently, the *RGG2* gene, encoding for a heterodimeric G protein, was also proven to be involved in the regulation of rice seed and organ size, via gibberellic acid (GA) signaling pathway (Miao et al., 2019). Similar studies were conducted also in bread wheat (*Triticum aestivum*), where the *TaGW2* and *TaGASR7* (a member of the Snakin/GASA gene family that acts as negative regulator of kernel width and weight) genes were targeted in all the three homoalleles, and the resulting products presented increased thousand-kernel weight (Zhang et al., 2016).

Aside from the use of CRISPR/Cas9 to induce mutations, a different study proposed knock-in inducible promoters to enhance gene expression (Shi et al., 2017). In this case, the native GOS2 promoter from maize (*Zea mays*), was inserted into the untranslated region of the ARGOS8 (Auxin Regulated Gene involved in Organ Size) gene, thus replacing its own promoter. The resulting maize plants presented an increase in grain yield under drought stress and no losses under well-watered conditions in a filed trial, thus combining both enhanced productivity and stress resistance in one product.

3. Improving seed nutritional content with genome editing tools

Since the start of the Green Revolution, at the beginning of the '60s, agricultural improvements focused mainly on increased yield. This however led to underestimating the nutritional content of most crops. Nutrient deficiency is a major challenge especially for people in third-world countries that lack access to a balanced diet and rely on staple food crops with low levels of micronutrients (e.g. vitamins, minerals, amino acids).

Hence, increasing efforts are now focusing on addressing also this complex trait through the use of biotechnology, including genome editing techniques (Yu and Tian, 2018). In this context, a special focus was dedicated to modifying the oil composition in several crops (**Table 3.1**). This is because, vegetable oils are important agricultural commodities, being used for both human direct consumption as well as industrial applications (e.g. production of lubricants and biofuels). One of the main targets to address seed oil content is the *FAD2* (fatty acid desaturase 2) gene, involved in the conversion of oleic to linoleic acid (Okuley et al., 1994). This is an important trait because oleic acid, a valuable monounsaturated fatty acid with high oxidative stability, can help in the prevention of several diseases, such as cardio-vascular associated disorders (Lopez-Huertas, 2010). The first crop targeted for the modification of oil seed content was soybean (*Glycine max*), as its oil contains high levels of polyunsaturated linoleic and linolenic acid. Soybean has three *FAD2* genes (*FAD2-1A*, *FAD2-1B*, *FAD2-2*) and two of them (*FAD2-1A* and *FAD2-1B*) were targeted by using TALENs to generate lines with increased levels of oleic acid (18:1) and decreased levels of linoleic (18:2) and linolenic acid (18:3) (Haun et al., 2014). Additionally, these lines were further modified by targeting the *FAD3A* (fatty acid desaturase 3A) gene to further decrease levels of linolenic acid (Demorest et al., 2016). Other species targeted for modifications in the seed oil content included peanuts (*Arachis hypogaea*) and rice (Wen et al., 2018; Abe et al., 2018). A particular example, in this case, is represented by the *Camelina sativa* (false flax), a polyploid species from the *Brassicaceae* family, for its short growing season and high productivity in diverse regions. *C. sativa* was engineered through the use of the CRISPR/Cas9 system by targeting several genes involved in the fatty acids biosynthetic pathway. For instance,

aside the *FAD2* (Jiang et al., 2017; Morineau et al., 2017), also the *FAE1* (fatty acid elongase) and *DGAT1* (diacylglycerol acyltransferase) genes were targeted to produce plants with altered seed oil composition (Ozseyhan et al., 2018; Aznar-Moreno and Durrett, 2017). Moreover, ample filed trials were also conducted to assess the productivity and safety of the generated lines while the authors strongly argue against the GM regulation of genome-edited crops in Europe (Faure and Napier, 2018).

A prodigious work was carried out to enhance the lycopene (a carotenoid synthesized during fruit ripening and considered as bioactive component responsible for lowering the risk of cancer and cardiovascular diseases) content in tomatoes (Li et al., 2018). The authors used a multiplex genome editing dedicated to boosting lycopene production by hindering the carotenoid biosynthetic pathway. One CRISPR/Cas9 vector was used to induce targeted mutations in five genes, namely *SGR1* (stay-green 1), *Blc* (β-lycopene cyclase), *LCY-E* (lycopene Ɛ-cyclase), *LCY-B1* (lycopene β-cyclase 1), and *LCY-B2*, that resulted in the loss of gene function. This strategy allowed to specifically increase by 5.1-fold the lycopene content in the fruits (Li et al., 2018). Another attempt to engineer the carotenoid pathway was performed in the rice grains in order to enhance the β-carotene content (Yang et al., 2017). Here, other five carotenoid catabolic genes were singularly targeted for the knockout, namely two carotene hydrolases (*OsCYP97A4*, *OsD5M2*) and three carotene dioxygenases (*OsCCD4a*, *OsCCD4b*, *OsCCD7*). Several types of mutated lines were identified and characterized but these did not accumulate more carotenoids in rice grains in neither the mono- or bi-allelic mutants obtained by targeting the five genes. On the other hand, homozygous rice plants for mutations in the *OsCCD7* gene presented

a dwarf phenotype with enhanced tiller number, hence increased seed productivity (Yang et al., 2017).

Rice is often targeted to improve nutritional aspects mainly because it is a staple food for more than half of the worlds' population while being poor in nutrient import. For this, multiple biofortification programs, using both traditional breeding and genetic engineering approaches, are being conducted to enhance rice vitamins and mineral contents (Slamet-Loedin et al., 2015; Trijatmiko et al., 2016; Singh et al., 2017; Descalsota et al., 2018). Additionally, genome editing tools, and precisely CRISPR/Cpf1, were used also to lower the concentration of damaging minerals such as cadmium (Tang et al., 2017). This was accomplished by knocking out the *NRAMP5* (Natural Resistance-Associated Macrophage Protein5) metal transporter, and the resulting lines grown under field conditions showed significantly lower Cd concentration (less than 0.05 mg/kg) in the grains, compared to wild-type lines (0.33-2.90 mg/kg). Genome editing techniques were also used to enhance rice flavor by targeting the *BADH2* (betaine aldehyde dehydrogenase) gene, encoding for an enzyme that obstructs the synthesis of 2-acetyl-1-pyrroline (2AP), responsible for the dominant rice fragrance (Shan et al., 2015; Shao et al., 2017). A more complex trait engineered in rice through the use of genome editing relates to the production of high-amylose lines (Sun et al., 2017). This was addressed because rice grains with high amylose content are considered as better sources of resistant starch and this was linked with a decreased incidence of gastrointestinal and cardiovascular disorders (Vonk et al., 2000). Hence, CRISPR/Cas9 was used to generate targeted mutagenesis in genes coding for starch branching enzymes (*SBEI* and *SBEIIb*, respectively), that catalyzes the cleavage of α-1,4-linked glucan chain to produce

branches in amylopectin (Syahariza et al., 2013). This strategy led to obtaining rice lines with an increased proportion of amylose and resistant starch only when the *SBEIIb* gene was silenced (Sun et al., 2017). In addition, in another study, the same gene was targeted using a deactivated Cas9 (nCas9-PBE) fused with a cytidine deaminase enzyme to generated specific cytidine (C) to thymine (T) base editing (point mutations) without inducing any cuts in the genome (Li J et al., 2017). Another gene targeted for the same trait in rice is *Waxy*, encoding for a granule-bound starch synthase (GBSS) responsible for the synthesis of amylose specifically in the endosperm. In this case, the loss-of-function mutants presented reduced amylose leading to the generation of low-glutinous rice without yield penalties (Zhang et al., 2018). A similar approach was also applied for maize lines developed by DuPont Pioneer (Waltz, 2016) whereas in potato all four *GBSS* alleles were silenced to produce tubers without amylose-containing starch (Andersson et al., 2017).

Aside from enhancing certain nutritional contents, other approaches were focused on lowering the amount of antinutritional elements, such as phytic acid or acrylamide. The development of low phytic acid maize seeds was among the first traits targeted with the use of genome editing tools. These products have a dual purpose because the *IPK1* (inositol-1,3,4,5,6-pentakisphosphate 2-kinase, involved in the final step of phytate biosynthesis) gene was disrupted while inserting within its open reading frame another gene (*PAT*, phosphinothricin N-acetyltransferase) that confers resistance to gluphosinate herbicides (Shukla et al., 2009; Liang et al., 2014). In another study, Holmes et al. (2017) used both TALEN and CRISPR/Cas9 to induce mutations in the promoter region of barley (*Hordeum vulgare*) phytase gene (*PAPhy*, the main contributor to phytase

metabolism in mature grains). In this case, the developed lines with very low phytate content were also associated with a significant delay in germination. Acrylamide, a potential carcinogen formed as a byproduct of the Maillard reaction, is another example of antinutritional qualities in processed (mainly heat) foods. To decrease the sugar contents (the main substrate for Maillard reaction) in potato (*Solanum tuberosum*) tubers, Clasen et al. (2016) used TALEN to knockout the *Vlnv* (vacuolar invertase) gene, responsible for the breakdown of sucrose into glucose and fructose, and succeeded to produced acrylamide-free potatoes. An interesting application of genome editing for nutritional purposes related to the development of low gluten wheat, designated for patients suffering from the coeliac disease, an autoimmune disorder triggered by the ingestion of gluten. In this case, the *α-gliadin* gene was targeted by CRISPR/Cas9 and the produced lines had a decrease of up to 85% in the gluten content (Sánchez-León et al., 2018).

4. Tackling seed physiology and development through genome editing applications

Due to their importance for both the natural biodiversity and agricultural purposes, seed development and related features are being targeted to optimize their quality at several levels spanning from the production process, to storage, seed maturation, and germination. However, because many basic molecular aspects related to seed quality are still meagerly understood (Macovei et al., 2016), engineering such complex traits is still a big challenge. Nonetheless, some studies where genome editing approaches were used to develop better products or to understand basic molecular functions, were recently published (**Table 4.1**). Among the targeted traits, hybrid seed production has intrinsic

value due to its relation to heterosis vigor, hence substantial yield enhancement. For this, approaches that lead to male pollen sterility are used to develop hybrid seed (Wu et al. 2016). CRISPR/Cas9 was used to target genes involved in pollen development to generate male sterile lines in two important cereal crops, namely maize and bread wheat (Chen et al., 2018; Singh et al., 2018). In the first case, the *MS8* (Male Sterility 8) gene, encoding a ß-1,3-galactosyltransferase enzyme involved in anther development (Chen et al., 2018), was targeted, while in the second case the *Ms45* (Male sterile 45), coding for a strictosidine synthase-like enzyme, was investigated (Singh et al., 2018). Because wheat is a hexaploidy crop, all the three gene homologs had to be knocked out, and only the triple mutants were able to achieve the desired trait. On the other hand, rice continues to remain a species often used in genome editing approaches related to seed development. Among the most known examples are the SWEET sugar transporters that have roles both in grain filling (Ma et al., 2017) and bacterial blight disease resistance (Zhou et al., 2015). In this case, however, silencing of the SWEET11 gene resulted in a deficient rice grain filling due to a decline of sucrose release from the maternal tissue (Ma et al., 2017). A base editing approach through the CRISPR/Cas9-APOBEC1 cytidine deaminase was used to create point mutations in the rice *SLR1* gene, encoding a DELLA protein with known roles in seed germination and plant development (Lu and Zhu, 2017). Differently, a multiplex CRISPR/Cas 9 was used to target the *Hd2*, *Hd4*, and *Hd5* genes that negatively affect heading date in rice (Li et al., 2017), resulting in the development of lines with shortened heading date.

Seed preservation during processing and storage is a key trait to promote sustainable agricultural practices without additional economic losses. Though, the molecular aspects

related to these traits are still undergoing. Nevertheless, two studies were conducted to target genes that resulted in reduced seed shattering during harvest and enhanced resistance during storage in rapeseed (*B. napus*) and rice, respectively (Ma et al., 2015; Braatz et al., 2017). In the first case, a CRISPR-Cas9 construct was designed to target the two *ALC* (ALCATRAZ) alleles in rapeseed plants. This gene, known to be involved in seed valve margin development, is responsible for seed shattering from mature fruits; hence, by silencing the *ALC* alleles, an enhanced shatter resistance trait was developed and this permitted to avert seed loss during mechanical harvest (Braatz et al., 2017). In another study, TALEN constructs were used to target the *LOX3* (lipoxygenase 3, involved in the deoxygenation of polyunsaturated fatty acids) gene in rice, leading to the development of lines showing improved seed storability (Ma et al., 2015).

Plant phytohormones have multiple roles in plant growth and development. Among them, abscisic acid (ABA) and gibberellins (GAs) are involved in maintaining the equilibrium between seed dormancy and germination (Rodríguez-Gacio et al., 2009). Within this context, the *NCED4* (9-cis-epoxycarotenoid dioxygenase 4) gene encodes a key enzyme in the ABA biosynthesis (Huo et al. 2013). CRISPR/Cas9 targeting the *NCED4* gene in lettuce (*Lactuca sativa*) resulted in the development of lines with an enhanced range of temperatures that condition seed germination. This study showed that more than 70% of the seeds were able to germinate at temperatures up to 37ºC, when the usual germination temperature in the particular case of lettuce range between 15ºC and 25ºC (Bertier et al., 2018). Still related to ABA biosynthesis, the pyrabactin resistance 1 (PYR1)/PYR1-like (PYL) genes encode for regulatory components of the ABA receptor (RCAR) family of proteins, involved in ABA sensing. CRISPR/Cas9 editing of the *PYL1-13* genes in rice

resulted in the generation of multiple lines with modified plant growth and seed dormancy (Miao et al., 2018). However, within the obtained mutations in single lines, the *pyl1* and *pyl12* mutants presented significant defects in seed dormancy whereas the *pyl1/4/6* triple mutant lines maintained a normal seed dormancy and showed improved plant growth and grain productivity under field conditions. When addressing seedling development, a transcription factor known to regulate the cold acclimation in *Arabidopsis thaliana* was shown to be involved also in other aspects of plant's life. This is the case of the C-repeat binding factors (CBF1, CBF2, and CBF3), coding proteins are involved in the regulation of lipid and carbohydrate metabolism, gene transcription, and cell wall modification. When all the three genes were targeted by a CRISPR/Cas9 construct, the *cbf* triple mutants resulted to be defective in seedling development and tolerance to salt stress (Zhao et al., 2016). Hence, to produce improved lines, a knock-in or transcription activation approach should be used instead of gene knockout.

5. Conclusions and future perspectives

In conclusion, the present book chapter emphasizes the implementation of genome editing tools to improve seed quality traits, covering aspects spanning from seed productivity and nutritional content to seed germination and dormancy. Further advances in both technical development and basal research will bring to light more application of these amazing tools to expand the number of targeted traits and frame the future of sustainable agriculture.

Table 2.1. Examples of genome editing use to improve crop yield.

Trait	Gene	Species	Tool	References
Seed size	*GW2*	*O. sativa* *T. aestivum*	CRISPR/Cas9	Xu et al., 2016 Wang et al., 2018a,b
	GW5	*O. sativa*		Xu et al., 2016
	TGW6	*O. sativa*		Xu et al., 2016
	RGG2	*O. sativa*		Miao et al., 2019
	gs3	*O. sativa*		Li et al., 2016
	GASR7	*T. aestivum*		Zhang et al., 2016
Seed number	*Gn1a*	*O. sativa*	CRISPR/Cas9	Li et al., 2016
	ARGOS8	*Z. mays*		Shi et al., 2017
	CLVTA3	*B. napus*		Yang et al., 2018
Plant architecture & productivity	*WUS*	*S. lycopersicum*	CRISPR/Cas9	Rodríguez-Leal et al., 2017
	SP	*S. lycopersicum*		Rodríguez-Leal et al., 2017
	SFT	*S. lycopersicum*		Rodríguez-Leal et al., 2017
	DEP1	*O. sativa*		Li et al., 2016
	IPA1	*O. sativa*		Li et al., 2016
Heterosis	*REC8*	*O. sativa*	CRISPR/Cas9	Wang et al., 2019
	PAIR1	*O. sativa*		Wang et al., 2019
	OSD1	*O. sativa*		Wang et al., 2019
	MTL	*O. sativa*		Wang et al., 2019

Table 3.1. Examples of genome editing use to improve see nutritional content

Trait	Gene	Species	Tool	References
Seed oil content	*FAD2*	*O. sativa*	CRISPR/Cas9	Abe et al., 2018
		A. hypogeaea	TALEN	Wen et al., 2018
		C. sativa	CRISPR/Cas9	Jiang et al., 2017; Morineau et al. 2017
		G. max	TALEN	Hain et al., 2014; Demorest et al., 2016
	DGAT1	*C. sativa*	CRISPR/Cas9	Aznar-Moreno and Durrett, 2017
	FAE1	*C. sativa*	CRISPR/Cas9	Ozseyhan et al., 2018
Carotenoids	*CYP97A4* *DSM2* *CCD4a* *OsCCD4b* *CCD7*	*O. sativa*	CRISPR/Cas9	Yang et al., 2017
	SGR1 *LCY-E* *Blc* *LCY-B1* *LCY-B2*	*S. lycopersicum*	CRISPR/Cas9	Li et al., 2018
Fragrance	*BADH2*	*O. sativa*	TALEN CRISPR/Cas9	Shan et al., 2015 Shao et al., 2017
Amylose content	*SBEIIb* *SBEI*	*O. sativa*	CRISPR/Cas9 nCas9-PBE	Sun et al., 2017 Li et al., 2017
	Waxy	*O. sativa* *Z. mays*	CRISPR/Cas9	Zhang et al., 2018 Waltz, 2016
	GBSS	*S. tuberosum*	CRISPR/Cas9	Andersson et al., 2017
Gluten	*α-gliadin*	*T. aestivum*	CRISPR/Cas9	Sánchez-León et al., 2018
Cadmium	*NRAMP5*	*O. sativa*	CRISPR/Cpf1	Tang et al., 2017
Acrylamide	*VInv*	*S. tuberosum*	TALEN	Clasen et al., 2016
Phytic acid	*IPK*	*Z. mays*	ZFN TALEN CRISPR/Cas9	Shukla et al., 2009 Liang et al., 2014
	PAPhy	*H. vulgare*	TALEN CRISPR/Cas9	Holme et al., 2017

Table 4.1. Examples of genome editing use to address seed physiology and development.

Trait	Gene	Species	Tool	References
Male sterility	*MS8*	*Z. mays*	CRISPR/Cas9	Chen et al., 2018
	Ms45	*T. aestivum*	CRISPR/Cas9	Singh et al., 2018
Seed development	*SWEET11*	*O. sativa*	CRISPR/Cas9	Zhou et al., 2015; Ma et al., 2017
	SLR1	*O. sativa*	CRISPR/Cas9-APOBEC1	Lu and Zhu, 2017
	Hd2,4,5	*O. sativa*	CRISPR/Cas9	Li et al., 2017
Seed storability & processing	*LOX*	*O. sativa*	TALEN	Ma et al., 2015
	ALC	*B. napus*	CRISPR/Cas9	Braatz et al., 2017
Seed germination & dormancy	*NCED4*	*L. sativa*	CRISPR/Cas9	Bertier et al., 2018
	PYL1-13	*O. sativa*	CRISPR/Cas9	Miao et al., 2018
Seedling development	*CDF*	*A. thaliana*	CRISPR/Cas9	Zhao et al., 2016

References

Abe K, Araki E, Suzuki Y, Toki S, Saika H. 2018. Production of high oleic/low linoleic rice by genome editing. Plant Physiol Biochem. 131:58-62. doi: 10.1016/j.plaphy.2018.04.033.

Allied Market Research, https://www.alliedmarketresearch.com/press-release/seed-market.htm

Andersson M, Turesson H, Nicolia A, Fält A-S, Samuelsson M, Hofvander P. 2017. Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. Plant Cell Rep. 36:117–128. doi: 10.1007/s00299-016-2062-3.

Aznar-Moreno JA, Durrett TP. 2017. Simultaneous targeting of multiple gene homeologs to alter seed oil production in *Camelina sativa*. Plant Cell Physiol. 58(7):1260-1267. doi: 10.1093/pcp/pcx058.

Baltes NJ, Gil-Humanes J, Voytas DF. 2017. Genome engineering and agriculture: opportunities and challenges. Prog Mol Biol Transl Sci. 149:1-26. doi: 10.1016/bs.pmbts.2017.03.011.

Baltes NJ, Voytas DF. 2015. Enabling plant synthetic biology through genome engineering. Trends Biotechnol. 33(2):120-31. doi: 10.1016/j.tibtech.2014.11.008.

Bertier LD, Ron M, Huo H, Bradford KJ, Britt AB, Michelmore RW. 2018. High-resolution analysis of the efficiency, heritability, and editing outcomes of CRISPR/Cas9-induced modifications of NCED4 in lettuce (*Lactuca sativa*). G3 (Bethesda). 8(5):1513-1521. doi: 10.1534/g3.117.300396.

Bogdanove AJ, Voytas DF. 2011. TAL effectors: customizable proteins for DNA targeting. Science 333: 1843–1846. doi: 10.1126/science.1204094.

Braatz J, Harloff HJ, Mascher M, Stein N, Himmelbach A, Jung C. 2017. CRISPR-Cas9 targeted mutagenesis leads to simultaneous modification of different homoeologous gene copies in polyploid oilseed rape (*Brassica napus*). Plant Physiol. 174(2):935-942. doi: 10.1104/pp.17.00426.

Brookes G, Barfoot P. 2017. Farm income and production impacts of using GM crop technology 1996-2015. GM Crops Food. 8(3):156-193. doi: 10.1080/21645698.2017.1317919.

Chen K, Gao C. 2014. Targeted genome modification technologies and their applications in crop improvements. Plant Cell Rep. 33(4):575-83. doi: 10.1007/s00299-013-1539-6.

Chen K, Wang Y, Zhang R, Zhang H, Gao C. 2019. CRISPR/Cas genome editing and precision plant breeding in agriculture. Annu Rev Plant Biol. doi: 10.1146/annurev-arplant-050718-100049.

Chen R, Xu Q, Liu Y, Zhang J, Ren D, Wang G, Liu Y. 2018. Generation of transgene-free maize male sterile lines using the CRISPR/Cas9 system. Front Plant Sci. 9:1180. doi: 10.3389/fpls.2018.01180.

Clasen BM, Stoddard TJ, Luo S, Demorest ZL, Li J, Cedrone F, Tibebu R, Davison S, Ray EE, Daulhac A, Coffman A, Yabandith A, Retterath A, Haun W, Baltes NJ, Mathis L, Voytas DF, Zhang F. 2016. Improving cold storage and processing traits in potato through targeted gene knockout. Plant Biotechnol. J. 14:169–176. doi: 10.1111/pbi.12370.

Debbarma J, Sarki YN, Saikia B, Boruah HPD, Singha DL, Chikkaputtaiah C. 2019. Ethylene Response Factor (ERF) family proteins in abiotic stresses and CRISPR-Cas9 genome editing of ERFs for multiple abiotic stress tolerance in crop plants: a review. Mol Biotechnol. 61(2):153-172. doi: 10.1007/s12033-018-0144-x.

Demorest ZL, Coffman A, Baltes NJ, Stoddard TJ, Clasen BM, Luo S, Retterath A, Yabandith A, Gamo ME, Bissen J, Mathis L, Voytas DF, Zhang F. 2016. Direct stacking of sequence-specific nuclease-induced mutations to produce high oleic and low linolenic soybean oil. BMC Plant Biol. 16(1):225. doi: 10.1186/s12870-016-0906-1.

Descalsota GIL, Swamy BPM, Zaw H, Inabangan-Asilo MA, Amparado A, Mauleon R, Chadha-Mohanty P, Arocena EC, Raghavan C, Leung H, Hernandez JE, Lalusin AB, Mendioro MS, Diaz MGQ, Reinke R. 2018. Genome-wide association mapping in a rice MAGIC Plus population detects QTLs and genes useful for biofortification. Front Plant Sci. 9:1347. doi: 10.3389/fpls.2018.01347.

Everari M. 1984. Seed physiology: its history from antiquity to the beginning of the 20th century. Bot Rev. 50:119-142.

FAO (Food and Agriculture Organization), http://www.fao.org/fileadmin/templates/agphome/documents/PGR/SoW2/tbs_Seed_Systems_081209.pdf

Farooq M, Hussain M, Usman M, Farooq S, Alghamdi SS, Siddique KHM. 2018. Impact of abiotic stresses on grain composition and quality in food legumes. J Agric Food Chem. 66(34):8887-8897. doi: 10.1021/acs.jafc.8b02924.

Faure JD, Napier JA. 2018. Europe's first and last field trial of gene-edited plants? E-life. 7: e42379. doi: 10.7554/eLife.42379.

Finch-Savage WE, Bassel GW. 2016. Seed vigour and crop establishment: extending performance beyond adaptation. J Exp Bot. 67(3):567-91. doi: 10.1093/jxb/erv490.

Haak DC, Fukao T, Grene R, Hua Z, Ivanov R, Perrella G, Li S. 2017. Multilevel regulation of abiotic stress responses in plants. Front Plant Sci. 8:1564. doi: 10.3389/fpls.2017.01564.

Haun W, Coffman A, Clasen BM, Demorest ZL, Lowy A, Ray E, Retterath A, Stoddard T, Juillerat A, Cedrone F, Mathis L, Voytas DF, Zhang F. 2014. Improved soybean oil quality by targeted mutagenesis of the fatty acid desaturase 2 gene family. Plant Biotechnol. J. 12:934–940. doi: 10.1111/pbi.12201.

Holme IB, Wendt T, Gil-Humanes J, Deleuran LC, Starker CG, Voytas DF, Brinch-Pedersen H. 2017. Evaluation of the mature grain phytase candidate HvPAPhy_a gene in barley (*Hordeum vulgare* L.) using CRISPR/Cas9 and TALENs. Plant Mol Biol. 95(1-2):111-121. doi: 10.1007/s11103-017-0640-6.

Huang X, Qian Q, Liu Z, Sun H, He S, Luo D, Xia G, Chu C, Li J, Fu X. 2009. Natural variation at the DEP1 locus enhances grain yield in rice. Nat Genet. 41(4):494-7. doi: 10.1038/ng.352.

Huo H, Dahal P, Kunusoth K, McCallum CM, Bradford KJ. 2013. Expression of 9-cis-EPOXYCAROTENOID DIOXYGENASE4 is essential for thermoinhibition of lettuce seed germination but not for seed development or stress tolerance. Plant Cell 25:884–900. doi: 10.1105/tpc.112.108902.

ISTA (International Seed Testing Association). 2018. International rules for seed testing. Basserdorf, Switzerland.

Jaganathan D, Ramasamy K, Sellamuthu G, Jayabalan S, Venkataraman G. 2018. CRISPR for crop improvement: an update review. Front Plant Sci. 9:985. doi: 10.3389/fpls.2018.00985.

Jiang WZ, Henry IM, Lynagh PG, Comai L, Cahoon EB, Weeks DP. 2017. Significant enhancement of fatty acid composition in seeds of the allohexaploid, *Camelina sativa*, using CRISPR/Cas9 gene editing. Plant Biotechnol J. 15(5):648-657. doi: 10.1111/pbi.12663.

Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 337(6096):816-21. doi: 10.1126/science.1225829.

Kausch AP, Nelson-Vasilchik K, Hague J, Mookkan M, Quemada H, Dellaporta S, Fragoso C, Zhang ZJ. 2019. Edit at will: Genotype independent plant transformation in

the era of advanced genomics and genome editing. Plant Sci. 281:186-205. doi: 10.1016/j.plantsci.2019.01.006.

Khatodia S, Bhatotia K, Passricha N, Khurana SM, Tuteja N. 2016. The CRISPR/Cas genome-editing tool: application in improvement of crops. Front Plant Sci. 7:506. doi: 10.3389/fpls.2016.00506.

Li J, Sun Y, Du J, Zhao Y, Xia L. 2017. Generation of targeted point mutations in rice by a modified CRISPR/Cas9 system. Mol Plant. 10:526–529. doi: 10.1016/j.molp.2016.12.001.

Li M, Li X, Zhou Z, Wu P, Fang M, Pan X, Lin Q, Luo W, Wu G, Li H. 2016. Reassessment of the four yield-related genes Gn1a, DEP1, GS3, and IPA1 in rice using a CRISPR/Cas9 system. Front Plant Sci. 7:377. doi: 10.3389/fpls.2016.00377.

Li X, Wang Y, Chen S, Tian H, Fu D, Zhu B, Luo Y, Zhu H. 2018. Lycopene is enriched in tomato fruit by CRISPR/Cas9-mediated multiplex genome editing. Front Plant Sci.9:559. doi: 10.3389/fpls.2018.00559.

Liang Z, Zhang K, Chen K, Gao C. 2014. Targeted mutagenesis in *Zea mays* using TALENS and the CRISPR/Cas system. J Genet Genomics. 41:63–68. doi: 10.1016/j.jgg.2013.12.001.

Linkies A, Graeber K, Knight C, Leubner-Metzger G. 2010. The evolution of seeds. New Phytol. 186(4):817-31. doi: 10.1111/j.1469-8137.2010.03249.x.

Liu C, Moschou PN. 2018. Phenotypic novelty by CRISPR in plants. Dev Biol. 435(2):170-175. doi: 10.1016/j.ydbio.2018.01.015.

Lopez-Huertas E. 2010. Health effects of oleic acid and long chain omega-3 fatty acids (EPA and DHA) enriched milks. A review of intervention studies. Pharmacol Res. 61:200–207. doi: 10.1016/j.phrs.2009.10.007.

Lu Y, Zhu JK. 2017. Precise editing of a target base in the rice genome using a modified CRISPR/Cas9 system. Mol Plant. 10:523–525. doi: 10.1016/j.molp.2016.11.013.

Ma L, Zhang D, Miao Q, Yang J, Xuan Y, Hu Y. 2017. Essential role of sugar transporter OsSWEET11 during the early stage of rice grain filling. Plant Cell Physiol. 58(5):863-873. doi: 10.1093/pcp/pcx040.

Ma L, Zhu F, Li Z, Zhang J, Li X, Dong J, Wang T. 2015. TALEN-based mutagenesis of lipoxygenase LOX3 enhances the storage tolerance of rice (*Oryza sativa*) seeds. PLoS One 10:e0143877. doi: 10.1371/journal.pone.0143877

Macovei A, Pagano A, Leonetti P, Carbonera D, Balestrazzi A, Araújo SS. 2017. Systems biology and genome-wide approaches to unveil the molecular players involved in the pre-germinative metabolism: implications on seed technology traits. Plant Cell Rep. 36(5):669-688. doi: 10.1007/s00299-016-2060-5.

Mahajan S, Tuteja N. 2005. Cold, salinity and drought stresses: an overview. Arch Biochem Biophys 444(2):139-58. doi: 10.1016/j.abb.2005.10.018.

Malzhan A, Lowder L, Qi Y. 2017. Plant genome editing with TALEN and CRISPR. Cell Biosci. 7:21. doi: 10.1186/s13578-017-0148-4.

Miao C, Xiao L, Hua K, Zou C, Zhao Y, Bressan RA, Zhu JK. 2018. Mutations in a subfamily of abscisic acid receptor genes promote rice growth and productivity. Proc Natl Acad Sci U S A. 115(23):6058-6063. doi: 10.1073/pnas.1804774115.

Miao J, Yang Z, Zhang D, Wang Y, Xu M, Zhou L, Wang J, Wu S, Yao Y, Du X, Gu F, Gong Z, Gu M, Liang G, Zhou Y. 2019. Mutation of RGG2, which encodes a type B heterotrimeric G protein γ subunit, increases grain size and yield production in rice. Plant Biotechnol J. 17(3):650-664. doi: 10.1111/pbi.13005.

Miura K, Ikeda M, Matsubara A, Song XJ, Ito M, Asano K, Matsuoka M, Kitano H, Ashikari M. 2010. OsSPL14 promotes panicle branching and higher grain productivity in rice. Nat Genet. 42(6):545-9. doi: 10.1038/ng.592.

Morineau C, Bellec Y, Tellier F, Gissot L, Kelemen Z, Nogué F, Faure JD. 2017. Selective gene dosage by CRISPR-Cas9 genome editing in hexaploid Camelina sativa. Plant Biotechnol J. 15(6):729-739. doi: 10.1111/pbi.12671.

Niklas KJ. 1997. The evolutionary biology of plants. Chicago, IL, USA: The University of Chicago Press.

Okuley J, Lightner J, Feldmann K, Yadav N, Lark E, Browse J. 1994. Arabidopsis FAD2 gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell. 6:147.

Osburn RM, Schroth MN. 1989. Effect of osmopriming sugar beet deed on exudation and subsequent damping-off caused by *Pythium ultimum*. Phytopathology 78:1246–1250.

Ozseyhan ME, Kang J, Mu X, Lu C. 2018. Mutagenesis of the FAE1 genes significantly changes fatty acid composition in seeds of *Camelina sativa*. Plant Physiol Biochem. 123:1-7. doi: 10.1016/j.plaphy.2017.11.021.

Paparella S, Araújo SS, Rossi G, Wijayasinghe M, Carbonera D, Balestrazzi A. 2015. Seed priming: state of the art and new perspectives. Plant Cell Rep. 34(8):1281-93. doi: 10.1007/s00299-015-1784-y.

Paul MJ, Nuccio ML, Basu SS. 2018. Are GM crops for yield and resilience possible? Trends Plant Sci. 23(1):10-16. doi: 10.1016/j.tplants.2017.09.007.

Puchta H. 2017. Applying CRISPR/Cas for genome engineering in plants: the best is yet to come. Curr Opin Plant Biol. 36:1-8. doi: 10.1016/j.pbi.2016.11.011.

Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. 2013. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 8(11):2281-2308. doi: 10.1038/nprot.2013.143.

Rodriguez-Gacio Mdel C1, Matilla-Vázquez MA, Matilla AJ. 2009. Seed dormancy and ABA signaling: the breakthrough goes on. Plant Signal Behav. 4(11):1035 - 49.

Rodríguez-Leal D, Lemmon ZH, Man J, Bartlett ME, Lippman ZB. 2017. Engineering quantitative trait variation for crop improvement by genome editing. Cell. 171(2):470-480.e8. doi: 10.1016/j.cell.2017.08.030.

Sánchez-León S, Gil-Humanes J, Ozuna CV, Giménez MJ, Sousa C, Voytas DF, Barro F. 2018. Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. Plant Biotechnol J. 16(4):902-910. doi: 10.1111/pbi.12837.

Schnable PS, Springer NM. 2013. Progress toward understanding heterosis in crop plants. Annu Rev Plant Biol. 64:71–88. doi: 10.1146/annurev-arplant-042110-103827

Sedeek KEM, Mahas A, Mahfouz M. 2019. Plant genome engineering for targeted improvement of crop traits. Front Plant Sci. 10:114. doi: 10.3389/fpls.2019.00114.

Shan Q, Voytas DF. 2018. Editing plant genes one base at a time. Nat Plants. 4(7):412-413. doi: 10.1038/s41477-018-0177-y.

Shan Q, Zhang Y, Chen K, Zhang K, Gao C. 2015. Creation of fragrant rice by targeted knockout of the OsBADH2 gene using TALEN technology. Plant Biotechnol J.13(6):791-800. doi: 10.1111/pbi.12312.

Shao G, Xie L, Jiao G, Wei X, Sheng Z, Tang S, et al. 2017. CRISPR/CAS9-mediated editing of the fragrant gene Badh2 in rice. Chin J Rice Sci. 31:216–222. doi: 10.16819/j.1001-7216.2017.6098

Shen L, Wang C, Fu Y, Wang J, Liu Q, Zhang X, Yan C, Qian Q2 Wang K. 2018. QTL editing confers opposing yield performance in different rice varieties. J Integr Plant Biol. 60:89–93. doi: 10.1111/jipb.12501.

Shi J, Gao H, Wang H, Lafitte HR, Archibald RL, Yang M, Hakimi SM, Mo H, Habben JE. 2017. ARGOS8 variants generated by CRISPR-Cas9 improve maize grain yield

under field drought stress conditions. Plant Biotechnol J. 15(2):207-216. doi: 10.1111/pbi.12603.

Shih PM, Liang Y, Loqué D. 2016. Biotechnology and synthetic biology approaches for metabolic engineering of bioenergy crops. Plant J. 87(1):103-17. doi: 10.1111/tpj.13176.

Shukla VK, Doyon Y, Miller JC, DeKelver RC, Moehle EA, Worden SE, Mitchell JC, Arnold NL, Gopalan S, Meng X, Choi VM, Rock JM, Wu YY, Katibah GE, Zhifang G, McCaskill D, Simpson MA, Blakeslee B, Greenwalt SA, Butler HJ, Hinkley SJ, Zhang L, Rebar EJ, Gregory PD, Urnov FD. 2009. Precise genome modification in the crop species *Zea mays* using zinc-finger nucleases. Nature. 459(7245):437-41. doi: 10.1038/nature07992.

Singh M, Kumar M, Albertsen MC, Young JK, Cigan AM. 2018. Concurrent modifications in the three homeologs of Ms45 gene with CRISPR-Cas9 lead to rapid generation of male sterile bread wheat (*Triticum aestivum* L.). Plant Mol Biol. 97(4-5):371-383. doi: 10.1007/s11103-018-0749-2.

Singh SP, Gruissem W, Bhullar NK. 2017. Single genetic locus improvement of iron, zinc and β-carotene content in rice grains. Sci Rep. 7(1):6883.

Slamet-Loedin IH, Johnson-Beebout SE, Impa S, Tsakirpaloglou N. 2015. Enriching rice with Zn and Fe while minimizing Cd risk. Front Plant Sci. Mar 6:121. doi: 10.3389/fpls.2015.00121.

Spillane C, Curtis MD, Grossniklaus U. 2004. Apomixis technology development virgin births in farmers' fields? Nat Biotechnol. 22:687–691. doi: 10.1038/nbt976.

Sun Y, Jiao G, Liu Z, Zhang X, Li J, Guo X, Du W, Du J, Francis F, Zhao Y, Xia L. 2017. Generation of high-amylose rice through CRISPR/Cas9-mediated targeted mutagenesis of starch branching enzymes. Front Plant Sci. 8:298. doi: 10.3389/fpls.2017.00298.

Sun Y, Li J, Xia L. 2016. Precise genome modification via sequence-specific nucleases-mediated gene targeting for crop improvement. Front Plant Sci. 7:1928. doi: 10.3389/fpls.2016.01928.

Syahariza Z, Sar S, Hasjim J, Tizzotti MJ, Gilbert RG. 2013. The importance of amylose and amylopectin fine structures for starch digestibility in cooked rice grains. Food Chem. 136:742–749. doi: 10.1016/j.foodchem.2012.08.053.

Tang X, Lowder LG, Zhang T, Malzahn AA, Zheng X, Voytas DF, Zhong Z, Chen Y, Ren Q, Li Q, Kirkland ER, Zhang Y, Qi Y. 2017. A CRISPR-Cpf1 system for efficient

genome editing and transcriptional repression in plants. Nat. Plants 3:17018. doi: 10.1038/nplants.2017.18.

Trijatmiko KR, Dueñas C, Tsakirpaloglou N, Torrizo L, Arines FM, Adeva C, Balindong J, Oliva N, Sapasap MV, Borrero J, Rey J, Francisco P, Nelson A, Nakanishi H, Lombi E, Tako E, Glahn RP, Stangoulis J, Chadha-Mohanty P, Johnson AA, Tohme J, Barry G, Slamet-Loedin IH. 2016. Biofortified indica rice attains iron and zinc nutrition dietary targets in the field. Sci Rep. 6:19792. doi: 10.1038/srep19792.

Vonk RJ, Hagedoorn RE, De Graaff R, Elzinga H, Tabak S, Yang Y-X, Stellaard F. 2000. Digestion of so-called resistant starch sources in the human small intestine. Am J Clin Nutr. 72:432–438. doi: 10.1093/ajcn/72.2.432.

Waltz E. 2016. CRISPR-edited crops free to enter market, skip regulation. Nat Biotechnol. 34:582. doi: 10.1038/nbt0616-582.

Wang C, Liu Q, Shen Y, Hua Y, Wang J, Lin J, Wu M, Sun T, Cheng Z, Mercier R, Wang K. 2019. Clonal seeds from hybrid rice by simultaneous genome engineering of meiosis and fertilization genes. Nat Biotechnol. 37(3):283-286. doi: 10.1038/s41587-018-0003-0.

Wang W, Pan Q, He F, Akhunova A, Chao S, Trick H, Akhunov E. 2018b. Transgenerational CRISPR-Cas9 activity facilitates multiplex gene editing in allopolyploid wheat. CRISPR J. 1(1):65-74. doi: 10.1089/crispr.2017.0010.

Wang W, Simmonds J, Pan Q, Davidson D, He F, Battal A, Akhunova A, Trick HN, Uauy C, Akhunov E. 2018a. Gene editing and mutagenesis reveal inter-cultivar differences and additivity in the contribution of TaGW2 homoeologues to grain size and weight in wheat. Theor Appl Genet. 131(11):2463-2475. doi: 10.1007/s00122-018-3166-7.

Wen S, Liu H, Li X, Chen X, Hong Y, Li H, Lu Q, Liang X. 2018. TALEN-mediated targeted mutagenesis of fatty acid desaturase 2 (FAD2) in peanut (*Arachis hypogaea* L.) promotes the accumulation of oleic acid. Plant Mol Biol. 97(1-2):177-185. doi: 10.1007/s11103-018-0731-z.

Wu Y, Fox TW, Trimnell MR, Wang L, Xu R-j, Cigan AM, Huffman GA, Garnaat CW, Hershey H, Albertsen MC. 2016. Development of a novel recessive genetic male sterility system for hybrid seed production in maize and other cross-pollinating crops. Plant Biotechnol J. 14:1046–1054. https://doi.org/10.1111/pbi.12477.

Xu R, Yang Y, Qin R, Li H, Qiu C, Li L, Wei P, Yang J. 2016. Rapid improvement of grain weight via highly efficient CRISPR/Cas9-mediated multiplex genome editing in rice. J Genet Genomics. 43(8):529-32. doi: 10.1016/j.jgg.2016.07.003.

Yang X, Chen L, He J, Yu W. 2017. Knocking out of carotenoid catabolic genes in rice fails to boost carotenoid accumulation, but reveals a mutation in strigolactone biosynthesis. Plant Cell Rep. 36(10):1533-1545. doi: 10.1007/s00299-017-2172-6.

Yang Y, Zhu K, Li H, Han S, Meng Q, Khan SU, Fan C, Xie K, Zhou Y. 2018. Precise editing of CLAVATA genes in *Brassica napus* L. regulates multilocular silique development. Plant Biotechnol J. 16(7):1322-1335. doi: 10.1111/pbi.12872.

Yu S, Tian L. 2018. Breeding major cereal grains through the lens of nutrition sensitivity. Mol Plant. 11(1):23-30. doi: 10.1016/j.molp.2017.08.006.

Zhang H, Zhang J, Lang Z, Botella JR, Zhu J-K. 2017. Genome editing - principles and applications for functional genomics research and crop improvement. Crit Rev Plant Sci. 36: 291-309. doi: 10.1080/07352689.2017.1402989

Zhang J, Zhang H, Botella JR, Zhu JK. 2018. Generation of new glutinous rice by CRISPR/Cas9-targeted mutagenesis of the Waxy gene in elite rice varieties. J Integr Plant Biol. 60(5):369-375. doi: 10.1111/jipb.12620.

Zhang Y, Liang Z, Zong Y, Wang Y, Liu J, Chen K, Qiu JL, Gao C. 2016. Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat Commun. 7:12617. doi: 10.1038/ncomms12617.

Zhao C, Zhang Z, Xie S, Si T, Li Y, Zhu JK. 2016. Mutational evidence for the critical role of CBF transcription factors in cold acclimation in Arabidopsis. Plant Physiol. 171(4):2744-59. doi: 10.1104/pp.16.00533.

Zhou J, Peng Z, Long J, Sosso D, Liu B, Eom JS, Huang S, Liu S, Vera Cruz C, Frommer WB, White FF, Yang B. 2015. Gene targeting by the TAL effector PthXo2 reveals cryptic resistance gene for bacterial blight of rice. Plant J. 82(4):632-43. doi: 10.1111/tpj.12838.

Zuo J, Li J. 2014. Molecular genetic dissection of quantitative trait loci regulating rice grain size. Annu Rev Genet. 48: 99–118. doi: 10.1146/annurevgenet-120213-092138.

Thank you for agreeing to contribute to **ADVANCEMENT IN CROP IMPROVEMENT TECHNIQUES** (the "**Work**") edited by **Narendra Tuteja, Renu Tuteja, Nishat Passricha** and **Shabnam K. Saifi** (the "**Editor**"). This Agreement between you (the "**Contributor**") and **Elsevier Inc.** (the "**Publisher**") regarding your contribution (the "**Contribution**") outlines the Contributor's and the Publisher's respective obligations and rights.

Molecular aspects of seed priming as a means of progress in crop improvement

Maraeva Gianella[1], Andrea Pagano[1], Chiara Forti[1], Filippo Guzzon[2,#], Andrea Mondoni[2], Susana de Sousa Araújo[3], Anca Macovei[1], Alma Balestrazzi[1,*]

[1] Department of Biology and Biotechnology "L. Spallanzani", University of Pavia, via Ferrata 9, Pavia 27100, Italy
[2] Department of Environment and Earth Sciences, University of Pavia, via S. Epifanio 14, Pavia 27100, Italy
[3] Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa (ITQB-NOVA), Avenida da República, Estação Agronómica Nacional, Oeiras 2780-157, Portugal
Present address: International Maize and Wheat Improvement Center (CIMMYT), Texcoco, Estado de Mexico 56237, Mexico

* *Corresponding author's email address:* alma.balestrazzi@unipv.it

Abstract: Seed germination is a critical parameter for the successful development of sustainable agricultural practices. However, it can be impaired by environmental constraints emerging from climate change. Seed priming is used to improve germination by managing a series of parameters during the initial stages of the process. During priming, seeds are advanced to an equal stage of the germination process to enable fast and uniform emergence when planted. Depending on plant species, seed morphology and physiology, different priming treatments can be used to enhance the 'pre-germinative metabolism'. Currently, seed priming methods are empirically applied by seed technologists, but the molecular aspects behind the seed response are not yet fully understood. This chapter presents the state-of-the-art of seed priming, including *i)* seed priming in the context of current challenges facing agriculture and crop production, *ii)* seed priming *versus* seed ageing in the context of seed bank storage, and *iii)* the molecular 'know-how' of seed priming.

Key Words: antioxidant response, DNA repair, longevity, pre-germinative metabolism seed germination, priming, omics

Introduction

Seed vigor is defined as '*the sum of those properties that determine the activity and performance of seed lots of acceptable germination in a wide range of environments*' (Finch-Savage and Bassel, 2016; ISTA, 2018) and quantified by parameters such as germination speed and rates, long-term and after-storage viability, seed lot homogeneity and purity, resistance to mechanical damage, lack of biological and chemical contaminants (Finch-Savage and Bassel, 2016). The notion that seed germination could be enhanced through specific treatments is historically attested starting from Theophrastus (371-287 B.C.), Gaius Plinius Secundus (A.D. 23-79), and later on by Oliver de Serres (1539-1619) and Charles Darwin (1809-1882). Nowadays, seed industries widely use priming on vegetables (Everari, 1984; Parera and Cantliffe, 1994; Paparella et al., 2015), ornamental species and herbs as well as on endangered species (Di Girolamo and Barbanti, 2012; Momin, 2013; Paparella et al., 2015). Different priming protocols have been optimized, varying temperature, aeration, oxygenation, and integrating protocols with additional treatments, to maximize and homogenize the priming effect and improve stress resistance (Paparella et al., 2015).

Since seed viability and vigor are strongly influenced by deterioration during storage, ageing is an issue in terms of seed lot quality, field establishment and crop productivity. Different priming methods have been used to overcome ageing-associated damages by improving germination rate, uniformity, and percentage. The promising availability of multiple kinds of 'omics' provide researchers with global views of their experimental systems at the genome, epigenome, transcriptome, proteome, metabolome, ionome, lipidome, etc. levels and this is true also in the field of seed biology where extensive studies are currently carried out to define the molecular profiles of seed priming.

1. Seed priming in the context of current challenges facing agriculture and crop production

The development of techniques to improve germination rates, speed, and consistency, as well as viability under stress conditions, has evolved in the modern concept of 'seed priming', that is broadly defined as any 'treatment that improves seed quality' (Osburn and Schroth, 1989; Paparella et al., 2015; Finch-Savage and Bassel, 2016). The temporal window useful for effective priming corresponds to the early stages of seed pre-germinative metabolism of desiccation-tolerant seeds. Indeed, an advanced or complete transition toward full germination leads to the loss of desiccation tolerance and makes desiccation deleterious for seed viability. Priming should be administered before the loss of desiccation tolerance and optimized according to the species or seed lot (Paparella et al., 2015).

1.1. Priming agents and treatments: an overview

From a historical perspective, hydropriming is the first protocol that has been developed. It consists of controlled pre-imbibition treatments particularly effective in improving

water uptake and germination in crops cultivated in dry areas. The main challenge of this technique is the selection of the best temperature and humidity values to avoid radicle protrusion, considering that water uptake depends mainly on the seed affinity for water (Taylor et al., 1998). Osmopriming involves the administration of solutions containing osmotic agents (e.g. polyethylene glycol, PEG) to delay water uptake. PEG is not able to enter inside the seed, due to its large molecular size (6000-8000 Daltons), avoiding any cytotoxic effects (Michel and Kaufman, 1973; Heydecker and Coolbear, 1977; Zhang et al., 2015). The use of PEG implies high costs and the extremely high viscosity reduces the oxygen transfer within the priming solution. Other compounds can be used, such as the inorganic salts of sodium, magnesium or potassium (e.g. NaCl, $NaNO_3$, $MnSO_4$, $MgCl_2$, K_3PO_4, and KNO_3), and this is known as 'halopriming' (Gholami et al., 2015). It overcomes the viscosity and aeration issues caused by PEG, despite the possible cytotoxic effects of excessive salt dosages However it is essential to assess the levels of ion accumulation that could result into cytotoxic effects and nutritional imbalance within the seed (Bradford, 1995; Balestrazzi et al., 2011; Gomes et al., 2016).

Physical treatments can be used to improve germination and stress resistance. Thermopriming consists of pre-sowing at specific temperatures to reduce thermo-inhibition of germination and its positive effects have been demonstrated especially for crops adapted to warm environments (Huang et al., 2008). Other physical techniques have been successfully utilized for seed invigoration. Magnetic fields, microwaves and electromagnetic radiations (UV rays, γ-rays, and X-rays) applied at specific intensities enhance the response to abiotic stresses in many model and crop species (Araújo et al., 2016).

Chemopriming involves the administration of disinfectants (e.g. sodium hypochlorite or hydrochloric acid), fungicides or pesticides to avoid the growth impairments due to contaminations (Paparella et al., 2015). Conversely, 'biopriming' integrates biologically active molecules, such as phytohormones involved in germination control and stress response (abscisic acid-ABA, gibberellins-GAs, salicylic acid-SA) (Hamayun et al., 2010; Radhakrishnan et al., 2013). Microbial strains, including *Bacillus*, *Enterobacter*, *Pseudomonas*, and *Trichoderma*, are also utilized in biopriming because of their ability to establish an endophytic relationship with the host plant, promoting its growth, stress resistance and hormone production (Niranjan et al., 2004; Waller et al., 2005).

Despite the diffusion and effectiveness of priming techniques in the seed industry, protocols need to be specifically optimized and the stress occurring during post-priming dehydration and under suboptimal storage conditions still, represents a common critical point.

2. Seed priming *versus* seed ageing in the context of seed bank storage

The term seed bank usually refers to a facility endowed with a system of collection, cleaning, packing, storing, testing and distribution of seeds. Conventional seed banking

exploits conditions of low temperature and moisture content to slow the seed ageing rate in order to extend the conservation time of collections (Hay, 2017). *Ex situ* conservation through seed banks allows medium and long-term seed storage and is a powerful tool to preserve large amounts of plant genetic resources both for ecological and economic purposes (Hong and Ellis, 1996; Walters et al., 2004). Although *ex situ* conservation is economically more convenient than *in situ* conservation, it presents some technical challenges for correct and long-lasting storage, due to the nature of conserved seeds (Li and Pritchard, 2009). They can be classified on the basis of the storage behavior, namely on their response to dehydration: desiccation-tolerant seeds are longer-lived and are called 'orthodox', while desiccation-sensitive and shorter-lived seeds are called 'recalcitrant'. Since orthodox seeds tolerate a higher degree of water loss than recalcitrant seeds (0.2 g H_2O g_{DW}^{-1} *vs* less than 0.07), they can be stored for longer periods under seed bank conventional conditions, i.e. at the dry state and at low temperatures (0°C and -20°C). With drying and cooling, the aqueous matrix inside seeds becomes glassy and this viscosity reduces or abates metabolic processes, thus seed longevity results extended (Ballesteros and Walters, 2011). Conversely, recalcitrant seeds cannot be preserved under conventional storage and cryopreservation is the safest approach, far less expensive than tissue culture or *in situ* conservation (Black and Pritchard, 2003; Walters et al., 2013). Some seeds present an intermediate behavior, as they are desiccation-tolerant but short-lived if stored at low temperatures (Roberts, 1973; Ellis et al., 1990; Pritchard, 2004).

Seed longevity is defined as the viability, or ability to germinate, retained by seeds over a period of dry storage. Although cryptobiotic organisms (they do not carry processes usually associated with living systems), seeds are exceptional examples of long-lived eukaryotes: radiocarbon dating sets the age of some still viable seeds at about 2000 or 1300 years ago (*Phoenix dactylifera* L. and *Nelumbo nucifera* Gaertn., respectively) (Walters, 1998; Rajjou and Debeaujon, 2008). Seed longevity is strongly influenced by several external factors, such as storage temperature and relative humidity (RH), but also by intrinsic features that determine seed quality and vigor (Walters, 1998; Walters et al., 2005). These factors vary among species but also among seed lots since different genotypes within the same species can differ in longevity even if stored under the same conditions (Probert et al., 2009; Nagel et al., 2010).

The assessment of seed longevity is functional to guarantee the viability of a seed collection over time, mainly predicting when collections should be regenerated and avoiding repetitive viability assays when the seed number is low (Niedzielski et al., 2009). Viability data are usually obtained through artificial ageing (AA) or controlled deterioration (CD) tests. These tests exploit high temperatures (40-60°C) and RH (75-100%) to accelerate the natural processes of ageing and the resulting data can be used to compare the estimated storage periods of different species and seed lots (Delouche and Baskin, 1973; Powell and Matthews, 1981; Newton et al., 2009). The electrical conductivity (EC) test for seed quality is based on leakage of solutes caused by damages

to lipidic membranes, that can be measured through electrodes, and it is used to assess damages during ageing, as EC is known to be negatively correlated with seed vigor (Powell, 1986). Elevated partial pressure of oxygen (EPPO) storage is another method that mimics ageing under seed bank conservation. It has been developed to avoid the use of high temperature and RH, thereby reducing their effect on seed deterioration (Groot et al., 2012).

'Ageing in all organisms is the sum total of the deteriorative processes that eventually lead to death' (Matthews, 1985). The ageing progress can be observed through survival curves, described by the viability equation by Ellis and Roberts (1980). Samples of the stored seed population are withdrawn at established time points and tested, then germination percentages are plotted against time. Seeds usually show two types of ageing trends: some species present an initial phase of low mortality followed by a subsequent phase of decrease in viability, while other species present only one phase of viability loss with sigmoidal shape (Bernal-lugo and Leopold, 1998). Apart from the decline in germination rate, other phenotypical signs of ageing can also be *i)* production, within aged seed lots, of smaller seedlings when compared with those produced by unaged seed lots *ii)* even if viable, i.e. seeds able to produce a radical protrusion, seedlings show abnormal phenotypes (Matthews, 1985). The primary processes of seed deterioration during ageing are thought to be oxidative and peroxidative reactions. Free radicals form spontaneously and, since water tends to quench these reactions and maintain antioxidant activity, they have the major effects when seeds are at the dry state or at low moisture levels. Lipid peroxidation is one of the main processes triggered by ROS and causes the breakdown of lipids and the formation of by-products that can damage other macromolecules such as proteins and nucleic acids (Bewley et al., 2013).

Since seed viability and vigor are strongly influenced by deterioration during storage, thus ageing is an issue in terms of seed lots quality, field establishment and crop productivity and identifying effective ageing hallmarks is therefore necessary to predict seed longevity and evaluate seed lots. Beside standard methods (AA, CD, EC tests and seedling establishment evaluations), new technologies from molecular biology, biotechnology, biophysics, and seedling imaging analysis have been exploited to detect ageing hallmarks and processes (Marcos-Filho, 2015). Several markers of quantitative trait loci (QTLs), detected after AA tests, are associated with stress response and ageing. In barley (*Hordeum vulgare* L.), it has been demonstrated that longevity is associated with different traits, such as floral and seed development, the ethylene and jasmonate pathways and antifungal activity (Nagel et al., 2009). In both barley and *Arabidopsis thaliana* (L.) Heynh. it has been underlined the role of DNA repair during ageing, with crucial players as DNA ligase 4 and 6 (Waterworth et al., 2010). In maize (*Zea mays* L.) some QTLs identified after AA are linked to energy metabolism, stress response, signal transduction and protein degradation pathways (Wang et al., 2016).

One of the main processes occurring during ageing is lipid peroxidation and it can be assessed with EC test and biochemical assays that measure its by-products, such as malondialdehyde (MDA) and proline, or tocopherols, that help preventing lipid peroxidation and decrease during ageing. Oxidative reactions can also be indirectly measured through gene expression analysis: up-regulation of genes encoding antioxidant enzymes can be viewed as a stress hallmark. Other genes related to stress response are involved in ageing processes, e.g. those coding for heat shock proteins and other defense proteins that prevent misfolding and protein aggregation (Bailly, 2004; Sattler et al., 2004; Rajjou and Debeaujon, 2008; Wang et al., 2018). Seed deterioration is also marked by the reduction of α-amylase activity and total soluble sugars content because the reduction in starch metabolism observed in aged seeds can impair germination (Wang et al., 2018; Pandey et al., 2017).

2.1. Seed priming as a tool to limit ageing-associated damage

Different priming methods have been used to overcome ageing-associated damages by improving germination rate, uniformity, and percentage. Seeds of different species have been submitted to several priming techniques and it has been suggested that the effectiveness of these treatments depends on repair mechanisms activated during the hydration phase, namely nucleic acids, lipids, and protein repair, reactivation of transcription and antioxidant enzymes scavenging activity (Probert et al., 1991; Kibinza et al., 2011; Parmoon et al., 2013; Pandey et al., 2017). For instance, it has been reported that halopriming with KH_2PO_4 and K_2HPO_4 in cucumber seeds (*Cucumis sativus* L.) enhances repair by stabilizing membrane integrity, as seed leachate measured through EC tests is reduced after the treatment (Pandey et al., 2017). However, it is not clear whether the damages repaired with priming are already present in aged seeds or priming plays a preventive role by recovering antioxidant and repair enzymes before the onset of germination and subsequent damages caused by ROS release. Although hydropriming and osmopriming are the most used treatments, other compounds such as salicylic acid or metallic nanoparticles have been used in aged seeds priming treatments with positive effects both on germination and seedling phenotype (Mahakham et al., 2017; Siavash Moghaddam et al., 2018). In some cases, seeds submitted to a priming treatment show a delayed loss of viability when stored, probably because the repair mechanisms activated by imbibition allow seeds to maintain germinability for longer periods (Dearman et al., 1986; Probert et al., 1991; Butler et al., 2009). Other studies report that primed seeds present reduced longevity when submitted to CD and AA tests (Chiu et al., 2002; Hill et al., 2007; Hussain et al., 2015). This loss of viability probably depends on the effects of high temperature and moisture content in aerobic conditions more than on the priming treatment *per se*. In fact, when longevity of primed rice seeds stored under different conditions was tested, seeds stored in an anaerobic environment remained viable for a longer period than seeds submitted to the same treatment but stored in aerobic conditions

(Wang et al., 2018). The viability of primed stored seeds can be partially restored with post-storage treatments, namely re-priming and heat shock, thereby maintaining the priming treatment benefits and allowing longer storage (Rao et al., 1987; Bruggink et al., 1999).

3. The molecular 'know-how' of seed priming and its implications in promoting new advances in the sector of seed biology and technology

The dynamics of water uptake and metabolic reactivation during seed imbibition has been proposed in the form of a triphasic temporal pattern (Bewley, 1997; Bewley et al., 2013). During seed dormancy, metabolic pathways are deactivated to prevent accumulation of damage and germination in unsuitable conditions. Cytoplasm is dehydrated to a 'glassy' state unsuitable for enzymatic reactions (Buitink et al., 2000; Buitink and Leprince, 2008). The first phase, imbibition, is characterized by a rapid water uptake physically-driven by seed coat permeability and tissue capillarity (Beweley et al., 1997; Kranner et al., 2010). Subsequently, water absorption slows down and water content remains constant, marking the starting point of the second phase. With rehydration, the optimal conditions for enzymatic reactions to occur are set, thus allowing processes essential for seed viability and germination, including DNA and membrane repair, protein synthesis and mitochondrial respiration. To fulfill these functions, new mRNAs must be transcribed and translated, although the dry seeds are endowed with residual maternal mRNAs (Beweley et al., 1997; Rajjou et al., 2006; Weitbrecht et al., 2011). More than 10.000 mRNAs able to resist desiccation during seed maturation have been identified in *Arabidopsis*, rice and other species. The most represented functions within the dry seed transcriptome include storage proteins and Late Embryogenesis Abundant proteins (LEA) (Weitbrecht et al., 2011). A small percentage of the dry seed transcriptome is involved in protein synthesis, protein degradation and hormonal responses (Holdsworth et al., 2008a, 2008b). The third stage culminates in the radicle emergence and features a new increase in water uptake and reserve consumption as cell division and elongation start (Bewley et al., 2013).

3.1. The redox context of pre-germinative metabolism and the harmful oxidative damage

Production and accumulation of ROS (Reactive Oxygen Species) and NRS (Nitrogen Reactive Species) in seeds has been documented in all developmental stages. These chemically reactive molecules have double-faced effects over seed viability and germination, causing oxidative damages to biological macromolecules but playing fundamental roles in several physiological processes (Chen et al., 2012). The recent scientific literature annotated the specific roles of the most abundant ROS and NRS, including hydroxyl radicals ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\cdot-}$), nitric oxide (NO) and other reactive molecules involved in seed and seedling metabolism

(Bailly et al., 2002, 2008; Bailly, 2004; Morohashi et al., 2002, Sarath et al., 2007). Additional ROS sources are found in seeds, e.g. oxidative reactions occurring in peroxisomes and glyoxysomes during the mobilization of seed reservoirs. ROS production, particularly at the mitochondrial level, are prominent in seed-specific developmental stages. Besides the physiological routes of ROS production, the occurrence of stress conditions can impair the ROS homeostasis and elicit different ROS production and distribution patterns (stress-associated 'ROS signatures') (Choudhury et al., 2016; Farooq et al., 2018; Rosbakh et al., 2018).

The occurrence of ROS-induced oxidative damage to different classes of biological macromolecules and cellular compartments has been reported (Osborne et al., 1984; Osborne, 1994; Bailly, 2004; Rajjou and Debeaujon, 2008; Rajjou et al., 2008) along with its negative effects on seed longevity and viability (Groot et al., 2012). In seeds, lipid oxidation can affect both membrane lipids and reservoir fatty acids in different contexts. In particular, lipid peroxidation taking place in peroxisomes and glyoxysomes is a major ROS source in metabolically active oily seeds that are degrading their lipidic reservoirs before seedling establishment (Del Rio et al., 2002; Corpas et al., 2001). Furthermore, self-oxidation processes such as Amadori and Maillard reactions can lead to oxidative damage of lipids in dormant or stored seeds, affecting seed long-term viability and shelf-life (Sun and Leopold, 1995; Murthy and Sun, 2000; Buitink and Leprince, 2008). Antioxidant molecules, e.g. tocopherol (Vitamin E) and tocotrienols, are involved in protection from lipid peroxidation (Horvath et al., 2006).

The functional groups of amino acids are particularly exposed to the oxidation caused by hydroxyl radical and hydrogen peroxide, and accumulation of functionally impaired proteins ultimately leads to irreversible cell damage (Charles and Halliwell, 1980). Beside the oxidation of functional groups, the specific redox state of each cellular compartment determines the stability of disulfide bonds necessary to maintain protein tertiary structure. Important changes in redox state and overall protein oxidation have been observed in both embryo and endosperm of imbibed seeds (Buchanan and Balmer, 2005). In this context, the preferential oxidation of specific classes of reserve proteins, like globulins and cruciferins, has been interpreted as ROS-scavenging activity to prevent or limit oxidative damage to other classes of proteins (Arc et al., 2011; Job et al., 2005; Rajjou et al., 2012). Specialized enzymes are involved in maintaining protein structure and integrity, in particular during hydration-dehydration cycles. Among them, thioredoxins, methionine sulfoxide reductases, and protein L-isoaspartyl methyltransferases are correlated with seed longevity in many species. Beside their main role, certain classes of seed storage proteins, in particular, 12S globulins, are particularly prone to oxidation, suggesting a possible role as ROS scavengers, as demonstrated in *Arabidopsis* (Rajjou et al., 2007; El-Maarouf-Bouteau et al., 2013; Nguyen et al., 2015). The presence of antioxidant enzymes has been documented both in dry mature seeds and in germinating seeds and constitutes an essential trait participating to seed vigor. Among

them, ascorbate peroxidase, catalase, dehydroascorbate reductase, glutathione peroxidase, glutathione reductase, monodehydroascorbate reductase, superoxide dismutase, contribute to the seed detoxification potential (Oracz et al., 2007). Beside them, reduced glutathione plays a pivotal role in maintaining the overall cytoplasmic redox state and preventing protein oxidation (Kranner et al., 2006, Nagel et al., 2015).

ROS accumulation is particularly deleterious for DNA integrity and genome stability. Oxidative damage of purines, pyrimidines and deoxyribose units is a primary cause of DNA strand breaks (Bray and West, 2005). One of the most common base modifications is guanine hydroxylation to 7,8-dihydro-8-oxoguanine (8-oxoG), whose mutagenic effects have been documented (Biedermann et al., 2011). The established correlation between the accumulation of strand breaks, genome instability, and seed vigor impairment makes the role of ROS a long-lasting and prolific field of study within seed biology and biotechnology. Also RNA can undergo oxidative damage and translational impairment due to these processes has been correlated to reduced seed longevity (Rajjou et al., 2008; Bazin et al., 2011).

3.2. Active DNA repair during the pre-germinative metabolism: a molecular 'know-how' for seed priming

Maintaining genome integrity during dry state is crucial for long-term seed survival. A significant reduction in the nucleus size and a notable chromatin condensation have been observed in dehydrated *Arabidopsis* seeds, suggesting for an adaptive response to dehydration also at the DNA level (van Zanten et al., 2011; Waterworth et al., 2015). Activation of DNA repair pathways, such as BER and NER, has been reported cross-specifically (Waterworth et al., 2010; Balestrazzi et al., 2011; Macovei et al., 2010). Base Excision Repair is a multistep pathway that is able to recognize and repair many kinds of nucleotide modifications, including oxidative damages, alkylation, deamination, apurinic/apyrimidinic (AP) sites and single-strand breaks. The first step of the process involves specific glycosylases that recognize the damaged deoxynucleoside and hydrolyze its N-glycosidic bond, thus removing the modified base. For example, 8-oxoguanine DNA glycosylase (OGG1), formamidopyrimidine DNA glycosylase (FPG) and uracil DNA glycosylase (UDG) are three enzymes specifically involved in this concern (Córdoba-Cañero et al., 2010). The resulting apurinic/apyrimidinic site is then processed by two alternative sub-pathways. The short-patch repair pathway is performed by the DNA polymerase β that inserts a single nucleotide whose ends are joined to the rest of the DNA strand by the DNA ligase III enzyme, whereas the long-patch repair pathway is carried out by the replicative DNA polymerases δ or ε that insert several nucleotides with the final intervention of the DNA ligase I that eventually repairs the DNA filament. The long-patch repair sub-pathway requires also the displacement and the removal of adjacent nucleotides while the synthesis occurs, this function is performed by the flap structure-specific endonuclease 1 (FEN-1) in association with the proliferating cell nuclear antigen

(PCNA) (Wu et al., 1996; Tsai et al., 2017). The involvement of BER components in stress response was confirmed in many plant tissues. Indeed, the expression levels of the genes encoding OGG1 and FPG were increased in response to copper- and polyethylene glycol (PEG)- induced stress in *Medicago truncatula* roots and aerial parts, while it was delayed in rehydrating seeds in presence of PEG, whose osmotic effects slow down water uptake and the subsequent metabolism reactivation (Macovei et al., 2011).

DNA ligase VI is essential to maintain seed longevity in *Arabidopsis thaliana* both under physiological and stress conditions (Waterworth et al., 2010). Other enzymes cover specific functions in the context of BER, e.g. tyrosyl-DNA phosphodiesterases (TDPs) (Pommier et al., 2014), that prevent the lesions caused by the stabilized covalent complexes DNA/topoisomerase thus preventing genotoxic effects occurring during transcription and replication, as well as under oxidative stress (El-Khamisy et al., 2005). The expression of the different *TDP* genes identified in plants (*TDP1α*, *TDP1β* and *TDP2*) is responsive to different stress conditions in different experimental systems, including cell suspensions, roots, aerial parts and seeds (Araújo et al., 2016b; Balestrazzi et al., 2011; Confalonieri et al., 2013; Donà et al., 2013a; Faè et al., 2014; Macovei et al., 2018) suggesting their recurrent involvement in plant responses to genotoxic stress.

Nucleotide Excision Repair, linked to the seed stress response (Macovei et al., 2010b, 2014), processes major DNA damages that imply covalent adducts or UV-photoproducts and it is specifically elicited during transcription (NER-TCR, NER-Transcription Coupled Repair) and in non-transcribed regions (NER-GGR, NER-Global Genome Repair) (Kunz et al., 2005; Conconi et al., 2002). Several factors are required to recruit and activate the NER machinery, among which the transcription elongation factor II-S (TFIIS) (Kuraoka et al., 2007) involved in the abiotic stress response in *M. truncatula* (Macovei et al., 2010b) and dormancy regulation in *Arabidopsis* seeds (Grasser et al., 2009). Because of their crucial effects on genome stability, DNA damage dynamics and DNA repair pathways represent a promising field of applied research to assess and improve seed quality, concerning seed priming, long-term storability, and harvest yields. This intricated network of molecular events has been envisaged as a source of novel hallmarks of seed vigor (**Fig. 1**) (Balestrazzi et al., 2011; Paparella et al., 2015; Pagano et al., 2017, 2018).

4. Technology advancement

4.1. Multilevel approaches to understand seed biology and assess seed quality

The possibility to associate genotyped markers to specific phenotypes is at the base of Genome-Wide Association Studies (GWAS). In the case of seed quality, this approach has lead to the identification of Quantitative Trait Loci (QTL) related to seed quality (Wang et al., 2018). Extensive transcriptomic studies have been carried out in many model systems focusing on seed metabolism and have led to the identification of the most

represented families of transcripts during the various stages of seed metabolism. In *Arabidopsis* seeds, genes related to protein turnover and cell wall plasticity are the most expressed during the early imbibition stage (Nakabayashi et al., 2005), while on overall increased expression of auxin-related genes was required for cell division and elongation in the subsequent phases (Holdsworth et al., 2008b), although substantial differences have been found between embryo and endosperm in pathways related to reserve mobilization and energy utilization (Penfield et al., 2006). The role of post-transcriptional regulation and silencing mediated by miRNA has been highlighted in seed germination and seedling development in many model species, including *Glycine max* and *Brassica napus* (Yu et al., 2018; Wei et al., 2018).

Through proteomic approaches, a global understanding of seed metabolism associated to an improved seed vigor has been achieved for model plants such as *Arabidopsis* (Gallardo et al., 2001; Rajjou et al., 2006) and crop plants as *Medicago sativa* (Yacoubi et al., 2013) and *Triticum aestivum* (Fercha et al., 2013, 2014). Furthermore, the combination of transcriptomic and proteomic data obtained from primed *Brassica napus* and *Hordeum vulgare* seeds had highlighted the overall upregulation of genes involved in water uptake (e.g. genes encoding aquaporins), cell cycle and division, cytoskeleton and response to oxidative stress in response to priming (Kubala et al., 2015; Mostek et al., 2016). Metabolomic profiling of germinating seeds has been successfully used to investigate the metabolic differences associated to different cultivars of *T. aestivum* (Das et al., 2017), *Zea mays* (Feenstra et al., 2017), *H. vulgare* (Gorzolka et al., 2016), highlighting the differential accumulation and distribution of specific classes of amino acids, lipids and carbohydrates. Metabolomic approaches have been also used to unveil the metabolic footprint of stress conditions in *A. thaliana* (Cohen et al., 2017) and *M. truncatula* (Pagano et al., 2018) seeds, identifying various putative hallmarks of seed germination and stress response. Metabolomic data can be deepened and integrated through specific analyses focused on particular classes of molecules, such as lipids or ions. Advancements in seed lipidomics are particularly promising, given the importance of lipids as structural components of membranes, carbon and energy reservoirs and signaling molecules, especially in oily seeds (Horn et al., 2014). Other high-throughput techniques are aimed to obtain a dynamic picture of the metabolic fluxes at the cell, organ and plant level. Through specific labeling and imaging techniques in more or less extended time lapses, 'Fluxomics' applied to seeds and embryos allows to trace the accumulation, compartmentalization and mobilization of starch, lipids and carbon, improving the current knowledge of seed metabolism and opening promising possibilities for the enhancement of seed nutritional properties (Salon et al., 2017).

5. Conclusion and future perspective

Despite the huge amount of data that can be obtained through 'omics' techniques and the possibilities of data integration offered by system biology, the notable variability of

biological system in their stress response strategies leads to unavoidable difficulties in finding common principles and conserved patterns able to explain complex phenomena. With these premises, the individuation of specific gene expression patterns, DNA or RNA modifications or metabolite accumulation that are recurrently associated with seed quality and vigor could have an interesting output for plant biotechnology and seed industry. The identification of such 'molecular markers of seed quality' will allow the biotechnologists to complement the empirical approaches currently used for seed priming and optimize invigoration protocols of agronomical relevance. Moreover, the availability of experimental systems for the study of seed deterioration will help understanding the impact of priming in preserving longevity whereas the so-called "priming memory" deserves as well more attention. The advanced avenues of genome editing are now open to explore the multifaceted aspects of seed vigour. The CRISPR (clustered, regularly interspaced, short palindromic repeat)-Cas9 technology appears as a promising tool to improve this complex trait.

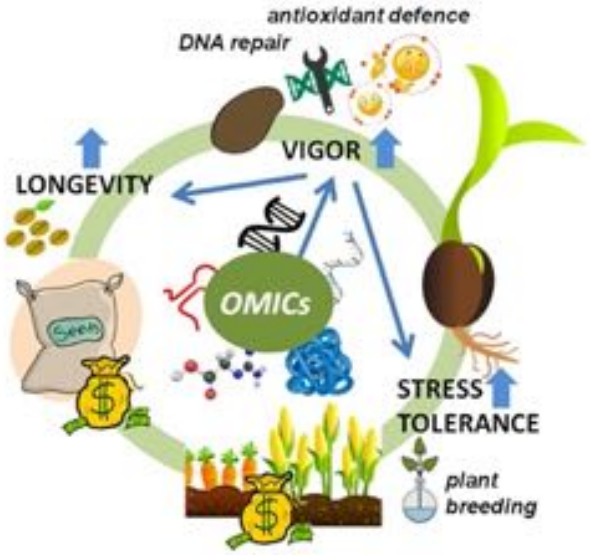


Fig. 1. Seed vigor and the underlying molecular mechanisms, particularly DNA repair and antioxidant defence, play a crucial role in maintaining high-standard seeds for both market and conservation purposes. The beneficial effects of seed priming range from

enhanced/synchronized germination to increased robustness or stress tolerance of seedlings, essential features for breeding. High vigor also supports seed longevity. In this context, novel molecular hallmarks useful to predict seed quality will be provided by high-throughput 'omics' technologies.

References

Araújo S, Paparella S, Dondi D, Bentivoglio A, Carbonera D, Balestrazzi A (2016a). Physical methods for seed invigoration: advantages and challenges in seed technology. *Front. Plant Sci.* 7, 646.

Araújo S, Balestrazzi A, Faè M, Morano M, Carbonera D, Macovei A (2016b) *MtTdp2α*-overexpression boosts the growth phase of *Medicago truncatula* cell suspension and increases the expression of key genes involved in the antioxidant response and genome stability. *Plant Cell Tissue Organ Cult.* 127, 675-680.

Arc E, Galland M, Cueff G, Godin B, Lounifi I, Job D, Rajjou L (2011) Reboot the system thanks to protein post-translational modifications and proteome diversity: how quiescent seeds restart their metabolism to prepare seedling establishment. *Proteomics* 11, 1606-1618.

Bailly C (2004) Active oxygen species and antioxidants in seed biology. *Seed Sci. Res.* 14, 93-107.

Bailly C, Benamar A, Corbineau F, Come D (1998) Free radical scavenging as affected by accelerated ageing and subsequent priming in sunflower seeds. *Physiol. Plant.* 104, 646-652.

Bailly C, Bogatek-Leszczynska R, Côme D, Corbineau F (2002) Changes in activities of antioxidant enzymes and lipoxygenase during growth of sunflower seedlings from seeds of different vigour. *Seed Sci. Res.* 12, 47-55.

Bailly C, El-Maarouf-Bouteau H, Corbineau F (2008) From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. *C R Biol.* 331, 806-814.

Balestrazzi A, Confalonieri M, Macovei A, Donà M, Carbonera D (2011a) Genotoxic stress and DNA repair in plants: emerging functions and tools for improving crop productivity. *Plant Cell Rep.* 30, 287-295.

Balestrazzi A, Confalonieri M, Macovei A, Carbonera D (2011b) Seed imbibition in *Medicago truncatula* Gaertn.: expression profiles of DNA repair genes in relation to PEG-mediated stress. *J. Plant Physiol.* 168, 706-713.

Ballesteros D, Walters C (2011) Detailed characterization of mechanical properties and molecular mobility within dry seed glasses: relevance to the physiology of dry biological systems. *Plant J.* 68, 607-619.

Bazin J, Langlade N, Vincourt P, Arribat S, Balzergue S, El-Maarouf-Bouteau H, Bailly C (2011) Targeted mRNA oxidation regulates sunflower seed dormancy alleviation during dry after-ripening. *Plant Cell* 23, 2196-2208.

Bernal-Iugo I, Leopold AC (1998) The dynamics of seed mortality. *J. Exp. Bot.* 49, 1455-1461.

Bewley JD (1997) Seed germination and dormancy. *Plant Cell* 9, 1055-1066.

Bewley JD, Bradford KJ, Hilhorst HWM, Nonogaki H (2013) Seeds: Physiology of Development, Germination and Dormancy, 3rd Edition. New York, NY: Springer Science+Business Media, LLC 2013.

Biedermann S, Mooney S and Hellmann H (2011) Recognition and repair pathways of damaged DNA in higher plants. In: Selected Topics in DNA Repair. Chen C (ed.) pp. 201-236. InTechOpen, London.

Black M, Pritchard HW (2003) Desiccation and survival in plants. Drying without dying. *Ann. Bot.* 91, 105.

Bradford KJ (1995) Water relations in seed germination. In: Seed development and germination. Kigel J and Galili G (eds.) Marcel Dekker, Inc., New York, pp 351-396.

Bray CM, West CE (2005) DNA repair mechanisms in plants: crucial sensors and effectors for the maintenance of genome integrity. *New Phytol.* 168, 511-528.

Bruggink GT, Ooms JJJ, Van Der Toorn P (1999) Induction of longevity in primed seeds. *Seed Sci. Res.* 9, 49-53.

Buchanan BB, Balmer Y (2005) Redox regulation: a broadening horizon. *Annu. Rev. Plant Biol.* 56, 187-220.

Buitink J, Leprince O, Hoekstra FA (2000) Dehydration-induced redistribution of amphiphilic molecules between cytoplasm and lipids is associated with desiccation tolerance in seeds. *Plant Physiol.* 124, 1413-1426.

Buitink J, Leprince O (2008) Intracellular glasses and seed survival in the dry state. *C R Biol.* 331, 788-795.

Butler LH, Hay FR, Ellis RH, Smith RD, Murray TB (2009) Priming and re-drying improve the survival of mature seeds of *Digitalis purpurea* during storage. *Ann. Bot.* 103, 1261-1270.

Charles SA, Halliwell B (1980) Effect of hydrogen peroxide on spinach (*Spinacia oleracea*) chloroplast fructose bisphosphatase. *Biochem. J.* 189, 373-376.

Chiu KY, Chen CL, Sung JM (2002) Effect of priming temperature on storability of primed sh-2 sweet corn seed. *Crop Sci.* 42, 1996-2003.

Choudhury FK, Rivero RM, Blumwald E, Mittler R (2017) Reactive oxygen species, abiotic stress and stress combination. *Plant J.* 90, 856-867.

Cohen H, Amir R (2017) Dose-dependent effects of higher methionine levels on the transcriptome and metabolome of transgenic *Arabidopsis* seeds. *Plant Cell Rep.* 36, 719-730.

Conconi A, Bespalov VA, Smerdon MJ (2002) Transcription-coupled repair in RNA polymerase I-transcribed genes of yeast. *Proc. Natl. Acad. Sci. U. S. A.* 99, 649-654.

Confalonieri M, Faè M, Balestrazzi A, Donà M, Macovei A, Valassi A, Giraffa G, Carbonera D (2013) Enhanced osmotic stress tolerance in *Medicago truncatula* plants overexpressing the DNA repair gene *MtTdp2α* (tyrosyl-DNA phosphodiesterase 2). *Plant Cell Tissue Organ Cult.* 116, 187-203.

Córdoba-Cañero D, Morales-Ruiz T, Roldán-Arjona T, Ariza RR (2009) Single-nucleotide and long-patch base excision repair of DNA damage in plants. *Plant J.* 60, 716-728.

Corpas FJ, Barroso JB, del Rio LA (2001) Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. *Trends Plant Sci.* 6, 145-150.

Das A, Kim DW, Khadka P, Rakwal R, Rohila JS (2017) Unraveling key metabolomic alterations in wheat embryos derived from freshly harvested and water-imbibed seeds of two wheat cultivars with contrasting dormancy status. *Front. Plant Sci.* 8, 1203.

Dearman J, Brocklehurst PA, Drew RLK (1986) Effects of osmotic priming and ageing on onion seed germination. *Ann. Appl. Biol.* 108, 639-648.

Del Rio LA, Corpas FJ, Sandalio LM, Palma JM, Gómez M, Barroso JB (2002) Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. *J. Exp. Bot.* 53, 1255-1272.

Delouche JC, Baskin CC (1973) Accelerated aging techniques for predicting the relative storability of seed lots. *Seed Sci. Tech.* 1, 427-452.

Di Girolamo G, Barbanti L (2012) Treatment conditions and biochemical processes influencing seed priming effectiveness. *Italian J. Agr.* 7, e25.

Diaz-Vivancos P, Barba-Espin G, Hernández JA (2013) Elucidating hormonal/ROS networks during seed germination: insights and perspectives. *Plant Cell Rep.* 32, 1491-1502.

Donà M, Confalonieri M, Minio A, Biggiogera M, Buttafava A, Raimondi E, Delledonne M, Ventura L, Sabatini ME, Giraffa G, Carbonera D, Balestrazzi A (2013) RNA-Seq analysis discloses early senescence and nucleolar dysfunction triggered by *Tdp1α* depletion in *Medicago truncatula*. *J. Exp. Bot.* 64, 1941–1951.

El-Khamisy SF, Saifi GM, Weinfeld M, Johansson F, Helleday T, Lupski JR, Caldecott KW. Defective DNA single-strand break repair in spinocerebellar ataxia with axonal neuropathy-1. Nature. 2005 Mar 3;434(7029):108-13.

El-Maarouf-Bouteau H, Meimoun P, Job C, Job D, Bailly C (2013) Role of protein and mRNA oxidation in seed dormancy and germination. *Front. Plant Sci.* 4, 77.

Ellis RH, Roberts EH (1980) Improved equations for the prediction of seed longevity. *Ann. Bot.* 45, 13-30.

Ellis RH, Hong TD, Roberts EH (1990) An intermediate category of seed storage. *J. Exp. Bot.* 41, 1167-1174.

Everari M (1984) Seed physiology: its history from antiquity to the beginning of the 20th century. *Bot. Rev.* 50, 119-142.

Faè M, Balestrazzi A, Confalonieri M, Donà M, Macovei A, Valassi A, Giraffa G, Carbonera D (2014) Copper-mediated genotoxic stress is attenuated by the overexpression of the DNA repair gene *MtTdp2α* (tyrosyl-DNA phosphodiesterase 2α) in *Medicago truncatula* plants. *Plant Cell Rep.* 33, 1071-1080.

Farooq M, Hussain M, Usman M, Farooq S, Alghamdi SS, Siddique KHM (2018) Impact of abiotic stresses on grain composition and quality in food legumes. *J. Agr. Food Chem.* 66, 8887-8897.

Feenstra AD, Alexander LE, Song Z, Korte AR, Yandeau-Nelson MD, Nikolau BJ, Lee YJ (2017) Spatial mapping and profiling of metabolite distributions during germination. *Plant Physiol.* 174, 2532-2548.

Fercha A, Capriotti AL, Caruso G, Cavaliere C, Gherroucha H, Samperi R, Stampachiacchiere S, Lagana A (2013) Gel-free proteomics reveal potential biomarkers of priming-induced salt tolerance in durum wheat. *J. Proteomics* 91, 486-499.

Fercha A, Capriotti AL, Caruso G, Cavaliere C, Samperi R (2014) Comparative analysis of metabolic proteome variation in ascorbate-primed and unprimed wheat seeds during germination under salt stress. *J. Proteomics* 108, 238-257.

Finch-Savage WE, Bassel GW (2016) Seed vigour and crop establishment: extending performance beyond adaptation. *J. Exp. Bot.* 67, 567-591.

Gallardo K, Job C, Groot SP, Puype M, Demol H, Vandekerckhove J, Job D (2001) Proteomic analysis of *Arabidopsis* seed germination and priming. *Plant Physiol.* 126, 835-848.

Gholami M, Mokhtarian F, Baninasab B (2015) Seed halopriming improves the germination performance of black seed (*Nigella sativa*) under salinity stress conditions. *J. Crop Sci. Biotech.* 18, 21-26.

Gorzolka K, Kölling J, Nattkemper TW, Niehaus K (2016) Spatio-temporal metabolite profiling of the barley germination process by MALDI MS imaging. *PLoS One* 11, e0150208.

Grasser M, Kane CM, Merkle T, Melzer M, Emmersen J, Grasser KD (2009) Transcript elongation factor TFIIS is involved in *Arabidopsis* seed dormancy. *J. Mol. Biol.* 386, 598-611.

Groot SP, Surki AA, de Vos RC, Kodde J (2012) Seed storage at elevated partial pressure of oxygen, a fast method for analysing seed ageing under dry conditions. *Ann. Bot.* 110, 1149-1159

Hamayun M, Khan SA, Khan AL, Tang DS, Hussain J, Ahmad B, Anwar Y, Lee IJ (2010) Growth promotion of cucumber by pure cultures of gibberellin-producing Phoma sp GAH7. *World J Microbiol. Biotechnol.* 26, 889-894.

Hay FR (2017) Seed banks. In: Encyclopedia of Applied Plant Sciences 2nd Edition. Thomas B, Murray BG and Murphy DJ (eds.) (Elsevier B.V. 2017), pp. 327-333.

Heydecker W, Coolbear P (1977) Seed treatments for improved performance-survey and attempted prognosis. *Seed Sci. Tech.* 5, 353-425.

Hill HJ, Cunningham JD, Bradford KJ (2007) Primed lettuce seeds exhibit increased sensitivity to moisture content during controlled deterioration. *Hort. Sci.* 42, 1436-1439.

Holdsworth MJ, Bentsink L, Soppe WJ (2008a) Molecular networks regulating *Arabidopsis* seed maturation, after-ripening, dormancy and germination. *New Phytol.* 179, 33-54.

Holdsworth MJ, Finch-Savage WE, Grappin P, Job D (2008b) Post-genomics dissection of seed dormancy and germination. *Trends Plant Sci.* 13, 7-13.

Hong TD, Ellis RH (1996) A protocol to determine seed storage behaviour. *IPGRI Tech. Bull.* 1, 1-62.

Horn PJ, Chapman KD (2014) Lipidomics in situ: insights into plant lipid metabolism from high resolution spatial maps of metabolites. *Prog. Lipid Res.* 54, 32-52.

Horvath G, Wessjohann L, Bigirimana J, Monica H, Jansen M, Guisez Y, Caubergs R, Horemans N (2006) Accumulation of tocopherols and tocotrienols during seed development of grape (*Vitis vinifera* L. cv. Albert Lavallée). *Plant Physiol. Biochem.* 44, 724-731.

Hussain S, Zheng M, Khan F, Khaliq A, Fahad S, Peng S, Huang J, Cui K, Nie L (2015) Benefits of rice seed priming are offset permanently by prolonged storage and the storage conditions. *Sci. Rep.* 5, 8101.

Huang Z, Boubriak I, Osborne DJ, Dong M, Gutterman Y (2008) Possible role of pectin-containing mucilage and dew in repairing embryo DNA of seeds adapted to desert conditions. *Ann. Bot.* 101, 277-283.

Kibinza S, Vinel D, Côme D, Bailly C, Corbineau F (2006) Sunflower seed deterioration as related to moisture content during ageing, energy metabolism and active oxygen species scavenging. *Physiol. Plant.* 128, 496-506.

Kranner I, Birtić S, Anderson KM, Pritchard HW (2006) Glutathione half-cell reduction potential: a universal stress marker and modulator of programmed cell death? *Free Radic Biol. Med.* 40, 2155-2165.

Kranner I, Minibayeva FV, Beckett RP, Seal CE (2010) What is stress? Concepts, definitions and applications in seed science. *New Phytol.* 188, 655-673.

Kubala S, Wojtyla L, Quinet M, Lechowska K, Lutts S, Garnczarska M (2015) Enhanced expression of the proline synthesis gene *P5CSA* in relation to seed osmopriming improvement of *Brassica napus* germination under salinity stress. *J Plant Physiol.* 183, 1-12.

Kunz BA, Anderson HJ, Osmond MJ, Vonarx EJ (2005) Components of nucleotide excision repair and DNA damage tolerance in *Arabidopsis thaliana*. *Environ. Mol. Mut.* 45, 115-127.

Kuraoka I, Suzuki K, Ito S, Hayashida M, Kwei JS, Ikegami T, Handa H, Nakabeppu Y, Tanaka K (2007) RNA polymerase II bypasses 8-oxoguanine in the presence of transcription elongation factor TFIIS. *DNA Repair (Amst)* 6, 841-851.

Job C, Rajjou L, Lovigny Y, Belghazi M, Job D (2005) Patterns of protein oxidation in Arabidopsis seeds and during germination. *Plant Physiol.* 138, 790-802.

ISTA, International Seed Testing Association. International rules for seed testing 2019.https://www.seedtest.org/.

Li D-Z, Pritchard H (2009) The science and economics of *ex situ* plant conservation. *Trends Plant Sci.* 14, 614-621.

Macovei A, Balestrazzi A, Confalonieri M, Carbonera D (2010) The *Tdp1* (Tyrosyl-DNA phosphodiesterase) gene family in barrel medic (*Medicago truncatula* Gaertn.): bioinformatic investigation and expression profiles in response to copper- and PEG-mediated stress. *Planta* 232, 393-407.

Macovei A, Balestrazzi A, Confalonieri M, Faè M, Carbonera D (2011a) New insights on the barrel medic *MtOGG1* and *MtFPG* functions in relation to oxidative stress response in planta and during seed imbibition. *Plant Physiol. Biochem.* 49, 1040-1050.

Macovei A, Balestrazzi A, Confalonieri M, Buttafava A, Carbonera D (2011b) The *TFIIS* and *TFIIS*-like genes from *Medicago truncatula* are involved in oxidative stress response. *Gene* 470, 20-30.

Macovei A, Garg B, Raikwar S, Balestrazzi A, Carbonera D, Buttafava A, Bremont JFJ, Gill SS, Tuteja N (2014) Synergistic exposure of rice seeds to different doses of gamma-ray and salinity stress resulted in increased antioxidant enzyme activities and gene-specific modulation of TC-NER pathway. *Biomed Res. Int.* 2014, 1-15.

Macovei A, Pagano A, Leonetti P, Carbonera D, Balestrazzi A, Araújo SS (2017) Systems biology and genome-wide approaches to unveil the molecular players involved in the pre-germinative metabolism: implications on seed technology traits. *Plant Cell Rep.* 36, 669-688.

Macovei A, Faè M, Biggiogera M, de Sousa Araújo S, Carbonera D, Balestrazzi A (2018) Ultrastructural and Molecular Analyses Reveal Enhanced Nucleolar Activity in *Medicago truncatula* Cells Overexpressing the *MtTdp2α* Gene. *Front. Plant Sci.* 9, 596.

Mahakham W, Sarmah AK, Maensiri S, Theerakulpisut P (2017) Nanopriming technology for enhancing germination and starch metabolism of aged seeds using phytosynthesized silver nanoparticles. *Sci. Rep.* 7, 8263.

Marcos-Filho J (2015) Seed vigor testing: an overview of the past, present and future perspective. *Sci. Agric.* 72, 363-374.

Matthews S (1985) Physiology of seed ageing. *Outlook Agric.* 14, 89-94.

Michel BE, Kauffmann MR (1973) The osmotic potential of polyethylene glycol 6000. *Plant Physiol.* 51, 914-916.

Momin KC (2013) Challenges of the flower seed industry. *Indian J. Appl. Res.* 3, 244-245.

Morohashi Y (2002) Peroxidase activity develops in the micropylar endosperm of tomato seeds prior to radicle protrusion. *J. Exp. Bot.* 53, 1643-1650.

Murthy UM, Sun WQ (2000) Protein modification by Amadori and Maillard reactions during seed storage: roles of sugar hydrolysis and lipid peroxidation. *J. Exp. Bot.* 51, 1221-1228.

Nagel M, Vogel H, Landjeva S, Buck-Sorlin G, Lohwasser U, Scholz U, Borner A (2009) Seed conservation in *ex situ* genebanks - genetic studies on longevity in barley. *Euphytica* 170, 5-14.

Nagel M, Arif MAR, Rosenhauer M, Börner A (2010) Longevity of seeds - intraspecific differences in the Gatersleben genebank collections. *Tagungsband* 179-181

Nagel M, Kranner I, Neumann K, Rolletschek H, Seal CE, Colville L, Fernández-Marín B, Börner A (2015) Genome-wide association mapping and biochemical markers reveal that seed ageing and longevity are intricately affected by genetic background and developmental and environmental conditions in barley. *Plant Cell Environ.* 38, 1011-1022.

Nakabayashi K, Okamoto M, Koshiba T, Kamiya Y, Nambara E (2005) Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: epigenetic and genetic regulation of transcription in seed. *Plant J.* 41, 697-709.

Newton R, Hay F, Probert R (2009) Protocol for comparative seed longevity testing. *Technical Information Sheet_01*, London, Royal Botanic Gardens Kew.

Niedzielski M, Walters C, Luczak W, Hill LM, Wheeler LJ, Puchalski J (2009) Assessment of variation in seed longevity within rye, wheat and the intergeneric hybrid triticale. *Seed Sci. Res.* 19, 213-224.

Niranjan Raj S, Shetty NP, Shetty HS (2004) Seed bio-priming with *Pseudomonas* fluorescens isolates enhances growth of pearl millet plants and induces resistance against downy mildew. *Int. J. Pest Manag.* 50, 41-48.

Nguyen TP, Cueff G, Hegedus DD, Rajjou L, Bentsink L (2015) A role for seed storage proteins in *Arabidopsis* seed longevity. *J. Exp. Bot.* 66, 6399-6413.

Oracz K, El-Maarouf Bouteau H, Farrant JM, Cooper K, Belghazi M, Job C, Job D, Corbineau F, Bailly C (2007) ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation. *Plant J.* 50, 452-465.

Osborne DJ, Dell'Aquila A, Elder RH (1984) DNA repair in plant cells. An essential event of early embryo germination in seeds. *Folia Biol. (Praha).* 1984;30 Spec. No, 155-169.

Osborne DJ (1994) DNA and desiccation tolerance. *Seed Sci. Res.* 4, 175-185.

Osburn RM, Schroth MN (1989) Effect of osmopriming sugar beet deed on exudation and subsequent damping-off caused by *Pythium ultimum*. *Phytopathology* 78, 1246-1250.

Pagano A, Susana Araújo S, Macovei A, Leonetti P, Balestrazzi A (2017) The seed repair response during germination: disclosing correlations between DNA repair, antioxidant response, and chromatin remodeling in *Medicago truncatula*. *Front. Plant Sci.* 8, 1972.

Pagano A, De Sousa Araújo S, Macovei A, Dondi D, Lazzaroni S, Balestrazzi A (2018) Metabolic and gene expression hallmarks of seed germination uncovered by sodium butyrate in *Medicago truncatula*. *Plant Cell Environ.*. doi: 10.1111/pce.13342.

Pandey, P., Bhanuprakash, K., Umesh (2017) Effect of seed priming on biochemical changes in fresh and aged seeds of cucumber. *I.J.E.A.B.* 2, 2261-2264.

Paparella S, Araújo SS, Rossi G, Wijayasinghe M, Carbonera D, Balestrazzi A (2015) Seed priming: state of the art and new perspectives. *Plant Cell Rep.* 34, 1281-1293.

Parera CA, Cantliffe DJ (1994) Pre-sowing seed priming. *Hort. Rev.* 16, 109-141.

Parmoon G, Ebadi A, Jahanbakhsh S, Davari M (2013) The effect of seed priming and accelerated aging on germination and physiochemical changes in milk thistle (*Silybum marianum*). *Not. Sci. Biol.* 5, 1-8.

Penfield S, Li Y, Gilday AD, Graham S, Graham IA (2006) *Arabidopsis* ABA INSENSITIVE4 regulates lipid mobilization in the embryo and reveals repression of seed germination by the endosperm. *Plant Cell* 18, 1887-1899.

Pommier Y, Huang S-Y N, Gao R, Das BB, Murai J, Marchand C (2014) Tyrosyl-DNA-phosphodiesterases (TDP1 and TDP2). *DNA Repair* 19, 114-129.

Powell AA, Matthews S (1981) Evaluation of controlled deterioration, a new vigour test for small seeded vegetables. *Seed Sci. Tech.* 9, 633-640.

Powell AA (1986) Cell membranes and seed leachate coductivity in relation to the quality of seed for sowing. *J. Seed Tech.* 10, 81-100.

Pritchard HW (2004) Classification of seed storage 'types' for *ex situ* conservation in relation to temperature and moisture. In: *Ex situ* Plant Conservation: Supporting Species Survival in the Wild (Guerrant EO, Havens K and Maunder M eds) (Island Press, Washington, D.C., USA), pp. 139-161.

Probert RJ, Bogh SV, Smith AJ, Wechsberg GE (1991) The effects of priming on seed longevity in *Ranunculus sceleratus* L. *Seed Sci. Res.* 1, 243-249.

Probert RJ, Daws MI, Hay FR (2009) Ecological correlates of *ex situ* seed longevity: a comparative study on 195 species. *Ann. Bot.* 104, 57-69.

Radhakrishnan R, Khan AL, Lee I-J (2013) Endophytic fungal pre-treatments of seeds alleviates salinity stress effects in soybean plants. *J. Microbiol.* 51, 850-857.

Rajjou L, Belghazi M, Huguet R, Robin C, Moreau A, Job C, Job D (2006) Proteomic investigation of the effect of salicylic acid on *Arabidopsis* seed germination and establishment of early defense mechanisms. *Plant Physiol.* 141, 910-923.

Rajjou L, Lovigny Y, Groot SP, Belghazi M, Job C, Job D (2008) Proteome-wide characterization of seed aging in *Arabidopsis*: a comparison between artificial and natural aging protocols. *Plant Physiol.* 148, 620-641.

Rajjou L, Debeaujon I (2008) Seed longevity: survival and maintenance of high germination ability of dry seeds. *C R Biol.* 331, 796-805.

Rajjou L, Duval M, Gallardo K, Catusse J, Bally J, Job C, Job D (2012) Seed germination and vigor. *Annu. Rev. Plant Biol.* 63, 507-33.

Rao NK, Roberts EH, Ellis RH (1987) The influence of pre and post-storage hydration treatments on chromosomal aberrations, seedling abnormalities, and viability of lettuce seeds. *Ann. Bot.* 60, 97-108

Roberts EH (1973) Predicting the storage life of seeds. *Seed Sci. Tech.* 1, 499-514.

Rosbakh S, Pacini E, Nepi M, Poschlod P (2018) An unexplored side of regeneration niche: seed quantity and quality are determined by the effect of temperature on pollen performance. *Front. Plant Sci.* 9, 1036.

Salon C, Avice JC, Colombié S, Dieuaide-Noubhani M, Gallardo K, Jeudy C, Ourry A, Prudent M, Voisin AS, Rolin D (2017) Fluxomics links cellular functional analyses to whole-plant phenotyping. *J. Exp. Bot.* 68, 2083-2098.

Sarath G, Hou G, Baird LM, Mitchell R (2007) Reactive oxygen species, ABA and nitric oxide interactions on the germination of warm-season C4-grasses. *Planta* 226, 697-708.

Sattler SE, Gilliland LU, Magallanes-Lundback M, Pollard M, DellaPenna D (2004) Vitamin E is essential for seed longevity and for preventing lipid peroxidation during germination. *Plant Cell* 16, 1419-1432.

Siavash Moghaddam S, Rahimi A, Noorhosseini SA, Heydarzadeh S, Mirzapour M (2018) Effect of seed priming with salycilic acid on germinability and seedling vigor of fenugreek (*Trigonella Foenum-Graecum*). Yüzüncü Yıl Üniversitesi Tarım Bilimleri Dergisi 28, 192-199.

Sun WQ, Leopold AC (1995) The Maillard reaction and oxidative stress during aging of soybean seeds. *Physiol. Plant.* 94, 94-104.

Taylor AG, Allen PS, Bennet MA, Bradford KJ, Burris JS, Misra MK (1998) Seed enhancements. *Seed Sci. Res.* 8, 245-256.

Tsai YC, Wang YH, Liu YC (2017) Overexpression of PCNA attenuates oxidative stress-caused delay of gap-filling during repair of UV-induced DNA damage. *J. Nucleic Acids* 2017, 8154646.

Ventura L, Donà M, Macovei A, Carbonera D, Buttafava A, Mondoni A, Rossi G, Balestrazzi A (2012) Understanding the molecular pathways associated with seed vigor. *Plant Physiol. Biochem.* 60, 196-206.

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Huckelhoven R, Neumann C, Von-Wettstein D (2005) The endophytic fungus *Piriformis indica*

reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc. Nat. Acad. Sci. USA* 102, 13386-13391.

Walters C (1998) Understanding the mechanisms and kinetics of seed ageing. *Seed Sci. Res.* 8, 223-244.

Walters C, Wheeler L, Stanwood PC (2004) Longevity of cryogenically stored seeds. *Cryobiology* 48, 229-244.

Walters C, Wheeler LM, Grotenhuis J (2005) Longevity of seeds stored in a genebank: species characteristics. *Seed Sci. Res.* 15, 1-20.

Walters C, Berjak P, Pammenter NW (2013) Preservation of recalcitrat seeds. *Science* 339, 915-916.

Wang B, Zhang Z, Fu Z, Liu Z, Hu Y, Tang J (2016) Comparative QTL analysis of maize seed artificial ageing between an immortalized F2 population and its corresponding RILs. *Crop J.* 4, 30-39.

Wang W, He A, Peng S, Huang J, Cui K, Nie L (2018a) The effect of storage condition and duration on the deterioration of primed rice seeds. *Front. Plant. Sci.* 9, 172.

Wang T, Hou L, Jian H, Di F, Li J, Liu L (2018b) Combined QTL mapping, physiological and transcriptomic analyses to identify candidate genes involved in *Brassica napus* seed ageing. *Mol. Genet. Genomics* 29, 1421-1435.

Waterworth WM, Masnavi G, Bhardwaj RM, Jiang Q, Bray CM, West CE (2010) A plant DNA ligase is an important determinant of seed longevity. *Plant J.* 63, 848-860.

Waterworth WM, Bray CM, West CE (2015) The importance of safeguarding genome integrity in germination and seed longevity. *J. Exp. Bot.* 66, 3549-3558.

Wei W, Li G, Jiang X, Wang Y, Ma Z, Niu Z, Wang Z, Geng X (2018) Small RNA and degradome profiling involved in seed development and oil synthesis of *Brassica napus*. *PLoS One* 13, e0204998.

Weitbrecht K, Müller K, Leubner-Metzger G (2011) First off the mark: early seed germination. *J. Exp. Bot.* 62, 3289-3309.

Wu X, Li J, Li X, Hsieh CL, Burgers PM, Lieber MR (1996) Processing of branched DNA intermediates by a complex of human FEN-1 and PCNA. *Nucleic Acids Res.* 24, 2036-2043.

van Zanten M, Koini MA, Geyer R, Liu Y, Brambilla V, Bartels D, Koornneef M, Fransz P, Soppe WJ (2011) Seed maturation in *Arabidopsis thaliana* is characterized by nuclear size reduction and increased chromatin condensation. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20219-20224.

Zhang F, Yu J, Johnston CR, Wang Y, Zhu K, Lu F, Zhang Z, Zhou J (2015) Seed priming with polyethylene glycol induces physiological changes in sorghum (*Sorghum bicolor* L. Moench) seedlings under suboptimal soil moisture environments. *PLoS One* 10, e0140620.