UNIVERSITÀ
DI PAVIA

Dipartimento di Biologia e Biotecnologie "L. Spallanzani"

Molecular and physiological hallmarks of seed longevity in crops and crop wild relatives

Maraeva Gianella

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXIV – A.A. 2018-2021

UNIVERSITÀ
DI PAVIA

Dipartimento di Biologia e Biotecnologie "L. Spallanzani"

Molecular and physiological hallmarks of seed longevity in crops and crop wild relatives

Maraeva Gianella

Supervised by Prof. Alma Balestrazzi

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXIV – A.A. 2018-2021

Table of contents

Abstract

Conserving the genetic richness embedded in plant genetic resources is of paramount importance for the selection of the high-yielding, improved varieties of the future. In the context of climate change, biodiversity conservation becomes a tool not only for crop improvement in breeding programmes, but also in reforestation and habitat restoration actions. Ex situ conservation within germplasm banks is considered as one of the most effective and convenient strategies for ensuring preservation and availability of this genetic richness. In particular, seeds can be stored in relatively small spaces and with low economic efforts, and their viability can be preserved for the long-term, at the same time providing a good sample of the genetic diversity within the conserved taxon and gene pool. It follows that the study of seed longevity, i.e., the ability of seeds to remain viable over time, allows the optimization of conservation techniques and viability monitoring, e.g., clarify differences among different species or accessions of the same species. Therefore, reliable artificial ageing techniques, as well as molecular and physiological markers associated to seed longevity can be used in quality control, to find materials more vulnerable to seed ageing and that therefore need more frequent monitoring and regeneration/recollection. The aim of this thesis was to study the different eco-physiological and molecular aspects of seed longevity, still poorly explored, in crops and wild relatives. The investigation focused on the following crop species: maize, garden pea (*Pisum sativum* L.) and wheat wild relatives, analysed in the context of seed banking. The different experimental systems were selected in order to obtain a wider picture of some common physiological processes involved in shaping seed longevity, i.e., oxidative stress in terms of antioxidant activity, ROS accumulation, oxidative damages and protective mechanisms. The different seed accessions were studied with a multidisciplinary approach, from the analyses of germination profiles and seed phenotype to the exploration of nuclear/nucleolar ultrastructure and gene expression. The obtained results enlarged the current knowledge about the ageing behaviour and mechanisms under genebank conditions, underlining the effectiveness of cold storage in preserving PGRs for the long term in all the three projects, involving material conserved in two of the world's major seed banks (IPK and CIMMYT). In the maize and wheat wild relatives' projects, it emerged the need of applying different viability monitoring intervals based on the accessions' characteristics (i.e., grain type in maize and morph in wheat wild relatives). The accuracy of artificial ageing as a predictive tool for longevity rankings was questioned by the results obtained comparing ageing in cold storage and artificial ageing in wheat wild relatives, adding novel information to the emerging literature on the topic. These results emphasize the current need for new ageing methods (alternative to cold storage and AA) and/or molecular and physiological hallmarks for fast and accurate predictions of seed lifespan and rankings in storage. Moreover, the biochemical, molecular and structural hallmarks used in this thesis improved the characterization of the seed longevity of the considered accessions in both the wheat wild relatives and the garden pea. Features related to the oxidative stress status and the antioxidant response correlated with the germination capability after storage, further strengthening the prominent role of ROS and ROS buffering in shaping seed longevity. In this work we tested approaches still poorly used in this research field, such as the measurement of

alternative ageing hallmarks (e.g. proline, reducing sugars) and the ultrastructural analysis of chromatin compaction and genome integrity. In particular, the preliminary results obtained with the TEM techniques appear very promising, and their application to larger and different experimental systems could add a deeper level of detail to studies about the seed longevity dynamics. Indeed, multidisciplinary approaches are fundamental in the characterization of such a multi-faceted biological process.

Abbreviations

A (Cold stored seeds)
AA (Artificial ageing)
ABA (Abscissic acid)
ABI3 (ABA-insensitive 3)
ACT (Actin)
APX (Ascorbate peroxidase)
ARF (ADP-ribosylation factor)
CAT (Controlled ageing test)
CAT (Catalase)
CD (Controlled deterioration)
CGIAR (Consortium of International Agricultural Research Centres)
CIMMYT (International Maize and Wheat Improvement Center)
CWR (Crop wild relative)
DCF-DA (2',7'-dichlorofluorescein diacetate)
DOG1 (Delay of germination 1)
DHAR (Dehydroascorbate reductase)
EC (electrical conductivity)
ELF (Elongation factor 1-alpha)
EPPO (Elevated partial pressure of oxygen)
F (Fresh seeds)
G (Germinability)
GA (Gibberellic acid)
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)
GHG (Greenhouse gases)
GLM (Generalized linear model)
GSH (Reduced glutathione)
GSR (Glutathione sulfo-reductase)
GSSG (Glutathione disulfide)
IPCC (Intergovernmental Panel on Climate Change)
IPK (Leibniz Institute of Plant Genetics and Crop Plant Research)
LEA (Late embryogenesis abundant protein)
MDA (Malondialdehyde)
MDAR (Monodehydroascorbate reductase)
MGR (Mean germination rate)
MGT (Mean germination time)
OLS (Ordinary Least Squares)
PAS (Periodic acid–Schiff)
PGR or PGRFA (Plant genetic resources for food and agriculture)
RFO (Raffinose family oligosaccharides)
RH (Relative humidity)
ROS (Reactive oxygen species)
SB (Gene bank storage)
SOD (Superoxide dismutase)
TGA (Thermogravimetric analysis)
UBI (Ubiquitin)
Z (Synchronization index)

1. Introduction

1.1 Food security, climate change and seed conservation

Climate shifts, rises in temperature and higher occurrences of extreme weather events marked the last few centuries at the global level, due to progressive industrialization and expansion of the world's population (Anderson et al., 2020). From the preindustrial period (1850 ca.) to present, land surface temperature has increased by 1.41 °C, with a 1°C-increase recorded in the last 30 years whereas by the end of this century it is predicted to increase by other 2.6-4.8 °C (Anderson et al., 2020; Leisner, 2020). Changes in precipitation events and drought periods can occur spatially and temporally, thus affecting seasonality and, eventually, regional climates (Anderson et al., 2020; IPCC, 2015; Orlowsky & Seneviratne, 2012): seasonal precipitation and evaporation are becoming more variable, with an increase of mean evaporation in all the projected scenarios (Konapala et al., 2020). By the end of the 21st century, the global land area in extreme terrestrial water storage drought is predicted to double due to climate change, and projections based on the Intergovernmental Panel on Climate Change (IPCC) Fifth Assessment estimate that more than 233 million people will suffer from water demands that exceed surface-water availability (Flörke et al., 2018; Pokhrel et al., 2021). Obviously, the food and agriculture sectors are majorly hit by the consequences of climate change, at the same time being major drivers of water and land consumption, causing massive deforestation and producing 20-35 % of the total anthropogenic greenhouse gases (GHG) (Clapp et al., 2018). Climate change, apart from increasing temperatures and the occurrence of extreme weather events, affect agricultural systems by altering the relationships among crops, pests, pathogens and weeds, directly by reducing pollination and increasing the ground-level ozone concentrations, but also indirectly by altering ecosystems and reducing biodiversity (Myers et al., 2017). Even though significant agricultural losses are expected worldwide, tropical areas and Africa are predicted to be the most affected by the negative impacts of climate change; these are often coincident with underdeveloped economic areas, highly dependent on agriculture and marked by land and water over-exploitation, undernutrition, and micronutrient deficiency (FAO, 2015; IPCC, 2015). Moreover, considering the constant increase of the global human population and the ongoing environmental changes, keeping up with the global food demand is a major challenge of this century (Myers et al., 2017). Climate change implications on food and nutrition security range from harvested area to food prices, posing at risk the accessibility and availability of the main staple crops: for instance, maize (*Zea mays* L.) production declines in most regions in most climate scenarios, and its price is predicted to rise by 42 up to 131% between 2010 and 2050 (Wiebe et al., 2019). To face all the challenges posed by climate change, adaptation and mitigation strategies need to be adopted. Agriculture and forestry play a major role, with focuses on GHG emissions reduction, diversification and sustainable intensification for higher production efficiency, water use optimization (Wiebe et al., 2019). A form of sustainable intensification is breeding new crop varieties able to cope with harsher or different climatic conditions, exploiting plant genetic diversity; this is complicated by the reduction of both genetic diversity and biodiversity caused by genetic erosion, selection operated on few traits of interest, and agricultural practices such as monoculture (Bhanu,

2017; Dempewolf et al., 2014). New breeding varieties require quality improvement and tolerance to various abiotic and biotic stresses: these genetic traits, in order to be explored and utilized, should be conserved in the form of germplasm resources (Bhanu, 2017). Ex situ conservation within genebanks is considered as one of the most effective strategies for ensuring preservation and availability of this genetic richness (Li & Pritchard, 2009). Worldwide there are >1750 genebanks, conserving >7.4 million accessions: the largest number of ex situ seed accessions are represented by wheat (*Triticum* spp.), rice (*Oryza sativa* L.), barley (*Hordeum vulgare* L.) and maize, accounting for the 77% of the total cereal and pseudo-cereal holdings. Among legumes, >138k accessions of common bean (including subspecies) and 98947 accessions of peas (including landraces, commercial cultivars, mutant or genetic stocks, and breeding lines). are currently conserved (https://www.genesys-pgr.org/, Coyne et al., 2020). The main genebanks belonging to CGIAR (Consortium of International Agricultural Research Centres) and conserving the largest collections of staple crops (FAO, 2010) are listed in **Table 1.1**

***Table 1.1**: List of the main CGIAR seed banks conserving crop genetic resources.*

Genebank	**Crop**	**Global collections (%)**	**Accessions (N°)**
CIMMYT (International Maize and Wheat Improvement Center)	Wheat Maize	13 8	> 140 000 > 28 000
IRRI (International Rice Research Institute)	Rice	14	> 130 000
ICRISAT (International Crops Research Institute for the Semi-Arid Tropics)	Sorghum Pearl Millet Chickpea Groundnut	16 33 20 12	> 42 000 > 24 000 > 20 000 > 15 000
ICARDA (International Center for Agricultural Research in the Dry Areas)	Lentil Faba Bean Vetches	19 21 16	- - -
CIAT (International Center for Tropical Agriculture)	Beans Cassava	14 17	> 37 000 > 6 000

The Svalbard Global Seed Vault, built under the Norwegian permafrost to resist even a nuclear war, was designed to protect duplicates of plant genetic material conserved in genebanks located worldwide and it now conserves more than 1 million seed samples (Asdal & Guarino, 2018). The Millennium Seed Bank (MSB), developed and managed by the Royal Botanic Gardens (RBG Kew, UK), is the world's largest seed bank and the MSB Project is the largest *ex situ* plant seed conservation program for wild species, involving 96 countries and territories and conserving > 98 000 seed accessions (data accessed in April 2021) (Liu et al., 2018).

1.2 Plant genetic resources and seed banks

Plant genetic resources for food and agriculture (PGRFA or PGRs) are defined as "any genetic material of plant origin of actual or potential value for food and agriculture (FAO 2009)". They are among the most important natural resources, and their preservation is of paramount importance in order to treasure this richness. *In situ* conservation (i.e., in the natural habitat) represents a dynamic form of conservation, as it allows species to evolve in their original place and to retain a higher genetic diversity compared to seed bank accessions. *Ex situ* conservation (i.e., outside the natural habitat) consists in sampling, transferring, and storing a population sample of a certain species away from the original location where it was collected. However, even if *ex situ* conservation of genetic resources is easy, cost effective and increase the accessibility of these resources; *in situ* conservation has the advantage of allowing species to evolve in their original place and to retain a higher genetic diversity compared to seed bank accessions. Several *ex situ*

conservation strategies are employed for different crops, e.g. *in vitro* storage, seed banking, field genebanks, DNA banks. The most used strategy is conventional seed banking, as it allows the storage of thousands seed accessions in relatively small spaces and with low economic efforts; seed collections can provide a good sample of the genetic diversity within the crop gene pool, usually remaining viable for the long-term (Li & Pritchard, 2009). However, conventional seed banking, namely drying and subsequent freezing at -15/-18°C, is efficient only when conserving orthodox seeds, i.e. seeds that are desiccation tolerant to very low moisture contents (MC) (Roberts, 1973). Storage of orthodox seeds increases logarithmically with decreasing water contents, with a limit beyond which no further gain in storability is reached (Ellis & Roberts, 1980). Under ideal conditions, orthodox seeds can retain their ability to germinate for years, decades or centuries. On the contrary, recalcitrant seeds are characterized by short post-harvest lifespans and sensitivity to desiccation, while intermediate seeds are desiccation tolerant that retain their germinability for short periods under conventional seed bank conditions (Black & Pritchard, 2003; Roberts, 1973). These non-orthodox seeds (or seed parts, e.g. embryonic axes) can be efficiently conserved by exploiting cryogenic technologies, that, despite being far more expensive than conventional freezing, represent a safer and cheaper alternative to tissue culture, greenhouses, or field plantings (Walters et al., 2013). All the conservation techniques currently employed for PGRs *ex situ* conservation are described, with detailed standards and guidelines, by the FAO Commission on Genetic Resources for Food and Agriculture (FAO, 2014). *Ex situ* seed conservation in genebanks can be divided into seven main activities: acquisition, seed drying, seed storage, viability monitoring, regeneration, characterization, and distribution. Following acquisition and seed drying, the storage phase is critical for an optimal conservation of genetic resources. Temperature and RH need to be set in order to maximize seeds' lifespan an minimize the costs for the conservation facilities. For long-term conservation, it is recommended to store dried seed accessions at a temperature of -18 ± 3 °C. In addition to the long-term ('base' collection), some banks have duplicate samples in an active short-medium term collection stored at a temperature range between -5 and 10 °C. Seed conserved in this 'active' collection are generally employed for regeneration, distribution and characterization, not to decrease the stocks conserved in the base collection. Viability monitoring, i.e. germination tests performed before and during storage, is fundamental for the correct management of a seed bank. Indeed, seed germination of stored accessions must be tested at regular intervals to understand the loss of viability in storage and to plan re-collection or schedule regeneration activities. Seed germination is generally tested using standard protocols (ISTA, 2019) with light and temperature-controlled incubators, using agar or filter paper as the germination medium. International standards recommend that initial germination percentage should exceed 85% for crop seed accessions stored for conservation purposes. Moreover, seed multiplication is required when seed longevity declines, i.e. germination drops below 85% of the initial value, or when the quantity of seeds has been depleted due to frequent use of the accession. A sufficient number of seeds needs to be used for regeneration activities in order to maintain the genetic variability within the accessions and 'trueness to type' of the regenerated material must be assessed. Beside some morphological and agronomical traits, trueness to type evaluation can be performed through molecular and physiological descriptors, e.g. RAPD, AFLP, microsatellites and genotyping techniques such as genotyping by sequencing or Diversity Arrays Technology sequencing - DArTseq (Börner et al., 2000; Chen et al., 2016).

1.3 Plant genetic resources, from endangered and wild species to crops

The definition and classification of PGRs started with the work by Harlan & de Wet (1971), where the bases of the gene pool concept were established and are nowadays globally recognized by breeders. There are three major gene pools based on the degree of sexual compatibility: Primary Gene Pool (GP-1) comprising the domesticated crop and the its closed wild forms with which the crop can cross producing fertile hybrids; Secondary Gene Pool (GP-2) which includes less closely related species, from which gene flow, even if difficult, is still possible using conventional breeding techniques; Tertiary Gene Pool (GP-3) which includes species from which gene transfer to the crop is impossible without the use of "rather extreme or radical measures" (Hammer & Teklu, 2008).

PGRs can be also grouped in the following biological/agronomic categories:

- Cultivated materials, consisting of:
  - Landraces (or primitive cultivars): "dynamic populations of a cultivated plant that have historical origin, distinct identity and lacks formal crop improvement, as well as often being genetically diverse, locally adapted and associated with traditional farming systems" (Camacho-Villa et al., 2005);
  - Old cultivars: sometimes known as obsolete cultivars, refers to cultivated varieties which have fallen into disuse;
  - Modern cultivated varieties (modern cultivars): agronomic varieties in current use and newly developed varieties;
  -Special stocks: such as advanced breeding lines (i.e. pre-released varieties developed by plant breeders), mapping populations, CRISPR-edited lines and cytogenetic stocks.
- Crop wild relatives (CWR): wild plant species that are genetically related to cultivated crops. CWR are not only the wild ancestors of the domesticated plant but also other more distantly related species.

Another category of PGR of significance are the neglected crops, also referred as underutilized or orphan crops: "crop species that have been ignored by science and development but are still being used in those areas where they are well adapted and competitive" (Hammer & Teklu, 2008). Among PGRs, CWRs represent the bulk of genetic diversity in crop gene pools. Given their relatively close relationships to a crop species and their adaptation to a wide diversity of habitats and range of environmental conditions, they are invaluable resources for plant breeding and improvement, through the introgression of genes into crop varieties to overcome biotic (e.g., pest resistance) and abiotic (e.g., drought tolerance) stresses, also in relation to climate change (Dempewolf et al., 2014; Nigel Maxted et al., 2006). Indeed, both CWRs and landraces are used to overcome the loss of genetic diversity that accompanies crop evolution and selection of modern cultivars. Genetic erosion, i.e., the loss of genetic diversity and variation within a crop, can be classified at the crop, variety and allele levels, with general reductions in allelic richness and evenness (Wouw et al., 2010). In order to perform the introgression, the following actions should be undertaken: i) CWR can be selected first based on

phenotypic, genotypic, or collection geographic data and are used in targeted crossing, and the evaluation is done in the offspring, or ii) a wide range of wild and domesticated species are crossed and selection of the trait of interest is made directly on the progeny in the domesticated background (Dempewolf et al., 2017). Some examples of successful CWR use in crop improvement can be found in **Table 1.2**.

Table 1.2: Examples of CWR use for crop improvement, from http://www.cropwildrelatives.org and Coyne et al., 2020

Crop	Donor species	Trait	US $worth
Tomato	*Lycopersicon peruvianum* (L.) Mill.	2.4% increase in solids content	250 million
Peanut	*Arachis batizocoi* Krapov. & W.C. Gregory, *A. cardenasii* Krapov & W.C. Gregory, *A. diogoi* Hoehne	Resistance to the root knot nematode	100 million/year
Maize	*Tripsacum dactyloides* L.	Resistance to corn blight, US$1,000 million economic loss in the 1970s	--
Bread and durum wheat	*Triticum dicoccoides* (Körn. ex Asch. & Graebn.) Schweinf.	Increased protein content	--
Potato	*Solanum demissum* Lindl.	Resistance to potato blight	--
Pea	*Pisum fulvum* Sibth. & Sm.	Bruchid resistance	--

Other single gene-controlled traits have been introduced from CWR to confer virus resistance in rice, powdery mildew resistance in wheat (*Triticum aestivum* L.) and Fusarium and nematode resistance in tomato (*Lycopersicon esculentum* Mill.), as well as different genes from wild *Brassica oleracea* L. have been used to increase the anti-cancer properties in broccoli (http://www.cropwildrelatives.org).

Apart from plant resistance to biotic and abiotic stresses, other traits are lost with domestication. The 'domestication syndrome', i.e., plant traits that mark the divergence of domesticated crops from their wild progenitors, often regards reproductive traits that affect yield, such as seed size and number or shattering at maturity (e.g., wheat) (Iriondo et al., 2018; Nave et al., 2016). For instance, when selecting crops for ready and uniform germination, dormancy (i.e., the inability of viable seeds to germinate under optimal environmental conditions) needs to be reduced or eliminated. Among the seed traits lost in this process diaspore heteromorphism is certainly one of the most frequent. Diaspore heteromorphism is the production by the same individual of two or more seed/fruit types (Baskin & Baskin, 1998). The difference between morphs can reside in different morphological traits, such as shape, colour and thickness of the seed coat, mass, as well

as in eco-physiological traits such as stress tolerance, longevity, dormancy, soil persistence and/or dispersal (Gianella et al., 2020). Heteromorphism is considered one of the main bet hedging strategies in flowering plants. The so-called 'bet hedging' strategy describes the adoption of a reproductive strategy that maximizes the long-term fitness to ensure survival when coping with an unpredictable environment or in the presence of fluctuating natural selection (Gianella et al., 2021). For instance, wild relatives of wheat show heteromorphism in terms of dormancy, longevity and antioxidant activity, possessing seed traits completely lost in their cultivated sister species. Moreover, CWRs as well as all native plants are gaining attention also for their value in revegetation and rewilding programmes, e.g., for ecological restoration, the process of assisting and managing the recovery of ecological integrity. Planting keystone species can indeed facilitate the immigration of other species to the restoration site and several wild relatives can serve the purpose also for their intrinsic adaptations to ruderal or harsh habitats (Maxted et al., 2007). In this context, ex situ conservation is the best tool for keeping these invaluable genetic resources viable and available for the long term, allowing them to be studied, used and distributed for agricultural and ecological purposes. One of the biggest CWR collecting efforts was concluded this year: the Global Crop Diversity Trust and the Millennium Seed Bank of the Royal Botanic Gardens, Kew embarked in 2011 on a global, long-term project to collect, conserve, and initiate the use of CWRs. This 10-year project, funded by the Government of Norway, was called ''Adapting Agriculture to Climate Change'' and focused on wild relatives of 29 crops of major importance for food security (Müller et al., 2021).

1.4 Seed longevity: definition and research approaches

Seeds are exceptional examples of long-lived eukaryotes: radiocarbon dating sets the age of some still viable seeds at about 2000 and 1300 years ago (*Phoenix dactylifera* L. and *Nelumbo nucifera* Gaertn., respectively) (Walters, 1998). Seed longevity is defined as the viability, or ability to germinate, retained by seeds over a period of dry storage (Rajjou & Debeaujon, 2008). Moreover, germination must be followed by the development of normal seedlings able to establish and complete their development into normal plants (Bewley et al., 2013). The assessment of seed longevity is functional to guarantee the viability of a seed collection within a seed bank over this period, mainly predicting when collections should be recollected/regenerated and avoiding repetitive viability assays if the seed number is low (Colville & Pritchard, 2019; Niedzielski et al., 2009; C Walters, 2003). Longevity estimates are usually obtained through artificial aging (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 & Baskin, 1973; Newton, 2009; Powell & Matthews, 1981). In general, there is a wide range of temperature/RH combinations during storage conditions that are used by laboratories and they are named in different ways, such as ambient or natural ageing, AA or CD (Zinsmeister et al., 2020). The electrical conductivity (EC) test for seed quality is based on the leakage of solutes caused by damage to lipidic membranes, which can be measured through electrodes and it is used to assess damage during aging, as EC is known to be negatively correlated with seed vigour (Ozden et al., 2017; Powell, 1986). Elevated

partial pressure of oxygen (EPPO) storage is another method that mimics ageing under seed bank conservation, elevating the pressure to 18 kPa using scuba diving tanks. It has been developed to avoid the use of high temperature and RH, thereby reducing their effect on seed deterioration, even though pressure itself has detrimental effects on seed vigour (Groot et al., 2012; Nagel et al., 2016). Both RH and temperature affect seed longevity in different ways at the species and population level: several species and genotypes show different behaviours under different storage conditions or ageing methods (Zinsmeister et al., 2020). Indeed, the accuracy of AA methods is being questioned, in particular when estimating ranks and profiles of seeds conserved within seed banks in cold storage (Buijs et al., 2020; Ozden et al., 2017; Roach et al., 2018; Schwember & Bradford, 2010). This also because the storage conditions will determine the type of ageing reactions: storage over water vapor at 100% relative humidity (RH) and 42–45°C will allow both the resumption of respiration, as seeds are progressively imbibing, and a heat stress leading to protein denaturation, whereas storage at 75% RH at 35°C does not allow respiration to occur because the cytoplasm is too viscous to allow molecular mobility. Thus, the different method used in different studies, even though used on the same species, make difficult the comparison, especially when studying the molecular processes driving seed ageing (Zinsmeister et al., 2020). Additionally, only a few seed longevity studies were performed on seed material under long-term conservation (cold storage between –15 and –20 °C), as well as few comparative studies between AA and seed ageing in seed banks are available (Desheva, 2016; Hay et al., 2013; Walters et al., 2005). Also, since most seed banks have a relatively short-term history, declines in seed viability of the conserved accessions are unlikely to be detected (van Treuren et al., 2018). Indeed, often monitoring data have right-skewed distributions, making predictions difficult or inaccurate, as many assumptions need to be done in order to plot the distribution of seed mortality over time. The viability equations widely used to model seed ageing rates assume that seed deaths are normally distributed over time (Ellis & Roberts, 1980; Roberts, 1973), because they were developed using crop species with homogenous germination profiles and characteristic peaks of mortality. Wild collections are, on the contrary, quite heterogenous in their seed traits, and often their ageing rates are not well-fitted by the viability equations; indeed, differences in flowering, maturation and dormancy are often observed among individuals in wild populations, and these genetically regulated traits are known to contribute to seed ageing profiles (Walters, 2003). According to Ellis & Roberts (1980), the loss of seed viability during storage generally follows a sigmoid pattern. Seed viability during storage is determined by transforming the viability percentage through link functions, among which the Probit function is the most commonly used and it assumes normality. Hay et al. (2014) proposed the use of the GLM-Probit, using the binomial distribution with germinated seeds as response and sown seeds as scale factor. With the diverse range of possible distribution families and link functions, one must take care in selecting the model, but it is generally not appropriate to analyze the cumulative germination data with Probit analysis because the observed germination counts at successive time-points during ageing are not independent (Hay et al., 2014). Other studies use ordinary least square analyses, and efforts are being made in order to provide more robust tools, and in particular link functions, in order to avoid the disregarding of model assumptions. Recently Logit (GLM), Cauchy-SSF (OLS), and Cauchy (GLM) functions were found to estimate seed longevity more robustly than the Probit function also in crop species, and Logit is being preferred over Probit as it allows for a more reliable estimate/prediction of viability loss especially in the tails of the

distribution (i.e., < 10 % or >80 % germination) (e.g., de Faria et al., 2020; dos Santos et al., 2019; Guzzon et al., 2021; Ozden et al., 2017). Other models exploit the Avrami kinetics, which describe co-operative reactions based on visco-elastic properties, adapting it to different equations in order to calculate time coefficient (introduced by Walters, 1998). However, also the Avrami models are being questioned, in particular being less plausible than other models in predictions of seed survival when considering after-ripening, i.e., complex enzymatic and biochemical process that certain plant embryos must undergo before germination in apparently mature seeds, often exploiting temperature and RH in a manner similar to ageing (Bewley et al., 2013; Trapp et al., 2012). In conclusion, further research is needed to improve the different methodologies used to mimic seed ageing and to model the survival rates used in different comparative studies. Additional studies need to be performed in order to obtain a standardized, efficient method to predict seed longevity.

1.5 Seed longevity and environment during development, maturation and storage

Longevity acquisition is regulated by hormonal and maternal factors and is under strong influence of the environment, during both maturation and storage. In general, longevity is developed during maturation, after the acquisition of desiccation tolerance, and the process spans between seed filling and weeks after storage (i.e. after-ripening) depending on the species. The environmental conditions that influence longevity include light, temperature, drought, and salinity, while temperature and water availability are the main driving factors (Zinsmeister et al., 2020, **Fig. 1.1**). These conditions are experienced by the seed or the mother plant, and longevity can vary also in different populations of the same species adapted to different habitats (Leprince et al., 2017). For instance, alpine plants from cool, wet climates are shorter-lived than those from warm, dry climates because of the low average temperature of the post-dispersal environment in alpine locations (Ellis et al., 1993; Mondoni et al., 2014). Indeed, longevity might be a long-term adaptation to arid environments, with possible selective pressures on future generations. Alternatively, intraspecific variations in longevity might represent phenotypic plasticity mediated by the mother plant as a response to the environment, as demonstrated in studies where mother plants were grown in greenhouses manipulating temperature and water availability, and significant differences in longevity in the offspring were observed (Zinsmeister et al., 2020). Indeed, the maternal-derived tissues of seeds can contribute to longevity, in particular the seed coat, the pericarp and the endosperm. The latter is a triploid tissue that can develop into a storage organ or into a thin layer around the embryo that can control germination, it has an increased dosage of the maternal genome, and its association with seed ageing has been demonstrated (Probert et al., 2009; Tausch et al., 2019). Transgenerational effects on seed longevity are not limited to the manipulation of temperature and water availability, but they also have been observed when taking in consideration ozone-induced oxidative stress and endophyte symbiosis, the latter increasing seed moisture content with a consequent detrimental impact on longevity (Ueno et al., 2020). Indeed, moisture content, partly influenced by relative humidity, is the major factor regulating seed ageing together with temperature and oxygen pressure (Sano et al., 2016; Walters, 1998, **Fig. 1.1**). Temperature can affect longevity, increasing or decreasing it, depending on species and

genotype. The mechanisms by which temperature acts on seed ageing at the maternal and zygotic levels are not yet fully understood, even if some studies demonstrated that it changes the seed coat (maternal tissue) permeability and it is probably involved in the hormonal regulation of seed dormancy (Zinsmeister et al., 2020). Also, water availability during seed maturation can positively or negatively influence seed longevity, either blocking or accelerating seed maturation and thus the acquisition of desiccation tolerance and then longevity. The plasticity that allows seeds to regain longevity depends on the degree of seed maturation itself at the time when water scarcity is experienced by the plant (Ellis & Yadav, 2016). Another factor that affects seed longevity in different manners is light. During maturation, seeds need to dismantle chloroplasts and degrade chlorophyll molecules, and the exposition to light can trigger chlorophyll production instead of facilitating its degradation. Indeed, failure in chlorophyll degradation in mature seeds is concomitant to a reduced shelf life. The photoperiod in which seeds are produced has an effect on seed longevity, as well as the intensity and the type of the light received by the plant (Zinsmeister et al., 2020). Finally, nutrient availability and thus the macro- and micro-nutrients uptake of the mother plant can alter the final germination performance in the offspring, as well as vigour and longevity (Nagel et al., 2015).

As already mentioned, RH and temperature play a fundamental role in determining the seeds shelf-life during storage. Indeed, consistent relationships among temperature, RH and seed longevity have originated some basic rules for seed storage, a commonly used practical rule for seed storage (known as James' Rule) is that the temperature (in Fahrenheit) plus the RH of the air (in percent) should total less than 100 for satisfactory seed storage. For example, if the RH is 50%, the storage temperature should be no greater than 50°F (10°C) for commercial (medium term) storage. Harrington's Rule states that storage life will approximately double for each 10°F (5.6°C) decrease in temperature and each 1% decrease in seed moisture content for temperatures between 0 and 40°C and moisture contents between 5 and 14%" (Bewley et al., 2013). In general, the combination of low temperature and low moisture content is fundamental in order to extend longevity in orthodox seeds. At threshold water contents that range between 0.03 and 0.07 g H_2O g^{-1} DW (dry weight), the tendency of increased longevity associated with drying is arrested: at lower moisture contents, longevity is unaffected or decreases for the excessive drying. Given the quantitative relationships between moisture content and the protection against ageing-induced damage, longevity is considered as a manifestation of desiccation tolerance (Walters, 2015). On the other hand, drying is not possible for the conservation of recalcitrant seeds, that cannot tolerate the classical drying and cooling approach as they often possess large, fleshy organs and thick covering layers surrounding the embryo as mechanisms to resist water loss. In this case, rather than desiccation tolerance, longevity is a manifestation of desiccation avoidance (Walters, 2015). Other abiotic and biotic factors can affect seed lifespan during storage, e.g., oxygen pressure and fungal/insect infestations, that in turn depend on, and act together with, temperature and relative humidity in the storage environment (Bewley et al., 2013)

1.6 Seed ageing physiology: the seed glassy state and the role of oxidative stress

Seed physiology, and all the biological processes implied, differ enormously depending on the hydration state, i.e., dry seeds stored at different RH or imbibed seeds. In ideal

storage conditions, below 50% RH, integrated cellular processes, including respiration, ATP generation, transcription, translation, and enzyme activity, are either completely blocked or can be active but slowed down in the fraction of hydrated cells within the seed. On the contrary, in many studies of AA, where RH is above 50%, seeds are partially hydrated and enzyme activity and some metabolic reactions are possible (Bewley et al., 2013). The seed hydration state is indeed involved in regulating the chemical-physical properties of the cytoplasm: during desiccation, cytoplasm changes its state from fluid to solid, forming the so-called "glassy state", where cellular components are stabilized and the molecular mobility is severely restricted, an indispensable requirement for desiccation tolerance development (Buitink & Leprince, 2008). When seeds are imbibed, the cytoplasm becomes fluid again, allowing molecular mobility and repair processes (Sano et al., 2016). The formation and stabilization of the glassy state depends on temperature and moisture content, that can alter the properties of different macromolecules. Among these molecules forming the glassy state, non-reducing oligosaccharides (5-10% of the dry mass), i.e., sucrose and raffinose family oligosaccharides (RFOs), replace water during desiccation and maintain lipids in a fluid state, thereby protecting membranes (Ballesteros et al., 2020; Sano et al., 2016). About 50% of the seed dry matter is composed of proteins, of which up to 20% contain intrinsically disordered proteins (IDPs), such as late embryogenesis abundant (LEA) and heat-shock proteins (HSP). LEAs undergo desiccation-induced folding during cell drying, suggesting differential functions under different imbibition states: as cells dry (<95% RH) LEAs form a gel together with sugars and ions, encapsulating other cell structures such as cell organelles for stabilization and protection (Ballesteros et al., 2020). On the other hand, heat-shock proteins (HSPs) are chaperones that stabilize neosynthesized proteins to enable correct folding, contributing to seed longevity by protecting proteins against oxidative damage and also by refolding proteins damaged by seed ageing (Sano et al., 2016b). The glassy state prevents damages induced by auto-oxidative processes and in particular RFOs could possess specific antioxidant activity possibly by scavenging hydroxyl radicals (Sano et al., 2016). Indeed, oxidative stress mediated by reactive oxygen species (ROS), affecting lipids, cell membranes, DNA, RNA and proteins, is considered as the main cause of seed deterioration during ageing (Bailly, 2004; Kurek et al., 2019; Rajjou & Debeaujon, 2008). Auto-oxidative processes that comprise the Amadori and Maillard reactions, lipid peroxidation and protein carbonylation are usually not catalysed by enzymes, thus require highly mobile molecules, ROS, that can diffuse through the cytosol and reach relatively distant targets (Ballesteros et al., 2020). Indeed, dry, mature seeds are quiescent, with little or null metabolic activity in terms of respiration and reserve mobilization from glyoxysomes, the two main ROS sources in metabolically active plant tissues (Bailly, 2004). Therefore, autoxidation is the main cause of ROS accumulation during ageing, and it acts as a progressive cascade during storage, producing peroxides, hydroperoxides, carbonyl and nitrosyl groups that can in turn react with other molecules, also forming adducts (Zinsmeister et al., 2020). Among the oxidation damages, lipid peroxidation is observed in aged seeds of a wide range of plant species, and it is measured through its by- and downstream products, e.g., malondialdehyde, propanal, butanal, hexanal, or by means of lipidomics and oxylipidomics (Bailly, 2004; Wiebach et al., 2020; Zinsmeister et al., 2020). Indeed, even little modifications in phospholipid composition during storage can drastically change the chemical-physical properties of the membrane upon imbibition, leading to embryonic damages and cell/seed death (Zinsmeister et al., 2020). It follows

that in order to extend their longevity, seeds need to prevent ROS accumulation and to scavenge them upon imbibition, exploiting their reserve of enzymatic and non-enzymatic antioxidants accumulated during maturation and development, also influenced by the mother plant environment (Sano et al., 2016). Among enzymatic antioxidants, superoxide dismutases, catalases, glutathione and ascorbate peroxidases, and monodehydroascorbate, dehydroascorbate and glutathione reductases are the most used by seeds, and they can act in the cytosol, mitochondria or choroplasts (Bailly, 2004). Other proteins that have ROS scavenging and signalling functions in seeds are thioredoxins, peroxiredoxins and glutaredoxins. The main non-enzymatic ROS scavenging mechanisms in seed metabolism involve seed storage proteins and low molecular weight antioxidants, such as polyphenols, tocopherols (Vitamin E), ascorbate and glutathione (Sano et al., 2016). Reduced glutathione (GSH) is a major regulator of the intracellular redox environment due to its abundance and negative redox potential. The measurement of oxidized glutathione (glutathione disulfide, GSSG)/GSH redox couple and the determination of the half-cell reduction potential (EGSSG/2GSH) are used as markers of seed longevity (Nagel et al., 2015; Roach et al., 2018). Also seed storage proteins (SSPs) contribute to ROS buffering, acting as primary targets of oxidation due to their affinity to ROS and their relative abundance, thereby protecting indirectly other cellular components necessary for the survival of the embryo (Sano et al., 2016). Another target of oxidation with a fundamental role in regulating seed longevity is RNA. A reduction in total RNA and in RNA integrity is associated with seed ageing, and in particular 18S and 25S rRNA degradation can be used as a seed longevity marker (Sano et al., 2020). Moreover, during storage, transcripts are broken by ROS attack at random bases, and the number of breaks is length-dependent: short mRNAs, often related to the transcriptional and translational machineries, remain mostly intact during storage, thus underlining the fundamental role of these biological processes for the germination process initiated with imbibition. Among different species subjected to natural and AA, significant reductions in transcripts of genes related to programmed cell death, antioxidants, seed storage proteins, heat shock transcription factors, and the glycolytic pathway are observed (Sano et al., 2020; Zinsmeister et al., 2020). Even though RNA is more vulnerable to oxidation because of its single-stranded structure, also DNA can be damaged by ROS accumulation during ageing (Kranner et al., 2010; Sano et al., 2015, 2020). DNA and RNA fragmentation can accumulate in aged seeds also as a consequence of programmed cell death (PCD), while strand breaks in DNA are primarily caused by ROS, directly with desaturation of deoxyribose units or indirectly, by covalent modifications of bases (the most common is the hydroxylation of the C-8 position of guanine, resulting in 7,8-dihydro-8-oxoguanine (8-oxoG)) (Biedermann et al., 2011; Bray & West, 2005). Indeed, OGG1, an apurinic/apyrimidinic DNA glycosylase/lyase that removes oxidatively damaged guanosines from DNA, is associated with seed longevity in *Arabidopsis thaliana* (L.) Heynh., and more generally, base excision repair (BER) is probably the main repair system used by seeds for ageing-associated DNA damage, together with repair enzymes such as ligases IV and VI, involved in strand breaks repair, and poly(ADP)polymerase 3 (PARP3) (Chen et al., 2012; Kurek et al., 2019; Waterworth et al., 2010, 2015). In general, seed ageing is concomitant with DNA damage accumulation, in terms of abasic sites, base modification, single strand breaks (SSBs) and double strand breaks (DSBs) (Cõrdoba-Cañero et al., 2014; Waterworth et al., 2015). Moreover, changes in nuclear/nucleolar structure can occur in aged seeds, often showing pyknotic phenotypes and reductions in heterochromatin density (Yan et

al., 2016). Finally, oxidation can alter protein synthesis, enzymatic activity and protein structure, e.g. transport protein receptors or ion channels, thus resulting in modified fluidity and extensive cellular dysfunction. Transcription factors, phosphatases and antioxidant enzymes are the most frequent targets of protein oxidation during ageing: their activity can be altered or blocked, leading to further oxidative stress and the inability to activate the metabolic processes necessary for germination, eventually resulting in seed death (Lehner et al., 2008; Sharma et al., 2012). Oxidation of methionine to methionine sulfoxide caused by ROS is one of the major forms of damage found in aged organisms and methionine sulfoxide reductase (MSR) activity is used as a seed longevity marker (Sano et al., 2016) (See **Fig. 1.1.** for a schematic representation of the physiology of seed longevity).

1.7 Hormonal regulation of seed longevity, relationships and negative association with seed dormancy and seed priming.

Dormancy and longevity possess distinct and shared signalling pathways, often related to endogenous hormonal balance that regulates physiological dormancy (Baskin & Baskin, 1998). Both traits contribute to seed survival and persistence, with dormancy delaying germination after dispersal to favour seedling establishment in optimal environmental conditions and reduce the risk of premature death (Bewley et al., 2013). At the hormonal level, abscisic acid (ABA), either generated by the cotyledons, or synthesized within the embryo axis, has the primary inhibitory role for seed germination, counteracted by the promotive action of gibberellic acid (GA). Both enzymes respond to environmental signals, and synthesis and deactivation, as well as their interaction with other hormones (e.g., brassinosteroids, ethylene) regulate their balance and therefore the seed entry in the germination phase (Bewley et al., 2013). The central role of ABA in regulating seed dormancy and longevity is demonstrated in the *Arabidopsis* mutants *aba-insensitive3* (*abi3*), that show reduced dormancy, intolerance to desiccation and rapid viability loss during dry storage, and also enhanced sensitivity to CD (Clerkx et al., 2004; Mao & Sun, 2015). Downstream ABI3, the seed-specific heat shock factor HSFA9 is expressed during late maturation, the acquisition of dormancy and desiccation tolerance, and possesses a central role in seed longevity-related signalling. It promotes the accumulation of HSPs and therefore thermoresistance, proved also in longevity studies using CD (Sano et al., 2016). ABA may also control water uptake and H_2O_2 accumulation, and therefore oxidative stress, through the modulation of aquaporins ABI3-dependent, thus further regulating seed longevity (Mao & Sun, 2015). Auxin signalling also acts downstream ABI3 in regulating seed longevity, enhancing the seed lifespan by contrasting the inhibition of HSFA9 (Carranco et al., 2010). Moreover, longevity genes of *Medicago truncatula* Gaertn. are enriched with auxin binding-sites, suggesting a potential additional role of auxins in seed longevity (Righetti et al., 2015). Despite the fact that the antagonistic action of ABA and GA in controlling dormancy and longevity is well known, the role of GA is not yet fully clarified. Indeed, even though *gibberellin-insensitive* (*gai*) mutants do not show losses in terms of seed viability during storage, the accumulation of gibberellins and enzymes related to their biosynthesis was proved to be correlated with enhanced longevity (Sano et al., 2016). Gibberellins could act by modifying the seed coat (*testa*), that provides chemical and mechanical protection to the embryo: its structure, thickness and composition are critical factors in seed longevity (Bueso et al., 2014; Debeaujon & Koornneef, 2000). The chemical composition

of the *testa* is defined during seed development by the accumulation of polyphenols, polysaccharides, suberin and cutin within the maternal-derived tissues of the coat, that, at the end of the maturation, are composed of dead cells (Sano et al., 2016). The maternal origin of the seed coat implies a dependence of the chemical composition on the environmental factors experienced during development, i.e. temperature, water and nutrient availability, that modify the synthetic pathways through gene and hormone activation (Zinsmeister et al., 2020). Polyphenols, divided into flavonoids, lignins and lignans, are associated with seed longevity. Flavonoids-deficient mutants of *Arabidopsis*, *Brassica napus* L. and *Linum usitatissimum* L. showed reduced longevity in long-term storage, CD and AA, and an increased permeability to tetrazolium salt, suggesting a potential role of these metabolites in protecting the embryo from external water and oxygen (Clerkx et al., 2004; Debeaujon & Koornneef, 2000; Zhang et al., 2006). Flavonoids possess antioxidant properties, thus they may scavenge ROS during seed ageing or imbibition. The oligomeric flavonoids proanthocyanidins (PAs) elicit ABA synthesis, thereby interacting with dormancy and longevity onset (Sano et al., 2016). Other components of the seed coat, such as defense-related proteins, polyphenol oxidases (PPOs, e.g. catechol oxidases and laccases), peroxidases (PODs) and chitinases (Moïse et al., 2005; Pourcel et al., 2005) can influence seed ageing. Lignin-deficient mutants show reduced seed longevity, probably due to the enhanced seed coat permeability and therefore the reduced mechanical protection from water and oxygen, while little or no data about the role of lignans in shaping seed longevity is available (Sano et al., 2016). Increased permeability of the seed coat is thought to be the primary cause of the fast seed ageing observed in primed seeds, i.e. seeds subjected to priming treatments and subsequently stored (Zinsmeister et al., 2020). Priming techniques are pre-sowing treatments that improve germination rates, speed, consistency, and viability, that can be followed by immediate sowing or by a period of storage (Paparella et al., 2015). In some cases, primed seeds show a delayed loss of viability when stored, probably because the repair mechanisms activated by imbibition allow them to maintain germinability for longer periods. Other studies report that primed seeds present reduced longevity when submitted to CD and AA tests, but this reduction in longevity compared to the unprimed seeds could be probably due to the high temperature and RH rather than on the priming treatment per se. The viability of primed stored seeds can be partially restored with post-storage treatments, e.g., re-priming (Gianella et al., 2020). On the other hand, different priming methods have been used to overcome aging-associated damage by improving the germination rate, uniformity, and percentage. It has been suggested that the effectiveness of these treatments depends on repair mechanisms activated during the hydration phase, acting on nucleic acids, lipids and proteins, and also on the reactivation of transcription and antioxidant enzyme scavenging activity. Although hydropriming and osmopriming are the most used treatments, other compounds such as salicylic acid or metallic nanoparticles have been used on aged seeds, with positive effects both on germination and the seedling phenotype (Gianella et al., 2020).

1.8 Genetic and epigenetic bases of seed longevity

Despite the mechanisms of seed ageing and the acquisition of longevity are still not fully clarified, several genetic and epigenetic factors are known to contribute to these complex traits. Transcription factors ABI3 (ABSCISIC ACID INSENSITIVE3), FUS3 (FUSCA3), and LEC2 (LEAFY COTYLEDON2) (B3 domain family) together with

LEC1 (LEAFY COTYLEDON1), (NFYB protein family) regulate seed maturation. ABI3 and LEC1 can influence the acquisition of longevity, by forming complexes with other proteins and acting redundantly (Zinsmeister et al., 2020). The reduction of longevity and dormancy in *abi3*, *lec* and *fus3* mutant seeds is due to their failure to acquire desiccation tolerance and induce dormancy during late seed development (North et al., 2010). Downstream the ABI3 function, several genes are implicated in seed longevity, e.g. the transcription factor HSFA9 or *stay-green* (*SGR*) genes involved in the catabolism of chlorophylls, fundamental for proper seed maturation and longevity acquisition (Sano et al., 2016). ABI3 and LEC1 regulate, either directly or indirectly, the expression of HSPs and LEAs, as well as SSPs that are associated with seed longevity (Sugliani et al., 2009). The alternative splicing of *ABI3* mRNA can also contribute to differential seed longevity profiles, with the prevalence of full-length transcripts at the beginning of seed development when seed longevity is acquired. In the dry seed, the spliced form *ABI3-β* increases and becomes prevalent. Such event is regulated by SUPRESSOR OF ABI3–5 (SUA1 to SUA4), a RNA binding protein involved in seed longevity (Zinsmeister et al., 2020). Alternative splicing is a frequent process in developing seeds, in particular in genes associated with mRNA catabolism. In general, RNA processing and translation are the functions associated with the most expressed genes during the acquisition of longevity in M. truncatula (Verdier et al., 2013). Another gene involved in the ABA signalling pathway, ABI5 is a regulator of seed longevity in legumes, and in turn it is regulated by DOG1 (DELAY OF GERMINATION 1), a heme binding protein that interacts with ABA HYPERSENSITIVE GERMINATION1 (AHG1, a group A type 2C protein phosphatase (PP2C)) to trigger an ABA response and dormancy. Together, *DOG1*, *ABI3*, *ABI5* and homologous *ABF* genes interact in the acquisition of longevity in *Arabidopsis* (Zinsmeister et al., 2020). Auxin-related genes are also involved in the complex network of seed longevity acquisition, e.g., the short-lived transcriptional repressor AUXIN-RESPONSIVE PROTEIN 27 (HaIAA27), the *cis*-regulatory element ARFAT (auxin response factor binding site), and *CYP79B2*, involved in a minor auxin biosynthesis pathway. Gibberellin-related proteins, such as the transcription factor HOMEOBOX25 (ATHB25; involved in GA biosynthesis control), or brassinosteroid (BR)-related proteins contribute to longevity. This is the case of the *Arabidopsis* BR-deficient mutants *cyp85a1*/*a2* and *det2* showing increased seed longevity (Zinsmeister et al., 2020).

QTLs for seed longevity have been investigated in several species subjected to different ageing conditions. Six *Arabidopsis* recombinant inbred line (RIL) populations were used to identify loci associated with seed longevity, with several *Germination Ability After Dry Storage* (*GAAS*) loci co-localized with *DOG* genes, showing an inverse correlation between storability and dormancy (Nguyen et al., 2012). Moreover, the study underlined a difference in longevity QTLs between the same accessions treated with long-term storage and CD, this indicating an incomplete overlapping of the genetic and metabolic pathways recruited in the seed response to these two ageing types (Nguyen et al., 2012), recently confirmed by another study that reveals that the EPPO method is the most similar to natural ageing when taking in consideration QTLs in *Arabidopsis* (Buijs et al., 2020). Also in barley, EPPO and CD show partially common QTLs but also distinct characteristic loci and genetic markers (Nagel et al., 2016). Moreover, loci associated with AA are related to floral and seed development, seed quality (germination, dormancy and yield), stress signalling, abiotic stress response and pathogen resistance in barley (Nagel et al., 2015). A QTL for seed longevity was found on chromosome 1A of bread

wheat (*Triticum aestivum* L.) and a QTL on chromosome 3B (*QRG.ipk-3B*, linked to number of grains per ear, thousand grain weight and coleoptile length, thus to seed and seedling vigour) of durum wheat (*Triticum durum* L.) (Arif & Börner, 2019; Börner et al., 2017). Regarding rice, within the QTL identified on chromosome 2, 45 genes potentially relevant to seed longevity mechanisms, such as antioxidant activity and DNA repair, were found in the *japonica* subspecies, and an additional QTL on chromosome 8 was recently found in a particularly long-lived variety of *O. sativa* (Liu et al., 2018; Raquid et al., 2021). Another study, taking in consideration both cultivated and semi-wild rice, unveiled that RNA modification, oxidation-reduction, protein-protein interactions and abscisic acid signal transduction play roles in seed longevity, with genetic markers on chromosomes 1, 3, 4 and 8 associating δ-tocopherol (vitamin E) with extended seed lifespan under AA (Lee et al., 2020). In maize, some QTLs identified after AA are linked to the energy metabolism, stress response, signal transduction, and protein degradation pathways (Wang et al., 2016). Interestingly, *Arabidopsis*, cabbage (*Brassica oleracea* L.) and tomato (*Solanum lycopersicum* L.) showed QTLs with the potential candidate gene *GolS* encoding galactinol synthase, a key enzyme in the synthesis of RFOs involved in the glassy state formation and stabilization, crucial for the acquisition of desiccation tolerance and longevity (de Souza Vidigal et al., 2016). The latter require a complex regulatory network associated with metabolic shutdown and storage material accumulation. These processes seem to be ABA-dependent in many species, and include the ABI3, FUS2, LEC1 and LEC2 functions (Kijak & Ratajczak, 2020).

Epigenetic stability is another aspect of seed longevity that still deserve exploration, as few data are currently available and only for a few species and ageing conditions. In general, cytosine DNA methylation (^{m}C) is an epigenetic modification fundamental for the regulation of gene expression in plants (Gehring & Henikoff, 2007). This kind of regulation allows plant cells to adapt to new conditions without affecting the DNA sequence, and to control development and responses to various environmental stresses (Mira et al., 2020). The DNA methylation profile changes during seed storage *in Mentha aquatica* L., in terms of both new methylation marks and demethylations, that could result in changes in gene expression (Mira et al., 2020). Also in rye (*Secale cereale* L.) increased epigenetic instability is observed in both aged seeds and seedlings (Pirredda et al., 2020). Interestingly, changes in the methylation states are detected also in plants regenerated from stored seeds, indicating that some of these alterations could be transmitted as adaptive transgenerational inheritance (Mira et al., 2020; Pirredda et al., 2020). In general, an increase in global DNA methylation level could prevent DNA damage, and therefore may contribute to desiccation tolerance, also observed when comparing the methylation profiles, of orthodox seeds subjected to conventional and cryogenic storage (Pérez et al., 2017; Plitta-Michalak et al., 2021). A recent study demonstrated that aged maize seeds possess increased DNA methylation levels, with genotype-dependent differences in methylation (Carvalho, 2020). Both the genetic and epigenetic processes involved in shaping seed longevity still need to be studied and fully clarified among the different plant species and ageing conditions.

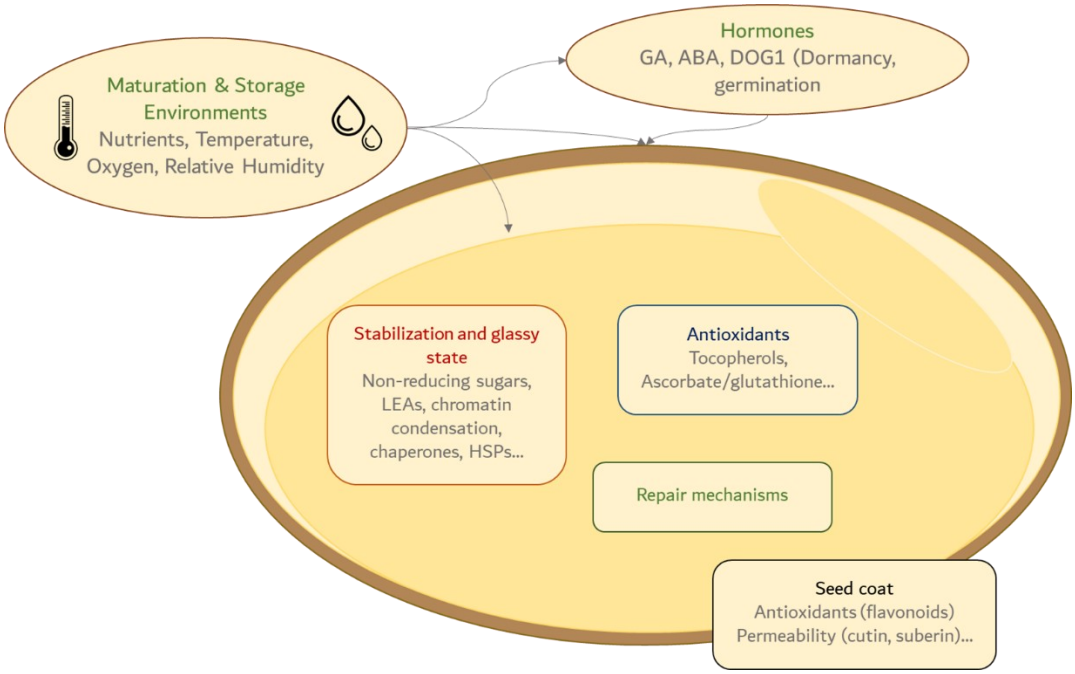


Figure 1.1 Schematic representation of the main factors, processes and biomolecules known to influence seed longevity (Adapted from Zinsmeister et al., 2020).

2. Aims of the work

The main goal of this PhD project was to study the different eco-physiological and molecular aspects of seed longevity, still poorly explored, in crops and crop wild relatives. The investigation focuses on the following crop species: maize, garden pea and wheat wild relatives, analysed in the context of seed banking, in order to expand the current knowledge about the dynamics of artificial and natural ageing. The experimental activity included three distinct research lines:

Project 1 – Comparison between AA and long-term storage in heteromorphic wheat wild relatives: *Aegilops tauschii* Coss., donor of the DD genome to the hexaploid bread wheat and *Triticum monococcum* subsp. *aegilopoides* Boiss., wild relative of the cultivated *Triticum monococcum* L. This activity was carried out at the Plant Biotechnology Laboratory-DBB (University of Pavia), in collaboration with Dr. Andreas Börner (IPK-Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany).

Project 2 - Screening of seed longevity in the CIMMYT maize collection. This activity was carried out during an 8-months Internship at the Genebank Viability Lab of the International Maize and Wheat Improvement Center, CIMMYT, Mexico (1st February-30th September 2020), part the Genetic Resources Program (GRP).

Project 3 – Molecular, physiological and ultrastructural aspects of seed longevity in *Pisum sativum* varieties subjected to different storage conditions. This activity was carried out at the Plant Biotechnology Laboratory-DBB (University of Pavia), in collaboration with Dr. Andreas Börner (IPK-Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany).

The different experimental systems were selected in order to obtain a wider picture of some common physiological processes involved in shaping seed longevity, i.e. oxidative stress in terms of antioxidant activity, ROS accumulation, oxidative damages and protective mechanisms. The different seed accessions were studied with a multidisciplinary approach, from the macroscopic analyses of germination profiles and seed phenotype to the microscopic exploration of nuclear/nucleolar ultrastructure and gene expression. Several biochemical compounds were measured in the different species, seed accessions and imbibition/ageing timepoints, with the purpose of exploring the ageing process over time and along with the activation of pre-germinative metabolism.

3. Materials and methods

3.1 The wheat wild relatives

3.1.1 Plant material

Caryopses (hereafter referred to as seeds) of *Ae. tauschii* and *T. monococcum* subsp. *aegilopoides* (for simplicity, hereafter referred to as *T. boeoticum*) were kindly provided by the Genebank Department of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben (Germany). In both species seed longevity loss has been investigated after 40 years of genebank bank storage at the IPK (-18 ± 2°C, 8 ± 2 % seed moisture content; hereafter referred to as "SB") and under AA condition (hereafter referred to as "AA"), using for the latter fresh seeds produced by plants of the same genotype held in genebank. Seed samples were regenerated at the experimental fields of IPK in Gatersleben (latitude 51° 49' 19.74'' N, longitude 11° 17' 11.80'' E, 110.5 m.a.s.l., black soil of clayey loamy type) and the collected seed lots are hereafter referred to as "fresh" or 0d. Seeds were extracted from the spikelets and sorted out according to the morph as previously described (Gianella et al., 2020): the larger, basal morph 'A' and the smaller, apical morph 'B'. After the cleaning process, seeds were kept in standard dry-room conditions at the Plant Germplasm Bank of the University of Pavia (Italy) at 15% relative humidity (RH) and15°C (ISTA, 2018) until use.

3.1.2 Germination tests and imbibition curves

Germination tests were carried out in triplicates of 20 seeds each in Petri dishes with 1% agar as substrate. Dishes were placed in temperature- and light-controlled incubators (LMS, Sevenoaks, UK) using a 12-h daily photoperiod (photosynthetically active radiation 40–50 µmol m^{-2} s^{-1}) at 20 °C (Guzzon et al., 2015). Petri dishes were checked every 12 h for germination, and seeds scored as germinated once the radicle had reached 2 mm length. At the completion of each germination test (4 weeks after sowing), non-germinated seeds were cut-tested to confirm whether they were empty. Imbibition curves were determined using 6 replicates of 5 seeds, placed in Petri dishes between double filter paper discs, moistened with dH_2O and sealed in plastic bags to avoid evaporation. Seeds were then withdrawn; the residual superficial water was removed with sterilized filter paper and seed mass was measured at 1h-intervals until radicle protrusion was observed. Water uptake was estimated by subtracting dry mass to the weight registered during imbibition and expressed as increase in % (Louf et al., 2018).

3.1.3 Controlled ageing test (CAT)

Seed longevity was tested with a modified protocol for AA from Newton (2009), according to Guzzon et al. (2018) Prior to storage, seeds were first rehydrated for 14 days in open Petri dishes over a non-saturated solution of LiCl in a sealed electrical enclosure box (Ensto UK, Southampton, UK) at 47% RH and 20 °C. Thereafter, seed equilibrium relative humidity (eRH) was verified with an AWDI0 water activity probe used in conjunction with a Hygro-Palm 3 display unit (Rotronic Instruments UK, Crawley, UK). Once the seeds had reached eRH, the initial germination was assessed using triplicates of

20 seeds and the protocol described above. Next, seed lots were stored in the dark in a sealed box over a non-saturated LiCl solution at 60% RH at 45 ± 2 °C, placed in a compact incubator (Binder FD53; Binder, Tuttlingen, Germany). When necessary, aliquots of distilled water were added to the LiCl to keep the RH at the required equilibrium, which was monitored inside the enclosure box (Tinytag View 2 Temperature/Relative Humidity Logger; Gemini Data Logger, Chichester, UK). At nine intervals during storage, three replicates of 20 seeds per lot were extracted and viability was tested with the same protocol as used for the preliminary germination test, until a complete viability loss was reached.

3.1.4 ROS detection

The fluorogenic dye 2',7'-dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich, Milan Italy) was used to quantify ROS levels released from dry 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 (DCF). The assay was carried as described by Forti et al. (2020) with the following modifications. Dry seeds were incubated in the dark for 30 min with 150 µl of a 10 µM DCF-DA solution. Subsequently, three replicates (50 µl each, per seed lot) were pipetted into 0.2 ml tubes and the emitted fluorescence was measured using the green channel (510 ± 5 nm) of a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia), after a single cycle of 30 s at 25°C. As negative control, three replicates containing only DCF-DA were used to subtract the baseline fluorescence. Relative fluorescence was calculated by normalizing samples to controls and expressed as Relative Fluorescence Units (R.F.U.).

3.1.5 qRT-PCR

The RNA extraction was performed according to Oñate-Sánchez & Vicente-Carbajosa, (2008) from dry, 1h- and 18h-imbibed seeds of *Ae. tauschii*, and from dry, 1h- and 14h-imbibed seeds of *T. boeoticum*. Retro-transcription was carried out using the iScript cDNA Synthesis kit (Bio-Rad, Milan, Italy), according to the manufacturer's instructions. qRT-PCRs were performed using SYBR Green qPCR Master Mix (2X) (Fermentas, Milan, Italy) and a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia). For oligonucleotide primer design, sequences were obtained from the online database EnsemblPlants: an alignment was performed between sequences from *Ae. tauschii* and *T. boeoticum* with the online software Multiple Sequence Alignment by CLUSTALW in order to select common and species-specific primer oligonucleotides used for the amplification of reference and target genes. The resulting oligonucleotide primers are listed in **Table 3.1.1**.

Table 3.1.1 List of oligonucleotide primers used for qRT-PCR analyses.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Species
Reference genes			
ACT Actin	CGACAGGATGAGCAAGGAGA	GAGGGAGGCGAGGATGGA	*Ae. tauschii*
ACT	TGCATAGAGGGAAAGCACG	AACCCAAAAGCCAACAGAGA	*T. boeoticum*
ARF ADP-ribosylation factor	CACCACCATCCCCACAATC	CCTCAACAACACGCTCTCTG	Both
GAPDH Glyceraldehyde-3-phosphate dehydrogenase	CGCAGAGATTCCTTGGGGTA	CACCACTTTCTTAGCACCACC	Both
ELF1 Elongation Factor 1 alpha	TGGTGGTTTTGAGGCTGGTA	TACTTGGGAGTGGTGGCATC	*Ae. tauschii*
ELF1	ATTAGGTTTGGTGGCGGGA	CGGTCGTTGTTGGAGTTGTC	*T. boeoticum*
RPT6 26S protease regulatory subunit	ATGTTGGCGAGGTTGTGAAG	GGGCAGGATAAGGTGAAGC	Both
UBI Ubiquitin	CGGTGGAGGTGGAGAGC	ACGAGGTGAAGCGTGGAC	Both
Target genes			
APX Ascorbate peroxidase	TCTCCTCCTTGATGGGCTC	GAAGAACTGCTCCCCGCTC	Both
CAT Catalase	AACTACCTGATGCTCCCCG	TCCTCGTCTTCTCCCTTTTTCC	*Ae. tauschii*
CAT	GCGTTGTCGTTGTTCCAGA	ATCGTTGCTTCCTCCGTTC	*T. boeoticum*
DHAR Dehydroascorbate reductase (cytosolic)	CCACACACACACAAGCAAGT	CCAAGGCGACCAAGGAGA	*Ae. tauschii*
DHAR	ATCACAGCAGGCATCTAAG	GCTCAGTATTTCCATCTTGTT	*T. boeoticum*
GSR Glutathione sulfo-reductase	ATCCTTGGTGGTTCTGGTGT	GTCGTGTGCCTTTGCTTTGA	Both
MDAR Monodehydroascorbate reductase (cytosolic)	ACCTTGCTCATCGCCACT	GCCTTCCCATCCTTCTTTGC	Both
SOD Superoxide dismutase	GTAAGCACAGCCACAGCC	CATCCCATCCCCCAAGTCAT	Both

To analyze the expression of genes encoding enzymes belonging to the ascorbate-glutathione ROS scavenging pathway (ascorbate peroxidase, APX, catalase, CAT, dehydro-ascorbate reductase, DHAR, glutathione reductase, GSR, mono-dehydro-ascorbate reductase, MDAR and superoxide dismutase, SOD) the geometric mean of two reference genes, was used as standard control. Quantification was carried out using the following reference genes for the experimental conditions (AA and SB) used in this work: *ARF* (*ADP-ribosylation factor*) and *ELF* (*elongation factor 1-alpha*) for *Ae. tauschii* AA and SB seeds, *ACT* (*actin*) and *ELF* for *T. boeoticum* SB, *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) and *UBI* (*ubiquitin*) for *T. boeoticum* SB. Selection was performed using the GeNorm algorithm (https://genorm.cmgg.be) (**Figures 3.1.1** and **3.1.2**). Oligonucleotide primers were designed with the online software Primer3Plus and thermodynamic parameters were checked with Oligoanalyzer (**Table 3.1.1**). qRT-PCR conditions were denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. Once aspecific fluorescence was subtracted, the raw fluorescence data obtained from the Software Rotor-Gene 6000 Series 1.7 (Corbett Robotics) were used to retrieve PCR Efficiency (E) and threshold cycle number (Ct) for each transcript quantification. The Pfaffl method (Pfaffl, 2001) was used for relative quantification of transcript accumulation and the two reference genes, selected for each species in the different ageing conditions, were used to normalize the values by calculating the ratio between target gene transcripts and the geometrical mean of the reference genes transcripts.

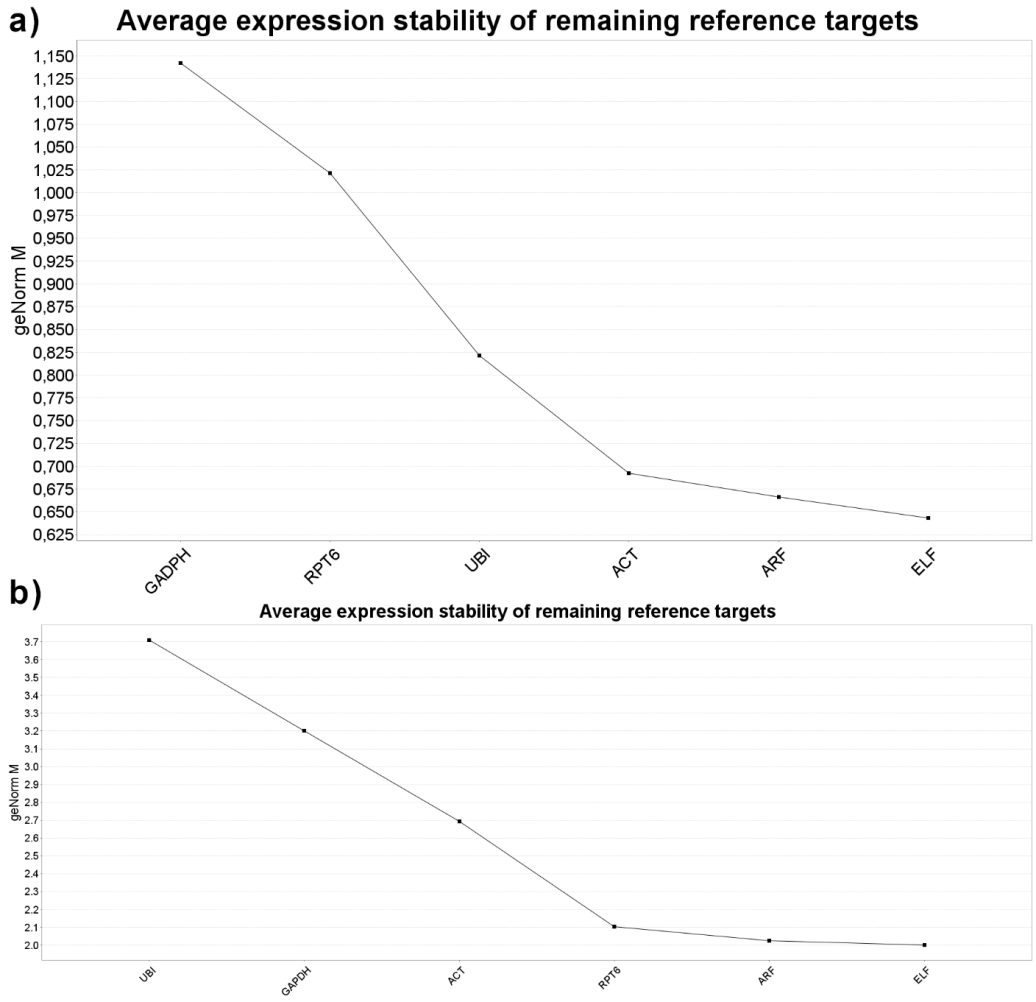


Figure 3.1.1 Selection of reference genes for qRT-PCR analysis in Ae. tauschii SB seeds (a) and AA seeds (b). Average expression stability values (M), calculated using GeNorm algorithm (https://genorm.cmgg.be), of the six putative reference genes analysed in dry and imbibed seeds throughout the tested time points of the experimental design. *ACT*, actin, *ARF*, ADP-rybosilation factor, *ELF*, elongation factor 1 alpha, *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase, *RPT6*, 26S protease regulatory subunit, *UBI*, ubiquitin. SB, seed bank ageing, AA, accelerated ageing.

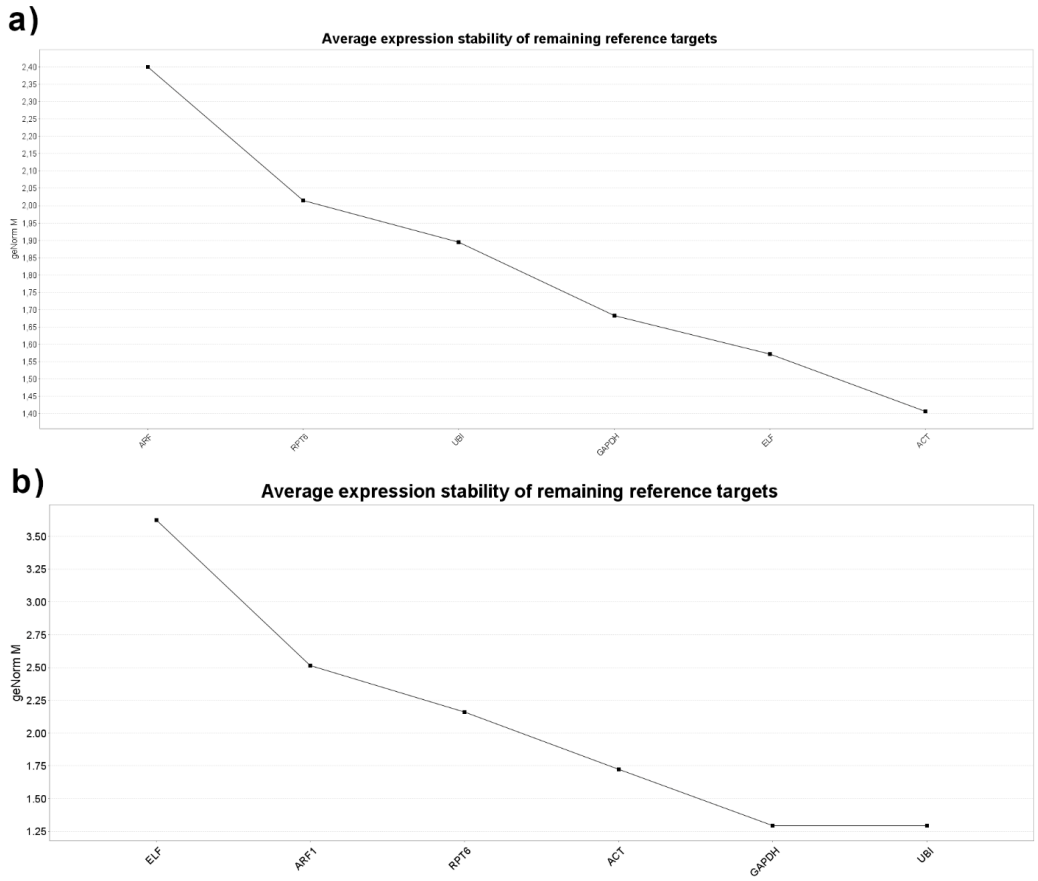


Figure 3.1.2 Selection of reference genes for qRT-PCR analysis in T. boeoticum SB seeds (a) and AA seeds (b). Average expression stability values (M), calculated using GeNorm algorithm (https://genorm.cmgg.be), of the six putative reference genes analysed in dry and imbibed seeds throughout the tested time points of the experimental design. *ACT*, actin, *ARF*, ADP-rybosilation factor, *ELF*, elongation factor 1 alpha, *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase, *RPT6*, 26S protease regulatory subunit, *UBI*, ubiquitin. SB, seed bank ageing, AA, accelerated ageing.

3.1.6 Statistical analysis

Statistical analysis was carried out in SPSS 21 and Genstat 9. Origin Pro 9.1 was used to build survival curves and heatmaps were plotted in the R software environment for statistical computing and graphics (v. 4.1.0). The R packages used are: '*tidyr*' (Wickham, 2021), '*dplyr*' (Wickham, 2021) and '*ggplot2*' (Wickham, 2016). Before analyses, data were checked for normality and homoscedasticity (Shapiro–Wilk's and Levene's tests, respectively). Probit analysis was carried out using GenStat 9 to obtain the time for viability to fall by 50% (p_{50}), used then as measure for seed longevity by fitting the viability equation (Ellis & Roberts, 1980) as previously described by (Gianella et al., 2020). P_{50}s were compared between seed lots belonging to the same species and subjected

to the same ageing conditions (AA or SB) using T student's tests. A GLM with gamma distribution, log link, was applied to compare the longevity profiles of the two morphs in the two species when subjected to the same ageing treatment. Heatmaps represent the Log2 fold change (Log2FC) of the relative gene expression and ROS levels of aged seed lots (AA and SB) compared to the controls, i.e., the maximum germination % of freshly harvested seeds (0d *Ae. tauschii*, 21d *T. boeoticum* due to after-ripening).

3.2. The maize collection at CIMMYT's genebank

3.2.1 Conservation conditions

Maize caryopses (hereafter referred to as 'seeds') were originally conserved in a seedbank located at the National School of Agriculture at Chapingo (Texcoco, Mexico) since 1943, in what can be considered the first germplasm bank of Latin America. After that, from the 1960s to 1971, the accessions included in this study were conserved in a temporary refrigerated seed storage facility (0 ± 5 °C) located in the basement of the National School of Agriculture at Chapingo University (Texcoco, Mexico), when they were moved to the first CIMMYT germplasm bank at its recently built headquarters in El Batán, Texcoco, Mexico. Here they were conserved in metallic cans with pressure lids at 0 °C and 45 % relative humidity, with the seeds dried below 10 % of moisture content prior to storage (CIMMYT, 1974, 1988). Starting in 1984, each seed collection was divided, and equal halves were moved to a base chamber (–15 °C) and an active chamber (0 °C) in metal cans. The two seed lots of the same accession conserved in the two chambers will be hereafter identified as: the 'active' seed lot and 'base' seed lot. In the second half of the 1980s, accessions in the base chamber were transferred to sealed aluminum envelopes. In 1996, the whole collection was moved to the current CIMMYT Germplasm Bank inside the Wellhausen-Anderson Plant Genetic Resources Center, where seeds are conserved in hermetic plastic flasks at –3 ± 2 °C (active chamber) and in hermetically sealed aluminum envelopes at –15 ± 3 °C (base chamber).

3.2.2 Study accessions

Germination tests of 987 seed lots from the base chamber were carried out for this study. Of these same accessions, 835 from the active chamber were also tested for germination. The number of seed lots tested for the active chamber is lower than that for the base chamber because some accessions in the active chamber did not have enough seeds to perform the germination test. Nineteen accessions were tested only in the active chamber since data from recent germination tests (carried out from 2016 to 2019) were available for the base chamber. Overall, a total of 1006 accessions from the base chamber were included in this study. One accession was tested only in the active chamber, due to lack of seeds in the base chamber. Overall, 855 accessions from the active chamber were tested in this experiment. The study accessions were chosen according to the following criteria: (1) having complete passport data including storage date, collecting site geographical information and information on the repropagation site); (2) having both initial and monitoring germination data; (3) being representative of the different grain types of maize (dent, floury, flint, popcorn and sweet) conserved in the CIMMYT collection; (4) being among the oldest collections for each grain type; and (5) maximizing the environmental range (in terms of latitude and elevation of the collecting sites) among

the study accessions. The study accessions originated from seed collections made in 33 different countries, covering an altitudinal range from 2 to 3919 m a.s.l. Considering the grain type of the study accessions: 363 were dent, 361 flint, 218 floury, 11 popcorn and 54 sweet. Popcorn and sweet accessions were less represented in the experiment in concordance with their overall lower representation in the collection. The ranges of storage dates for each grain type were as follows: dent (1965–1971), flint (1960–1974), floury (1969–1978), popcorn (1970–1996) and sweet (1968–2002). The average age of the accessions tested for this study was 48 years. More recent accessions of popcorn and sweet maize were used in the study due to the scarcity of older accessions of these grain types in the collection. The study seed lots are identified in the CIMMYT GRIN-Global database as well as marked with a special label (*Longevity project*) in the conservation chambers; those seed lots will not be substituted with new regenerations, with the hope that researchers can continue, in the next decades, to study the viability of this set. Only one accession was represented by seeds from its original collection. All other accessions had been regenerated in a Mexican or US location prior to conservation (11 regeneration locations in total). Historical initial germination data collected prior to storage were available for the study accessions, and the average initial germination was 99%. Moreover, results of an additional germination monitoring test were available, but only for the active chamber, since systematic germination monitoring of seeds conserved in the CIMMYT base chamber only began in 2012. Prior to the germination tests carried out for this study, seed mass and moisture content of the accessions (from both the active and base chambers) were measured. Seed mass was determined by weighing three replicates of 20 seeds, kept in one of the dry rooms of the CIMMYT Germplasm Bank at 9–15 °C and 10–20 % RH, randomly sampled from each seed lot, using an analytical balance (Adventurer Pro AV 8101, OHAUS, Parsippany, NJ, USA). The seed moisture content (MC) of the accessions was tested using a moisture meter (SL95, Steinlite, Atchison, KS, USA). The accession passport data considered in this study, besides the historical germination data, were grain type and colour, regeneration site, co-ordinates (latitude and longitude), elevation and climatic zone of the original collection site. Climatic zones of the collecting sites were based on the Köppen–Geiger climatic classification system (Bryant et al., 2017).

3.2.3 Germination tests

Three replicates of 20 seeds of each accession were sown in rolled filter paper (16.6 × 16.6 cm) moistened with distilled water. It was not possible to use a higher number of seeds or replicates in the germination tests due to the low seed number of some accessions. Filter paper rolls were inserted in transparent plastic trays, and the trays were randomly dispersed in an incubator (Biotronette plant growth chamber 844, Lab-Line) at a constant temperature of 25 °C and a 12 h photoperiod. Distilled water was added to the trays as needed, to avoid desiccation. Germination scoring was performed 1 week after sowing. A seed was considered to be germinated if it had developed into a normal seedling, according to ISTA (2018) criteria. A label with a unique, identifier QR code was attached to each paper roll. The germination scoring was performed through the app GB zone, that is connected to the GRIN-Global genebank database, by means of QR code scanners (two models: CS 4070, Symbol, Zebra, USA; and S740 2D, Socketmobile, USA) connected to a laptop or tablet device. The germination experiments were performed in March and April 2020 in the Seed Viability Laboratory of the CIMMYT

Germplasm Bank (Texcoco, Mexico).

3.2.4 Statistical analysis

Data analysis and graphic representation were carried out in the R software environment for statistical computing and graphics (v. 3.6.2). The R packages used for the analyses are: '*corrplot*' (Taiyun and Viliam, 2017), '*dplyr*' (Wickham et al., 2021), '*ecotox*', '*fmsb*' (Nakazawa, 2019), '*ggplot2*' (Wickham, 2016), '*ggpubr*' (Kassambara, 2020), '*lsmeans*' (Lenth, 2016), '*kgc*' (Bryant et al., 2017), '*multcomp*' (Hothorn et al. 2008), '*psych*' (Revelle, 2019) and '*statmod*' (Giner and Smyth, 2016). Before analyses, data were checked for normality and homoscedasticity (Shapiro–Wilk's and Levene's tests, respectively). Two Scheirer–Ray–Hare tests were applied to determine if mass and moisture content differed among accessions and conservation chambers (active and base). A Kruskal–Wallis test was used to determine if there were differences among grain types in terms of seed mass. Pairwise comparisons were carried out with the Bonferroni test. Multiple parameters were used to characterize seed longevity: final germination (germinated/sown seeds at the end of the germination test); and p_{50} and p85, corresponding to the time for viability to fall to 50 % and 85 % of the initial value (retrieved from historical, pre-storage data), respectively, estimated or predicted by logit analyses. Generalized linear models (GLMs) with binomial distributions, with logit as the link function, were carried out for p_{50} and p_{85} predictions of the accessions conserved in the active chamber. Logit was preferred over probit as link function, since logit showed a higher goodness of fit, compared by means of analysis of variance (ANOVA; $\chi 2$ test) and AIC (Akaike information criterion) scores, when compared with probit analysis (d.f. 0, deviance = –462.23, $P < 0.001$; AIC logit—probit: –460). Moreover, logit function, as previously observed by dos Santos et al. (2019) and de Faria et al. (2020), allows for a more reliable estimate/prediction of viability loss when compared with probit, especially in the tails of the distribution (i.e., < 10 % or >80 % germination), thus making it more suitable for the p_{85} estimation. Smaller differences between logit and probit models are observed in the central points of the function (50% germination, p_{50}). Longevity estimates were filtered for those accessions that showed a final germination lower than the initial and a p_{85} lower than the years of storage. This was done to estimate the p_{85} based only on observed data and not predictions, in order to avoid unrealistic values driven by the absence of viability loss in a significant percentage of the seed lots. Yamasaki et al. (2020) also highlighted this issue, suggesting that only longevity estimates within the storage period should be considered, while the reliability of a prediction extrapolated beyond the observation period needs further data accumulation and verification, especially considering that viability loss does not follow a linear pattern, but rather a sigmoidal one (Walters et al., 2005). Another parameter used to characterize seed longevity was the ageing rate (named L), calculated using the following formula:

$$L = \frac{G_i - G_f}{G_i} x \frac{1}{Y}$$

Where G_i is the initial germination in percentage, tested before storage; G_f is the most recent germination result in percentage; and Y corresponds to the storage time experienced by the seed accession, expressed in years. A GLM, with a binomial distribution and logit as link function, was applied to determine the effect of the

conservation in the two different chambers (active or base) on final germination, and a post-hoc Bonferroni test was used for pairwise comparisons of the same seed accession in the two conservation conditions. A Kendall's tau b correlation test was performed on germination in the active and base chambers, in order to assess if there was a correlation between the decline in viability in the two chambers, and, in particular, whether the decline in seed viability of any accession in the active chamber was similar to its decline in the base chamber. Monitoring intervals were calculated as one third of the p_{85}, with an upper limit set to 40 years as suggested by the Genebank Standards for Plant Genetic Resources for Food and Agriculture (FAO, 2014). A Kruskal–Wallis test was used to determine if there were differences among grain types in terms of monitoring intervals. Pairwise comparisons were carried out with the Wilcoxon rank sum test. A correlation plot was built based on a mixed correlation matrix for both active- and base-conserved seed lots, considering the following variables: ageing rate (L), seed mass and moisture content, grain type and colour, and regeneration site, as well as latitude, longitude, elevation and climatic zone (Köppen– Geiger) of the original collection sites. Moreover, a partial correlation analysis, using Kendall's test, was performed, for both the chambers, in order to evaluate the relationship of each single variable with the ageing rate (L), adjusting for the effect of the other variables. A GLM with a compound Poisson-gamma tweedie distribution was applied to assess the effect of grain type on the ageing rate (L).

3.3 *Pisum sativum* accessions from the IPK collection

3.3.1 Plant material and germination tests

Seeds of four yellow pea (*Pisum sativum*) accessions (PIS 2, PIS 8, PIS 15, and PIS 224) and four green pea accessions (PIS 686, PIS 706, PIS 783, and PIS 2865) were kindly provided by the Genebank Department of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben (Germany). Yellow and green accessions were renamed Y1, Y2, Y3, Y4 and G1, G2, G3, G4, respectively (**Table 3.3.1**).

Table 3.3.1 Main features of the eight *Pisum sativum* L. accessions investigated in this study. Y, yellow. G, green

Seed lot	Accession number (IPK)	Seed coat colour	Accession name	Country of origin	Biological status
Y1	PIS 2	Yellow	-	Greece	Landrace
Y2	PIS 8	Yellow	Nordost Kleine Weiße	Germany	Improved Cultivar
Y3	PIS 15	Yellow	Kronenerbse	Germany	Improved Cultivar
Y4	PIS 224	Yellow	Sperba	Germany	Improved Cultivar
G1	PIS 686	Green	Fügeder Erbse Monte Express	Unknown	Improved Cultivar
G2	PIS 706	Green	Mauthner-Fall Express	Germany	Improved Cultivar
G3	PIS 783	Green	Grüne Saxa	Germany	Improved Cultivar
G4	PIS 2865	Green	Frogel	Unknown	Mutant

For each accession, both fresh (F) seeds harvested in 2019, and stored seeds, harvested in 2001, were tested. As for stored seeds, one lot (A) was kept at cold chamber under controlled conditions (-18 ± 2°C, 8 ± 2 % seed moisture content) and another lot (R) was conserved at room temperature conditions (20 ± 2°C, 9 ± 2 % seed moisture content). Seed moisture content was measured using a moisture meter (Precisa XM 66, Switzerland). For each seed lot, three replicates of 20 seeds were sown in Petri dishes with 1% agar as substrate and then placed in a growth chamber (22 ± 2°C, 70-80 % RH, 150 μmol m^{-2} s^{-1} photon density, 16/8 h photoperiod). Petri dishes were checked every 12 h for germination and seeds scored as germinated once the radicle had reached 2 mm length. Germination was evaluated using the following parameters: G (germinability), MGT (mean germination time), MGR (mean germination rate), and Z (synchronization index), as described by Ranal & Santana (2006) (**Table 3.3.2**).

Table 3.3.2 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. N: total seed number; n_i: number of seeds germinated at the i^{th} observation; t_i: time at the i^{th} observation (hours); k: time at the end of the experiment; f_i: relative frequency of germination; $C_{ni,2}$: combination of the seeds germinated in the time i, two together.

Parameter	Definition	Formula	Limits	Unit
G	Germinability	$\boldsymbol{G} = \frac{(\sum_{i=1}^{k} \boldsymbol{n_i}) * \boldsymbol{100}}{\boldsymbol{N}}$	$0 \leq G \leq 100$	%
MGT	Mean germination time	$\boldsymbol{MGT} = \frac{\sum_{i=1}^{k} \boldsymbol{n_i t_i}}{\sum_{i=1}^{k} \boldsymbol{n_i}}$	$0 < MGT \leq k$	Hours
CVG	Coefficient of velocity of germination	$\boldsymbol{CVG} = \frac{\sum_{i=1}^{k} \boldsymbol{f_i}}{\sum_{i=1}^{k} \boldsymbol{f_i n_i}} * \boldsymbol{100}$	$0 \leq CVG \leq 100$	%
MGR	Mean germination rate	$\boldsymbol{MGR} = \frac{\boldsymbol{CVG}}{\boldsymbol{100}}$	$0 < MGR \leq 1$	$hour^{-1}$
Z	Synchronization index	$\boldsymbol{Z} = \frac{\sum_{i=1}^{k} \boldsymbol{C_{n_i,2}}}{\boldsymbol{C_{\sum n_i,2}}}$	$0 \leq Z \leq 1$	Unit-less

3.3.2 ROS detection

The fluorogenic dye 2',7'-dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich, Milan Italy) was used to quantify ROS levels released from dry 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 (DCF). The assay was carried out as described by Forti et al. (2020), with the following modifications. Dry seeds were incubated in the dark for 30 min with 500 µl of a 10 µM DCF-DA solution. Subsequently, three replicates (50 µl each) per seed lot were pipetted into 0.2 ml tubes and the emitted fluorescence was measured using the green channel (510 nm) of a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia), after a single cycle of 30 s at 25°C. As negative control, three replicates containing only DCF-DA were used to subtract the baseline fluorescence. Relative fluorescence was calculated by normalizing samples to controls and on the seed mass, then expressed as Relative Fluorescence Units (R.F.U.).

3.3.3 Determination of MDA levels

Malondialdehyde (MDA) levels were quantified according to Sari et al. (2012) and Zeb et al. (2016), with the following modifications. Dry seeds were grinded in a Retsch Mixer Mill M 301 (Retsch-Allee, Haan, Germany) three times for 30 s at the vibrational frequency of 30 Hz s^{-1}. For each lot, the resulting powder was divided into three replicates

(0.2 g each) that were resuspended with 5 ml of a H_2O: 0.5 M $HClO_4$ solution (4:1) with 2 % BHT (butylated hydroxytoluene, Sigma-Aldrich) in ethanol to precipitate proteins. Samples were subsequently centrifuged (4°C, 10 min). MDA was determined as a thiobarbituric acid reactive substance (TBARS), following its reaction with thiobarbituric acid (TBA, Sigma-Aldrich) at high temperature. For each sample, an aliquot of 100 µl was mixed with 100 µl of TBA in 1 ml dH_2O and the mixture was heated in a boiling water bath at 95°C for 60 min. Test tubes were cooled at room temperature and absorbance was measured at 254 nm using an UV-visible spectrophotometer (UV-1800, Shimadzu, U.K.). A standard MDA (Sigma-Aldrich) solution (100 µl, in a range of 0.025-0.1 mg ml-1) was added in a 1 ml test tube and mixed with TBA (100 µl) as previously described (**Figure 3.3.1a**).

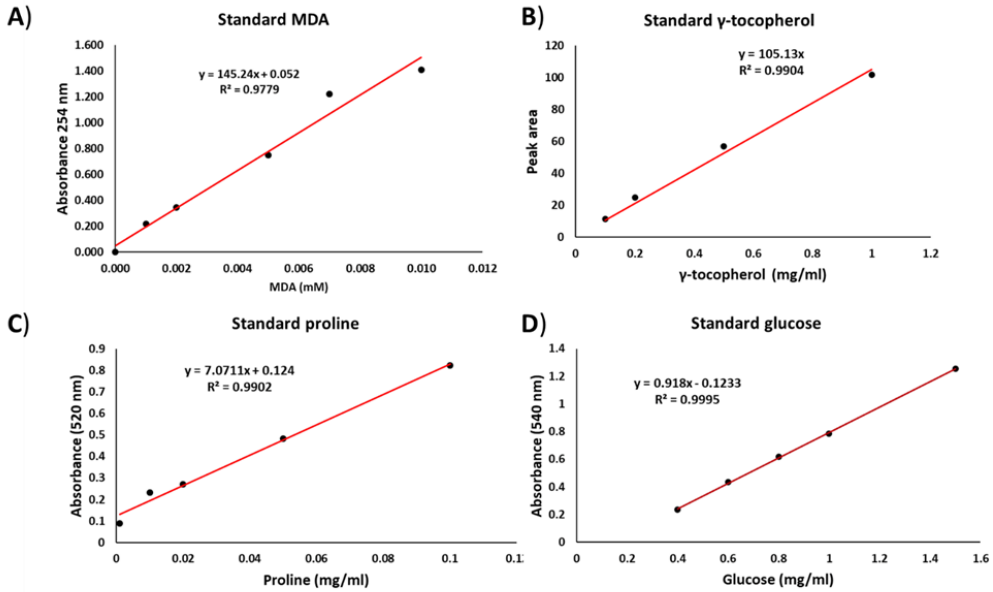


Figure 3.3.1 a) Malondialdehyde (MDA) standard curve measured at 254 nm using an UV-visible spectrophotometer (UV-1800, Shimadzu, U.K.). **b**) The γ-tocopherol standard curve measured using the HPLC system (Kontron Instrument 420 system) equipped with a C18 column (250 x 4.6 mm, 5 mm). **c**) Proline standard curve measured at 520 nm using an UV-visible spectrophotometer (UV-1800, Shimadzu, U.K.). **d**) Glucose standard curve measured at 540 nm using an UV-visible spectrophotometer (UV-1800, Shimadzu, U.K.).

3.3.4 Extraction and analysis of tocopherols

The extraction procedure was performed as described by Kurilich & Juvic (1999) and Doria et al. (2009) with the following modifications. Dry seeds were grinded as previously described. For each lot, an aliquot (0.5 g) of seed powder was added to 5 ml of ethanol containing 0.1 % butylated hydroxytoluene (BHT, Sigma-Aldrich) and the mixture was incubated for 10 min at 85°C. Subsequently, samples were subjected to saponification by adding 150 µl of 80 % KOH and incubated for 10 min. After adding 3 ml of H_2O, samples were placed on ice for 3 min, then 3 ml of pure hexane were added. After shaking 10 min at 800 rpm and centrifuging at 12.000 rpm, the upper phase was transferred into a separate test tube, and pellet was re-extracted using 2 ml hexane. The

combined hexane fractions were washed with 3 ml of dH_2O, vortexed, centrifuged for 10 min and transferred into another test tube. Hexane fractions were dried used a vacuum evaporator and the residue dissolved in 200 µl acetonitrile:methanol : dichloromethane 45:20:35 (v/v/v) prior to injection into a HPLC system (Kontron Instrument 420 system, Kontron Instruments, Munich, Germany) equipped with a C18 column (Zorbax ODS column 250 x 4.6 mm, 5 µl, Agilent Technologies). The isocratic mobile phase consisted of acetonitrile:methanol (60:40) (v/v), flow rate was 1.0 ml min-1 at room temperature and absorbance was measured at 220 nm. As standard, γ-tocopherol (Sigma-Aldrich) was used for a calibration curve and identified in the chromatogram (**Figure 3.3.1b**).

3.3.5 Determination of free proline content

Free proline content was measured as described by Abrahám et al. (2010) with the following modifications. Dry seeds were grinded as previously described. The seed powder (0.1 g) was added to 500 µl of 3 % sulfosalicylic acid (Sigma-Aldrich). Following centrifugation at 13.000 rpm for 5 min, a 100 µl aliquot of the extract was added to 500 µl of 3 % sulfosalicylic acid: glacial acetic acid: acidic ninhydrin (1:2:2) (v/v/v). The reaction of ninhydrin with free proline was carried out at 96°C for 60 min and stopped on ice. Samples were then extracted with 1 ml of toluene. After 20 s vortex, phases were allowed to separate. The upper phase was transferred to quartz cuvettes and absorbance was read at 520 nm using an UV-visible spectrophotometer (UV-1800, Shimadzu) and toluene as reference. A standard proline solution (100 µl, in a range of 0.001-0.1 mg ml^{-1}) was prepared, added to a 2 ml test tube and mixed with the ninhydrin solution as previously described (**Figure 3.3.1c**).

3.3.6 Spectrophotometric determination of reducing sugars

The content of reducing sugars was measured as described by Miller (1959).Dry seeds were grinded as previously described. The seed powder (0.5 g) was added to 5 ml of dH2O, vortexed and incubated 2 h at 80°C in a water bath. After centrifugation at 3500 rpm for 15 min, the upper phase was transferred in new test tubes and the content of reducing sugars was quantified using DNS (3,5-dinitrosalycilic acid, Sigma-Aldrich) solution. Absorbance was read at 540 nm using an UV-visible spectrophotometer (UV-1800, Shimadzu) and dH2O as reference. Standard solutions of glucose in the range of 0.4-1.5 mg ml^{-1} were used (**Figure 3.3.1d**).

3.3.7 Thermogravimetric analysis and differential scanning calorimetry

Inorganic content and thermal decomposition of the pea seed biomass were investigated by thermogravimetric analysis (TGA) in an air and nitrogen environment, respectively, using a Mettler Toledo TGA 1 instrument with a fixed heating rate of 20°C min^{-1}. The temperature range was from 25 to 800°C with a gas flow in the oven (air or nitrogen) of 4 l h^{-1}. The samples were grounded into powder and sieved to a size of 100 µm. About 5 mg of sample was used in each test.

3.3.8 Nuclear staining with Toluidine Blue

Embryos excised from dry seeds of Y1 and G4 accessions were fixed with 2 %

paraformaldehyde/0.2 % glutaraldehyde (Sigma-Aldrich) for 3 h at 4°C. Embryos were rinsed in phosphate-buffered saline (PBS, pH 7.2) overnight, and then incubated in 0.5 M NH4Cl for 30 min at room temperature. Semithin sections (500 nm in thickness) were cut using an ultramicrotome, embedded in acrylic LR-White resin (Agar Scientific, Stansted, UK) and allow to harden at 60°C overnight. Toluidine blue staining was performed by covering the tissue sections, prepared as previously described, with a drop of the die and incubating for 5 min at 100°C. Sections were then washed thoroughly with dH2O to remove die excess, airdried, mounted in Mowiol (Sigma Aldrich) and finally imaged using Zeiss Axioskop 2 plus microscope.

3.3.9 Immunodetection of γH2AX foci

In order to detect the occurrence of γH2AX foci in the nucleus, sections prepared as previously described were subjected to indirect immunohistochemical reaction by incubating them with the primary antibody Phospho-Histone H2A.X (Ser139) Polyclonal Antibody from rabbit (ThermoFisher Scientific, Milan, Italy) according to the Supplier's suggestions and subsequently with a secondary antibody coupled with 12 nm colloidal gold grain. Sections were stained by EDTA regressive technique and observed with a Jeol JEM-2100Plus electron microscope equipped with a 30 mm objective aperture and operating at 80 kV. Images were submitted to morphometric analyses using the software ImageJ (https://imagej.nih.gov/ij). The results are expressed as mean values ± SEM. For each sample, 10 nuclei were scored for the presence of γH2AX foci. The density of foci was calculated as follows: 100 squares (each one with an area of 400 nm2) were identified and the number of foci per single area was counted. The measurement was performed considering 10 cells for each sample and 10 squares per single cell.

3.3.10 Statistical analysis

Statistical analysis was performed in IBM SPSS 21.0 and in R environment for statistical computing and graphics (studio version 4.0.2). The following packages were used: '*dplyr*' (Wickham et al., 2021), '*ggplot2*' (Wickham, 2016), '*corrplot*' (Wei & Simko, 2017), '*multcomp*' (Hothorn et al., 2008), and '*lsmeans*' (Lenth, 2016). After checking data for normality and homoscedasticity, GLMs were applied to evaluate the effect of accession, type of conservation, their interaction on different variables (germination parameters, ROS levels, chlorophyll content, tocopherols, MDA, free proline, reducing sugars); post-hoc Tukey's or Bonferroni tests were used to perform multiple comparisons. A two-way ANOVA was used to determine the effect of accession and conservation on the temperature of glass transition (Tg). A heatmap was used to represent the Log2 fold changes (Log2FC) between fresh (F) and aged (A, R) seeds in terms of mean MDA, tocopherols, free proline and reducing sugars contents. Correlations were performed with Pearson or Kendall's Tau-b tests. Statistical analysis to quantify the occurrence of γH2AX foci in the nucleus was performed using the two-tailed paired Student's t test.

4. Results

4.1 Wheat wild relatives

4.1.1 Imbibition and germination

Fresh seeds of Ae. tauschii and T. boeoticum were subjected to imbibition and germination tests in order to select the appropriate timepoints for subsequent analyses. Both species showed the quickest water absorption in the time interval between imbibition and 1 h of soaking (**Figure 4.1.1**).

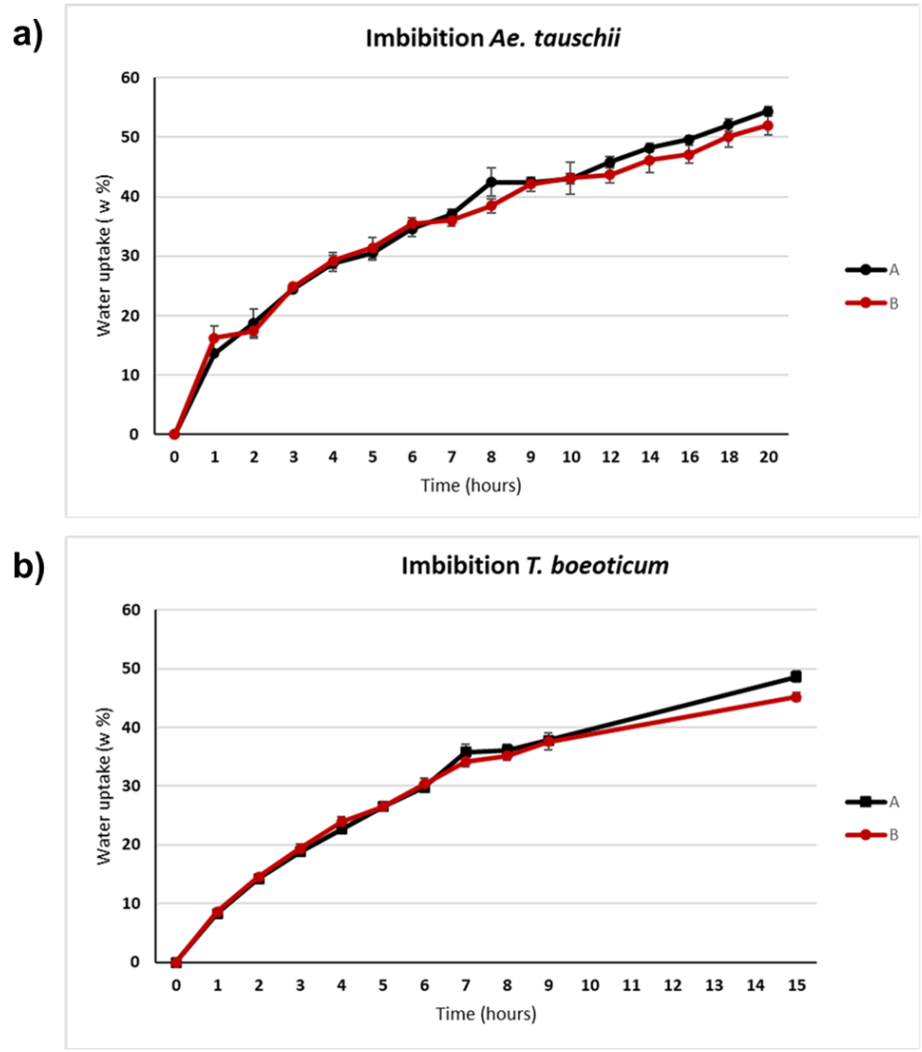


Figure 4.1.1 Imbibition curves of (**a**) *Ae. tauschii* and (**b**) *T. boeoticum* seeds. Seeds were imbibed with dH_2O on filter paper, then withdrawn 1 hour-intervals until radical protrusion was observed. Water uptake was estimated by subtracting dry mass to the weight registered during imbibition after residual superficial water was removed, and then expressed as increase in %.

Thus, the 1 h-interval was selected in order to analyze the ROS content and gene expression after the activation of the pre-germinative metabolism induced by water absorption. 1h imbibition is also the endpoint of the so-called 'abrupt imbibition' in wheat, corresponding to the embryo water uptake, followed by the water absorption in the other seed parts (Lev & Blahovec, 2017). Another time point was selected, corresponding to two hours before the first radicle protrusions were observed for *Ae. tauschii* (18 hours after imbibition) and for *T. boeoticum* (14 hours after imbibition). This was done in order to select an appropriate timepoint for the analysis of the late stage pre-germinative metabolism, while avoiding the comparisons among different phenological states (seeds vs radical protrusions). Unlike *Ae. tauschii*, which did not show any after-ripening requirement, both morphs of *T. boeoticum* reached the maximum germination percentage after 21 days of AA treatment. Therefore, this timepoint was chosen as control for the subsequent analyses, while 0 days was chosen as control for *Ae. tauschii*. See **Table 4.1.1** for the controls and ageing intervals of the different seed accessions tested in this study.

Table 4.1.1: **a**) ageing intervals of the *Ae. tauschii* and *T. boeoticum* seed accessions tested in this study: seeds were tested fresh, artificially aged, AA (method: controlled ageing test, CAT) and aged in cold storage (SB, seed bank). d=days; y=years. **b**) harvest year of the accessions aged in the cold chambers. Control=seed lots used as controls in the molecular analyses, fresh (0d) for *Ae. tauschii*, after-ripened (21d) for *T. boeoticum*.

a)	***Ae. tauschii* (AE 278)**	***T. boeoticum* (TRI 10061)**
	Ageing time	
Control (d)	0	21
Artificially aged, AA (d)	14, 21, 29, 35, 42, 49, 56, 63, 70	14, 21, 28, 35, 42, 49, 56, 63, 70
Aged in cold storage, SB (y)	10, 11, 17, 19, 40	14, 17, 38
b)	***Ae. tauschii* (AE 278)**	***T. boeoticum* (TRI 10061)**
Cold-stored accessions	1978, 1999, 2001, 2007, 2008	1980, 2002, 2005

4.1.2 SB and AA

P_{50} was calculated for the two species in the SB and AA conditions and the survival curves fitting the viability equations are represented in **Figure 4.1.2**.

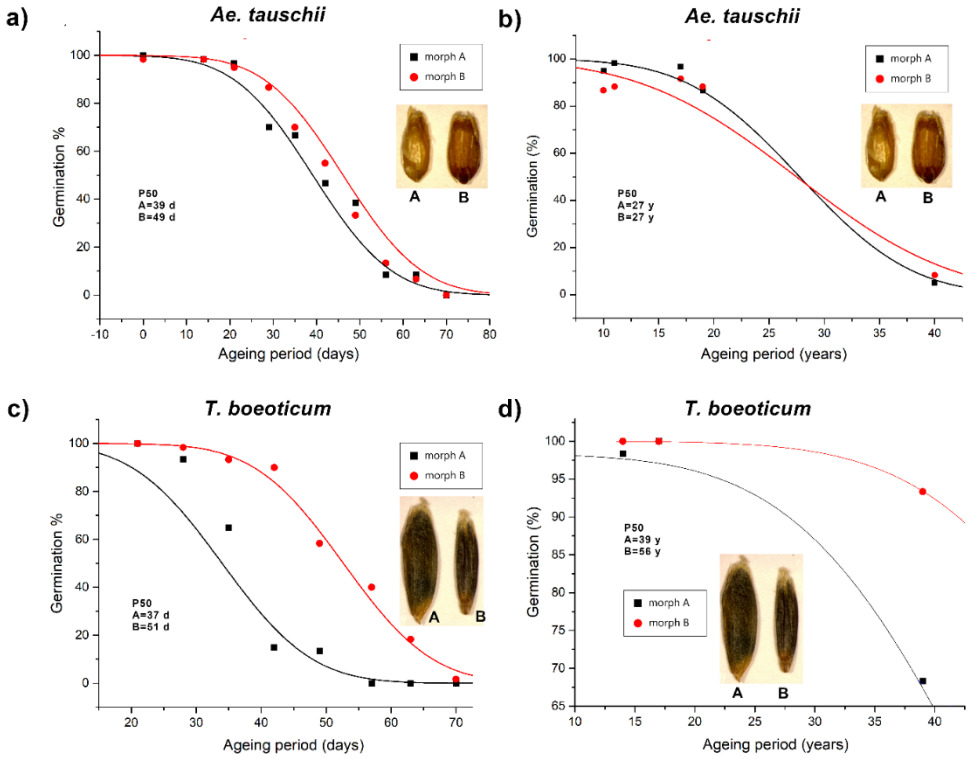


Figure 4.1.2 Survival curves fitted by probit analysis of *Ae. tauschii* **a**) AA seeds, **b**) SB seeds, and *T. boeoticum* **c**) AA seeds, **d**) SB seeds. AA = AA, SB = ageing in seed bank conditions (cold storage)

When subjected to AA, both species showed significant differences in terms of p_{50} among morphs: *Ae. tauschii*, A = 39.66 d, B = 49.55 d, $P < 0.05$, **Figure 4.1.2a**; *T. boeoticum*, A = 37.02 d, B = 51.49 d, $P < 0.001$, **Figure 4.1.2c**. In SB conditions, *Ae. tauschii* showed no differences among the two morphs (A = 27.76 y, B = 27.49 y, P = 0.665, **Figure 4.1.2b**), while the two morphs of *T. boeoticum* showed significantly different longevity profiles (A = 38.95 y, B = 55.66 y, $P < 0.001$, **Figure 4.1.2d**). Thus, *Ae. tauschii* morphs showed different longevity profiles when subjected to SB and AA. Also, the two species showed similar lifespans when subjected to AA (GLM, species*morph Wald Chi-squared=3.071, P=0.08), while both morphs of *T. boeoticum* were largely longer-lived than those of *Ae. tauschii* during SB storage: the species*morph interaction had a statistically significant effect on p_{50} (Wald Chi-squared=27.402, $P<0.001$), with morph A and B of *T. boeoticum* significantly longer-lived than morph A and B of *Ae. tauschii*, respectively (Bonferroni post hoc, both $P<0.001$).

4.1.3 ROS accumulation in aged seeds

ROS content was evaluated in AA and SB seeds, and the accumulation relative to the controls was represented by means of heatmaps in **Figure 4.1.3** (AA) and **Figure 4.1.4** (SB). Hereafter, when referring to 'lower' or 'higher' contents, "increase" or "decrease", a statistically significant difference was found ($P < 0.05$).

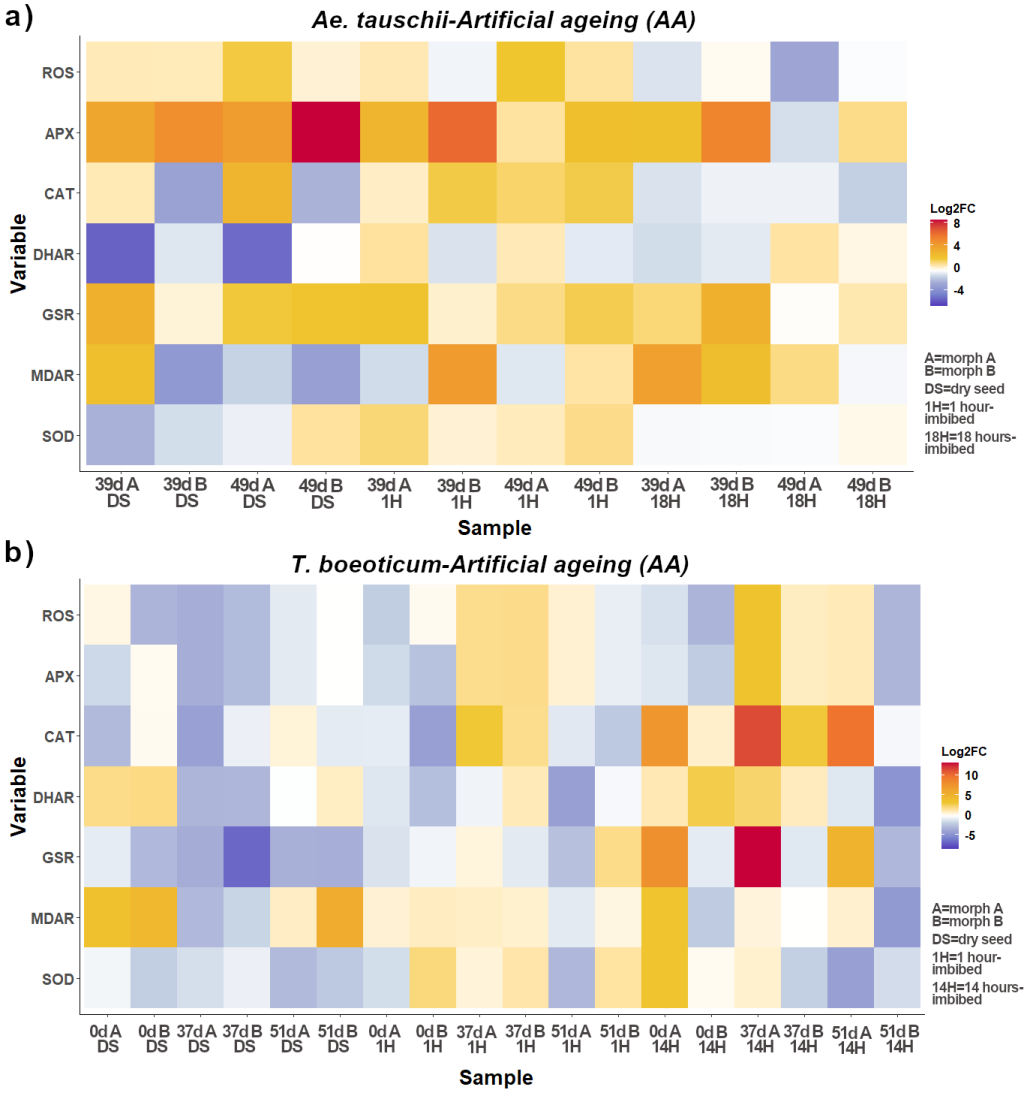


Figure 4.1.3 Heatmaps representing the Log2 fold-changes, i.e, ratios, of ROS levels and relative gene expression in **a**) Ae. tauschii and **b**) T. boeoticum artificially aged (AA) seeds compared to the fresh controls (a, 0d; b, 21d). In **b**), ROS levels and gene expression were compared to controls also for 0d seeds, i.e., before after-ripening. APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; GSR, glutathione sulfo-reductase; MDAR, mono-dehydroascorbate reductase; SOD, superoxide dismutase. a) 39d, 39 days of AA, corresponding to the p_{50} of morph A. 49d, 49 days of AA, corresponding to the p_{50} of morph B. **b**) 0d, fresh, dormant seeds before the AA treatment. 37d, 37 days of AA, corresponding to the p_{50} of morph A. 51d, 51 days of AA, corresponding to the p50 of morph B.

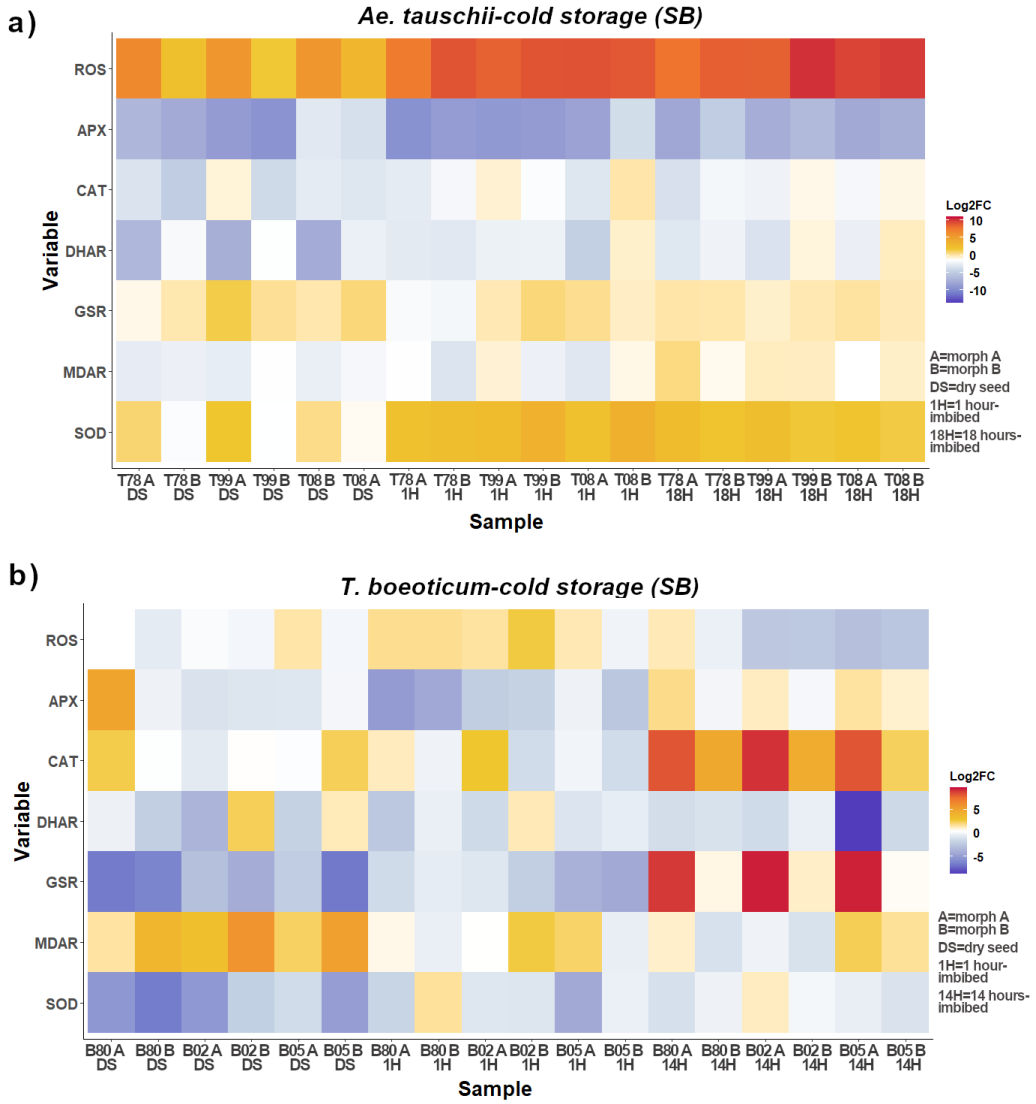


Figure 4.1.4 Heatmaps representing the Log2 fold-changes, i.e., ratios, of ROS levels and relative gene expression in **a**) *Ae. tauschii* and **b**) *T. boeoticum* seeds aged in seed bank conditions (cold storage, SB) compared to the fresh controls (a, 0d; b, 21d). *APX*, ascorbate peroxidase; *CAT*, catalase; *DHAR*, dehydroascorbate reductase; *GSR*, glutathione sulfo-reductase; *MDAR*, mono-dehydroascorbate reductase; *SOD*, superoxide dismutase. **a**) *Ae. tauschii*: T78, seed accession harvested and stored in 1978; T99, 1999 seed accession; T08, 2008 seed accession. **b**) *T. boeoticum*: B80, 1980 seed accession; B02, 2002 seed accession; B05, 2005 seed accession.

In both species, ROS accumulation was observed along with AA compared to the controls (**Figure 2a, b**) at 1h after imbibition in both morphs, while at the end of the pre-germinative metabolism a significant decrease was observed except for aged seeds A of *T. boeoticum*. Also, in dry seeds of *Ae. tauschii* a higher ROS accumulation was observed when compared to the controls (**Figure 4.1.3a**), while in *T. boeoticum* both fresh and aged seeds showed lower ROS levels compared to the after-ripened controls (**Figure 4.1.3b**). Regarding SB, a very strong ROS accumulation was observed in both morphs of

Ae. tauschii, slightly less evident in morph B and maximum at the beginning of the pre-germinative metabolism (**Figure 4.1.4a**). In *T. boeoticum* SB seeds the highest increase was again observed at 1 h after imbibition, while ROS levels showed a decrease in the late stages of pre-germinative metabolism in the more recent seed lots (**Figure 4.1.4b**). Thus, a more consistent pattern among timepoints and imbibition states was observed during SB ageing, while AA conditions showed more variables consequences in terms of ROS accumulation. In general, in all the ageing conditions a higher ROS accumulation was observed after the activation of the pre-germinative metabolism (1 h after imbibition), while at the late stage a lower level was observed in *T. boeoticum* SB seeds and *Ae. tauschii* AA seeds. Indeed, high ROS levels were observed in SB seeds of *Ae. tauschii* also at 18 h after imbibition.

4.1.4 Expression profiles of genes involved in H_2O_2 scavenging in AA seeds

Hereafter, when referring to 'lower' or 'higher' contents, "increase" or "decrease", a statistically significant difference was found ($P < 0.05$). The expression levels of the genes belonging to the H_2O_2 scavenging pathway were measured in AA seeds of *Ae. tauschii* (**Figure 4.1.3a**). *GSR* and *APX* transcripts showed an increase in all the imbibition states in the two morphs during ageing compared to the controls. *CAT* mRNA was detected at higher levels in dry seeds A at 39 and 49 d of AA (0.37 and 1.75 Log2FC, respectively), with a subsequent increase at 1h after imbibition in both morphs followed by a decrease in the late stage of pre-germinative metabolism. *DHAR* mRNA showed lower levels in morph A dry seeds compared to the control (39d=-2.65 and 49d=-2.05 Log2FC), and little variations at 1h and 18h after imbibition in both morphs compared to the controls. Also, the *SOD* transcript showed little variation at the late stage of imbibition, while accumulation was observed at 1h in both morphs (in the range of 0.6-0.7 Log2FC). The *MDAR* gene showed very variable expression profiles in the different morphs, imbibition stages and ageing timepoints.

In *T. boeoticum* (**Figure 4.1.3b**) dry seeds at 0d, i.e. before the after-ripening in the incubator, only *MDAR* (2.88 Log2FC) and *DHAR* (0.36 Log2FC) genes showed higher expression levels compared to the controls, while the other four genes revealed lower transcripts levels (in the range –0.67 to -1.74 Log2FC). At 1h after imbibition seed A and B 0d showed generally lower gene expression levels compared to the control (-0.14 to -1.82 Log2FC). At 14 h after imbibition, seed A showed higher transcript levels for all the tested genes except *APX*, while seed B showed only upregulation of *DHAR* gene (3.10 Log2FC). At p_{50} of seed A, 37d, both morphs at the dry state showed lower transcript levels compared to the control, while in seed A higher expression levels were observed at both the start and the end of the pre-germinative metabolism, with a notably higher expression of *GSR* at 14 h (11.37 Log2FC). Morph B showed a higher quantity of *SOD* transcripts at 1h after imbibition, and an upregulation of *DHAR* and *CAT* genes at 14 h. At p_{50} of seed B, 51d, little variation was observed in dry seeds compared to the controls, except for *MDAR* gene, more expressed in morph B (3.09 Log2FC). At 1h after imbibition transcript levels were higher in seed B and lower in seed A compared to the controls, while, on the contrary, at 14 h gene expression was higher in seed A (especially for *GSR* and *CAT* genes) and lower in seed B.

4.1.5 Expression profiles of genes involved in H_2O_2 scavenging in SB seeds

The expression levels of the genes involved in the H_2O_2 scavenging pathway were measured in SB seeds of *Ae. tauschii* (**Figure 4.1.4a**). Notably, both *APX* and *SOD* gene expression patterns were consistent with the ROS levels measured in aged seeds. Compared to the controls, *APX* transcript levels resulted lower (in the range -2.87 to -9.05 Log2FC) whereas *SOD* transcript levels were higher (in the range 1.27 to 3.79 Log2FC), proportionally decreasing or increasing, respectively, along with ROS levels and imbibition states. In dry seeds, *CAT, DHAR* (only morph A) and *MDAR* genes showed lower expression levels compared to the controls, while *GSR* showed higher transcript levels. At 1 h after imbibition, expression decreased in the oldest seed lots compared to the fresh controls, except for the more recent seed lots (T08, 2008), that showed higher levels especially in morph B, and except for the *GSR* gene, more expressed also in the T99 (1999) seed lots (0.63-0.82 Log2FC). *GSR* was also highly expressed at 18 h after imbibition, while *DHAR* and *CAT* genes revealed lower expression levels except for morph B of the T99 and T08 seed lots. Also, the *MDAR* gene showed higher expression levels but with a more variable trend in the different morphs of the aged seed lots.

In *T. boeoticum* (**Figure 4.1.4b**) dry seeds aged in SB conditions, *SOD* and *GSR* transcript levels were lower compared to the controls, while the *MDAR* gene showed higher expression levels. *CAT* and *APX* transcript levels showed low variations compared to the controls, except for morph A of the oldest seed lot (B80, 1980), that showed higher expression levels (*APX*=2.31 and 2.69 *CAT*=Log2FC). *DHAR* gene expression was generally lower except for morph B of the 2002 seed lot (B02). At 1H after imbibition, lower transcript levels were observed compared to the controls, except for *CAT* in morph A of the1980 and 2002 seed lots, *SOD* and *DHAR* in the morph B of 1980 and 2002 seed lots, respectively, and for *DHAR* in morph B of the 2002 lot and morph A in the 2005 lot. At 14 h after imbibition, higher *GSR* and *CAT* transcript levels were observed more evident in morph A (3.15 to 9.42 Log2FC). *DHAR* and *SOD* mRNA levels were lower in all the aged seed lots, except for *SOD* in morph B of the 2002 seed lot. *MDAR* gene expression was higher in the more recent seed lot (B05) compared to the controls (A=1.64, B=1.91 Log2FC).

In *Ae. tauschii* a consistent pattern of the *GSR* gene expression among ageing treatments and imbibition states was observed. In *T. boeoticum* the expression pattern is similar among imbibition states, in particular in the higher expression of *GSR* and *CAT* at 18 h after imbibition.

4.2. The maize collection at CIMMYT's genebank

4.2.1 Seed mass and moisture content

Seed mass differed among accessions (d.f. 841, $H = 4844.662$, $P < 0.001$), ranging from 1.43 ± 0.05 to 14.53 ± 0.15 g in the different study accessions. Seed mass was not significantly different between the seed lots of the same accession conserved in the two chambers (d.f. 841, $H = 99.64$, $P = 1$). Seed mass was different among grain types (d.f. 4, $H = 1091.7$, $P < 0.001$); the highest values of seed mass were detected in floury

accessions (mean seed mass 7.3 ± 1.2 g), followed by dent (6.6 ± 1.5 g), flint (4.7 ± 1.2 g), sweet (4.7 ± 0.9 g) and popcorn (2 ± 0.4 g). Moisture content differed among accessions and between chambers (respectively d.f. 841, H = 3110.262, P < 0.001; d.f. 1, H = 982.336, P < 0.001), ranging from 4.98 to 15.16 % (average = 9.42 ± 1.95 %) in the base chamber and from 5.15 to 15.48 % (average = 11.00 ± 1.46 %) in the active chamber.

4.2.2. Final germination: active and base chambers

The final germination data obtained from the experiment showed significant differences among accessions, the two conservation chambers (active and base) and their interaction (**Table 4.2.1**).

Table 4.2.1 Analysis of deviance of generalized linear model (GLM) with binomial distribution and logit link function: model effects on germination percentage of 835 maize accessions conserved in 2 different chambers.

	Df	Deviance	Resid. Df	Resid. Dev	P value
Accession	834	10003.77	4173	9868.27	<0.001
Chamber	1	3261.02	4172	6607.25	<0.001
Accession*Chamber	834	2923.84	3337	3678.53	<0.001

Among the 835 accessions tested from both active and base chambers in this experiment, 284 showed a significant difference in germination between the two chambers (34.01 % of total). Of the 284 statistically significant pairwise comparisons, 275 accessions showed a higher germination in the base chamber (96.83 % of the comparisons). The remaining nine accessions, with a better performance in the active chamber, accounted for only 3.17 % of comparisons. Considering all accessions tested in this experiment from both chambers, the average germination was 92.1 ± 9.1 % for the seed lots conserved in the base chamber and 81.4 ± 16.3 % for the seed lots conserved in the active chamber (**Figure 4.2.1**).

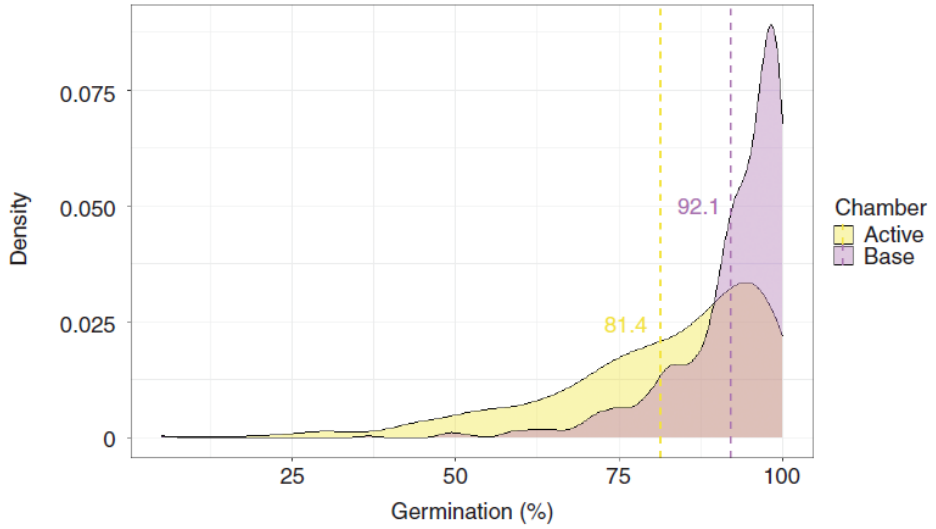


Figure 4.2.1 Density plot representing the frequency of seed lots showing different values of germination percentage on a continuous scale, divided by conservation chamber (active and base). Dashed lines represent the average germination percentage for the two chambers (intercept).

In the base chamber, 84.5 % of the seed lots showed a final germination percentage above the threshold of 85 % of the initial germination value (FAO, 2014), while in the active chamber the final germination of 53 % of the seed lots was above this threshold. Moreover, in the active chamber, we detected twice the number of seed lots in the final germination range between 70 and 85 % of the initial compared with the base chamber, and about seven times the number in the base chamber between 50 and 70 %. Only four seed lots below 50 % of the initial germination were found in the base chamber (accounting for 0.4 % of the total), while 44 seed lots were found in the active chamber (5.2 %, **Table 4.2.2**).

Table 4.2.2 Frequencies of the tested accessions in active and base chambers divided into 4 classes based on germination % ranges.

Germination %	% of acc. Active	% of acc. Base
>85%	53	84.5
70-85%	27.3	12.8
50-70%	14.6	2.2
<50%	5.2	0.4
Total	100	100

Considering the 835 accessions tested for both the active and base chambers, 47.8 and 13.9 % of the seed lots in the active and base chambers, respectively, showed a final germination below the 85 % threshold of the initial germination and therefore need regeneration (FAO, 2014). Based on a Kendall's test, a positive correlation was found between the germination of the seed lots of the same accession in the active and base chambers (Tau b = 0.35, $P < 0.01$), indicating a similar behaviour, in terms of viability loss, of the same accession conserved in the two different chambers.

4.2.3 P_{50} and p_{85}

The p_{50} and p_{85} predictions were performed only for the active chamber for which historical data on seed germination were available. Three viability data points were available and used in the analysis: initial germination, an intermediate point (corresponding to a viability monitoring test performed between 1985 and 2011, depending on the accession) and the germination data obtained from the current experiment. Of the total 855 active accessions, logit analysis successfully predicted p_{85} and p_{50} for 400 accessions (46.78 % of the total). p_{85} spanned between 4.2 and 54.4 years, with an average of 37.6 years; p50 values of the same accessions were between 16.5 and 91 years, with an average of 60 years. The observed differences among accessions in longevity estimates were statistically significant (d.f. = 854, residual deviance = 8245.112, $P < 0.001$). Due to the fact that reliable longevity estimates (p_{85} and p_{50}) could not be calculated for all of the accessions, the ageing rate (L) was selected as the longevity indicator for subsequent analyses, making it possible to also include accessions that did not show a decrease in terms of germination across time (L = 0).

4.2.4 Correlations

Correlation plots were made for both 'active' and 'base' seed lots, based on mixed correlation matrices (**Figure 4.2.2**).

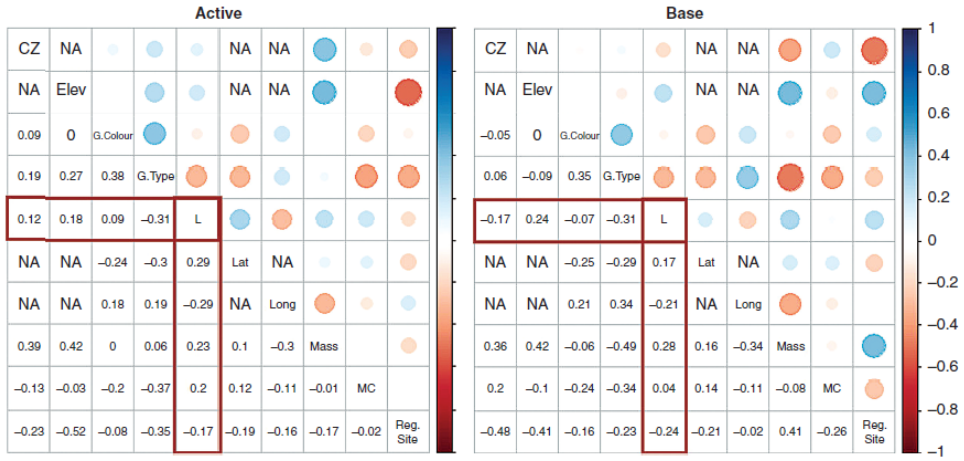


Figure 4.2.2 Correlation plots of active and base chambers. Coefficients of correlation are represented by numbers in the lower part of the graph, and by colours in the upper part. Continuous variables: elevation (Elev), ageing rate (L), latitude (Lat), longitude (Long), moisture content (MC) and mass. Polytomous variables: Köppen–Geiger climatic zone (CZ), grain colour (G.Colour), type (G.Type) and regeneration site (Reg.Site). Correlations among geographical variables are indicated as NA.

Correlations between L and all the other variables were statistically significant ($P < 0.5$), except for moisture content in the base chamber ($P = 0.23$). The ageing rate L showed the strongest correlation with the polytomous variable 'grain type' consistently in both conservation chambers (**Figure 4.2.2**). The partial correlation analysis confirmed that the variable with the strongest correlation with L was grain type, followed by seed mass. Correlations with geographical coordinates were also significant, and a cautious

interpretation of this correlation is reported in the Manuscript (section 7, page 97), as accessions were collected in different years and in limited areas within the same country.

4.2.5 Effect of grain type

Since grain type showed the strongest correlation with the ageing rate (L), a GLM was performed to understand how L differed among the grain types. Grain type showed a significant effect on the ageing rate L both in the active (d.f. 4, residual deviance = 162.7146, $P < 0.001$) and in the base chamber (d.f. 4, residual deviance = 223.7943, $P < 0.001$). In particular, in the active chamber, flint and floury seeds, without significant differences between them ($P = 0.22$), showed the lowest L, and therefore the lowest loss of seed viability across time, when compared with dent seeds ($P < 0.001$). In the base chamber, flint seeds showed the lowest L ($P < 0.001$), while floury and dent seeds did not show differences ($P = 0.32$). Overall, flint seeds showed a lower ageing rate when compared with the other grain types in terms of both the average and mode of the ageing rate L (**Figure 4.2.3**).

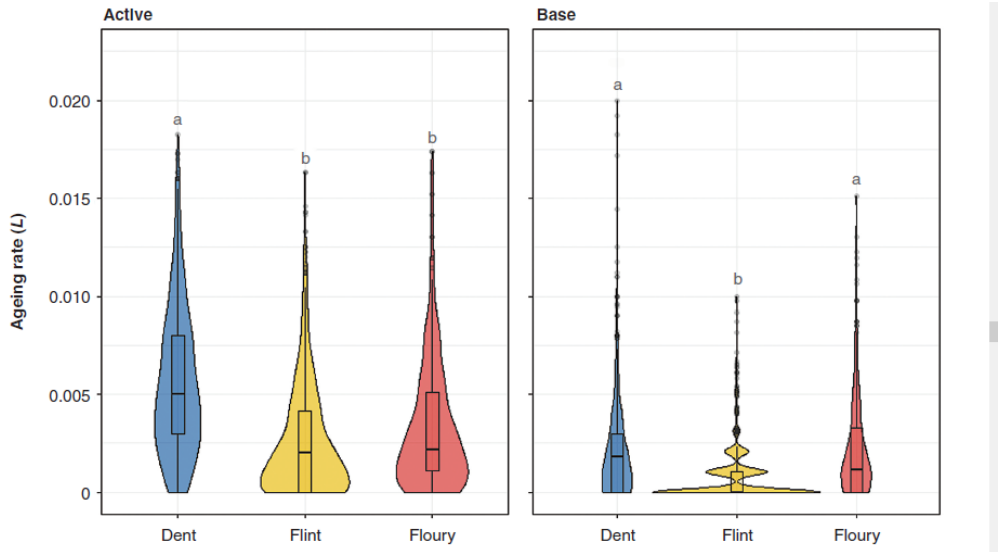


Figure 4.2.3 Violin and box plots representing the ageing rate (L) in the three most represented grain types, in both the active and base chambers. Letters above violins represent statistically significant differences.

In this latter analysis, only the three major grain types, floury, flint and dent, were considered since the sample sizes were much lower for sweet and popcorn in the set of accession used in this study (see 'Study accessions' in **Materials and Methods 3.2.2**).

4.2.6 Monitoring intervals

Viability monitoring intervals were calculated as one-third of the p_{85}, with a maximum interval of 40 years between monitoring points, for all the active seed lots, as suggested by FAO (2014). For this calculation, no filter on the p_{85} was applied; all the p_{85}s, extrapolated from the logit model, were employed in this analysis, since, following FAO (2014) a maximum interval of 40 years is adopted, even when p_{85} is >120 years. The

average viability monitoring interval for the accessions conserved in the active chamber is 17.5 ± 7.7 years. Monitoring intervals (measured in years) were significantly different among the three main grain types, flint, dent and floury (d.f. = 2, Kruskal–Wallis χ 2 = 80.30, P < 0.001), as follows: flint accessions (20.3 ± 9 years, P < 0.001), significantly longer than dent (14.6 ± 4.9 years) and floury (18.0 ± 7.6 years; P < 0.001). The difference in monitoring intervals between dent and floury was also significant (P < 0.01).

4.3 *Pisum sativum* accessions from the IPK collection

4.3.1 Germination profiles reveal genotype-dependent changes in pea seed longevity

Germination tests were performed with seeds from the yellow (Y1, Y2, Y3, Y4) and green (G1, G2, G3, G4) pea accessions (**Figure 4.3.1a**).

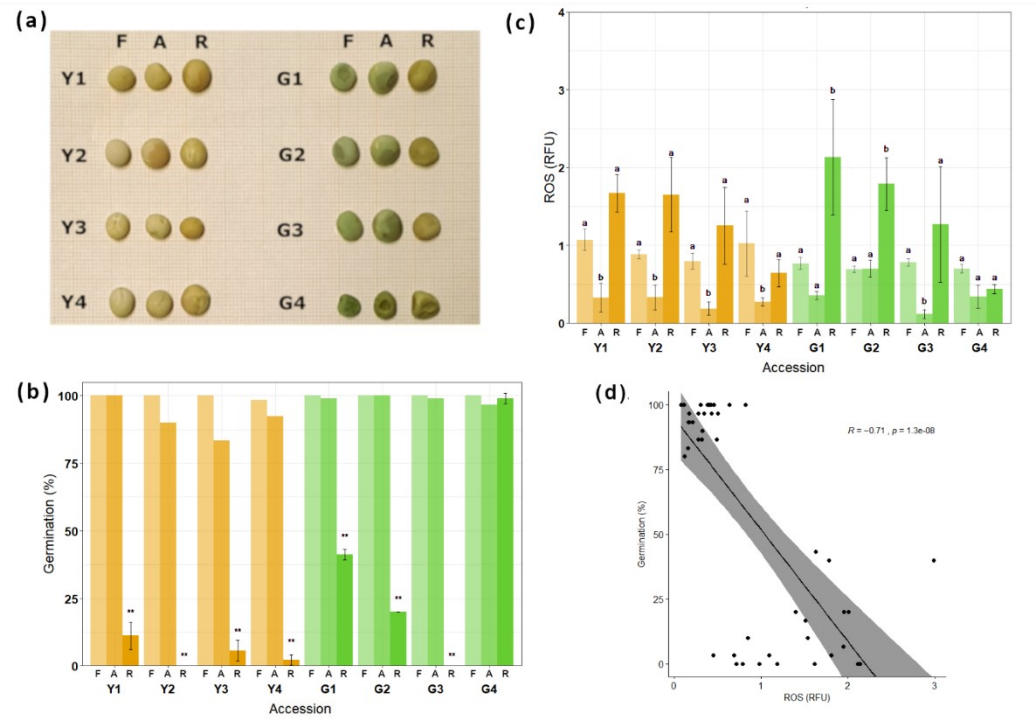


Figure 4.3.1 a) Seed lots used in this study. Y, yellow. G, green. F, harvested in 2019 (fresh). A, harvested in 2001 and kept in cold storage (aged). R, harvested in 2001 and conserved at room temperature conditions. **b**) Germination percentage of the 8 pea accessions (fresh seeds; seeds aged in cold storage and at room temperature conditions). **c**) ROS levels measured in dry pea seeds using the DCF-DA fluorescent dye. Letters above bars represent statistically significant differences (GLM with Tukey post-hoc test, P < 0.05) within the same accession. R.F.U., relative fluorescence unit. ROS, reactive oxygen species. DCF-DA, dye 2',7'-dichlorofluorescein diacetate. Asterisks represent statistically significant differences between aged and fresh seeds within the same accession (** = P < 0.01). **d**) Kendall's Tau-b correlation between ROS levels and germination profile of dry seeds from the yellow (Y1, Y2, Y3, Y4) and green (G1, G2, G3, G4) accessions.

Both fresh seeds (F) and seeds stored under different environments (A, cold chamber; R,

room-temperature conditions) were analyzed (**Figure 4.3.1b**). No significant difference in terms of germination percentage was observed between F and A seeds in all the tested accessions. However, significantly lower germination percentages were observed in R seeds when compared to F ($P < 0.01$), with the exception of G4 variety. The latter conserved nearly 100 % germination in F, A and R conditions, revealing an impressive longevity profile (**Figure 4.3.1b**). Accessions and storage conditions (F, A and R) had a statistically significant effect on germination percentage, as did the accession*conservation interaction ($P < 0.01$) (see **Table 4.3.1** for the corresponding Wald χ-squared values and d.f.).

Parameter	Factors	Wald χ-squared	d.f.	*P* value
G	**Accession**	1842.469	6	<0.001
	Conservation	146.002	2	<0.001
	Accession*Conservation	1482.498	14	<0.001
MTG	**Accession**	71.755	6	<0.001
	Conservation	13.539	2	<0.001
	Accession*Conservation	97.988	14	<0.001
MGR	**Accession**	148.311	6	<0.001
	Conservation	455.482	2	<0.001
	Accession*Conservation	137.744	14	<0.001
Z	**Accession**	4.98	6	0.546
	Conservation	7.151	2	0.007
	Accession*Conservation	15.025	14	0.02
MC	**Accession**	438.199	6	<0.001
	Conservation	1845.743	2	<0.001
	Accession*Conservation	398.148	13	<0.001
ROS	**Accession**	42.261	6	<0.001
	Conservation	246.989	2	<0.001
	Accession*Conservation	78.308	14	<0.001

Overall, MGT and MGR showed a significant increase in A and R seeds when compared to F while Z did not show significant differences among accessions or conservation conditions (**Table 4.3.1**). In order to figure out any possible effects related to chlorophyll content on seed germination, chlorophyll a and b levels were measured in dry and imbibed seeds of the green pea accessions. No correlation was found between chlorophyll a and b total contents or their ratio and germination percentage.

4.3.2 Increased ROS levels correlate with seed deterioration

ROS accumulation was determined in yellow and green pea seeds and the different conditions, namely fresh seeds (F) and seeds stored in cold chamber (A) or at room temperature (R) were analyzed (**Figure 4.3.1c**). In the yellow accessions (Y1, Y2, Y3,

Y4) and in the green accession G3, the F seeds showed a higher ROS content, compared to A seeds whereas F and R seeds shared similar ROS content ($P < 0.05$). When considering the different storage conditions, R seeds showed a significantly ($P < 0.05$) higher ROS content compared to A seeds in all the tested accessions, except for G4. The latter showed similar ROS levels in F, A and R seed lots (**Figure 4.3.1c**). Overall, ROS content in aged seeds (both A and R) showed a negative correlation with germination percentage (Tau-b: -0.76) (**Figure 4.3.1d**). Additional measurements were carried out on seeds collected at 24 h and 48 h of imbibition. ROS contents detected in seeds at 24 and 48 h of imbibition showed a negative correlation with germination percentage (Tau-b: -0.57, -0.59, respectively). Accessions, storage conditions (F, A and R) and their interaction had a statistically significant effect on ROS content ($P < 0.01$) (See **Table 4.3.1** for the corresponding Wald χ-squared values and d.f.). Given the exceptional behaviour of G4 in terms of seed longevity and ROS profiles, this accession was selected for subsequent evaluations. The G1 and Y1 accessions, showing an intermediate (about 40 % germination in R seeds) and low (about 10 % germination in R seeds) longevity profile, respectively, and the Y2 accession whose R seeds did not germinate were also chosen for this study.

4.3.3 Wrinkled seed longevity correlates with reduced lipid peroxidation and enhanced tocopherols content

In-depth analyses were carried out to assess oxidative damage at the level of lipid membranes in Y1, Y2, G1 and G4 accessions showing contrasting longevity profiles. MDA contents were measured as hallmark of lipid peroxidation. Results are shown in **Figure 4.3.2** as heatmap where values represent the Log2 FC of MDA levels monitored in A and R seeds compared with F seeds.

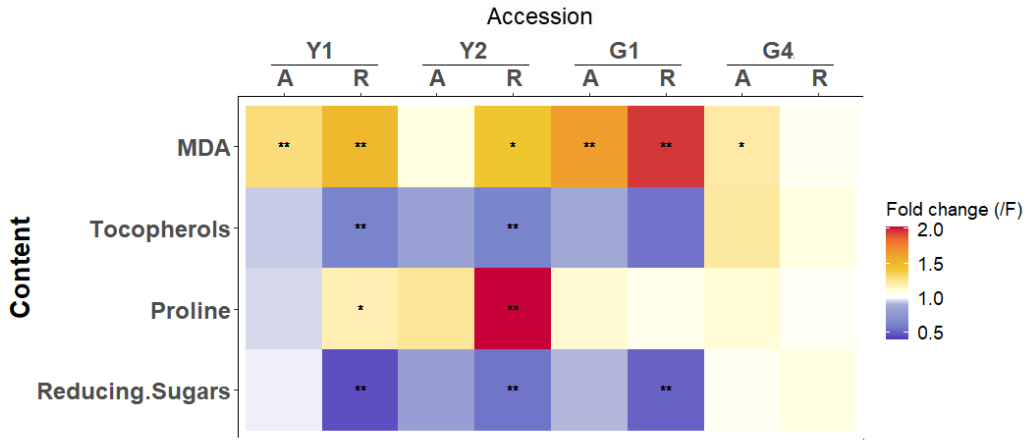


Figure 4.3.2 Levels of malondialdehyde (MDA), tocopherols, free proline, and reducing sugars in dry seeds of the yellow (Y) and green (G) pea accessions. F, harvested in 2019 (fresh). A, harvested in 2001 and kept in cold storage (aged). R, harvested in 2001 and conserved at room temperature conditions. Heatmaps represent changes of relevant metabolites in A and R seeds, compared to F seeds. For each metabolite, the mean values are available in Figure **4.3.3**. Asterisks represent statistically significant differences between

fresh and aged seeds within the same accession, showed by the post-hoc Bonferroni test (* = $P < 0.05$; ** = $P < 0.01$).

Mean values are available in **Figure 4.3.3a**.

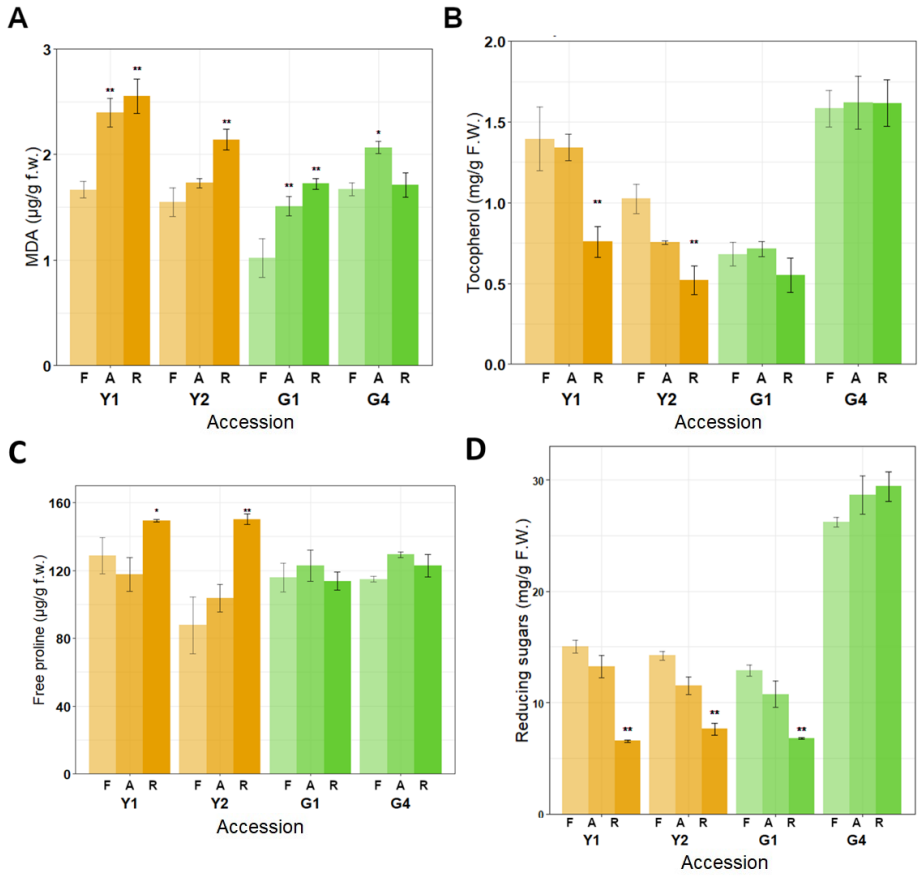


Figure 4.3.3 Levels of **A**) malondialdehyde (MDA), **B**) tocopherols, **C**) free proline, **D**) reducing sugars measured in pea seeds of the yellow (Y1, Y2) and green (G1, G4) accessions. F, fresh seeds harvested in 2019. A, seeds harvested in 2001 and kept in cold storage. R, seeds harvested in 2001 and conserved at room temperature conditions. Asterisks above bars represent statistically significant differences, comparisons made between F and A/R seeds (GLM with post-hoc Bonferroni test, * $P < 0.05$, ** $P < 0.01$) within the same accession.

Lipid peroxidation, measured in terms of MDA contents, was significantly higher in A seeds of Y1 ($P < 0.01$), G1 ($P < 0.01$), and G4 ($P < 0.05$). It was also significantly higher in R seeds in Y1 ($P < 0.01$), Y2 ($P < 0.05$) and G1 ($P < 0.01$) accessions with the exception of G4 (**Figure 4.3.2**, MDA). In this accession, characterized by high seed longevity, the estimated MDA content of R seeds (1.7 ± 0.11 µg/gFW) was similar to that found in F seeds (1.66 ± 0.06 µg/gFW) (Figure 4.3.3a). Accessions, storage conditions (F, A, R) and their interaction had a statistically significant effect on MDA levels (**Table 4.3.2**).

Table 4.3.2 Results of the generalized linear models (GLMs) performed on the levels of biochemical compounds MDA (malondialdehyde), tocopherols, free proline, and reducing sugars measured in the four selected accessions in their three conservation states. F, fresh seeds, harvested in 2019. A, seeds harvested in

2001 and kept in cold storage. R, seeds harvested in 2001 and conserved at room temperature. Y, yellow. G, green.

MDA content showed a negative correlation with germination (Tau-b: -0.52; P < 0.01) (**Figure 4.3.4a**).

Parameter	Factors	Wald χ-squared	d.f.	*P* value
MDA	**Accession**	177.029	3.000	<0.001
	Conservation	139.220	2.000	<0.001
	Accession*Conservation	54.472	6.000	<0.001
Tocopherol	**Accession**	349.369	3.000	<0.001
	Conservation	69.546	2.000	<0.001
	Accession*Conservation	43.455	6.000	<0.001
Proline	**Accession**	24.779	3.000	<0.001
	Conservation	46.787	2.000	<0.001
	Accession*Conservation	77.307	6.000	<0.001
Reducing sugars	**Accession**	2097.451	3.000	<0.001
	Conservation	517.180	2.000	<0.001
	Accession*Conservation	272.290	6.000	<0.001

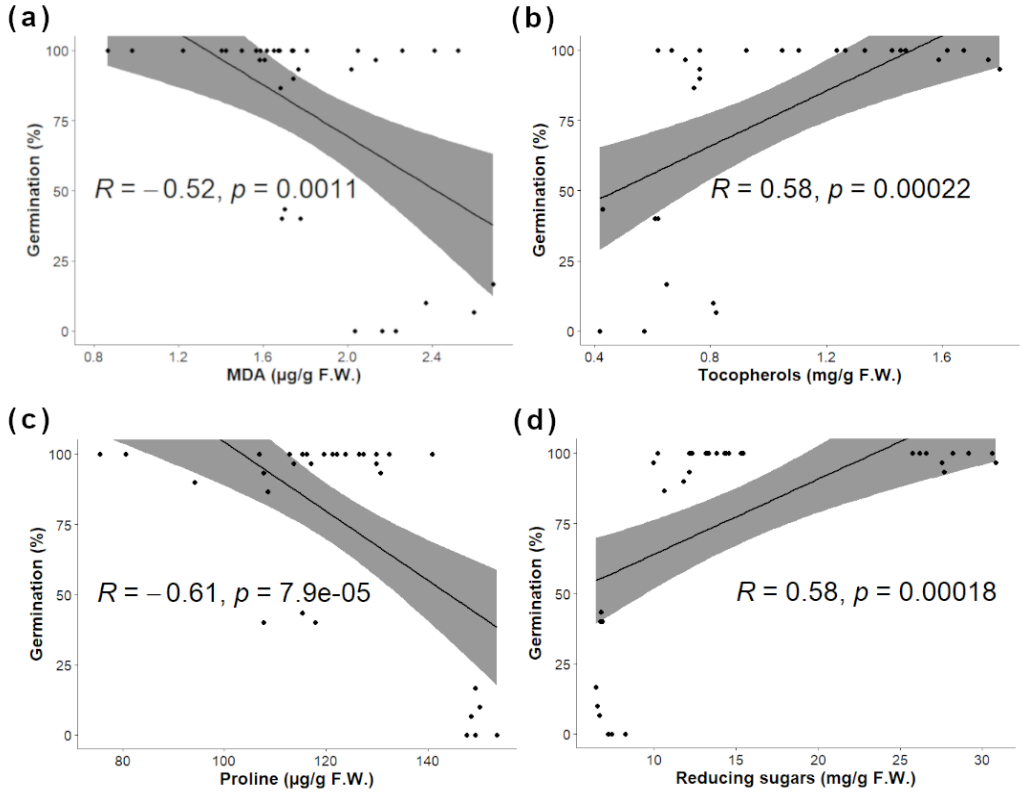


Figure 4.3.4 Kendall's Tau-b correlations between germination percentage and **a**) malondialdehyde (MDA), **b**) tocopherols, **c**) free proline, and **d**) reducing sugars contents of seeds from the yellow (Y1, Y2) and green (G1, G4) accessions, in both fresh and aged (cold storage, room temperature conditions) seeds.

The reduced levels of lipid peroxidation detected in the R seeds of G4 suggest for the presence of protective mechanisms, unique to this accession.

The total tocopherols content was determined in order to assess the possible contribution to the seed antioxidant response. Results are shown in **Figure 4.3.2** as heatmap where values represent the Log2 FC of tocopherols content monitored in A and R seeds compared with F seeds. Mean values are available in **Fig. 4.3.3b**. In all the tested accessions, no significant difference in terms of total tocopherols content was observed in A seeds compared to F seeds. A significant ($P < 0.01$) decrease was found in R seeds of Y1 and Y2, compared with F seeds. No significant ($P = 1$) decrease in total tocopherols content was detected in both G1 and G4 seeds stored at room temperature conditions (R), compared to F seeds. In the long-lived G4 accession, the estimated total tocopherols content of F seeds (1.58 ± 0.11 mg/gFW) was similar to that found in A (1.62 ± 0.16 mg/gFW) and R seeds (1.62 ± 0.14 mg/gFW) (**Figure 4.3.3b**). Accessions, storage conditions (F, A and R) and their interaction had a statistically significant effect on tocopherols levels (**Table 4.3.2**). Total tocopherols content was positively correlated to germination (Tau-b: 0.58; $P < 0.01$) (**Figure 4.3.4b**), suggesting for a role of these antioxidant compounds in the high-longevity profile of the wrinkled seeds.

4.3.4 Increased free proline content is a stress-induced hallmark of pea seed deterioration

Proline accumulation, typically observed in planta under oxidative stress conditions, has been also reported during prolonged seed storage (Kong et al., 2015). In order to further assess the metabolic response of the Y1, Y2, G1 and G4 varieties showing contrasting longevity profiles, the free proline content was measured. Results are shown in **Figure 4.3.2** as heatmap where values represent the Log2 FC of proline levels monitored in A and R seeds compared with F seeds. Mean values are available in **Figure 4.3.3c**. The metabolite amount was significantly higher only in R seeds of Y1 ($P < 0.05$) and Y2 ($P < 0.01$), compared to F. The estimated amount of free proline was 149.34 ± 0.84 µg/gFW (R seeds, Y1) and 150.18 ± 3.05 µg/gFW (R seeds, Y2) (**Figure 4.3.3c**). Variety, storage conditions (F, A, R) and their interaction had a statistically significant effect on proline levels (**Table 4.3.2**). A negative correlation with germination percentage was observed (Tau-b: -0.61; $P < 0.01$) (**Figure 4.3.4c**), thus strengthening the role played by this low-molecular weight osmolyte as hallmark of seed ageing.

4.3.5 High levels of reducing sugars are found in wrinkled seed

Considering the documented role of reducing sugars in seed deterioration (Murthy & Sun, 2000) the levels of these metabolites were measured in the Y1, Y2, G1 and G4 accessions showing contrasting longevity profiles. Results are shown in **Figure 4.3.2** as a heatmap where values represent the Log2FC of reducing sugars monitored in A and R seeds compared with F seeds. Mean values are available in **Figure 4.3.3d**. A significant ($P < 0.01$) reduction of reducing sugars was observed in the R seeds of Y1, Y2, and G1 accessions. In G4, showing the highest longevity in R seeds, the estimated total reducing sugars content of F seeds (26.19 ± 0.41 mg/gFW) was similar to that found in A seeds (28.61 ± 1.72 mg/gFW) and R seeds (29.40 ± 1.32 mg/gFW), being these levels significantly higher than those observed in the other accessions (**Figure 4.3.3d**). Accessions, storage conditions (F, A, and R) and their interaction had a statistically significant effect on the reducing sugars contents (**Table 4.3.2**). A positive correlation with germination percentage was observed (Tau-b 0.58; $P < 0.01$) (**Figure 4.3.4d**), thus suggesting a relationship between the availability of reducing sugars and seed longevity in wrinkled seeds.

4.3.6 Thermodynamical properties of the pea wrinkled seeds suggest for the presence of low molecular weight components

Temperatures of glass transition (Tg) were measured using differential scanning calorimetry. Even though Tg was significantly different among accessions ($F = 28.634$; d, f = 3; $P < 0.01$) and conservation states ($F = 6.597$; d, f = 2; $P < 0.01$), no clear association with longevity was observed. Indeed, Tg was not significantly correlated with germination percentage (Tau-b = - 0.04, $P = 0.806$). Moisture content was measured in all the pea accessions, for the different conservation conditions (F, A, R). Although the accession*conservation interaction showed a significant effect on moisture content, no significant differences among accessions were consistent with their longevity profiles (**Table 4.3.1**). Moisture content was also evaluated by thermogravimetric analysis (**Figure 4.3.5**).

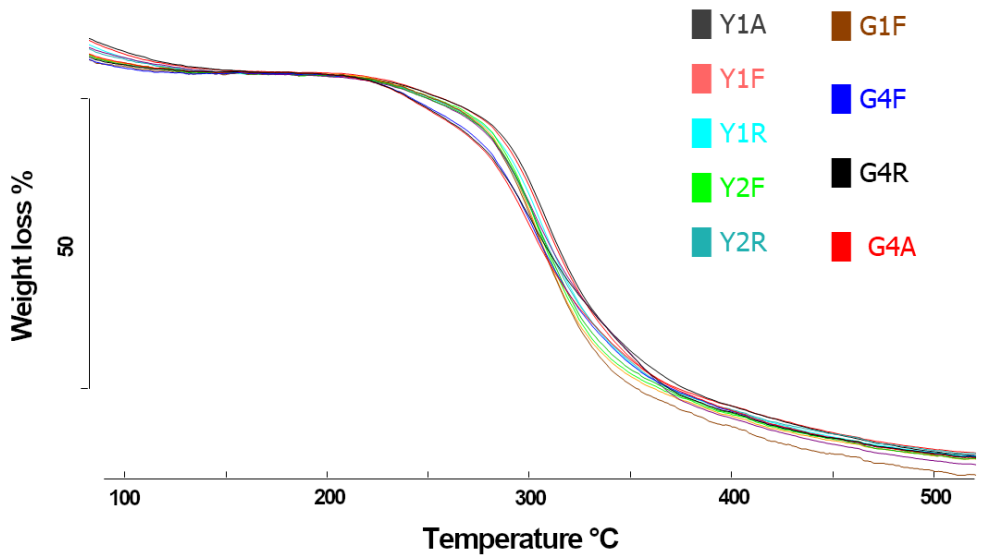


Figure 4.3.5 Weight loss (%) TG curves for dry seeds of the yellow (Y) and green (G) pea accessions.

The moisture content of the pea dry seeds was 10 ± 1 %, visible as a weight loss close to 100°C. TGA measurements were made both in air and in nitrogen atmosphere. Interestingly, under nitrogen atmosphere, curves showed a thermal decomposition profile including two steps: the first one occurred close to 250°C and the second one, more evident, was detected at 300°C. In order to better evidence the differences in composition between seeds, curves were translated to match the same dry weight at 170°C. This point was chosen because at this temperature the loss of water was completed, and thermal decomposition had not started yet. As shown in **Figure 4.3.5**, it is evident that those curves corresponding to the G4 seeds in the three different tested conditions started to lose material close to 200°C. This is indicated by the different slope observed for the G4 seeds in the range 200-300°C. This profile might correspond to the decomposition and/or evaporation of relatively small molecules exclusively found in the wrinkled seeds. Periodic acid-Schiff (PAS) staining combined with transmission electron microscopy was used to localize the occurrence of polysaccharides in the pea embryo axes. The progression of the oxidation reaction within polysaccharides, and the consequent generation of aldehyde groups, is delayed in these high molecular weight polymers, featuring the occurrence of dark-stained dots. By contrast, oxidation is accelerated when low molecular weight oligosaccharides are the predominant substrates, resulting in staining of weak intensity. As for the G4 wrinkled seeds, in the cytoplasm of cell embryos the PAS reaction highlighted the occurrence of dots of low intensity (**Figure 4.3.6, a and b**) whereas dark-stained dots were observed in the PAS-treated Y1 cells (**Figure 4.3.7**).

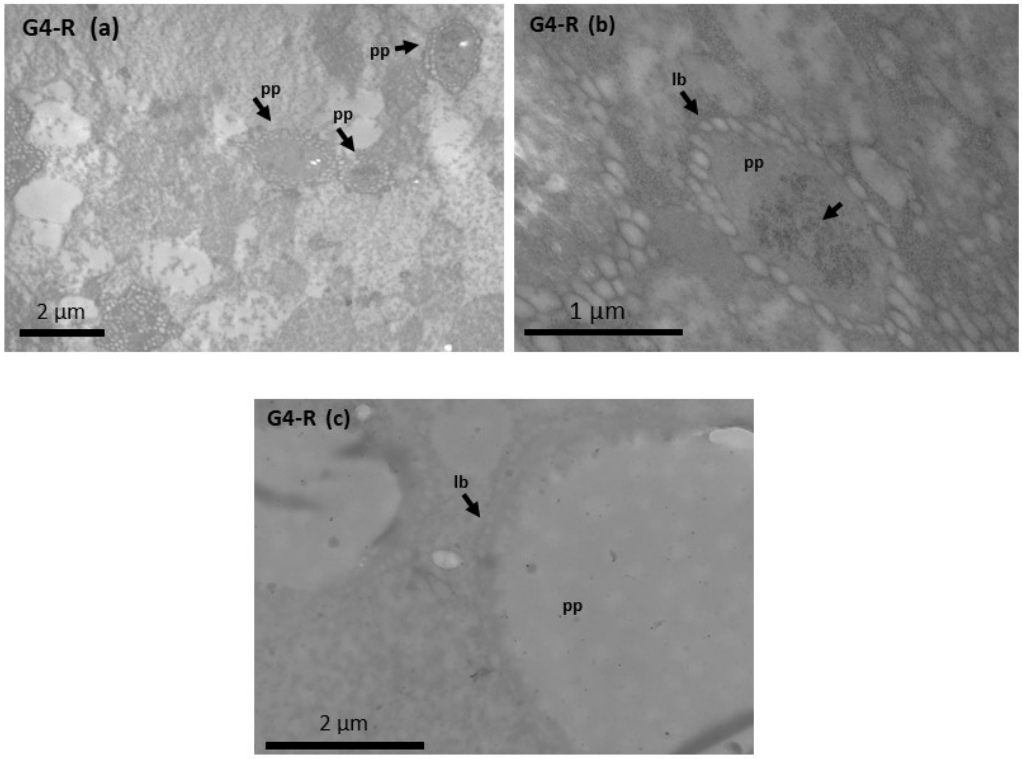


Figure 4.3.6 Transmission electron microscopy analysis of sections of dry embryos excised from the G4 wrinkled seeds and subjected to periodic acid-Schiff (PAS) staining for 30 min in order to localize the occurrence of polysaccharides *versus* oligosaccharides in the pea embryo axes. **(a)** Cytoplasm region of a PAS-stained cell showing the distribution of some proplastids (pp, arrows). **(b)** Enlarged section of a PAS-stained cell showing a proplastid (pp) surrounded by several lipid bodies (lb) and the occurrence of weak dots inside the proplastid (arrow), resulting from the PAS mediated oxidation of polysaccharides. (c) Negative control (osmium ammine staining, without periodic acid treatment): enlarged section of a cell showing a proplastid (pp) and the surrounding lipid bodies (lb, arrow).

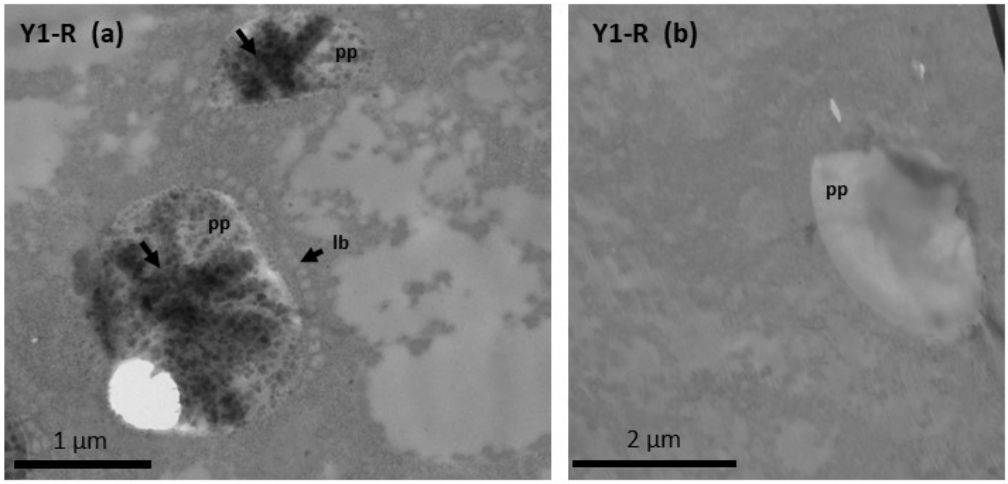


Figure 4.3.7 Transmission electron microscopy analysis of sections of dry embryos excised from the Y1 seeds and subjected to periodic acid-Schiff (PAS) staining for 30 min in order to localize the occurrence of polysaccharides *versus* oligosaccharides in the pea embryo axes. **(a)** Cytoplasm region of a PAS-stained cell showing the distribution of some proplastids (pp), surrounded by several lipid bodies (lb) and the occurrence of intense precipitates inside the proplastid (arrow), resulting from the PAS mediated oxidation of polysaccharides. (c) Negative control (osmium ammine staining, without periodic acid treatment): enlarged section of a cell showing a proplastid (pp).

Dark dots reflect the ongoing oxidation of polysaccharides in the Y1 cells while the poor signal detected in the G4 sample indicates that the same process was already concluded. The different reactivity hereby observed might reflect for different carbohydrate composition profiles in the seeds of the two pea accessions, particularly the occurrence of low molecular weight oligosaccharides in the G4 seeds, as suggested by the TGA measurements.

4.3.7 The high longevity profile of the wrinkled seeds features expanded heterochromatic areas and reduced occurrence of γH2AX foci

To assess the impact of almost 20 years of storage at room temperature on the nuclear architecture of the wrinkled seeds, the nuclear compartment was explored in the G4 embryos. A comparison was made with the Y1 embryos. Preliminary nuclear staining with Toluidine Blue carried out on sections of pea embryo axes revealed consistent nuclear shrinkage, with an expansion of the area of heterochromatin domains, in the wrinkled seeds (**Figure 4.3.8, a and b**).

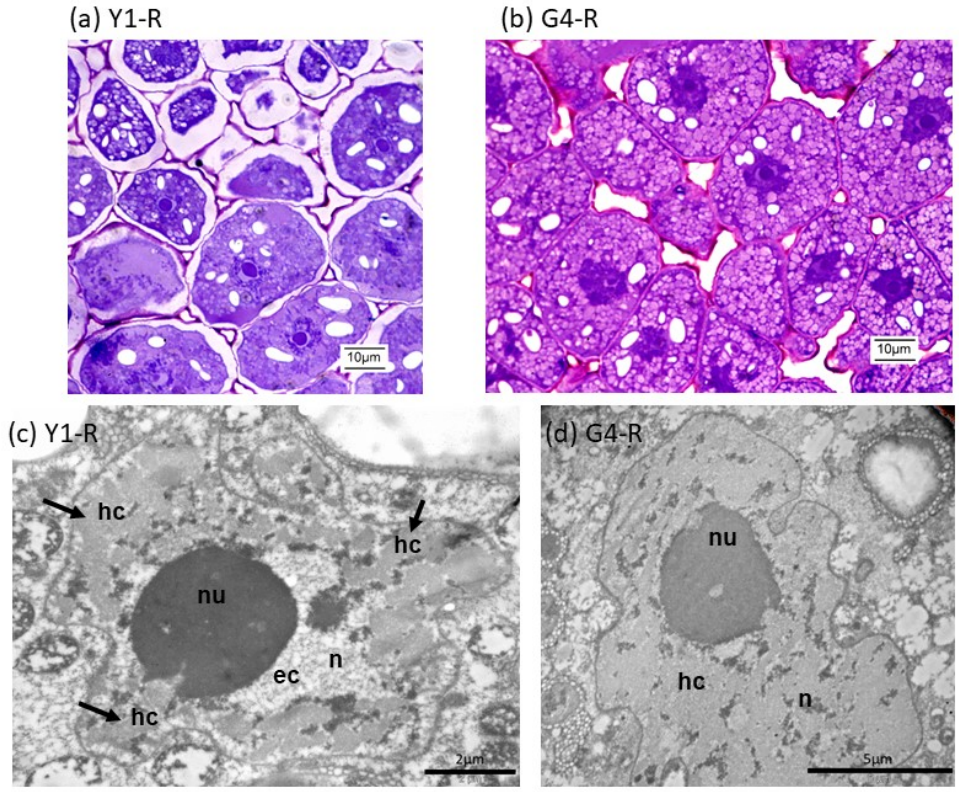


Figure 4.3.8 Nuclear staining with Toluidine Blue. Ultrastructural profile of Y1 **a**) and G4 **b**) nuclei of embryo axis excised from seeds stored at room temperature (Y1-R, and G4-R). Ultrastructural changes in chromatin distribution highlighted in Y1 **c**) and G4 **d**) nuclei subjected to osmium ammine staining for TEM analysis. hc, heterochromatin. ec, euchromatin. nu, nucleolus. n, nucleus.

In order to investigate changes in chromatin distribution that might represent ultrastructural hallmarks related to the high-longevity profile of the G4 accession, sections of pea embryo axes underwent osmium ammine staining for TEM analysis. The ultrastructural profile of Y1 and G4 nuclei is shown in **Figure 4.3.8** (**c and d**). In the Y1 nuclei, large heterochromatin areas are visible, as expected when dehydration occurs (**Figure 4.3.8c**, hc, arrows), as well as regions of decondensed euchromatin (**Figure 4.3.8c**, ec). Some of these condensed heterochromatin regions are located close to the nucleolus. In the G4 nuclei, chromatin condensation patterns were remarkably enhanced, covering the entire nuclear area (**Figure 4.3.8d**, hc). Such an unusual expansion of the packed chromatin might be indicative of an effective strategy driving genome maintenance in the G4 accession, possibly limiting long-term DNA damage accumulation in these extremely long-lived seeds. To assess this hypothesis, the distribution of γH2AX foci was investigated.

Upon DNA damage, ATM phosphorylates the histone variant H2AX on Ser139 (Burma

et al., 2001) and such modification (γH2AX) can spread for up to 1 Mb away from the break site, acting as a platform to recruit the DNA repair enzymes (Iacovoni et al., 2010). In order to map the γH2AX foci in the Y1 and G4 pea nuclei, immunocytochemical and TEM analyses were performed, using an antibody raised against the human histone variant H2AX on Ser139. Representative examples of the distribution of γH2AX foci in the nuclei of R seeds, in both Y1 and G4 accessions are shown in **Figure 4.3.9** (**a** and **b**).

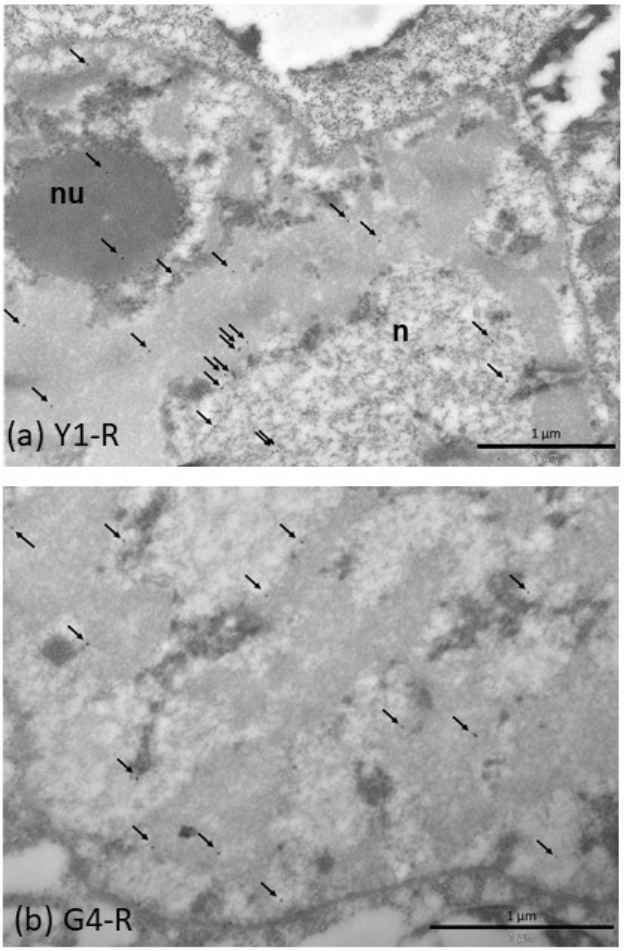


Figure 4.3.9 Distribution of γH2AX foci in the nucleus of embryo axis excised from seeds stored at room temperature: **a**) Y1-R and **b**) G4-R. Detection of γH2AX foci was performed by immunocytochemical and TEM analyses. n, nucleus. nu, nucleolus

The estimated density of γH2AX foci, expressed as n° foci per 400 nm2 of nuclear area, was significantly higher ($P < 0.05$) in the Y1 nuclei (1.6), compared to G4 nuclei (1.1). Thus, the limited genotoxic impact exerted by long-term storage on the wrinkled seeds might be the consequence of the highly compacted chromatin conformation previously described.

5. Discussion

Even though seed longevity is currently one of the main focus of seed biology, many aspects concerning its underlying mechanisms still remain unexplored or not sufficiently clarified. Indeed, given the importance of seed lifespan for food security, habitat restoration and biodiversity conservation, current studies are focusing on seed longevity at the intra- and inter-specific level, as well as in optimal vs. stressful storage conditions, and at different levels of analysis, i.e., eco-physiological, genetic, and molecular processes. In this context, the present work focused on the analysis of seed longevity in crops and wild relatives, with the aim of expanding the current knowledge about the dynamics of artificial and natural ageing in seed bank, long-term storage.

Few studies addressed the existing differences in longevity profiles between different accessions of the same plant species, and still fewer data are available when considering different morphs belonging to the same genotype (Gianella et al., 2021; Guzzon et al., 2021). Understanding how these biological entities age in seed bank conditions is crucial for the definition of appropriate monitoring and regeneration intervals in order to secure the invaluable richness of the plant genetic resources (PGRs) we are conserving for the future of our planet and the tomorrow's human generations. Intra-crop and intra-genotype comparisons were performed in maize, garden pea and wheat wild relatives. The wheat wild relatives experiment confirmed the presence of heteromorphism in terms of seed longevity (Gianella et al., 2020; Guzzon et al., 2018), but for the first time it was observed that the two morphs age differently also in seed bank (and not artificial) ageing conditions. This finding highlighted different needs in terms of viability monitoring also within the same genotype. Regarding the maize project, the seed viability and longevity estimates were significantly different among accessions considered, e.g., the p50 varied from 16.5 to 91 years. Within a large seed bank such as CIMMYT's, where the experiments took place, it is of particular importance to identify group of accessions that show different longevity profiles, in particular the ones that mostly require regeneration efforts, in order to optimize human resources and funding allocations (Hay et al., 2013). In the pea project, even though involving few accessions, significant differences were found, thus confirming the differential behaviour in terms of seed ageing within the same crop species. Indeed, in both the yellow and green pea accessions, seed viability was maintained in the samples kept under cold storage conditions whereas a significant decrease occurred in the samples maintained at room temperature, except for the G4 accession. According to the seed bank database, the G4 accession was classified as a mutant of the pea variety named 'Frogel'. Among the different accessions, the observed decline in seed viability varied, spanning between complete (0% germination), intermediate (40%) or null (100%).

The second level of analysis was the comparison of the longevity profiles among different ageing conditions, namely cold storage (base chamber, -18°C; active chamber, 3 °C), room conditions and artificial ageing (AA). In the wheat wild relatives project, different behaviours were observed when the two species were subjected to seed bank ageing and AA. Morph B of *T. boeoticum* was significantly longer-lived than morph A in both conditions. Differently, *Ae. tauschii* did not show significantly different p_{50} values among morphs when aged in cold storage, while morph B was significantly longer-lived when subjected to AA. These differences in the longevity estimates and slope of the curves

detected between the two ageing conditions can be due to several factors, such as the different number of time-intervals between storage conditions or environmental factors at regeneration stage that influenced seed heteromorphism and longevity. Nevertheless, the possibility that these differences are due to the fact that AA and cold storage affected seed survival in different ways cannot be ruled out (Walters et al., 2010; Colville & Pritchard, 2019). It follows that predictions made with AA should be used with caution to infer ranks of longevity under cold-storage and, therefore, to make subsequent decision on seed bank management (e.g., change seed processing methodologies, prioritization of accessions for regeneration and/or viability monitoring). Maize seed accessions conserved at CIMMYT genebank for up to 60 years showed a very high viability, higher than previously reported from maize accessions stored in similar conditions (Walters et al. 2005; Nagel and Börner 2010; Yamasaki et al. 2020). Given that, the germination percentage of the accessions conserved in active conditions was significantly lower and more variable than that observed in samples stored in the base chamber. The positive correlation between the germination profiles of the same accession in the active and in the base collection highlights an accession-specific behaviour in terms of viability loss. This indicates that the conservation conditions are the cause of the steeper decline in viability observed in the active chamber, where temperature and RH are higher compared to the base. Regarding the pea accessions, no significant decline in the final viability was observed in seeds conserved in cold storage for 20 years. On the contrary, all the accessions except G4 showed a pronounced viability loss when conserved at room conditions. This underlines the importance and effectiveness of cold storage in preserving PGRs: even though not all the different genotypes survived to 20 years in conditions of 'natural' ageing, the germination performance was optimal for all the accessions conserved in cold storage.

Among the seed traits correlating with seed longevity, morphology was a leitmotif in all the three projects composing this thesis. Seed dimensions were correlated with longevity in both maize and wheat wild relatives. In previous studies we demonstrated that different morphs of the same species respond differently to AA in several wheat wild relatives, with smaller seeds possessing higher longevity, dormancy and antioxidant profiles (Guzzon *et al.*, 2018; Gianella *et al.*, 2020). These seed traits are known as part of a bet-hedging evolutionary strategy that provides smaller seeds with a longer soil persistence (Arshad et al., 2019; Gianella et al., 2021), thereby reducing the risk of germination failure over time. The results of the experiment carried out for this thesis show for the first time that differences in seed longevity due to heteromorphism may occur also in seeds held under genebank conditions, which may have important implications for the *ex situ* conservation practices. In the maize project, the ageing rate (L) of the study accessions correlated with seed-related traits, namely seed mass and grain type. The ageing rate was positively correlated with seed mass, a trait that showed a great variation among the studied accessions, meaning that larger seeds aged faster than smaller ones (Fig. 2). This has been observed in other cereal crop gene pools such as rice (Rao et al., 1996) and the aforementioned wheat wild relatives. The biological bases for this inverse correlation in maize are not fully clarified yet and further research is needed to verify this observation. In the pea experiments, even though seed mass was not correlated with longevity, the role played by seed colour was the first research question raised at the IPK's seed bank, as green seeds seemed to possess a longer lifespan. However, in the pool of accessions considered in this study, a variable pattern was encountered, and a

possible role of chlorophyll content in shaping the different longevity profiles was ruled out. Results hereby provided evidence that the outstanding viability retained by G4 seeds after 20 years of room storage is associated more with the seed coat wrinkled phenotype rather than its colour.

Oxidative stress levels and the seed antioxidant response were explored in both the experimental systems of wheat wild relatives and pea seeds. The conditions applied to AA or cold storage affect the cytoplasmic viscoelastic properties and the biochemical processes taking place within it, in turn influencing the physiological pH and the redox state (Nagel et al., 2015). Oxidative stress is considered as the main cause of seed ageing, and it arises due to an imbalance between the accumulation of ROS, also functioning as signalling molecules, and the cellular antioxidant capacity. The latter plays an essential role in order to avoid cellular damages induced by the oxidative reaction that affect nucleic acids, lipids and proteins (Kurek et al., 2019). In the wheat wild relative study, the oxidative stress status was explored in order to compare AA and ageing in cold storage. The two studied species did not show similar patterns in terms of ROS accumulation within the same ageing conditions. In the dry state, in *Ae. tauschii* an increase in ROS levels was observed, compared to the fresh controls in both ageing treatments, while *T. boeoticum* showed generally lower ROS levels in the comparison with the after-ripened controls. ROS accumulation in the after-ripened controls could be due to the fact that after-ripening was performed within the ageing box (45°C, 60% RH), and even if germination was higher, the conditions of high temperature and RH used for AA could have triggered ROS production. Indeed, the conditions that determine the loss of dormancy during after-ripening are the same, i.e., increased temperature and RH, and it is considered as the first stage of seed ageing in seeds with primary dormancy (Bewley et al., 2013). Indeed, ROS levels are generally higher in non-dormant seeds than in dormant seeds (Bailly et al., 2008). No consistent patterns of ROS accumulation were observed between morphs, in terms of response to ageing treatments and timepoints as well as imbibition states. A and B did not show consistent ROS accumulation. *Ae. tauschii* showed lower ROS contents in morph B only at the dry state of SB aged seeds. The possibility that a differential imbalance between ROS accumulation and antioxidant capacity, might occur in the two morphs, linked to their different lifespan, cannot be ruled out. Also, a different antioxidant capacity that could rescue seeds from irreversible oxidative stress could explain the lower ROS accumulation in morph B compared to morph A at the end of pre-germinative metabolism. Indeed, this was observed in *T. boeoticum* in both ageing conditions, and in the AA seeds of *Ae. tauschii*. In these three conditions a dimorphism in longevity was observed, with morph B significantly longer-lived than morph A. This antioxidant response could be linked partly to a differential endowment of antioxidant molecules accumulated during maturation and then depleted with ageing, but also to a differential production of newly synthesized molecules during pre-germinative metabolism (Bewley et al., 2013; Sano et al., 2016). A general increase of ROS accumulation was observed at 1 h of imbibition, the first stage of pre-germinative metabolism, in all the species and ageing treatment combinations compared to the dry seed lots. This observation is in agreement with the finding that water uptake during seed imbibition triggers metabolism resumption, and the conversion of oxygen into superoxide and H_2O_2 at the level of mitochondria (Bailly et al., 2008). The comparative analysis of the eight pea accessions confirmed the inverse correlation between ROS levels and seed longevity. As for the fresh seeds (F), all the tested accessions displayed ROS contents

varying within a limited range. Indeed, ROS production in fresh seeds during post-harvest storage has been documented (Bailly et al., 2008). The low ROS levels observed in seeds aged for about 20 years under controlled conditions (A), compared to fresh seeds, well correlated with the high germinability profiles of all the investigated accessions. The long-term storage at room temperature conditions (R) resulted in significant ROS accumulation in all the yellow and green pea accessions showing a drop in germination, except for G4. This finding posed the question about the mechanisms underlying the ability of G4 wrinkled seeds to control ROS levels.

Following imbibition, all the biological processes associated with germination are reactivated, including respiration, reserve mobilization, DNA synthesis and repair, translation and degradation of stored mRNAs, transcription and translation of newly synthesized mRNAs (Bewley et al., 2013). In the wheat wild relatives project, transcript levels of genes coding for the enzymes with ROS scavenging activity or belonging to the glutathione-ascorbate pathway were evaluated in aged seeds at the dry state and during pre-germinative metabolism triggered by imbibition. The lowest variations compared to the controls were at 1 h after imbibition. A general decrease in transcript levels was observed at this stage compared to the dry seeds, as expected for the initial period of phase II of germination, when transcripts are degraded or translated in order to generate the ROS scavenging enzymes (Bewley et al., 2013). Considering all the six genes analysed, no clear pattern between morphs was observed. This could be due to several reasons, e.g., different kind of ROS produced and accumulated, similar levels of oxidative stress, different enzymes needed at the same timepoint of imbibition (thus different genes need to be expressed). At the late stage of pre-germinative metabolism, an increase in transcript levels was detected, compared to controls, suggesting that storage and AA might require higher antioxidant activities. When taking in consideration the expression of single genes among ageing treatments, *GSR* showed a consistent pattern among all the imbibition states in *Ae. tauschii*, while in *T. boeoticum* both *GSR* and *CAT* showed consistent profiles at 18 h after imbibition in the two ageing treatments. The GSH/ GSSG redox couple is a viability marker associated with seed longevity in barley (Nagel et al., 2015; Roach et al., 2018). Glutathione scavenging activity is particularly important in seeds with lower oil contents like cereals, as it is water-soluble compared to other lipid-soluble antioxidants (e.g., tocopherols) (Nagel et al., 2015). The consistent expression of *GSR* detected in this study could be linked to the enzymatic activity necessary to the GSSG to GSH re-conversion in the glutathione-ascorbate pathway. Moreover, GSR and CAT activity has been reported to be higher in the late stage of pre-germinative metabolism in sunflower for H_2O_2 scavenging and limitation of lipid peroxidation (Bailly, 2004). In the maize project, a GWAS is in progress, taking in consideration the 6 longevity parameters used in the study: germination (active and base), L (active and base) and p_{85} and p_{50}. Preliminary results indicate that 120 genes are annotated by all the significant SNPs, and the SNPs were mainly annotated in transcript regions. Following Gene Ontology analysis, results show that most genes are involved in intracellular processes, especially related to the nucleus and cytoplasm (intracellular and intracellular part), including organized structures of distinctive morphology and function occurring within the cell, i.e., nucleus, mitochondria, plastids, vacuoles, vesicles, ribosomes and the cytoskeleton. Further analyses are ongoing with the aim of clarifying the specific pathways in which these genes are involved. One issue encountered in these analyses was that the results of the prediction of the trait performance by the significant

SNPs, to report how much phenotypic variance the significant SNPs could cover, was low (<15%). This might indicate a low heritability of these traits that are known to be greatly influenced by environmental factors during regeneration and storage. It will be important to evaluate whether these factors can be incorporated into analysis to account for G x E x M (Genome X Environment X Management) and subsequently enhance prediction accuracies. Overall, these preliminary results further underline the complexity of the biological and environmental framework that must be considered when dealing with seed longevity studies.

In this thesis, oxidative stress-linked hallmarks were further investigated in the pea experimental system. To address the research question about the exceptional longevity of G4 and the observed limited oxidative damage, specific metabolites associated with the seed ability to scavenge the toxic free radicals were measured. The investigation was restricted to four pea accessions, namely Y1, Y2, G1, and G4, showing contrasting germination and ROS profiles. ROS-driven oxidation mainly targets polyunsaturated fatty acids, generating lipid peroxides responsible for membrane disruption, further ROS production and, after additional degradation into reactive compounds, cross-linking with proteins and nucleic acids (Gaschler & Stockwell, 2017). Similar levels of lipid peroxidation were detected in both F and R seeds of the G4 variety, confirming its unique long-term oxidative stress resilience, in contrast with the progressive enhancement of lipid peroxidation observed during ageing in all the other pea varieties. The G4 seeds displayed the highest levels of the lipophilic antioxidant tocopherols, known for their ability to interact with polyunsaturated acyl groups and scavenge lipid peroxyl radicals (Fritsche et al., 2017), independent on their conservation state (F, A, R). Thus, it appears that there was no need to exploit the tocopherols pool in G4 seeds, since lipid peroxidation did not overcome a critical damage threshold. In the G1 accession, characterized by a low tocopherols content (approximately 50% less, compared to G4), longevity was compromised following long-term storage at room temperature conditions. The comparative analysis showed that tocopherols were utilized by the Y1 and Y2 varieties to face ROS toxicity, however this was not sufficient to avoid the dramatic drop in germinability observed in R seeds. Among other non-enzymatic antioxidants, free proline is an efficient ROS scavenger and a compatible osmolyte involved in the response to various abiotic stresses such as drought or salinity (Hayat et al., 2012; Liang et al., 2013). Increased free proline content contributed to oxidative stress adaptation in oat (*Avena sativa* L.) seeds with higher moisture content, stored for up to one year (Kong et al., 2015). A similar free proline content was detected in both G1 and G4 accessions, in all the tested treatments (F, A, R) whereas a significant accumulation occurred in the Y1 and Y2 seeds stored under room temperature conditions (R). This finding corroborates the role of free proline as a seed-specific oxidative damage marker for the first time during long-term ageing under seed bank conditions. Future in-depth gene expression studies might help defining the role of proline in the longevity response of wrinkled seeds. The fresh seeds of the G4 accession displayed significantly higher levels of reducing sugars, compared to Y1, Y2, and G1, and this is a characteristic of the wrinkled seed phenotype (Stickland & Wilson, 1983). The reducing sugars content of A and R wrinkled seeds did not decrease upon long-term storage, differently from what occurred in Y1, Y2, and G1. Reducing sugars participate in non-enzymatic protein glycosylation (Maillard reaction) that, together with lipid peroxidation, is indicative of the biochemical deterioration associated with seed ageing (Murthy & Sun, 2000). Evidently, the low ROS

content of G4 seeds prevented the occurrence of this type of damage whereas reducing sugars are engaged in the Maillard process triggered by the oxidative environment of Y1, Y2, and G1 seeds. The research question that arises from the reported data is how the G4 seeds can maintain constitutive low ROS levels despite 20 years of storage, considering that ROS are continuously generated in an oxygenic environment and the activity of ROS scavenging enzymes is restricted in the glassy state of dry cytoplasm (Nagel et al., 2019). In such environment, the dry seed exploits the pool of available antioxidant compounds to withstand oxidative deterioration, as hereby observed for the Y and G accessions. The study of mechanical properties within the dry cytoplasm of pea embryonic axes has revealed low molecular mobility over a broad range of moisture contents and temperatures, possibly due to steric hindrance between adjacent macromolecules, and such features might contribute to seed longevity (Ballesteros & Walters, 2019). However, there is scanty information concerning the mechanical properties of the dry cytoplasm in wrinkled pea seeds and their possible role in longevity. Results of this multidisciplinary investigation, aimed at dissecting the high-longevity phenotype of G4 seed, point at their ability to maintain a reducing cellular environment. The TGA profiles recorded in the G4 seeds, independent on treatments, suggested for the presence of relatively small molecules. Indeed, in wrinkled pea seeds carrying mutations at the *r* and *rb* loci, alterations in the starch biosynthetic pathway result in pleiotropic effects such as accumulation of the raffinose family oligosaccharides (Gawlowska et al., 2017), previously associated with membrane stability (Crowe et al., 1992). At the moment, we cannot rule out the possibility that G4 seeds use specific low molecular-weight antioxidant molecules, e.g., glutathione and L-ascorbic acid, as redox buffer to maintain ROS within a threshold critical to ensure longevity. It has been reported that mutations at the r locus altering seed composition and hygroscopic properties, can affect seed longevity (Lyall et al., 2003). However, to our knowledge, this is the first study providing evidence of high longevity under long-term storage conditions in a wrinkled seed accession.

Structural peculiarities linked to the glassy state are thought to influence the viscoelastic properties of the cytoplasm, therefore influencing the molecular mobility and the ability to buffer ROS accumulation (Buitink and Leprince, 2008; Ballesteros & Walters, 2011). The latter is expected to restrict genotoxic damage but certainly some structural rearrangements within the nucleus might also provide protection against nucleic acid deterioration (Lee et al., 2020). In the pea project, almost all the nuclear area of the G4 accession is filled with heterochromatin. Chromatin condensation occurring upon severe water loss might be promoted by increased levels of cations and changes of histone variants. In addition, larger nucleoprotein grains and thicker fibrils, found in nuclei of quiescent embryo cells, disappeared at the onset of germination (Deltour, 1985; Washio, 2014). This aspect is still poorly explored in the context of seed longevity and the G4 accession might provide a unique working system for further investigations on the dynamics of nuclear architecture in response to desiccation. According to Bhattacharyya et al. (1993), the high sucrose content of pea wrinkled seeds enhances the embryo osmotic potential, increasing water up-take during seed development. Subsequently, during desiccation, excess water is lost causing the wrinkled phenotype. These shrinkage dynamics might lead to a tighter chromatin conformation useful for genome maintenance (Bhattacharyya et al., 1993). The G4 seeds aged at room temperature also showed a significantly lower frequency of the γH2AX foci, compared to the Y1 seeds. It has been

reported that chromatin compaction protects DNA from damage, but it also blocks the expansion of H2AX phosphorylation (Cann & Dellaire, 2011; Nair et al., 20127). It is possible that the high-longevity profile of tee G4 seeds and the associated resilience to genotoxic stress were positively influenced by such chromatin dynamics. Results hereby obtained in the pea seeds are in agreement with those described in both yeast and mammalian cells revealing that the γH2AX foci at double strand break sites were formed at lower levels in heterochromatin, when compared to euchromatin (Cowell et al., 2007; Kim et al., 2007). On the other hand, the protective effect of chromatin compaction might be related to non-histone chromatin proteins that physically shield the genomic DNA (Falk et al., 2008).

In the maize project, grain type was the trait more strongly correlated with the longevity rate (L).. Flint accessions were the longest lived among the three main grain types (dent, flint, floury). This confirmed the observation of Bewley and Black (1994) that seeds of flint varieties are longer-lived when compared to other grain types. Grain-type is a qualitative trait based mainly on the seed coat morphology and endosperm texture: grains of varieties of flint maize have mostly hard, glassy endosperm compared to the softer and starchier endosperms typical of dent and floury varieties (Zilic et al., 2011). Several landraces can show intermediate appearance between two different grain types. Therefore, more quantitative measures will be needed to study the effect of the grain type on seed longevity and eventually organize viability monitoring intervals of accessions of different grain types showing different longevity estimates. We can therefore hypothesize that the higher seed longevity detected in flint varieties could be due to structural (i.e. glassy endosperm) or physiological (e.g. seed coat, antioxidant capacity) peculiarities, but further investigations, e.g. metabolomic and antioxidant profiling, and analyses of visco-elastic properties of the endosperm, will be performed to clarify the biological basis of the differences in seed longevities detected among grain types.

In conclusion, different aspects of seed longevity were dissected, and the obtained results enlarged the current knowledge about the ageing behaviour and mechanisms under genebank conditions. The effectiveness of cold storage in preserving PGRs for the long term was evidenced in all the three projects, involving material conserved in two of the world's major seed banks (IPK and CIMMYT). Although conservation in base chambers proved to be more effective, and therefore less resource-consuming, than that in active chambers, different viability monitoring intervals should be applied based on the accessions' characteristics (i.e., grain type in maize and seed morphs in wheat wild relatives). The accuracy of AA as a predictive tool for longevity rankings was further questioned by the results obtained comparing cold storage ageing and AA in wheat wild relatives, adding novel information to the emerging literature on the topic. These results underline the current need for new ageing methods (alternative to cold storage and AA) and/or molecular and physiological hallmarks for fast and accurate predictions of seed lifespan and rankings in storage. The biochemical, molecular and structural hallmarks used in this thesis improved the profiling of the considered accessions in both the wheat wild relatives and the garden pea projects. Features related to the oxidative stress status and the antioxidant response correlated with the germination capability after storage, further strengthening the prominent role of ROS and ROS buffering in shaping seed longevity. In this work we tested approaches still poorly used in this research field, such as the measurement of alternative ageing hallmarks (e.g., proline, reducing sugars) and

the ultrastructural analysis of chromatin compaction and genome integrity. In particular, the preliminary results obtained with the TEM techniques appear very promising, and their application to larger experimental systems could add a deeper level of detail to studies about the seed longevity dynamics. Indeed, multidisciplinary approaches are fundamental in the characterization of such a multi-faceted biological process.

6. References

Abdur M., Arif R, Börner A. Mapping of QTL associated with seed longevity in durum wheat (Triticum durum Desf.). *J Appl Genet*. 2019; 60(1), 33–36. https://doi.org/10.1007/s13353-018-0477-y

Ábrahám E, Hourton-Cabassa C, Erdei L, Szabados L. Methods for determination of proline in plants. In: Sunkar R, ed. *Plant stress tolerance: methods and protocols*. Springer New York Dordrecht Heidelberg London. 2010. 316-331.

Anderson R, Bayer PE, Edwards D. Climate change and the need for agricultural adaptation. *Curr Opin Plant Biol*. 2020; 56, 197–202. https://doi.org/10.1016/j.pbi.2019.12.006

Arshad W, Sperber K, Steinbrecher T, Nichols B, Jansen VAA, Leubner-Metzger G, Mummenhoff K. Dispersal biophysics and adaptive significance of dimorphic diaspores in the annual Aethionema arabicum (Brassicaceae). *New Phytol*. 2019; 221, 1434–1446.

Asdal Å, Guarino L. The svalbard global seed vault: 10 Years - 1 million samples. *Biopreserv and Biobank*. 2018; 16(5), 391–392. https://doi.org/10.1089/bio.2018.0025

Bailly C. (2004). Active oxygen species and antioxidants in seed biology. *Seed Sci Res*. 2004; 14 (2), 93-107. https://doi.org/10.1079/SSR2004159

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*. 2008; 331, 806–814. https://doi.org/10.1016/j.crvi.2008.07.022

Ballesteros D, Walters C. Solid-state biology and seed longevity: a mechanical analysis of glasses in pea and soybean embryonic axes. *Front Plant Sci*. 2019, 10, 920.

Ballesteros D, Pritchard HW, Walters C. Dry architecture: Towards the understanding of the variation of longevity in desiccation-tolerant germplasm. *Seed Sci Res*. 2020, 30 (2), 142–155. https://doi.org/10.1017/S0960258520000239

Baskin CC, Baskin JM. Seeds: ecology, biogeography, and evolution of dormancy and germination. Elsevier. 1998.

Bewley JD, Bradford KJ, Nonogaki H, Hilhorst HWM. Seeds Physiology of Development, Germination and Dormancy, 3rd Edition. Springer. 2013.

Bhanu AN. (2017). Assessment of genetic diversity in crop plants - an overview. *Adv Plants Agric Res*. 2019; 7(3): 0025. https://doi.org/10.15406/apar.2017.07.00255

Bhattacharyya MK, Smith AM, Ellis THN, Hedley C, Martin C. The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a

gene encoding a starch-branching enzyme. *Cell*. 1990; 60, 115-122.

Biedermann S, Mooney S, Hellmann H. Recognition and repair pathways of damaged DNA in higher plants. In Chen C, ed. Selected Topics in DNA Repair. InTech Open. 2011. 201-236. https://doi.org/10.5772/21380

Black M, Pritchard HW. Desiccation and survival in plants. Drying without dying. In: Black M, Pritchard, HW, eds. *Annals of Botany Vol. 91, Issue 1*. Oxford University Press. 2003. https://doi.org/10.1093/aob/mcg004

Börner A, Chebotar S, Korzun V. Molecular characterization of the genetic integrity of wheat (Triticum aestivum L.) germplasm after long-term maintenance. *Theor Appl Genet*. 2000; 100(3–4), 494–497. https://doi.org/10.1007/s001220050064

Börner A, Nagel M, Agacka-Mołdoch M, Gierke PU, Oberforster M, Albrecht T, Mohler V. QTL analysis of falling number and seed longevity in wheat (Triticum aestivum L.). *J Appl Gen*. 2017; 59(1), 35–42. https://doi.org/10.1007/S13353-017-0422-5

Bray CM, West CE. DNA repair mechanisms in plants: crucial sensors and effectors for the maintenance of genome integrity. *New Phytol*. 2005; 168(3), 511–528. https://doi.org/10.1111/J.1469-8137.2005.01548.X

Bryant C, Wheeler NR, Rubel F, French RH. kgc: Koeppen–Geiger climatic zones. R package version 1.0.0.2. 2017. https://CRAN.R-project.org/ package=kgc.

Bueso E, Muñoz-Bertomeu J, Campos F, Brunaud V, Martínez L, Sayas E, Ballester P, Yenush L, Serrano R. ARABIDOPSIS THALIANA HOMEOBOX25 uncovers a role for gibberellins in seed longevity. *Plant Physiol*. 2014; 164(2), 999–1010. https://doi.org/10.1104/pp.113.232223

Buijs G, Willems LAJ, Kodde J, Groot SPC, Bentsink L. Evaluating the EPPO method for seed longevity analyses in Arabidopsis. *Plant Sci*. 2020; 301, 110644. https://doi.org/10.1016/j.plantsci.2020.110644

Buitink J, Leprince O. Intracellular glasses and seed survival in the dry state. *C R-Biol*. 2008; 331(10), 788–795. https://doi.org/10.1016/J.CRVI.2008.08.002

Burma S, Chen BP, Murphy M, Kurimasa A. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem*. 2001; 276, 42462-42467

Camacho-Villa TC, Maxted N, Scholten M, Ford-Lloyd B. Defining and identifying crop landraces. *Plant Genet Resour*. 2005; 3(3), 373–384. https://doi.org/10.1079/pgr200591

Cann KL, Dellaire G. Heterochromatin and the DNA damage response: the need to relax. *Biochem Cell Biol*. 2011; 89, 45-60.

Carranco R, Espinosa JM, Prieto-Dapena P, Almoguera C, Jordano J. Repression by an

auxin/indole acetic acid protein connects auxin signaling with heat shock factor-mediated seed longevity. *PNAS*. 2010; 107(50), 21908–21913. https://doi.org/10.1073/PNAS.1014856107

Carvalho RF. Heterochromatic knobs gene-like effects on flowering time, and the seed aging epigenetic-genetic program in maize. Biblioteca Digital de Teses e Dissertações da Universidade de São Paulo. PhD thesis. 2020. https://doi.org/10.11606/T.11.2020.TDE-14012021-140924

Chen H, Chu P, Yuliang Z, Li Y, Liu J, Ding Y, Tsang EWT, Jiang L, Wu K, Huang S. Overexpression of AtOGG1, a DNA glycosylase/AP lyase, enhances seed longevity and abiotic stress tolerance in Arabidopsis. *J Exp Bot*. 2012; 63(11), 4107–4121. https://doi.org/10.1093/jxb/ers093

Chen J, Zavala C, Ortega N, Petroli C, Franco J, Burgueño J, Costich DE, Hearne SJ. The development of quality control genotyping approaches: A case study using elite maize lines. PLoS ONE. 2016; 11(6), 1–17. https://doi.org/10.1371/journal.pone.0157236

CIMMYT. Proceedings: World-wide maize improvement in the 70's and the role for CIMMYT. Texcoco: International Maize and Wheat Improvement Center (CIMMYT). 1974.

CIMMYT. Recent advances in the conservation and utilization of genetic resources. Proceedings of the Global Maize Germplasm Workshop. Texcoco: International Maize and Wheat Improvement Center (CIMMYT).1988.

Clapp J, Newell P, Brent ZW. The global political economy of climate change, agriculture and food systems. *J Peasant Stud*. 2018; 45(1), 80–88. https://doi.org/10.1080/03066150.2017.1381602

Clerkx EJM., Vries HB-D, Ruys GJ, Groot SPC, Koornneef M. Genetic differences in seed longevity of various Arabidopsis mutants. *Physiol Plant*. 2004; 121(3), 448–461. https://doi.org/10.1111/J.0031-9317.2004.00339.X

Colville L, Pritchard HW. Seed life span and food security. *New Phytol.* 2019; 224(2), 557–562. https://doi.org/10.1111/nph.16006

Cõrdoba-Cañero D, Roldán-Arjona T, Ariza RR. Arabidopsis ZDP DNA 3'-phosphatase and ARP endonuclease function in 8-oxoG repair initiated by FPG and OGG1 DNA glycosylases. *Plant J*. 2014; 79(5), 824–834. https://doi.org/10.1111/tpj.12588

Cowell IG, Sunter NJ, Singh PB, Austin CA, Durkacz BW, Tilby MJ. GammaH2AX foci form preferentially in euchromatin after ionising-radiation. *PLoS ONE*. 2017; 2, e1057.

Coyne CJ, Kumar S, von Wettberg EJB, Marques E, Berger JD, Redden RJ, Ellis THN, Brus J, Zablatzká L, Smýkal P. Potential and limits of exploitation of crop wild

relatives for pea, lentil, and chickpea improvement. *Legume Sci*. 2020; 2: e36. https://doi.org/10.1002/leg3.36

Crowe JH, Hoekstra FA, Crowe LM. Anhydrobiosis. *Annu Rev Physiol*. 1992; 54, 579-599.

de Faria RQ, dos Santos ARP, Amorim DJ, Cantão RF, da Silva EAA, Sartori MMP. Probit or Logit? Which is the better model to predict the longevity of seeds*? Seed Sci Res*. 2010; 30(1), 49–58. https://doi.org/10.1017/S0960258520000136

de Souza Vidigal D, Willems L, van Arkel J, Dekkers BJW, Hilhorst HWM, Bentsink L. Galactinol as marker for seed longevity. *Plant Sci*. 2016; 246, 112–118. https://doi.org/10.1016/J.PLANTSCI.2016.02.015

Debeaujon I, Koornneef M. Gibberellin requirement for Arabidopsis seed germination is determined both by testa characteristics and embryonic abscisic acid. *Plant Physiol*. 200; 122(2), 415–424. https://doi.org/10.1104/PP.122.2.415

Delouche JC, Baskin CC. Accelerated aging techniques for predicting the relative storability of seed lots. *Seed Sci Technol*. 1997; 427-452. .https://ir.library.msstate.edu/bitstream/handle/11668/13316/F-4.pdf?sequence=1&isAllowed=y

Deltour R. Nuclear activation during early germination of the higher plant embryo. *J Cell Sci*. 1985; 75, 43-83.

Dempewolf H, Baute G, Anderson J, Kilian B, Smith C, Guarino L. Past and future use of wild relatives in crop breeding. *Crop Sci*. 2017; 57(3), 1070–1082. https://doi.org/10.2135/CROPSCI2016.10.0885

Dempewolf H, Eastwood RJ, Guarino L, Khoury CK, Müller JV, Toll J. Adapting agriculture to climate change: a global initiative to collect, conserve, and use crop wild relatives. *Agroecol Sustain Food Syst*. 2014; 38(4), 369–377. https://doi.org/10.1080/21683565.2013.870629

Desheva G. The longevity of crop seeds stored under long-term condition in the National Gene Bank of Bulgaria. *Agric*. 2016; 62(2), 90–100. https://doi.org/10.1515/AGRI-2016-0010

Doria E, Galleschi L, Calucci L, Pinzino C, Pilu R, Cassani E, Nielsen E. Phytic acid prevents oxidative stress in seeds: evidence from a maize (Zea mays L.) low phytic acid mutant. *J Exp Bot*. 2009; 60, 967-978.

dos Santos ARP, de Faria RQ, Amorim DJ, Giandoni VCR, da Silva EAA, Sartori MMP. Cauchy, cauchy–santos–sartori–faria, logit, and probit functions for estimating seed longevity in soybean. *Agron J*. 2019; 111(6), 2929–2939. https://doi.org/10.2134/agronj2018.11.0700

Ellis RH, Hong TD, Jackson MT. Seed production environment, time of harvest, and the potential longevity of seeds of three cultivars of rice (Oryza sativa L.). *Ann Bot*. 1993; 72(6), 583–590. https://doi.org/10.1006/ANBO.1993.1148

Ellis RH, Roberts EH. Improved equations for the prediction of seed longevity. *Ann Bot*. 1980; 45(1), 13–30. https://doi.org/10.1093/oxfordjournals.aob.a085797

Ellis RH, Yadav G. Effect of simulated rainfall during wheat seed development and maturation on subsequent seed longevity is reversible. *Seed Sci Res*. 2016; 26(1), 67–76. https://doi.org/10.1017/S0960258515000392

Falk M, Lukasova E, Kozubek S. Chromatin structure influences the sensitivity of DNA to gamma-radiation. *Biochim Biophys Acta*. 2008; 1783, 2398-2414.

FAO. International treaty on plant genetic resources for food and agriculture. Rome: FAO. 2009

FAO. The second report on the state of the world's plant genetic resources for food and agriculture. Commission on Genetic Resources for Food and Agriculture, Food and Agriculture Organization of the United Nations, Rome. 2010.

FAO. Genebank standards for plant genetic resources for food and agriculture. 2014.

FAO. Climate change and food systems: global assessments and implications for food security and trade. 2015. http://www.fao.org/policy-support/resources/resources-details/en/c/435192/

Flörke M, Schneider C, McDonald RI. Water competition between cities and agriculture driven by climate change and urban growth. *Nat Sustain*. 2018; 1(1), 51–58. https://doi.org/10.1038/s41893-017-0006-8

Forti C, Ottobrino V, Bassolino L, Toppino L, Rotino GL, Pagano A, Macovei A, Balestrazzi A. Molecular dynamics of pre-germinative metabolism in primed eggplant (Solanum melongena L.) seeds. *Hortic Res*. 2020; 7(1), 87. https://doi.org/10.1038/s41438-020-0310-8

Fritsche S, Wang X, Jung C. Recent advances in our understanding of tocopherol biosynthesis in plants: an overview of key genes, functions, and breeding of vitamin E improved crops. *Antioxidants*. 2017; 6, 99.

Gaschler MM, Stockwell BR. Lipid peroxidation in cell death. *Biochem Biophy Res Comm*. 2017; 482, 419-425.

Gawlowska M, Swiecicki W, Lahuta L, Kaczmarek Z. Raffinose family oligosaccharides in seeds of Pisum wild taxa, type lines for seed genes, domesticated and advanced breeding materials. Genet Resour Crop Evol. 2017; 64, 569-578.

Gehring M, Henikoff S. DNA methylation dynamics in plant genomes. *Biochim Biophys*

Acta - Gene Struct Expression. 2007; 1769(5–6), 276–286. https://doi.org/10.1016/J.BBAEXP.2007.01.009

Gianella M, Balestrazzi A, Pagano A, Müller JV, Kyratzis AC, Kikodze D, Canella M, Mondoni A, Rossi G, Guzzon F. (2020). Heteromorphic seeds of wheat wild relatives show germination niche differentiation. *Plant Biol*. 2020; 22(2), 191-202. https://doi.org/10.1111/plb.13060

Gianella M, Bradford KJ, Guzzon F. Ecological, (epi)genetic and physiological aspects of bet-hedging in angiosperms. *Plant Reprod*. 2021; 34, 21–36. https://doi.org/10.1007/s00497-020-00402-z

Gianella M, Pagano A, Forti C, Guzzon F, Mondoni A, de Sousa Araújo S, Macovei A, Balestrazzi A. Molecular aspects of seed priming as a means of progress in crop improvement. In Tuteja N, Tuteja R, Passricha N, Saifi SK, eds. *Advancement in Crop Improvement Techniques*. Woodhead Publishing. 2020; 89–100. https://doi.org/10.1016/B978-0-12-818581-0.00006-1

Giner G, Smyth GK. statmod: probability calculations for the inverse Gaussian distribution. *R J*. 2016; 8, 339–351.

Groot SPC, Surki AA, de Vos RCH, Kodde J. Seed storage at elevated partial pressure of oxygen, a fast method for analysing seed ageing under dry conditions. *Ann Bot*. 2012; 110(6), 1149–1159. https://doi.org/10.1093/aob/mcs198

Guzzon F, Gianella M, Velazquez Juarez JA, Sanchez Cano C, Costich DE. Seed longevity of maize conserved under germplasm bank conditions for up to 60 years. *Ann Bot*. 2021; 127(6), 775–785. https://doi.org/10.1093/aob/mcab009

Guzzon F, Müller JV, Abeli T, Cauzzi P, Ardenghi NMG, Balestrazzi A, Rossi G, Orsenigo S. Germination requirements of nine European Aegilops species in relation to constant and alternating temperatures. *Acta Bot Gall*. 2015; 162(4), 349–354. https://doi.org/10.1080/12538078.2015.1088793

Guzzon F, Orsenigo S, Gianella M, Müller JV, Vagge I, Rossi G, Mondoni A. Seed heteromorphy influences seed longevity in Aegilops. *Seed Sci Res*. 2018; 28(4). https://doi.org/10.1017/S096025851800034X

Hammer K, Teklu Y. Plant genetic resources: Selected issues from genetic erosion to genetic engineering. *J Agric Rural Dev Trop Subtrop*. 2008; 109(1), 15–50.

Harlan JR, de Wet JMJ. Toward a rational classification of cultivated plants. *Taxon*. 1971; 20(4), 509–517. https://doi.org/10.2307/1218252

Hay FR, de Guzman F, Ellis D, Makahiya H, Borromeo T, Hamilton NRS. Viability of Oryza sativa L. seeds stored under genebank conditions for up to 30 years. *Genet Resour Crop Evol*. 2013; 60(1), 275–296. https://doi.org/10.1007/s10722-012-9833-7

Hay FR, Mead A, Bloomberg M. Modelling seed germination in response to continuous variables: use and limitations of probit analysis and alternative approaches. *Seed Sci Res*. 2014; 24(3), 165–186. https://doi.org/10.1017/S096025851400021X

Hayat S, Hayat Q, Nasser A, Wani AS, Pichtel J, Ahmad A. Role of proline under changing environments. *Plant Signal Behav*. 2012; 7, 1456-1466.

Hothorn T, Bretz F, Westfall P. Simultaneous inference in general parametric models. *Biom J*. 2008; 50, 346–363.

Iacovoni JS, Caron P, Lassadi I, Nicolas E, Massip L, Trouche D, Legube G. High-resolution profiling of gammaH2AX around DNA double strand breaks in the mammalian genome. *EMBO J*. 2010; 29, 1446-1457.

Intergovernmental Panel on Climate Change. Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the IPCC. 2015.

Iriondo JM, Milla R, Volis S, Rubio de Casas R. Reproductive traits and evolutionary divergence between Mediterranean crops and their wild relatives. *Plant Biol*. 2018; 20, 78–88. https://doi.org/10.1111/plb.12640

ISTA. International rules for seed testing. Bassersdorf, Switzerland, International Seed Testing Association. 2019.

Kassambara A. ggpubr: 'ggplot2' based publication ready plots. R package version 0.4.0. 2020. https://CRAN.R-project.org/package=ggpubr.

Kijak H, Ratajczak E. What do we know about the genetic basis of seed desiccation tolerance and longevity? *Int J Mol Sci*. 2020; Vol. 21, Page 3612, 21(10), 3612. https://doi.org/10.3390/IJMS21103612

Kim JA, Kruhlak M, Dotiwala F, Nussenzweig A, Haber JE. Heterochromatin is refractory to gamma-H2AX modification in yeast and mammals. *J Cell Biol*. 2007; 178, 209-218.

Konapala G, Mishra AK, Wada Y, Mann ME. Climate change will affect global water availability through compounding changes in seasonal precipitation and evaporation. *Nat Comm*. 2020; 11(1), 1–10. https://doi.org/10.1038/s41467-020-16757-w

Kong L, Huo H, Mao P. Antioxidant response and related gene expression in aged oat seed. *Front Plant Sci*. 2020; 6,158.

Kranner I, Chen H, Pritchard HW, Pearce SR, Birtić S. Inter-nucleosomal DNA fragmentation and loss of RNA integrity during seed ageing. *Plant Growth Regul*. 2010; 63(1), 63–72. https://doi.org/10.1007/S10725-010-9512-7

Kurek K, Plitta-Michalak B, Ratajczak E. Reactive oxygen species as potential drivers of

the seed aging process. *Plants*. 2019;8(6), 174. https://doi.org/10.3390/plants8060174

Kurilich C, Juvik J. Quantification of carotenoid and tocopherol antioxidants in Zea mays. *J Agric Food Chem*. 2009; 47, 1948-1955

Lee JH, Kim EW, Croteau DL, Bohr VA. Heterochromatin: an epigenetic point of view in aging. *Exp Mol Med.* 2020; 52, 1466-1474.

Lee J-S, Kwak J, Hay FR. Genetic markers associated with seed longevity and vitamin E in diverse Aus rice varieties. *Seed Sci Res*. 2020; 30(2), 133–141. https://doi.org/10.1017/S0960258520000173

Lehner A, Mamadou N, Poels P, Côme D, Bailly C, Corbineau F. Changes in soluble carbohydrates, lipid peroxidation and antioxidant enzyme activities in the embryo during ageing in wheat grains. *J Cereal Sci*. 2008; 47(3), 555–565. https://doi.org/10.1016/J.JCS.2007.06.017

Lenth RV. Least-squares means: the R package lsmeans. *J Stat Softw*. 2016; 69, 1–33.

Lev J, Blahovec J. Imbibition of wheat seeds: Application of image analysis. *Intern Agrophys*. 2017; 31, 475–481. https://doi.org/10.1515/intag-2016-0072

Leisner CP. Review: Climate change impacts on food security- focus on perennial cropping systems and nutritional value. *Plant Sci*. 2020; 293, 110412. https://doi.org/10.1016/j.plantsci.2020.110412

Leprince O, Pellizzaro A, Berriri S, Buitink J. Late seed maturation: drying without dying. *J Exp Bot*. 2017; 68(4), 827–841. https://doi.org/10.1093/JXB/ERW363

Li D-Z, Pritchard HW. The science and economics of ex situ plant conservation. *Trends Plant Sci*. 2009; 14(11), 614–621. https://doi.org/10.1016/J.TPLANTS.2009.09.005

Liang X, Zhang L, Natarajan SK, Becker DF. Proline mechanisms of stress survival. *Antiox Redox Signal*. 2013; 19, 998-1011.

Liu U, Breman E, Cossu TA, Kenney S. The conservation value of germplasm stored at the Millennium Seed Bank, Royal Botanic Gardens, Kew, UK. Biodiv Conserv. 2018; 27(6), 1347–1386. https://doi.org/10.1007/s10531-018-1497-y

Liu W, Pan X, Li Y, Duan Y, Min J, Liu S, Sheng X, Li X. Detection and validation of QTLs associated with seed longevity in rice (Oryza sativa L.). *Plant Breed.* 2018; 137(4), 546–552. https://doi.org/10.1111/PBR.12611

Louf J-F, Zheng Y, Kumar A, Bohr T, Gundlach C, Harholt J, Poulsen HF, Jensen KH. Imbibition in plant seeds. *Phys Rev*. 2018; 98, 042403.

Lyall TW, Ellis RH, John P, Hedley CL, Wang TL. Mutant alleles at the rugosus loci in pea affect seed moisture sorption isotherms and the relations between seed longevity

and moisture content. *J Exp Bot*. 2003; 54, 445-450.

Mao Z, Sun W. Arabidopsis seed-specific vacuolar aquaporins are involved in maintaining seed longevity under the control of ABSCISIC ACID INSENSITIVE 3. *J Exp Bot*. 2015; 66(15), 4781–4794. https://doi.org/10.1093/jxb/erv244

Maxted N, Kell SP, Ford-Lloyd B, Iriondo JM, Turok J, Dulloo E. Crop wild relative conservation and use. 2007. CABI.

Maxted N, Ford-Lloyd B, Jury S, Kell S, Scholten M. Towards a definition of a crop wild relative. *Biodiv Conserv*. 2006; 15(8), 2673–2685. https://doi.org/10.1007/s10531-005-5409-6

Miller GL. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal Chem*. 1959; 31(3), 426–428. https://doi.org/10.1021/ac60147a030

Mira S, Pirredda M, Martín-Sánchez M, Marchessi JE, Martín C. DNA methylation and integrity in aged seeds and regenerated plants. *Seed Sci Res*. 2020; 30(2), 92–100. https://doi.org/10.1017/S0960258520000021

Moïse JA, Han S, Gudynaitę-Savitch L, Johnson DA, Miki BLA. Seed coats: Structure, development, composition, and biotechnology. *In Vitro Cell Devel Biol – Plant*. 2005; 41(5), 620–644. https://doi.org/10.1079/IVP2005686

Mondoni A, Orsenigo S, Donà M, Balestrazzi A, Probert RJ, Hay FR, Petraglia A, Abeli T. Environmentally induced transgenerational changes in seed longevity: maternal and genetic influence. *Ann Bot*. 2014; 113(7), 1257–1263. https://doi.org/10.1093/AOB/MCU046

Müller JV, Cockel CP, Gianella M, Guzzon F. Treasuring crop wild relative diversity: analysis of success from the seed collecting phase of the 'Adapting Agriculture to Climate Change' project. *Genet Resour Crop Evol*. 2021; 0123456789. https://doi.org/10.1007/s10722-021-01229-x

Murthy UMN, Sun WQ. Protein modification by the Amadori and Maillard reactions during seed storage: roles of sugar hydrolysis and lipid peroxidation. *J Exp Bot*. 2000; 51, 1221-1228.

Myers SS, Smith MR, Guth S, Golden CD, Vaitla B, Mueller ND, Dangour AD, Huybers P. Climate Change and Global Food Systems: Potential Impacts on Food Security and Undernutrition. *Annu Rev Public Health*. 2017; 38(1), 259–277. https://doi.org/10.1146/annurev-publhealth-031816-044356

Nagel M, Börner. The longevity of crop seeds stored under ambient conditions. *Seed Sci Res*. 2010; 20, 1–20.

Nagel M, Kodde J, Pistrick S, Mascher M, Börner A, Groot SPC. Barley seed aging: genetics behind the dry elevated pressure of oxygen aging and moist controlled

deterioration. *Front Plant Sci*. 2016; 7, 388. https://doi.org/10.3389/FPLS.2016.00388

Nagel M, Kranner I, Neumann K, Rolletschek H, Seal CE, Colville L, Fernández-Marín, B, Börner A. 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*. 2015; 38(6), 1011–1022. https://doi.org/10.1111/pce.12474

Nagel M, Seal CE, Colville L, Rodenstein A, Un S, Richter J, Pritchard HW, Börner A, Kranner I. Wheat seed ageing viewed through the cellular redox environment and changes in pH. *Free Radic Res*. 2019; 53, 641-654.

Nair N, Shoaib M, Storgaard Sorensen C. Chromatin dynamics in genome stability: roles in suppressing endogenous DNA damage and facilitating DNA repair. *Int J Mol Sci*. 2017; 18, 1486.

Nakazawa M. fmsb: functions for medical statistics book with som demographic data. R package version 0.7.0. 2019. https://CRAN.R-project.org/package=fmsb.

Nave M, Avni R, Ben-Zvi B, Hale I, Distelfeld A. QTLs for uniform grain dimensions and germination selected during wheat domestication are co-located on chromosome 4B. *Theor Appl Genet*. 2016; 129(7), 1303–1315. https://doi.org/10.1007/s00122-016-2704-4

Newton R, Hay F, Probert R. Protocol for comparative seed longevity testing. *Technical Information Sheet_01.* London, Royal Botanic Gardens Kew. 2009.

Nguyen T-P, Keizer P, Eeuwijk F, Smeekens S, Bentsink L. Natural variation for seed longevity and seed dormancy are negatively correlated in Arabidopsis. *Plant Physiol*. 2012; 160(4), 2083. https://doi.org/10.1104/PP.112.206649

Niedzielski M, Walters C, Luczak W, Hill LM, Wheeler LJ, Puchalski J. Assessment of variation in seed longevity within rye, wheat and the intergeneric hybrid triticale. *Seed Sci Res*. 2009; 19(4), 213–224. https://doi.org/10.1017/S0960258509990110

North H, Baud S, Debeaujon I, Dubos C, Dubreucq B, Grappin P, Jullien M, Lepiniec L, Marion-Poll A, Miquel M, Rajjou L, Routaboul JM, Caboche M. Arabidopsis seed secrets unravelled after a decade of genetic and omics-driven research. *Plant J*. 2010; 61(6), 971–981. https://doi.org/10.1111/j.1365-313X.2009.04095.x

Oñate-Sánchez L, Vicente-Carbajosa J. DNA-free RNA isolation protocols for Arabidopsis thaliana, including seeds and siliques. *BMC Res Notes*. 2008; 1(1), 93. https://doi.org/10.1186/1756-0500-1-93

Orlowsky B, Seneviratne SI. Global changes in extreme events: Regional and seasonal dimension. *Clim Change*. 2012; 110(3–4), 669–696. https://doi.org/10.1007/s10584-011-0122-9

Ozden E, Albayrak Delialioglu R, Hussein S, Demir I. Prediction of germination of commercially available seed lots by regression models developed by artificial and natural ageing and electrical conductivity in leek. *Seed Sci Technol*. 2017; 45(2), 465–474. https://doi.org/10.15258/sst.2017.45.2.01

Paparella S, de Sousa Araújo S, Rossi G, Wijayasinghe M, Carbonera D, Balestrazzi A. Seed priming: state of the art and new perspectives. *Plant Cell Rep*. 2015; 34(8), 1281–1293. https://doi.org/10.1007/S00299-015-1784-Y

Pérez J, Araya-Valverde E, Garro G, Abdelnour-Esquivel A. Analysis of stress indicators during cryopreservation of seeds of landrace maize (Zea mays). *Cryo Letters*. 2017; 38(6), 445–454.

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*. 2001; 29(9). https://doi.org/10.1093/nar/29.9.e45

Pirredda M, González-Benito ME, Martín C, Mira S. Genetic and Epigenetic Stability in Rye Seeds under Different Storage Conditions: Ageing and Oxygen Effect. *Plants*. 2020; 9(3), 393. https://doi.org/10.3390/PLANTS9030393

Plitta-Michalak BP, Naskręt-Barciszewska MZ, Barciszewski J, Chmielarz P, Michalak, M. Epigenetic integrity of orthodox seeds stored under conventional and cryogenic conditions. *Forests*. 2021; 12(3), 288. https://doi.org/10.3390/F12030288

Pokhrel Y, Felfelani F, Satoh Y, Boulange J, Burek P, Gädeke A, Gerten D, Gosling SN, Grillakis M, Gudmundsson L, Hanasaki N, Kim H, Koutroulis A, Liu J, Papadimitriou L, Schew L, Müller Schmied H, Stacke T, Telteu CE, Wada Y. Global terrestrial water storage and drought severity under climate change. *Nat Clim Change*. 2021 11(3), 226–233. https://doi.org/10.1038/s41558-020-00972-w

Pourcel L, Routaboul J-M, Kerhoas L, Caboche M, Lepiniec L, Debeaujon I. TRANSPARENT TESTA10 encodes a laccase-like enzyme involved in oxidative polymerization of flavonoids in Arabidopsis seed coat. *Plant Cell*. 2005; 17(11), 2966. https://doi.org/10.1105/TPC.105.035154

Powell AA. Cell membranes and seed leachate conductivity in relation to the quality of seed for sowing. *J Seed Technol*. 2006; 10(2), 81–100.

Powell AA, Matthews S. Evaluation of controlled deterioration, a new vigour test for small seeded vegetables. *Seed Sci Technol*. 1981; 9(2), 633-640.

Probert RJ, Daws MI, Hay FR. Ecological correlates of ex situ seed longevity: a comparative study on 195 species. *Ann Bot*. 2009; 104(1), 57–69. https://doi.org/10.1093/aob/mcp082

Rajjou L, Debeaujon I. Seed longevity: survival and maintenance of high germination ability of dry seeds. *C R – Biol*. 2008; 331(10), 796–805. https://doi.org/10.1016/J.CRVI.2008.07.021

Ranal MA, Garcia de Santana, D. How and why to measure the germination process? *Braz J Bot*. 2006; 29(1), 1–11. https://doi.org/10.1590/S0100-84042006000100002

Rao NK, Hanson J, Dulloo ME, Ghosh N, Nowell D, Larinde. Manual of seed handling in genebanks. Handbook for genebank No.8. Bioversity International, Rome, Italy. 2006.

Raquid R, Kohli A, Reinke R, Dionisio-Sese M, Kwak J, Chebotarov D, Mo Y, Lee J-S. Genetic factors enhancing seed longevity in tropical japonica rice. *Curr Plant Biol*. 2021; 26, 100196. https://doi.org/10.1016/J.CPB.2021.100196

Revelle W. psych: procedures for personality and psychological research, Northwestern University, Evanston, Illinois, USA. 2019. https://CRAN.Rproject. org/package=psych Version =1.9.12.

Righetti K, Vu JL, Pelletier S, Vu BL, Glaab E, Lalanne D, Pasha A, Patel R, Provart NJ, Verdier J, Leprince O, Buitink J. Inference of longevity-related genes from a robust coexpression network of seed maturation identifies regulators linking seed storability to biotic defense-related pathways. *Plant Cell*. 2015; 27(10), 2692–2708. https://doi.org/10.1105/tpc.15.00632

Roach T, Nagel M, Börner A, Eberle C, Kranner I. Changes in tocochromanols and glutathione reveal differences in the mechanisms of seed ageing under seedbank conditions and controlled deterioration in barley. *J Environ Exp Bot*. 2018; 156, 8–15. https://doi.org/10.1016/j.envexpbot.2018.08.027

Roberts EH. Predicting the storage life of seeds. *Seed Sci Technol*. 1973; 1, 499–514. http://ci.nii.ac.jp/naid/10030093916/en/

Sano N, Ono H, Murata K, Yamada T, Hirasawa T, Kanekatsu M. Accumulation of long-lived mRNAs associated with germination in embryos during seed development of rice. *J Exp Bot*. 2015; 66(13), 4035–4046. https://doi.org/10.1093/JXB/ERV209

Sano N, Rajjou L, North HM. Lost in translation: physiological roles of stored mRNAs in seed germination. *Plants*. 2020, 9(3), 347. https://doi.org/10.3390/plants9030347

Sano N, Rajjou L, North HM, Debeaujon I, Marion-Poll A, Seo M. Staying alive: molecular aspects of seed longevity. *Plant Cell Physiol*. 2016; 57(4), 660–674. https://doi.org/10.1093/pcp/pcv186

Sari A, Kursat M, Civelek S. Determination of MDA levels in the plant (some Salvia L. Taxa growing in Turkey). *J Drug Metabol Toxicol*. 2012; 3, 3.

Schwember AR, Bradford KJ. Quantitative trait loci associated with longevity of lettuce seeds under conventional and controlled deterioration storage conditions. *J Exp Bot.* 2010; 61(15), 4423–4436. https://doi.org/10.1093/jxb/erq248

Sharma P, Jha AB, Dubey RS, Pessarakli M. Reactive oxygen species, oxidative damage,

and antioxidative defense mechanism in plants under stressful conditions. *J Bot*. 2012; 1–26. https://doi.org/10.1155/2012/217037

Stickland RG, Wilson KE. Sugars and starch in developing round and wrinkled pea seeds. *Ann Bot*. 1983; 52, 919-992.

Sugliani M, Rajjou L, Clerkx EJM, Koornneef M, Soppe WJJ. Natural modifiers of seed longevity in the Arabidopsis mutants abscisic acid insensitive3-5 (abi3-5) and leafy cotyledon1-3 (lec1-3). *New Phytol*. 2009; 184(4), 898–908. https://doi.org/10.1111/j.1469-8137.2009.03023.x

Taiyun W, Viliam S.R package 'corrplot': visualization of a correlation matrix. 2017. https://github.com/taiyun/corrplot.

Tausch S, Leipold M, Reisch C, Poschlod P. Dormancy and endosperm presence influence the ex situ conservation potential in central European calcareous grassland plants. *AoB PLANTS*. 2019; 11(4), 1–12. https://doi.org/10.1093/AOBPLA/PLZ035

Trapp A, Dixon P, Widrlechner MP, Kovach DA. Scheduling viability tests for seeds in long-term storage based on a Bayesian multi-level model. *J Agric Biol Environ Stat*. 2012; 17(2), 192–208. https://doi.org/10.1007/S13253-012-0085-Y

Ueno AC, Gundel PE, Seal CE, Ghersa CM, Martínez-Ghersa MA. The negative effect of a vertically-transmitted fungal endophyte on seed longevity is stronger than that of ozone transgenerational effect. *J Environ Exp Bot*. 2020; 175, 104037. https://doi.org/10.1016/J.ENVEXPBOT.2020.104037

van Treuren R, Bas N, Kodde J, Groot SPC, Kik C. Rapid loss of seed viability in ex situ conserved wheat and barley at 4°C as compared to -20°C storage. *Conserv Physiol*. 2018, 6(1). https://doi.org/10.1093/conphys/coy033

Verdier J, Lalanne D, Pelletier S, Torres-Jerez I, Righetti K, Bandyopadhyay K, Leprince O, Chatelain E, Vu BL, Gouzy J, Gamas P, Udvardi MK, Buitink J. A regulatory network-based approach dissects late maturation processes related to the acquisition of desiccation tolerance and longevity of medicago truncatula seeds. *Plant Physiol*. 2013; 163(2), 757–774. https://doi.org/10.1104/pp.113.222380

Walters C. Understanding the mechanisms and kinetics of seed aging. Seed Sci Res. 1998; 8(02). https://doi.org/10.1017/S096025850000413X

Walters C. Optimising seed banking procedures. In: Smith RD, Dickie JB, Linington SH, Pritchard HW, Probert RJ, eds. *Seed Conservation: Turning Science into Practice*. London, Royal Botanic Gardens, Kew., 723–743. 2003.

Walters C. Orthodoxy, recalcitrance and in-between: describing variation in seed storage characteristics using threshold responses to water loss. *Planta*. 2015; 242(2), 397–

406. https://doi.org/10.1007/s00425-015-2312-6

Walters C, Berjak P, Pammenter N, Kennedy K, Raven P. Preservation of recalcitrant seeds. *Science*, 2013, 339(6122), 915–916). https://doi.org/10.1126/science.1230935

Walters C, Wheeler LM, Grotenhuis JM. Longevity of seeds stored in a genebank: species characteristics. *Seed Sci Res*. 2005; 15(1), 1–20. https://doi.org/10.1079/SSR2004195

Wang B, Zhang Z, Fu Z, Liu Z, Hu Y, Tang J. Comparative QTL analysis of maize seed artificial aging between an immortalized F2 population and its corresponding RILs. *Crop J*. 2016; 4(1), 30–39. https://doi.org/10.1016/J.CJ.2015.07.004

Washio K. The chromatin structure and seed function of land plants. *Int J Plant Biol Res*. 2014; 2, 1015.

Waterworth W, Bray CM, West CE. The importance of safeguarding genome integrity in germination and seed longevity. *J Exp Bot*. 2015; 66(12), 3549–3558. https://doi.org/10.1093/JXB/ERV080

Waterworth WM, Masnavi G, Bhardwaj RM, Jiang Q, Bray CM, West CE. A plant DNA ligase is an important determinant of seed longevity. *Plant J*. 2010; 63(5), 848–860. https://doi.org/10.1111/j.1365-313X.2010.04285.x

Wei T, Simko V. R package "corrplot": visualization of a correlation matrix (version 0.84). 2017.

Wickham H. ggplot2: elegant graphics for data analysis. Springer-Verlag New York. 2016.

Wickham H. Tidy messy data [R package tidyr version 1.1.3]. 2021. https://CRAN.R-project.org/package=tidyr

Wickham H, François R, Henry L, Müller K. A grammar of data manipulation [R package dplyr version 1.0.7]. 2021. https://cran.r-project.org/package=dplyr

Wiebach J, Nagel M, Börner A, Altmann T, Riewe D. Age-dependent loss of seed viability is associated with increased lipid oxidation and hydrolysis. *Plant Cell Environ*. 2020; 43(2), 303–314. https://doi.org/10.1111/PCE.13651

Wiebe K, Robinson S, Cattaneo A. Climate change, agriculture and food security. in sustainable food and agriculture. Elsevier. 2019.

Wouw M, Kik C, Hintum T, van Treuren R, & Visser B. Genetic erosion in crops: concept, research results and challenges. *Plant Genet Resour*. 2010; 8(1), 1–15. https://doi.org/10.1017/S1479262109990062

Yamasaki F, Domon E, Tomooka N, Baba-Kasai A, Nemoto H, Ebana K. Thirty-year

monitoring and statistical analysis of 50 species' germinability in genebank medium-term storage suggest specific characteristics in seed longevity. *Seed Sci Technol*. 2020; 48, 2, 269-287.

Yan HF, Mao PS, Sun Y, Li ML. Impacts of ascorbic acid on germination, antioxidant enzymes and ultrastructure of embryo cells of aged Elymus sibiricus seeds with different moisture contents. *Int J Agric Biol*. 2016; 18(1), 176–183. https://doi.org/10.17957/IJAB/15.0092

Zeb A, Ullah F. A simple spectrophotometric method for the determination of thiobarbituric acid reactive substances in fried fast foods. *J Anal Methods Chem*. 2016; 1-5.

Zhang XK, Yang GT, Chen L, Yin JM, Tang ZL, Li JN. Physiological differences between yellow-seeded and black-seeded rapeseed (Brassica napus L.) with different testa characteristics during artificial ageing. *Seed Sci Technol*. 2006; 34(2), 373–381. https://doi.org/10.15258/SST.2006.34.2.13

Zilic S, Milasinovic M, Terzic D, Barac M, Ignjatovic-Micic D. Grain characteristics and composition of maize specialty hybrids. *Span J Agric Res*. 2011; 9, 230–241.

Zinsmeister J, Leprince O, Buitink J. Molecular and environmental factors regulating seed longevity. *Biochem J*. 2020; 477(2), 305–323. https://doi.org/10.1042/BCJ20190165

7. Original manuscripts

7.1 Research articles

Gianella M, Balestrazzi A, Pagano A, Müller JV, Kyratzis AC, Kikodze D, Canella M, Mondoni A, Rossi G, Guzzon F. Heteromorphic seeds of wheat wild relatives show germination niche differentiation. *Plant Biol*. 2020; 22(2), 191–202. https://doi.org/10.1111/plb.13060

plant biology

Plant Biology ISSN 1435-8603

RESEARCH PAPER

Heteromorphic seeds of wheat wild relatives show germination niche differentiation

M. Gianella[1], A. Balestrazzi[1], A. Pagano[1], J. V. Müller[2], A. C. Kyratzis[3], D. Kikodze[4], M. Canella[5], A. Mondoni[5], G. Rossi[5] & F. Guzzon[6]

1 Department of Biology and Biotechnology "L. Spallanzani", University of Pavia, Pavia, Italy
2 Millennium Seed Bank, Conservation Science Department, Royal Botanic Gardens Kew, Wakehurst Place, UK
3 Vegetable Crops Sector, Agricultural Research Institute of Cyprus, Nicosia, Cyprus
4 Institute of Botany, Ilia State University, Tbilisi, Georgia
5 Department of Earth and Environmental Sciences, University of Pavia, Pavia, Italy
6 International Maize and Wheat Improvement Center (CIMMYT), Texcoco, Estado de Mexico, Mexico

Keywords
Aegilops; antioxidants; controlled ageing test; functional traits; seed dimorphism; seed germination; seed longevity.

Correspondence
Filippo Guzzon, International Maize and Wheat Improvement Center (CIMMYT), Texcoco, Estado de Mexico 56237, Mexico.
E-mail: f.guzzon@cgiar.org

Editor
D. Byers

Received: 6 August 2019; Accepted: 12 October 2019

doi: 10.1111/plb.13060

ABSTRACT

- Crop wild relatives are fundamental genetic resources for crop improvement. Wheat wild relatives often produce heteromorphic seeds that differ in morphological and physiological traits. Several *Aegilops* and *Triticum* species possess, within the same spikelet, a dimorphic seed pair, with one seed being larger than the other. A comprehensive analysis is needed to understand which traits are involved in seed dimorphism and if these aspects of variation in dimorphic pairs are functionally related.
- To this end, dispersal units of *Triticum urartu* and five *Aegilops* species were X-rayed and the different seed morphs weighed. Germination tests were carried out on seeds, both dehulled and left in their dispersal units. Controlled ageing tests were performed to detect differences in seed longevity among seed morphs, and the antioxidant profile was assessed in terms of antioxidant compounds equipment and expression of selected antioxidant genes. We used PCA to group seed morphs sharing similar patterns of germination traits, longevity estimates and antioxidant profile.
- Different seed morphs differed significantly in terms of mass, final germination, germination timing, longevity estimates and antioxidant profile in most of the tested species. Small seeds germinated slower, had lower germination when left in their dispersal units, a higher antioxidant potential and were longer-lived than large seeds. The antioxidant gene expression varied between morphs, with different patterns across species but not clearly reflecting the phenotypic observations.
- The results highlight different trait trade-offs in dimorphic seeds of *Aegilops* and *T. urartu*, affecting their germination phenology and longevity, thereby resulting in recruitment niche differentiation.

INTRODUCTION

Seed heteromorphy is defined as the production of different types of seeds by the same individual. The difference between morphs can reside in different morphological traits, such as shape, colour and thickness of the seed coat, mass, as well as in ecophysiological and molecular traits such as stress tolerance, dormancy, soil persistence, dispersal or gene expression (Matilla *et al.*, 2005). Some aspects of seed heteromorphy reflect ecological strategies with an evolutionary significance, *e.g.*, a different germination timing associated with soil persistence allows the avoidance of competition between siblings in relatively short-lived species with small or absent dispersal areas (Venable & Burquez, 1989). The scaled germination of different seed morphs, spreading seedling emergence over several seasons, is therefore linked with the so-called 'bet hedging strategy', which occurs when an individual has to lower its variance in fitness between years in order to maximise its long-term fitness (Seger & Brockmann, 1987; Olofsson *et al.*, 2009); this enhances species survival in environments with unpredictable climate conditions (Matilla *et al.*, 2005; Volis, 2016). Seed heteromorphy is often present in crop wild relatives (CWR, a glossary can be found in the Data S1) of important cereals such as oat (*Avena sativa* L.), rye (*Secale cereale* L.) and bread wheat (*Triticum aestivum* L.) (Hutchinson, 1984; Volis, 2016; Guzzon personal observation). With domestication, the adaptive role of seed heteromorphy tends to be eliminated by means of artificial selection for uniform and ready germination and yield; heteromorphy is usually controlled by few major loci and their modification results in monomorphism within crops (Nave *et al.*, 2016).

Several wild species in the genera *Aegilops* L. and *Triticum* L. show seed heteromorphy (Datta, Evenari, & Gutterman, 1970; van Slageren, 1994; Kilian *et al.*, 2011; Marcussen *et al.*, 2014; Dizkirici, Kansu, & Onde, 2016). *Aegilops* is a genus of annual grasses that comprises 23 species arranged in five sections. The genus *Triticum* includes six species arranged in three sections (van Slageren 1994). *Aegilops* and *Triticum* are so similar in several traits that they are sometimes grouped in the same genus by some taxonomists (Petersen *et al.* 2006). These two genera include the most economically important wheat wild

relatives (WWR), and the *ex situ* conservation of their seeds is fundamental to safeguard agrobiodiversity with the aim of preserving useful traits for crop improvement (Dempewolf *et al.*, 2014; Warschefsky *et al.*, 2014). Indeed, several *Aegilops* and *Triticum* species have been used as gene donors in plant breeding to improve resistance to biotic and abiotic stresses (Chhuneja *et al.*, 2008; Maxted *et al.*, 2008; Kilian *et al.*, 2011; Redden *et al.*, 2015) and, as more recently found, to facilitate the antioxidant response to water stress (Ahmadi *et al.*, 2018).

The seed heteromorphy of these WWR is generally characterised by the presence of two caryopses (the dry indehiscent fruits with the pericarp adherent to the seed coat, typical of grasses) per spikelet, one large and one small. In many species, the two caryopses differ in the colour of their seed coat, one being darker than the other (Marañón, 1989; Dyer, 2004). Germination phenology also differs between sibling seeds in *Aegilops* and *Triticum*. In many species, the larger and brighter seed germinates early after dispersal, while the smaller and darker seed may germinate even more than 1 year later (Onnis *et al.*, 1995; Volis, 2016; Guzzon *et al.*, 2018), probably due to secondary dormancy caused by a water-soluble inhibitor in the glumes (Wurzburger & Leshem, 1967; Datta *et al.*, 1970; Fandrich & Mallory-Smith, 2005). The delay in the germination of one morph reduces the competition among sibling seedlings and spreads germination over two consecutive years, thereby improving diversity and chances of recruitment success (Nave *et al.*, 2016). More recently, Guzzon *et al.* (2018) highlighted significant differences in longevity across seed morphs in several *Aegilops* species when exposed to a controlled ageing test (CAT), with the smaller seed being consistently longer-lived than its larger sibling, thereby confirming previous observations in *T. aestivum* (Calucci *et al.*, 2004) and reinforcing the hypothesis of a correlation between seed longevity and soil seed bank persistence, i.e., there may be reasonable levels of correspondence of longevity estimates obtained through CAT with field seed persistence (Long *et al.*, 2008).

In this context, several studies have reported a strong correlation between seed longevity and the antioxidant system (Zhu & Chen, 2007; Demirkaya *et al.*, 2010; Bewley *et al.*, 2013; Donà *et al.*, 2013). In fact, during storage and ageing, seeds produce ROS (reactive oxygen species) through auto-oxidation reactions. To combat damage produced by ROS release, seeds have evolved a complex system of detoxification that involves both an enzymatic and a non-enzymatic response (Bailly, 2004). However, during natural or accelerated ageing, levels of antioxidant enzymes and antioxidant molecules decrease, leading to loss of viability, mainly caused by biochemical interactions between ROS and biological macromolecules like lipids, proteins and nucleic acids (Yin *et al.*, 2017; Wang *et al.*, 2018). Consequently, the possibility that longevity differences across seed morphs (see above) could be due to related differences of antioxidant system cannot be ruled out.

Current studies highlight that some aspects of variation in dimorphic pairs are well characterised. However, a more comprehensive analysis is needed to understand whether these aspects are functionally related and the way (if any) they may contribute to fitness, *e.g.*, through niche differentiation between seed morphs. To this end, we characterised seed heteromorphy in five *Aegilops* species: *Aegilops bicornis* (Forssk.) Jaub. & Spach, *Ae. cylindrica* Host, *Ae. neglecta* Req. ex Bertol., *Ae. tauschii* Coss., *Ae. triuncialis* L. and in *T. urartu* Thumanjan ex Gandilyan, considering different traits: (i) anatomy of the spikelets, (ii) germination response of both seed enclosed in their dispersal units and dehulled seeds, (iii) longevity using CAT, and (iv) antioxidant profiles, in terms of parental equipment in dry caryopses and expression of selected antioxidant genes. For three of the species (*Ae. bicornis*, *Ae. tauschii* and *T. urartu*) this is the first assessment of seed heteromorphy. Our research hypothesis was that dimorphic pairs within the same species differ in ecophysiological traits, but the same single morph across species share similar germination, longevity and antioxidant profiles, and that these traits are functionally related to maximise niche differentiation between seed morphs.

MATERIALS AND METHODS

Plant material

Dispersal units of the six species were collected in the wild in Italy, Cyprus and the Republic of Georgia at the time of natural dispersal, between May and July 2016 (Table 1), with the exception of the spikelets of *T. urartu*, which were collected from an accession cultivated under common garden conditions at the MUSA botanic garden (Zibido San Giacomo, Italy).

Anatomical analysis of heteromorphism

A sample of five dispersal units for each accession was X-ray scanned (Faxitron Biopticts, Tucson, AZ, USA) to determine the quantity, relative position and eventual dimorphism of the caryopses, hereafter referred to as 'seeds', within the dispersal units of the different species. After collection, seeds were extracted from the dispersal units and the different seed morphs encountered were separated and considered as different seed lots in the following experiments. For those two *Aegilops* species (*Ae. neglecta* and *Ae. triuncialis*) whose spikelets do not disarticulate at maturity and therefore the whole spike is the dispersal unit, the two basal fertile spikelets were separated from the upper ones and the dimorphic seed pairs were extracted from them. After the cleaning process, seeds were kept in standard conditions at 15% relative humidity (RH) and 15 °C (ISTA, 2018) until use.

Seed mass was determined by weighing 20 individual seeds for each seed morph of each species (using individual seeds as replicates), kept at 15% RH, randomly sampled from each seed lot, using a microbalance (UMT2, Mettler Toledo, Columbus, OH, USA).

Germination tests of dehulled seeds

Three replicates of 20 seeds of each seed lot and of each species, extracted from their dispersal units, were sown on two layers of filter paper (Thermo Fisher Scientific, Waltham, MA, USA), soaked with 9 ml distilled water, held in 90-mm diameter Petri dishes. The Petri dishes were put in transparent plastic bags to avoid evaporation and placed in temperature- and light-controlled incubators (LMS, Sevenoaks, UK) using a 12-h daily photoperiod (photosynthetically active radiation 40–50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 20 °C, the constant temperature in which *Aegilops* species achieve the highest final germination according to Guzzon *et al.* (2015). Petri dishes were checked every 12 h for germination, and seeds scored as germinated

Table 1. Provenance, coordinates of collecting locations, type of dispersal unit and seed morphs of the six species used in this study.

Species	Provenance	Coordinates	Section (according to Van Slageren, 1994)	Dispersal Unit	Seed morphs
Ae. bicornis	Akrotiri, Cyprus	34° 58′ 51.5″ N 33°42′ 52.4″ E	*Sitopsis*	Spikelet	A, B, S
Ae. cylindrica	Chambave, Italy	45° 44′ 47.3″ N 7° 32′ 23″ E	*Cylindropyrum*	Spikelet	A, B, S
Ae. neglecta	Fortunago, Italy	44° 55′ 24.5″ N 9° 12′ 58.3″ E	*Aegilops*	Spike	1A, 1B, 2A, 2B
Ae. tauschii	Dedoplistskaro, Georgia	41° 29′ 5.0″ N 46° 8′ 13.2″ E	*Vertebrata*	Spikelet	A, B
Ae. triuncialis	Melissano, Italy	39° 58′ 6.9″ N 18° 6′ 20.7 E	*Aegilops*	Spike	1A, 1B, 2A, 2B
T.urartu	Zibido San Giacomo, Italy (cultivated)	45° 22′ 8″ N 09° 7′ 12″ E	(*Triticum* genus) *Monococcon*	Spikelet	A, B

once the radicle had reached 2 mm length. At the completion of each germination test (4 weeks after sowing), non-germinated seeds were cut-tested to confirm whether they were empty. Non-germinated empty seeds were excluded from the subsequent analyses.

The mean time to germination (MTG) was calculated using the formula:

$$MTG = \sum(nT)/N$$

where n is the number of seeds that germinated within consecutive intervals of time, T is the time (in hours) between the beginning of the test and the end of a particular interval of measurement, and N is the total number of germinated seeds. The MTG was calculated using the hour of sowing as initial time and it is expressed in hours.

Germination tests of seeds enclosed in the dispersal unit

To determine a possible effect of the spikelet tissue on seed germination, germination tests of seeds enclosed in their dispersal units were performed. Three replicates of 20 dispersal units of each species were placed on sand. For this, 500 g fine sand (Hustridge Garden Products) was sterilised for 1 h at 103 °C and sifted. It was then put in transparent plastic boxes of 16 cm × 6 cm × 10.5 cm and wetted with 70 ml water. The plastic boxes were placed in temperature- and light-controlled incubators (LMS, Sevenoaks, UK) as previously described (12-h daily photoperiod and 20 °C). The dispersal units were sown resembling the natural dispersal strategy of the species, *i.e.*, the dispersal units were inserted in the first cm of sand, so that the entire dispersal unit was in contact with the substrate. The only exception was made for *Ae. cylindrica* and *Ae. tauschii*, whose dispersal units are spikelets of cylindrical shape that were simply laid onto the surface of the wet sand. Seeds were considered as germinated when radicle emergence was evident outside the dispersal units. Seed germination was checked every 5 days. In the scoring of the germination, the dispersal units were carefully removed from the sand and then re-sown after the scoring as previously described (inserted in the sand or laid onto the surface). At the end of the experiment, after 4 weeks, the dispersal units were opened to see which seeds had germinated.

To check if the germination medium (filter paper or wet sand) influenced the germination of dehulled seeds (performed on filter paper) and the germination of seed enclosed in the dispersal units (performed on sand), three replicates of 20 seeds per morph (per species) were extracted from their dispersal units and put on wet sand as described above.

All tests performed on freshly harvested seeds were conducted in the laboratory of the Millennium Seed Bank of the Royal Botanical Gardens, Kew, in August and September 2016 (UK).

Controlled ageing test (CAT)

Accelerated ageing

Seed longevity was tested with a modified protocol for artificial ageing (Guzzon *et al.*, 2018 modified from Newton *et al.*, 2009). Prior to storage, seeds were first rehydrated for 14 days in open Petri dishes over a non-saturated solution of LiCl in a 300 × 300 × 130 mm sealed electrical enclosure box (Ensto UK, Southampton, UK) at 47% RH and 20 °C. Thereafter, seed equilibrium relative humidity (eRH) was verified with an AW-DI0 water activity probe used in conjunction with a Hygro-Palm 3 display unit (Rotronic Instruments UK, Crawley, UK). Once the seeds had reached eRH, the initial germination was assessed using triplicates of 20 seeds and the protocol described above. Next, seed lots were stored in the dark in a sealed box over a non-saturated LiCl solution at 60% RH at 45 ± 2 °C, placed in a compact incubator (Binder FD53; Binder, Tuttlingen, Germany). When necessary, aliquots of distilled water were added to the LiCl to keep the RH at the required equilibrium, which was monitored inside the enclosure box (Tinytag View 2 Temperature/Relative Humidity Logger; Gemini Data Logger, Chichester, UK) (Hay *et al.*, 2008). At three to eight intervals during storage, three replicates of 20 seeds per lot were extracted and viability was tested with the same protocol as used for the preliminary germination test. Different seed lots were extracted at different time points according to their lifespan, until a complete viability loss was reached (Table S1).

The CAT was performed in the Laboratory of Seed Ecology at the University of Pavia (Italy) starting in September 2017 and lasting for 4 months.

Analyses of the antioxidant components

Dry seed extracts were prepared as described by Li *et al.* (2008). Samples (100 mg each) were homogenised at room temperature with 1 ml 80% acetone, then incubated overnight in the dark at 26 °C and stored at −18 °C. Free radical scavenging activity was assessed as described by Braca *et al.* (2001). Aliquots of seed extracts (0.1 ml each) were added to 3 ml 100 μM DPPH (Sigma-Aldrich, Milan, Italy) dissolved in methanol and the reaction was carried out in the dark. A calibration curve was built using 0.1 ml ascorbic acid in the 2.00–0.125 mM concentrations. DPPH reduction was measured at λ = 517 nm with a spectrophotometer (Jasco, Easton, MD, USA). The

antioxidant potential was expressed as Ascorbic Acid Equivalents (AAE) μg^{-1} fresh weight.

The total phenolic content was measured using the Folin-Ciocalteu's reagent (Sigma-Aldrich). Aliquots (0.02 ml each) of seed extracts were added to distilled water (1.58 ml) and to 0.1 ml of reagent. After 8 min incubation, the reaction was neutralised with 0.3 ml 7.5% w/v Na_2CO_3 (Sigma-Aldrich). Samples were then incubated in the dark for 120 min. A standard curve was built using gallic acid in the 500–50 mg^{-1} concentration range. Absorbance was measured at $\lambda = 765$ nm. Total phenolic content was expressed as Gallic Acid Equivalents (GAE) mg^{-1} fresh weight. The Specific Antioxidant Activity (SAA), defined as the ratio between antioxidant potential and total phenolic content, was calculated and expressed as mg AAE mg^{-1} GAE.

Gene expression analysis

The RNA extraction was performed according to Oñate-Sánchez & Vicente-Carbajosa (2008) from dry and 12-h imbibed seeds of *Ae. cylindrica*, *Ae. tauschii* and *Ae. triuncialis*. Retro-transcription was carried out using the iScript cDNA Synthesis kit (Bio-Rad, Milan, Italy), according to the manufacturer's instructions. qRT-PCR was performed using SYBR Green qPCR Master Mix (2X) (Fermentas, Milan, Italy) and a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia). For oligonucleotide primer design, sequences were obtained from the online database EnsemblPlants: an alignment was performed between sequences from *Ae. tauschii* and *T. urartu* with the online software Multiple Sequence Alignment by CLUSTALW. To analyse the expression of *SOD* (superoxide dismutase) and *APX* (ascorbate peroxidase) genes, the geometric mean of two reference genes, *ACT* (actin) and *UBI* (ubiquitin), was used as standard control, since they showed the most stable expression in different tissues and treatments in wheat and in *Aegilops* spp. (Kashlush *et al.*, 2003; Paolacci *et al.*, 2009; Mizuno *et al.*, 2010; Koyama *et al.*, 2012). Oligonucleotide primers were designed with the online software Primer3Plus and thermodynamic parameters were checked with Oligoanalyzer (Table 2). qRT-PCR conditions were denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. Once a specific fluorescence was subtracted, the raw fluorescence data obtained from the Software Rotor-Gene 6000 Series 1.7 (Corbett Robotics) were used to retrieve 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 the two reference genes were used to normalise the values by calculating the ratio between target gene transcripts and the geometrical mean of the reference gene transcripts.

The DPPH test, Folin-Ciocalteu's reagent assay and qRT-PCR were performed at the Laboratory of Plant Biotechnology of the University of Pavia (Italy) in parallel with CAT.

Statistical analysis

Normality of distribution of variables was checked with Shapiro–Wilk tests.

As seed mass was not normally distributed, non-parametric Kruskal–Wallis tests were applied to evaluate if the differences in seed masses was significant among different species and if the mass of the different seed lots varied significantly within species.

Probit analysis was carried out using GenStat 9 (VSN International, Oxford, UK) to obtain the time for viability to fall by 50% (*p50*), used then as measure for seed longevity by fitting the viability equation (Ellis & Roberts, 1980):

$$v = K_i - p/\sigma.$$

where v is the viability in normal equivalent deviates (NED) at time p (days); Ki is the initial viability or the intercept on the y-axis (NED), and σ is the standard deviation of the normal distribution of seed deaths in time.

To find differences in longevity between species and seed lots, different *p50* were compared using a generalised linear model (GLM) using the statistical software SPSS 21 (Chicago, IL, USA). Normal distribution and identity as link function were applied, with *post-hoc* pairwise comparisons between seed lots belonging to the same species.

The GLM were also used to compare other germination parameters: germination of dehulled seeds and seeds enclosed in the dispersal unit and MTG. Binomial distribution and logit link function were used in models regarding germination; gamma distribution with identity as link function was used in the MTG model. Seed lot and species were tested as fixed factors. Pairwise comparisons were employed to compare seed lots within species. GLM were also applied to evaluate the statistical significance of differences between morphs in antioxidant profiles and gene expression: normal distribution and identity as link function were used for AAE and GAE, while for SAA and gene expression a gamma distribution with identity as link function was applied. Samples were then compared with *post-hoc* pairwise comparisons. Correlations between AAE, GAE, SAA and *p50* were performed using two non-parametrical tests: Kendall's Tau b and Spearman's Rho.

A principal components analysis (PCA) using the FactoMineR (Lê *et al.*, 2008) package in R environment for statistical computing and graphics (studio version 3.5.2) was performed

Table 2. List of oligonucleotide primers used for qRT-PCR analyses. For each oligonucleotide set; PCR efficiency is reported. Quantification was carried out using ACT and UBI as reference genes for the experimental conditions (dry versus imbibed) used in this work. ACT, actin; UBI, ubiquitin; APX, ascorbate peroxidase; SOD, superoxide dismutase.

Gene (*PlantEnsembl* accession)	Forward primer (5'-3')	Reverse primer (5'-3')	Efficiency
ACT (F775_26523)	CGACAGGATGAGCAAGGAGA	GAGGGAGGCGAGGATGGA	1.78
UBI (F775_29337)	CGGTGGAGGTGGAGAGC	ACGAGGTGAAGCGTGGAC	1.82
APX (F775_32980)	TCTCCTCCTTGATGGCTC	GAAGAACTGCTCCCCGCTC	1.84
SOD (F775_26675)	ACGAGGTGAAGCGTGGAC	TGTCAGCATCAAGCACCAGT	1.70

to assess which traits better explained seed heteromorphy among germination and longevity parameters, seed mass and antioxidant profile variables. The input data were arithmetic means of replicates after standardisation of variables by means of z-score transformation.

RESULTS

Seed dimorphism

Based on the morphology of the spike, the six species were divided into two groups (Figure 1), and the identified seed morphs were considered as different seed lots during the germination experiment:

1 In four species (*T. urartu*, *Ae. bicornis*, *Ae. cylindrica* and *Ae. tauschii*), the spike disarticulates in several spikelets at maturity; therefore, the spikelet is the dispersal unit of those species. Two seed morphs were detected, seed A, the larger one, and seed B, the smaller one. A third category, S (= single seed), was established in cases where just one seed was enclosed in the spikelet.
2 In the other two species (*Ae. neglecta* and *Ae. triuncialis*), the spike does not disarticulate at maturity, therefore, the whole spike can be considered as the dispersal unit. In these two species the following morphs were considered, the large and the small seeds from the first basal spikelet were named 1A and 1B, respectively, while seeds from the second spikelet were named 2A and 2B. In the experiments, these different seed morphs were considered as different seed lots.

The relative position of the two seeds within the same spikelet has been clarified through the X-ray scanning analysis. In all six studied species, the lower floret is occupied by the smaller seed (Figure 1).

Seed mass varied significantly both among species ($\chi^2 = 234{,}616$, df = 5, $P < 0.001$) and within all the species among seed lots ($\chi^2 = 159{,}625$, df = 6, $P < 0.001$).

Larger seeds from the dimorphic pairs are defined as 'primary seeds' (morphs A, 1A, 2A), while the smaller ones are 'secondary seeds' (morphs B, 1B, 2B).

Germination of dehulled seeds

Both the species and the seed lot had an effect on the germination of dehulled seeds (species: Wald $\chi^2 = 22{,}90$, df = 4, $P < 0.001$; seed lot: Wald $\chi^2 = 54{,}602$, df = 5, $P < 0.001$). No statistically significant difference was found between morphs in the germination of dehulled seeds of *Ae. bicornis* (A/B $P = 0.420$, A/S $P = 0.32$, B/S $P = 0.186$), *Ae. cylindrica* (A/B $P = 0.601$, A/S $P = 0.76$, B/S $P = 0.205$) and *Ae. tauschii* (A/B $P = 0.063$), while dehulled seeds A of *T. urartu* showed higher germination percentage than seeds B ($P < 0.001$). On the other hand, in the two species whose whole spikes serve as the dispersal unit (*Ae. triuncialis* and *Ae. neglecta*), even if extracted and dehulled, germination of sibling seeds was significantly

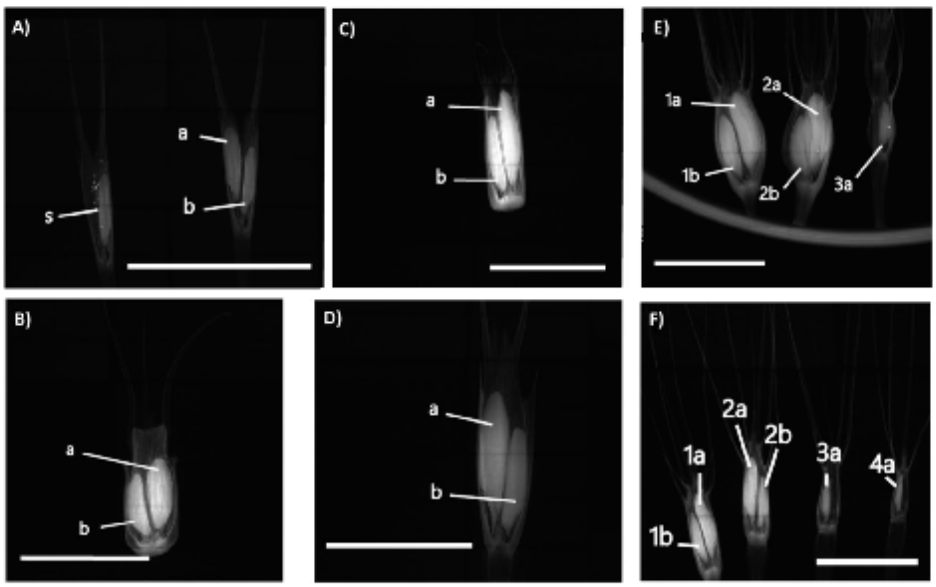


Fig. 1. Images of X-ray scanned samples. On the left, species with single spikelet as dispersal unit: (A) *Ae. bicornis*, (B) *Ae. tauschii*, (C) *Ae. cylindrica*, (D) *T. urartu*. On the right, species with whole spike as dispersal unit: (E) *Ae. neglecta*, (F) *Ae. triuncialis*. In *Ae. neglecta* and *Ae. triuncialis* spikelet were manually disarticulated to obtain a clearer view of the seed morphs in each spikelet. In *Ae. neglecta* and *Ae. triuncialis* note a third and in *Ae. triuncialis* also a fourth fertile spikelet (with seed lots 3a and 4a), not tested in the experiments due to lack of seed material. White bars represent 1 cm.

different, with a higher final germination in primary seeds (1A and 2A) than in secondary seeds (1B and 2B) ($P < 0.001$; Figure 2, Table 3).

Also for the MTG, both the species and the seed lot had an effect on dehulled seeds (species: Wald $\chi^2 = 14{,}583$, df = 4, $P < 0.01$; seed lot: Wald $\chi^2 = 51{,}362$, df = 5, $P < 0.001$). In *T. urartu* the MTG of the two morphs did not show a significant difference ($P = 0.094$), in *Ae. tauschii*, *Ae. cylindrica*, *Ae. bicornis*, *Ae. neglecta* and *Ae. triuncialis* primary seeds showed a lower MTG than secondary ones ($P < 0.05$). In *Ae. cylindrica* and *Ae. bicornis*, seed S did not differ in MTG from seed A ($P = 0.071$ and $P = 0.501$), nor from seed B ($P = 0.129$ and $P = 0.080$) (Table 3).

Germination of seeds enclosed in their dispersal unit

When left in their dispersal unit, imitating natural dispersal, significant differences in seed germination were observed for all species. Both the species and the seed lot had an effect on germination of enclosed seeds (species: Wald $\chi^2 = 35{,}083$, df = 4, $P < 0.001$; seed lot: Wald $\chi^2 = 202{,}740$, df = 5, $P < 0.001$). In all four species where the spikelet serves as the dispersal unit (*Ae. bicornis*, *Ae. cylindrica* and *Ae. tauschii*, *T. urartu*), seed A showed a higher final germination percentage than seed B ($P < 0.001$), and when present, seed S showed intermediate behaviour with no statistically significant difference to the other two morphs ($P > 0.05$). In more detail, in the primary seeds of all species, when left in the dispersal unit, the

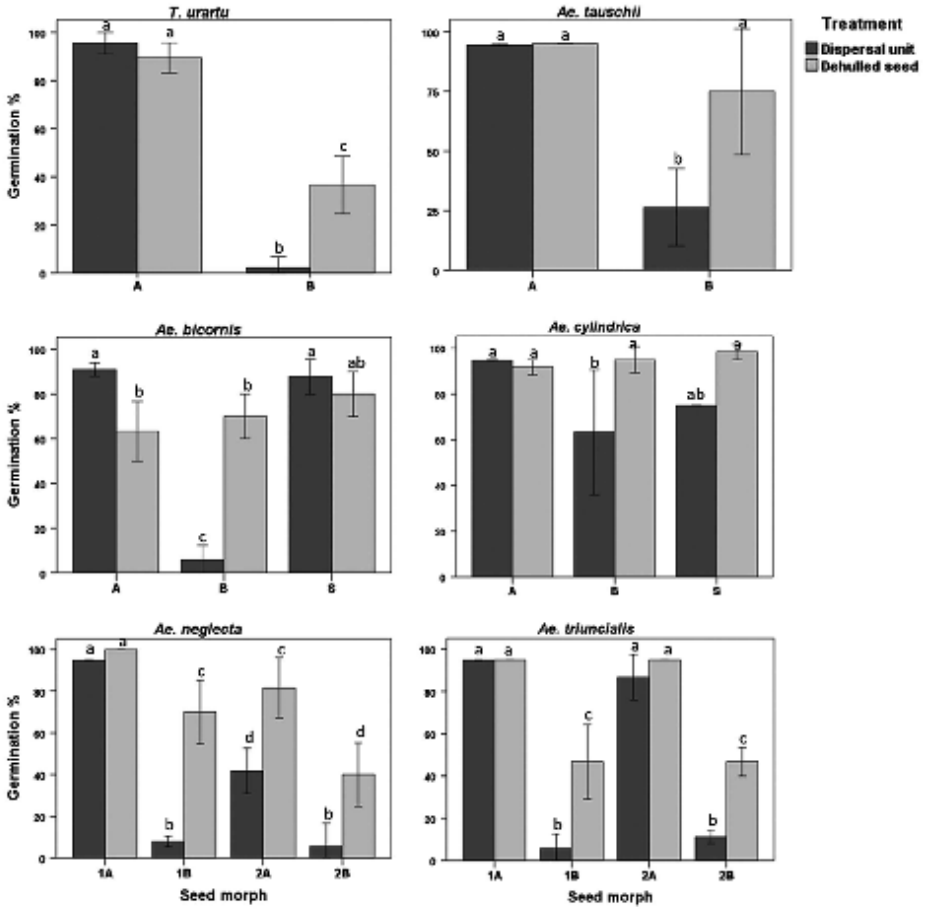


Fig. 2. Germination percentages of different species and morphs of dehulled seeds (light grey) and seed enclosed in their dispersal unit (dark grey). Different letters above bars represent statistically significant differences between morphs ($P < 0.05$).

Table 3. Mean values and standard deviations of MTG and germination percentage of dehulled seeds in all the species and seed lots used in this study.

Species	Seed Lot	MTG (h)	Germination (%)
T. urartu	A	29.34 ± 1.14	89.56 ± 5.39
	B	210.03 ± 186.68	36.67 ± 10.41
Ae. bicornis	A	80.69 ± 17.66	63.33 ± 11.55
	B	166.44 ± 68.21	70.00 ± 8.66
	S	104.21 ± 26.80	80.00 ± 8.66
Ae. cylindrica	A	32.73 ± 4.89	91.67 ± 2.89
	B	61.07 ± 13.65	95.00 ± 5.00
	S	48.12 ± 10.91	98.33 ± 2.89
Ae. tauschii	A	89.09 ± 10.86	95.00 ± 0.00
	B	260.94 ± 49.24	75.00 ± 22.91
Ae. neglecta	1A	57.13 ± 17.43	100.00 ± 0.00
	1B	115.95 ± 46.65	70.00 ± 13.23
	2A	45.47 ± 2.63	81.67 ± 12.58
	2B	177.7 ± 33.50	40.00 ± 13.23
Ae. triuncialis	1A	25.41 ± 1.54	95.00 ± 0.00
	1B	232.20 ± 83.19	46.67 ± 15.28
	2A	28.43 ± 3.40	95.00 ± 0.00
	2B	189.42 ± 85.77	46.67 ± 5.77

final germination percentage was higher than 90%, while in the secondary seeds it was always lower than 30%, with the exception of *Ae. cylindrica* B which showed a final germination percentage of 60%. In *Ae. triuncialis* and *Ae. neglecta*, where the dispersal unit is the whole spike, differences were found between seeds 1A and 1B, and between seed 2A and 2B; in both spikelets, the morph A had a higher germination percentage than morph B ($P < 0.001$). Primary seed, 1A and 2A, had a similar final germination which was higher than 90%. Also, secondary seeds, secondary seeds 1B and 2B, showed a similar final germination lower than 15% ($P > 0.05$).

No significant differences in germination were found between dehulled seeds sown on filter paper and on sand (Wald $\chi^2 = 0.00$, df = 1, $P = 1.00$); this allows us to rule out the effect of the medium on the germination experiments and to compare the two different germination experiments (*i.e.*, with dehulled seeds and with seeds enclosed in the dispersal unit).

A significant difference was detected betweeen the final germination of dehulled seeds (*i.e.*, extracted from their dispersal units) and of seeds enclosed in their dispersal units (Wald $\chi^2 = 9{,}567$, df: 2, $P < 0.001$). In all species, secondary seeds (B, 1B, 2B) showed significant higher germination when dehulled than when kept in their dispersal units ($P < 0.001$; Figure 2). No significant differences in the germination between dehulled seeds and seeds enclosed in their dispersal units was detected in primary seeds (A, 1A, 2A), with the exception of *Ae. bicornis*, where dehulled seed A showed a lower germination than the enclosed one ($P < 0.001$). In both species with seed S (*Ae. bicornis* and *Ae. cylindrica*), no significant differences in final germination were dectected between the two conditions, *i.e.*, dehulled versus enclosed in the dispersal units ($P > 0.05$).

Controlled ageing test (CAT)

The *p50* varied greatly in the different seed accessions (Table 4). Days required for germination to drop by 50% ranged from 24.5 days in *T.urartu* A to 72 days in *Ae. bicornis* B. Species and seed lot (seed A or B) had a significant effect on *p50* (df = 4, Wald $\chi^2 = 330.916$, $P < 0.001$; df = 5, Wald $\chi^2 = 110.086$, $P < 0.001$). In *Ae. tauschii* no significant difference was observed in terms of *p50* between the two seed morphs.

In the other species, secondary seeds were significantly longer-lived than primary ones ($P < 0.05$). In *Ae. bicornis* and *Ae. cylindrica* seed S showed an intermediate behaviour between seed A and B ($P > 0.05$).

In the species whose whole spike is the dispersal unit, *Ae. neglecta* and *Ae. triuncialis*, seeds 2B were the longest-lived, while 1A seeds were the shortest-lived (Table 4). In *Ae. triuncialis*, no significant difference in *p50* was observed between 1A and 2A, nor between 1B and 2B ($P = 0.687$, $P = 641$, respectively), while in *Ae neglecta* those differences were significant (1A/2A $P < 0.001$, 1B/2B $P < 0.05$).

Table 4. Mean (±SE) values of longevity parameters (*p50*, σ^{-1}, K_i), MTG and weight in all the seed lot used for the CAT.

Species	Seed Lot	*p50* (days)	σ-1	Ki	Weight (mg)
T. urartu	A	24.54 ± 2.56	0.059 ± 0.008	1.441 ± 0.266	18.00 ± 2.297
	B	31.63 ± 3.17	0.046 ± 0.007	1.463 ± 0.246	10.57 ± 1.672
Ae. bicornis	A	64.13 ± 4.84	0.027 ± 0.003	1.705 ± 0.211	4.196 ± 0.919
	B	72.03 ± 6.30	0.023 ± 0.003	1.671 ± 0.215	3.332 ± 0.517
	S	61.63 ± 4.07	0.031 ± 0.003	1.929 ± 0.234	3.869 ± 0.637
Ae. cylindrica	A	49.04 ± 3.18	0.068 ± 0.011	3.348 ± 0.526	16.32 ± 3.269
	B	64.58 ± 4.02	0.063 ± 0.013	4.100 ± 0.826	9.700 ± 1.759
	S	53.66 ± 4.28	0.083 ± 0.007	4.448 ± 0.392	15.16 ± 4.355
Ae. tauschii	A	58.76 ± 3.46	0.068 ± 0.013	3.965 ± 0.709	15.21 ± 3.884
	B	59.71 ± 4.28	0.042 ± 0.007	2.498 ± 0.392	10.56 ± 2.322
Ae. neglecta	1A	45.30 ± 2.34	0.088 ± 0.015	3.993 ± 0.667	48.64 ± 4.769
	1B	69.20 ± 9.3	0.143 ± 0.05	9.890 ± 3.14	30.32 ± 5.849
	2A	52.66 ± 3.04	0.108 ± 0.023	5.670 ± 1.14	29.18 ± 10.870
	2B	69.94 ± 5.4	0.127 ± 0.015	8.890 ± 1.09	20.22 ± 5.923
Ae. triuncialis	1A	50.67 ± 2.07	0.108 ± 0.018	5.470 ± 0.945	19.62 ± 4.878
	1B	61.99 ± 3.22	0.073 ± 0.012	4.527 ± 0.784	12.23 ± 3.537
	2A	49.16 ± 2.05	0.109 ± 0.018	5.379 ± 0.921	18.36 ± 4.640
	2B	59.34 ± 3.57	0.066 ± 0.013	3.913 ± 0.89	8.059 ± 2.719

Antioxidant profiles

Species and seed lots had an effect on AAE values (Species: Wald $\chi^2 = 41{,}672$, df = 4, $P < 0.001$; seed lot: Wald $\chi^2 = 60{,}91$, df = 5, $P < 0.001$). Significant differences in the seed antioxidant potential measured through the DPPH test were found in three species (*Ae. bicornis*, *Ae. triuncialis*, *Ae. neglecta*). *Ae. bicornis* seed A showed a lower potential ($P < 0.01$) than B and S. In both *Ae. neglecta* and *Ae. triuncialis* comparisons between sibling seeds from spikelet 1 and 2 showed a statistically significant difference in AAE, with lower levels in primary seeds (1A and 2A) compared to secondary seeds (1B and 2B) (1A/1B, $P < 0.01$; 2A/2B, $P < 0.01$): Among the tested accessions, seed 2B showed the highest antioxidant potential in both species.

Species and seed lot had an effect on GAE (Species: Wald $\chi^2 = 18{,}696$, df = 4, $P < 0.01$; seed lot: Wald $\chi^2 = 180{,}119$, df = 5, $P < 0.001$). All species except *Ae. tauschii and Ae. bicornis* showed significant differences between morphs in terms of total phenolic content. In the two species with a single spikelet as the dispersal unit (*Ae. cylindrica* and *T. urartu*), seed B had a higher phenolic content than seed A (*Ae. cylindrica* A/B $P < 0.01$; *T. urartu* A/B $P < 0.01$). In the species with the whole spike as the dispersal unit (*Ae. neglecta* and *Ae. triuncialis*), levels of phenolic compounds of small seeds (1B and 2B) were higher than those of the larger sibling seeds within the spikelet (1A and 2A, respectively) ($P < 0.01$; Figure 3). Both species and seed lot had an effect on SAA (Species: Wald $\chi^2 = 38{,}493$, df = 4, $P < 0.01$: Seed lot: Wald $\chi^2 = 17{,}949$, df = 5, $P < 0.01$). Comparisons of Specific Antioxidant Activities (SAA) between morphs were statistically significant only in *Ae. triuncialis*, where SAA of seed 2A was lower than that of seed 2B and in *T. urartu*, where seed A showed a higher SAA than seed B ($P < 0.01$; Figure 3).

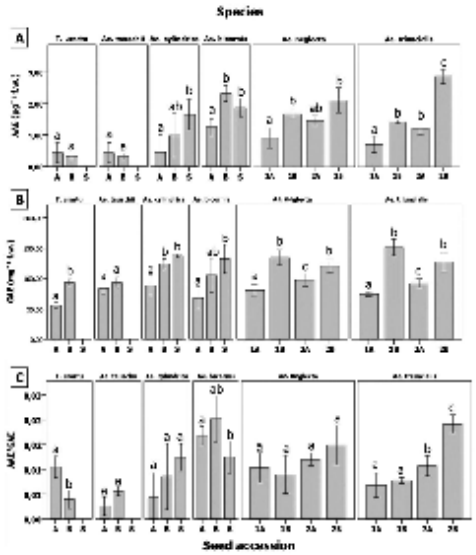


Fig. 3. Antioxidant profile of the different seed accessions. (A) DPPH radical-scavenging activity expressed as μg^{-1} fresh weight. (B) Total phenolic content, evaluated using the Folin-Ciocalteu reagent method, expressed as GAE mg^{-1} fresh weight. (C) Specific Antioxidant Activity is expressed as AAE/GAE. Different letters above bars represent statistically significant differences between morphs: comparisons have been done only within the same species.

Correlations between longevity estimates and antioxidant profiles

Longevity estimates (in terms of *p50*) and the antioxidant profile (in terms of AAE, GAE and SAA) appeared to be positively correlated in both non-parametric tests (Kendal's Tau B and Spearman's Rho, Table S2). Antioxidant potential, expressed as AAE, revealed a significant correlation with *p50* in both tests ($P < 0.001$; Tau B = 0.412; Rho = 0.580). Similarly, the correlation between total phenolic compounds, expressed as GAE, with *p50* was significant (Tau B = 0.302, $P < 0.01$; Rho = 0.430, $P < 0.001$). Also, the correlation of *p50* with specific antioxidant activity (SAA) was statistically significant (Tau B = 0.257, $P < 0.05$; Rho = 0.154, $P < 0.05$). These correlations are represented in two 2-axes graphs (Figure S1).

Gene expression of APX and SOD is modulated in seed morphs

Expression profiles of *SOD* and *APX* genes, which are molecular indicators of the seed antioxidant response, were investigated in *Ae. cylindrica*, *Ae. tauschii* and *Ae. triuncialis*. *Ae. tauschii* was selected because of the availability of the genome sequence and since no differences in terms of antioxidant activity had been detected between seeds A and B during the initial screening. *Ae. cylindrica* and *Ae. triuncialis* were selected as they showed similar antioxidant profiles in dry seeds, although characterised by a different type of dispersal unit. Expression was analysed in dry seeds and 12-h rehydrated seeds, to assess the equipment in terms of transcripts both after seed maturation (dry seeds) and before germination but after the onset of the pre-germinative metabolism (12-h imbibed seeds). The relative gene expression values are shown in Table S3. Species and imbibition had an effect on *SOD* transcript levels, while seed lot did not (species: Wald $\chi^2 = 33.494$, df = 2, $P < 0.001$; imbibition: Wald $\chi^2 = 122{,}081$, df = 1, $P < 0.001$; seed lot: Wald $\chi^2 = 0.00$, df = 1, $P = 1.000$). Species, imbibition and seed lot had an effect on *APX* transcript levels (species: Wald $\chi^2 = 6.475$, df = 2, $P < 0.05$; imbibition: Wald $\chi^2 = 16.766$, df = 1, $P < 0.001$; seed lot: Wald $\chi^2 = 4.182$, df = 1, $P < 0.05$). In *Ae. cylindrica*, the level of *SOD* transcripts was significantly higher in seed B in the dry state ($P < 0.05$), while in imbibed seeds no significant difference was observed ($P = 0.573$); expression of *APX* gene was higher in imbibed seed B ($P < 0.01$), while no difference was observed between dry seeds ($P = 0.473$). In *Ae. tauschii* a significantly higher level of *SOD* transcripts was detected in seed A both dry and imbibed when compared to seed B ($P < 0.01$), whereas the *APX* transcripts peaked in imbibed seed A ($pP < 0.01$) and no difference was found between dry seeds ($P = 0.213$). In *Ae. triuncialis SOD* gene expression showed statistically significant differences in imbibed seeds, with

higher transcript levels in seed B ($P < 0.05$), while dry seeds showed no difference ($P = 0.403$). Also for *APX* expression, no difference between morphs was found in dry seeds ($P = 0.491$), while imbibed seed A showed a higher trancript level compared to that of seed B ($P < 0.01$). To underline differences in antioxidant gene expression between morphs, the ratio between transcript levels measured in seed B and A was calculated and presented in Figure 4. As shown, *SOD* gene expression did not differ in any of the tested accessions, except for *Ae. tauschii*, where imbibed seed A revealed higher transcript levels. *APX* gene expression, conversely, showed variable behaviour in the different species, namely up-regulation in *Ae. cylindrica* in both dry and imbibed seed B, down-regulation in *Ae. tauschii* seed B at both stages. Finally in *Ae. triuncialis* up-regulation in dry seed B and down-regulation in imbibed seed B was detected.

Principal components analysis

The total variance explained by PCA on the two axes was 71.23% (Figure 5). Axis 1 (PC1), representing 54% of the total variance, was mainly explained by the germination of seeds enclosed in the dispersal unit, by the germination of dehulled seeds ('Dehulled'), by AAE and GAE. Axis 2 (PC2), representing 17.2% of the total variance, was explained mostly by the Specific Antioxidant Activity (SAA) and AAE of fresh seeds (for eigenvalues and the relative contributions of variables to the variance, see Figure S2). When seed lots were clustered by morph, the 95% confidence level ellipses demonstrated a clear separation between morphs A and B.

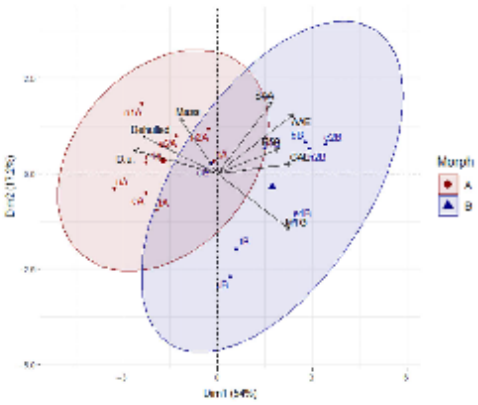


Fig. 5. Principal components analysis (PCA) using germination and longevity parameters, seed mass and antioxidant profile variables. Seed accessions were clustered by morph A or B, without considering the position within the spike.

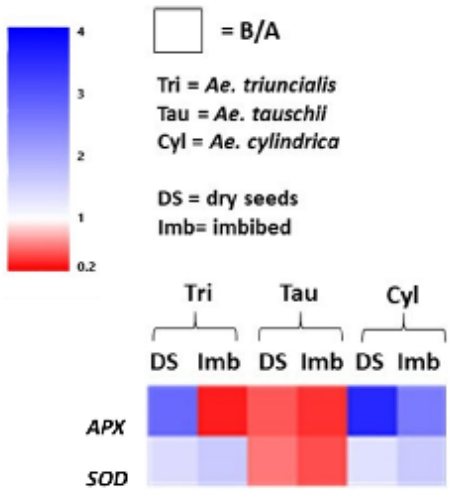


Fig. 4. Heatmap representing expression changes of antioxidant genes in dry and 1-h imbibed seeds. Expression mean values are available in Table S1. Heatmap values represent fold-changes of transcript levels monitored by qRT-PCR occurring in seed B compared to seed A (B/A). Red tones represent genes down-regulated while blue tones represent genes up-regulated at the specific comparison stated. Legend: APX, ascorbate peroxidase; SOD, superoxide dismutase.

DISCUSSION

In this study, all species showed seed heteromorphy, especially within-spikelet seed dimorphism, with primary seeds being significantly bigger and heavier than secondary seeds, which were always located in the lower floret inside the spikelet. Intriguingly, unlike previous reports, which had described *T. urartu* as having only one seed per each spikelet (Nave *et al.*, 2016), most of the spikelets of the accession studied here were double-seeded and showed within-spikelet seed dimorphism.

Primary and secondary seeds were characterised by different germination patterns, longevity estimates and antioxidant profiles, as shown in the multivariate space where they clustered separately regardless of species. In particular, all species primary seeds when left in their dispersal unit possessed a higher final germination than secondary seeds. The final germination of secondary seeds significantly improved when seeds were extracted from their dispersal units, confirming that the latter play a crucial role in the staggered germination of the two morphs, imposing dormancy on the seeondary seeds (Wurzburger & Leshem, 1969; Lavie *et al.*, 1974; Cooper, Levy, & Lavie, 1977; Dyer, 2004). Primary seeds also showed faster germination than secondary ones. Moreover, secondary seeds were longer-lived than primary ones (except in *Ae. tauschii*) and had a significantly higher content of antioxidant molecules, particularly polyphenols. Longevity changes have been directly correlated with their antioxidant activities in seeds from populations growing in different climate conditions (Probert *et al.* 2009; Mondoni *et al.*, 2011; Donà *et al.*, 2013), while this study provides the first evidence of such a correlation in seed morphs of the same mother plants. It is interesting to note that in the two species with solitary seeds (seed S), these seeds showed intermediate values between primary and secondary in several traits (germination, longevity estimates, MTG). A possible

explanation for this intermediate behaviour is that, during maturation, one of the two sibling seeds fails to develop or dies during formation; if this hypothesis is true, the S seed lot would actually be composed of one fraction of seeds belonging to the A morph, while the other fraction corresponds to the B morph.

The higher antioxidant activity, longevity and dormancy found here in secondary compared to primary seeds can be considered as adaptive traits related to the different persistence of these seeds in the soil after dispersal, as the latter germinate soon after the dispersal, while the former germinate only 1 year later (as shown by Volis, 2016 and Guzzon *et al.*, 2018; Dyer, 2017; Lewandrowski *et al.*, 2018). Indeed, secondary seeds, showing delayed germination, need a larger provision of antioxidants to enhance their longevity and survive longer in the soil (Bailly, 2004; Jeevan Kumar *et al.*, 2015; Sano *et al.*, 2016). Phenols, beside their antioxidant activity, are also related to seed impermeability (Marbach & Mayer, 1974; Slattery *et al.*, 1982), a feature that becomes fundamental for seed persistence until the onset of the appropriate climate conditions by avoiding imbibition when the soil water content is insufficient. Moreover, non-enzymatic antioxidants such as ascorbic acid or glutathione have been proved to be related to soil persistence and are a marker of seed longevity (Long *et al.*, 2015; Kranner *et al.*, 2010). The higher antioxidant content detected in secondary seeds could therefore allow their longer survival within the soil seed bank and perhaps also under *ex situ* storage conditions. As highlighted by Guzzon *et al.* (2018), the significant differences in seed longevity among heteromorphic seeds detected in most of the wheat wild relatives suggest that different seed morphs are also likely to have different longevities under *ex situ* seed bank storage, with secondary seeds being longer lived than primary seeds. It follows that, with the aim of capturing all the possible adaptive traits in the seed samples (Warschefsky *et al.*, 2014), different seed morphs of wheat wild relatives should be held separately in storage, and their viability, regeneration or re-collection intervals (Hay & Probert, 2013) should follow different timings in order to avoid important loss of genetic resources. However, further research about the impact on *ex situ* seed conservation of different longevities in heteromorphic seeds is needed. Our results on the characterisation of the germination strategy of *Aegilops* and *Triticum* are also important to obtain a better understanding of the ecology of those species in order to implement successful *in situ* conservations strategies, considering that some of the study species are endangered at continental level (*e.g.*, *Ae. tauschii* is listed as Endangered and *Ae. bicornis* as Vulnerable in the European Red List of Vascular Plants, Bilz *et al.*, 2011).

We can hypothesise that the differences in size among the different seed morphs detected in this study could be due to different flowering times of the different florets, with the largest seeds developing from the first flowering spikelets as observed in several cereal species (Evers & Millar, 2002; Nave *et al.*, 2016). In most angiosperms, the allocation of resources occurs after egg fertilisation, and the quantitative dosage of different compounds can be influenced by epigenetic mechanisms such as imprinted genes (Sadras & Denison, 2009; Cailleau *et al.*, 2018). These mechanisms could explain the different dosage within the endosperm of sibling seeds in terms of both non-enzymatic antioxidants and of transcripts for antioxidant enzymes detected in this study. Even if with different patterns across species the antioxidant gene expression varied between morphs, which indicates the presence of a differential transcription that might contribute to the differences among seed morphs detected in *Aegilops* and *Triticum*. In *Ae. tauschii* the antioxidant genes were more expressed in primary seeds, both in the dry and imbibed states, and this could be related to the absence of differences in longevity between morphs detected in this species. This, together with the fact that primary seeds are always larger, could indicate a greater investment by the mother plant into these seeds rather than the secondary ones. The *SOD* expression in *Ae. triuncialis* and *Ae. tauschii* did not differ visibly between morphs, neither at the dry nor at the imbibed state. Conversely, the up-regulation of *APX* transcription in dry secondary seeds, detected both in *Ae. cylindrica* and *Ae. triuncialis*, could mean that a greater provision of the antioxidant enzyme coded by this gene is needed in morph B to assure a longer lifespan, coherently with the increased endowment of non-enzymatic antioxidants observed in this study. The expression of *APX* in the two species at the imbibed stage showed an opposite behaviour, probably indicating that the transcription of this gene during pre-germinative metabolism is differently regulated at the time point chosen for the analysis.

In annual species with seed heteromorphism, differences in seed mass, dormancy, longevity and polymorphic physiological and genetic traits allow the maintenance of diversity, assure a fitness advantage in environments with unpredictable climate conditions and reduce competition in *taxa* with a small dispersal area (De Pace *et al.*, 2011; Dyer, 2017; Hughes, 2018; Arshad *et al.*, 2019); for the first time all these traits have been studied with the same seed accessions and with several species, highlighting that primary and secondary seeds in *Aegilops* and *Triticum* show a consistent shift in their recruitment niches, likely driven by different trade-offs in term of seed mass, dormancy, longevity and antioxidant activity. Further experiments are needed to explore the presence and extent of trade-off mechanisms among different seed traits in wheat wild relatives. In future research, more populations of the same species will need to be analysed to better evaluate intraspecific trait variation in seed heteromorphy that has already been observed in the widespread species *Aegilops geniculata* Roth (see Onnis *et al.*, 1995; Orsenigo *et al.*, 2017). A better knowledge of seed heteromorphy of wild wheats will also help to clarify the domestication syndrome that led to the uniform germination that can be observed in domesticated wheats (Nave *et al.* 2016). Further investigations are needed also to disentangle the effects and the relative importance of the different traits contributing to the fascinating germination strategies of these wheat wild relatives, to enhance their usage in plant breeding programmes, considering that it is likely that different morphs might show different behaviours also in traits of interest for breeding (*e.g.*, abiotic stress resistance, Datta *et al.*, 1970; Orsenigo *et al.*, 2017).

ACKNOWLEDGEMENTS

This work was supported by CARIPLO Foundation in the frame of the WAKE-APT project (Code 2016-0723) 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 M.G., A.P., A.B.). The authors are indebted to

Silvia Bodino and Chiara Cianferoni (University of Pavia) for their help with seed cleaning and germination tests. The authors would like to thank Paolo Cauzzi (Pavia Botanical Garden) and Nicola M.G. Ardenghi (University of Pavia) for the priceless help in collecting some of the tested accessions. We are indebted to Paolo Cauzzi for cultivation of the *T. urartu* accession used in this study. We are grateful to Pablo Gómez Barreiro (Millennium Seed Bank, Kew Gardens) for his supervision with the X-ray scans. Special thanks are due to Manana Khutsishvili (Institute of Botany, Ilia State University) for help in the field to locate the populations of *Ae. tauschii*. The authors would also like to thank Prof. Diane Byers and two anonymous reviewers for their invaluable comments on an earlier version of the manuscript.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Double-y axis graphs of correlation between longevity and antioxidant profile in dry seeds.

Figure S2. Results and variable factor map of the PCA built with germination and antioxidant activity parameters of all the seed lots.

Table S1 Time points of the viability tests used to build the survival curves of each seed lot. Time points were chosen according to seed lots lifespan.

Table S2 Values of the two non-parametric tests (Kendal's tau b and Spearman's rho) applied to check the correlation between longevity estimates (P50) and the antioxidant profiles (AAE, GAE, SAA).

Table S3 Relative gene expression values measured in *Ae. triuncialis*, *Ae. tauschii* and *Ae. cylindrica* seeds A and B at the dry state and at 1 h of imbibition.

Data S1 Glossary of all the abbreviation and acronyms present in the text.

REFERENCES

Ahmadi J., Pour-Aboughadareh A., Ourang S.F., Mehrabi A.A., Siddique K.H.M. (2018) Wild relatives of wheat: *Aegilops-Triticum* accessions disclose differential antioxidative and physiological responses to water stress. *Acta Physiologiae Plantarum*, 40, 1–14.

Arshad W., Sperber K., Steinbrecher T., Nichols B., Jansen V.A.A., Leubner-Metzger G., Mummenhoff K. (2019) Dispersal biophysics and adaptive significance of dimorphic diaspores in the annual *Aethionema arabicum* (Brassicaceae). *New Phytologist*, 221, 1434–1446.

Bailly C. (2004) Active oxygen species and antioxidants in seed biology. *Seed Science Research*, 14, 93–107.

Bewley J.D., Bradford K.J., Hilhorst H.W.M., Nonogaki H. (2013) Longevity, storage, and deterioration. In: Bewley J. D., Bradford K. J., Hilhorst H. W. M., Nonogaki H. (Eds), *Seeds: physiology of development, germination and dormancy*. Springer, New York, NY, USA, pp 341–376.

Bilz M., Kell S.P., Maxted N., Lansdown R.V. (2011) *European red list of vascular plants*. Publications Office of the European Union, Luxembourg.

Bisca A., De Tommasi N., Basi L.Di, Pizza C., Politi M., Morelli I. (2001) Antioxidant principles from *Bauhinia tarapotensis*. *Journal of Natural Products*, 64, 892–895.

Caillaux A., Blanchet E., Cheptou P.-O., Lenormand T., Grimanelli D. (2018) Dividing a maternal pie among half-sibs: genetic conflicts and the control of resource allocation to seeds in maize. *The American Naturalist*, 192, 577–592.

Calucci L., Capocchi A., Calleschi I., Chiringhelli S., Pinzino C., Saviozzi F., Zandomeneghi M. (2004) Antioxidants, free radicals, storage proteins, puroindolines, and proteolytic activities in bread wheat (*Triticum aestivum*) seeds during accelerated aging. *Journal of Agricultural and Food Chemistry*, 52, 4274–4281.

Chhuneja P., Kaur S., Goel R.K., Aghaee-Sarbarzeh M., Prashar M., Dhaliwal H.S. (2008) Transfer of leaf rust and stripe rust resistance from *Aegilops umbellulata* Zhuk. to bread wheat (*Triticum aestivum* L.). *Genetic Resources and Crop Evolution*, 55, 849–859.

Cooper R., Levy E.C., Lavie D. (1977) Novel germination inhibitors from *Aegilops ovata* L. *Journal of the Chemical Society, Chemical Communications*, 21, 794–795.

Datta S.C., Evenari M., Gutterman Y. (1970) The heteroblasty of *Aegilops ovata*. *Israel Journal of Botany*, 19, 463–483.

De Pace C., Vaccino P., Giorgio Cionini P., Pasquini M., Bizzarri M., Qualset C. (2011) Dasypyrum. In: Kole C. (Ed), *Wild crop relatives: genomic and breeding resources. Cereals*. Springer, Berlin, Germany, pp 185–292.

Demidkaya M., Dietz K.J., Sivitskye H.O. (2010) Changes in antioxidant enzymes during ageing of onion seeds. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 38, 49–52.

Dempewolf H., Eastwood R.J., Guarino L., Khoury C.K., Müller J.V., Toll J. (2014) Adapting agriculture to climate change: a global initiative to collect, conserve, and use Crop Wild Relatives. *Agroecology and Sustainable Food Systems*, 38, 369–377.

Dizkirisi A., Kansu C., Onde S. (2016) Molecular phylogeny of *Triticum* and *Aegilops* genera based on ITS and MATK sequence data. *Pakistan Journal of Botany*, 48, 143–153.

Donà M., Balestrazzi A., Mondoni A., Rossi G., Ventura L., Buttafava A., Macovei A., Sabatini M.E., Valassi A., Carbonera D. (2013) DNA profiling, telomere analysis and antioxidant properties as tools for monitoring ex situ seed longevity. *Annals of Botany*, 111, 987–998.

Dyer A.R. (2004) Maternal and sibling factors induce dormancy in dimorphic seed pairs of *Aegilops triuncialis*. *Plant Ecology*, 172, 211–218.

Dyer A.R. (2017) The seed ecology of *Aegilops triuncialis*: linking trait variation to growing conditions. *Seed Science Research*, 27, 183–198.

Ellis R.H., Roberts E.H. (1980) Improved equations for the prediction of seed longevity. *Annals of Botany*, 45, 13–30.

Evers T., Millar S. (2002) Cereal grain structure and development: some implications for quality. *Journal of Cereal Science*, 36(3), 261–284.

Fandrich L., Malloy-Smith C. (2005) Temperature effects on jointed goatgrass (*Aegilops cylindrica*) seed germination. *Weed Science*, 53, 594–599.

Guzzon F., Müller J.V., Abeli T., Cauzzi P., Ardenghi N.M.G., Balestrazzi A., Rossi G., Orsenigo S. (2015) Germination requirements of nine European *Aegilops* species in relation to constant and alternating temperatures. *Acta Botanica Gallica*, 162, 349–354.

Guzzon F., Orsenigo S., Gianella M., Müller J.V., Vagge I., Rossi G., Mondoni A. (2018) Seed heteromorphy influences seed longevity in *Aegilops*. *Seed Science Research*, 28, 277–285.

Hay F.R., Probert R.J. (2013) Advances in seed conservation of wild plant species: a review of recent research. *Conservation Physiology*, 1, 1, cot030.

Hay F.R., Adams J., Manger K., Probert R. (2008) The use of non-saturated lithium chloride solutions for experimental control of seed water content. *Seed Science and Technology*, 36, 737–746.

Hughes P.W. (2018) Minimal-risk seed heteromorphism: proportions of seed morphs for optimal risk-averse heteromorphic strategies. *Frontiers in Plant Science*, 9, 1412.

Hutchinson E.S. (1984) Seed size and quantitative characters in *Avena barbata*. *Heredity*, 52, 25–33.

ISTA (2018) *International Rules for Seed Testing*. International Seed Testing Association, Bassersdorf, Switzerland.

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, 663–668.

Kashkush K., Feldman M., Levy A.A. (2003) Transcriptional activation of retrotransposons alters the expression of adjacent genes in wheat. *Nature Genetics*, 33, 102–106.

Kilian B., Mammen K., Millet E., Sharma R., Graner A., Salamini F., Hammer K., Özkan H. (2011) Aegilops. In: Kole C. (Ed), *Wild crop relatives: genomic and breeding resources. Cereals*. Springer, Berlin, Germany, pp 1–76.

Koyama K., Hatano H., Nakamura J., Takumi S. (2012) Characterization of three VERNALIZATION INSENSITIVE3-like (VIL) homologs in wild wheat, *Aegilops tauschii* Coss. *Hereditas*, 149, 62–71.

Kramer I., Panetta F.D., Steadman K.J., Bittic S., Long R.L., Adkins S.W. (2010) Wet–dry cycling extends seed persistence by re-instating antioxidant capacity. *Plant and Soil*, 338, 511–519.

Iavic D., Levy E.C., Cohen A., Evenari M., Gutterman Y. (1974) New germination inhibitor from *Aegilops ovata* L. *Nature*, **249**, 388.

Lê S., Josse J., Husson F. (2008) FactoMineR : an R package for multivariate analysis. *Journal of Statistical Software*, **25**, 1–18.

Lewandrowski W., Erickson T.E., Dalziell E.L., Stevens J.C. (2018) Ecological niche and bet-hedging strategies for Triodia (R.Br.) seed germination. *Annals of Botany*, **121**, 367–375.

Li B.-Y., Cheng M., Gao H.-Q., Ma Y.-B., Xu L., Li X.-H., Li X.L., You R.-A. (2008) Back-regulation of six oxidative stress proteins with grape seed proanthocyanidin extracts in rat diabetic nephropathy. *Journal of Cellular Biochemistry*, **104**, 668–679.

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*, **90**, 31–59. https://doi.org/10.1111/brv.12095.

Long R.L., Panetta F.D., Steadman K.J., Probert R., Bekker R.M., Brooks S., Adkins S.W. (2008) Seed persistence in the field may be predicted by laboratory-controlled aging. *Weed Science*, **56**, 523–528.

Marañón T. (1989) Variations in seed size and germination in three *Aegilops* species. *Seed Science and Technology*, **17**, 583–588.

Marbach I., Mayer A.M. (1974) Permeability of seed coats to water as related to drying conditions and metabolism of phenolics. *Plant Physiology*, **54**, 817–820.

Marcussen T., Sandve S.R., Heier L., Spannagl M., Pfeifer M. (2014) A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome: Ancient hybridizations among the ancestral genomes of bread wheat. *Science*, **345**, 6194.

Matilla A., Gallardo M., Puga-Hermida M.I. (2005) Structural, physiological and molecular aspects of heterogeneity in seeds : a review. *Seed Science Research*, **15**, 63–76.

Maxted N., Ford-Lloyd B.V., Jury S., Kell S., Scholten M. (2008) Towards a definition of a crop wild relative. *Biodiversity and Conservation*, **15**, 2673–2685.

Minaro N., Hosogi N., Park P., Takumi S. (2010) Hypersensitive response-like reaction is associated with hybrid necrosis in interspecific crosses between tetraploid wheat and *Aegilops tauschii* Coss. *PLoS ONE*, **5**, e11326.

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. *Annals of Botany*, **107**, 171–179.

Nave M., Avni R., Ben-Zvi B., Hale I., Distelfeld A. (2016) QTLs for uniform grain dimensions and germination selected during wheat domestication are co-located on chromosome 4B. *Theoretical and Applied Genetics*, **129**, 1303–1315.

Newton R., Hay F., Probert R. (2009) *Protocol for comparative seed longevity testing. Technical Information Sheet_01*. Royal Botanic Gardens Kew, London, UK.

Olofsson H., Ripa J., Jonzén N. (2009) Bet-hedging as an evolutionary game: the trade-off between egg size and number. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, **276**, 2963–2969. doi:https://doi.org/10.1098/rspb.2009.0500.

Oñate-Sánchez L., Vicente-Carbajosa J. (2008) DNA-free RNA isolation protocols for *Arabidopsis thaliana*, including seeds and siliques. *BMC Research Notes*, **1**, 93.

Onnis A., Bertacchi A., Lombardi T., Stefani A. (1995) Morphology and germination of yellow and brown caryopses of *Aegilops geniculata* Roth (Gramineae) population from Italy. *Giornale Botanico Italiano*, **129**, 813–821.

Orsenigo S., Guzzon F., Abeli T., Rossi G., Vagge I., Balestrazzi A., Mondoni A., Müller J.V. (2017) Comparative germination responses to water potential across different populations of Aegilops geniculata and cultivar varieties of Triticum durum and Triticum aestivum. *Plant Biol J*, **19**, 165–171.

Paolacci A.R., Tanzarella O.A., Porceddu E., Ciaffi M. (2009) Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. *BMC Molecular Biology*, **10**, 1–27.

Petersen G., Seberg O., Yde M., Berthelsen K. (2006) Phylogenetic relationships of *Triticum* and *Aegilops* and evidence for the origin of the A, B, and D genomes of common wheat (*Triticum aestivum*). *Molecular Phylogenetics and Evolution*, **39**, 70–82.

Pfaffl M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, **29**, e45.

Probert R.J., Daws M.I., Hay F.R. (2009) Ecological correlates of ex situ seed longevity: a comparative study on 195 species. *Annals of Botany*, **104**, 57–69.

Redden R., Yadav S. S., Maxted N., Dulloo M. E., Guarino L., Smith P. (Eds) (2015) Crop Wild Relatives and climate change. *Plant genetic resources and climate change*. Wiley-Blackwell, Chichester, UK.

Sadras V.O., Denison R.F. (2009) Do plant parts compete for resources? An evolutionary viewpoint. *New Phytologist*, **183**, 565–574.

Sano N., Rajjou L., North H.M., Debeaujon I., Marion-Poll A., Seo M. (2016) Staying alive: molecular aspects of seed longevity. *Plant and Cell Physiology*, **57**, 660–674.

Seger J., Brockmann H.J. (1987) What is bet-hedging. In: Harvey P. H., Partridge L. (Eds), *Oxford surveys in evolutionary biology*. Oxford University Press, Oxford, UK, pp 182–211.

Slattery H.D., Atwell B.J., Kuo J. (1982) Relationship between colour, phenolic content and impermeability in the seed coat of various *Trifolium subterraneum* L. genotypes. *Annals of Botany*, **50**, 373–378.

Van Slageren M.W. (1994) Wild wheats: a monograph of Aegilops L. and Amblyopyrum (Jaub. & Spach) Eig (Poaceae). *Wageningen Agricultural University Papers*, **7**, 94–97.

Venable D.L., Buzquez A.M. (1989) Quantitative genetics of size, shape, life-history, and fruit characteristics of the seed-heteromorphic composite *Heterosperma pinnatum*. I. Variation within and among populations. *Evolution*, **43**, 113–124.

Volis S. (2016) Seed heteromorphism in *Triticum dicoccoides*: association between seed positions within a dispersal unit and dormancy. *Oecologia*, **181**, 401–412.

Wang W., He A., Peng S., Huang J., Cui K., Nie L. (2018) The effect of storage condition and duration on the deterioration of primed rice seeds. *Frontiers in Plant Science*, **9**, 172.

Warschefsky E., Penmetsa R.V., Cook D.R., von Wettberg E.J.B. (2014) Back to the wilds: tapping evolutionary adaptations for resilient crops through systematic hybridization with crop wild relatives. *American Journal of Botany*, **101**, 1791–1800.

Wurzburger J., Leshem Y. (1967) Gibberellin and hull controlled inhibition of germination in *Aegilops kotschyi* Boiss. *Israel Journal of Botany*, **16**, 181–186.

Wurzburger J., Leshem Y. (1969) Physiological action of the germination inhibitor in the husk of *Aegilops kotschyi* Boiss. *New Phytologist*, **68**, 337–341.

Yin G., Xin X., Fu S., An M., Wu S., Chen X., Zhang J., He J., Whelan J., Lu X. (2017) Proteomic and Carbonylation profile analysis at the critical node of seed ageing in *Oryza sativa*. *Scientific Reports*, **7**, 40611.

Zhu C., Chen J. (2007) Changes in soluble sugar and antioxidant enzymes in peanut seeds during ultra dry storage and after accelerated aging. *Seed Science and Technology*, **35**, 387–401.

Guzzon F, **Gianella M**, Velazquez Juarez JA, Sanchez Cano C, Costich DE. Seed longevity of maize conserved under germplasm bank conditions for up to 60 years. *Ann Bot*. 2021; 127(6), 775-785. https://doi.org/10.1093/aob/mcab009

Annals of Botany **127**: 775–785, 2021
doi: 10.1093/aob/mcab009, available online at www.academic.oup.com/aob

ANNALS OF BOTANY

Seed longevity of maize conserved under germplasm bank conditions for up to 60 years

Filippo Guzzon[1,*], Maraeva Gianella[2], Jose Alejandro Velazquez Juarez[1], Cesar Sanchez Cano[1] and Denise E. Costich[1]

[1]International Maize and Wheat Improvement Center (CIMMYT), Texcoco, 56237, Mexico State, Mexico and [2]Department of Biology and Biotechnology 'L. Spallanzani', University of Pavia, 27100 Pavia, Italy
** For correspondence. E-mail f.guzzon@cgiar.org*

Received: 28 September 2020 Returned for revision: 21 December 2020 Editorial decision: 8 January 2021 Accepted: 13 January 2021
Electronically published: 13 February 2021

• **Background and Aims** The long-term conservation of seeds of plant genetic resources is of key importance for food security and preservation of agrobiodiversity. Nevertheless, there is scarce information available about seed longevity of many crops under germplasm bank conditions.
• **Methods** Through germination experiments as well as the analysis of historical monitoring data, we studied the decline in viability manifested by 1000 maize (*Zea mays* subsp. *mays*) seed accessions conserved for an average of 48 years at the CIMMYT germplasm bank, the largest maize seedbank in the world, under two cold storage conditions: an active (−3 °C; intended for seed distribution) and a base conservation chamber (−15 °C; for long-term conservation).
• **Key Results** Seed lots stored in the active chamber had a significantly lower and more variable seed germination, averaging 81.4 %, as compared with the seed lots conserved in the base chamber, averaging 92.1 %. The average seed viability detected in this study was higher in comparison with that found in other seed longevity studies on maize conserved under similar conditions. A significant difference was detected in seed germination and longevity estimates (e.g. p_{85} and p_{50}) among accessions. Correlating seed longevity with seed traits and passport data, grain type showed the strongest correlation, with flint varieties being longer lived than floury and dent types.
• **Conclusions** The more rapid loss of seed viability detected in the active chamber suggests that the seed conservation approach, based on the storage of the same seed accessions in two chambers with different temperatures, might be counterproductive for overall long-term conservation and that base conditions should be applied in both. The significant differences detected in seed longevity among accessions underscores that different viability monitoring and regeneration intervals should be applied to groups of accessions showing different longevity profiles.

Key words: Corn, *ex situ* conservation, germination, grain type, maize, plant genetic resources, seedbank, seed viability, *Zea mays*.

INTRODUCTION

The genetic diversity intrinsic to plant genetic resources (PGRs), especially in crop landraces, is fundamental for the selection of the high-yielding, improved cultivars of the future, able to cope with climatic changes and pests, in order to increase agricultural production and sustainability (Guarino and Lobell, 2011; Vincent *et al.*, 2013; Warschefsky *et al.*, 2014). In this scenario, it is of key importance to conserve PGRs for the long term, as well as to keep them readily available for users worldwide (McCouch *et al.*, 2013). Conservation of PGRs is also important to prevent genetic erosion (i.e. the loss of genetic diversity in the form of alleles and genotypes) that has been observed at very high percentages in the last decades in several areas of the world (Hammer *et al.*, 1996; Veteläinen *et al.*, 2009; van de Wouw *et al.*, 2010).

Ex situ seed storage in seedbanks is considered to be one of the most effective strategies for ensuring the conservation and availability of plant species with orthodox seeds (i.e. seeds that can tolerate drying to low moisture content and subsequent freezing; Roberts *et al.*, 1973). Collections of thousands of seed accessions can be stored in relatively small spaces, providing adequate samples of the genetic diversity within plant populations and species. They will remain viable for the long term, but only if properly conserved (Li and Pritchard, 2009; Riviere and Müller, 2017). Worldwide there are >1750 genebanks, conserving >7.4 million accessions (FAO, 2010; Colville and Pritchard, 2019). There have been several incidents of less than expected longevity at conventional seed bank storage conditions (Li and Pritchard, 2009; Colville and Pritchard, 2019). This raises the question of how well seed banks are carrying out their mission of conserving plant genetic resources for the long term.

The CIMMYT (International Maize and Wheat Improvement Center) Germplasm Bank (located at CIMMYT's headquarters in Texcoco, Mexico) is the largest seedbank in the world, dedicated to conserving genetic resources of maize (*Zea mays* subsp. *mays*) focusing on the tropical and sub-tropical areas of the world. The CIMMYT Germplasm Bank stores for the long term >28 000 maize seed accessions, mainly landraces, but also the genetic

legacy of CIMMYT's maize breeding programme, from the diverse pools and populations to the inbred lines (known as CMLs or CIMMYT maize lines). It also conserves maize wild relatives: the 'teosintes' that include all undomesticated taxa in the genus *Zea*, and species from the sister genus, *Tripsacum*. More than 14 000 samples of maize seeds conserved at CIMMYT are shipped yearly to breeders, researchers and farmers worldwide. As in many international seedbanks, seeds of the same accessions are conserved in two chambers under different temperature regimes, called 'active' (–3 °C) and 'base' (–15 °C). In the base chamber, seeds are conserved for the long term (several decades), while in the active chamber, seed samples are used for regeneration, distribution and characterization, to avoid using the stocks conserved in the base, which is considered to be the first level of safety backup for the collection (FAO, 2014).

The Genebank Standards for Plant Genetic Resources for Food and Agriculture recommends that the regeneration or recollection of a seed accession should occur when seed viability drops below 85 % of the initial viability. Intervals of viability monitoring need to be calculated according to the decline of seed viability in the target species (FAO, 2014). Within this framework, it emerges that understanding seed longevity differences among accessions (see, for example, Walters *et al.*, 2005; Probert *et al.*, 2009; Mondoni *et al.*, 2011; Guzzon *et al.*, 2018) is crucial for the management of *ex situ* seed collections, as these data inform the planning of re-collection or regeneration intervals (Walters, 2003). This is particularly relevant for a crop such as maize that shows, in its diverse landraces, several adaptations to local environments and climatic conditions (Huang *et al.*, 2018). It has been demonstrated that seed longevity varies significantly across different populations of the same species, influenced by their climate of origin (Mondoni *et al.*, 2011, 2018; Zani and Müller, 2017). Moreover, this high degree of local adaptations makes the regeneration of seed accessions of maize landraces challenging, because each accession should be repropagated in sites with environmental conditions that are optimal for its growth and seed production. Therefore, understanding seed longevity of the conserved maize accessions is crucial not only to carefully plan their regeneration intervals and allocate adequate resources to the repropagation activities, but also to assess if the achieved repropagation success (i.e. the percentage of accessions with low viability that are successfully repropagated in the field every year) is sufficient to ensure the long-term conservation of high-quality seeds in the entire collection.

Seed longevity is a complex trait, which is influenced by several factors such as taxonomy, seed structure, germination phenology and environmental factors, including climatic conditions experienced by the seeds during the post-zygotic phase (Zani and Müller, 2017), as well as temperature and relative humidity during seed storage (Guzzon *et al.*, 2020). In addition, various molecular and physiological aspects controlling protection and repair mechanisms are important (Walters *et al.*, 2005; Probert *et al.*, 2009; Mondoni *et al.*, 2011; Gianella *et al.*, 2020). Most of the available publications on seed longevity are comparative studies among different species (e.g. Merritt *et al.*, 2014; Zani and Müller, 2017; Solberg *et al.*, 2020), while only a few have addressed differences in seed longevity among accessions of a single plant species, such as Hay *et al.* (2013) on rice (*Oryza sativa*); Mondoni *et al.* (2018) on *Silene suecica*; and Van Treuren *et al.* (2018) on wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). These latter studies revealed that within the same taxon, different populations can show considerable differences in seed longevity. Additionally, only a few seed longevity studies were performed on seed material under long-term conservation (cold storage between –15 and –20 °C; Walters *et al.*, 2005; Hay *et al.*, 2013; Desheva, 2016). Since most seedbanks have relatively short histories, declines in seed viability of the conserved accessions are unlikely to be detected (Van Treuren *et al.*, 2018). For this reason, few empirical data on seed longevity under germplasm bank conditions are available for many important crop species. Interestingly, Van Treuren *et al.* (2018) found that barley and wheat accessions stored in active conditions (4 °C) for 23–33 years showed a noticeable decline in seed viability in terms of seed germination (–30 %), compared with base conditions (–20 °C), where the same accessions maintained very high germination (95 %). This underscores the need to assess seed longevity under the different storage conditions used in any germplasm bank.

In the case of maize, the data on seed longevity under germplasm bank conditions and intraspecific differences in seed longevity are few and contradictory. Roos and Davidson (1992) estimated an average p_{50} for maize (the time for viability to fall by 50 %) of 65 years, considering the loss of viability in five maize accessions under long-term storage at –18 °C in the USDA's National Laboratory for Genetic Resource Preservation in Fort Collins, Colorado, USA. Walters *et al.* (2005) estimated an average p_{50} of 49 years for >2000 maize accessions also conserved at the USDA genebank. Deseheva (2016) found a very small (<5 %) loss of seed viability in 364 seed accessions stored for 23 years at –18 °C in the National Genebank of Bulgaria. Yamasaki *et al.* (2020) recently calculated a mean survival time (MST; the estimated time for half of the seed lots to fall below 85 % of the initial germination) of 21.2 years for 3953 maize accessions, conserved at –1 °C and 30 % relative humidity at NARO genebank (Japan) for up to 29 years.

The objective of this study was to fill a tremendous gap in our understanding of seed longevity in maize *ex situ* collections under long-term conservation. The current study is, to the best of our knowledge, one of the largest seed longevity experiments, using original data (together with historical data), ever performed. We aimed to (1) evaluate the viability of 1000 maize seed accessions conserved under germplasm bank conditions for up to 60 years; (2) compare seed life span of the same accessions conserved in the active and base chambers of CIMMYT; and (3) investigate the relationship between maize seed traits and seed longevity under germplasm bank conditions.

MATERIALS AND METHODS

Conservation conditions

Maize caryopses (hereafter referred to as 'seeds') were originally conserved in a seedbank located at the National School of Agriculture at Chapingo (Texcoco, Mexico) since 1943, in what can be considered the first germplasm bank of Latin America. After that, from the 1960s to 1971, the accessions included in this study were conserved in a temporary refrigerated seed storage facility (0 ± 5 °C) located in the basement of the National School of Agriculture at Chapingo University (Texcoco, Mexico), when they were moved to the first CIMMYT germplasm bank at its recently built headquarters in El Batán, Texcoco, Mexico. Here they were conserved in metallic cans with pressure lids at 0 °C and 45 % relative humidity, with the seeds dried below 10 % of moisture content prior to storage (CIMMYT, 1974, 1988). Starting in 1984, each seed collection was divided, and equal halves were moved to a base chamber (–15 °C) and an active chamber (0 °C) in metal cans. The two seed lots of the same accession conserved in the two chambers will be hereafter identified as: the 'active' seed lot and 'base' seed lot. In the second half of the 1980s, accessions in the base chamber were transferred to sealed aluminium envelopes. In 1996, the whole collection was moved to the current CIMMYT Germplasm Bank inside the Wellhausen-Anderson Plant Genetic Resources Center, where seeds are conserved in hermetic plastic flasks at –3 ± 2 °C (active chamber) and in hermetically sealed aluminium envelopes at –15 ± 3 °C (base chamber).

Study accessions

Germination tests of 987 seed lots from the base chamber were carried out for this study. Of these same accessions, 835 from the active chamber were also tested for germination. The number of seed lots tested for the active chamber is lower than that for the base chamber because some accessions in the active chamber did not have enough seeds to perform the germination test. Nineteen accessions were tested only in the active chamber since data from recent germination tests (carried out from 2016 to 2019) were available for the base chamber. Overall, a total of 1006 accessions from the base chamber were included in this study. One accession was tested only in the active chamber, due to lack of seeds in the base chamber. Overall, 855 accessions from the active chamber were tested in this experiment. The study accessions were chosen according to the following criteria: (1) having complete passport data (including storage date, collecting site geographical information and information on the repropagation site); (2) having both initial and monitoring germination data; (3) being representative of the different grain types of maize (dent, floury, flint, popcorn and sweet) conserved in the CIMMYT collection; (4) being among the oldest collections for each grain type; and (5) maximizing the environmental range (in terms of latitude and elevation of the collecting sites) among the study accessions. The study accessions originated from seed collections made in 33 different countries, covering an altitudinal range from 2 to 3919 m above sea level. Considering the grain type of the study accessions: 363 were dent, 361 flint, 218 floury, 11 popcorn and 54 sweet. Popcorn and sweet accessions were less represented in the experiment in concordance with their overall lower representation in the collection. The ranges of storage dates for each grain type were as follows: dent (1965–1971), flint (1960–1974), floury (1969–1978), popcorn (1970–1996) and sweet (1968–2002). The average age of the accessions tested for this study was 48 years. More recent accessions of popcorn and sweet maize were used in the study due to the scarcity of older accessions of these grain types in the collection. The study seed lots are identified in the CIMMYT GRIN-Global database as well as marked with a special label (Longevity project) in the conservation chambers; those seed lots will not be substituted with new regenerations, with the hope that researchers can continue, in the next decades, to study the viability of this set. Only one accession was represented by seeds from its original collection. All other accessions had been regenerated in a Mexican or US location prior to conservation (11 regeneration locations in total). Historical initial germination data collected prior to storage were available for the study accessions, and the average initial germination was 99 %. Moreover, results of an additional germination monitoring test were available, but only for the active chamber, since systematic germination monitoring of seeds conserved in the CIMMYT base chamber only began in 2012. Prior to the germination tests carried out for this study, seed mass and moisture content of the accessions (from both the active and base chambers) were measured. Seed mass was determined by weighing three replicates of 20 seeds, kept in one of the dry rooms of the CIMMYT Germplasm Bank at 9–15 °C and 10–20 % relative humidity, randomly sampled from each seed lot, using an analytical balance (Adventurer Pro AV 8101, OHAUS, Parsippany, NJ, USA). The seed moisture content (MC) of the accessions was tested using a moisture meter (SL95, Steinlite, Atchison, KS, USA). The accession passport data considered in this study, besides the historical germination data, were: grain type and colour; regeneration site; and co-ordinates (latitude and longitude), elevation and climatic zone of the original collection site. The most relevant passport data and information (accession ID number, grain type, country of collection, final germination in the active and base chamber, seed mass and ageing rate) for all of the study accessions tested can be found in Supplementary data Table S1. Climatic zones of the collecting sites were based on the Köppen–Geiger climatic classification system (Bryant *et al.*, 2017).

Germination tests

Three replicates of 20 seeds of each accession were sown in rolled filter paper (16.6 × 16.6 cm) moistened with distilled water. It was not possible to use a higher number of seeds or replicates in the germination tests due to the low seed number of some accessions. Filter paper rolls were inserted in transparent plastic trays, and the trays were randomly dispersed in an incubator (Biotronette plant growth chamber 844, Lab-Line) at a constant temperature of 25 °C and a 12 h photoperiod. Distilled water was added to the trays as needed, to avoid

desiccation. Germination scoring was performed 1 week after sowing. A seed was considered to be germinated if it had developed into a normal seedling, according to ISTA (2018) criteria. A label with a unique, identifier QR code was attached to each paper roll. The germination scoring was performed through the app GB zone, that is connected to the GRIN-Global genebank database, by means of QR code scanners (two models: CS 4070, Symbol, Zebra, USA; and S740 2D, Socketmobile, USA) connected to a laptop or tablet device. The germination experiments were performed in March and April 2020 in the Seed Viability Laboratory of the CIMMYT Germplasm Bank (Texcoco, Mexico). Germination data obtained in this experiment are available in Supplementary data Table S1.

Statistical analysis

Data analysis and graphic representation were carried out in the R software environment for statistical computing and graphics (v. 3.6.2). The R packages used for the analyses are: 'corrplot' (Taiyun and Viliam, 2017), 'dplyr' (Wickham *et al.*, 2020), 'ecotox', 'fmsb' (Nakazawa, 2019), 'ggplot2' (Wickham, 2016), 'ggpubr' (Kassambara, 2020), 'lsmeans' (Lenth, 2016), 'kgc' (Bryant *et al.*, 2017), 'multcom' (Hothorn *et al.* 2008), 'psych' (Revelle, 2019) and 'statmod' (Giner and Smyth, 2016). Before analyses, data were checked for normality and homoscedasticity (Shapiro–Wilk's and Levene's tests, respectively).

Two Scheirer–Ray–Hare tests were applied to determine if mass and moisture content differed among accessions and conservation chambers (active and base). A Kruskal–Wallis test was used to determine if there were differences among grain types in terms of seed mass. Pairwise comparisons were carried out with the Bonferroni test.

Multiple parameters were used to characterize seed longevity: final germination (germinated/sown seeds at the end of the germination test); and p_{50} and p_{85}, corresponding to the time for viability to fall to 50 % and 85 % of the initial value (retrieved from historical, pre-storage data), respectively, estimated or predicted by logit analyses. Generalized linear models (GLMs) with binomial distributions, with logit as the link function, were carried out for p_{50} and p_{85} predictions of the accessions conserved in the active chamber. Logit was preferred over probit as link function, since logit showed a higher goodness of fit, compared by means of analysis of variance (ANOVA; χ^2 test) and AIC (Akaike information criterion) scores, when compared with probit analysis (d.f. 0, deviance = –462.23, $P < 0.001$; AIC logit—probit: –460). Moreover, logit function, as previously observed by dos Santos *et al.* (2019) and de Faria *et al.* (2020), allows for a more reliable estimate/prediction of viability loss when compared with probit, especially in the tails of the distribution (i.e. < 10 % or >80 % germination), thus making it more suitable for the p_{85} estimation. Smaller differences between logit and probit models are observed in the central points of the function (50 % germination, p_{50}). Longevity estimates were filtered for those accessions that showed a final germination lower than the initial and a p_{85} lower than the years of storage. This was done to estimate the p_{85} based only on observed data and not predictions, in order to avoid unrealistic values driven by the absence of viability loss in a significant percentage of the seed lots. Yamasaki *et al.* (2020) also highlighted this issue, suggesting that only longevity estimates within the storage period should be considered, while the reliability of a prediction extrapolated beyond the observation period needs further data accumulation and verification, especially considering that viability loss does not follow a linear pattern, but rather a sigmoidal one (Walters *et al.*, 2005).

Another parameter used to characterize seed longevity was the ageing rate (named L), calculated using the following formula:

$$L = \frac{(G_i - G_f)}{G_i} \times \frac{1}{Y}$$

Where G_i is the initial germination in percentage, tested before storage; G_f is the most recent germination result in percentage; and Y corresponds to the storage time experienced by the seed accession, expressed in years.

A GLM, with a binomial distribution and logit as link function, was applied to determine the effect of the conservation in the two different chambers (active or base) on final germination, and a post-hoc Bonferroni test was used for pairwise comparisons of the same seed accession in the two conservation conditions. A Kendall's correlation test was performed on germination in the active and base chambers, in order to assess if there is a correlation between the decline in viability in the two chambers, and, in particular, whether the decline in seed viability of any accession in the active chamber is similar to its decline in the base chamber.

Monitoring intervals were calculated as one third of the p_{85}, with an upper limit set to 40 years as suggested by the Genebank Standards for Plant Genetic Resources for Food and Agriculture (FAO, 2014). A Kruskal–Wallis test was used to determine if there were differences among grain types in terms of monitoring intervals. Pairwise comparisons were carried out with the Wilcoxon rank sum test.

A correlation plot was built based on a mixed correlation matrix for both active- and base-conserved seed lots, considering the following variables: ageing rate (L), seed mass and moisture content, grain type and colour, and regeneration site, as well as latitude, longitude, elevation and climatic zone (Köppen–Geiger) of the original collection sites. Moreover, a partial correlation analysis, using Kendall's test, was performed, for both the chambers, in order to evaluate the relationship of each single variable with the ageing rate (L), adjusting for the effect of the other variables. A GLM with a compound Poisson-gamma tweedie distribution was applied to assess the effect of grain type on the ageing rate (L).

RESULTS

Seed mass and moisture content

Seed mass differed among accessions (d.f. 841, H = 4844.662, $P < 0.001$), ranging from 1.43 ± 0.05 to 14.53 ± 0.15 g in the different study accessions (see Supplementary data Table S1). Seed mass was not significantly different between the seed lots of the same accession conserved in the two chambers (d.f. 841, H = 99.64, $P = 1$). Seed mass was different among grain types (d.f. 4, H = 1091.7, $P < 0.001$); the highest values of

seed mass were detected in floury accessions (mean seed mass 7.3 ± 1.2 g), followed by dent (6.6 ± 1.5 g), flint (4.7 ± 1.2 g), sweet (4.7 ± 0.9 g) and popcorn (2 ± 0.4 g).

Moisture content differed among accessions and between chambers (respectively d.f. 841, H = 3110.262, $P < 0.001$; d.f. 1, H = 982.336, $P < 0.001$), ranging from 4.98 to 15.16 % (average = 9.42 ± 1.95 %) in the base chamber and from 5.15 to 15.48 % (average = 11.00 ± 1.46 %) in the active chamber.

Final germination: active and base chambers

The final germination data obtained from the experiment showed significant differences among accessions, the two conservation chambers (active and base) and their interaction (see Table 1).

Among the 835 accessions tested from both active and base chambers in this experiment, 284 showed a significant difference in germination between the two chambers (34.01 % of total).

Of the 284 statistically significant pairwise comparisons, 275 accessions showed a higher germination in the base chamber (96.83 % of the comparisons). The remaining nine accessions, with a better performance in the active chamber, accounted for only 3.17 % of comparisons.

Considering all accessions tested in this experiment from both chambers, the average germination was 92.1 ± 9.1 % for the seed lots conserved in the base chamber and 81.4 ± 16.3 % for the seed lots conserved in the active chamber (Fig. 1). In the base chamber, 84.5 % of the seed lots showed a final germination percentage above the threshold of 85 % of the initial germination value (FAO, 2014), while in the active chamber the final germination of 53 % of the seed lots was above this threshold. Moreover, in the active chamber, we detected twice the number of seed lots in the final germination range between 70 and 85 % of the initial compared with the base chamber, and about seven times the number in the base chamber between 50 and 70 %. Only four seed lots below 50 % of the initial germination were found in the base chamber (accounting for 0.4 % of the total), while 44 seed lots were found in the active chamber (5.2 %, Table 2). Considering the 835 accessions tested for both the active and base chambers, 47.8 and 13.9 % of the seed lots in the active and base chambers, respectively, showed a final germination below the 85 % threshold of the initial germination and therefore need regeneration (FAO, 2014).

Based on a Kendall's test, a positive correlation was found between the germination of the seed lots of the same accession in the active and base chambers (Tau b = 0.35, $P < 0.01$, Supplementary data Fig. S1), indicating a similar behaviour, in terms of viability loss, of the same accession conserved in the two different chambers.

p_{50} *and* p_{85}

The p_{50} and p_{85} predictions were performed only for the active chamber for which historical data on seed germination were available. Three viability data points were available and used in the analysis: initial germination, an intermediate point

TABLE 1. *Analysis of deviance using a generalized linear model (GLM) with binomial distribution and the logit link function: model effects on germination percentage of 835 maize accessions conserved in the two different chambers (active and base)*

	d.f.	Deviance	Residual d.f.	Residual deviance	*P*-value
Accession	834	10 003.77	4173	9868.27	<0.001
Chamber	1	3261.02	4172	6607.25	<0.001
Accession × Chamber	834	2923.84	3337	3678.53	<0.001

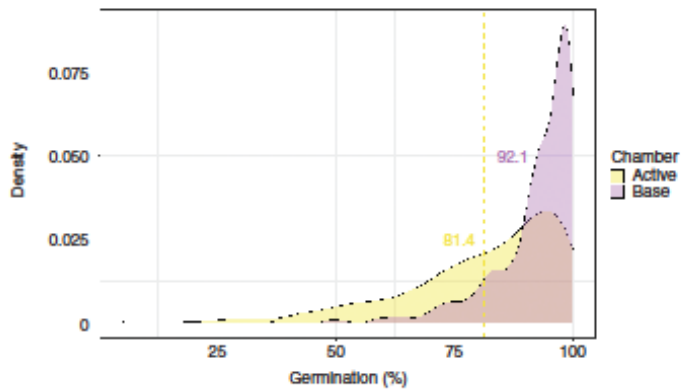


FIG. 1. Density plot representing the frequency of seed lots showing different values of germination percentage on a continuous scale, divided by conservation chamber (active and base). Dashed lines represent the average germination percentage for the two chambers (intercept).

(corresponding to a viability monitoring test performed between 1985 and 2011, depending on the accession) and the germination data obtained from the current experiment.

Of the total 855 active accessions, logit analysis successfully predicted p_{85} and p_{50} for 400 accessions (46.78 % of the total). p_{85} spanned between 4.2 and 54.4 years, with an average of 37.6 years; p_{50} values of the same accessions were between 16.5 and 91 years, with an average of 60 years. The observed differences among accessions in longevity estimates were statistically significant (d.f. = 854, residual deviance = 8245.112, $P < 0.001$).

Due to the fact that reliable longevity estimates (p_{85} and p_{50}) could not be calculated for all of the accessions, the ageing rate (L) was selected as the longevity indicator for subsequent analyses, making it possible to also include accessions that did not show a decrease in terms of germination across time ($L = 0$).

Correlations

Correlation plots were made for both 'active' and 'base' seed lots, based on mixed correlation matrices (Fig. 2). Correlations between L and all the other variables were statistically significant ($P < 0.5$), except for moisture content in the base chamber ($P = 0.23$).

The ageing rate L showed the strongest correlation with the polytomous variable 'grain type' consistently in both conservation chambers (Fig. 2). The partial correlation analysis (Supplementary data Table S2) confirmed that the variable with the strongest correlation with L was grain type, followed by seed mass.

Table 2. *Frequencies of the tested seed lots in active and base chambers divided into four classes based on germination percentage range*

Germination %	% of active accessions	% of base accessions
>85 %	53	84.5
70–85 %	27.3	12.8
50–70 %	14.6	2.2
<50 %	5.2	0.4
Total	100	100

Effect of grain type

Since grain type showed the strongest correlation with the ageing rate (L), a GLM was performed to understand how L differed among the grain types. Grain type showed a significant effect on the ageing rate L both in the active (d.f. 4, residual deviance = 162.7146, $P < 0.001$) and in the base chamber (d.f. 4, residual deviance = 223.7943, $P < 0.001$). In particular, in the active chamber, flint and floury seeds, without significant differences between them ($P = 0.22$), showed the lowest L, and therefore the lowest loss of seed viability across time, when compared with dent seeds ($P < 0.001$). In the base chamber, flint seeds showed the lowest L ($P < 0.001$), while floury and dent seeds did not show differences ($P = 0.32$). Overall, flint seeds showed a lower ageing rate when compared with the other grain types in terms of both the average and mode of the ageing rate L (Fig. 3). In this latter analysis, only the three major grain types, floury, flint and dent, were considered since the sample sizes were much lower for sweet and popcorn in the set of accession used in this study (see 'Study accessions' in the Materials and Methods).

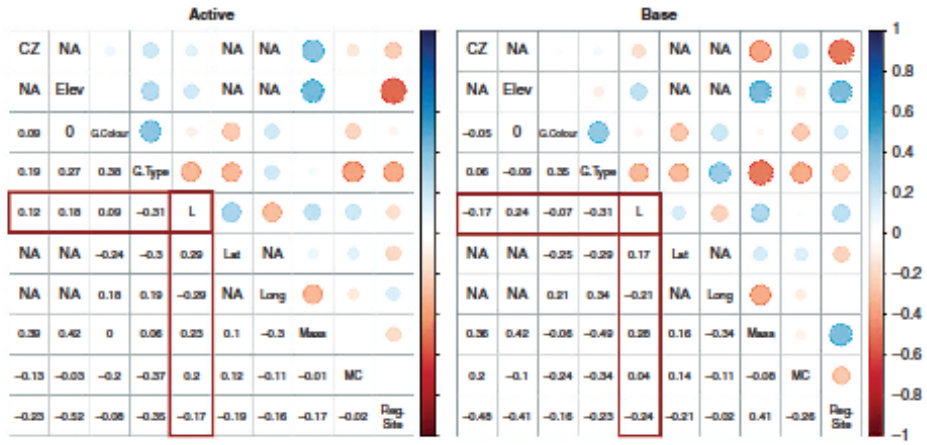


Fig. 2. Correlation plots of active and base chambers. Coefficients of correlation are represented by numbers in the lower part of the graph, and by colours in the upper part. Continuous variables: elevation (Elev), ageing rate (L), latitude (Lat), longitude (Long), moisture content (MC) and mass. Polytomous variables: Köppen–Geiger climatic zone (CZ), grain colour (G.Colour), type (G.Type) and regeneration site (Reg.Site). Correlations among geographical variables are indicated as NA.

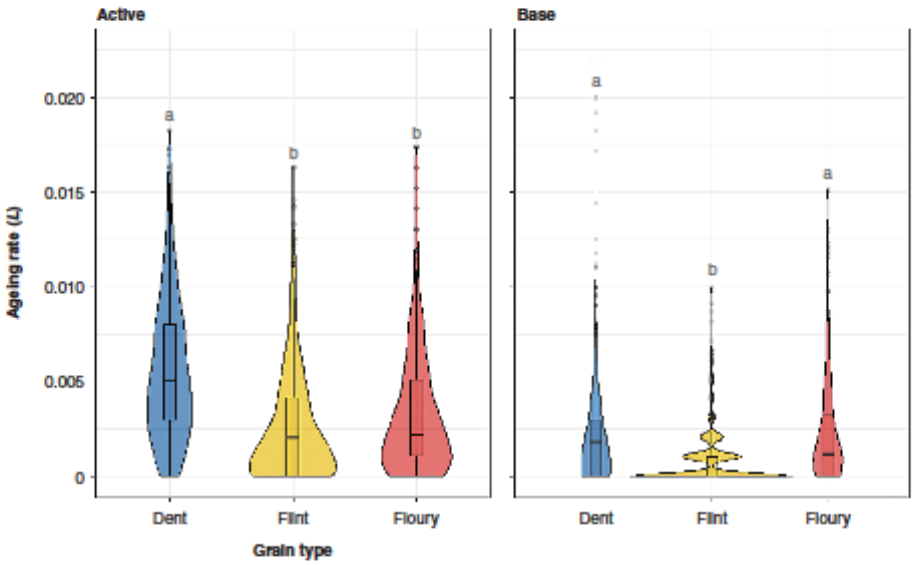


FIG. 3. Violin and box plots representing the ageing rate (L) in the three most represented grain types, in both the active and base chambers. Letters above violins represent statistically significant differences.

Monitoring intervals

Viability monitoring intervals were calculated as one-third of the p_{85}, with a maximum interval of 40 years between monitoring points, for all the active seed lots, as suggested by FAO (2014). For this calculation, no filter on the p_{85} was applied; all the p_{85}, extrapolated from the logit model, were employed in this analysis, since, following FAO (2014) a maximum interval of 40 years is adopted, even when p_{85} is >120 years.

The average viability monitoring interval for the accessions conserved in the active chamber is 17.5 ± 7.7 years. Monitoring intervals (measured in years) were significantly different among the three main grain types, flint, dent and floury (d.f. = 2, Kruskal–Wallis χ^2 = 80.30, $P < 0.001$, see Supplementary data Fig. S2), as follows: flint accessions (20.3 ± 9 years, $P < 0.001$), significantly longer than dent (14.6 ± 4.9 years) and floury (18.0 ± 7.6 years; $P < 0.001$). The difference in monitoring intervals between dent and floury was also significant ($P < 0.01$).

DISCUSSION

This set of >1000 maize accessions, stored for as long as 60 years in the CIMMYT germplasm bank, the largest and most diverse collection of maize genetic resources worldwide, provided a unique source of data to study seed longevity. Given the current lack of knowledge on seed longevity of maize accessions conserved under germplasm bank conditions, the data here presented, results of one of the largest seed longevity experiments ever performed, are of great importance to guide *ex situ* seed conservation of one of the crop species that plays the greatest role in the food security of humanity. Moreover, our results provide new data on seed traits connected to longevity under long-term storage, which merit further investigation.

The results obtained from this study, in terms of both final germination and longevity estimates (p_{50} and p_{85}), confirmed that maize seeds conserved under germplasm bank conditions are long lived (Walters *et al.*, 2005; Nagel and Börner 2010; Yamasaki *et al.*, 2020; Solberg *et al.*, 2020), showing p_{50} values of >50 years under long-term storage (Roos and Davidson, 1992; Solberg *et al.*, 2020). Nevertheless, we obtained different longevity values from those reported by previous studies of maize seeds stored under similar conditions (see, for example, Roos and Davidson, 1992; Walters *et al.*, 2005; Deseheva, 2016). Using historical germination data from >2000 maize accessions, Walters *et al.*, (2005) estimated a p_{50} for maize seeds of 49 years under long-term conservation. The germination results as well as the longevity estimates of the current study indicate that maize accessions conserved at CIMMYT showed a higher seed longevity. The average age of the maize seed accessions considered herein was 48 years, and their average germination was 92.1 % in the base chamber (–15 °C) and 81.4 % in the active chamber (–3 °C; Fig. 1). The average p_{50} of the seed lots conserved in the active chamber was 60 years; this estimate was calculated only for those seed lots (400 out of 855) showing a decline of viability, thus the overall p_{50} is likely to be higher, considering the seed longevity of all accessions in both active and base chambers. Moreover, other studies evaluating maize seed longevity under long-term storage conditions similar to those used at the CIMMYT germplasm bank provided different p_{50} values, e.g. Deseheva (2016) 141 years,

Roos and Davidson (1992) 65 years. Future research should aim at understanding those differences in seed longevity found among different conservation facilities to determine whether they are due to different seed regeneration, processing, conservation or data analysis methodologies, or seed-related traits, all of which can influence seed longevity estimates. Many of the seed accessions used in this experiment have been subjected to several changes of locations, containers and conservation conditions in their history (see 'Conservation conditions' in the Materials and Methods). Moreover, the MC detected in the study seed lots (9.42 and 11.00 % for the base and active chambers, respectively) was higher than the current standards of conservation (5–8 %), meaning that current seed lots stored at CIMMYT might show even higher longevity than those considered in this experiment. Remedial drying in the drying rooms of the CIMMYT germplasm bank will be performed for those seed lots with MC higher than the current standards of conservation.

Active chambers conserve seed lots that are going to be used and/or distributed so as to not affect the conservation (due to frequent opening of the containers and extraction of the seeds from the chambers) of the seeds stored for the long term in the base chamber (FAO, 2014). Hay *et al.* (2013) and Van Treuren *et al.* (2018) observed a significant decline in the germination of barley, rice and wheat seeds conserved in the active chamber (4 °C) compared with the base chamber (–20 °C) after up to 30 years of storage. In the current study, we also detected a significant difference in the final germination of the same accessions in the active vs. the base chamber: 34 % of the seed lots stored in the active chamber had a lower viability than those of the same accessions stored in the base chamber. Moreover, we detected more variable germination percentages among seed lots in the active chamber (Fig. 1; Table 2). These differences in seed viability between active and base conservation conditions may be explained by the fact that seed longevity increases as the storage temperature decreases (Ellis *et al.*, 1992). Additionally, seed lots conserved in the active chamber showed on average a higher moisture content, probably due to more frequent opening of the hermetic containers. This is confirmed by the distribution data of the CIMMYT Germplasm Bank from 1987 until 2020. Considering the study accessions, 'active' seed lots were retrieved from the chamber for distribution from three up to 84 times (on average 14 times). On the other hand, 'base' seed lots are not used for distribution and are retrieved from the chamber only for viability monitoring. Together with temperature, seed moisture content is the major factor contributing to seed ageing (Zinsmeister *et al.*, 2020)

The more rapid loss of seed viability in the active when compared with the base chamber questions the effectiveness of a seed conservation strategy based on two different thermal storage conditions for the same accessions. Almost half of the seed lots tested in the active chamber will need to be regenerated, being below the 85 % threshold of their initial viability (FAO, 2014), while only 14 % of those same accessions conserved in the base chamber would require regeneration.

The conservation of the same accessions in two separate chambers (active and base) certainly has some advantages; the 'active' seed lots are likely to have a higher moisture content, as detected in this study, and could be exposed to frequent changes in temperatures due to more frequent retrieval from the conservation chamber; both factors could affect seed viability in the long term and would occur to a lesser extent in the 'base' seed lots. In order to minimize the changes in temperature and relative humidity experienced by 'active' seed lots every time an accession is ordered, a few replicates of seeds intended for distribution, both internal and external, could be pre-packed in hermetically sealed containers (e.g. aluminium pouches), avoiding the retrieval of the entire seed lot. Nevertheless, if the 'active' seed lots had been stored at the lower temperature conditions of the base chamber, it is safe to assume that the number of 'active' seed lots needing regeneration would be significantly lower, improving the overall conservation status of the entire collection and reducing the investments made for monitoring and regeneration (Singh *et al.*, 2012).

We detected a positive correlation between the germination of the same accession from both the active and the base chamber, highlighting an accession-specific behaviour in terms of viability loss. This means that seed accessions with lower viability after storage in the active chamber are likely to also have lower viability after storage in the base chamber (see Supplementary data Fig. S1), as previously observed by Hay *et al.*, (2013) in rice accessions. This can be very important for seedbank management, since the seed lots conserved in the active chamber, i.e. those intended for characterization, distribution, multiplication and research, are more often tested for their viability. Therefore, when a seed lot in the active chamber shows a low viability, the 'base' seed lot of the same accession should also be tested to ensure the conservation of high-quality seeds and avoid the loss of genetic resources of inestimable value.

The seed viability and longevity estimates were significantly different among accessions considered in this study; for example, the p_{50} varied from 16.5 to 91 years. This underscores that within the same plant species, large differences in the longevity profile can be found in different populations, as already observed in several other domesticated and wild species (e.g. Hay *et al.*, 2013; Mondoni *et al.*, 2018; Van Trueren *et al.*, 2018). Due to the increasing age of many historical seedbanks (such as CIMMYT) and therefore the age in storage of many seed accessions, the number of accessions that will need to be regenerated is only going to increase. For this reason, it is important to understand those within-species differences in seed longevity by finding groups of accessions characterized by similar longevity profiles and groups that are particularly affected by seed ageing, in order to plan viability monitoring intervals and regeneration efforts (Hay *et al.*, 2013, Guzzon *et al.*, 2018).

The ageing rate (L) of the study accessions was found to be correlated with seed-related traits, namely seed mass and grain type. The ageing rate was positively correlated with seed mass, which means that larger seeds aged faster than smaller ones (Fig. 2; Supplementary data Table S2). This has been observed in other cereal crop genepools, such as rice (Rao and Jackson, 1996) and wheat wild relatives (Guzzon *et al.*, 2018; Gianella *et al.*, 2020). While there is considerable evidence that seed size has an influence on seed longevity in soil seed banks and in controlled ageing experiments (see, for example, Shutte *et al.*, 2008; Guzzon *et al.*, 2018), the relationship between seed size and longevity under germplasm bank conditions

is less understood (Probert *et al.*, 2009). Further research is needed to shed light on this correlation under long-term cold storage.

Among seed-related traits, grain type showed the highest correlation with the ageing rate (Fig. 2; Supplementary data Table S2). Flint accessions are the longest lived among the three main grain types (dent, flint and floury; Fig. 3). This confirmed the observation of Bewley and Black (1994) that seeds of flint varieties are longer lived when compared with other grain types. A similar observation was made by Ortega Paczka (1971), who tested > 4500 maize accessions, conserved at the INIA genebank (Mexico). Grains of varieties of flint maize have mostly hard, glassy endosperm compared with the softer and starchier endosperms typical of dent and floury varieties (Zilic *et al.*, 2011). A glassy endosperm reduces molecular mobility and is directly involved in slowing seed ageing (Buitink and Leprince, 2008; Ballesteros and Walters, 2011). Additionally, we detected that flint seeds have a significantly lower seed mass than dent and floury seeds. We hypothesize that the higher seed longevity detected in flint varieties is due to structural (i.e. glassy endosperm, seed coat and seed mass) or physiological (e.g. antioxidant capacity) traits, but further investigations, e.g genome-wide association studies (GWAS), antioxidant profiling and analyses of viscoelastic properties of the endosperm, need to be carried out, to clarify the biological bases of the differences in seed longevities detected among grain types.

Yamasaki *et al.* (2020), following the Genebank Standards of the FAO (2014), calculated an average monitoring interval of 7 years for 3953 maize accessions conserved at the NARO genebank in Japan at – 1 °C and 30 % relative humidity. In the current experiment, we calculated an average monitoring interval of 17 years from our study seed lots conserved in the active chamber (–3 °C), within the interval currently used at the CIMMYT Germplasm Bank where 'active' seed lots are tested for their viability after 15–20 years of storage. Moreover, significantly different monitoring intervals were obtained for the main grain types in the active chamber. i.e. dent, 15 years; flint, 20 years; floury, 18 years (see Supplementary data Fig. S2). This suggests that different grain types should have different monitoring intervals. Grain type is a qualitative trait based mainly on the seed coat morphology and endosperm texture. Maize landrace kernels can show intermediate or multiple phenotypes between two different grain types. Therefore, more quantitative measures will be needed to study the effect of the grain type on seed longevity and to fine-tune viability monitoring intervals of accessions of different grain types showing different longevity estimates.

Significant correlations were also found between the ageing rate (L) and geographical data of the original collecting sites of the accessions: co-ordinates and elevation data (Fig. 2). It is known that the seed longevity of different populations of the same species can be influenced by the provenance of the populations, as described by latitude and elevation, probably due to adaptive responses to different environmental conditions; see, for example, Probert *et al.* (2009), Mondoni *et al.* (2011) and Zani and Müller (2017). It is interesting to see how maize accessions from high altitudes showed on average lower seed longevity, a pattern also observed in other plant species (Fig. 2; Mondoni *et al.*, 2011). These correlations were only partly confirmed by the partial correlation coefficients (Supplementary data Table S2), and further research is needed to understand if those differences are really due to environmental adaptations or to other factors (e.g. differences in moisture content or the geographical distribution of the different grain types; see Supplementary data Table S2). To clarify these possible environmental adaptations, the longevity of similar genotypes collected at different latitudes and altitudes should be tested after the same seed processing (see, for example, Mondoni *et al.*, 2011, 2014).

In conclusion, maize seed accessions conserved at the CIMMYT germplasm bank for up to 60 years showed a high viability (on average >80 %), confirming that maize can be considered a long-lived species under germplasm bank conditions. There have been several debates about the effectiveness of *ex situ* conservation of large germplasm collections. Some experts raised the concern that large plant germplasm collections cannot be characterized and regenerated fast enough and therefore are merely 'seed morgues' (CIMMYT, 1988; Goodman, 1990). We have demonstrated that maize seeds, if properly conserved, can maintain a high viability for decades, providing sufficient time to seed bank curators and researchers for the characterization and regeneration of the accessions, as well as the organization of the composition of the collections, based on genetic diversity, cultural significance and agronomic considerations. Nevertheless, the germination of the seed lots conserved in the active chamber was significantly lower and more variable than in the base chamber. This raises doubts about the current strategy, employed by several international seedbanks, of conserving the same accessions in two chambers with different temperature conditions. Our data indicate that base chamber conditions (cold storage between –15 and –20 °C) should be preferred for the conservation of long-term collections of maize seeds, also for 'active' seed lots (intended for regeneration, distribution and characterization) to decrease their loss of viability and, therefore, the frequency of their regeneration. Future revisions of manuals on genebank standards (e.g. FAO, 2014) should consider these results, and similar ones obtained in other crop species (see, for example, Hay *et al.*, 2013, Van Treuren *et al.*, 2018), and direct seedbanks towards the preference of all seed conservation in base chamber conditions. Significant differences were detected in seed longevity among accessions; in particular, flint varieties were longer lived than dent and floury varieties. This underscores the importance of further study of within-crop differences in seed longevity to provide a sound, scientific basis for both viability monitoring and regeneration intervals, on a crop-by-crop basis.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Table S1: principal passport data and ageing rate of the accessions tested in the experiment. Table S2: results of the partial correlation analysis for the variables included in the correlation plot. Figure S1: scatterplot of germination percentage in the active and base

chambers of the same accessions with regression line. Figure S2: monitoring intervals calculated as one-third of the p_{85} for the three main grain types, dent, flint and floury.

ACKNOWLEDGMENTS

We are indebted to the maize germplasm bank team for their work on this experiment, especially the members of the Viability Unit: Ángel Jesús Limón Gallegos, Bryan Sánchez Cordero, Irene Nallely Benítez Hernández and Valeria González Meraz. We are indebted to Suketoshi Taba, Jean Hanson, Rafael Ortega Paczka and Garrison Wilkes for the precious information on the history of the CIMMYT maize collection. We thank Antonio da Costa Teixeira (University of Minho, Portugal), Eduardo Fernandez Pascual (University of Oviedo, Spain) and Juan Burgueño (CIMMYT) for fruitful discussions on the analysis of seed longevity data. We thank Dr Susanne Barth and two anonymous reviewers for their invaluable comments on an earlier version of the manuscript. We are extremely grateful to all of the past and present staff members of the CIMMYT Germplasm Bank for the excellent job they have done in conserving the CIMMYT maize collection over all these years. We dedicate this paper to Edwin Wellhausen, the man who started the CIMMYT maize germplasm collection, as the Director of the 'Races of Maize' Project, the first Head of the CIMMYT Germplasm Bank, and the first Director General of CIMMYT. The authors have no conflict of interest.

Author contributions: F.G. designed the experiment, carried out the lab activities, analysed the data and wrote the manuscript; M.G. carried out lab activities, analysed the data and wrote the manuscript; J.A.V.J. organized the database and retrieved historical data, organized the data capturing and approved the final version of the manuscript; C.S.C. co-ordinated and carried out the lab activities and approved the final version of the manuscript; D.E.C. designed the experiment, wrote the manuscript and performed the linguistic revision.

FUNDING

This work of the CIMMYT Germplasm Bank is supported by the CGIAR Genebank Platform. M.G. was supported by a mobility grant for PhD students of the University of Pavia for carrying out research activities at CIMMYT.

LITERATURE CITED

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. *The Plant Journal* **68**: 607–619.

Bewley JD, Black M. 1994. *Seeds: physiology of development and germination*. Berlin/Heidelberg: Springer Science & Business Media.

Bryant C, Wheeler NR, Rubel F, French RH. 2017. *kgc: Koeppen–Geiger climatic zones. R package version 1.0.0.2.* https://CRAN.R-project.org/package=kgc. (15 August 2020).

Buitink J, Leprince O. 2008. Intracellular glasses and seed survival in the dry state. *Comptes Rendus Biologies* **331**: 788–795.

CIMMYT. 1974. *Proceedings: World-wide maize improvement in the 70's and the role for CIMMYT*. Texcoco: International Maize and Wheat Improvement Center (CIMMYT).

CIMMYT. 1988. *Recent advances in the conservation and utilization of genetic resources. Proceedings of the Global Maize Germplasm Workshop*. Texcoco: International Maize and Wheat Improvement Center (CIMMYT).

Colville L, Pritchard HW. 2019. Seed life span and food security. *New Phytologist* **224**: 557–562.

Desheva G. 2016. The longevity of crop seeds stored under long-term condition in the National Gene Bank of Bulgaria. *Agriculture (Pol'nohospodárstvo)* **62**: 90–100.

Ellis RH, Hong TD, Roberts EH. 1992. The low-moisture content limit to the negative logarithmic relation between seed longevity and moisture content in three subspecies of rice. *Annals of Botany* **69**: 53–58.

FAO. 2010. *The second report on the state of the world's plant genetic resources for food and agriculture*. Rome: FAO.

FAO. 2014. *Genebank standards for plant genetic resources for food and agriculture*. Rome: FAO.

de Faria RQ, dos Santos ARP, Amorim DJ, Cantão RF, da Silvia EAA, Sartori MMP. 2020. Probit or logit? Which is the better model to predict the longevity of seeds? *Seed Science Research* **30**: 49–58.

Gianella M, Balestrazzi A, Pagano A, *et al.* 2020. Heteromorphic seeds of wheat wild relatives show germination niche differentiation. *Plant Biology (Stuttgart, Germany)* **22**: 191–202.

Giner G, Smyth GK. 2016. statmod: probability calculations for the inverse Gaussian distribution. *R Journal* **8**: 339–351.

Goodman MM. 1990. What genetic and germplasm stocks are worth conserving? In: McGuire PE, Qualset CO, eds. *Genetic resources at risk: scientific issues, technologies, and funding policies*. Davis, CA: University of California, Davis, 1–9.

Guarino L, Lobell DB. 2011. A walk on the wild side. *Nature Climate Change* **1**: 374–375.

Guzzon F, Bello P, Bradford KJ, Mérida Guzman MA, Costich DE. 2020. Enhancing seed conservation in rural communities of Guatemala by implementing the dry chain concept. *Biodiversity and Conservation* **29**: 3997–4017.

Guzzon F, Orsenigo S, Gianella M, *et al.* 2018. Seed heteromorphy influences seed longevity in *Aegilops*. *Seed Science Research* **28**: 277–285.

Hammer K, Knüpffer H, Xhuveli L, Perrino P. 1996. Estimating genetic erosion in landraces – two case studies. *Genetic Resources and Crop Evolution* **43**: 329–336.

Hay FR, de Guzman F, Ellis D, Makahiya H, Borromeo T, Sackville Hamilton NR. 2013. Viability of *Oryza sativa* L. seeds stored under genebank conditions for up to 30 years. *Genetic Resources and Crop Evolution* **60**: 275–296.

Hothorn T, Bretz F, Westfall P. 2008. Simultaneous inference in general parametric models. *Biometrical Journal* **50**: 346–363.

Huang C, Sun H, Xu D, *et al.* 2018. ZmCCT9 regulates maize flowering time. *Proceedings of the National Academy of Sciences, USA* **115**: 334–341.

ISTA. 2018. *ISTA handbook on seedling evaluation*, 4th edn. Bassersdorf, Switzerland: International Seed Testing Association.

Kassambara A. 2020. *ggpubr: 'ggplot2' based publication ready plots. R package version 0.4.0.* https://CRAN.R-project.org/package=ggpubr. (8 December 2020).

Lenth RV. 2016. Least-squares means: the R package lsmeans. *Journal of Statistical Software* **69**: 1–33.

Li DZ, Pritchard HW. 2009. The science and economics of ex situ plant conservation. *Trends in Plant Science* **14**: 614–621.

McCouch S, Baute GJ, Bradeen J, *et al.* 2013. Feeding the future. *Nature* **499**: 23–24.

Merritt DJ, Martyn AJ, Ainsley P, *et al.* 2014. A continental-scale study of seed lifespan in experimental storage examining seed, plant, and environmental traits associated with longevity. *Biodiversity and Conservation* **23**: 1081–1104.

Mondoni A, Probert RJ, Rossi G, Vegini E, Hay FR. 2011. Seeds of alpine plants are short lived: implications for long-term conservation. *Annals of Botany* **107**: 171–179.

Mondoni A, Orsenigo S, Donà M, *et al.* 2014. Environmentally induced transgenerational changes in seed longevity: maternal and genetic influence. *Annals of Botany* **113**: 1257–1263.

Mondoni A, Orsenigo S, Müller JV, Carlsson-Granér U, Jiménez Alfaro B, Abeli T. 2018. Seed dormancy and longevity in subarctic and alpine populations of *Silene suecica*. *Alpine Botany* **128**: 71–81

Nagel M, Börner A. 2010. The longevity of crop seeds stored under ambient conditions. *Seed Science Research* **20**: 1–20.

Nakazawa M. 2019. *fmsb: functions for medical statistics book with some demographic data. R package version 0.7.0.* https://CRAN.R-project.org/package=fmsb. (8 December 2020).

Ortega Paczka RA. 1971. *Estudio de algunos factores que afectan el poder germinativo de las semillas de maíz del banco de germoplasma del INIA.* Thesis, Chapingo Autonomous University, Mexico.

Probert RJ, Daws MI, Hay FR. 2009. Ecological correlates of *ex situ* seed longevity: a comparative study on 195 species. *Annals of Botany* **104**: 57–69.

Rao NK, Jackson MT. 1996. Seed longevity of rice cultivars and strategies for their conservation in genebanks. *Annals of Botany* **77**: 251–260.

Revelle W. 2019. *psych: procedures for personality and psychological research,* Northwestern University, Evanston, Illinois, USA. https://CRAN.R-project.org/package=psych Version =1.9.12. (8 December 2020).

Rivière S, Muller JV. 2017. Contribution of seed banks across Europe towards the 2020 Global Strategy for plant conservation targets, assessed through the ENSCONET database. *Oryx* **52**: 464–470.

Roberts EH. 1973. Predicting the storage life of seeds. *Seed Science and Technology* **1**: 499–514.

Roos EE, Davidson DA. 1992. Record longevities of vegetable seeds in storage. *HortScience* **27**: 393–396.

dos Santos ARP, de Faria RQ, Amorim DJ, Giandoni VCR, da Silva EAA, Sartori MMP. 2019. Cauchy, Cauchy–Santos–Sartori–Faria, logit, and probit functions for estimating seed longevity in soybean. *Agronomy Journal* **111**: 2929–2939.

Shutte BJ, Regnier EE, Harrison SK. 2008. The association between seed size and seed longevity among maternal families in *Ambrosia trifida* L. populations. *Seed Science Research* **18**: 201–211.

Singh AK, Varaprasad KS, Venkateswaran K. 2012. Conservation costs of plant genetic resources for food and agriculture: seed genebanks. *Agricultural Research* **1**: 223–239.

Solberg SØ, Yndgaard F, Andreasen C, von Bothmer R, Loskutov IG, Asdal Å. 2020. Long-term storage and longevity of orthodox seeds: a systematic review. *Frontiers in Plant Science* **11**: 1007.

Taiyun W, Viliam S. 2017. *R package 'corrplot': visualization of a correlation matrix.* https://github.com/taiyun/corrplot. (8 December 2020).

van Treuren R, Bas N, Kodde J, Groot SPC, Kik C. 2018. Rapid loss of seed viability in ex situ conserved wheat and barley at 4°C as compared to –20°C storage. *Conservation Physiology* **6**: coy033.

Van de Wouw M, Kik C, Van Hintum T, Van Treuren R, Visser B. 2010. Genetic erosion in crops: concept, research results and challenges. *Plant Genetic Resources* **8**: 1–15.

Veteläinen M, Negri V, Maxted N. 2009. *European landraces: on-farm conservation management and use. Bioversity technical bulletin No. 15.* Rome: Bioversity International.

Vincent H, Wiersema J, Kell SP, *et al.* 2013. A prioritised crop wild relative inventory as a first step to help underpin global food security. *Biological Conservation* **167**: 265–275.

Walters C. 2003. Optimising seed banking procedures. In: Smith RD, Dickie JB, Linington SH, Pritchard HW, Probert RJ, *Seed conservation: turning science into practice*. Kew, London: Royal Botanic Gardens, 723–743.

Walters C, Wheeler LM, Grothenius JM. 2005. Longevity of seeds stored in a genebank: species characteristics. *Seed Science Research* **15**: 1–20.

Warschefsky E, Varma Penmetsa R, Cook DR, von Wettberg EJB. 2014. Back to the wild: tapping evolutionary adaptations for resilient crops through systematic hybridization with crop wild relatives. *America Journal of Botany* **101**: 1791–1800.

Wickham H. 2016. *ggplot2: elegant graphics for data analysis.* New York: Springer-Verlag.

Wickham H, François R, Henry L, Müller K. 2020. *dplyr: a grammar of data manipulation. R package version 0.8.5.* https://CRAN.R-project.org/package=dplyr. (8 December 2020).

Yamasaki F, Domon E, Tomooka N, Baba-Kasai A, Nemoto H, Ebana K. 2020. Thirty-year monitoring and statistical analysis of 50 species' germinability in genebank medium-term storage suggest specific characteristics in seed longevity. *Seed Science and Technology* **48**: 269–287.

Zani D, Müller JV. 2017. Climatic control of seed longevity of *Silene* during the post-zygotic phase: do seeds from warm, dry climates possess higher maturity and desiccation tolerance than seeds from cold, wet climates? *Ecological Research* **32**: 983–994.

Zilic S, Milasinovic M, Terzic D, Barac M, Ignjatovic-Micic D. 2011. Grain characteristics and composition of maize specialty hybrids. *Spanish Journal of Agricultural Research* **9**: 230–241

Zinsmeister J, Leprince O, Buitink J. 2020. Molecular and environmental factors regulating seed longevity. *The Biochemical Journal* **477**: 305–323.

Gualtieri C, **Gianella M**, Pagano A, Cadeddu T, Araújo S, Balestrazzi A. Exploring microRNA signatures of DNA damage response using an innovative system of genotoxic stress in Medicago truncatula seedlings. *Front Plant Sci*. 2021; 12, 347. https://doi.org/10.3389/fpls.2021.645323

frontiers in Plant Science

ORIGINAL RESEARCH
published: 09 March 2021
doi: 10.3389/fpls.2021.645323

Exploring microRNA Signatures of DNA Damage Response Using an Innovative System of Genotoxic Stress in *Medicago truncatula* Seedlings

*Carla Gualtieri††, Maraeva Gianella††, Andrea Pagano†, Tiziano Cadeddu†, Susana Araújo‡,§, Alma Balestrazzi† and Anca Macovei†**

†Plant Biotechnology Laboratory, Department of Biology and Biotechnology "L. Spallanzani", University of Pavia, Pavia, Italy, ‡Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Oeiras, Portugal, §Association BLC3, Technology and Innovation Campus, Centro BIO- R&D Unit, Lagares da Beira, Portugal

OPEN ACCESS

Edited by:
Paola Vittorioso, Sapienza University of Rome, Italy

Reviewed by:
Giovanna Frugis, National Research Council (CNR), Italy
Manuel Bacana, Consejo Superior de Investigaciones Científicas, Spain

***Correspondence:**
Anca Macovei
anca.macovei@unipv.it

†These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science

Received: *22 December 2020*
Accepted: *15 February 2021*
Published: *09 March 2021*

Citation:
Gualtieri C, Gianella M, Pagano A, Cadeddu T, Araújo S, Balestrazzi A and Macovei A (2021) Exploring microRNA Signatures of DNA Damage Response Using an Innovative System of Genotoxic Stress in Medicago truncatula Seedlings. Front. Plant Sci. 12:645323. doi: 10.3389/fpls.2021.645323

One of the challenges that living organisms face is to promptly respond to genotoxic stress to avoid DNA damage. To this purpose, all organisms, including plants, developed complex DNA damage response (DDR) mechanisms. These mechanisms are highly conserved among organisms and need to be finely regulated. In this scenario, microRNAs (miRNAs) are emerging as active players, thus attracting the attention of the research community. The involvement of miRNAs in DDR has been investigated prominently in human cells whereas studies in plants are still scarce. To experimentally investigate the involvement of plant miRNAs in the regulation of DDR-associated pathways, an *ad hoc* system was developed, using the model legume *Medicago truncatula*. Specific treatments with camptothecin (CPT) and/or NSC120686 (NSC), targeting distinct components of DDR, namely topoisomerase I (Topl) and tyrosyl-DNA phosphodiesterase 1 (TDP1), were used. Phenotypic (germination percentage and speed, seedling growth) and molecular (cell death, DNA damage, and gene expression profiles) analyses demonstrated that the imposed treatments impact DDR. Our results show that these treatments do not influence the germination process but rather inhibit seedling development, causing an increase in cell death and accumulation of DNA damage. Moreover, treatment-specific changes in the expression of suppressor of gamma response 1 (*SOG1*), master-regulator of plant DDR, were observed. Additionally, the expression of multiple genes playing important roles in different DNA repair pathways and cell cycle regulation were differentially expressed in a treatment-specific manner. Subsequently, specific miRNAs identified from our previous bioinformatics approaches as putatively targeting genes involved in DDR processes were investigated alongside their targets. The obtained results indicate that under most conditions when a miRNA is upregulated the corresponding candidate target gene is downregulated, providing an indirect evidence of miRNAs action over these targets. Hence, the present study extends the present knowledge on the information available regarding the roles played by miRNAs in the post-transcriptional regulation of DDR in plants.

Keywords: DNA damage response, microRNA, genotoxicity, camptothecin, NSC120686, tyrosyl-DNA phosphodiesterase 1, seedling development

INTRODUCTION

During their lifespan, plants continuously face stressful conditions (caused by exogenous and endogenous factors) that affect plant growth and development. Considering their sessile lifestyle, plants are provided with incredible genomic plasticity. For instance, the metaphorical "perceptron," defined as an information-processing system composed of several processing units with biochemical connections, enables the selection of the most suitable options for coping with the changing environment (Scheres and Van der Putten, 2017). Linked to this, DNA damage response (DDR) is among the main strategies used by plant cells to safeguard their genome and therefore plant growth and development. To briefly define it, DDR is an intricated signal transduction network involving many players that act as DNA damage sensors, signal transducers, mediators, and effectors, working together to coordinate appropriate responses depending on the type of stimuli. A recent bibliometric study illustrates that DDR is generally far less studied in plants as compared to mammals but the interest in plant DDR research is expanding in view of future agricultural applications (Gimenez and Manzano-Agugliaro, 2017). Coincidently, it is also opportune to pinpoint that DDR is an evolutionarily conserved pathway in eukaryotes, although kingdom-specific variations are encountered (see reviews by Yoshiyama et al., 2013a; Nikitaki et al., 2018; Nisa et al., 2019). Just to cite some differences, suppressor of gamma response 1 (SOG1) is the proposed master-regulator of plant DDR, acting as a functional homolog of the mammalian p53 (Yoshiyama et al., 2009, 2013a,b). As a transcription activator, SOG1 regulates the expression of DNA repair- and cell cycle-related genes (Bourbousse et al., 2018; Ogita et al., 2018). Besides, SOG1-independent pathways have been also proposed to work in plant DDR; though the molecular mechanism is not yet fully understood, it is believed that these may include the E2F-RBR1 (RetinoBlastoma Related 1) complex, comprising transcription regulators that control the entry in the S-phase of the cell cycle (Berckmans and De Veylder, 2009). The E2Fa transcription factor also participates in DNA replication and DNA damage repair (Roa et al., 2009; Gutzat et al., 2012).

Ultimately, DDR enables the activation of cell cycle checkpoints as well as specific DNA repair mechanisms (Hu et al., 2016). Hence, the recognition and repair of DNA damage involve both the activation of DNA repair processes as well as the regulation of the cell cycle arrest, allowing the necessary time for DNA lesions to be corrected prior to cell cycle initiation. If DNA repair processes are impaired, changes in the cell cycle, transcription, and protein synthesis are encountered as well (Britt, 1999; Bray and West, 2005). Among the DNA damage repair mechanisms, some are highly specialized for specific types of damage whereas others work in a more generalized manner. It is also important to recognize that different DNA repair pathways have overlapping functions and can share key enzymes. For instance, Tyrosyl-DNA-phosphodiesterase 1 (TDP1), involved in the removal of topoisomerase I (TopI)-DNA covalent complexes (Yang et al., 1996; Pouliot et al., 1999), has been associated with both base excision repair (BER; Lebedeva et al., 2011; Donà et al., 2013) and DNA-protein crosslink (DPC) repair (Enderle et al., 2019a,b). Studies in model plants like *Arabidopsis thaliana* and *Medicago truncatula* showed that the lack of TDP1 function led to the development of dwarf genotypes sensitive to DNA damage with impaired DNA repair and cell cycle activities (Lee et al., 2010; Kim et al., 2012; Donà et al., 2013; Sabatini et al., 2016). The presence of a small subfamily of *TDP1* genes (composed of *TDP1α* and *TDP1β*) was highlighted in plants and it has been shown that the two genes do not have overlapping functions and they are differentially expressed in a species-, tissue-, and stress-specific manner (Macovei et al., 2010; Donà et al., 2013; Sabatini et al., 2017; Mutti et al., 2020).

To take place properly, the DDR system requires advanced regulatory mechanisms, which are not yet fully understood. In this context, microRNAs (miRNAs), a class of small, non-coding RNAs (~21–22 nt) that play key regulatory roles in gene expression at a post-transcriptional level (Bartel, 2004), may participate in the regulation of DDR. This aspect is quite recent and insufficiently explored, especially within the plant kingdom. Studies in human cells demonstrated that miRNAs are involved in the regulation of different components of the DDR machinery (Zhang and Peng, 2015). For instance, miR-24, miR-138, miR-182, miR-101, miR-421, miR-125b, and miR-504 were identified as crucial regulators of H2AX, BRCA1, ATM, or p53. Other such examples include miR-96, miR-155, miR-506, miR-124, miR-526, and miR-622b, shown to be involved in homologous recombination (HR) or nonhomologous end-joining (NHEJ) repair by targeting RAD51 or KU70/80 (Choi et al., 2014; Thapar, 2018). The presence of DNA lesions influences miRNA degradation as well as their expression. In both plants and animals, it has been demonstrated that miRNAs are responsive to irradiation (IR)-induced oxidative stress and may be responsible for the post-transcriptional regulation of some DDR genes (Joly-Tonetti and Lamartine, 2012; Kim et al., 2016). Plant specific miRNAs responsive to genotoxic stress include the IR-induced Arabidopsis miR840 and miR850, which remain to be further characterized in terms of their roles in DDR and DNA repair (Kim et al., 2016). Few rice miRNAs (osa-miR414, osa-miR164e, and osa-miR408) demonstrated to target specific helicases (Macovei and Tuteja, 2012) were also found to be responsive to γ-irradiation (Macovei and Tuteja, 2013). Predictive studies were employed as well; Liang et al. (2017) reported that MUTL-homolog 1 (MLH1) and MRE11 were putatively targeted by miR5176 and miR5261 in *Citrus sinensis* whereas the *Brachypodium distachyon* novel_mir_69 was identified to putatively target the RAD50 mRNA (Lv et al., 2016). Based on recent reviews of literature, an interrelation between DDR, redox systems, and miRNAs, has been proposed (Cimini et al., 2019). Nonetheless, specific hurdles have been pinpointed to explain the poorly represented examples in plants. Namely, this may be because DDR is significantly less studied in plants compared to animals (probably due to plant genome complexity) combined with limited information on miRNA targets specifically involved in coping with genotoxic stress (Chowdhury and Basak, 2019).

Considering the implications of DDR in plant genome stability, it is worth investigating deeper these fine-tuning aspects to gain novel insights on this complex topic. To address the existing gaps-of-knowledge, the current study proposes to explore the role of post-transcriptional regulation mediated by miRNAs in plant DDR. To do so, the first step consisted of setting up an original experimental system. This involved the administration of two compounds, namely, camptothecin (CPT, a well-known inhibitor of TopI enzyme) and NSC120686 (2-chloro-6-fluorobenzaldehyde 9H-fluoren-9-ylidenehydrazone). The latter was identified by Weidlich et al. (2010) as a substrate mimetic of the human TDP1. Together with topoisomerase inhibitors, NSC120686 has been used as a pharmacophoric model to suppress the TDP1 activity as part of a synergistic treatment for cancer therapies (Perego et al., 2012) whereas, in plants, dose-dependent genotoxicity was evidenced (Macovei et al., 2018a). As an experimental model, we have chosen to work on *M. truncatula*, because it is emerging as an informative and versatile system to investigate DDR during seed germination (Macovei et al., 2019). Moreover, DDR is an essential component of the seed repair response during germination (Waterworth et al., 2019) when active cell proliferation is determinant for the development of healthy seedlings and DNA damage must be repaired before the start of cell division to ensure the generation of robust plants. Phenotypic (germination percentage and speed, seedling growth) and molecular (cell death, DNA damage, and gene expression profiles) analyses demonstrated that the imposed treatments impact DDR. Subsequently, a list of miRNAs and putative target genes identified in a previous bioinformatics approach as being involved in DDR-associated biological processes (Bellato et al., 2019), were investigated in the developed system in terms of expression profiles. The results hereby presented show that miRNA/target gene expression is treatment-specific, thus confirming that miRNAs can be affected by DNA damage and that their targeted genes may have a contribution in the response to DNA damage.

MATERIALS AND METHODS

Experimental Design

Medicago truncatula (cv. Jemalong) seeds, kindly provided by Fertiprado L.d.a. (Portugal), were used for this study. Seeds were treated with 25 µM CPT (Sigma-Aldrich, Milan, Italy), and 25 µM NSC120686 (NSC) provided by the National Cancer Institute (Bethesda, United States). A combined CPT + NSC treatment was implemented as well. The concentrations of the genotoxic agents were selected based on preliminary phenotypic results (**Supplementary Figure S1**) and previous studies (Macovei et al., 2018a). Because these compounds are dissolved in 100% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Milan, Italy), specific DMSO controls, corresponding to each concentration used in the indicated treatments, were included. Specifically, DMSO 0.29% (subsequently denominated as DMSO_C) corresponds to the concentration used for the CPT treatments, DMSO 0.17% to NSC treatments (DMSO_N), and DMSO 0.23% to CPT + NSC treatments (DMSO_CN). The DMSO concentrations differ for CPT and NSC because the stock solutions (compounds dissolved in 100% DMSO) were prepared at different molarities (CPT 8.61 M and NSC 14.71 M), according to the manufacturer's instructions. This affected also the combined treatment, where CPT and NSC were mixed 1:1. A non-treated control (NT) was used for all experiments. The designated treatments were applied to *M. truncatula* seeds placed in Petri dishes (30 seeds per dish) containing a filter of blotting paper moistened with 2.5 ml H_2O (NT) or indicated solutions. Each sample/treatment was performed at least in triplicates. Petri dishes sealed with parafilm were kept in a growth chamber at 22°C 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 experiment was followed for 7 days and subsequently, the harvested plant material was used fresh or frozen in liquid nitrogen (N_2) for designated analyses.

Phenotypic Evaluation

Treated and non-treated *M. truncatula* seeds were monitored for 7 days and data concerning germination percentage (%) and speed (T_{50}), seedling length, and fresh weight (FW) were determined at the end of the experiment. The germination % parameter was assessed as the percentage of total germinated seeds in which the radicle protrusion reaches at least 1 mm of length. The time required for 50% of seeds to germinate (T_{50}) was calculated according to the formula developed by 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 seed germination, n_i, n_j represent the cumulative number of seeds that germinated by adjacent counts at times t_i and t_j when $n_i < N/2 < n_j$. Seedling length (millimeters, mm) was measured using millimetric paper whereas FW (grams, g) was measured using an analytical weight scale (Mettler AJ100, Mettler Toledo, Germany). Data are represented as mean ± SD of at least three independent measurements.

Single Cell Gel Electrophoresis

The single cell gel electrophoresis (SCGE) protocol was implemented to *M. truncatula* radicles as previously described (Pagano et al., 2017; Araújo et al., 2019). Nuclei were extracted from treated/untreated radicles isolated from freshly harvested 7-day-old seedlings. For nuclei extraction, liquid N_2 frozen radicles in Tris HCl EDTA (0.4 M Tris HCl pH 7.0, 1 mM EDTA pH 8) were finely sliced. The solution containing extracted nuclei was mixed with 1% low melting point (LMP) agarose and pipetted onto glass slides previously coated with 1% LMP. For alkaline SCGE, the glass slides containing isolated nuclei were subjected to electrophoresis (25 V, 300 A) in an alkaline buffer (0.3 M NaOH, 1 mM EDTA, and pH > 13) for 25 min at 4°C. For neutral SCGE, the slides were subjected to electrophoresis (20 V, 10 mA) in Tris-borate-EDTA (TBE; 89 mM Tris Base, 89 mM Boric Acid, 2 mM EDTA, and pH 8.3) for 8 min at 4°C. Subsequently, the slides were washed twice with Tris-HCl pH 7.5 for 5 min and rinsed in 70%

ethanol (v/v) for 12 min. For nuclei count, the slides were stained with 20 µl 4′,6- diamidino-2-phenylindole (DAPI, 1 µgml^{-1} stock solution; Sigma-Aldrich) and visualized at a fluorescence microscope (Olympus BX51, Olympus, Germany) with an excitation filter of 340–380 nm and a barrier filter of 400 nm. For each slide, about 100 nuclei were scored and analyses were performed in triplicates. The results were expressed in arbitrary units (a.u) calculated according to the formula proposed by Collins (2004): $[\Sigma(N_c \times c) \times 100]/N_{tot}$, where N_c is the number of nuclei of each class, c is the class number (e.g., 0, 1, 2, 3, and 4), and N_{tot} is the total number of counted nuclei.

DNA Diffusion Assay

The DNA diffusion assay was performed to evaluate cell death events and distinguish cells subjected to PCD or necrosis from viable cells as described by Macovei et al. (2018b). Nuclei extraction was performed from radicles of 7-day-old seedlings using the same methodology described for SCGE. The glass slides containing nuclei embedded in 1% LMP agarose were incubated in high salt lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, and pH 7.5) for 20 min at 4°C to disrupt the nuclear membrane and permit DNA diffusion. The slides were immersed in neutral TBE for 5 min for three consecutive times to remove lysis solution and rinsed in 70% ethanol for 5 min at 4°C. Following DAPI staining, about 100 nuclei were scored (in triplicate samples) under the fluorescent microscope. The overall cell death level is given as a.u. while an additional analysis was used to represent the percentage of each class of nuclei (0-nuclei from viable cells, 1-nuclei from PCD cells, and 2-nuclei from necrotic cells).

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Total RNA was isolated from treated and untreated *M. truncatula* seedlings as described (Pagano et al., 2017; Araújo et al., 2019). Briefly, liquid N_2 grinded material was mixed with 550 µl Extraction Buffer (0.4 M LiCl, 0.2 M Tris pH 8.0, 25 mM EDTA, and 1% SDS) and 550 µl chloroform. Samples were centrifuged at 10,000 rpm for 3 min at 4°C. A phenol-chloroform solution was added to the supernatant followed by same centrifuge step. A 1/3 volume of 8 M LiCl was added to the supernatant, incubated at 4°C for 1 h, and subsequently centrifuged. The resulting pellet was washed with 70% ethanol, air-dried, and suspended in diethylpyrocarbonate (DEPC) water. The RNA was subsequently treated with DNase (Thermo Scientific), as indicated by the manufacturer. Finally, RNA was quantified with a NanoDrop spectrophotometer (Biowave DNA, WPA, ThermoFisher Scientific).

The complementary DNAs (cDNAs) were obtained using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific) according to the manufacturer's suggestions.

The quantitative real-time PCR (RT-*q*PCR) reactions were performed with the Maxima SYBR Green qPCR Master Mix (2X; ThermoFisher Scientific) according to the supplier's indications, using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics Pty Ltd., Brisbane, Queensland 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 60 s. Oligonucleotide primers (**Supplementary Table S1**) were designed using Primer3Plus[1] and verified with Oligo Analyzer.[2] The relative quantification was carried out using actin-related protein 4A (*Act*) and elongation factor 1α (*ELF1α*) as reference genes since they resulted the most stable under the tested conditions following geNorm (Vandesompele et al., 2002) analysis (**Supplementary Figure 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 (Pfaffl, 2001) was used for the relative quantification of transcript accumulation. All reactions were performed in triplicate. The data are presented as fold change (FC), where values for each treatment were normalized to their corresponding DMSO control. Heatmaps were constructed using the Shinyheatmap tool (Khomtchouk et al., 2017).

microRNAs Expression Analysis

For miRNAs expression, total RNA was isolated using TRIzol (ThermoFisher Scientific), as indicated by the supplier. The two-tailed RT-*q*PCR technique (Androvic et al., 2017) was performed to quantify miRNA accumulation. The miRNAs expression profiles were analyzed in 7-day-old untreated and treated seedlings. Different sets of primers were used to perform reverse transcription (RT) and RT-*q*PCR for each mature miRNA, one to synthesize the cDNA and two for the SYBR *q*PCR amplification. cDNAs were obtained using the qScript® Flex cDNA Synthesis Kit (QIAGEN, Beverly, Massachusetts). The RT primers (**Supplementary Table S2**) were designed to have a two-tailed structure as indicated by Androvic et al. (2017). RNAfold WebServer[3] was used to predict the stable secondary structure. To obtain the cDNA, a forward primer specific for the designed region in the 5′-terminus of the two-tailed RT-primer and a reverse primer specific for the miRNA target sequence were used. Subsequently, RT-*q*PCR was performed as described in the above paragraph using the oligonucleotide primers shown in **Supplementary Table S3**.

Statistical and Integrative Data Analyses

For phenotypic evaluation, the significance of mean differences was determined using the Student's *t*-test. For gene/miRNA expression data, following the normality test (Shapiro-Wilk), a one way ANOVA on ranks was performed using the Kruskal-Wallis test in an R (software version 4.0.2) background.

Principal components analysis (PCA) was performed on the phenotypic and molecular variables quantified across the study using the FactoMineR (Lê et al., 2008) and factoextra (Kassambara and Mundt, 2020) packages in R environment for statistical computing and graphical design. Values were

[1]https://primer3plus.com/
[2]https://eu.idtdna.com/calc/analyzer
[3]http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi

standardized by means of z-score using the default scaling settings of the PCA function. The included variables were: germination %, T_{50}, seedling length (divided as aerial part and radicles), DNA damage levels, all gene expression data, and miRNA expression profiles.

RESULTS

CPT and NSC Treatments Do not Affect Seed Germination but Impair Seedling Development

The CPT and NSC120686 inhibitors require to be dissolved in DMSO, which, at certain concentrations, can impair plant development (Zhang et al., 2016). Thus, it was necessary to first identify the inhibitor concentrations at which minimal or null DMSO effects are evident at a phenotypic level. In the case of the CPT treatments, the selected concentration was 25 µM dissolved in 0.29% DMSO (**Supplementary Figure S1**). The selection of NSC concentration (25 µM dissolved in 0.17% DMSO) was based on previous results (Macovei et al., 2018a). The last treatment consisted of synergistically exposing *M. truncatula* seeds to CPT 25 µM and NSC 25 µM (treatment denominated as CPT + NSC), dissolved in 0.23% DMSO. As described in "Materials and Methods," each corresponding DMSO concentrations (denominated as DMSO_C, DMSO_N, and DMSO_CN) were tested along with the non-treated (water) control (NT).

To verify whether CPT and NSC influence seed germination, a phenotypic characterization was performed by evaluating germination % and speed (T_{50}), seedling length, and FW after 7 days of treatment. While seed germination % and T_{50} were not significantly affected by any of the imposed treatments at the end of the indicated timeframe (**Supplementary Figure S3**), CPT impacted mostly on seedling development. **Figure 1A** shows the morphology of the 7-day-old seedlings, grown in the presence of CPT, NSC, and CPT + NSC, and their corresponding DMSO controls. Treatment with the NSC inhibitor did not result in a visible change in seedling morphology while seedlings treated with CPT and CPT + NSC appeared shorter and stockier than the relative controls. These observations are supported by the registered significant ($p < 0.05$) differences when measuring the

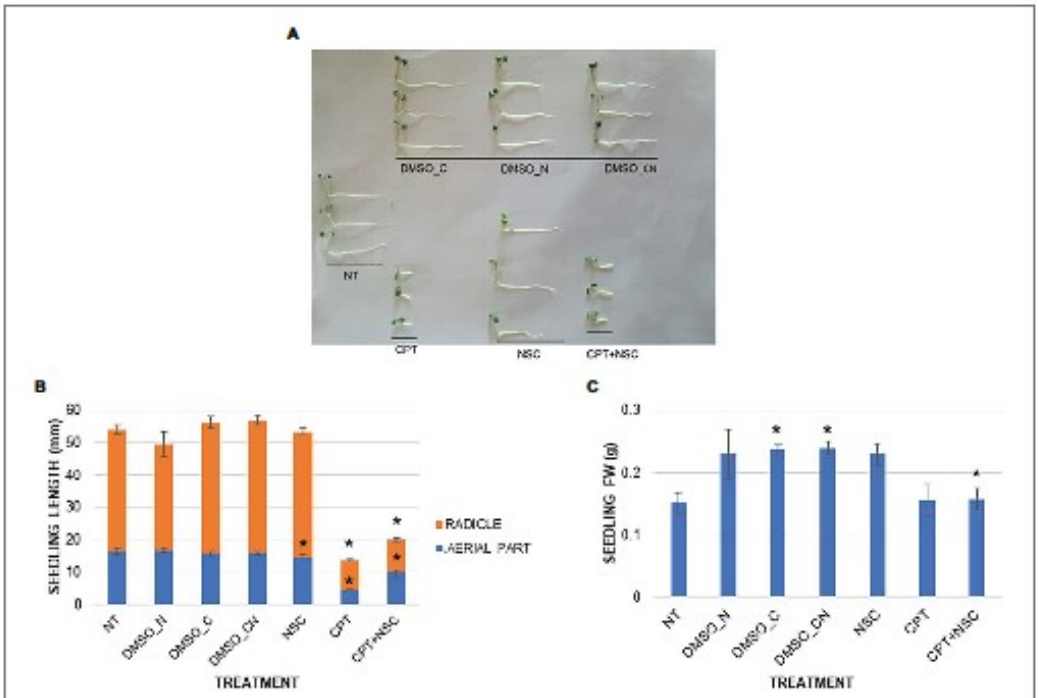


FIGURE 1 | Phenotypic effect of CPT, NSC, and CPT + NSC treatments, and corresponding DMSO concentrations (DMSO_N, DMSO_C, and DMSO_CN) on *Medicago truncatula* seed germination. **(A)** Representative image of 7-day-old seedlings. **(B)** Seedling length (mm). **(C)** Fresh weight, FW (g). Data are represented as mean ± SD of three independent replicates. Statistically significant ($p < 0.05$) differences between treatments and control (NT) are represented with an asterisk (*). CPT, camptothecin; NSC, TDP1 inhibitor NSC120686; and DMSO, dimethyl sulfoxide.

seedling length and FW (**Figure 1B**). A reduction in seedling length was caused by the CPT and CPT + NSC treatments, with radicles being more severely affected than the aerial parts. A minor, although still significant impact, was observed in the case of the NSC-treated seedlings. When considering the FW parameter, an increase in seedling weight was detected for DMSO_C and DMSO_N, while FW was significantly decreased in the NSC + CPT-treated seedlings (**Figure 1C**).

Overall, these results show that the imposed treatments do not affect germination *per se* but inhibit seedling growth, especially in the CPT- and CPT + NSC-treated samples. This may lead to assume that CPT contributes the most to the impairment of the seedling growth since a lesser effect was observed when the NSC compound was delivered alone.

The Imposed Treatments Induce Different Cell Death Profiles

A DNA diffusion assay was performed to evaluate cell death events in 7-day-old *M. truncatula* seedlings subjected to CPT and NSC treatments (**Figure 2**). The results of the diffusion assay were expressed both as arbitrary units (a.u.) to indicate the overall level of cell death and as percentage of nuclei per class to indicate the different types of cell death events (class 0 – viable cells, class 1 – programmed cell death events, and class 2 – necrosis events). Enhanced levels of cell death are evident in the imposed treatments when compared to NT, with the highest values registered during the CPT treatment (**Figure 2A**). Cell death significantly increased also in samples treated with DMSO_C and DMSO_CN but at a substantially lesser degree than when compared to the CPT/NSC system. When looking at the different types of nuclei classes, the data show that the NT and DMSO_N samples are both characterized by a high percentage of viable nuclei (86.36 ± 6.00 and 83.63 ± 3.16%, respectively) and a low percentage of PCD and necrosis (**Figure 2B**). Seedlings treated with DMSO_C and DMSO_CN started to show a decrease in viable nuclei (47.60 ± 3.40, 55.74±4.74%) toward PCD, while the nuclei classified as belonging to necrotic cells (class 2) are not present.

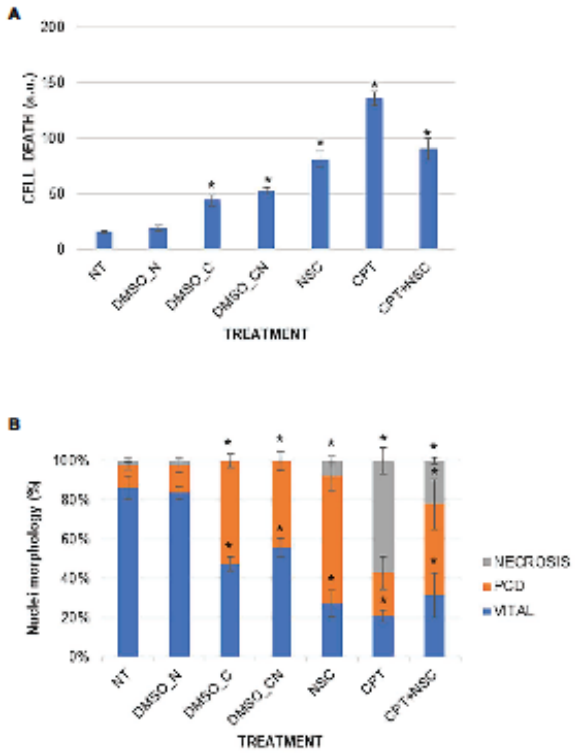


FIGURE 2 | Cell death induced by the imposed treatments in *M. truncatula* 7-day-old seedlings. **(A)** Overall cell mortality scores represented as arbitrary units (a.u.). **(B)** Cell death represented as percentage of nuclei per class. Values are expressed as mean ± SD of three replicates. Statistically significant ($p < 0.05$) differences between treatments and control (NT) are represented with an asterisk (*). CPT, camptothecin; NSC, TDP1 inhibitor NSC120686; and DMSO, dimethyl sulfoxide.

Class 2 nuclei are mostly present in CPT and CPT + NSC samples, while the NSC treatments evidence the presence of class 1 nuclei characteristic for PCD events (**Figure 2B**). Concerning the NSC- and CPT + NSC-treated samples, a marked decrease in the percentage of viable nuclei (27.18 ± 6.76, 31.52 ± 11.18%) is observed with a concomitant increase in the percentage of nuclei subjected to PCD (52.12 ± 5.49, 46.53 ± 12.7%) and necrosis (27.38 ± 6.20, 21.9 ± 6.20%). Similarly, a reduction in the percentage of viable nuclei is observed for CPT-treated samples (21.05 ± 2.91%), where the most represented nuclei belong to class 2 (57.13 ± 6.82%), characteristic for the presence of necrotic events.

Overall, the imposed treatments decrease cell vitality and induce different types of cell death events. The most severe effects are observed with the CPT treatment, characterized by a high level of necrosis whereas PCD events prevail in the NSC treatment. In the CPT + NSC combination, both PCD and necrosis events are registered at similar levels.

The Imposed Treatments Cause Accumulation of DNA Damage

To quantitatively measure DNA damage, SCGE was performed using both the alkaline and neutral versions of the assay. Representative images for each nuclei class (0–4) are provided (**Figure 3A**). The neutral version generally detects double-stranded breaks (DSBs) whereas the alkaline version includes different types of breaks such as single-strand breaks (SSBs) formed from alkali-labile sites, DNA-DNA, or DNA-protein cross-links (Ventura et al., 2013). Compared to NT, the NSC-treated samples showed a 7.22-fold increase in the level of DNA damage under alkaline conditions while only a 1.99-fold increase was observed under neutral conditions (**Figure 3B**). A 5.86- and 5.79-fold increase in the level of DNA damage was observed in the CPT-treated samples under alkaline and neutral conditions, respectively. The CPT + NSC-treated samples showed a 13.7-fold increase in the level of DNA damage in alkaline conditions while an 8.4-fold increase was detected under neutral conditions. Considering the DMSO controls, no significant differences in the accumulation of DNA damage as DSBs are evident under neutral conditions. However, a small but significant increase in the levels of DNA damage was registered under alkaline conditions. This may suggest that DMSO could generate SSBs, alkali-labile sites, incomplete excision repair sites, and DNA-DNA/DPCs rather than more extensive damage like DSBs.

Overall, the observed results indicate that the administration of CPT/NSC agents cause an accumulation of both SSBs and DSBs, but at different degrees depending on the type of treatment. While in the case of NSC, SSBs and associated damage types are prevalent, for the CPT treatments an additional increase in the presence of DSBs is observed. The combination of the two agents (CPT + NSC) resulted in the highest level of DNA damage, combining DSBs, SSBs, and associated damage, the latter being most prevalent.

CPT/NSC Treatments Trigger Differential Expression of DDR-Related Genes

Given that CPT/NSC treatments resulted in reduced seedling growth, increased cell mortality, and accumulation of DNA damage, the next step consisted in the evaluation of DDR-related gene expression profiles using RT-*q*PCR. The following genes were selected:

1. *SOG1*, as the master-regulator of plant DDR;
2. *TDP1α*, *TDP1β*, *TDP2α*, *Top1α*, and *Top2*, as genes that encode for proteins most probably affected by the CPT and NSC inhibitors;
3. *MRE11*, *RAD50*, *NBS1*, *PARP1*, *ERCC1*, and *MUS81*, as genes that encode for proteins involved in repair processes considered as alternative to the function of *TDP1* genes. The genes belonging to the MNR complex were selected as they represent the frontline players in the detection and

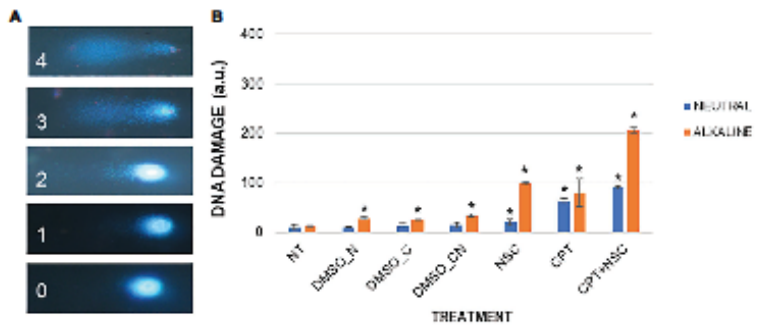


FIGURE 3 | DNA damage induced by the imposed treatments in *M. truncatula* 7-day-old seedlings. **(A)** Nucleus morphology and its related class identification number. **(B)** DNA damage scores represented as a.u. Values are expressed as mean ± SD of three replicates. Statistically significant ($p < 0.05$) differences between treatments and control (NT) are represented with an asterisk (*). CPT, camptothecin; NSC, TDP1 inhibitor NSC120686; and DMSO, dimethyl sulfoxide.

signaling of DSBs, thus HR and NHEJ repair pathways. On the other side, *PARP1*, *ERCC1*, and *MUS81* are associated with both BER and DPC repair. All selected genes were already validated in *M. truncatula* calli exposed to NSC120686 (Macovei et al., 2018a).

4. *TOR*, *CDKA1*, *CycB1*, *CycD2*, and *CycD3*, as genes that encode for proteins known to be involved in the regulation of the cell cycle.

Because the expression of the genes appears to be influenced by DMSO (**Supplementary Figure S4**), and to evaluate the real effect that CPT and NSC treatments may induce at the level of gene expression, the data are presented as FC to control, where the control is represented by each corresponding DMSO concentration. The FC values were used to generate a heatmap (**Figure 4**), where blue color indicates downregulated genes and red color indicates upregulated genes compared to their respective controls. The ANOVA analysis show statistical differences ($p < 0.05$) between treatments and controls for the majority of investigated genes (**Supplementary Table S4**). These results show that the *SOG1* gene is upregulated by CPT and downregulated by NSC, suggesting a contrasting effect of the two treatments at the level of DDR. This contrasting trend is maintained as well when looking at the expression levels of most investigated genes. *TDP1α*, *TDP1β*, and *TopIα* are upregulated by NSC and downregulated by CPT treatments. Conversely, most of the genes involved in alternative DNA repair pathways (*PARP1*, *ERCC1*, *MUS81*, *MRE11*, and *NBS1*) are upregulated by CPT and downregulated by NSC treatments. Within the genes involved in the regulation of the cell cycle, *Cdka1*, *Cycd3*, and *TOR* are upregulated during CPT treatments whereas *Cycb1* is upregulated by NSC. The concomitant administration of CPT + NSC had a different response compared to the individual CPT or NSC treatments; namely, most of the investigated genes are downregulated and the only upregulated genes are *TDP2α*, *MUS81*, and *Cycd2*.

Overall, the gene expression data indicate a contrasting effect for the single administration of NSC and CPT treatments along with a distinct response in case of the synergistic exposure to both compounds where most investigated genes appeared downregulated.

Expression Analyses of Selected microRNAs and Their Putative Targets

Since the main goal of this work was to identify miRNAs able to regulate DDR-associated processes, we proceeded with the investigation of different miRNA-target gene pairs, previously identified from bioinformatics analyses as being related to DDR processes (Bellato et al., 2019). The expression profiles of selected

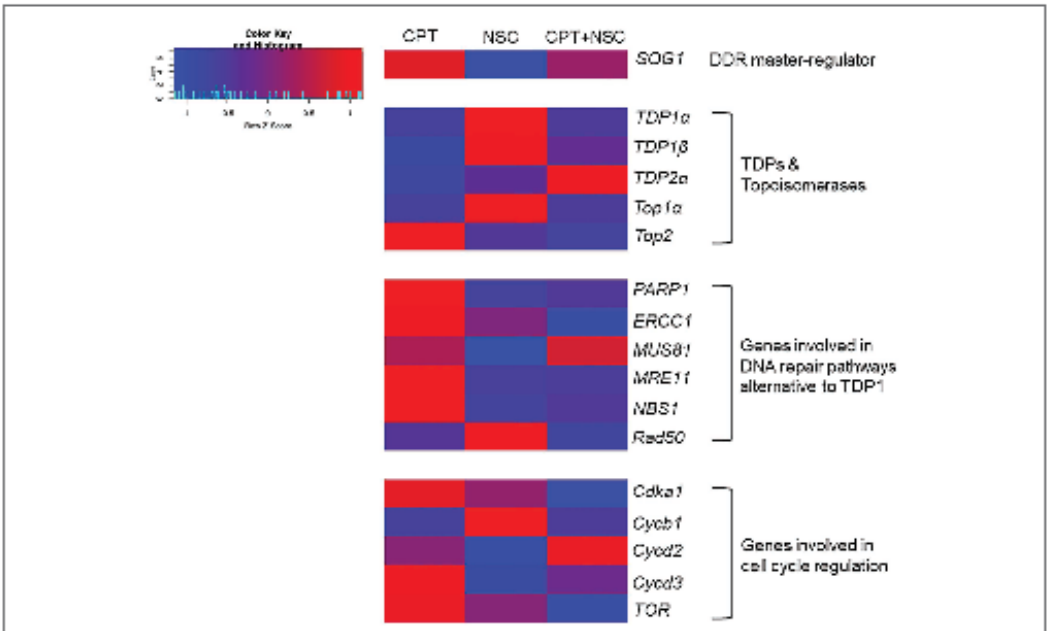


FIGURE 4 | Heatmap representing fold changes (FCs) in gene expression values in response to CPT, NSC, and CPT + NSC treatments in 7-day-old *M. truncatula* seedlings. For each treatment, the values were normalized to their corresponding DMSO controls. The heatmap was constructed using the Shinyheatmap. CPT, camptothecin; NSC, TDP1 inhibitor NSC120686 (http://shinyheatmap.com/) application.

miRNAs and putative target genes were investigated in the CPT/NSC system, proven to affect DDR. Specifically, the following miRNA-gene pairs were considered:

1. mtr-miR156a, identified as putatively targeting *UBE2A* (ubiquitin-conjugating enzyme, Medtr4g108080), involved in histone modification processes.
2. mtr-mir172c-5p, putatively targeting *RAD54-like* (DNA repair and recombination RAD54-like protein, Medtr5g004720), involved in DSBs repair.
3. mtr-miR2600e, putatively targeting *5AT* (anthocyanin 5-aromatic acyltransferase, Medtr2g089765), involved in antioxidant defense.
4. mtr-mir395e, putatively targeting *DMAP1* (DNA methyltransferase 1-associated protein, Medtr1g086590), associated with histone modifications.
5. mtr-miR5741a, putatively targeting *E2FE-like* (E2F transcription factor-E2FE-like protein, Medtr4g106540), involved in DNA-dependent DNA replication.
6. mtr-miR168a, targeting *AGO1A* (Argonaute protein 1, Medtr6g477980), used as a control since the relation between this miRNA and target gene has already been experimentally validated (Vaucheret et al., 2004, 2006).

The expression profiles of miRNAs and putative target genes are shown in **Figure 5** while associated statistics are given in **Supplementary Table S4**. First, their expression in non-treated (NT) samples was monitored to evaluate their behavior under physiological conditions. As shown in **Figure 5A**, while the majority of the tested miRNAs are highly expressed (except for mtr-miR395e), the expression of their putative target gene is significantly reduced, thus corroborating the expected trend where miRNAs activity inhibits the expression of the targeted gene. The ability of miR168a to target *AGO1A* gene is a well-known fact to the scientific community (Vaucheret et al., 2004, 2006), therefore, this miRNA was chosen as quality control for function/target validation. Indeed, a low level of *AGO1A* expression corresponds to a high level of miR168a expression in NT samples (**Figure 5A**). Looking into the expression of this specific miRNA and its target gene during the imposed treatments, it is evidenced that when the expression of miR168 is low, the expression of *AGO1A* is high, and vice-versa (**Figure 5B**).

Since gene expression is influenced by DMSO, also in this case, data are represented as FC to respective controls and gathered in a heatmap (**Figure 5C**) where blue color represents downregulation and red color represents upregulation. Overall, the heatmap shows that under most conditions when a miRNA is upregulated the corresponding candidate target gene is downregulated. Looking at the miRNAs expression according to each treatment, it is possible to observe a treatment-specific behavior where different miRNAs expression is triggered by different treatments. Namely, mtr-miR156a and mtr-miRA5671 are upregulated by CPT, mtr-miR172c-5p is upregulated by NSC, and mtr-miR2600e are upregulated by CPT+NSC.

Overall, an indirect evidence of miRNA action over these targets is provided; the contrasting profiles between miRNA-predicted target abundances support the evidence that these miRNAs could repress the expression of these targets.

Principal Component Analysis for Data Integration

Principal components analysis was used to investigate the differences between samples and which variables most contributed to these differences (**Figure 6**). The *X*-axis and *Y*-axis show the principal dimension Dim1 and Dim2 that explain 29.1 and 21.5% 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. The orientation of the ellipses shows that the most different samples are those treated with CPT and CPT + NSC whereas the NSC treatment is located in the proximity of DMSO_CN- and DMSO_N-treated samples (**Figure 6A**). Other distinctive groups are formed by the NT and DMSO_C samples located in the upper-right panel. Hence, the plotted data allow a clear separation of the majority of the samples according to the imposed treatments. The observed vicinity among replicates is indicative of data reliability. The variables that most contributed to the group differentiation are represented in a light blue color (**Figure 6B**). Among the phenotypic parameters, the most representative variables include seedling length, cell death, and DNA damage. Amidst the investigated genes, DMAP1, E2FE-like, PARP1, Cycd3, and Cycd2 had the highest contribution but also TDP1β, Top1α, Top2, and NBS1 are well-represented. When considering the miRNAs, it is relevant to underline that these had an important contribution to the differentiation of the samples and the most representative ones are mtr-miR2600e and mtr-miR5741.

DISCUSSION

In this work, CPT and NSC120686 were used alone or in combination to develop an original experimental system in which plant specific DDR functions would be altered so that miRNAs associated with DDR pathways could be revealed. CPT is a widely used agent much employed in anticancer therapies due to its activity as TopI inhibitor since it intercalates between DNA breaks flanking the TopI-cleavage complex (Pommier et al., 2010). CPT is known for its cytotoxic effects also in plants (Buta and Worley, 1976; Takahashi et al., 2002) where enhanced levels of cell death had been registered (Locato et al., 2006; Iakimova et al., 2020) presumably through the accumulation of TopI-covalent complexes as in the case other eukaryotes. On the other side, the NSC120686 compound was recently identified based on virtual screening of pharmacophores able to inhibit human Tdp1 (Weidlich et al., 2010) and subsequently used in combination with CPT-derivates to inhibit the growth of different cancer cell lines (Perego et al., 2012). *Medicago truncatula* calli treated with different concentrations of NSC120686 displayed enhanced levels of cell mortality and DNA damage (Macovei et al., 2018a). So far, combined application of the two agents has not been reported in plants.

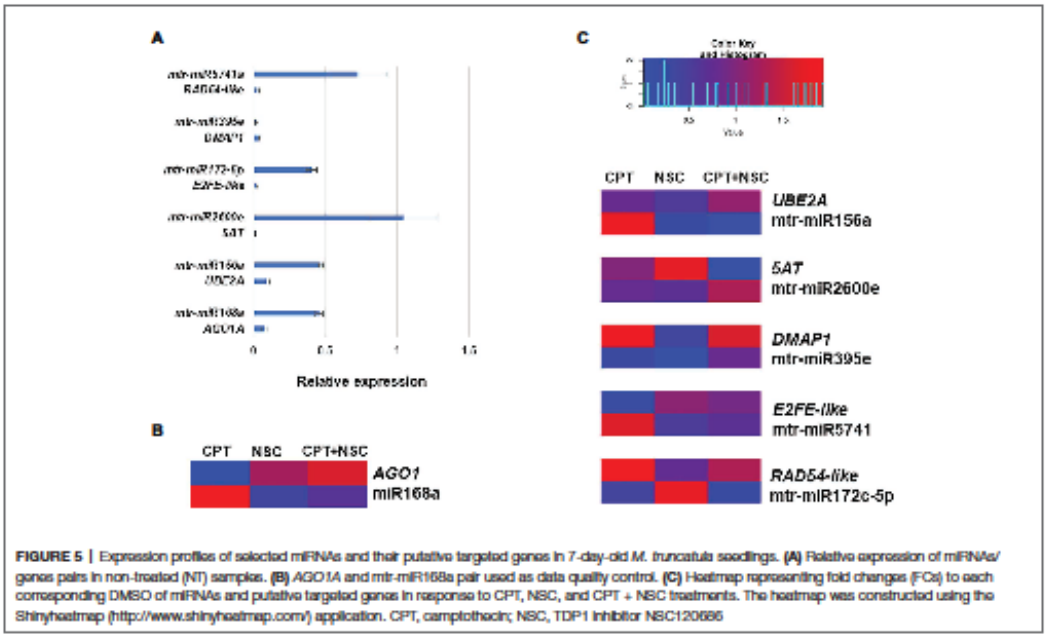


FIGURE 5 | Expression profiles of selected miRNAs and their putative targeted genes in 7-day-old *M. truncatula* seedlings. **(A)** Relative expression of miRNAs/genes pairs in non-treated (NT) samples. **(B)** *AGO1A* and mtr-miR168a pair used as data quality control. **(C)** Heatmap representing fold changes (FCs) to each corresponding DMSO of miRNAs and putative targeted genes in response to CPT, NSC, and CPT + NSC treatments. The heatmap was constructed using the Shinyheatmap (http://www.shinyheatmap.com/) application. CPT, camptothecin; NSC, TDP1 inhibitor NSC120686

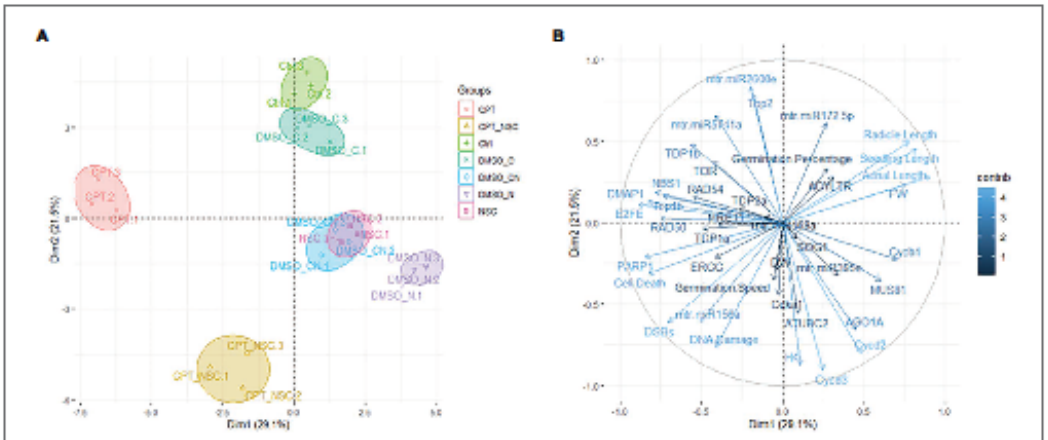


FIGURE 6 | Principal component analyses (PCA). **(A)** Loading plot explaining the distribution of samples focusing on the imposed treatments. **(B)** Loading plot explaining the contribution of each measured variable (germination %, T_{50}, seedling length, FW, gene, and miRNAs expression profiles).

Before evaluating the possible involvement of miRNAs in this system, it was first necessary to prove that it targets DDR-associated processes. The phenotypic investigation revealed that CPT and CPT + NSC had a major effect on seedling development mostly by inhibiting radicle growth while the single administration of NSC had a milder effect (**Figure 1**). Hence, the phenotypic changes could be mostly attributed to CPT, as in agreement with previous studies, where 25 μM of CPT substantially inhibited the growth of *Arabidopsis* plantlets while concentrations higher than 50 μM resulted

in a strong impairment of both roots and shoots at young seedling stages (Takahashi et al., 2002). In accordance with the observed phenotypes, enhanced levels of cell death and accumulation of DNA damage were evidenced (**Figures 2, 3**). Interestingly, different types of cell death events and DNA damage were encountered according to the imposed treatments. While CPT administration resulted in enhanced levels of necrosis and accumulation of DSBs, the delivery of NSC was accompanied by PCD and accumulation of SSBs, DNA-DNA, or DPCs. For the CPT + NSC combination, both PCD and necrosis events are present at similar levels while the high levels of DNA damage indicate the most genotoxic effect. Previous literature reports that CPT results in the accumulation of DPCs (Enderle et al., 2019b) and DSBs (Ferrara and Kmiec, 2004; Berniak et al., 2013), lesions know to be associated with necrotic events in plant cells (Rowan et al., 2010; Song and Bent, 2014). On the other hand, low concentrations of NSC120686 resulted in enhanced levels of PCD in *M. truncatula* calli (Macovei et al., 2018a).

The outlined distinction between treatments was maintained when considering the expression profiles of selective genes belonging to different DNA repair pathways and cell cycle regulation (**Figure 4**). In addition to *TDP1*, *α*, and *β*, and *Top1α* genes, *TDP2α*, and *Top2* genes were investigated because of the closed connection between these two, as TDP2 enzyme is involved in the removal of DNA TopII-mediated DNA damage and cell proliferation/differentiation signaling (Cortes Ledesma et al., 2009). Moreover, the overexpression of *TDP2α* gene in *M. truncatula* was correlated with a decrease in the accumulation of DSBs, increased cell proliferation, and enhanced resistance to stress (Confalonieri et al., 2014; Faè et al., 2014; Araújo et al., 2016). Genes involved in DNA repair alternative to TDP1 (Pommier et al., 2014) include the MNR complex, composed of *MRE11*, *NBS1*, and *Rad50*, known to be involved in the detection of DBSs and HR (Manova and Gruszka, 2015) as well as *PARP1*, *MUS81*, and *ERCC1* involved in BER and DPC repair (Enderle et al., 2019b; Roldán-Arjona et al., 2019). Since DDR includes a response from both DNA repair and cell cycle regulation, several cyclins (*Cdka1*, *Cycb1*, *Cycd2*, and *Cycd3*) were investigated alongside the master-regulators *TOR* and *SOG1*. The observed changes in the expression profiles of *SOG1* gene indicate that DDR is truly affected by the imposed treatments; hence, we can conclude that the developed system has an impact on DDR. To briefly summarize the behavior of the tested genes in association with the phenotypic observations, the following assumptions are taken into consideration (**Figure 7**):

1. During the CPT treatment, TopI enzyme is presumably blocked, TopI-DNA covalent complexes would accumulate and high levels of DNA damage and cellular mortality are registered, resulting in substantial inhibition of seedling growth. In this situation, *TDP1* and *Top1* genes are inhibited while genes involved in DNA repair pathways alternative to TDP1 are highly active. Based on the expression of genes involved in the cell cycle, this is delayed presumably to allow the repair of the induced DNA damage.

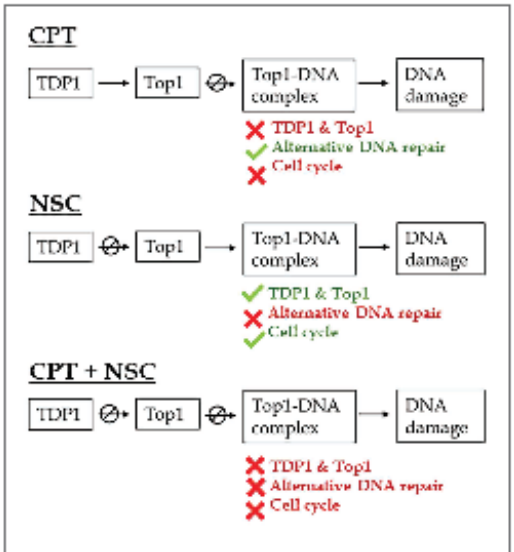


FIGURE 7 | Schematic representation of the proposed effects of CPT and NSC, inhibitors of Top1 and TDP1 enzymes, on DNA repair pathways and cell cycle regulation during *M. truncatula* early seedling development. CPT, camptothecin; NSC, TDP1 inhibitor NSC120686.

2. When NSC is given, the TDP1 enzyme would interact with this mimicking compound, thus being prevented from engaging with its substrate and hydrolyze the crosslink between TopI and DNA. In turn, this may again lead to the accumulation of these complexes and the subsequently observed enhancement in the levels of cell death and DNA damage, although at a lesser extent, in agreement with the phenotypic observations. In this case, the *TDP1* and *Top1* genes are active, the alternative DNA repair is inhibited, and the cell cycle is progressing.
3. The CPT + NSC combination may target both TDP1 and TopI functions and this leads to the highest cytotoxic and genotoxic effects, corresponding to the obstructed seedling development. In terms of gene expression, this treatment induced the downregulation of most of the investigated genes, affecting both DNA repair and cell cycle progression.

In a previous bioinformatics investigation, we have identified specific miRNAs (mtr-miR156a, mtr-mir172c-5p, mtr-miR2600e, mtr-mir395e, and mtr-miR5741) putatively targeting genes associated with DDR processes (Bellato et al., 2019). Among these, miR156 is an evolutionarily conserved family, although diversification in its members, sequence, and functions are present (Sunkar and Jagadeeswaran, 2008; Cui et al., 2017). Others, like miRNA172 family has been associated with seed development alongside with other regulatory functions (Smoczynska and Szweykowska-Kulinska, 2016). High-throughput sequencing of *M. truncatula* seedlings found

that miR156 and miR172 are involved in salinity stress (Cao et al., 2018). MiR395 is involved in sulfate assimilation regulatory network (Matthewman et al., 2012) whereas miR5741 has been associated with roles in the defense response (Siemens et al., 2006). It is therefore clear that these miRNAs have been studied mainly in relation to plant development and response to biotic/abiotic stress. The RT-*q*PCR analyses performed in this work indicate that they are also involved in the response to genotoxic stress, as indicated by their differential expression induced by the CPT/NSC treatments. For example, mtr-miR172c-5p is upregulated in NSC treated samples and downregulated in CPT treated samples. By observing the expression profiles of its putatively targeted gene *E2FE-like*, it is shown that an upregulation of the miRNA is accompanied by a downregulation of the gene predicted to be its target. Importantly, this gene is a homolog of the *Arabidopsis* E2F transcription regulator shown to be involved in cell cycle regulation, DNA replication, and DNA damage repair, in pathways alternative to SOG1 (Berckmans and De Veylder, 2009; Roa et al., 2009; Gutzat et al., 2012).

In conclusion, by inducing seedling growth inhibition, accumulation of cell death, and DNA damage, along with the differential expression of genes involved in DDR, the developed CPT/NSC system actively influence DDR-associated processes. Above all, we demonstrated that specific miRNA-target gene pairs, identified from a bioinformatics approach, are responsive to the imposed treatments, thus showing that these miRNAs have a role to play in DDR. This study extends the knowledge regarding the roles played by miRNAs in the post-transcriptional regulation of DDR in plants. This may disclose new regulatory networks with further possibilities regarding biotech application relevant to enhance crop adaptation to genotoxic stresses. Given the complexity of regulatory networks and since miRNAs can repress multiple targets, further functional validation studies are needed to corroborate these suggested roles in DDR. This is particularly relevant to clarify if other regulatory mechanisms might be responsible for the observed downregulation of target genes expression.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AM conceptualized the study. CG, MG, AP, and TC performed the treatments and conducted the designed analyses. AM, MG, CG, and SA analyzed and interpreted the data. AM and AB wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research received support from the Italian Ministry of Education, University and Research (MIUR): *Dipartimenti di Eccellenza* Program (2018-2022) – Department of Biology and Biotechnology "L. Spallanzani," University of Pavia. AP is supported by a Departmental Fellowship (*assegno di ricerca tipo A* – COD. DBB2020-A01). SA acknowledges the support of Fundação para a Ciência e Tecnologia throughout the Research Unit "GREEN-IT" (UID/Multi/04551/2020), as well as, the support the 31 Bioeconomy project POCI-01-0246-FEDER-026758, funded by the Fundo Europeu de Desenvolvimento Regional (FEDER), under the "Programa Operacional Temático Competitividade e Internacionalização"-COMPETE 2020 and the program NORTE 2020 through the project NORTE-06-3559-FSE-000103 funded by the Fundo Social Europeu (FSE).

ACKNOWLEDGMENTS

We are thankful to Dr. Alexander Martinkosky (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, United States) for providing the NSC120686 compound. We would also like to thank Dr. José Parreira (ITQB-NOVA, Portugal) for the support provided during the setting-up of the analysis of miRNA expression.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.645323/full#supplementary-material

REFERENCES

Androvic, P., Valihrach, L., Elling, J., Sjoback, R., and Kubista, M. (2017). Two-tailed RT-qPCR: a novel method for highly accurate miRNA quantification. *Nucleic Acids Res.* 45:e144. doi: 10.1093/nar/gkx588

Araújo, S., Balestrazzi, A., Faè, M., Morano, M., Carbonera, D., and Macovei, A. (2016). MITdp2a-overexpression boosts the growth phase of *Medicago truncatula* cell suspension and increases the expression of key genes involved in antioxidant response and genome stability. *PCTOC* 127, 675–680. doi: 10.1007/s11240-016-1075-5

Araújo, S., Pagano, A., Dondi, D., Lazzaroni, S., Pinela, E., Macovei, A., et al. (2019). Metabolic signatures of germination triggered by kinetin in *Medicago truncatula*. *Sci. Rep.* 9:10466. doi: 10.1038/s41598-019-46866-6

Bartel, D. P. (2004). microRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297. doi: 10.1016/S0092-8674(04)00045-5

Bellato, M., De Marchi, D., Gualtieri, C., Sauta, E., Magni, P., Macovei, A., et al. (2019). A bioinformatics approach to explore microRNAs as tools to bridge pathways between plants and animals. Is DNA damage response (DDR) a potential target process? *Front. Plant Sci.* 10:1535. doi: 10.3389/fpls.2019.01535

Berckmans, B., and De Veylder, L. (2009). Transcriptional control of the cell cycle. *Curr. Opin. Plant Biol.* 12, 599–605. doi: 10.1016/j.pbi.2009.07.005

Berniak, K., Rybak, P., Bernas, T., Zarębski, M., Biela, E., Zhao, H., et al. (2013). Relationship between DNA damage response, initiated by camptothecin or oxidative stress, and DNA replication, analyzed by quantitative 3D image analysis. *Cytometry A* 83, 913–924. doi: 10.1002/cyto.a.22327

Bourbousse, C., Vegesna, N., and Law, J. A. (2018). SOG1 activator and MYB3R repressors regulate a complex DNA damage network in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* 115, E12453–E12462. doi: 10.1073/pnas.1810582115

Bray, C. M., and West, C. E. (2005). DNA repair mechanisms in plants: crucial sensors and effectors for the maintenance of genome integrity. *New Phytol.* 168, 511–528. doi: 10.1111/j.1469-8137.2005.01548.x

Britt, A. B. (1999). Molecular genetics of DNA repair in higher plants. *Trends Plant Sci.* 4, 20–25. doi: 10.1016/S1360-1385(98)01355-7

Buta, J. G., and Worley, J. F. (1976). Camptothecin, a selective plant growth regulator. *J. Agric. Food Chem.* 24, 1085–1086. doi: 10.1021/jf60207a016

Cao, C., Long, R., Zhang, T., Kang, J., Wang, Z., Wang, P., et al. (2018). Genome-wide identification of microRNAs in response to salt/alkali stress in *Medicago truncatula* through high-throughput sequencing. *Int. J. Mol. Sci.* 19:4076. doi: 10.3390/ijms19124076

Chot, Y. E., Pan, Y., Park, E., Konstantinopoulos, P., De, S., D'Andrea, A., et al. (2014). microRNAs down-regulate homologous recombination in the G1 phase of cycling cells to maintain genomic stability. *eLife* 3:e02445. doi: 10.7554/eLife.02445

Chowdhury, M. R., and Basak, J. (2019). Tiny yet indispensable plant microRNAs are worth to explore as key components for combating genotoxic stresses. *Front. Plant Sci.* 10:1197. doi: 10.3389/fpls.2019.01197

Cimini, S., Gualtieri, C., Macovei, A., Balestrazzi, A., De Gara, L., and Locato, V. (2019). Redox balance-DDR-miRNA triangle: relevance in genome stability and stress responses in plants. *Front. Plant Sci.* 10:989. doi: 10.3389/fpls.2019.00989

Collins, A. R. (2004). The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol. Biotechnol.* 26, 249–261. doi: 10.1385/MB:26:3:249

Confalonieri, M., Faè, M., Balestrazzi, A., Donà, M., Macovei, A., Giraffa, G., et al. (2014). Enhanced osmotic stress tolerance in *Medicago truncatula* plants overexpressing the DNA repair gene *MtTdp2α* (tyrosyl-DNA-phosphodiesterase 2). *PCTOC* 116, 187–203. doi: 10.1007/s11240-013-0395-y

Cortes Ledesma, F. C., El Khamisy, S. F., Zuma, M. C., Osborn, K., and Caldecott, K. W. (2009). A human tyrosyl-DNA phosphodiesterase that repairs topoisomerase-mediated DNA damage. *Nature* 461, 674–678. doi: 10.1038/nature08444

Cui, J., You, C., and Chen, X. (2017). The evolution of microRNAs in plants. *Curr. Opin. Plant Biol.* 35, 61–67. doi: 10.1016/j.pbi.2016.11.006

Donà, M., Confalonieri, M., Minio, A., Biggiogera, M., Buttafava, A., Raimondi, E., et al. (2013). RNA-Seq analysis discloses early senescence and nucleolar dysfunction triggered by Tdp1 depletion in *Medicago truncatula*. *J. Exp. Bot.* 64, 1941–1951. doi: 10.1093/jxb/ert063

Enderle, J., Dorn, A., Beying, N., Trapp, O., and Puchta, H. (2019a). The protease WSS1A, the endonuclease MUS81, and the phosphodiesterase TDP1 are involved in independent pathways of DNA-protein crosslink repair in plants. *Plant Cell* 31, 775–790. doi: 10.1105/tpc.18.00824

Enderle, J., Dorn, A., and Puchta, H. (2019b). DNA- and DNA-protein-crosslink repair in plants. *Int. J. Mol. Sci.* 20:4304. doi: 10.3390/ijms20174304

Faè, M., Balestrazzi, A., Confalonieri, M., Donà, M., Macovei, A., Valassi, A., et al. (2014). Copper-mediated genotoxic stress is attenuated by the overexpression of the DNA repair gene *MtTdp2α* (tyrosyl-DNA phosphodiesterase 2 alpha) in *Medicago truncatula* plants. *Plant Cell Rep.* 33, 1071–1080. doi: 10.1007/s00299-014-1595-6

Farooq, M., Basra, S. M. A., Ahmad, N., and Hafeez, K. (2005). Thermal hardening: a new seed vigor enhancement tool in rice. *J. Integr. Plant Biol.* 47, 187–193. doi: 10.1111/j.1744-7909.2005.00031.x

Ferrara, L., and Kmiec, E. B. (2004). Camptothecin enhances the frequency of oligonucleotide-directed gene repair in mammalian cells by inducing DNA damage and activating homologous recombination. *Nucleic Acids Res.* 32, 5239–5248. doi: 10.1093/nar/gkh822

Gimenez, E., and Manzano-Agugliaro, F. (2017). DNA damage repair system in plants: a worldwide research update. *Genes* 8:299. doi: 10.3390/genes8110299

Gutzat, R., Borghi, L., and Gruissem, W. (2012). Emerging roles of RETINOBLASTOMA-RELATED proteins in evolution and plant development. *Trends Plant Sci.* 17, 139–148. doi: 10.1016/j.tplants.2011.12.001

Hu, Z., Cools, T., and De Veylder, L. (2016). Mechanisms used by plants to cope with DNA damage. *Annu. Rev. Plant Biol.* 67, 439–462. doi: 10.1146/annurev-arplant-043015-111902

Iakimova, E. T., Yordanova, Z. P., Cristescu, S. M., Harren, F. F. M., and Woltering, E. J. (2020). Cell death associated release of volatile organic sulphur compounds with antioxidant properties in chemical-challenged tobacco BY-2 suspension cultured cells. *J. Plant Physiol.* 251:153223. doi: 10.1016/j.jplph.2020.153223

Joly-Tonetti, N., and Lamartine, J. (2012). "The role of microRNAs in the cellular response to ionizing radiations" in *Current topics in ionizing radiation research*. ed. M. Nenoi (Croatia: InTechOpen).

Kassambara, A., and Mundt, F. (2020). factoextra: extract and visualize the results of multivariate data analyses. R package version 1.0.7. Available at: https://CRAN.R-project.org/package=factoextra

Khomtchouk, B. B., Hennessy, J. R., and Wahlestedt, C. (2017). shinyheatmap: Ultra fast low memory heatmap web interface for big data genomics. *PLoS One* 12:e0176334. doi: 10.1371/journal.pone.0176334

Kim, J. H., Go, Y. S., Kim, J. K., and Chung, B. Y. (2016). Characterization of microRNAs and their target genes associated with transcriptomic changes in gamma-irradiated Arabidopsis. *Genet. Mol. Res.* 15:gmr8386. doi: 10.4238/gmr.15038386

Kim, H., Na, S. H., Lee, S. Y., Jeong, Y. M., Hwang, H. J., Hur, J. Y., et al. (2012). Structure-function studies of a plant tyrosyl-DNA phosphodiesterase provide novel insights into DNA repair mechanisms of *Arabidopsis thaliana*. *Biochem. J.* 443, 49–56. doi: 10.1042/BJ20111308

LÊ, S., Josse, J., and Husson, F. (2008). FactoMineR: an R package for multivariate analysis. *J. Stat. Softw.* 25, 1–18. doi: 10.18637/jss.v025.i01

Lebedeva, N. A., Rechkunova, N. I., and Lavrik, O. (2011). AP-site cleavage activity of tyrosyl-DNA phosphodiesterase 1. *FEBS Lett.* 585, 683–686. doi: 10.1016/j.febslet.2011.01.032

Lee, S. Y., Kim, H., Hwang, H. J., Jeong, Y. M., Na, S. H., Woo, J. C., et al. (2010). Identification of tyrosyl-DNA phosphodiesterase as a novel DNA damage repair enzyme in Arabidopsis. *Plant Physiol.* 154, 1460–1469. doi: 10.1104/pp.110.165068

Liang, W. -W., Huang, J. -H., Li, C. -P., Yang, L. -T., Ye, X., Lin, D., et al. (2017). microRNA-mediated responses to longterm magnesium-deficiency in *Citrus sinensis* roots revealed by Illumina sequencing. *BMC Genomics* 18:657. doi: 10.1186/s12864-017-3999-5

Locato, V., Balestrazzi, A., De Gara, L., and Carbonera, D. (2006). Reduced expression of top1beta gene induces programmed cell death and alters ascorbate metabolism in *Daucus carota* cultured cells. *J. Exp. Bot.* 57, 1667–1676. doi: 10.1093/jxb/erj194

Lv, D. -W., Zhen, S., Zhu, G. -R., Bian, Y. -W., Chen, G. -X., Han, C. -X., et al. (2016). High-throughput sequencing reveals H_2O_2 stress-associated microRNAs and a potential regulatory network in *Brachypodium distachyon* seedlings. *Front. Plant Sci.* 7:1567. doi: 10.3389/fpls.2016.01567

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. doi: 10.1007/s00425-010-1179-9

Macovei, A., Donà, M., Carbonera, D., and Balestrazzi, A. (2018b). "DNA diffusion assay applied to plant cells" in *Plant Programmed Cell Death*. eds. L. De Gara and V. Locato (New York, NY: Springer), 107.

Macovei, A., Pagano, A., Forti, C., Araújo, S., and Balestrazzi, A. (2019). "*Medicago truncatula*, an informative model to investigate the DNA damage response during seed germination" in *Medicago Handobook*. ed. F. J. de Bruijn (Hoboken, NJ, USA: Wiley Online Library).

Macovei, A., Pagano, A., Sabatini, M. E., Grandi, S., and Balestrazzi, A. (2018a). The human tyrosyl-DNA phosphodiesterase 1 (hTdp1) inhibitor NSC120686 as an exploratory tool to investigate plant Tdp1 genes. *Gene* 9:186. doi: 10.3390/genes9040186

Macovei, A., and Tuteja, N. (2012). microRNAs targeting DEAD-box helicases are involved in salinity stress response in rice (*Oryza sativa* L.). *BMC Plant Biol.* 12:183. doi: 10.1186/1471-2229-12-183

Macovei, A., and Tuteja, N. (2013). Different expression of miRNAs targeting helicases in rice in response to low and high dose rate γ-ray treatments. *Plant Signal. Behav.* 8:e25128. doi: 10.4161/psb.25128

Manova, V., and Gruszka, D. (2015). DNA damage and repair in plants—from models to crops. *Front. Plant Sci.* 6:885. doi: 10.3389/fpls.2015.00885

Matthewman, C. A., Kawashima, C. G., Huska, D., Csorba, T., Dalmay, T., and Kopriva, S. (2012). miR395 is a general component of the sulfate assimilation regulatory network in Arabidopsis. *FEBS Lett.* 586, 3242–3248. doi: 10.1016/j.febslet.2012.06.044

Mutti, G., Raveane, A., Pagano, A., Bertolini, F., Semino, O., Balestrazzi, A., et al. (2020). Plant TDP1 (Tyrosyl-DNA Phosphodiesterase 1): a phylogenetic perspective and gene expression data mining. *Gene* 11:1465. doi: 10.3390/genes11121465

Nikitaki, Z., Holá, M., Donà, M., Pavlopoulou, A., Michalopoulos, I., Angelis, K. J., et al. (2018). Integrating plant and animal biology for the search of novel DNA damage biomarkers. *Mutat. Res.* 775, 21–38. doi: 10.1016/j.mrrev.2018.01.001

Nisa, M. U., Huang, Y., Benhamed, M., and Raynaud, C. (2019). The plant DNA damage response: signaling pathways leading to growth inhibition and putative role in response to stress conditions. *Front. Plant Sci.* 10:653. doi: 10.3389/fpls.2019.00653

Ogita, N., Okushima, Y., Tokizawa, M., Yamamoto, Y. Y., Tanaka, M., Seki, M., et al. (2018). Identifying the target genes of SUPPRESSOR OF GAMMA RESPONSE 1, a master transcription factor controlling DNA damage response in Arabidopsis. *Plant J.* 94, 439–453. doi: 10.1111/tpj.13866

Pagano, A., de Sousa Araújo, S., Macovei, A., Leonetti, P., and 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. doi: 10.3389/fpls.2017.01972

Perego, P., Cossa, G., Tinelli, S., Corna, E., Carenini, N., Gatti, L., et al. (2012). Role of tyrosyl-DNA phosphodiesterase 1 and inter-players in regulation of tumor cell sensitivity to topoisomerase I inhibition. *Biochem. Pharmacol.* 83, 27–36. doi: 10.1016/j.bcp.2011.09.021

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45. doi: 10.1093/nar/29.9.e45

Pommier, Y., Huang, S. Y., Gao, R., Das, B. B., Murai, J., and Marchand, C. (2014). Tyrosyl-DNA-phosphodiesterases (TDP1 and TDP2). *DNA Repair* 19, 114–129. doi: 10.1016/j.dnarep.2014.03.020

Pommier, Y., Leo, E., Zhang, H., and Marchand, C. (2010). DNA topoisomerases and their poisoning by anticancer and antibacterial drugs. *Chem. Biol.* 17, 421–433. doi: 10.1016/j.chembiol.2010.04.012

Pouliot, J. J., Yao, K. C., Robertson, C. A., and Nash, H. A. (1999). Yeast gene for a Tyr-DNA phosphodiesterase that repairs topoisomerase I complexes. *Science* 286, 552–555. doi: 10.1126/science.286.5439.552

Roa, H., Lang, J., Culligan, K. M., Keller, M., Holec, S., Cognat, V., et al. (2009). Ribonucleotide reductase regulation in response to genotoxic stress in Arabidopsis. *Plant Physiol.* 151, 461–471. doi: 10.1104/pp.109.140053

Roldán-Arjona, T., Ariza, R. R., and Córdoba-Cañero, D. (2019). DNA base excision repair in plants: an unfolding story with familiar and novel characters. *Front. Plant Sci.* 10:1055. doi: 10.3389/fpls.2019.01055

Rowan, B. A., Oldenburg, D. J., and Bendich, A. J. (2010). RecA maintains the integrity of chloroplast DNA molecules in Arabidopsis. *J. Exp. Bot.* 61, 2575–2588. doi: 10.1093/jxb/erq088

Sabatini, M. E., Donà, M., Leonetti, P., Minio, A., Delledonne, M., Carbonera, D., et al. (2016). Depletion of tyrosyl-DNA phosphodiesterase 1 (MtTdp1) affects transposon expression in *Medicago truncatula*. *J. Integr. Plant Biol.* 58, 618–622. doi: 10.1111/jipb.12457

Sabatini, M. E., Pagano, A., Araújo, S., Balestrazzi, A., and Macovei, A. (2017). The tyrosyl-DNA phosphodiesterase 1β (Tdp1β) gene discloses an early response to abiotic stresses. *Gene* 8:305. doi: 10.3390/genes8110305

Scheres, B., and Van der Putten, W. H. (2017). The plant perceptron connects environment to development. *Nature* 543, 337–345. doi: 10.1038/nature22010

Siemens, J., Keller, I., Sarx, J., Kunz, S., Schuller, A., Nagel, W., et al. (2006). Transcriptome analysis of Arabidopsis clubroots indicate a key role for cytokinins in disease development. *Mol. Plant-Microbe Interact.* 19, 480–494. doi: 10.1094/MPMI-19-0480

Smoczynska, A., and Szweykowska-Kulinska, Z. (2016). microRNA-mediated regulation of flower development in grasses. *Acta Biochim. Pol.* 63, 687–692. doi: 10.18388/abp.2016_1358

Song, J., and Bent, A. F. (2014). Microbial pathogens trigger host DNA double-strand breaks whose abundance is reduced by plant defense responses. *PLoS Pathog.* 10:e1004030. doi: 10.1371/journal.ppat.1004030

Sunkar, R., and Jagadeeswaran, G. (2008). In silico identification of conserved microRNAs in large number of diverse plant species. *BMC Plant Biol.* 8:37. doi: 10.1186/1471-2229-8-37

Takahashi, T., Matsuhara, S., Abe, M., and Komeda, Y. (2002). Disruption of a DNA topoisomerase I gene affects morphogenesis in Arabidopsis. *Plant Cell* 14, 2085–2093. doi: 10.1105/tpc.001925

Thapar, R. (2018). Regulation of DNA double-strand break repair by non-coding RNAs. *Molecules* 23:2789. doi: 10.3390/molecules23112789

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., et al. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 3:research0034.1. doi: 10.1186/gb-2002-3-7-research0034

Vaucheret, H., Mallory, A. C., and Bartel, D. P. (2006). AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of miR168 by AGO1. *Mol. Cell* 22, 129–136. doi: 10.1016/j.molcel.2006.03.011

Vaucheret, H., Vazquez, F., Crété, P., and Bartel, D. P. (2004). The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev.* 18, 1187–1197. doi: 10.1101/gad.1201404

Ventura, L., Giovannini, A., Savio, M., Donà, M., Macovei, A., Buttafava, A., et al. (2013). Single cell gel electrophoresis (Comet) assay with plants: research on DNA repair and ecogenotoxicity testing. *Chemosphere* 92, 1–9. doi: 10.1016/j.chemosphere.2013.03.006

Waterworth, W. M., Bray, C. M., and West, C. E. (2019). Seeds and the art of genome maintenance. *Front. Plant Sci.* 10:706. doi: 10.3389/fpls.2019.00706

Weidlich, I. E., Dexheimer, T., Marchand, C., Antony, S., Pommier, Y., and Nicklaus, M. C. (2010). Virtual screening using ligand-based pharmacophores for inhibitors of human tyrosyl-DNA phospodiesterase (hTdp1). *Bioorg. Med. Chem.* 18, 2347–2355. doi: 10.1016/j.bmc.2010.02.009

Yang, S. W., Burgin, A. B. Jr., Huizenga, B. N., Robertson, C. A., Yao, K. C., and Nash, H. A. (1996). A eukaryotic enzyme that can disjoin dead-end covalent complexes between DNA and type I topoisomerases. *Proc. Natl. Acad. Sci. U. S. A.* 93, 11534–11539. doi: 10.1073/pnas.93.21.11534

Yoshiyama, K., Conklin, P. A., Huefner, N. D., and Britt, A. B. (2009). Suppressor of gamma response 1 (SOG1) encodes a putative transcription factor governing multiple responses to DNA damage. *Proc. Natl. Acad. Sci. U. S. A.* 106, 12843–12848. doi: 10.1073/pnas.0810304106

Yoshiyama, K. O., Kobayashi, J., Ogita, N., Ueda, M., Kimura, S., Maki, H., et al. (2013b). ATM-mediated phosphorylation of SOG1 is essential for the DNA damage response in Arabidopsis. *EMBO Rep.* 14, 817–822. doi: 10.1038/embor.2013.112

Yoshiyama, K. O., Sakaguchi, K., and Kimura, S. (2013a). DNA damage response in plants: conserved and variable response compared to animals. *Biology* 2, 1338–1356. doi: 10.3390/biology2041338

Zhang, C., and Peng, G. (2015). Non-coding RNAs: an emerging player in DNA damage response. *Mutat. Res. Rev. Mutat. Res.* 763, 202–211. doi: 10.1016/j.mrrev.2014.11.003

Zhang, X., Yu, X., and Yue, D. (2016). Phytotoxicity of dimethyl sulfoxide (DMSO) to rice seedlings. *Int. J. Environ. Sci. Technol.* 13, 607–614. doi: 10.1007/s13762-015-0899-6

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Gianella M, Balestrazzi A, Ravasio A, Mondoni A, Börner, Guzzon F. Comparative seed longevity profiles under genebank storage and artificial ageing: a case study in heteromorphic wheat wild relatives. *Plant Biol*. Submitted.

Comparative seed longevity profiles under genebank storage and artificial ageing: a case study in heteromorphic wheat wild relatives.

Journal:	*Plant Biology*
Manuscript ID	PlaBio-2021-07-0357-RP
Manuscript Type:	Research Paper
Date Submitted by the Author:	26-Jul-2021
Complete List of Authors:	Gianella, Maraeva; Universita degli Studi di Pavia Balestrazzi, Alma; University of Pavia, Biology and Biotechnology Ravasio, Adriano; Universita degli Studi di Pavia Mondoni, Andrea; University of Pavia, Scienze della Terra e dell'Ambiente Börner, Andreas Guzzon, Filippo; CIMMYT,
Keyword:	Aegilops, Triticum, seed bank, artificial ageing, oxidative stress, gene expression, antioxidant response

Comparative seed longevity profiles under genebank storage and artificial ageing: a case study in heteromorphic wheat wild relatives.

Maraeva Gianella[1], Alma Balestrazzi[1*], Adriano Ravasio[1,2], Andrea Mondoni[2], Andreas Börner[3], Filippo Guzzon[4*]

[1] Department of Biology and Biotechnology 'L. Spallanzani', University of Pavia, via Ferrata 9, 27100, Pavia, Italy;

[2] Department of Earth and Environmental Sciences, University of Pavia, via Ferrata 1, 27100, Pavia, Italy;

[3] Genebank Department, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, D-06466 Seeland, OT Gatersleben, Germany;

[4] International Maize and Wheat Improvement Center (CIMMYT), Carretera México-Veracruz, Km. 45, El Bátan, Texcoco, Mexico State, Mexico

*Corresponding author: Filippo Guzzon, email: f.guzzon@cgiar.org, phone and fax number: +52 5951923197. Co-corresponding author: Alma Balestrazzi, email: address: alma.balestrazzi@unipv.it, phone and fax number: +39 0382 985435.

Short title

Genebank storage and artificial ageing in heteromorphic wheat wild relatives

Keywords

Aegilops, *Triticum*, seed bank, artificial ageing, oxidative stress, gene expression, antioxidant response

Key message

Two heteromorphic wheat wild relatives show different longevity profiles when exposed to artificial ageing and gene bank storage, with ROS accumulation in aged seed lots and differential expression of genes coding for enzymes related to the hydrogen peroxide scavenging pathway.

List of abbreviations

AA=Artificial ageing

ACT=Actin

APX=Ascorbate peroxidase

ARF=ADP-ribosylation factor

CAT=Controlled ageing test

CAT=Catalase

DCF-DA=2',7'-dichlorofluorescein diacetate

DHAR=Dehydroascorbate reductase

ELF=Elongation factor 1-alpha

GAPDH=Glyceraldehyde-3-phosphate dehydrogenase

GSR=Glutathione sulfo-reductase

MDAR=Monodehydroascorbate reductase

ROS=Reactive oxygen species

SB=Gene bank storage

SOD=Superoxide dismutase

UBI=Ubiquitin

Abstract

-Seed longevity is a complex trait that depends on numerous factors. Seed longevity varies among species and populations, and also within different seed morphs produced by the same plant. Little is known about variation in longevity profiles in different seed morphs as well as the physiological and molecular bases of these differences.

-In this study, we compared controlled ageing tests (seed storage at 45°C and 60% of relative humidity, a method of accelerated ageing used to estimate longevity in genebank conditions) with storage in a genebank for more than forty years (-18°C and 8% of seed moisture content). We employed as study species two wild wheats characterized by seed heteromorphism: *Aegilops tauschii* and *Triticum boeoticum*. Additionally, since oxidative stress is considered the main cause of seed ageing, we estimated the reactive oxygen species (ROS) content and the expression of genes coding for enzymes related to the hydrogen peroxide scavenging pathway.

-Results confirm that seed longevity varies between different seed morphs. Different storage environments resulted in different longevity profiles and survival curves. ROS levels, even if with variable patterns, were higher in several aged seed lots, and we observed consistency in the expression of two genes (*GSR* and *CAT*) related to ROS scavenging in the late phase of pre-germinative metabolism.

Differences in seed longevity between morphs were observed for the first time under genebank conditions. Our results suggest also that controlled ageing tests should be used with caution to infer ranks of longevity under cold storage.

Introduction

Seed lifespan is an extremely variable trait that depends on genetic and environmental factors. The exploration of the mechanisms regulating seed ageing is one of the central topics of seed science (Zinsmeister et al., 2020). The ability of orthodox seeds to retain vigour and germination capacity,

i.e. seed longevity, relies on desiccation tolerance and the anhydrobiotic state developed in the late stages of seed maturation, and these features allow seeds to overcome environmental conditions unfavourable for germination and establishment (Black & Pritchard, 2003; Pritchard, 2004; Ballesteros *et al.*, 2020). Temperature and relative humidity can modify the dynamics within this anhydrobiotic state, thus influencing seed longevity. Indeed, dry and cold conditions are exploited to extend seed lifespan in *ex situ* conservation within genebanks, while high RH and temperature can be used to artificially accelerate seed ageing in order to study their longevity profiles and ageing dynamics in laboratory conditions (Walters, 1998; Groot *et al.*, 2012; Hay et *al.*, 2019). However, both humidity and temperature affect seed longevity in different ways at the species and population level, with different species and genotypes showing different behaviour under different storage conditions (Zinsmeister et al., 2020). Moreover, the accuracy of laboratory methods of accelerated ageing (AA), such as controlled artificial ageing test (CAT) or controlled deterioration test (CDT), used to estimate longevity under genebank conditions, are being questioned for their accuracy (Schwember & Bradford, 2010; Roach *et al.*, 2018; Buijs *et al.*, 2020). Despite this, few seed longevity studies on long-term, cold-stored material are available, as well as comparative studies between artificial ageing and seed ageing in genebanks (Guzzon *et al.*, 2021).

Oxidative stress is considered as the main driving force of seed ageing, depending in large part on reactive oxygen species (ROS) accumulation and their mobility through membranes, accompanied by a decrease in antioxidant capacity (Kurek *et al.*, 2019). To cope with oxidative stress plants put in place a complex antioxidant machinery that can be divided into an enzymatic response, mainly consisting of enzymes belonging to the glutathione-ascorbate cycle, and a non-enzymatic one, composed of low molecular weight antioxidants (Bailly, 2004; Kurek *et al.*, 2019). Among the antioxidant enzymes, superoxide dismutase (SOD) dismutates superoxide radicals into oxygen and H_2O_2, which in turn is directly eliminated by the action of catalase (CAT). The ascorbate-glutathione cycle is also involved in the H_2O_2 scavenging, and it comprises ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR, or sulpho-reductase, GSR), all involved in the regeneration of antioxidants such as ascorbate, glutathione and α-tocopherol (Bailly, 2004). Protection and repair

are thought to be the two main mechanisms regulating seed longevity (Sano *et al.*, 2016). Indeed, even if endowed with the aforementioned protective mechanisms, seeds are subjected to cellular damages during ageing that are accumulated, together with ROS, during storage. However, during the pre-germinative metabolism induced upon imbibition the redox balance can be restored and oxidative damages are repaired before the completion of the germination process (Sano *et al.*, 2016).

Understanding the molecular mechanisms involved in shaping seed longevity is still challenging, as this trait is a complex biological process that depends on numerous environmental, physiological and genetic factors. Indeed, even if different seed lots are stored in the same conditions (e.g. cold storage, accelerated ageing), seed longevity greatly varies among species, but also within the same taxon, e.g. different populations and cultivars (Mondoni *et al.*, 2018, Guzzon *et al.*, 2021). On the other hand, also within the same genotype, and within different seeds produced by the same mother plant, differences in longevity can be observed. Several wheat wild relatives of the genera *Aegilops* and *Triticum* show seed heteromorphism, i.e. the production of different types of seeds by the same individual (Gianella *et al.*, 2021). Different seed morphs produced by the same mother plants show significant differences in terms of longevity when subjected to artificial ageing but also in terms of soil seed bank persistence (Calucci *et al.*, 2004; Guzzon *et al.*, 2018; Gianella *et al.*, 2020). These heteromorphic species provide an excellent system to study seed longevity.

Even though efforts are being made in order to disentangle the molecular and physiological drivers of seed longevity, and recent studies have shed light on intraspecific variation in longevity profiles (e.g. Nagel *et al.*, 2011; Hay *et al.*, 2013; van Treuren *et al.*, 2018; Guzzon *et al.*, 2021), still little is known about the behaviour in terms of longevity in heteromorphic species. Moreover, fewer data are available on the behaviour of the same genotype responding to different ageing conditions, i.e. cold storage *vs* artificial ageing (Nagel et al., 2015). Therefore, here we explored the longevity profiles of two dimorphic wheat wild relatives subjected to artificial ageing and to long-term genebank storage: *Aegilops tauschii* Coss., donor of the DD genome to the hexaploid bread wheat (*Triticum aestivum* L.), and *Triticum monococcum* subsp. *aegilopoides* (Link) Thell. (= *Triticum*

boeoticum Boiss.), wild ancestor of cultivated einkorn wheat (*Triticum monococcum* L. subsp. *monococcum*). We compared the survival curves between morphs and ageing conditions, and we further explored the seeds lifespan by evaluating the ROS accumulation and the expression of genes coding for enzymes involved in H_2O_2 scavenging. We further characterized the germination profiles of fresh and aged seeds along with imbibition and the activation of the pre-germinative metabolism. We hypothesized that different morphs respond differently to the ageing treatments, namely artificial ageing and genebank storage, possibly with differential oxidative stress degrees in terms of ROS accumulation and antioxidant gene expression depending on the ageing and imbibition timepoint.

Materials and methods

Plant Material

Caryopses (hereafter referred to as seeds) of *Ae. tauschii* (AE 278) and *T. monococcum* subsp. *aegilopoides* (for simplicity, hereafter referred to as *T. boeoticum*) (TRI 10061) were kindly provided by the Genebank Department of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben (Germany). AE 278 was originated from Afghanistan and supplied by the VIR genebank St. Peterburg (Russia) in 1974 whereas TRI 10061, with geographical origin Soviet Union, was obtained from the Botanical Garden Brno (Czech Republic) in 1972. In both species seed longevity loss has been investigated after 40 years of genebank bank storage at the IPK (-18 ± 2°C, 8 ± 2 % seed moisture content; hereafter referred to as "SB") and under artificial ageing condition (hereafter referred to as "AA"), using for the latter fresh seeds produced by plants of the same genotype held in genebank (**Table 1**). Seed samples were regenerated at the experimental fields of IPK in Gatersleben (latitude 51° 49' 19.74" N, longitude 11° 17' 11.80" E, 110.5 m.a.s.l., black soil of clayey loamy type) and the collected seed lots are hereafter referred to as "fresh" or 0d. Seeds were extracted from the spikelets and sorted out according to the morph as previously described (Gianella *et al.*, 2020): the larger, basal morph 'A' and the smaller, apical morph 'B'. After the cleaning process, seeds were kept in standard dry-room conditions at the Plant

148 Germplasm Bank of the University of Pavia (Italy) at 15% relative humidity (RH) and15°C (ISTA,
149 2018) until use.

150 *Germination tests and imbibition curves*

151 Germination tests were carried out in triplicates of 20 seeds each in Petri dishes with 1% agar as
152 substrate. Dishes were placed in temperature- and light-controlled incubators (LMS, Sevenoaks,
153 UK) using a 12-h daily photoperiod (photosynthetically active radiation 40–50 µmol m^{-2} s^{-1}) at 20 °C
154 (see Guzzon et al., 2015). Petri dishes were checked every 12 h for germination, and seeds scored
155 as germinated once the radicle had reached 2 mm length. At the completion of each germination
156 test (4 weeks after sowing), non-germinated seeds were cut-tested to confirm whether they were
157 empty. Imbibition curves were determined using 6 replicates of 5 seeds, placed in Petri dishes
158 between double filter paper discs, moistened with dH_2O and sealed in plastic bags to avoid
159 evaporation. Seeds were then withdrawn, the residual superficial water was removed with sterilized
160 filter paper and seed mass was measured at 1h-intervals until radical protrusion was observed.
161 Water uptake was estimated by subtracting dry mass to the weight registered during imbibition and
162 expressed as increase in % (Louf et al. 2018).

163 *Controlled ageing test (CAT)*

164 Seed longevity was tested with a modified protocol for artificial ageing from Newton (2009),
165 according to Guzzon *et al.*, (2018). Prior to storage, seeds were first rehydrated for 14 days in
166 open Petri dishes over a non-saturated solution of LiCl in a sealed electrical enclosure box (Ensto
167 UK, Southampton, UK) at 47% RH and 20 °C. Thereafter, seed equilibrium relative humidity (eRH)
168 was verified with an AWDI0 water activity probe used in conjunction with a Hygro-Palm 3 display
169 unit (Rotronic Instruments UK, Crawley, UK). Once the seeds had reached eRH, the initial
170 germination was assessed using triplicates of 20 seeds and the protocol described above. Next,
171 seed lots were stored in the dark in a sealed box over a non-saturated LiCl solution at 60% RH at
172 45 ± 2 °C, placed in a compact incubator (Binder FD53; Binder, Tuttlingen, Germany). When
173 necessary, aliquots of distilled water were added to the LiCl to keep the RH at the required
174 equilibrium, which was monitored inside the enclosure box (Tinytag View 2 Temperature/Relative

Humidity Logger; Gemini Data Logger, Chichester, UK). At nine intervals during storage, three replicates of 20 seeds per lot were extracted and viability was tested with the same protocol as used for the preliminary germination test, until a complete viability loss was reached (**Table 1**).

ROS detection

The fluorogenic dye 2',7'-dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich, Milan Italy) was used to quantify ROS levels released from dry 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 (DCF). The assay was carried as described by Macovei et al. (2016) with the following modifications. Dry seeds were incubated in the dark for 30 min with 150 µl of a 10 µM DCF-DA solution. Subsequently, three replicates (50 µl each, per seed lot) were pipetted into 0.2 ml tubes and the emitted fluorescence was measured using the green channel (510 ± 5 nm) of a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia), after a single cycle of 30 s at 25°C. As negative control, three replicates containing only DCF-DA were used to subtract the baseline fluorescence. Relative fluorescence was calculated by normalizing samples to controls and expressed as Relative Fluorescence Units (R.F.U.).

qRT-PCR

The RNA extraction was performed according to Oñate-Sánchez & Vicente-Carbajosa (2008) from dry, 1h- and 18h-imbibed seeds of *Ae. tauschii*, and from dry, 1h- and 14h-imbibed seeds of *T. boeoticum*. Retro-transcription was carried out using the iScript cDNA Synthesis kit (Bio-Rad, Milan, Italy), according to the manufacturer's instructions. qRT-PCRs were performed using SYBR Green qPCR Master Mix (2X) (Fermentas, Milan, Italy) and a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia). For oligonucleotide primer design, sequences were obtained from the online database EnsemblPlants: an alignment was performed between sequences from *Ae. tauschii* and *T. boeoticum* with the online software Multiple Sequence Alignment by CLUSTALW in order to select common and species-specific primer oligonucleotides

used for the amplification of reference and target genes. The resulting oligonucleotide primers are listed in **Table S1**.

To analyse the expression of genes encoding enzymes belonging to the ascorbate-glutathione ROS scavenging pathway (ascorbate peroxidase, *APX*, catalase, *CAT*, dehydro-ascorbate reductase, *DHAR*, glutathione reductase, *GSR*, mono-dehydro-ascorbate reductase, *MDAR* and superoxide dismutase, *SOD*) the geometric mean of two reference genes, was used as standard control. Quantification was carried out using the following genes as reference genes for the experimental conditions (AA and SB) used in this work: *ARF* (ADP-ribosylation factor) and *ELF* (elongation factor 1-alpha) for *Ae. tauschii* AA and SB seeds, *ACT* (actin) and *ELF* for *T. boeoticum* SB, *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *UBI* (ubiquitin) for *T. boeoticum* SB. Selection was performed using the GeNorm algorithm (https://genorm.cmgg.be) (**Figure S1 and S2**). Oligonucleotide primers were designed with the online software Primer3Plus and thermodynamic parameters were checked with Oligoanalyzer (**Table S1**). qRT-PCR conditions were denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. Once aspecific fluorescence was subtracted, the raw fluorescence data obtained from the Software Rotor-Gene 6000 Series 1.7 (Corbett Robotics) were used to retrieve PCR Efficiency (E) and threshold cycle number (Ct) for each transcript quantification. The Pfaffl method (Pfaffl, 2001) was used for relative quantification of transcript accumulation and the two reference genes, selected for each species in the different ageing conditions, were used to normalise the values by calculating the ratio between target gene transcripts and the geometrical mean of the reference genes transcripts.

Statistical analysis

Statistical analysis was carried out in SPSS 21 and Genstat 9. Origin Pro 9.1 was used to build survival curves and heatmaps were plotted in the R software environment for statistical computing and graphics (v. 4.1.0). The R packages used are: *tidyr* (Wickham, 2021), dplyr (Wickham et al., 2021), and *ggplot2* (Wickham, 2016). Before analyses, data were checked for normality and homoscedasticity (Shapiro–Wilk's and Levene's tests, respectively). Probit analysis was carried out

using GenStat 9 to obtain the time for viability to fall by 50% (p_{50}), used then as measure for seed longevity by fitting the viability equation (Ellis & Roberts, 1980) as previously described by Gianella *et al.* (2020). P_{50s} were compared between seed lots belonging to the same species and subjected to the same ageing conditions (AA or SB) using T student's tests. A GLM with gamma distribution, log link, was applied to compare the longevity profiles of the two morphs in the two species when subjected to the same ageing treatment. Heatmaps represent the Log_2 fold change (Log2FC) of the relative gene expression and ROS levels of aged seed lots (AA and SB) compared to the controls, i.e. the maximum germination % of freshly harvested seeds (0d *Ae. tauschii*, 21d *T. boeoticum* due to after-ripening).

Results

Imbibition and germination

Fresh seeds of *Ae. tauschii* and *T. boeoticum* were subjected to imbibition and germination tests in order to select the appropriate timepoints for subsequent analyses. Both species showed the quickest water absorption in the time interval between imbibition and 1 h of soaking (**Figure S3**). Thus, the 1 h-interval was selected in order to analyze the ROS content and gene expression after the activation of the pre-germinative metabolism induced by water absorption. 1h imbibition is also the endpoint of the so-called 'abrupt imbibition' in wheat, corresponding to the embryo water uptake, followed by the water absorption in the other seed parts (Lev & Blahovec, 2017). Another time point was selected, corresponding to two hours before the first radicle protrusions were observed for *Ae. tauschii* (18 hours after imbibition, **Figure S3a**), for *T. boeoticum* (14 hours after imbibition, **Figure S3b**). This was done in order to select an appropriate timepoint for the analysis of the late stage pre-germinative metabolism, while avoiding the comparisons among different phenological states (seeds vs radical protrusions). Unlike *Ae. tauschii*, which did not show any after-ripening requirement, both morphs of *T. boeoticum* reached the maximum germination percentage after 21 days of AA treatment (**Table S2**). Therefore, this timepoint was chosen as control for the subsequent analyses, while 0 days was chosen as control for *Ae. tauschii*.

Seed bank and artificial ageing

P_{50} was calculated for the two species in the SB and AA conditions and the survival curves fitting the viability equations are represented in **Figure1**. When subjected to AA, both species showed significant differences in terms of p_{50} among morphs: *Ae. tauschii*, A = 39.66 d, B = 49.55 d, $P < 0.05$, **Figure 1a**; *T. boeoticum,* A = 37.02 d, B = 51.49 d, $P < 0.001$, **Figure 1c**. In SB conditions, *Ae. tauschii* showed no differences among the two morphs (A = 27.76 y, B = 27.49 y, $P = 0.665$, **Figure 1b**), while the two morphs of *T. boeoticum* showed significantly different longevity profiles (A = 38.95 y, B = 55.66 y, $P < 0.001$, **Figure 1d**). Thus, *Ae. tauschii* morphs showed different longevity profiles when subjected to SB and AA. Also, the two species showed similar lifespans when subjected to AA (GLM, species*morph Wald Chi-squared=3.071, P=0.08), while both morphs of *T. boeoticum* were largely longer-lived than those of *Ae. tauschii* during SB storage: the species*morph interaction had a statistically significant effect on p_{50} (Wald Chi-squared=27.402, P<0.001), with morph A and B of *T. boeoticum* significantly longer-lived than morph A and B of *Ae. tauschii*, respectively (Bonferroni post hoc, both P<0.001).

For subsequent analyses, different conditions were chosen in order to evaluate ROS contents and gene expression in dry seeds, and at the starting and ending points of pre-germinative metabolism at different ageing timepoints. For AA, p_{50} of seed A and B for both species (PA and PB) and 0d for *T. boeoticum*, freshly harvested. For SB, three different years of harvest per species were considered for further analyses: *Ae. tauschii* 1978 (T78), 1999 (T99) and 2008 (T08); *T. boeoticum*, 1980 (B80), 2002 (B02), 2005 (B05).

ROS accumulation in aged seeds

ROS content was evaluated in AA and SB seeds, and the accumulation relative to the controls was represented by means of heatmaps in **Figure 2** (AA) and **Figure 3** (SB). See **Table S3** for mean values and standard deviations. In both species, ROS accumulation was observed along with AA compared to the controls (**Figure 2 a, b**) at 1h after imbibition in both morphs, while at the end of the pre-germinative metabolism a significant decrease was observed except for aged seeds A of *T. boeoticum*. Also, in dry seeds of *Ae. tauschii* a higher ROS accumulation was observed when compared to the controls (**Figure 2a**), while in *T. boeoticum* both fresh and aged seeds showed

lower ROS levels compared to the after-ripened controls (**Figure 2b**). Regarding SB, a very strong ROS accumulation was observed in both morphs of *Ae. tauschii*, slightly less evident in morph B and maximum at the beginning of the pre-germinative metabolism (**Figure 3a**). In *T. boeoticum* SB seeds the highest increase was again observed at 1 h after imbibition, while ROS levels showed a decrease in the late stages of pre-germinative metabolism in the more recent seed lots (**Figure 3b**). Thus, a more consistent pattern among timepoints and imbibition states was observed during SB ageing, while AA conditions showed more variables consequences in terms of ROS accumulation.

In general, in all the ageing conditions a higher ROS accumulation was observed after the activation of the pre-germinative metabolism (1 h after imbibition), while at the late stage a lower level was observed in *T. boeoticum* SB seeds and *Ae. tauschii* AA seeds. Indeed, high ROS levels were observed in SB seeds of *Ae. tauschii* also at 18 h after imbibition.

Expression profiles of genes involved in H_2O_2 scavenging in AA seeds

The expression levels of the genes belonging to the H_2O_2 scavenging pathway were measured in AA seeds of *Ae. tauschii* (**Figure 2a**). See **Table S4** for mean values and standard deviations. *GSR* and *APX* transcripts showed an increase in all the imbibition states in the two morphs during ageing compared to the controls. *CAT* mRNA was detected at higher levels in dry seeds A at 39 and 49 d of AA (0.37 and 1.75 Log2FC, respectively), with a subsequent increase at 1h after imbibition in both morphs followed by a decrease in the late stage of pre-germinative metabolism. *DHAR* mRNA showed lower levels in morph A dry seeds compared to the control (39d=-2.65 and 49d=-2.05 Log2FC), and little variations at 1h and 18h after imbibition in both morphs compared to the controls. Also, the *SOD* transcript showed little variation at the late stage of imbibition, while accumulation was observed at 1h in both morphs (in the range of 0.6-0.7 Log2FC). The *MDAR* gene showed very variable expression profiles in the different morphs, imbibition stages and ageing timepoints.

In *T. boeoticum* (**Figure 2b**) dry seeds at 0d, i.e. before the after-ripening in the incubator, only *MDAR* (2.88 Log2FC) and *DHAR* (0.36 Log2FC) genes showed higher expression levels

compared to the controls, while the other four genes revealed lower transcripts levels (in the range –0.67 to -1.74 Log2FC). At 1h after imbibition seed A and B 0d showed generally lower gene expression levels compared to the control (-0.14 to -1.82 Log2FC). At 14 h after imbibition, seed A showed higher transcript levels for all the tested genes except *APX*, while seed B showed only upregulation of *DHAR* gene (3.10 Log2FC). At p_{50} of seed A, 37d, both morphs at the dry state showed lower transcript levels compared to the control, while in seed A higher expression levels were observed at both the start and the end of the pre-germinative metabolism, with a notably higher expression of *GSR* at 14 h (11.37 Log2FC). Morph B showed a higher quantity of *SOD* transcripts at 1h after imbibition, and an upregulation of *DHAR* and *CAT* genes at 14 h. At p_{50} of seed B, 51d, little variation was observed in dry seeds compared to the controls, except for *MDAR* gene, more expressed in morph B (3.09 Log2FC). At 1h after imbibition transcript levels were higher in seed B and lower in seed A compared to the controls, while, on the contrary, at 14 h gene expression was higher in seed A (especially for *GSR* and *CAT* genes) and lower in seed B.

Expression profiles of genes involved in H_2O_2 scavenging in SB seeds

The expression levels of the genes involved in the H_2O_2 scavenging pathway were measured in SB seeds of *Ae. tauschii* (**Figure 3a**). See **Table S4** for mean values and standard deviations. Notably, both *APX* and *SOD* gene expression patterns were consistent with the ROS levels measured in aged seeds. Compared to the controls, *APX* transcript levels resulted lower (in the range -2.87 to -9.05 Log2FC) whereas *SOD* transcript levels were higher (in the range 1.27 to 3.79 Log2FC), proportionally decreasing or increasing, respectively, along with ROS levels and imbibition states. In dry seeds, *CAT, DHAR* (only morph A) and *MDAR* genes showed lower expression levels compared to the controls, while *GSR* showed higher transcript levels. At 1 h after imbibition, expression decreased in the oldest seed lots compared to the fresh controls, except for the more recent seed lots (T08, 2008), that showed higher levels especially in morph B, and except for the *GSR* gene, more expressed also in the T99 (1999) seed lots (0.63-0.82 Log2FC). *GSR* was also highly expressed at 18 h after imbibition, while *DHAR* and *CAT* genes revealed lower expression levels except for morph B of the T99 and T08 seed lots. Also, the *MDAR* gene showed

higher expression levels but with a more variable trend in the different morphs of the aged seed lots.

In *T. boeoticum* (**Figure 3b**) dry seeds aged in SB conditions, *SOD* and *GSR* transcript levels were lower compared to the controls, while the *MDAR* gene showed higher expression levels. *CAT* and *APX* transcript levels showed low variations compared to the controls, except for morph A of the oldest seed lot (B80, 1980), that showed higher expression levels (*APX*=2.31 and 2.69 *CAT*=Log2FC). *DHAR* gene expression was generally lower except for morph B of the 2002 seed lot (B02). At 1H after imbibition, lower transcript levels were observed compared to the controls, except for *CAT* in morph A of the1980 and 2002 seed lots, *SOD* and *DHAR* in the morph B of 1980 and 2002 seed lots, respectively, and for *DHAR* in morph B of the 2002 lot and morph A in the 2005 lot. At 14 h after imbibition, higher *GSR* and *CAT* transcript levels were observed more evident in morph A (3.15 to 9.42 Log2FC). *DHAR* and *SOD* mRNA levels were lower in all the aged seed lots, except for *SOD* in morph B of the 2002 seed lot. *MDAR* gene expression was higher in the more recent seed lot (B05) compared to the controls (A=1.64, B=1.91 Log2FC).

In *Ae. tauschii* a consistent pattern of the *GSR* gene expression among ageing treatments and imbibition states was observed. In *T. boeoticum* the expression pattern is similar among imbibition states, in particular in the higher expression of *GSR* and *CAT* at 18 h after imbibition.

Discussion

The prediction of seed lifespan, and the understanding of the physiological mechanisms involved in determining it, are crucial for an efficient genebank management, i.e. for planning the monitoring and regeneration intervals of long-term conservation collections (Walters *et al.*, 2005; Probert *et al.*, 2009). However, seed longevity varies largely among taxa, populations and even among seed morphs produced by the same mother plant. Seed longevity is highly influenced by the storage conditions (mainly temperature and RH) or the laboratory methods used to infer longevity ranks among species and evaluate their storability (Zinsmeister *et al.*, 2020). Also, the shapes and slopes of the sigmoid survival curves extrapolated from the longevity data, and the predicted survival

parameters such as p_{85} and p_{50}, greatly depend on genotype, and also on the functions chosen to fit the data (Bernal-Lugo & Leopold, 1998; de Faria *et al.*, 2020).

In this study, *Ae. tauschii* and *T. boeoticum* showed variable behaviours in terms of heteromorphism when subjected to the two storage environments (AA, SB). In previous studies we demonstrated that different morphs of the same species respond differently to AA in several wheat wild relatives, with smaller seeds possessing higher longevity, dormancy and antioxidant profiles (Guzzon *et al.*, 2018; Gianella *et al.*, 2020). These seed traits are known as part of a bet-hedging evolutionary strategy that allow smaller seeds a longer persistence in the soil (Arshad *et al.*, 2019), thereby spreading the risk of germination failure in time. In this paper we went further, showing for the first time that differences of seed longevity due to heteromorphism may occur also on seeds held under genebank conditions, which may have important implications for the *ex situ* conservation practices. Indeed, the main aim of seed collections of plant genetic resources for food and agriculture (PGRFA) is to capture all the possible adaptive traits in the seed samples (Warschefsky *et al.*, 2014). Several differences have been detected at the different levels (e.g. biochemical, genetic and morphological levels, as well as in abiotic stress resistance) among different seed morphs in species of Poaceae (Datta, 1970; De Gara *et al.*, 1991; Cremonini *et al.*, 1994; Frediani *et al.*, 1994; Orsenigo *et al.*, 2017). It follows that different morphs with different longevity profiles should be conserved separately in order to better monitor their viability over time and to avoid an unconscious selection of plants originating only from longer-lived, morph B seeds (Guzzon *et al.*, 2018). Alternatively, assuming that conservation of cleaned, separated morphs could be costly for the time-consuming cleaning process, lower regeneration thresholds could be applied in order to avoid loss of genetic material belonging to the shorter-lived morph A pool.

While morph B of *T. boeoticum* was significantly longer-lived than morph A in both conditions, *Ae. tauschii* did not show significantly different p_{50s} among morphs when aged in cold storage, while morph B was significantly longer-lived when subjected to AA. These differences in the longevity estimates and slope of the curves detected between the two ageing conditions can be due to several factors, such as the different number of time-intervals between storage conditions or

environmental factors at regeneration stage that influenced seed heteromorphism and longevity. Nevertheless, the possibility that these differences are due to the fact that artificial ageing and cold storage affected seed survival in different ways cannot be ruled out (see e.g. Walters *et al.*, 2010; Colville & Pritchard, 2019). It follows that predictions made with AA should be used with caution to infer ranks of longevity under cold-storage, and therefore to make subsequent decision on seed bank management (e.g. change seed processing methodologies, prioritization of accessions for regeneration and/or viability monitoring).

The conditions applied to artificial ageing or cold storage affect the cytoplasmic viscoelastic properties and the biochemical processes taking place within it, in turn influencing the physiological pH and the redox state (Nagel *et al.*, 2015). Oxidative stress is considered as the main cause of seed ageing, and it arises due to an imbalance between the accumulation of ROS, also functioning as signalling molecules and therefore regulating several biological processes, and the antioxidant capacity necessary to avoid cellular damages induced by the oxidative reaction that affect nucleic acids, lipids and proteins (Kurek *et al.*, 2019). In this study, the two species did not show similar patterns in terms of ROS accumulation within the same ageing conditions. At the dry state, in *Ae. tauschii* an increase in ROS levels was observed, compared to the fresh controls in both ageing treatments, while *T. boeoticum* showed generally lower levels in the comparison with the after-ripened controls. The accumulation of ROS in the after-ripened controls could be due to the fact that after-ripening was performed within the ageing box (45°C, 60% RH), and even if germination was higher, the conditions of high T and RH used for AA could have triggered ROS production. Indeed, the conditions that determine the loss of dormancy during after-ripening are the same, i.e. temperature and RH, and it is considered as the first stage of seed ageing in seeds with primary dormancy (Bewley *et al.*, 2013). Indeed, ROS levels are generally higher in non-dormant seeds than in dormant seeds (Bailly *et al.*, 2008). No consistent patterns of ROS accumulation were observed between morphs, in terms of response to ageing treatments and timepoints as well as imbibition states. A and B did not show consistent ROS accumulation. *Ae. tauschii* showed lower ROS contents in morph B only at the dry state of SB aged seeds. The possibility that a differential imbalance between ROS accumulation and antioxidant capacity, might occur in the two morphs,

linked to their different lifespan, cannot be ruled out. Also, a different antioxidant capacity that could rescue seeds from irreversible oxidative stress could explain the lower accumulation of ROS in morph B compared to morph A at the end of pre-germinative metabolism. Indeed, this was observed in *T. boeoticum* in both ageing conditions, and in the AA seeds of *Ae. tauschii*. In these three conditions a dimorphism in longevity was observed, with morph B significantly longer-lived than morph A. This antioxidant response could be linked partly to a differential endowment of antioxidant molecules accumulated during maturation and then depleted with ageing, but also to a differential production of newly synthesized molecules during pre-germinative metabolism (Bewley *et al.*, 2013; Sano *et al.*, 2016). A general increase of ROS accumulation was observed at 1 h of imbibition, the first stage of pre-germinative metabolism, in all the species and ageing treatment combinations compared to the dry seed lots. This observation is in agreement with the finding that water uptake during seed imbibition triggers metabolism resumption, and the conversion of oxygen into superoxide and H_2O_2 at the level of mitochondria (Bailly *et al.*, 2008).

Following imbibition, all the biological processes associated with germination are reactivated, including respiration, reserve mobilization, DNA synthesis and repair, translation and degradation of stored mRNAs, transcription and translation of newly synthesized mRNAs (Bewley *et al.*, 2013). We evaluated the transcript levels of genes coding for the enzymes with ROS scavenging activity or belonging to the glutathione-ascorbate pathway in aged seeds at the dry state and during pre-germinative metabolism triggered by imbibition. The lowest variations compared to the controls were at 1h after imbibition, and a general decrease in transcript levels was observed at this stage compared to the dry seeds, this being in accordance with the initial period of phase II of germination, where transcripts are degraded or translated in order to generate the enzymes necessary for the ROS scavenging (Bewley *et al.*, 2013). Considering all the six genes analysed, no clear pattern between morphs was observed. This could be due to several reasons, e.g. different kind of ROS produced and accumulated, similar levels of oxidative stress, different enzymes needed at the same timepoint of imbibition (thus different genes need to be expressed). At the late stage of pre-germinative metabolism, an increase in transcript levels was detected, also compared to the controls suggesting that storage and artificial ageing might require higher

antioxidant activities. When taking in consideration the expression of single genes among ageing treatments, *GSR* showed a consistent pattern among all the imbibition states in *Ae. tauschii*, while in *T. boeoticum* both *GSR* and *CAT* showed consistent profiles at 18 hours after imbibition in the two ageing treatments. The GSH/ glutathione disulphide (GSSG) redox couple is a viability marker associated with seed longevity in barley (Nagel *et al.*, 2015; Roach *et al.*, 2018). Glutathione scavenging activity is particularly important in seed with lower oil contents like cereals, as it is water-soluble compared to other lipid-soluble antioxidants (e.g. tocopherols) (Nagel *et al.*, 2015). The consistent expression of *GSR* detected in this study could be linked to the enzymatic activity necessary to the GSSG to GSH re-conversion in the glutathione-ascorbate pathway. Moreover, GSR and CAT activity has been reported to be higher in the late stage of pre-germinative metabolism in sunflower for H_2O_2 scavenging and limitation of lipid peroxidation (Bailly, 2004).

In conclusion, we found that heteromorphism in seed longevity can be observed in both cold storage and in artificially aged seeds, but that the different ageing treatments result in different longevity profiles and survival curves, in terms of shapes and slopes. ROS levels, even if with variable patterns, were higher in several aged seed lots, and in after-ripened seeds compared to their dormant counterpart, in accordance with the literature. The expression of genes coding for enzymes with ROS scavenging activity was variable depending on the seed lot, ageing timepoint and imbibition stage, with consistency among ageing treatments detected for *GSR* and *CAT*: both genes are associated with seed response to abiotic stresses and with ROS scavenging in the late phase of pre-germinative metabolism before radicle protrusion. Further studies are necessary in order to better understand the dynamics of oxidative stress and antioxidant response in the context of heteromorphic seeds artificially aged and stored within seed banks.

Supporting information

Table S1 List of oligonucleotide primers used for qRT-PCR analyses.

Table S2 Germination percentage of *T. boeoticum* during the AA treatment. Morph B shows after-ripening requirements, with maximum percentage of both morphs at 21 days.

Table S3 ROS content in both the considered species, in fresh seeds and in the two storage treatments (AA and SB), considering different imbibition points.

Table S4 Expression levels of the genes involved in the H_2O_2 scavenging pathway in both the considered species, in fresh seeds and in the two storage treatments (AA and SB), considering different imbibition points.

Figure S1 Selection of reference genes for qRT-PCR analysis in *Ae. tauschii* SB seeds (a) and AA seeds (b). Average expression stability values (M), calculated using GeNorm algorithm (https://genorm.cmgg.be), of the six putative reference genes analysed in dry and imbibed seeds throughout the tested time points of the experimental design. *ACT*, actin, *ARF*, ADP-rybosilation factor, *ELF*, elongation factor 1 alpha, *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase, *RPT6*, 26S protease regulatory subunit, *UBI*, ubiquitin.

Figure S2 Selection of reference genes for qRT-PCR analysis in *T. boeoticum* SB seeds (a) and AA seeds (b). Average expression stability values (M), calculated using GeNorm algorithm (https://genorm.cmgg.be), of the six putative reference genes analysed in dry and imbibed seeds throughout the tested time points of the experimental design. *ACT*, actin, *ARF*, ADP-rybosilation factor, *ELF*, elongation factor 1 alpha, *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase, *RPT6*, 26S protease regulatory subunit, *UBI*, ubiquitin.

Figure S3 Imbibition curves of *(a) Ae. tauschii* and *(b) T. boeoticum* seeds. Seeds were imbibed with dH_2O on filter paper, then withdrawn at 1 hour-intervals until radical protrusion was observed. Water uptake was estimated by subtracting dry mass to the weight registered during imbibition after residual superficial water was removed, and then expressed as increase in percentage.

Acknowledgements

This work was supported by the University of Pavia Crowdfunding (UNIVERSITIAMO) campaign "The other side of the seed" (https://universitiamo.eu/en/campaigns/the-other-side-of-theseed/), and 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 A. B.).

References

Arshad W., Sperber K., Steinbrecher T., Nichols B., Jansen V.A.A., Leubner-Metzger G., Mummenhoff K. (2019) Dispersal biophysics and adaptive significance of dimorphic diaspores in the annual *Aethionema arabicum* (Brassicaceae). *New Phytologist*, **221**, 1434–1446.

Bailly C. (2004) Active oxygen species and antioxidants in seed biology. *Seed Science Research*, **14**, 93-107. https://doi.org/10.1079/SSR2004159

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. *Comptes Rendus – Biologies*, **331**, 806–814. https://doi.org/10.1016/j.crvi.2008.07.022

Ballesteros D., Pritchard H.W., Walters C. (2020) Dry architecture: Towards the understanding of the variation of longevity in desiccation-tolerant germplasm. *Seed Science Research*, **30**, 142–155. https://doi.org/10.1017/S0960258520000239

Bernal-Lugo I., Leopold, A. C. (1998) The dynamics of seed mortality. *Journal of Experimental Botany*, **49**, 1455–1461. https://doi.org/10.1093/jxb/49.326.1455

Bewley J. D., Bradford K. J., Hilhorst H. W. M., Nonogaki, H. (2013) Seeds Physiology of Development, Germination and Dormancy, 3rd Edition. Springer. https://doi.org/10.15713/ins.mmj.3

Black M., Pritchard H. W. (2003) Desiccation and survival in plants. Drying without dying. In M. Black & H. W. Pritchard (Eds.), *Annals of Botany*, **91**, 105. Oxford University Press. https://doi.org/10.1093/aob/mcg004

519 Buijs G., Willems L. A. J., Kodde J., Groot S. P. C., Bentsink L. (2020) Evaluating the EPPO
520 method for seed longevity analyses in *Arabidopsis*. *Plant Science*, **301**, 110644.
521 https://doi.org/10.1016/j.plantsci.2020.110644

522 Calucci L., Capocchi A., Galleschi L., Ghiringhelli S., Pinzino C., Saviozzi F., Zandomeneghi M.
523 (2004) Antioxidants, free radicals, storage proteins, puroindolines, and proteolytic activities in
524 bread wheat (*Triticum aestivum*) seeds during accelerated aging. *Journal of Agricultural and Food*
525 *Chemistry*, **52**, 4274–4281 https://doi.org/10.1021/jf0353741

526 Colville L., Pritchard H. W. (2019) Seed life span and food security. *New Phytologist*, **224**, 557–
527 562. https://doi.org/10.1111/nph.16006

528 Cremonini R., Colonna N., Stefani A., Galasso I., Pignone D. (1994) Nuclear DNA content,
529 chromatin organization and chromosome banding in brown and yellow seeds of *Dasypyrum*
530 *villosum* (L.) Candargy. *Heredity*, **72**, 365–373.

531 Datta S.C., Evenari M., Gutterman Y. (1970) Heteroblasty of *Aegilops ovata* L. *Israel Journal of*
532 *Botany*, **19**, 463–483.

533 De Gara L., Paciolla C., Liso R., Stefani A., Arrigoni O. (1991) Correlation between ascorbate
534 peroxidase activity and some anomalies of s,dlings from aged caryopses of *Dasypyrum villosum*
535 (L.) Borb. *Journal of Plant Physiology*,**137**, 697–700.

536 de Faria R. Q., dos Santos A. R. P., Amorim D. J., Cantão R. F., da Silva E. A. A., Sartori, M. M. P.
537 (2020). Probit or Logit? Which is the better model to predict the longevity of seeds? *Seed Science*
538 *Research*, **30**, 49–58. https://doi.org/10.1017/S0960258520000136

539 Ellis R. H., Roberts E. H. (1980) Improved Equations for the Prediction of Seed Longevity. *Annals*
540 *of Botany*, **45**, 13–30. https://doi.org/10.1093/oxfordjournals.aob.a085797

541 Frediani M., Colonna N., Cremonini R., De Pace C., Delre V., Caccia C., Cionini P.G. (1994)
542 Redundancy modulation of nuclear DNA sequences in *Dasypyrum villosum*. *Theoretical and*
543 *Applied Genetics*, **88**, 167–174.

544 Gianella M., Balestrazzi A., Pagano A., Müller J. V., Kyratzis A. C., Kikodze D., Canella M.,
545 Mondoni A., Rossi G., Guzzon F. (2020) Heteromorphic seeds of wheat wild relatives show
546 germination niche differentiation. *Plant Biology*, **22**, 191-202. https://doi.org/10.1111/plb.13060

547 Gianella M., Bradford K.J., Guzzon F. (2021) Ecological, (epi)genetic and physiological aspects of
548 bet-hedging in Angiosperms. *Plant Reproduction*, **34**, 21-36. DOI: 10.1007/s00497-020-00402-z.

549 Groot S. P. C., Surki A. A., de Vos R. C. H., Kodde J. (2012) Seed storage at elevated partial
550 pressure of oxygen, a fast method for analysing seed ageing under dry conditions. *Annals of*
551 *Botany*, **110**, 1149–1159. https://doi.org/10.1093/aob/mcs198

552 Guzzon F., Orsenigo S., Gianella M., Müller J. V., Vagge I., Rossi G., Mondoni A. (2018) Seed
553 heteromorphy influences seed longevity in *Aegilops*. *Seed Science Research*, **28**, 277 - 285.
554 https://doi.org/10.1017/S096025851800034X

555 Guzzon F., Gianella M., Velazquez Juarez J. A., Sanchez Cano C., Costich D. E. (2021) Seed
556 longevity of maize conserved under germplasm bank conditions for up to 60 years. *Annals of*
557 *Botany*, **127**, 775–785. https://doi.org/10.1093/aob/mcab009

558 Guzzon F., Müller J. V., Abeli T., Cauzzi P., Ardenghi N. M. G., Balestrazzi A., Rossi G., Orsenigo
559 S. (2015). Germination requirements of nine European *Aegilops* species in relation to constant and
560 alternating temperatures. *Acta Botanica Gallica*, **162**, 349–354.
561 https://doi.org/10.1080/12538078.2015.1088793

562 Hay F. R., de Guzman F., Ellis D., Makahiya H., Borromeo T., Hamilton N. R. S. (2013) Viability of
563 *Oryza sativa* L. seeds stored under genebank conditions for up to 30 years. *Genetic Resources*
564 *and Crop Evolution*, **60**, 275–296. https://doi.org/10.1007/s10722-012-9833-7

565 Hay F. R., Valdez R., Lee J. S., Sta Cruz P. C. (2019) Seed longevity phenotyping:
566 recommendations on research methodology. In Journal of experimental botany, **70**, 425–434.
567 https://doi.org/10.1093/jxb/ery358

568 ISTA (2018) International Rules for Seed Testing. International Seed Testing Association,
569 Bassersdorf, Switzerland.

570 Kurek K., Plitta-Michalak B., Ratajczak E. (2019) Reactive oxygen species as potential drivers of
571 the seed aging process. *Plants*, **8**, 174. https://doi.org/10.3390/plants8060174

572 Lev J., Blahovec J. (2017) Imbibition of wheat seeds: Application of image analysis. International
573 *Agrophysics*, **31**, 475–481. https://doi.org/10.1515/intag-2016-0072

574 Louf J.-F., Zheng Y., Kumar A., Bohr T., Gundlach C., Harholt J., Poulsen H. F., Jensen K. H.
575 (2018) Imbibition in plant seeds. *Physical Review E*, **98**, 1–6.
576 https://doi.org/10.1103/PhysRevE.98.042403

577 Macovei A., Caser M., Doná M., Valassi A., Giovannini A., Carbonera D., Scariot V., Balestrazzi A.
578 (2016) Prolonged cold storage affects pollen viability and germination along with hydrogen
579 peroxide and nitric oxide content in rosa hybrida. *Notulae Botanicae Horti Agrobotanici Cluj-*
580 *Napoca*, **44**, 6–10. https://doi.org/10.15835/nbha44110357

581 Mondoni A., Orsenigo S., Müller J.V., Carlsson-Graner U., Jiménez Alfaro B., Abeli T. (2018) Seed
582 dormancy and longevity in subarctic and alpine populations of *Silene suecica*. *Alpine Botany*, **128**,
583 71–81. DOI: 10.1007/s00035-017-0194-x

584 Nagel M., Kranner I., Neumann K., Rolletschek H., Seal C. E., Colville L., Fernández-Marín B.,
585 Börner A. (2015) Genome-wide association mapping and biochemical markers reveal that seed
586 ageing and longevity are intricately affected by genetic background and developmental and
587 environmental conditions in barley. *Plant, Cell and Environment*, **38**, 1011–1022.
588 https://doi.org/10.1111/pce.12474

589 Nagel M., Rosenhauer M., Willner E., Snowdon R. J., Friedt W., Börner A. (2011) Seed longevity in
590 oilseed rape (Brassica napus L.)-genetic variation and QTL mapping. *Plant Genetic Resources:*
591 *Characterisation and Utilisation*, **9**, 260–263. https://doi.org/10.1017/S1479262111000372

592 Newton R., Hay F., Probert, R. (2009) Protocol for comparative seed longevity testing. Technical
593 Information Sheet_01. London, Royal Botanic Gardens Kew.

594 Oñate-Sánchez L., Vicente-Carbajosa J. (2008) DNA-free RNA isolation protocols for Arabidopsis
595 thaliana, including seeds and siliques. *BMC Research Notes*, **1**, 93. https://doi.org/10.1186/1756-
596 0500-1-93

597 Orsenigo S., Guzzon F., Abeli T., Rossi G., Vagge I., Balestrazzi A., Mondoni A., Müller J.V. (2017)
598 Comparative germination responses to water potential across different populations of *Aegilops*
599 *geniculata* and cultivar varieties of *Triticum durum* and *Triticum aestivum*. *Plant Biology*, **19**, 165–
600 171. https://doi.org/10.1111/plb.12528

601 Pfaffl M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR.
602 *Nucleic Acids Research*, **29**, e45. https://doi.org/10.1093/nar/29.9.e45

603 Pritchard H. W. (2004) Classification of seed storage 'types' for ex situ conservation in relation to
604 temperature and moisture. In E. O. Guerrant, K. Havens, & M. Maunder (Eds.), *Ex situ Plant*

Conservation: Supporting Species Survival in the Wild, Island Press, Washington, D.C., USA: 139–161.

Probert R. J., Daws M. I., Hay F. R. (2009) Ecological correlates of ex situ seed longevity: a comparative study on 195 species. *Annals of Botany*, **104**, 57–69. https://doi.org/10.1093/aob/mcp082

Roach T., Nagel M., Börner A., Eberle C., Kranner I. (2018) Changes in tocochromanols and glutathione reveal differences in the mechanisms of seed ageing under seedbank conditions and controlled deterioration in barley. *Environmental and Experimental Botany*, **156**, 8–15. https://doi.org/10.1016/j.envexpbot.2018.08.027

Sano N., Rajjou L., North H. M., Debeaujon I., Marion-Poll A., Seo M. (2016) Staying alive: Molecular aspects of seed longevity. *Plant and Cell Physiology*, **57**, 660–674. https://doi.org/10.1093/pcp/pcv186

Schwember A. R., Bradford K. J. (2010) Quantitative trait loci associated with longevity of lettuce seeds under conventional and controlled deterioration storage conditions. *Journal of Experimental Botany*, **61**, 4423–4436. https://doi.org/10.1093/jxb/erq248

van Treuren R., Bas N., Kodde J., Groot S. P. C., Kik C. (2018) Rapid loss of seed viability in ex situ conserved wheat and barley at 4°C as compared to -20°C storage. *Conservation Physiology*, **6**. https://doi.org/10.1093/conphys/coy033

Walters C. (1998) Understanding the mechanisms and kinetics of seed aging. *Seed Science Research*, **8**, 223-244. https://doi.org/10.1017/S096025850000413X

Walters C., Ballesteros D., Vertucci V. A. (2010) Structural mechanics of seed deterioration: Standing the test of time. *Plant Science*, **176**, 565–573. https://doi.org/10.1016/j.plantsci.2010.06.016

Walters C., Wheeler L. M., Grotenhuis J. M. (2005). Longevity of seeds stored in a genebank: species characteristics. *Seed Science Research*, **15**, 1–20. https://doi.org/10.1079/SSR2004195

Wickham H. (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

Wickham H, François R., Henry L., Müller K. (2021) A Grammar of Data Manipulation [R package dplyr version 1.0.7]. https://cran.r-project.org/package=dplyr

Wickham H. (2021). Tidy Messy Data [R package tidyr version 1.1.3]. https://CRAN.R-project.org/package=tidyr

Zinsmeister J., Leprince O., Buitink J. (2020) Molecular and environmental factors regulating seed longevity. *Biochemical Journal*, **477**, 305–323. https://doi.org/10.1042/BCJ20190165

638 **Figure Legends**

639 **Figure 1** Survival curves fitted by probit analysis of *Ae. tauschii* a) AA seeds, b) SB seeds, and *T.*
640 *boeoticum* c) AA seeds, d) SB seeds. AA = artificial ageing, SB = ageing in seed bank conditions
641 (cold storage)

642 **Figure 2** Heatmaps representing the Log2 fold-changes, i.e. ratios, of ROS levels and relative
643 gene expression in **a)** *Ae. tauschii* and **b)** *T. boeoticum* artificially aged (AA) seeds compared to
644 the fresh controls (**a**, 0d; **b**, 21d). In **b)**, ROS levels and gene expression were compared to
645 controls also for 0d seeds, i.e. before after-ripening. *APX*, ascorbate peroxidase; *CAT*, catalase;
646 *DHAR*, dehydroascorbate reductase; *GSR*, glutathione sulfo-reductase; *MDAR*, mono-
647 dehydroascorbate reductase; *SOD*, superoxide dismutase. **a)** 39d, 39 days of AA, corresponding
648 to the p_{50} of morph A. 49d, 49 days of AA, corresponding to the P50 of morph B. **b)** 0d, fresh,
649 dormant seeds before the AA treatment. 37d, 37 days of AA, corresponding to the p_{50} of morph A.
650 51d, 51 days of AA, corresponding to the p_{50} of morph B.

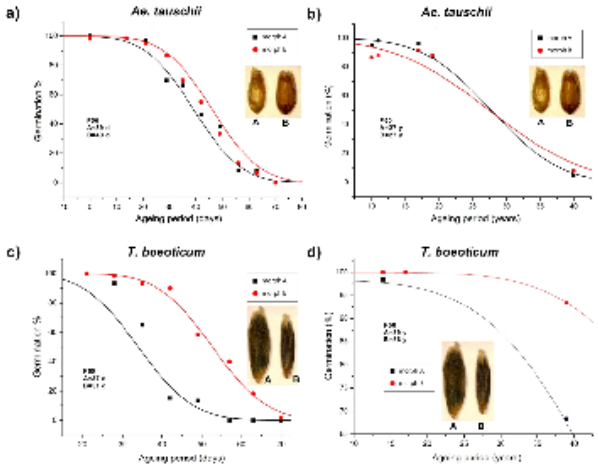
651 **Figure 3** Heatmaps representing the Log2 fold-changes, i.e. ratios, of ROS levels and relative
652 gene expression in **a)** *Ae. tauschii* and **b)** *T. boeoticum* seeds aged in seed bank conditions (cold
653 storage, SB) compared to the fresh controls (**a**, 0d; **b**, 21d). *APX*, ascorbate peroxidase; *CAT*,
654 catalase; *DHAR*, dehydroascorbate reductase; *GSR*, glutathione sulfo-reductase; *MDAR*, mono-
655 dehydroascorbate reductase; *SOD*, superoxide dismutase. **a)** *Ae. tauschii*: T78, seed accession
656 harvested and stored in 1978; T99, 1999 seed accession; T08, 2008 seed accession. **b)** *T.*
657 *boeoticum*: B80, 1980 seed accession; B02, 2002 seed accession; B05, 2005 seed accession.

658

Tables

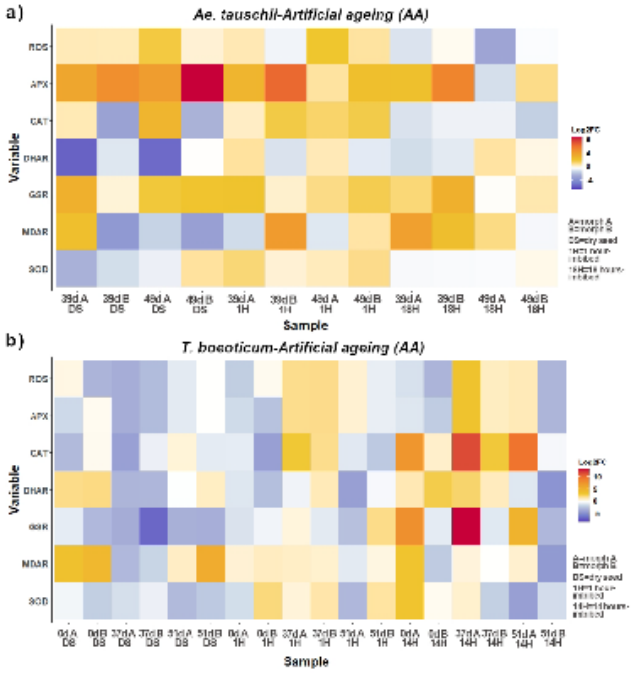
Table 1: a) ageing times of the *Ae. tauschii* and *T. boeoticum* seed accessions tested in this study: seeds were tested fresh, artificially aged, AA (method: controlled ageing test, CAT) and aged in cold storage (SB, seed bank). d=days; y=years. b) harvest year of the accessions aged in the cold chambers. Control=seed lots used as controls in the molecular analyses, fresh (0d) for Ae. tauschii, after-ripened (21d) for T. boeoticum.

a)	*Ae. tauschii* (AE 278)	*T. boeoticum* (TRI 10061)
	Ageing time	
Control (d)	0	21
Artificially aged, AA (d)	14, 21, 29, 35, 42, 49, 56, 63, 70	14, 21, 28, 35, 42, 49, 56, 63, 70
Aged in cold storage, SB (y)	10, 11, 17, 19, 40	14, 17, 38
b)	*Ae. tauschii* (AE 278)	*T. boeoticum* (TRI 10061)
Cold-stored accessions	1978, 1999, 2001, 2007, 2008	1980, 2002, 2005



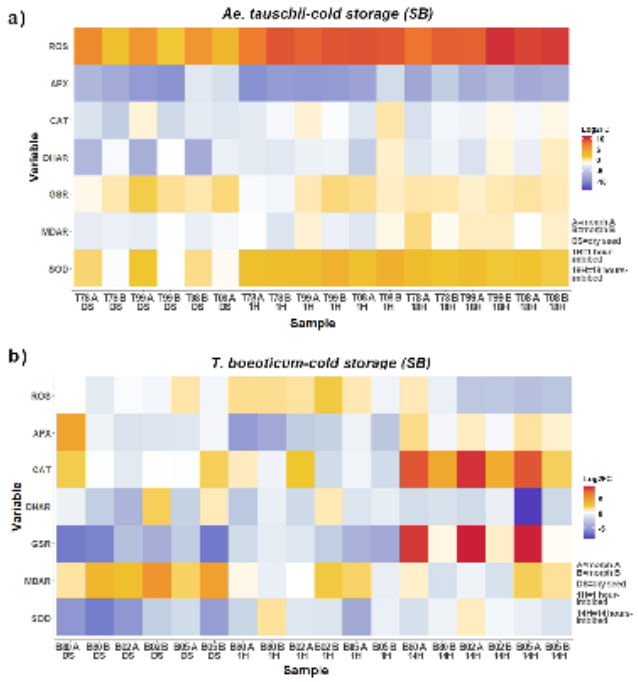
Survival curves fitted by probit analysis of Ae. tauschii a) AA seeds, b) SB seeds, and T. boeoticum c) AA seeds, d) SB seeds. AA = artificial ageing, SB = ageing in seed bank conditions (cold storage)

263x208mm (300 x 300 DPI)



Heatmaps representing the Log2 fold-changes, i.e. ratios, of ROS levels and relative gene expression in a) Ae. tauschii and b) T. boeoticum artificially aged (AA) seeds compared to the fresh controls (a, 0d; b, 21d). In b), ROS levels and gene expression were compared to controls also for 0d seeds, i.e. before after-ripening. APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; GSR, glutathione sulfo-reductase; MDAR, mono-dehydroascorbate reductase; SOD, superoxide dismutase. a) 39d, 39 days of AA, corresponding to the p50 of morph A. 49d, 49 days of AA, corresponding to the P50 of morph B. b) 0d, fresh, dormant seeds before the AA treatment. 37d, 37 days of AA, corresponding to the p50 of morph A. 51d, 51 days of AA, corresponding to the p50 of morph B.

116x126mm (300 x 300 DPI)



Heatmaps representing the Log2 fold-changes, i.e. ratios, of ROS levels and relative gene expression in a) Ae. tauschii and b) T. boeoticum seeds aged in seed bank conditions (cold storage, SB) compared to the fresh controls (a, 0d; b, 21d). APX, ascorbate peroxidase; CAT, catalase; DHAR, dehydroascorbate reductase; GSR, glutathione sulfo-reductase; MDAR, mono-dehydroascorbate reductase; SOD, superoxide dismutase. a) Ae. tauschii: T78, seed accession harvested and stored in 1978; T99, 1999 seed accession; T08, 2008 seed accession. b) T. boeoticum: B80, 1980 seed accession; B02, 2002 seed accession; B05, 2005 seed accession.

117x127mm (300 x 300 DPI)

Gianella M, Doria E, Dondi D, Milanese C, Gallotti L, Börner A, Zannino L, Macovei A, Pagano A, Guzzon F, Biggiogera M, Balestrazzi A. Physiological and molecular aspects of seed longevity: a case study of a Pisum sativum L. accession with wrinkled seeds. *Ann Bot*. Submitted

Original Article

Physiological and molecular aspects of seed longevity: a case study of a *Pisum sativum* L. accession with wrinkled seeds

Maraeva Gianella[1], Enrico Doria[1], Daniele Dondi[2], Chiara Milanese[2], Lucia Gallotti[2], Andreas Börner[3], Lorena Zannino[1], Anca Macovei[1], Andrea Pagano[1], Filippo Guzzon[4], Marco Biggiogera[1], Alma Balestrazzi[1,*]

[1] Department of Biology and Biotechnology 'L. Spallanzani', University of Pavia, via Ferrata 9, 27100, Pavia, Italy [2] Department of Chemistry, University of Pavia, Viale Taramelli 12, 27100 Pavia, Italy [3] Genebank Department, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) Correnssstr. 3, D-06466 Seeland, OT Gatersleben, Germany [4] International Maize and Wheat Improvement Center (CIMMYT), Carretera México-Veracruz, Km. 45, El Bátan, Texcoco, Mexico State, Mexico

** For correspondence. E mail alma.balestrazzi@unipv.it*

Running Title: Long-term longevity of pea wrinkled seeds

- **Background and Aims** Conservation of plant genetic diversity is fundamental for crop improvement and for increasing agricultural production and sustainability, especially in the face of climatic changes. Although seed longevity is essential for the management of seed banks, only few studies have so far addressed differences in this trait among the accessions of a single crop species. Eight *Pisum sativum* L. (pea) accessions were investigated to study the impact of long-term (~ 20 years) ageing, revealing contrasting seed longevity profiles. The outstanding longevity observed in the wrinkled seeds of G4 accession provided a unique experimental system.
- **Methods** Reactive oxygen species, lipid peroxidation, tocopherols, free proline and reducing sugars were measured. Thermogravimetric, differential scanning calorimetry, and transmission electron microscopy combined with immunohistochemical analysis were performed.
- **Key Results** Differently from the other tested accessions, the long-lived G4 seeds neither consumed tocopherols during storage nor showed free proline accumulation, as deterioration hallmark, whereas reducing sugars were not affected. Thermal decomposition suggested a biomass profile compatible with the presence of low molecular weight molecules. Expansion of heterochromatic areas and reduced occurrence of γH2AX foci were highlighted in the nucleus of G4 seeds.
- **Conclusions** The long-lived G4 phenotype correlates with the occurrence of a reducing cellular environment and a nuclear ultrastructure favourable to genome stability. This work brings novelty to the study of within-species variations in seed longevity, underlining the relevance of multidisciplinary approaches in seed longevity studies.

Key words: seed longevity, long term storage, *Pisum sativum* L., reactive oxygen species, lipid peroxidation, nuclear ultrastructure, γH2AX foci

INTRODUCTION

Agricultural production must be enhanced to cope with the growing human population and, at the same time, it has to become more sustainable to minimize detrimental impacts of agricultural activities on ecosystems (Godfray *et al*., 2010; Asseng *et al*., 2015; Nelson *et al*., 2018). To this end, conservation, accessibility and use of genetic diversity in crop improvement are key factors to improve crop varieties and increase agricultural production and sustainability in the face of climatic changes (Esquinas-Alcázar, 2005; McCouch *et al*., 2013). One of the most effective strategies to ensure availability of plant genetic resources is *ex situ* conservation in seed banks. Seedbanking allows the long-term conservation of large numbers of orthodox species with limited costs (Li and Pritchard, 2009; McCouch *et al*., 2013; Davies and Allender, 2017; Riviere and Muller, 2017).

Despite the fundamental relevance of seed longevity for the management of seed banks (Colville and Pritchard, 2019), there are few available studies addressing differences in seed longevity among the accessions of a single crop species, especially in relation to different seed phenotypes (Guzzon *et al*., 2021). Furthermore, the physiological and molecular bases of such a complex trait, shaped by genetic and environmental factors, still need to be fully clarified (Sano *et al*., 2015; Lee *et al*. 2019; Zinsmeister *et al*., 2020). Temperature and moisture content are the major exogenous factors influencing seed longevity in storage (Roberts, 1973; Ellis and Roberts 1980; Dickie *et al*., 1990). Oxidative stress, the main driving force of seed ageing, results from reactive oxygen species (ROS) accumulation and decreased antioxidant capacity (Kurek *et al*., 2019). Seed deterioration can be monitored measuring the by-products of lipid peroxidation (Bailly *et al*., 1996; Wiebach *et al*., 2020). Among the cellular antioxidants, tocopherols prevent membrane deterioration by scavenging lipid peroxy radicals during seed storage and modifications in their biosynthesis result in

reduced longevity (Chen *et al*., 2016). Oligosaccharides, favoring the seed glassy state by increasing cytoplasmatic viscosity, can also improve storability (Buitink *et al*., 2000; Lehner *et al*., 2008; Ebone *et al*., 2019). However, according to Gurusinghe and Bradford (2001) changes in oligosaccharide contents alone cannot account for poor seed longevity particularly in the context of post-priming storage whereas a negative correlation between monosaccharide levels and desiccation tolerance has been reported (Hoekstra *et al*., 2001).

Seed longevity is tightly linked to genome maintenance, being the loss of DNA integrity a typical hallmark of seed deterioration (Kranner *et al*., 2010; El-Maarouf-Bouteau *et al*., 2011; Córdoba-Cañero et al., 2014; López-Fernández *et al*., 2018). Following long-term storage, successful germination is dependent on the DNA damage response, namely a plethora of DNA damage sensing, signaling and repair pathways activated during imbibition (Balestrazzi *et al*., 2011; Diaz and Pecinka, 2017; Waterworth *et al*., 2019). In deteriorated seeds, prolonged DNA repair is required before the onset of DNA replication (Elder et al., 1987). The cellular repair pathways can also influence seed longevity in response to environmental changes (Mondoni et al., 2014). In both animals and plants, phosphorylation of histone H2AX in proximity of DNA double strand breaks (DSBs) sites is regarded as the earliest DDR hallmark which triggers DSB repair (Branzei and Foiani, 2008; Waterworth *et al*., 2011). The phosphorylated histone variant γ-H2AX reflects DNA damage accumulation and it has been used to assess the kinetics of DSB repair in actively dividing plant tissues (Charbonnel *et al*., 2010; Hirakawa and Matsunaga, 2019). According to Waterworth *et al.* (2019), *Arabidopis thaliana h2ax* mutants showed increased sensitivity to seed ageing, highlighting the role of histone H2AX in promoting genome maintenance in the context of seed germination and longevity. Changes in chromatin structure also contribute to genome maintenance in the desiccated state as well as during rehydration (Waterworth *et al*., 2019). High chromatin condensation provides protection against oxidative damage and double-strand breakage (Falk *et al*., 2010; Takata *et al*., 2013). Changes in nuclear structure found in aged seeds, as reduced

heterochromatin density (Begnami and Cortelazzo, 1996; Burrieza *et al*., 2016) resembled the chromatin compaction profiles observed during seed maturation (van Zanten *et al*. 2011).

In the present work, eight *Pisum sativum* L. (pea) accessions were investigated using a multidisciplinary approach to dissect the impact of 20 years of ageing on cold-stored and room-temperature stored seeds, compared to freshly collected seeds. Pea is a crop of major relevance worldwide, with an estimated global harvested area of 4.3% and 10.8% of the total area of vegetables and legumes, respectively. Its production accounts for 2.7% and 30.6% of the global vegetables and legumes production (FAOSTAT, 2018). The long-term conservation of pea genetic resources is fundamental to keep available accessions that could serve as sources of favorable alleles (Holdsworth *et al*., 2017). The storage behaviour of pea seeds has been investigated in a few studies (Nagel *et al*., 2010; Ellis *et al*., 2018; Redden and Partington, 2019; Ballesteros and Walters, 2019) but differences in seed longevity observed among different genotypes still need to be fully clarified. A comparative, multidisciplinary analysis was performed in different pea accessions in order to define the contribution of different cellular and metabolic factors to their seed longevity profiles.

MATERIALS AND METHODS

Plant material and germination tests

Seeds of four yellow pea (*Pisum sativum* L.) accessions (PIS 2, PIS 8, PIS 15, and PIS 224) and four green pea accessions (PIS 686, PIS 706, PIS 783, and PIS 2865) were kindly provided by the Genebank Department of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben (Germany). Yellow and green accessions were renamed Y1, Y2, Y3, Y4 and G1, G2, G3, G4, respectively (Fig. 1a; Supplementary Table S1). For each accession, both fresh (F) seeds harvested in 2019, and stored seeds, harvested in 2001,

were tested. After harvest, seeds were transferred to a drying chamber (22 ± 2°C, 11 ± 3 % relative humidity). After three weeks, samples were split into A and R lots. As for stored seeds, one lot (A) was kept at cold chamber under controlled conditions (-18 ± 2°C, 8 ± 2 % seed moisture content) and another lot (R) was conserved at room temperature conditions (20 ± 2°C, 9 ± 2 % seed moisture content). For determining the seed moisture content, 30 seeds per replicate were grinded and analysed using a moisture meter (Precisa XM 66, Switzerland). For each seed lot, three replicates of 20 seeds were sown in Petri dishes with 1% agar as substrate and then placed in a growth chamber (22 ± 2°C, 70-80 % RH, 150 µmol m^{-2} s^{-1} photon density, 16/8 h photoperiod). Petri dishes were checked every 12 h for germination for 14 days and seeds scored as germinated once the radicle had reached 2 mm length. Germination was evaluated using the following parameters: *G* (germinability), *MGT* (mean germination time), *MGR* (mean germination rate), and *Z* (synchronization index), as described by Ranal and Garcia De Santana (2006) (Supplementary Table S2).

ROS detection

The fluorogenic dye 2',7'-dichlorofluorescein diacetate (DCF-DA; Sigma-Aldrich, Milan Italy) was used to quantify ROS levels released from dry 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 (DCF). The assay was carried out as described by Forti *et al.* (2020), with the following modifications. Dry seeds (five seeds per replicate) were incubated in the dark for 30 min with 500 µl of a 10 µM DCF-DA solution. Subsequently, three replicates (50 µl each) per seed lot were pipetted into 0.2 ml tubes and the emitted fluorescence was measured using the green channel (510 ± 5 nm) of a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia), after a single cycle of 30 s at 25°C. As negative control, three replicates containing only DCF-DA were used to

subtract the baseline fluorescence. Relative fluorescence was calculated by normalizing samples to controls and on the seed mass, then expressed as Relative Fluorescence Units (R.F.U.).

Determination of MDA levels

Malondialdehyde (MDA) levels were quantified as reported (Doria *et al.*, 2019). For each accession, 30 dry seeds were grinded in a Retsch Mixer Mill M 301 (Retsch-Allee, Haan, Germany) three times for 30 s at the vibrational frequency of 30 Hz s^{-1}. For each lot, the resulting powder was divided into three replicates (0.2 g each) that were resuspended with 5 ml of a H_2O: 0.5 M $HClO_4$ solution (4:1) with 2 % BHT (butylated hydroxytoluene, Sigma-Aldrich) in ethanol to precipitate proteins. Samples were subsequently centrifuged (4°C, 10 min). MDA was determined as a thiobarbituric acid reactive substance (TBARS), following its reaction with thiobarbituric acid (TBA, Sigma-Aldrich) at high temperature. For each sample, an aliquot of 100 µl was mixed with 100 µl of TBA in 1 ml dH_2O and the mixture was heated in a boiling water bath at 95°C for 60 min. Test tubes were cooled at room temperature and absorbance was measured at 254 nm using an UV-visible spectrophotometer (UV-1800, Shimadzu, U.K.). A standard MDA (Sigma-Aldrich) solution (100 µl, in a range of 0.025-0.1 mg ml^{-1}) was added in a 1 ml test tube and mixed with TBA (100 µl) as previously described (Supplementary Fig. S1a).

Extraction and analysis of tocopherols

The extraction procedure was performed as described by Doria *et al.* (2019). For each accession, 30 dry seeds were grinded as previously described. For each lot, an aliquot (0.5 g)

of seed powder was added to 5 ml of ethanol containing 0.1 % butylated hydroxytoluene (BHT, Sigma-Aldrich) and the mixture was incubated for 10 min at 85°C. Subsequently, samples were subjected to saponification by adding 150 µl of 80 % KOH and incubated for 10 min. After adding 3 ml of H_2O, samples were placed on ice for 3 min, then 3 ml of pure hexane were added. After shaking 10 min at 800 rpm and centrifuging at 12.000 rpm, the upper phase was transferred into a separate test tube, and pellet was re-extracted using 2 ml hexane. The combined hexane fractions were washed with 3 ml of dH_2O, vortexed, centrifuged for 10 min and transferred into another test tube. Hexane fractions were dried used a vacuum evaporator and the residue dissolved in 200 µl acetonitrile:methanol:dichloromethane 45:20:35 (v/v/v) prior to injection into a HPLC system (Kontron Instrument 420 system, Kontron Instruments, Munich, Germany) equipped with a C18 column (Zorbax ODS column 250 x 4.6 mm, 5 µ, Agilent Technologies). The isocratic mobile phase consisted of acetonitrile:methanol (60:40) (v/v), flow rate was 1.0 ml min^{-1} at room temperature and absorbance was measured at 220 nm. As standard, γ-tocopherol (Sigma-Aldrich) was used for a calibration curve and identified in the chromatogram (Supplementary Fig. S1b).

Determination of free proline content

Free proline content was measured as described by Abrahám *et al.* (2010) with the following modifications. For each accession, 30 dry seeds were grinded as previously described. The seed powder (0.1 g) was added to 500 µl of 3 % sulfosalicylic acid (Sigma-Aldrich). Following centrifugation at 13.000 rpm for 5 min, a 100 µl aliquot of the extract was added to 500 µl of 3 % sulfosalicylic acid: glacial acetic acid: acidic ninhydrin (1:2:2) (v/v/v). The reaction of ninhydrin with free proline was carried out at 96°C for 60 min and stopped on ice.

Samples were then extracted with 1 ml of toluene. After 20 s vortex, phases were allowed to separate. The upper phase was transferred to quartz cuvettes and absorbance was read at 520 nm using an UV-visible spectrophotometer (UV-1800, Shimadzu) and toluene as reference. A standard proline solution (100 µl, in a range of 0.001-0.1 mg ml^{-1}) was prepared, added to a 2 ml test tube and mixed with the ninhydrin solution as previously described (Supplementary Fig. S1c).

Spectrophotometric determination of reducing sugars

The content of reducing sugars was measured as described by Miller (1959). For each accession, 30 dry seeds were grinded as previously described. The seed powder (0.5 g) was added to 5 ml of dH_2O, vortexed and incubated 2 h at 80°C in a water bath. After centrifugation at 3500 rpm for 15 min, the upper phase was transferred in new test tubes and the content of reducing sugars was quantified using DNS (3,5-dinitrosalycilic acid, Sigma-Aldrich) solution. Absorbance was read at 540 nm using an UV-visible spectrophotometer (UV-1800, Shimadzu) and dH_2O as reference. Standard solutions of glucose in the range of 0.4-1.5 mg ml^{-1} were used (Supplementary Fig. S1d).

Thermogravimetric analysis and differential scanning calorimetry

Inorganic content and thermal decomposition of the pea seed biomass were investigated by thermogravimetric analysis (TGA) in an air and nitrogen environment, respectively, using a Mettler Toledo TGA 1 instrument with a fixed heating rate of 20°C min^{-1}. The temperature range was from 25 to 800°C with a gas flow in the oven (air or nitrogen) of 4 l h^{-1}. For each

accession, 30 dry seeds were grounded into powder and sieved to a size of 100 μm. About 5 mg of sample was used in each test.

Nuclear staining with Toluidine Blue

Embryos excised from four dry seeds of Y1 and G4 accessions, respectively, were fixed with 2 % paraformaldehyde/0.2 % glutaraldehyde (Sigma-Aldrich) for 3 h at 4°C. Embryos were rinsed in phosphate-buffered saline (PBS, pH 7.2) overnight, and then incubated in 0.5 M NH_4Cl for 30 min at room temperature. Semithin sections (500 nm in thickness) were cut using an ultramicrotome, embedded in acrylic LR-White resin (Agar Scientific, Stansted, UK) and allow to harden at 60°C overnight. Toluidine blue staining was performed by covering the tissue sections, prepared as previously described, with a drop of the die and incubating for 5 min at 100°C. Sections were then washed thoroughly with dH_2O to remove die excess, airdried, mounted in Mowiol (Sigma Aldrich) and finally imaged using Zeiss Axioskop 2 plus microscope.

Immunodetection of γH2AX foci

In order to detect the occurrence of γH2AX foci in the nucleus, sections prepared as previously described were subjected to indirect immunohistochemical reaction by incubating them with the primary antibody Phospho-Histone H2A.X (Ser139) Polyclonal Antibody from rabbit (ThermoFisher Scientific, Milan, Italy) according to the Supplier's suggestions and subsequently with a secondary antibody coupled with 12 nm colloidal gold grain. Sections were stained by EDTA regressive technique (Berhard, 1969) and observed with a Jeol JEM-2100Plus electron microscope equipped with a 30 mm objective aperture and operating at 80 kV. Images were submitted to morphometric analyses using the software ImageJ

(https://imagej.nih.gov/ij). The results are expressed as mean values ± SEM. For each sample, 10 nuclei were scored for the presence of γH2AX foci. The density of foci was calculated as follows: 100 squares (each one with an area of 400 nm^2) were identified and the number of foci per single area was counted. The measurement was performed considering 10 cells for each sample and 10 squares per single cell.

Statistical analysis

Statistical analysis was performed in IBM SPSS 21.0 and in R environment for statistical computing and graphics (studio version 4.0.2). The following packages were used: *plyr* (Wickham, 2011), *ggplot2* (Wickham, 2016), *corrplot* (Wei and Simko, 2017), *ppcor* (Kim, 2015), *multcomp* (Hothorn *et al*., 2008), and *lsmeans* (Lenth, 2016). After checking data for normality and homoscedasticity, generalized linear models (GLM) were applied to evaluate the effect of accession, type of conservation, their interaction on different variables (germination parameters, ROS levels, chlorophyll content, tocopherols, MDA, free proline, reducing sugars); post-hoc Tukey's or Bonferroni tests were used to perform multiple comparisons. A two-way ANOVA was used to determine the effect of accession and conservation on the temperature of glass transition (Tg). A heatmap was used to represent the Log2 fold changes (Log2FC) between fresh (F) and aged (A, R) seeds in terms of mean MDA, tocopherols, free proline and reducing sugars contents. Correlations were performed with Pearson or Kendall's Tau-b tests. Partial correlations were computed to evaluate the correlation between ROS content and germination percentage controlling for the effect of other variables (MC, Accession, Conservation).Statistical analysis to quantify the occurrence of γH2AX foci in the nucleus was performed using the two-tailed paired Student's *t* test.

RESULTS

Germination profiles reveal genotype-dependent changes in pea seed longevity

Germination tests were performed with seeds from the yellow (Y1, Y2, Y3, Y4) and green (G1, G2, G3, G4) pea accessions. Both fresh seeds (F) and seeds stored under different environments (A, cold chamber; R, room-temperature conditions) were analyzed (Fig. 1b). No significant difference in terms of germination percentage was observed between F and A seeds in all the tested accessions. However, significantly lower germination percentages were observed in R seeds when compared to F ($P < 0.01$), with the exception of G4 variety. The latter conserved nearly 100 % germination in F, A and R conditions, revealing an impressive longevity profile (Fig. 1b, Supplementary Table S3). Accessions and storage conditions (F, A and R) had a statistically significant effect on germination percentage and *MGT*, as did the accession*conservation interaction ($P < 0.01$) (see Supplementary Table S4 for the corresponding Wald χ-squared values and d.f.). *MGT* of F seeds did not show significant differences among accessions ($P = 1$). Moreover, within the same accession no statistically significant difference was found in terms of *MGT* between F and A conditions ($P = 1$). On the other hand, a significant increase in MGT was observed for R seeds of all the accessions, when compared to their F and A counterparts ($P < 0.01$), except for G4, that did not show differences among the three conditions ($P = 1$). Overall, *MGR* showed a significant increase in A and R seeds when compared to F while *Z* did not show significant differences among accessions or conservation conditions (Supplementary Tables S3 and S4). In order to figure out any possible effects related to chlorophyll content on seed germination, chlorophyll *a* and *b* levels were measured in dry and imbibed seeds of the green pea accessions (Supplementary Fig. S2). No correlation was found between chlorophyll *a* and *b* total contents or their ratio and germination percentage (Supplementary Fig. S3).

F seeds. In the case of Y2, only the R seeds showed increased MDA contents ($P < 0.05$) (Supplementary Fig. S5a). In the long-lived G4 accession, the estimated MDA content of R seeds (1.7 ± 0.11 $\mu g/g_{FW}$) was similar to that found in F seeds (1.66 ± 0.06 $\mu g/g_{FW}$) (Fig. S5a).

In all the tested accessions, no significant difference in terms of total tocopherols content was observed in A seeds compared to F seeds. A significant ($P < 0.01$) decrease was found in R seeds of Y1 and Y2, compared with F seeds whereas in G4, the estimated total tocopherols content of F seeds (1.58 ± 0.11 mg/g_{FW}) was similar to that found in A (1.62 ± 0.16 mg/g_{FW}) and R seeds (1.62 ± 0.14 mg/g_{FW}) (Fig. 2; Supplementary Fig. S5b).

Proline accumulation, typically observed *in planta* under oxidative stress conditions, has been also reported during prolonged seed storage (Kong et al., 2015). Proline levels were significantly higher only in R seeds of Y1 ($P < 0.05$) and Y2 ($P < 0.01$), compared to F (Fig. 2; Supplementary Fig. S5c). Considering the documented role of reducing sugars in seed deterioration (Murthy and Sun, 2000), the levels of these metabolites were measured in the Y1, Y2, G1 and G4 accessions. A significant ($P < 0.01$) reduction of reducing sugars was observed in the R seeds of Y1, Y2, and G1 accessions (Fig. 2; Supplementary Fig. S5d). In G4, the reducing sugars content of F seeds (26.19 ± 0.41 mg/g_{FW}) was similar to that found in A (28.61 ± 1.72 mg/g_{FW}) and R seeds (29.40 ± 1.32 mg/g_{FW}) and these levels were significantly higher than those observed in the other accessions (Supplementary Fig. S5d). Accessions, storage conditions (F, A, R) and their interaction had a statistically significant effect on all the tested metabolites (Supplementary Table S5). A negative correlation with germination was observed for MDA content (Tau-b: -0.52; $P < 0.01$) and free proline levels (Tau-b: -0.61; $P < 0.01$) whereas a positive correlation was evidenced for total tocopherols content (Tau-b: 0.58; $P < 0.01$) and reducing sugars (Tau-b 0.58; $P < 0.01$). Overall, the metabolites hereby tested as hallmarks of oxidative damage and antioxidant response show accumulation profiles aligned with the germination phenotype and seed viability of the different pea accessions, indicating the impact of ageing at the cellular level. Despite ageing,

limited oxidative damage was detected in the G4 seeds, and this finding is consistent with the observed long-lived phenotype.

Thermal decomposition profiles suggest for the presence of low molecular weight components in G4 seeds

Temperatures of glass transition (Tg) were measured using differential scanning calorimetry. As shown in Supplementary Table S6, even though Tg was significantly different among accessions ($F = 28.634$; d, f = 3; $P < 0.01$) and conservation states ($F = 6.597$; d, f = 2; $P < 0.01$), Tg was not significantly correlated with germination percentage (Tau-b = - 0.04, $P = 0.806$). Moisture content was measured in all the pea accessions, for the different conservation conditions (F, A, R) (Supplementary Table S3). Although the accession*conservation interaction showed a significant effect on moisture content, no significant differences among accessions were consistent with their longevity profiles (Supplementary Table S4, Supplementary Fig. S6). Moisture content was also evaluated by thermogravimetric analysis (Fig. 3). The moisture content of the pea dry seeds was 10 ± 1 %, visible as a weight loss close to 100°C. TGA measurements were made both in air and in nitrogen atmosphere. Interestingly, under nitrogen atmosphere, curves showed a thermal decomposition profile including two steps: the first one occurred close to 250°C and the second one, more evident, was detected at 300°C. In order to better evidence the differences in composition between seeds, curves were translated to match the same dry weight at 170°C. This point was chosen because at this temperature the loss of water was completed, and thermal decomposition had not started yet. As shown in Fig. 3 and Supplementary Fig. S7, it is evident that those curves corresponding to the G4 seeds in the three different tested conditions started to lose material close to 200°C. This is indicated by the different slope observed for the G4 seeds in the range 200-300°C. This profile might correspond to the

decomposition and/or evaporation of relatively small molecules exclusively found in the wrinkled seeds. Periodic acid-Schiff (PAS) staining combined with transmission electron microscopy was used to localize the occurrence of polysaccharides in the pea embryo axes. The progression of the oxidation reaction within polysaccharides, and the consequent generation of aldehyde groups, is delayed in these high molecular weight polymers, featuring the occurrence of dark-stained dots. By contrast, oxidation is accelerated when low molecular weight oligosaccharides are the predominant substrates, resulting in staining of weak intensity. As for the G4 wrinkled seeds, in the cytoplasm of cell embryos the PAS reaction highlighted the occurrence of dots of low intensity (Supplementary Fig. S8, a and b) whereas dark-stained dots were observed in the PAS-treated Y1 cells (Supplementary Fig. S9a). Dark dots reflect the ongoing oxidation of polysaccharides in the Y1 cells while the poor signal detected in the G4 sample indicates that the same process was already concluded. The different reactivity hereby observed might reflect for different carbohydrate composition profiles in the seeds of the two pea accessions, particularly the occurrence of low molecular weight oligosaccharides in the G4 seeds, as suggested by the TGA measurements.

Nuclear architecture and DNA damage levels in aged seeds

The previously reported biochemical data evidenced the contrasting impact of ageing, in terms of oxidative damage, in the long-lived G4 seeds compared to Y1, Y2, and G1 accessions. In order to assess whether the observed oxidative stress profiles were associated with changes in nuclear ultrastructure and DNA damage levels, TEM-base analyses were carried out. This investigation was restricted to embryos excised from R seeds of the G4 and Y1 accessions, both subjected to the same ageing conditions but bearing significant differences in the cellular oxidative stress hallmaks. Preliminary nuclear staining with Toluidine Blue carried out on sections of pea embryo axes revealed distinctive nuclear

morphologies. The Y1 nuclei showed a compacted nucleolus (Fig. 4a, nu, arrow) surrounded by some dark blue-stained areas corresponding to heterochromatin domains (Fig. 4a, hc, arrow). Such morphology, recurrent in Y1 embryos, was different from that observed in G4 embryos. As shown in Fig. 4b (n), each G4 nucleus of the section contained larger and more uniform dark blue-stained areas, suggesting for an expansion of the heterochromatin regions. Such morphologies were further evidenced by TEM analysis. In the Y1 nuclei, large heterochromatin areas are visible, as expected when dehydration occurs (Fig. 4c, hc, arrows), as well as regions of decondensed euchromatin (Fig. 4c, ec). Some of these condensed heterochromatin regions are located close to the nucleolus. In the G4 nuclei, chromatin condensation patterns were remarkably enhanced, covering the entire nuclear area (Fig. 4d, hc). Such an unusual expansion of the packed chromatin might be indicative of an effective strategy driving genome maintenance in the G4 accession, possibly limiting long-term DNA damage accumulation in these long-lived seeds. Morphological profiles were then combined with the investigation of the γH2AX foci distribution.

Upon DNA damage, ATM phosphorylates the histone variant H2AX on Ser139 (Burma et al., 2001) and such modification (γH2AX) can spread for up to 1 Mb away from the break site, acting as a platform to recruit the DNA repair enzymes (Iacovoni et al., 2010). In order to map the γH2AX foci in the Y1 and G4 pea nuclei, immunocytochemical and TEM analyses were performed, using an antibody raised against the human histone variant H2AX on Ser139. Representative examples of the distribution of γH2AX foci in the nuclei of R seeds, in both Y1 and G4 accessions are shown in Fig. 5 (a and b). The estimated density of γH2AX foci, expressed as n° foci per 400 nm^2 of nuclear area, was significantly higher ($P < 0.05$) in the Y1 nuclei (1.6), compared to G4 nuclei (1.1). Thus, the limited genotoxic impact exerted by long-term storage on the wrinkled seeds might be the consequence of the highly compacted chromatin conformation previously described.

DISCUSSION

Assessment of seed longevity is crucial for the effective management of genebank collections. The pea collection analyzed in the present work provided a unique opportunity to investigate different physiological and molecular aspects of seed longevity under seed bank storage. Pea seed viability was maintained in samples stored under controlled cold storage conditions (-18°C) whereas a significant decrease occurred in samples kept at room temperature (20°C), except for the G4 accession, classified as a mutant of the variety named 'Frogel' (Auld et al., 1988). G4 seeds showed a wrinkled phenotype, frequently associated with the *r* (*rugosus*) locus in the *rr* configuration (Bhattacharyya et al., 1990; Rayner et al., 2017).

The comparative analysis of the eight pea accessions confirmed the inverse correlation between ROS levels and seed longevity. Fresh seeds, collected from plants of the different accessions regenerated at IPK in 2019, were also included in the study to provide information on pre-storage viability and compare results with long-term stored material (Porteous et al., 2019). Even if all accessions and seed lots were regenerated in the same location, we cannot rule out that different environmental factors (mainly temperature and rainfall), experienced by the mother plants of the same accessions in different regeneration years, might have influenced different seed traits (Mondoni et al., 2014). Given that, high and uniform germination of the F seeds of all accessions was observed, and therefore a comparison of germination and ROS levels of fresh and aged seeds was performed. In fresh seeds (F), all the accessions displayed ROS contents varying within a limited range. Indeed, ROS production in fresh seeds during post-harvest storage has been documented (Bailly et al., 2008). The low ROS levels observed in seeds aged for about 20 years under controlled conditions (A) correlated with the high germinability profiles of all the investigated accessions. The long-term storage at room temperature conditions (R) resulted in significant ROS accumulation in all the accessions showing a drop in germination, except for G4. This finding poses the

question about the mechanisms underlying the ability of G4 wrinkled seeds to control ROS levels. To address such issue, specific metabolites associated with the seed ability to scavenge the toxic free radicals were measured. The investigation was restricted to four pea accessions, namely Y1, Y2, G1, and G4, showing contrasting germination and ROS profiles.

ROS-driven oxidation targets polyunsaturated fatty acids, generating lipid peroxides (Gaschler and Stockwell, 2017). Similar levels of lipid peroxidation were detected in F and R seeds of the G4 accession, confirming its long-term oxidative stress resilience. The G4 seeds displayed the highest levels of the lipophilic antioxidant tocopherols, known for their ability to scavenge lipid peroxyl radicals (Fritsche et al., 2017), independent on their conservation state. Thus, it appears that there was no need to exploit the tocopherols pool in G4 seeds, since lipid peroxidation did not overcome a critical threshold. In the G1 accession, characterized by a low tocopherols content (approximately 50% less, compared to G4), longevity was compromised following long-term storage at room temperature conditions. Although tocopherols were utilized by the Y1 and Y2, this was not sufficient to avoid the germinability drop in R seeds. Free proline is an efficient ROS scavenger and a compatible osmolyte (Hayat et al., 2012; Liang et al., 2013). Increased free proline content contributed to oxidative stress adaptation in oat (*Avena sativa* L.) seeds with higher moisture content, stored for up to one year (Kong et al., 2015). A similar free proline content was detected in G1 and G4 accessions, in all the tested treatments whereas a significant accumulation occurred in the Y1 and Y2 seeds stored under room temperature conditions (R). This finding corroborates the role of free proline as a seed-specific oxidative damage marker for the first time during long-term ageing under seed bank conditions.

Fresh G4 seeds displayed significantly higher levels of reducing sugars, a feature of the wrinkled phenotype (Stickland and Wilson, 1983). The reducing sugars content of A and R wrinkled seeds did not decrease upon long-term storage, differently from what occurred in Y1, Y2, and G1. Reducing sugars participate in non-enzymatic protein glycosylation

(Maillard reaction) that, together with lipid peroxidation, is indicative of the biochemical deterioration associated with seed ageing (Murthy and Sun, 2000). The low ROS content of G4 seeds prevented damage whereas reducing sugars were engaged in the Maillard process triggered by the oxidative environment of Y1, Y2, and G1 seeds.

The research question that arises from the reported data is how the G4 seeds can maintain constitutive low ROS levels despite 20 years of storage, considering that ROS are continuously generated in an oxygenic environment and the activity of ROS scavenging enzymes is restricted in the glassy state of dry cytoplasm (Nagel et al., 2019). In such environment, the dry seed exploits the pool of available antioxidant compounds as observed for the Y and G accessions. The study of mechanical properties within the dry cytoplasm of pea embryonic axes has revealed low molecular mobility over a broad range of moisture contents and temperatures, possibly due to steric hindrance between adjacent macromolecules, and such features might contribute to seed longevity (Ballesteros and Walters, 2019). However, there is scanty information concerning the mechanical properties of the dry cytoplasm in wrinkled pea seeds and their possible role in longevity.

The TGA profiles recorded in the G4 seeds, independent on treatments, suggested for the presence of relatively small molecules. Indeed, in wrinkled pea seeds carrying mutations at the *r* and *rb* loci, alterations in the starch biosynthetic pathway result in pleiotropic effects such as accumulation of the raffinose family oligosaccharides (Gawlowska et al., 2017), associated with membrane stability (Crowe et al., 1992). At the moment, we cannot rule out the possibility that G4 seeds use specific low molecular-weight antioxidant molecules, e.g. glutathione and L-ascorbic acid, as redox buffer to maintain ROS within a threshold critical to ensure longevity. It has been reported that mutations at the *r* locus altering seed composition and hygroscopic properties, can affect seed longevity (Lyall et al., 2003). However, to our knowledge, this is the first study providing evidence of high longevity under long-term storage conditions in a wrinkled seed accession.

The ability to buffer ROS accumulation is expected to restrict genotoxic damage but certainly nuclear rearrangements might provide protection (Lee et al., 2020). In the G4 accession, almost all the nuclear area is filled with heterochromatin. Chromatin condensation occurring upon severe water loss might be promoted by increased levels of cations and changes of histone variants (Deltour, 1985; Washio, 2014). This aspect is still poorly explored in the context of seed longevity and the G4 accession might provide a unique working system for further investigations on the dynamics of nuclear architecture in response to desiccation. According to Bhattacharyya et al. (1993), the high sucrose content of pea wrinkled seeds enhances the embryo osmotic potential, increasing water up-take during seed development. Subsequently, during desiccation, excess water is lost causing the wrinkled phenotype. These shrinkage dynamics might lead to a tighter chromatin conformation useful for genome maintenance (Bhattacharyya et al., 1993).

The G4 seeds aged at room temperature showed a significantly lower frequency of the γH2AX foci, compared to the Y1 seeds. Chromatin compaction protects DNA from damage, but it also blocks the expansion of H2AX phosphorylation (Cann and Dellaire, 2011; Nair et al., 20127). Possibly, the high-longevity profile of G4 seeds and genotoxic stress resilience were positively influenced by such chromatin dynamics. These findings are in agreement with those described in both yeast and mammalian cells revealing that the γH2AX foci at double strand break sites were formed at lower levels in heterochromatin, when compared to euchromatin (Cowell et al., 2007; Kim et al., 2007). On the other hand, the protective effect of chromatin compaction might be related to non-histone chromatin proteins that physically shield the genomic DNA (Falk et al., 2008). Results from ultrastructural and immunohistochemical analyses should be also critically assessed considering the effect of seed moisture content, which was lower for the G4 sample (7.87 % for R seeds) compared to other samples (8.52-8.81 %). According to the Harrington's rule, even 1.0 % decrease in water content might double longevity (Harrington, 1960) whereas van Zanten et al. (2011)

showed that chromatin compaction is influenced by moisture content. At the moment, the possibility that the observed differences between G4 and Y1 seeds in nuclear shrinkage and γH2AX density might be due to a difference in moisture content cannot be ruled out.

The present work underlines the relevance of multidisciplinary approaches in seed longevity studies, particularly when addressing intra-species variations in longevity. The case study hereby reported brings to the forefront the redox context and some aspects of the genotoxic stress response as that contribute to the long-lived pea seed phenotype. Investigating the biological bases of these intra-species variations will be fundamental to identify groups of seed accessions with different longevity profiles, and therefore to plan seed banks activities in terms of viability monitoring and regeneration efforts, in the attempt not to lose potentially priceless genetic resources.

SUPPLEMENTARY DATA

Supplementary Data include: Table S1. Main features of the eight *Pisum sativum* L. accessions.

Table S2. Germination parameters. Table S3. Germination parameters and seed moisture content of the eight pea accessions. Table S4. Results of generalized linear models applied to germination parameters, moisture contents, and reactive oxygen species. Table S5. Results of generalized linear models applied to malondialdehyde, tocopherols, proline and reducing sugars content. Table S6. Results of differential scanning calorimetry analysis. Fig. S1. Standard curves (MDA, γ-tocopherol, proline, glucose). Fig. S2. Chlorophyll *a* and chlorophyll *b* contents measured in the four green pea accessions. Fig. S3. Results of Kendall's Tau-b test highlighting correlations between germination percentage and chlorophyll content. Fig. S4. ROS levels measured in pea seeds during imbibition, and results

of Kendall's Tau-b test highlighting correlations between ROS levels and germination percentage. Fig. S5. Mean values of malondialdehyde, tocopherols, proline, and reducing sugars contents in pea seeds. Fig. S6. Moisture content measured in pea seeds. Fig. S7. Representative example of thermogravimetric measurements for F seeds of G4 accession. Fig. S8. Transmission electron microscopy analysis of G4 dry embryos subjected to periodic acid-Schiff (PAS) staining. Fig. S9. Transmission electron microscopy analysis of Y1 dry embryos subjected to periodic acid-Schiff (PAS) staining.

ACKNOWLEDGMENT

This work was supported by the University of Pavia Crowdfunding (UNIVERSITIAMO) campaign "The other side of the seed" (https://universitiamo.eu/en/campaigns/the-other-side-of-theseed/), and 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 A.M., M.B., and A. Balestrazzi). A.P. is supported by a Departmental Research Fellowship (type A - COD. DBB2020-A01).

Author contributions: A.Börner, M.G, F.G., and A.Balestrazzi conceived and designed the study. M.G., E.D., D.D., C.M., L.G., and L.Z. performed the experiments. A.Balestrazzi and M.G. wrote the manuscript. E.D., D.D., C.M., A. Börner, A.M., A.P., F.G. and M.B. reviewed the manuscript. All authors read and approved the final manuscript.

LITERATURE CITED

Ábrahám M, Hourton-Cabassa C, Erdei L, Szabados L. 2010. Methods for determination of proline in plants. In: Sunkar R, ed. *Plant stress tolerance: methods and protocols*. Springer New York Dordrecht Heidelberg London, 316-331.

Assaker K, Rima J. 2019. A new spectrophotometric method for the analysis of fructose, glucose and sucrose, using 2-thiobarbituric acid and zero-valent iron powder (ZVIP). *Journal of Food Research* **8**: 48-55.

Asseng SF, Ewert P, Martre RP, et al. 2015. Rising temperatures reduce global wheat production. *Nature Climate Change* **5**: 143-147.

Auld DL, Erickson DA, Field LA, Dial MJ, Crock JC, Heikkeinen MK. 1988. Registration of foliar near isoline germplasms of *af*, *st* and *tl* in three cultivars and one breeding line of winter pea. *Crop Science* **28**: 579-580.

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. *Comptes Rendus Biologies* **331**: 806-814.

Bailly C, Benamar A, Corbineau F, Côme D. 1996. Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathione reductase activities in sunflower seeds as related to deterioration during accelerated aging. *Physiologia Plantarum* **97**: 104-110.

Balestrazzi A, Confalonieri M, Macovei A, Carbonera D. 2011. 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.

Ballesteros D, Walters C. 2019. Solid-state biology and seed longevity: a mechanical analysis of glasses in pea and soybean embryonic axes. *Frontiers in Plant Science* **10**, 920.

Begnami CN, Cortelazzo AL. 1996. Cellular alteration during accelerated ageing of French bean seeds. *Seed Science Technolology* **24**: 295-303.

Bhattacharyya MK, Smith AM, Ellis THN, Hedley C, Martin C. 1990. The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding a starch-branching enzyme. *Cell* **60**: P115-P122.

Branzei D, Foiani M. 2008. Regulation of DNA repair throughout the cell cycle. *Nature Reviews Molecular Cell Biology* **9**: 297-308.

Buitink J, Hemminga MA, Hoekstra FA. 2000. Is there a role for oligosaccharides in seed longevity? An assessment of intracellular glass stability. *Plant Physiology* **122**: 1217-1224.

Burma S, Chen BP, Murphy M, Kurimasa A. 2001. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *Journal of Biological Chemistry* **276**: 42462-42467.

Burrieza HP, Sanguinetti A, Michieli CT, Bertero HD, Maldonado S. 2016. Death of embryos from 2300-year-old quinoa seeds found in an archeological site. *Plant Science* **253**: 107-117.

Cann KL, Dellaire G. 2011. Heterochromatin and the DNA damage response: the need to relax. *Biochemistry and Cell Biology* **89**, 45-60.

Charbonnel C, Gallego ME, White CI. 2010. Xrcc1-dependent and Ku-dependent DNA double-strand break repair kinetics in Arabidopsis plants. *The Plant Journal* **64**: 280-290

Chen D, Li Y, Fang T, Shi X, Chen X. 2016. Specific roles of tocopherols and tocotrienols in seed longevity and germination tolerance to abiotic stress in transgenic rice. *Plant Science* **244**: 31-39.

Cowell IG, Sunter NJ, Singh PB, Austin CA, Durkacz BW, Tilby MJ. 2007. GammaH2AX foci form preferentially in euchromatin after ionising-radiation. *PLoS ONE* **2**: e1057.

Crowe JH, Hoekstra FA, Crowe LM. 1992. Anhydrobiosis. *Annual Reviews in Physiology* **54**: 579-599.

El-Maarouf-Bouteau H, Mazuy C, Corbineau F, Bailly C. 2011. DNA alteration and programmed cell death during ageing of sunflower seed. *Journal of Experimental Botany* **62**: 5003-5011.

Esquinas-Alcázar J. 2005. Science and society: protecting crop genetic diversity for food security: political, ethical and technical challenges. *Nature Review Genetics* **6**: 946-953.

Falk M, Lukasova E, Kozubek S. 2008. Chromatin structure influences the sensitivity of DNA to gamma-radiation. *Biochimica Biophysica Acta* **1783**: 2398-2414.

Falk M, Lukasova E, Kozubek S. 2010. High-order chromatin structure in DSB induction, repair and misrepair. *Mutation Research* **704**: 88-100.

FAOSTAT. 2018. http://www.fao.org/faostat/

Forti C, Ottobrino V, Bassolino L, et al. 2020. Molecular dynamics of pre-germinative metabolism in primed eggplant *(Solanum melongena* L.) seeds. *Horticulture Research* 7: 87.

Fritsche S, Wang X, Jung C. 2017. Recent advances in our understanding of tocopherol biosynthesis in plants: an overview of key genes, functions, and breeding of vitamin E improved crops. *Antioxidants* **6**: 99.

Gaschler MM, Stockwell BR. 2017. Lipid peroxidation in cell death. *Biochemical and Biophysical Research Communications* **482**: 419-425.

Gawlowska M, Swiecicki W, Lahuta L, Kaczmarek Z. 2017. Raffinose family oligosaccharides in seeds of *Pisum* wild taxa, type lines for seed genes, domesticated and advanced breeding materials. *Genetic Resources and Crop Evolution* **64**: 569-578.

Godfray HC, Beddington JR, Crute IR, et al. 2010. Food security: the challenge of feeding 9 billion people. *Science* **327**: 812-818.

Gurusinghe S, Bradford KJ. 2001. Galactosyl-sucrose oligosaccharides and potential longevity of primed seeds. *Seed Science Research* **11**: 121-133.

1 **Guzzon F, Gianella M, Velazquez Juarez JA, Sanchez Cano C, Costich DE. 2021.** Seed
2 longevity of maize conserved under germplasm bank conditions for up to 60 years. *Annals*
3 *of Botany* **127**: 775-785.
4 **Harrington GT. 1960.** Thumb rules of drying seed. *Crops and Soils* **13**: 16-17.
5 **Hayat S, Hayat Q, Nasser A, Wani AS, Pichtel J, Ahmad A. 2012.** Role of proline under
6 changing environments. **Plant Signaling and Behaviour 7**: 1456-1466.
7 **Hirakawa T, Matsunaga S. 2019.** Characterization of DNA repair foci in root cells
8 of *Arabidopsis* in response to DNA damage. *Frontiers in Plant Science* **10**: 990.
9 **Hoekstra FA, Golovina EA, Tetteroo FAA, Wolkers WF. 2001.** Induction of desiccation
10 tolerance in plant somatic embryos: how exclusive is the protective role of
11 sugars? *Cryobiology* **43**: 140-150.
12 **Holdsworth W, Gazave E, Cheng P, et al. 2017.** A community resource for exploring and
13 utilizing genetic diversity in the USDA pea single plant plus collection. *Horticulture*
14 *Research* **4**: 17017.
15 **Hothorn T, Bretz F, Westfall P. 2008.** Simultaneous inference in general parametric models.
16 *Biometrical Journal* **50**: 346-363.
17 **Kim JA, Kruhlak M, Dotiwala F, Nussenzweig A, Haber JE. 2007.** Heterochromatin is
18 refractory to γ-H2AX modification in yeast and mammals. *Journal of Cell Biology* **178**:
19 209-218.
20 **Kim S. 2015.** ppcor: An R package for a fast calculation to semi-partial correlation
21 coefficients. *Communications for Statistical Applications and Methods*, **22**: 665-674.
22 **Kong L, Huo H, Mao P. 2015.** Antioxidant response and related gene expression in aged oat
23 seed. *Frontiers in Plant Science* **6**:158.
24 **Kranner I, Minibayeva FV, Beckett RP, Seal CE. 2010.** What is stress? Concepts,
25 definitions and applications in seed science. *New Phytologist* **188**: 655-673.

1 **Kurek K, Plitta-Michalak B, Ratajczak E. 2019.** Reactive oxygen species as potential drivers
2 of the seed aging process. *Plants* **8**: 174.
3 **Iacovoni JS, Caron P, Lassadi I, et al. 2010.** High-resolution profiling of gammaH2AX
4 around DNA double strand breaks in the mammalian genome. *EMBO Journal* **29**: 1446-
5 1457.
6 **Lee JS, Velasco-Punzalan M, Pacleb M, et al. 2019.** Variation in seed longevity among
7 diverse Indica rice varieties. *Annals of Botany* **124**: 447-460.
8 **Lee JH, Kim EW, Croteau DL, Bohr VA. 2020.** Heterochromatin: an epigenetic point of
9 view in aging. *Experimental and Molecular Medicine* **52**: 1466-1474.
10 **Lehner A, Mamadou N, Poels P, Côme D, Bailly C, Corbineau F. 2008.** Changes in soluble
11 carbohydrates, lipid peroxidation and antioxidant enzyme activities in the embryo during
12 ageing in wheat grains. *Journal of Cereal Science* **47**: 555-565.
13 **Lenth RV. 2016.** Least-squares means: the R package lsmeans. *Journal of Statistical Software*
14 **69**: 12347.
15 **Li DZ, Pritchard HW. 2009.** The science and economics of *ex situ* plant conservation. *Trends*
16 *in Plant Science* **14**: 614-621.
17 **Liang X, Zhang L, Natarajan SK, Becker DF. 2013.** Proline mechanisms of stress survival.
18 *Antioxidant & Redox Signaling* **19**: 998-1011.
19 **López-Fernández MP, Moyano L, Correa MD, Vasile F, Burrieza HP, Maldonado S.**
20 **2018.** Deterioration of willow seeds during storage. *Scientific Reports* **8**: 17207.
21 **Lyall TW, Ellis RH, John P, Hedley CL, Wang TL. 2003.** Mutant alleles at the *rugosus* loci
22 in pea affect seed moisture sorption isotherms and the relations between seed longevity and
23 moisture content. *Journal of Experimental Botany* **54**: 445-450.
24 **McCouch S, Baute G, Bradeen J, et al. 2013.** Feeding the future. *Nature* **499**: 23-24.

Mondoni A, Orsenigo S, Donà M, et al. 2014. Environmentally induced transgenerational changes in seed longevity: maternal and genetic influence. *Annals of Botany* **113**: 1257-1263.

Murthy UMN, Sun WQ. 2000. Protein modification by the Amadori and Maillard reactions during seed storage: roles of sugar hydrolysis and lipid peroxidation. *Journal of Experimental Botany* **51**: 1221-1228.

Nagel M, Börner A. 2010. The longevity of crop seeds stored under ambient conditions. *Seed Science Research* **20**: 1-12.

Nagel M, Seal CE, Colville L, et al. 2019. Wheat seed ageing viewed through the cellular redox environment and changes in pH. *Free Radical Research* **53**: 641-654.

Nair N, Shoaib M, Storgaard Sorensen C. 2017. Chromatin dynamics in genome stability: roles in suppressing endogenous DNA damage and facilitating DNA repair. International *Journal of Molecular Science* **18**: 1486.

Nelson G, Bogard J, Lividini K, et al. 2018. Income growth and climate change effects on global nutrition security to mid-century. *Nature Sustainability* **1**: 773-781.

Porteous G, Nesbitt M, Kendon JP, et al. 2019. Assessing extreme seed longevity: the value of historic botanical collections to modern research. *Frontiers in Plant Science* **10**:1181.

Ranal MA, Garcia de Santana D. 2006. How and why to measure the germination process? *Revista Brasileira de Botânica* **29**: 1-11.

Rayner T, Moreau C, Ambrose M, Isaac PG, Ellis N, Domoney C. 2017. Genetic variation controlling wrinkled seed phenotypes in *Pisum*: how lucky was Mendel? *International Journal of Molecular Science* **18**: 1205.

Redden R, Partington D. 2019. Gene bank scheduling of seed regeneration: interim report on a long term storage study. *Journal of Integrative Agriculture* **18**: 1529-1540.

Rivière S, Müller JV. 2018. Contribution of seed banks across Europe towards the 2020 Global Strategy for plant conservation targets, assessed through the ENSCONET database. *Oryx* **52**: 464-470.

Roberts EH. 1973. Predicting the storage life of seeds. *Seed Science Technology* **1**: 499-514.

Sano N, Rajjou L, North HN, Debeaujon I, Marion-Poll A, Seo M. 2015. Staying alive: molecular aspects of seed longevity. *Plant and Cell Physiology* **57**: 660-674.

Stickland RG, Wilson KE. 1983. Sugars and starch in developing round and wrinkled pea seeds. *Annals of Botany* **52**: 919-921.

Takata H, Hanafusa T, Mori T, et al. 2013. Chromatin compaction protects genomic DNA from radiation damage. *PLoS One* **8**: e75622

Verbruggen N, Hermans C. 2008. Proline accumulation in plants: a review. *Amino Acids* **35**: 753-759.

Washio K. 2014. The chromatin structure and seed function of land plants. *International Journal of Plant Biology & Research* **2**: 1015.

Waterworth WM, Masnavi G, Bhardwaj RM, Jiang Q, Bray CM, West CE. 2010. A plant DNA ligase is an important determinant of seed longevity. *The* Plant Journal **63**: 848-860.

Waterworth WM, Drury GE, Bray CM, West CE. 2011. Repairing breaks in the plant genome: the importance of keeping it together. *New Phytologist* **192**: 805-822.

Waterworth WM, Bray CM, West CE. 2019. Seeds and the art of genome maintenance. *Frontiers in Plant Science* **10**: 706.

Waterworth WM, Wilson M, Wang D, et al. 2019. Phosphoproteomic analysis reveals plant DNA damage signaling pathways with a functional role vfor histone H2AX phosphorylation in plant growth under genotoxic stress. *The Plant Journal* **100**: 1007-1021

Wei T, Simko V. 2017. R package "corrplot": visualization of a correlation matrix (version 0.84). Available from https://github.com/taiyun/corrplot

Wickham H. 2011. ggplot2. *WIREs Computational Statistics* **3**: 180-185.

Wickham H. 2016. ggplot2. Elegant graphics for data analysis. Springer.

Wiebach J, Nagel M, Börner A, Altmann T, Riewe D. 2020. Age-dependent loss of seed viability is associated with increased lipid oxidation and hydrolysis. *Plant, Cell & Environment* **43**: 303-314.

van Zanten M, Koini MA, Geyer R, et al. 2011. Seed maturation in *Arabidopsis thaliana* is characterized by nuclear size reduction and increased chromatin condensation. *Proceedings of the National Academy of Sciences U S A.* **108**: 20219-20224.

Zinsmeister J, Leprince O, Buitink J. 2020. Molecular and environmental factors regulating seed longevity. *Biochemistry Journal* **477**: 305-323.

FIGURE LEGENDS

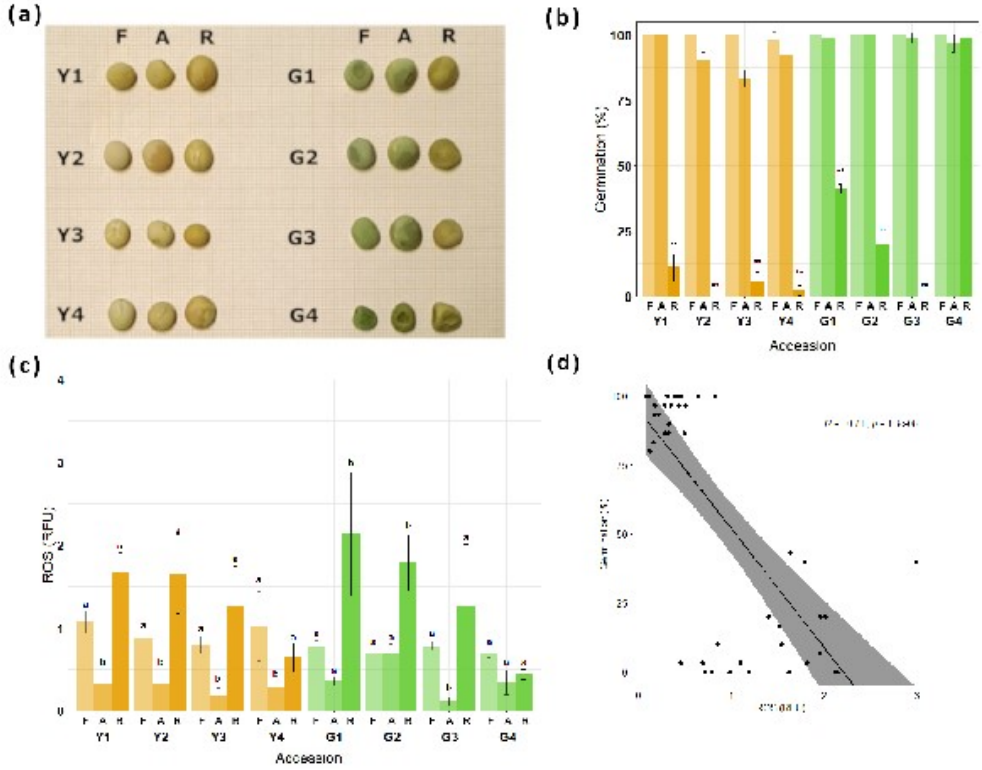
FIG. 1. (A) Seed lots used in this study. Y, yellow. G, green. F, harvested in 2019 (fresh). A, harvested in 2001 and kept in cold storage (aged). R, harvested in 2001 and conserved at room temperature conditions. (B) Germination percentage of the 8 pea accessions (fresh seeds; seeds aged in cold storage and at room temperature conditions). Error bars represent the standard deviation of three biological replicates (20 seeds each). (C) ROS levels measured in dry pea seeds using the DCF-DA fluorescent dye. Letters above bars represent statistically significant differences (GLM with Tukey post-hoc test, $P < 0.05$) within the same accession. R.F.U., relative fluorescence unit. ROS, reactive oxygen species. DCF-DA, dye 2',7'-dichlorofluorescein diacetate. Asterisks represent statistically significant differences between aged and fresh seeds within the same accession (** = $P < 0.01$). Error bars represent the standard deviation of three biological replicates (5 seeds each). (D) Kendall's Tau-b correlation between ROS levels and germination profile of dry seeds from the yellow (Y1, Y2, Y3, Y4) and green (G1, G2, G3, G4) accessions.

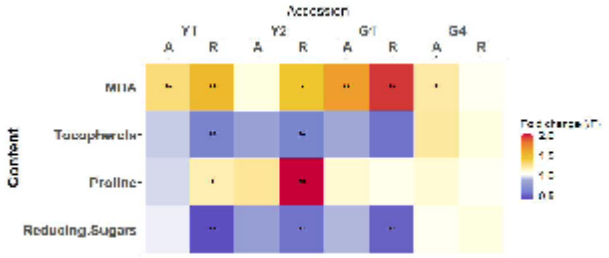
FIG. 2. Levels of malondialdehyde (MDA), tocopherols, free proline, and reducing sugars in dry seeds of the yellow (Y) and green (G) pea accessions. F, harvested in 2019 (fresh). A, harvested in 2001 and kept in cold storage (aged). R, harvested in 2001 and conserved at room temperature conditions. Heatmaps represent changes of relevant metabolites in A and R seeds, compared to F seeds. For each metabolite, the mean values are available in Supplementary Fig. S5. Asterisks represent statistically significant differences between fresh and aged seeds within the same accession, showed by the post-hoc Bonferroni test (* = $P < 0.05$; ** = $P < 0.01$).

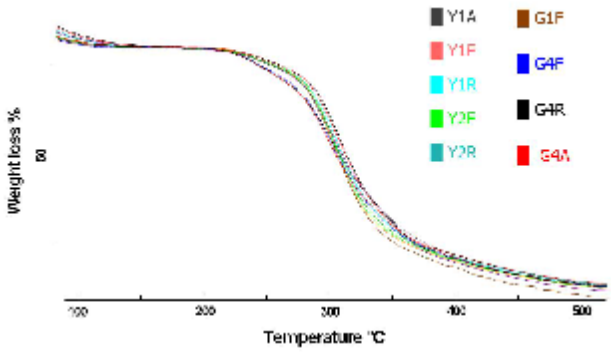
FIG. 3. Weight loss (%) TG curves for dry seeds of the yellow (Y) and green (G) pea accessions.

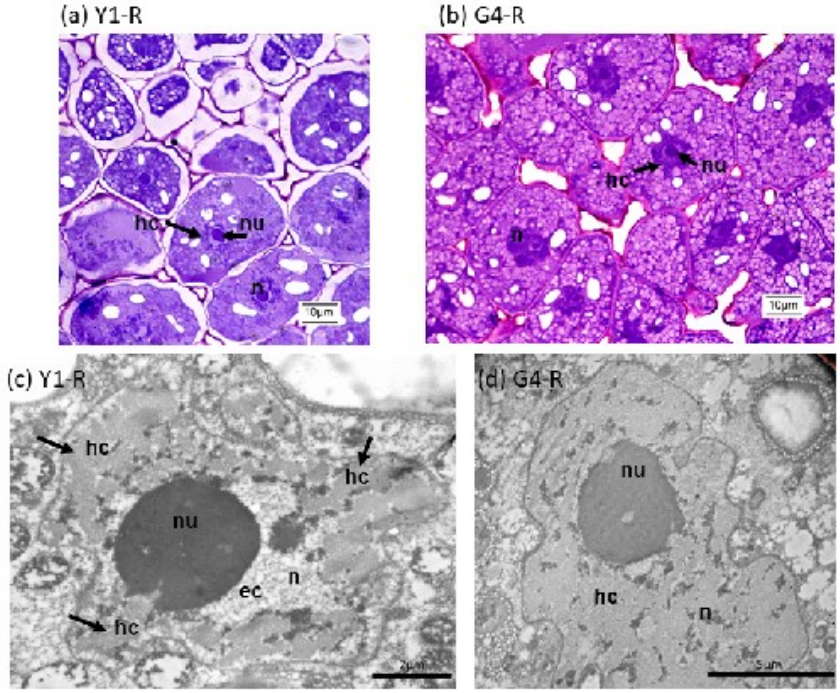
FIG. 4. Nuclear morphology in embryo axes excised from R seeds of Y1 and G4 accessions with contrasting longevity profiles. (A) and (B). Staining with Toluidine Blue. (C) and (D). Ultrastructural changes in chromatin distribution highlighted in Y1 and G4 nuclei subjected to EDTA regressive staining for TEM analysis. hc, heterochromatin. ec, euchromatin. nu, nucleolus. n, nucleus.

FIG. 5. Distribution of γH2AX foci in the nucleus of embryo axis excised from seeds stored at room temperature: (A) Y1-R and (B) G4-R. Detection of γH2AX foci was performed by immunocytochemical and TEM analyses. n, nucleus. nu, nucleolus.


(a)
F A R
Y1
Y2
Y3
Y4
G1
G2
G3
G4
(b)
Germination (%)
Accession
(c)
ROS (RFU)
Accession
(d)


Accession
Y1
Y2
G1
G4
A
R
A
R
A
R
A
R
MDA
Tocopherols
Proline
Reducing.Sugars
Content


Y1A
Y1F
Y1R
Y2F
Y2R
G1F
G4F
G4R
G4A
Weight loss %
Temperature °C


(a) Y1-R
hc
nu
n
(b) G4-R
hc
nu
n
(c) Y1-R
hc
hc
nu
ec
n
hc
(d) G4-R
nu
hc
n

7.2 Review

Gianella M, Bradford KJ, Guzzon F. Ecological, (epi)genetic and physiological aspects of bet-hedging in angiosperms. *Plant Reprod*. 2021; 34(5), 21-36. https://doi.org/10.1007/s00497-020-00402-z

Plant Reproduction
https://doi.org/10.1007/s00497-020-00402-z

REVIEW

Ecological, (epi)genetic and physiological aspects of bet-hedging in angiosperms

Maraeva Gianella[1] · Kent J. Bradford[2] · Filippo Guzzon[3]

Received: 28 September 2020 / Accepted: 28 December 2020

Abstract

Key message **Bet-hedging is a complex evolutionary strategy involving morphological, eco-physiological, (epi)genetic and population dynamics aspects. We review these aspects in flowering plants and propose further research needed for this topic.**

Bet-hedging is an evolutionary strategy that reduces the temporal variance in fitness at the expense of a lowered arithmetic mean fitness. It has evolved in organisms subjected to variable cues from the external environment, be they abiotic or biotic stresses such as irregular rainfall or predation. In flowering plants, bet-hedging is exhibited by hundreds of species and is mainly exerted by reproductive organs, in particular seeds but also embryos and fruits. The main example of bet-hedging in angiosperms is diaspore heteromorphism in which the same individual produces different seed/fruit morphs in terms of morphology, dormancy, eco-physiology and/or tolerance to biotic and abiotic stresses in order to 'hedge its bets' in unpredictable environments. The objective of this review is to provide a comprehensive overview of the ecological, genetic, epigenetic and physiological aspects involved in shaping bet-hedging strategies, and how these can affect population dynamics. We identify several open research questions about bet-hedging strategies in plants: 1) understanding ecological trade-offs among different traits; 2) producing more comprehensive phylogenetic analyses to understand the diffusion and evolutionary implications of this strategy; 3) clarifying epigenetic mechanisms related to bet-hedging and plant responses to environmental cues; and 4) applying multi-omics approaches to study bet-hedging at different levels of detail. Clarifying those aspects of bet-hedging will deepen our understanding of this fascinating evolutionary strategy.

Keywords Bet-hedging · Heteromorphism · Fitness · Seed dormancy · Eco-physiology · Soil seed bank

Introduction

Organisms must cope with a variety of threats to their survival, ranging from abiotic stresses, such as the lack of resources or unfavourable climatic conditions, to biotic stresses such as predation and infections. Plants, being sessile organisms, evolved different strategies to overcome these environmental factors, depending on their temporal variability. When environmental variation is consistent, such as seasonal regularity, physiological or developmental plasticity is sufficient to allow individuals to adapt their phenotypes in response to the prevailing conditions. When, on the contrary, environmental variation is unpredictable, organisms apply diverse options without predicting how it will affect their future fitness, thereby 'hedging their bets' (Childs et al. 2010; Slatkin 1974). Bet-hedging occurs when a population lowers its mean fitness over time (across years) by decreasing also its annual variance in survival, thus improving its long-term fitness (Olofsson et al. 2009; Seger and Brockmann 1987). The canonical example of bet-hedging in plants is seed dormancy, i.e. the temporary failure of a seed to complete germination under favourable conditions (Bewley et al. 2013). When seeds germinate, the probability of survival

Communicated by Thomas Dresselhaus.

✉ Maraeva Gianella

✉ Filippo Guzzon
f.guzzon@cgiar.org

[1] Department of Biology and Biotechnology "L. Spallanzani", University of Pavia, 27100 Pavia, Italy

[2] Department of Plant Sciences, Seed Biotechnology Center, University of California, Davis, USA

[3] International Maize and Wheat Improvement Center (CIMMYT), Carretera México-Veracruz, Km. 45, El Batán, 56237 Texcoco, Mexico State, Mexico

Published online: 15 January 2021

for the seedlings is heavily dependent on the environmental conditions at that time. If a dry period occurs, the seedling is almost certainly bound to perish, while, if germination occurs during a wet period, survival is more likely. When the occurrence of dry/wet periods is not predictable, seed dormancy allows a distribution of germination events that, just like placing bets, enhances the probability that a proportion of the seed cohort germinates during a wet period (Cohen 1966). When different seed phenotypes are produced at the same time, only the ones adapted to the ongoing conditions survive. This way, survival of part of the progeny is assured, even if the mean fitness of the mother plant for that particular timeframe is not at its maximum (here referred to the maximum fitness possible, when a high proportion of the seeds produced survive because of specialized adaptation to that particular condition). Indeed, bet-hedging strategies tend to lower the arithmetic mean of fitness of single generations, at the same time lowering the temporal variance of fitness over time, thus improving the long-term fitness for the whole population over several generations (Philippi & Seger 1989).

Bet-hedging is a widespread strategy in flowering plants and can be observed in different phases of the plant reproductive cycle, mainly at the seed stage but also at the gametic level (pollen/ovules), in the embryos or fruits, and occasionally also in vegetative tissues such as buds (Fig. 1a) (Charlesworth 1989; Cohen 1966; Nilsson et al. 1996; Peters et al. 2011; Thurlby et al. 2012). It is particularly evident in wild plant species adapted to unpredictable environments, in terms of abiotic factors such as rainfall or soil salinity (e.g. ruderal areas or intertidal zones) or biotic components (e.g. host-parasite cycles, predation) (see, e.g. Guzzon et al. 2018; Long et al. 2015; Verin & Tellier 2018; Volis and Bohrer 2013). On the contrary, bet-hedging has been subjected to negative selection during crop domestication in favour of rapid and uniform germination and field establishment even under sub-optimal conditions (Mitchell et al. 2017).

Given the importance of bet-hedging in angiosperms, we review its ecological and evolutionary implications with a focus on seed dormancy and heteromorphism, but also considering special cases such as bud dormancy, apomixis and serotiny. Despite a conspicuous number of studies describing bet-hedging at the ecological level, especially investigating how morphology and germination patterns affect fitness of different plant populations and taxa, still little is known about the molecular mechanisms underlying this phenomenon. We start by providing a resume on the different definitions and categorizations of bet-hedging, moving to its diffusion in flowering plants and its consequences for the dynamics of population genetics. We then review the genetic bases of seed heteromorphism, citing the most recent works linking different loci to bet-hedging strategies. We also describe how transcription and its regulation can drive bet-hedging in plants, including via epigenetic mechanisms triggered by environmental cues. Finally, we provide information on the physiological aspects that are linked to bet-hedging, such as hormonal regulation and antioxidant responses, and we conclude by suggesting further research that is needed on this subject.

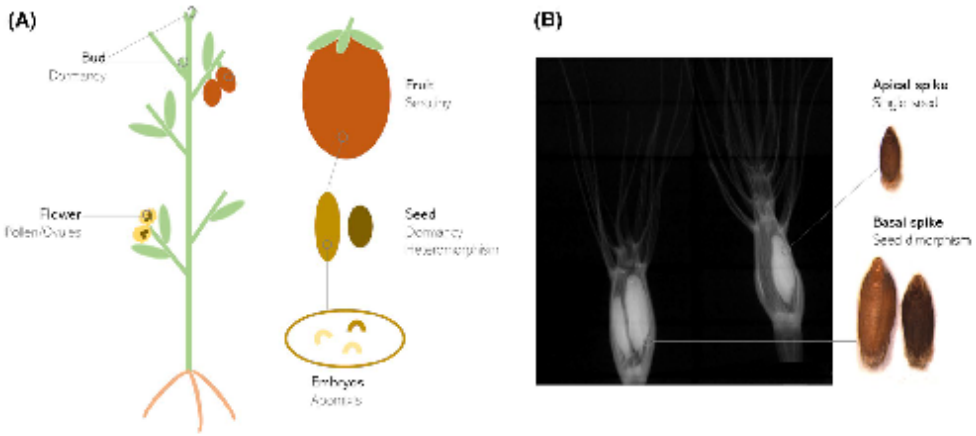


Fig. 1 **a** Examples of plant anatomical parts showing bet-hedging strategies, **b** Example of caryopses heteromorphism in Poaceae: *Aegilops geniculata* Roth (photo and X-ray scan: F Guzzon)

The bet-hedging strategy: definitions and ecology

In a stable environment with predictable fluctuations, the fitness of phenotypes that are specialized to those conditions is maximized and constant over time, and thus, variance of fitness is low or null. In the same environment, generalists are disadvantaged as, even if with low variance, their mean fitness is lower (Fig. 2a). On the contrary, in an unstable environment, specialization can maximize fitness only in the timeframe of particular conditions, while increasing the variance of fitness over time because fitness dramatically decreases when those particular conditions are not met (Olofsson et al. 2009). In such cases, generalist strategies sacrifice the mean fitness in order to lower the variance over time as fitness remains constant over most environmental conditions (Fig. 2b). The so-called 'bet-hedging' strategy describes the adoption of a reproductive strategy that maximizes the long-term fitness to ensure survival when coping with an unpredictable environment (Philippi and Seger 1989; Slatkin 1974) or in the presence of fluctuating natural selection (Simons 2009).

In other words, bet-hedging is a trade-off between the mean and the variance of fitness (Philippi and Seger 1989); it results in a reduction in the temporal variance in fitness and a lowered arithmetic mean fitness but in a higher overall (geometric mean) fitness over time under unstable conditions (Fig. 2). Moreover, a strategy that favours diversification within the same generation results in a reduction of competition among siblings, thus uncoupling the survival and fitness of one individual from those of its siblings. Correlation of fitness among individuals is therefore decreased, making bet-hedging a triple trade-off among mean, variance and correlations of fitness (Starrfelt and Kokko 2012).

Olofsson et al. (2009) defined four main types of bet-hedging strategies: (i) *conservative*, or 'playing safe': a consistent, low-risk, generalist strategy, e.g. large seeds produced yearly; (ii) *diversified*, several specialized strategies at once but fixed, e.g. variable seed sizes drawn from a fixed distribution; (iii) *'adaptive coin flipping'*, e.g. randomly

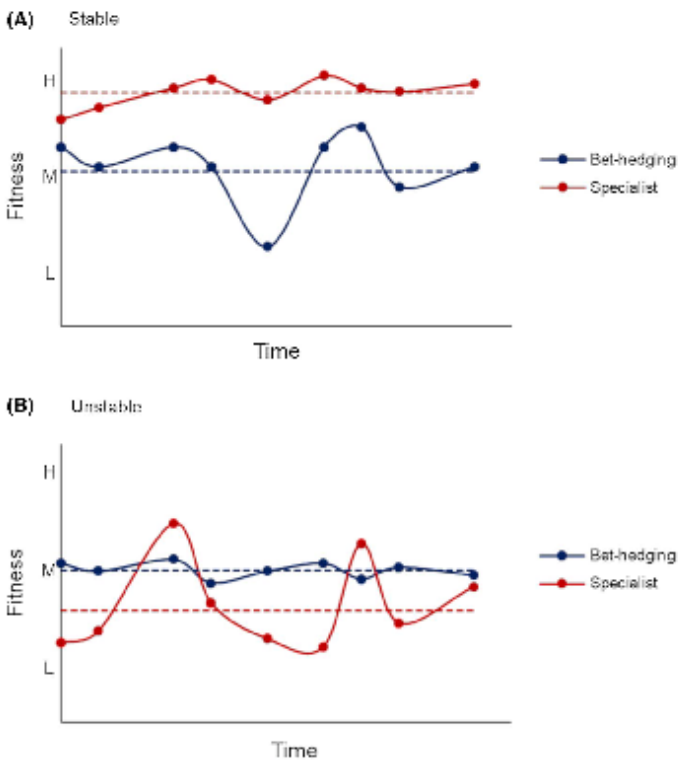


Fig. 2 Trends of fitness in bet-hedging and specialization strategies in **a** stable and **b** unstable environments over time L = low; M = medium; H = high fitness. Dashed lines represent the geometric mean of fitness for each strategy. Comparisons are made between strategies, not environments

produced seed sizes; and (iv) *combined*, any combination of the above.

Diaspore heteromorphism, or the production by the same individual of two or more seed/fruit types, is considered one of the main bet-hedging strategies in flowering plants (see, e.g. Fig. 1b) (Imbert 2002; Venable 1985). Baskin and Baskin (2014) divide seed/fruit heteromorphism in two main categories: heterodiaspory and amphicarpy. Heterodiaspory occurs when 'two or more types of diaspores are produced above ground and differ in ecological function, e.g. dispersal and dormancy'. Heterocarpic species show different fruit types, with heteroanthrocarpic species possessing segmented fruits bearing different seed morphs and amphi-basicarpic species producing flowers and fruits on both aerial and basal parts of the plant. Heterospermy is another type of heterodiaspory that involves the production of different seed morphs in one or more fruit types. Amphicarpy occurs in plants producing 'one or more than one type of fruit both above- and below-ground that differ in ecological function' (Baskin and Baskin 2014).

Venable (1985) described two types of heteromorphism based on the mean fitness and its variance. In the *high risk/high risk* (HRHR) type, two seed morphs are specialized for two different conditions (e.g. wet/dry years), thus maximizing the mean and the variance of fitness of both seeds. On the contrary, in the *high-risk/low-risk* (HRLR) heteromorphic species both seed types are specialized for the same conditions (e.g. wet years), but the fitness of one morph is higher in optimal conditions and lower in different conditions, thus increasing both its mean and variance of fitness (*high risk*) compared to the other morph (*low risk*), whose mean fitness is lower but less variable over time. Seed heteromorphism is common in soil seed banking, in which a fraction of seeds remains dormant after dispersal within the soil or on its surface (Imbert 2002; Venable 2007). A proportion of one morph's seed cohort remains dormant for a period, influencing fitness in a temporal way by reducing sibling competition, overcrowding and/or inbreeding. In general, the formation of persistent soil seed banks is a bet-hedging strategy that enhances survival and decreases the probability of reproductive failure when the environmental conditions are not favourable (Cohen 1966).

Diaspore heteromorphism can affect fitness also in a spatial manner, for instance, with the endowment of different dispersion capabilities depending on morphology in order to reduce competition. As an example of this, *Picris echioides* L. (Asteraceae) produces two types of achenes: the peripheral ones are dispersed by mammals and remain enclosed in the involucral bract, while the central achenes are wind-dispersed (Imbert 2002). Dispersal itself can be considered a type of diversified bet-hedging, as it can reduce competition among siblings and correlation in fitness between individuals, diversifying their fitness in a spatial manner within the same generation (Hopper et al. 2003; Starrfelt and Kokko 2012). In general, dormancy and dispersal provide bet-hedging strategies to plants experiencing variable environmental conditions in space and time, with the two traits often negatively associated (Buoro and Carlson 2014; de Casas et al. 2015). Indeed, trade-offs in dispersal and dormancy are observed in dimorphic species, where one morph shows a high dispersal (HDi) capability coupled with low or no dormancy levels (LDo), while the other is characterized by low dispersal ability (LDi) and high dormancy (HDo). This is not limited to true dimorphism, as a gradient of strategies following this kind of trade-off was observed along the continuum of diaspores produced from the basal to the aerial parts of the plant by the amphi-basicarpic *Ceratocarpus arenarius* L. (Lu et al. 2013). However, where dormancy and dispersal coevolve, they can become positively associated, so HDo/HDi and LDo/LDi strategies are observed (e.g. in *Aethionema arabicum* (L.) Andrz. ex DC) (Arshad et al. 2019). This species produces two fruit morphs, one being dehiscent and bearing quickly germinating, mucilaginous seeds, and the other fruit being indehiscent with dormant seeds. Mucilaginous seeds germinate quickly after anchoring to the soil near the mother plant, while the fruit with the dormant morph is dispersed for long distances, mainly with rainfall but also by anemochory in the case of hydrothermal stress, due to its winged pericarp (Arshad et al. 2019; Bhattacharya et al. 2019). Moreover, different site-specific offspring ratios are produced, shifting the dimorphic fruits' migration based on the fluctuations of environmental temperature sensed by the plant, as modelled mathematically by Nichols et al. (2020).

Therefore, heteromorphic systems can adopt within- or between-generations bet-hedging strategies, as diaspores with different behaviours can be produced within the same cohort or in different reproductive seasons. For instance, seed types produced in heteromorphic species can differ in several traits, such as:

- Morphological characteristics: seed mass, colour, hardness of the seed coat (Guzzon et al. 2018; Xu et al. 2016);
- Tolerance to abiotic stresses: salt stress, osmotic stress (Bhatt and Santo 2016; Datta et al. 1970);
- Susceptibility to predation (Hulme 1998);
- Longevity and persistence in the soil seed bank (Guzzon et al. 2018; Zinsmeister et al. 2020);
- Seed dormancy levels (Philippi 1993).

Obviously, several of the aforementioned traits interact in shaping bet-hedging strategies. In wheat wild relatives of the genera *Aegilops* L. and *Triticum* L.(Poaceae), heteromorphic caryopses are produced within the same spike and variations in colour, mass, dormancy, abiotic stress tolerance, longevity and phenology can be observed among the different morphs,

contributing to the adaptation to ruderal or disturbed environments (Datta et al. 1970; Guzzon et al. 2018). Similarly, the seed morphs produced by the halophyte *Suaeda salsa* (L.) Pall. (Chenopodiaceae) differ in several traits that allow the adaptation of this species to saline soils, including different dormancy levels, seed coat thickness and colour, and resistance to saline stress (Xu et al. 2016; Zhao et al. 2018). In this species, the variance in seed size in the offspring depends on the interaction between the maternal seed morph and the offspring seed morph. Moreover, the seed types ratio observed in the offspring is influenced by the seed type of the mother plant (Jiang et al. 2019). Recently, also in *Suaeda aralocaspica* (Bunge) Freitag & Schütze variations in seed heteromorphism, in terms of plant size, seed number and heteromorphic seeds ratios, have been associated with maternal environmental factors (Cao et al. 2020).

As pointed out by Long et al. (2015), plants that produce large numbers of seeds per generation should persist better in the soil seed bank, given the fact that a larger number enters the soil and that they tend to be smaller and longer-lived. On the other hand, Huxman et al. (2008) emphasized that high seed production is observed in those species that perform well in favourable seasons but that do not survive in other conditions, e.g. due to low tolerance to drought stress, thus showing high variance in fitness over different years/reproductive seasons. Further research is needed to clarify the possible trade-offs among seed number, persistence and fitness.

Seed sensitivity to environmental factors such as temperature (T) and water potential (Ψ) can be modelled to derive the threshold values that permit germination in a fraction of the seed population (Bradford 2018). These models, called 'population-based thresholds' (PBT), can be good descriptors for bet-hedging strategies in plant communities as they allow quantitative evaluations of the seed response to environmental fluctuations. For example, higher germination plasticity in a desert community can be achieved when the median value of base water potential, $\Psi_b(50)$, is lower and its standard deviation is higher. Indeed, larger water potential ranges allow larger differences in the germination fraction over different years or rain events within the same year, thus leading to plastic germination and bet-hedging strategies (Liu et al. 2020). Moreover, high variance in year-to-year seed production per each seedling, hence higher variance in fitness, has been associated with small seeds and hydrothermal traits (Ψ and T) linked to slow and fractional germination (Huang et al. 2016). A special case of bet-hedging can be observed in plants that show mixed strategies in terms of embryo production, generating diploid embryos sexually and asexually (i.e. agamospermy or apomixis). Indeed, the co-option of these two mechanisms, which can also lead to the formation of polyembryonic seeds, can provide several advantages. While sexual reproduction leads to gains in genetic diversity due to its intrinsic recombination processes, asexual embryos can assure survival when sexual reproduction is energetically costly and can better preserve genomes that are well adapted to certain environmental conditions (Niklas and Cobb 2017; Thurlby et al. 2012).

Another form of bet-hedging is serotiny, or retention of seeds upon the mother plant at least until the ripening of another seed cohort. Seed expulsion usually happens when the environmental conditions are favourable or in a gradual fashion, e.g. in different seasons, to increase the chances for the seedlings to find an appropriate time window for establishment (Peters et al. 2011). Serotiny is present within the genus *Mammillaria* (Cactaceae): in *M. pectinifera* F.A.C. Weber seeds are expulsed actively during rainy periods and passively over years in order to spread the chances of seedling establishment over time (Peters et al. 2011). The serotinous species *M. hernandezii* Glass and Foster retains a fraction of seeds to protect them from predation and prepares them for dispersal via a rainfall-induced priming process: seed hydration enables a phase of pre-germinative metabolism followed by dehydration that speeds germination and seedling establishment after expulsion from the fruit (Santini and Martorell 2013). In fire-prone environments, 'weak' serotiny, i.e. seed retention for < 10 years, is an effective strategy when the interval between two fires exceeds the plant lifespan and there is stochastic variability in fire occurrence. On the other hand, when fires are predictable, the opposite strategies 'strong' serotiny (retention > 10 years) and nonserotiny become advantageous specialist adaptations (Lamont et al. 2020).

The production of a diversified population of offspring, in terms of morphs, sizes and number among generations, which is a combination of diversified and adaptive coin-flipping bet-hedging, is theorized as the optimal reproductive strategy for an individual (Olofsson et al. 2009). In this situation, different generations are subjected to different selective pressures, while seeds belonging to same generation experience more or less the same conditions. On the other hand, within-generational bet-hedging is favoured when seeds belonging to the same generation are subjected to different selective pressures, e.g. predation affecting some individuals and not others (Hopper et al. 2003). Indeed, predation affects the more common seed phenotypes more strongly compared to the rare ones (Horst and Venable 2017). Also, predatory pressure can be heavier in certain time windows, for example, some rodents are more active from late spring to autumn. This can favour a within-generational bet-hedging strategy with the production of seeds possessing different dormancy behaviours and therefore a scattered germination pattern during the year (Gremer and Venable 2014). Indeed, assuming that dormancy and within-season germination phenology evolve independently, variance in the latter can favour earlier phenology when dealing with biotic stresses,

while later phenology is selected when managing abiotic stresses. Moreover, when modelling within-season mortality and continuous reproduction in these systems, evolutionary branching can arise, with multiple individuals presenting different germination strategies, coexisting but reproducing at different timings (ten Brink et al. 2020).

Bud dormancy enables the repeated phase of rest that punctuates periods of growth in the life cycle of many perennial species (Cooke et al. 2012). It has also been proposed to be a bet-hedging strategy in response to herbivory predation, if it results in lower seeds production in years of low herbivore pressure and reduces the variance of seed production in time with compensatory effects in years of intense pressure (Nilsson et al. 1996).

Moreover, also among different populations of the same species there can be variation of seed production depending on the environment experienced by the population, thus again depending on the selective pressure(s) exerted by the environment (Dyer 2017; Philippi 1993). However, Starrfelt & Kokko (2012) suggest that bet-hedging is a continuum of strategies rather than divided into distinct categories, in terms of degree of conservation/diversification among different populations of the same taxa and also within/among generations. This latter model has been demonstrated mathematically by Haaland et al. (2020), also predicting a continuum in terms of among- versus within-individual phenotypic variation affected by the amount of environmental stochasticity experienced and tuned in order to maximize the genotype fitness.

Phylogenesis and distribution in the Angiosperms

Bet-hedging strategies are diffused in many plant families in different plant development phases and anatomical parts, but to the best of our knowledge, it has been phylogenetically quantified only in terms of seed heteromorphism and persistence within the soil seed bank. Lamont and colleagues (2020) describe the phylogenetic distribution of serotiny, present in eight angiosperm families; however, this distribution is not yet resolved at the species level. Heteromorphism was reported to be present in 218 plant species and is more frequent in dicotyledonous plants (16 families out of 18 are dicots; Imbert 2002) (Fig. 3). Wang and colleagues in 2010 listed additional heteromorphic species, raising the total count to 292. Recent work by Scholl and colleagues (2020) examined the presence of seed heteromorphism in 101 angiosperm species, distributed across 51 genera and 9 families, mainly within Asteraceae and Boraginaceae (Fig. 3). This analysis is the first to directly link heteromorphism to bet-hedging strategies at the phylogenetic level by considering the association with different factors: aridity, coefficient of variation (CV) of precipitation, life-span (annual/perennial) and weediness of the species. A significant correlation was found between heteromorphism and aridity, while weediness and annual life cycle were not significant predictors, in contrast with previous hypotheses (Imbert 2002; Scholl et al. 2020). Even if the resolution of the phylogenetic analysis could be biased by the occurrence locations of the considered taxa, as the authors predominantly considered North America, this paper gives insight on the diffusion of seed heteromorphism as a bet-hedging strategy in the Angiosperms.

Recently, another phylogenetic analysis highlighted the presence of soil seed banking in more than 2500 angiosperm taxa having different life cycles (annual/perennial, weedy/woody), habitats and seed traits such as dormancy. Persistent seed banks, suggesting bet-hedging strategies, are mostly diffused in weedy taxa with annual life cycles

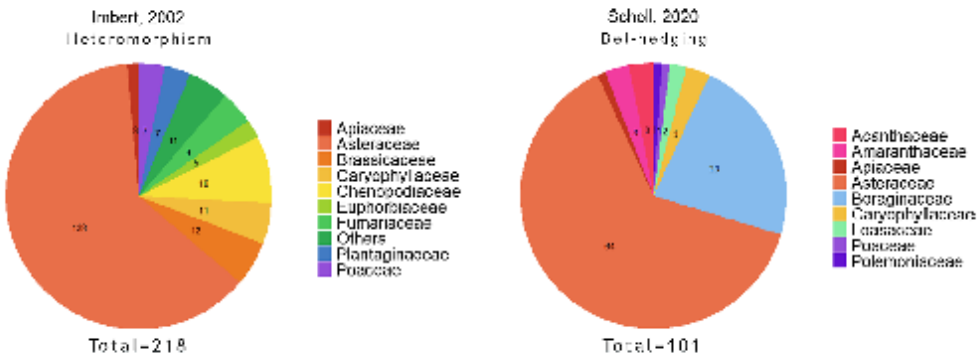


Fig. 3 Number of heteromorphic species and of species showing bet-hedging strategies distributed in different plant families as reported by Imbert (2002) and Scholl et al (2020), respectively.

living in disturbed and ruderal habitats (Gioria et al. 2020). Given the lack of association with life cycle and weediness found by Scholl et al. (2020) in heteromorphic bet-hedgers, a comprehensive phylogenetic analysis that considers both persistence of the seed bank and heteromorphism is needed.

Diaspore heteromorphism, while observed in crop wild relatives (e.g. wheat wild relatives: Gianella et al. 2020; oat wild relatives, *Avena sterilis* L.: Volis 2014; wild *Polygonum erectum* L.: Mueller 2017; lupin wild relatives, *Lupinus angustifolius* L.: Moncalvillo et al. 2019), is often lost in crops as a result of selection for uniform and quick germination as part of the domestication process. Indeed, the traits that characterize the 'domestication syndrome', i.e. plant traits that mark the divergence of domesticated crops from their wild progenitors, are often reproductive traits that affect yield, such as seed size and number or shattering at maturity (e.g. wheat) (Iriondo et al. 2018; Nave et al. 2016). In agricultural contexts, bet-hedging strategies are indeed disadvantageous as they result in staggered germination and seedling establishment, reducing yield and complicating pest and growth management (Mitchell et al. 2017). Hence, comprehensive analyses of the genetic bases of bet-hedging strategies are of great importance, in particular in genetic drift studies about crop domestication by comparing presence/absence of this trait in crops and their wild relatives.

Population dynamics

Long-term soil seed banking connected with bet-hedging strategies can modify the dynamics of population genetics in different ways. These include (Tellier 2018): (i) reducing the extinction rate in unpredictable environments, thereby influencing population size and genetic drift; (ii) through persistence in the soil, lengthening the time to the point where two lineages coalesce in their most recent common ancestor (MRCA); (iii) increasing the genetic recombination rate since the coalescent time is lengthened, leading to higher genetic diversity and lower linkage disequilibrium; (iv) potentially increasing the mutation rate due to the time spent in the soil; (v) reducing both the fixation rate of favourable alleles and the risk of allele loss by random drift, thereby affecting the rates and signatures of natural selection; and (vi) reducing inbreeding.

In particular, it has been demonstrated that long-term soil seed banking seeds show an equal or higher nucleotide substitution rate when compared to less persistent seeds, and that the majority of seed bank-borne mutations are neutral or nearly neutral, in accordance with Otha and Kimura's neutral theory of molecular evolution (Dann et al. 2017). Moreover, differences in substitution rates can be observed at the interspecific level but also intra-taxa at the same locus, depending on specific population traits such as generation time or altitude and latitude (Dann et al. 2017).

The evolution and diffusion of soil seed banking in different taxa and populations is not only linked to unpredictable climatic conditions but also to unstable co-evolutionary dynamics between host and parasites: seed banking is evolutionarily favoured when the cost of alleles for resistance to parasites and the disease severity are high (Verin and Tellier 2018). This is more likely to happen in more stable environments (e.g. temperate areas), where infections are favoured and host-parasite cycles can be chaotic, making the host's biotic environment unstable (Verin and Tellier 2018). Predation is an evolutionary driving force for the constitution of persistent seed banks; for instance, in grasslands the predatory pressure exerted by rodents favours the establishment of seed banks in grasses and legumes (Hulme 1998). This selective force acts not only on the degree of permanence within the soil but also on seed size, existing as a negative correlation between these two seed traits (i.e. smaller seeds persist more in the soil seed bank) (Volis & Bohrer 2013). Indeed, larger seeds are predated preferentially in both pre- and post-dispersal stages and represent a higher metabolic cost when remaining dormant in the soil compared to small seeds. These two features constrain larger seeds to evolve, on average, a quicker germination strategy and transient seed banks (Hulme 1998).

Genetic bases of bet-hedging

Although the adaptive significance of bet-hedging in seeds and fruits has been studied quite extensively from the point of view of morpho-ecology, little is known about its basis at the molecular level.

Some early studies focused on macro-differences in molecular features of heteromorphic species. For example, karyotypic variations in terms of chromosomal morphology and length, together with differences in electrophoretic profiles of total seed proteins, have been linked to different morphs in the achenes of *Calendula micrantha* Tineo & Guss. (Asteraceae) (Soliman 2003). The morphologic variance of distinct seed sets of *Primula vulgaris* Hudson (Primulaceae) was explained, in part, by genes linked to flower morphology and the influence of the light and temperature environment experienced by the female parent during the flowering period (Vaerbak & Andersen 2004). Another approach used was the utilization of amplified fragment length polymorphisms (AFLP) in *Packera tomentosa* (Asteraceae) to genetically differentiate clones (genets) that showed cryptic heteromorphism, i.e. a variable seed behaviour such as dormancy that is not accompanied by a discriminant morphological variation (Imbert 2002). While differences in morphology were not evident, seed mass and germination performance differed among genets and seed positions within the flowers (Leverett and Jolls 2013).

More recently, Nave and colleagues (2016) analysed the molecular bases of within-spikelet heteromorphic caryopses exploiting a recombinant inbred line (RIL) population originated from durum wheat (*Triticum turgidum* subsp. *durum* (Desf.) Husn.) and its progenitor, wild emmer wheat (*Triticum turgidum* subsp. *dicoccoides* (Asch. & Graebn.) Thell.) (Poaceae), in order to characterize heteromorphism to clarify the domestication syndrome in wheat. While domesticated emmer shows uniform grain size and germination, wild emmer bears, within the same spikelet, bigger caryopses located in the upper part that germinate more rapidly when compared to their smaller siblings located in the lower part of the spikelet. A quantitative trait locus (QTL) for uniform grain size and germination on chromosome 4B (*QGD-4BL*) explained a high proportion of within-spikelet variation in terms of caryopses dimensions and dormancy, indicating a modification selected during early stages of domestication. (Nave et al. 2016).

On chromosomes 3 and 5 of *Arabidopsis thaliana* L. (Brassicaceae), two loci, one overlapping with *DELAY OF GERMINATION 6 (DOG6)* and the other with *DOG1* (two of the loci underlying the extent of dormancy under different environmental conditions; Bentsink et al. 2010) have been associated with a bet-hedging strategy in seeds belonging to the same siliques when coping with environmental stresses. Seeds subjected to short strong stresses, e.g. heat shock at 49 °C for 30 min, showed differential germination timing that allows part of the seed cohort to survive an unpredictable period of unfavourable environmental conditions (Abley et al. 2020). Also, two proteins encoded by *FLOWERING LOCUS C (FLC)* and *FLOWERING LOCUS T (FT)* enable *A. thaliana* mother plants to modulate seed dormancy in the progeny depending on the external temperature, sensed through the epigenetic state of these genes. This mechanism allows the modification of dormancy levels through the regulation of seed coat development and hormone production in response to environmental conditions during seed maturation to diversify the behaviour of the progeny, presumably to maximize fitness in the following generation (Chen and Penfield 2018; Springthorpe and Penfield 2015). Sensitivity to environmental signals for dormancy release also occurs when other key germination-repressing genes are expressed at low levels (e.g. *DOG1*, *CIPK23—CBL-interacting serine/threonine-protein kinase 23*, *PHYA—Phytochrome A*), and subtle differences in the seed response contribute to bet-hedging via the formation of transient or persistent seed banks (Footitt et al. 2014). The formation of seed banks in *A. thaliana* can be linked to the differential response to chilling in terms of primary dormancy release, with mixed autumn- and spring-germinating cohorts observed within populations in the same year. This system has been subjected to genetic and molecular analyses that support a role for *DOG1* in determining the depth of dormancy, but identified other loci more closely associated with dormancy cycling (Footitt et al. 2019). Recent work by Martínez-Berdeja and colleagues (2020) linked primary dormancy release induced by chilling in *A. thaliana* seeds to *DOG1* haplotype identities through a genome-wide association study.

Two populations of *Brassica oleracea* L. (Brassicaceae), genetically identical except for two loci involved in abscisic acid (ABA) catabolism (*RABA1—Reduced ABA 1*) and sensitivity (*SOG1—Suppressor of gamma response 1*), showed a bet-hedging strategy based on a continuum of ABA-dependent dormancy. Allelic differences at these loci were correlated with dormancy release when seeds were subjected to different temperatures, with the two lines showing different lower limits of tolerance (Awan et al. 2018).

A special case of bet-hedging is exhibited by *Syzygium paniculatum* Gaertn (Myrtaceae). This rare tree species relies on a mixed reproductive strategy utilizing both sexual embryonic production and agamospermy (Thurlby et al. 2012). Seeds are polyembryonic, and when dimorphic, the sexual embryo is bigger than the asexual one, whereas, when polymorphic, the embryos are the same size, apparently due to the increased competition from multiple asexual siblings. Overall, genetic diversity in this species is low, as observed when different populations were compared using nuclear simple sequence repeat (nSSR) markers. This mixed reproductive strategy can be seen as a form of bet-hedging, as the sexual embryos represent an adaptive advantage for maintaining genetic diversity, while asexual embryos assure dispersion and survival even when sexual reproduction fails or is too costly (Thurlby et al. 2012).

Although there is some evidence for single dominant loci being responsible for apomeiosis and parthenogenesis in some grasses, several genes have been associated with apomixis in Poaceae: *ASGR-BABY BOOM-like (PsASGR-BBML)* gene from *Pennisetum squamulatum* (L.) R.Br. and *BABY BOOM1* (*BBM1*) in rice (*Oryza sativa* L.) (Conner et al. 2015; Khanday et al. 2019). Khanday and colleagues (2019) demonstrated that the ectopic expression of *BBM1* in egg cells of rice is sufficient for parthenogenesis but that fertilization is still required for seed endosperm production; notably, this system shows the feasibility of clonal propagation through apomictic seeds in crops. In addition to a genetic basis, epigenetic regulation of apomixis has also been hypothesized, since the latter could explain the facultative nature and reversibility to sexual seed production (Kumar 2017; Rodrigues and Koltunow 2005).

Bet-hedging and non-genetic bases: transcription

Non-genetic variation could play a major role in terms of phenotypic variation in the context of bet-hedging. Selection for diversification often implies low heritability of fixed genetic adaptations because the latter is the ratio of additive

genetic variance to the total phenotypic variance (Simons and Johnston 2006). On the other hand, a major source of molecular variation is differential gene expression and its regulation. Transcriptional variability (often referred to as 'transcriptional noise') can be caused by environmental fluctuation or other constraints (e.g. at the cellular level by physical position in a cell population, differential cell functions) and can result in detrimental or beneficial phenotypic variability (Mitchell et al. 2017). Wide variation in transcript levels, when interpreted as noise, suggests poor regulatory control or unavoidable stochastic 'error'. The development of technologies to assess transcriptomes at the single-cell level has provided different interpretations of what has been designated as transcriptional 'noise' (Stadler and Eisen 2017). Pooled samples inevitably bulk together the transcripts from populations of individuals, whether of seeds or cells. However, biological variation can be interpreted in terms of populations of individuals in which response thresholds and physiological/transcriptional activities vary in reproducible or programmed ways. Thus, transcriptional variability may represent differences among cells in a population that is part of the regulatory process, rather than representing uncontrollable error. In fact, population models based on threshold-dependent dynamics have successfully described diverse aspects of seed germination behaviour, including responses to temperature, water potential, hormones, dormancy, ageing, respiration rates and other parameters (Bradford 2018). Similarly, recent single-cell in vivo transcriptional studies revealed that 'plant tissues respond to external signals by modulating the number of cells engaged in transcription rather than the transcription rate of active cells' (Alamos et al. 2020). An important implication of this is that experimental designs for examining seed molecular biology will need to move toward analyses of individual seeds (e.g. Still and Bradford 1997) rather than pooling multiple seeds per sample, which inevitably combines seeds in different physiological or developmental states, e.g. dormant and non-dormant or germinating vs non-germinating, at a given time.

It is evident that seed-to-seed variation is a fundamental component of bet-hedging strategies. For instance, beneficial variability can be observed in the halophyte *S. salsa* which shows differential transcription to adapt to environmental fluctuations in soil salinity (Xu et al. 2017). This species produces two seed morphs: brown seeds germinate faster and tolerate higher salt concentrations, while black seeds remain dormant, tolerate lower salt concentrations and constitute the soil seed bank. The transcriptomic profiling performed by Xu and colleagues (2017) on mature dry seeds revealed that the two morphs show differential expression of 4648 genes, mainly belonging to pathways related to embryo development, fatty acids metabolism, osmotic equilibrium and hormonal regulation, indicating their different developmental trajectories. In particular, genes involved in the metabolism of two organic osmolytes, betaine and proline, were upregulated, the latter being not only involved in osmotic regulation but also in the prevention of germination in stressful conditions by maintaining the embryo axis in a resting state.

In *Ae. arabicum* (Brassicaceae), fruit dimorphism (dehiscent or indehiscent) is a 'last-minute' developmental decision, happening after fertilization. In this species, flowers that already possess the structures typical of the dehiscent morph (four to six ovules and a segment) can degrade those structures and instead form indehiscent fruits (Lenser et al. 2018). This bet-hedging strategy allows plastic responses to environmental unpredictability in the current season. Indeed, the ratio of single-seeded indehiscent fruits, bearing dormant seeds, to multi-seeded dehiscent fruits, bearing quickly and uniformly germinating seeds, increases in adverse growth conditions such as defoliation or shading. Lenser and colleagues (2018) demonstrated that the production of these two fruit morphs is regulated by the transcript levels of *BRANCHED1 (BRC1)*, belonging to the family of transcription factors *TB1 CYCLOIDEA PCF (TCP)*. *BRC1* transcription is indeed particularly important in hormonal production and is thought to integrate the regulatory roles of developmental hormones such as auxin, strigonolactone and cytokinin that are involved in fruit development (Lenser et al. 2018). Moreover, a transcriptome analysis revealed differentially expressed genes (DEGs) in dry seeds belonging to the two fruit morphs. The 'less dormant' morph showed higher levels of transcripts of genes whose expression increases with maturation, such as genes encoding ribosomal proteins, LEAs, NYE1 protein (chlorophyll degradation) and HSFA9 (heat shock protein), and lower expression of genes that decrease during maturation such as *ABI3* (abscisic acid-mediated regulation and embryo degreening) and *WRI1* (embryo development). These results, suggesting different degrees of after-ripening in the two morphs, are in accordance with the already known dormancy behaviour of the two morphs, while other DEGs (related to ROS detoxification, late embryo abundant proteins-LEAs) suggest also the possibility of different longevity behaviour, so far unknown (Wilhelmsson et al. 2019).

Bet-hedging and non-genetic bases: epigenetics

Changes in environmental cues can modify gene expression through epigenetic mechanisms (i.e. methylation, histone modifications and non-coding RNAs-mediated), generating plastic phenotypic variation that can be heritable or not. Phenotypic plasticity can thus be individual (within-generation) or be inherited as an effect of the epigenetic changes exerted by the environment on the parents that is meiotically transmitted (trans-generational). The latter can be defined as 'heritable bet-hedging' and can maintain or

increase the genetic potential of a population across generations, acting as a means of evolutionary rescue from extinction (Herman et al. 2013; O'dea et al. 2016). Epigenetic stability across several generations can be seen as a trade-off between short-term, within-generation epigenetic changes and long-term, genetically fixed adaptations (Alvarez et al. 2020). Random switching between epigenetic states is indeed advantageous in situations of high unpredictability, where there is not an optimal phenotype adapted to all the possible environmental conditions, while genetically fixed adaptations are advantageous for phenotypes close to the optimum, in less variable environments. When intermediate situations occur, i.e. variations in the environment exist but are not so frequent, transgenerational heritability of randomly generated epiallelic variants is advantageous as an evolutionary strategy that maintains adaptive phenotype-environment matching at an intermediate temporal scale (Herman et al. 2013). Thus, persistent trans-generational effects become adaptive when a response to long-term, multigenerational environmental changes is necessary. In this context, 'fixed' epigenetic states are not directly inherited as epigenetic signatures, rather they are re-induced through a feedback between phenotype and the environmental cues/conditions (Alvarez et al. 2020). The proportion of variation in seed traits related to dispersal that cannot be explained by genetic or environment variability and that can be transmitted to subsequent generations has its origins in epigenetics, which can be both heritable and adaptive (Johnson et al. 2019). The heritability of epigenetic marks was explored in four different *A. thaliana* inbred lines after a heat stress induced in an 'ancestral' generation and then studied in the third generation. One genotype (Cvi) showed the establishment of a bet-hedging strategy possibly induced by the inheritance, within the same generation, of random epiallelic variants caused by the heat stress experienced by the ancestor (Suter & Widmer 2013). In another study on four *A. thaliana* lines, no transgenerational epigenetic heritability was found when plants were subjected to water stress. Rather, the differences in responses to drought stress observed between generations were based on phenotypic plasticity rather than maternal effects and were exerted in a within-generational fashion (van Dooren et al. 2020).

Epigenetically induced variability can be advantageous in coping with environmental changes, but it can also be maladaptive when random epialleles are generated. Nevertheless, maladaptive epigenetic marks are often negatively selected at the seedling stage, and therefore they result in more tolerance in plants that produce large seed numbers, where the probability of extinction caused by maladaptive marks in the offspring is lower (Minow & Colasanti 2020). So, even with the possible insurgence of maladaptive marks, epigenetically variable populations can be advantageous, as a fraction of individuals is likely to be more suited to certain environmental conditions, thus providing buffering capacity to the total population (Alonso et al. 2018).

Cytosine methylation is a key component of epigenetic regulation in plants and it has been associated with functional changes in gene expression and genomic stability (Alonso et al. 2014, 2018). Genome-wide variations in cytosine methylation are associated with fecundity-related traits in the evergreen shrub *Lavandula latifolia* Medik (Lamiaceae) and in the perennial herb *Helleborus foetidus* Moench (Ranunculaceae) (Alonso et al. 2018; Herrera et al. 2014). In *L. latifolia*, sub-individual epigenetic mosaicism, in which different parts of the same genetic individual differ in DNA methylation patterns, was hypothesized to be related to variations in the exposure of different plant anatomical parts to one or more environmental factors that trigger epigenetic changes as a plastic response, resulting in the differential production of seeds in terms of mass and number (Alonso et al. 2018). In *H. foetidus*, variation in the individual transgenerational transmission of epigenetic marks (mainly methylation) was related to within-plant variance in seed size (Johnston & Bassel 2018), suggesting a complex mechanism, not only related to mosaicism but also to transmissibility that could link epigenetic changes to sub-individual heterogeneity of reproductive organs.

Rapid changes in the dormancy status of *A. thaliana* seeds in response to environmental fluctuations, especially temperature, can also be driven by epigenetic modifications. Genome-wide chromatin remodelling induces changes in gene expression that enable seeds to respond to seasonal variation through different dormancy behaviours, in particular via histone modification of the *DOG1* chromatin with the H3K4me3 and H3K27me3 marks that cause a reduction of DOG1 protein production in late spring and therefore dormancy release (Footitt et al. 2015).

While full details remain to be uncovered, it is clear that inducible changes in genetic networks can increase the range of possible molecular interactions, thereby allowing the expression of plasticity required for the adaptation to biotic or environmental stochasticity.

Impact on seed physiology

A positive-feedback regulatory motif involved in ABA synthesis and degradation was modelled in relation to germination patterns in *A. thaliana* (Abley et al. 2020; Johnston and Bassel 2018). This motif belongs to a larger regulatory network that involves also gibberellin (GA), which interacts antagonistically with ABA in regulating germination and dormancy (Topham et al. 2017); this system can be tuned in response to environmental cues and provides regulation of transcriptional factors that control germination and dormancy as an adaptation to stressful conditions (Abley et al.

2020). Indeed, a type of bet-hedging strategy observed in seeds of *A. thaliana* subjected to stress has been recently associated with loci overlapping with several genes involved in the regulation of GA and ABA sensitivity/degradation. This regulation, of ABA levels in particular, in turn generates downstream transcriptional variation possibly involved in phenotypic plasticity. Moreover, the authors ruled out a previously hypothesized positional regulatory gradient in the ovary, thus uncoupling a possible developmental influence on dormancy as a confirmation of the effective presence of bet-hedging (Abley et al. 2020).

Differences in physiological responses to oxidative stress have also been observed in heteromorphic species with bet-hedging strategies. In *Arthrocnemum macrostachyum* (Moric.) K.Koch and *Arthrocnemum indicum* (Willd.) Moq. (Chenopodiaceae), two halophytes with black/brown and large/small heteromorphic seeds, respectively, differential levels of antioxidant activity and oxidation markers (H_2O_2 and malondialdehyde-MDA) were found during seed germination under increasing salinity. In both species, the salt-tolerant morphs did not show changes in their antioxidant activity nor in H_2O_2 and MDA levels, while the morphs with lower salt tolerance also showed a less resilient antioxidant machinery, resulting in higher levels of oxidative damage (Nisar et al. 2019). Similarly, when subjected to accelerated ageing, heteromorphic caryopses of *Aegilops* and of *Triticum urartu* Thum. ex Gandilyan (Poaceae) showed different lifespans and antioxidant activities: the shorter-lived, larger morphs possess lower antioxidant activities when compared to their smaller, darker and longer-lived counterparts (Gianella et al. 2020).

In both studies, darker morphs showed higher phenolic contents and delayed germination. Some polyphenols indeed act as germination inhibitors and slow down water uptake by thickening the seed coat, thus allowing a longer persistence in the soil. For instance, higher phenolic contents in black seeds of *S. salsa* have been associated with a population-dependent bet-hedging strategy linked to persistence in intertidal soils subjected to waterlogging, and also to longevity (Xu et al. 2016). Brown seeds in this species, with lower phenolic contents in their seed coats, germinate quickly due to more rapid water uptake and showed higher contents of free sugars and enzymes related to lipid and pre-germinative metabolism (Xu et al. 2016; Zhao et al. 2018). Similarly, *Atriplex centralasiatica* Iljin (Amaranthaceae) possesses two morphs, black and brown, in which there is differential accumulation of phenolics in the seed coat. The black morph, with slower water uptake, constitutes the soil seed bank, whereas the brown one is salt tolerant and shows a more rapid germination, due to its permeable coat and a higher GA content (Li et al. 2011).

It has been postulated that seeds with deeper dormancy could be less damaged by reactive oxygen species (ROS) due to the 'collateral' antioxidant activity of chemicals involved in dormancy regulation, e.g. phenols (Lepiniec et al. 2006). Flavonoids, lignins and lignans found in the seed coat are also associated with seed dormancy and longevity (Long et al. 2015), and polyphenols in general act as protective chemicals fundamental for the persistence in the soil, being antioxidant and also antimicrobial compounds (Hradilová et al. 2019). Differential levels of proanthocyanidins (PAs), a class of polyphenols, were observed in the seed coats of differently pigmented populations of wild pea (*Pisum sativum* subsp. *elatius* (M.Bieb.) Asch. & Graebn. (Fabaceae)) collected across the Mediterranean area, south-eastern Europe and the Middle East. The soluble to insoluble PAs ratio and coat thickness correlated with different dormancy levels in differently distributed populations, this in turn correlating with different seasonality and climatic conditions and thus indicating a bet-hedging strategy in wild peas (Hradilová et al. 2019). An association between seed coat properties and bet-hedging was found also in *Medicago truncatula* Gaertn (Fabaceae), showing plasticity in dormancy release along with an aridity gradient. A genome-wide association study revealed that four genes related to flavonoid metabolism and seven peroxidases and thio-/peroxiredoxins are associated with differential dormancy release depending on the environmental conditions (Renzi et al. 2020).

Conclusions and future perspectives

Bet-hedging strategies result from ecological adaptions and are driven by different genetic, epigenetic and physiological processes that in turn modify the dynamics of population genetics (Fig. 4):

- Bet-hedging strategies, diffused in probably hundreds of angiosperm species, can be categorized depending on the degree of specialization or risk taken. Different plant anatomical parts (e.g. seeds, fruits and buds) can embody these strategies by exhibiting physical (e.g. seed size) or physiological (e.g. seed dormancy) heteromorphism (Olofsson et al. 2009; Scholl et al. 2020).
- Several traits are influenced by bet-hedging strategies, including abiotic and biotic stress resistance, germination phenology, susceptibility to predation, seed dormancy, seed morphology and seed longevity (Bhatt and Santo 2016; Datta et al. 1970; Guzzon et al. 2018).
- The presence of bet-hedging alters the dynamics of population genetics, in particular modifying the substitution rates and influencing also host-parasite coevolution (Dann et al. 2017; Verin and Tellier, 2018).
- Different loci have been associated with seed heteromorphism and bet-hedging, mainly comprising genes involved in pathways linked to dormancy (e.g. *DOG1* and ABA-related genes). Bet-hedging is also reflected

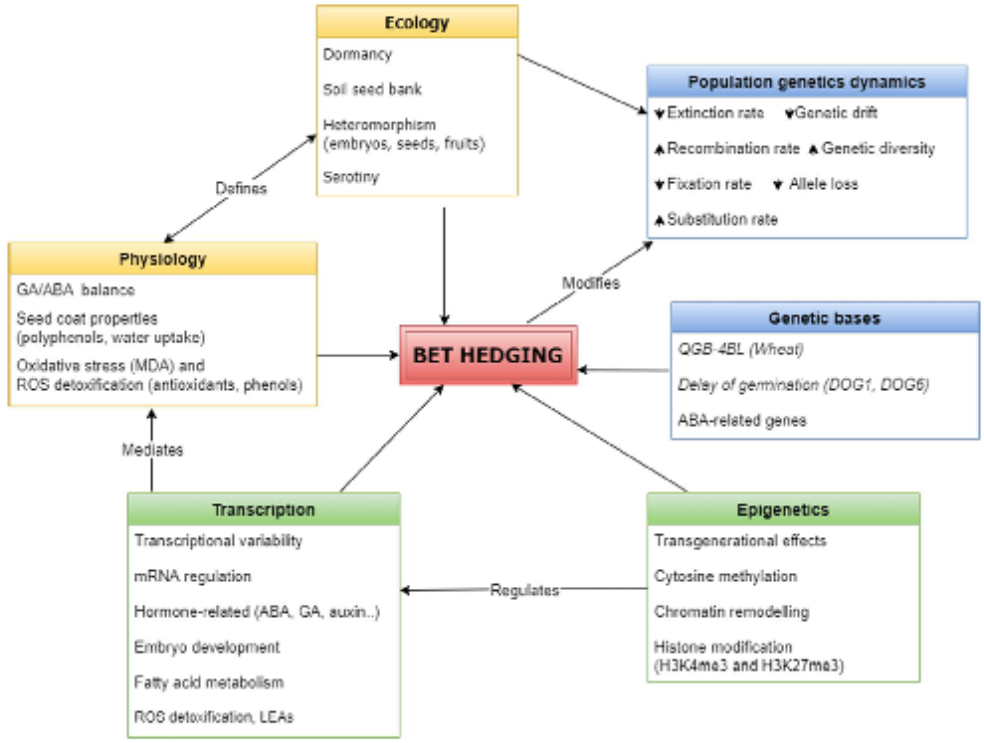


Fig. 4 Network of processes related to bet-hedging

in differential transcription patterns of genes belonging to different metabolic and developmental processes, including embryo development, fatty acids and sugar metabolism, ROS detoxification and late embryogenesis abundant proteins-LEAs (Footitt et al. 2014; Nave et al. 2016; Wilhelmsson et al. 2019; Zinsmeister et al. 2020).

- Epigenetics can drive bet-hedging via trans- and intergenerational transmission by regulating gene expression through genome-wide methylation marks in response to environmental cues. The regulation of different pathways affects physiology through differential hormone levels, antioxidant responses and seed coat properties in heteromorphic seeds (Abley et al. 2020; Alonso et al. 2018; Hradilová et al. 2019).

Many aspects of bet-hedging in flowering plants still need to be explored. From an ecological point of view, most of the studies on bet-hedging have focused on just one phenotypic trait, such as seed longevity (Guzzon et al. 2018) or resistance to abiotic stresses during germination (Bhatt and Santo 2016). Further studies are needed to consider simultaneously the different traits influenced by bet-hedging in order to clarify the ecological trade-offs involved in this strategy in different plant species (Gianella et al. 2020). Moreover, a more comprehensive phylogenetic analysis is needed to assess the presence of bet-hedging and its evolutionary significance in the whole phylum, considering taxa from all over the globe (Scholl et al. 2020; Gioria et al. 2020). Additionally, the genetic bases of bet-hedging are poorly known, particularly in those plant families where it is less distributed. Indeed, studies that could identify the loci underlying bet-hedging strategies in different taxa are necessary, such as studying loci negatively selected for diaspore and germination uniformity during domestication, as elegantly done by Nave and colleagues in durum wheat (2016). Given the important role of non-genetic mechanisms in the plant responses to environmental cues, a focus on epigenetics is also necessary and could complement multigenerational germination studies where seeds are subjected to different stresses resembling the environmental variations

that are suspected to be the trigger of the evolution of bet-hedging. Since differential gene expression and different patterns of metabolites have already been observed in some taxa showing bet-hedging strategies (e.g. Nisar et al. 2019; Xu et al. 2017), multi-omics approaches could shed light on the dynamics of pre-germinative metabolism in heteromorphic seeds that show bet-hedging strategies. Indeed, with the recent advancements and the increasing cost-effectiveness of molecular and physiological assays, bet-hedging could be explored at different levels of detail also in non-model organisms, in order to obtain a clearer picture of this fascinating evolutionary strategy.

Author contribution statement M.G. conceived and wrote the manuscript, performed the literature search and developed the figures, K.J.B. wrote some sections of the manuscript, critically revised earlier versions and performed linguistic revision, F.G. wrote some sections and critically revised earlier versions of the manuscript.

Funding Open Access funding provided by Università degli Studi di Pavia. The authors did not receive support from any organization for the submitted work.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and material Not applicable.

Code availability Not applicable.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent to Publication Not applicable.

References

Abley K, Formosa-Jordan P, Tavares H, Chan E, Leyser O (2020) LockeJCW (2020) An ABA-GA bistable switch can account for natural variation in the variability of *Arabidopsis* seed germination time. BioRxiv 06(05):135681

Alamos S, Reimer A, Niyogi KK (2020) Garcia HG (2020) Quantitative imaging of RNA polymerase II activity in plants reveals the single-cell basis of tissue-wide transcriptional dynamics. BioRxiv 2008(2030):274621

Alonso C, Perez R, Bazaga P, Medrano M, Herrera CM (2014) Individual variation in size and fecundity is correlated with differences in global DNA cytosine methylation in the perennial herb *Helleborus foetidus* (Ranunculaceae). Am J Bot 101:1309–1313. https://doi.org/10.3732/ajb.1400126

Alonso C, Pérez R, Bazaga P, Medrano M, Herrera CM (2018) Within-plant variation in seed size and inflorescence fecundity is associated with epigenetic mosaicism in the shrub *Lavandula latifolia* (Lamiaceae). Ann Bot 121:153–160. https://doi.org/10.1093/aob/mcx140

Alvarez M, Bleich A, Donohue K (2020) Genotypic variation in the persistence of transgenerational responses to seasonal cues. Evolution 74:2265–2280. https://doi.org/10.1111/evo.13996

Arshad W, Sperber K, Steinbrecher T, Nichols B, Jansen VAA, Leubner-Metzger G, Mummenhoff K (2019) Dispersal biophysics and adaptive significance of dimorphic diaspores in the annual *Aethionema arabicum* (Brassicaceae). New Phytol 221:1434–1446. https://doi.org/10.1111/nph.15490

Awan S, Footitt S, Finch-Savage WE (2018) Interaction of maternal environment and allelic differences in seed vigour genes determines seed performance in *Brassica oleracea*. Plant J 94:1098–1108. https://doi.org/10.1111/tpj.13922

Baskin CC, Baskin JM (2014) Seeds-Ecology, biogeography, and evolution of dormancy and germination, 2nd edn. Academic Press, Cambridge

Bentsink L, Hanson J, Hanhart CJ, Blankestijn-de Vries H, Coltrane C, Keizer P, El-Lithy M, Alonso-Blanco C, de Andres MT, Reymond M, van Eeuwijk F, Smeekens S, Koornneef M (2010) Natural variation for seed dormancy in Arabidopsis is regulated by additive genetic and molecular pathways. Proc Natl Acad Sci USA 107:4264–4269. https://doi.org/10.1073/pnas.1000410107

Bewley JD, Bradford KJ, Nonogaki HWM (2013) Seeds: Physiology of Developenent, Germination and Dormancy, 3rd Edition. Springer https://doi.org/10.15713/ins.mmj.3

Bhatt A, Santo A (2016) Germination and recovery of heteromorphic seeds of *Atriplex canescens* (Amaranthaceae) under increasing salinity. Plant Ecol 217:1069–1079. https://doi.org/10.1007/s11258-016-0633-6

Bhattacharya S, Sperber K, Ozudogru B, Leubner-Metzger G, Mummenhoff K (2019) Naturally-primed life strategy plasticity of dimorphic *Aethionema arabicum* facilitates optimal habitat colonization. Sci Rep 9:16108. https://doi.org/10.1038/s41598-019-52520-y

Bradford KJ (2018) Interpreting biological variation: seeds, populations and sensitivity thresholds. Seed Sci Res 28:158–167. https://doi.org/10.1017/S0960258518000156

Buoro M, Carlson SM (2014) Life-history syndromes: integrating dispersal through space and time. Ecol Lett 17:756–767

Cao L, Chen L, Wang J, Xing J, Lv X, Maimaitijiang T, Lan H (2020) Effects of genetic and environmental factors on variations of seed heteromorphism in *Suaeda aralocaspica*. AoB PLANTS 12:44. https://doi.org/10.1093/aobpla/plaa044

Charlesworth D (1989) Evolution of low female fertility in plants: Pollen limitation, resource allocation and genetic load. Trends Ecol Evol 4:289–292. https://doi.org/10.1016/0169-5347(89)90023-2

Chen M, Penfield S (2018) Feedback regulation of *COOLAIR* expression controls seed dormancy and flowering time. Science 360:1014–1017. https://doi.org/10.1126/science.aar7361

Childs DZ, Metcalf CJE, Rees M (2010) Evolutionary bet-hedging in the real world: empirical evidence and challenges revealed by plants. Proc R Soc Lond [Biol] 277:3055–3064. https://doi.org/10.1098/rspb.2010.0707

Cohen D (1966) Optimizing reproduction in a randomly varying environment. J Theor Biol 12:119–129. https://doi.org/10.1016/0022-5193(66)90188-3

Conner JA, Mookkan M, Huo H, Chae K, Ozias-Akins P (2015) A parthenogenesis gene of apomict origin elicits embryo formation from unfertilized eggs in a sexual plant. PNAS 112:11205–11210

Cooke JEK, Eriksson ME, Junttila O (2012) The dynamic nature of bud dormancy in trees: Environmental control and molecular mechanisms. Plant Cell Environ 35:1707–1728. https://doi.org/10.1111/j.1365-3040.2012.02552.x

Dann M, Bellot S, Schepella S, Schaefer H, Tellier A (2017) Mutation rates in seeds and seed-banking influence substitution rates across the angiosperm phylogeny. bioRxiv preprint 156398.https://doi.org/10.1101/156398

Datta SC, Evenari M, Gutterman Y (1970) The heteroblasty of *Aegilops ovata*. Israel J Bot 19:463–483

de Casas RR, Donohue K, Venable DL, Cheptou P-O (2015) Gene-flow through space and time: dispersal, dormancy and adaptation to changing environments. Evol Ecol 29:813–831

Dyer AR (2017) The seed ecology of *Aegilops triuncialis*: linking trait variation to growing conditions. Seed Sci Res 27:183–198. https://doi.org/10.1017/S0960258517000174

Footitt S, Clay HA, Dent K, Finch-Savage WE (2014) Environment sensing in spring-dispersed seeds of a winter annual Arabidopsis influences the regulation of dormancy to align germination potential with seasonal changes. New Phytol 202:929–939. https://doi.org/10.1111/nph.12694

Footitt S, Müller K, Kermode AR, Finch-Savage WE (2015) Seed dormancy cycling in Arabidopsis: chromatin remodelling and regulation of *DOG1* in response to seasonal environmental signals. Plant J 81:413–425. https://doi.org/10.1111/tpj.12735

Footitt S, Walley PG, Lynn JR, Hambidge AJ, Penfield S, Finch-Savage WE (2019) Trait analysis reveals *DOG1* determines initial depth of seed dormancy, but not changes during dormancy cycling that result in seedling emergence timing. New Phytol 225:2035–2047. https://doi.org/10.1111/nph.16081

Gianella M, Balestrazzi A, Pagano A, Müller JV, Kyratzis AC, Kikodze D, Canella M, Mondoni A, Rossi G, Guzzon F (2020) Heteromorphic seeds of wheat wild relatives show germination niche differentiation. Plant Biol 22:191–202. https://doi.org/10.1111/plb.13060

Gioria M, Pyšek P, Baskin CC, Carta A (2020) Phylogenetic relatedness mediates persistence and density of soil seed banks. J Ecol 108:2121–2131. https://doi.org/10.1111/1365-2745.13437

Gremer JR, Venable DL (2014) Bet hedging in desert winter annual plants: Optimal germination strategies in a variable environment. Ecol Lett 17:380–387. https://doi.org/10.1111/ele.12241

Guzzon F, Orsenigo S, Gianella M, Müller JV, Vagge I, Rossi G, Mondoni A (2018) Seed heteromorphy influences seed longevity in *Aegilops*. Seed Sci Res 28:277–285. https://doi.org/10.1017/S096025851800034X

Haaland TR, Wright J, Ratikainen II (2020) Generalists versus specialists in fluctuating environments: a bet-hedging perspective. Oikos 129:879–890. https://doi.org/10.1111/oik.07109

Herman JJ, Spencer HG, Donohue K, Sultan SE (2013) How stable "should" epigenetic modifications be? Insights from adaptive plasticity and bet hedging. Evol 68:632–643. https://doi.org/10.1111/evo.12324

Herrera CM, Medrano M, Bazaga P (2014) Variation in DNA methylation transmissibility, genetic heterogeneity and fecundity-related traits in natural populations of the perennial herb *Helleborus foetidus*. Mol Ecol 23:1085–1095. https://doi.org/10.1111/mec.12679

Hopper KR, Rosenheim JA, Prout T, Oppenheim SJ (2003) Within-generation bet hedging: a seductive explanation? Oikos 101:219–222. https://doi.org/10.1034/j.1600-0706.2003.12051.x

Horst JL, Venable LD (2017) Frequency-dependent seed predation by rodents on Sonoran Desert winter annual plants. Ecol 99:196–203. https://doi.org/10.1002/ecy.2066

Hradilová I, Duchoslav M, Brus J, Pechanec V, Hýbl M, Kopecký P, Smržová L, Štefelová N, Vaclávek T, Bariotakis M, Machalová J, Hron K, Pirintsos S, Smýkal P (2019) Variation in wild pea (Pisum sativum subsp elatius) seed dormancy and its relationship to the environment and seed coat traits. PeerJ 7:e6263. https://doi.org/10.7717/peerj.6263

Huang Z, Liu S, Bradford KJ, Huxman TE, Venable DL (2016) The contribution of germination functional traits to population dynamics of a desert plant community. Ecol 97:250–261. https://doi.org/10.1890/15-0744.1

Hulme PE (1998) Post-dispersal seed predation and seed bank persistence. Seed Sci Res 8:513–519. https://doi.org/10.1017/s0960258500004487

Huxman TE, Barron-Gafford G, Gerst KL, Angert AL, Tyler AP, Venable LD (2008) Photosynthetic resource-use efficiency and demographic variability in desert winter annual plants. Ecol 89:1554–1563. https://doi.org/10.1890/06-2080.1

Imbert E (2002) Ecological consequences and ontogeny of seed heteromorphism. Perspect Plant Ecol Evol Syst 5:13–36. https://doi.org/10.1078/1433-8319-00021

Iriondo JM, Milla R, Volis S, Rubio de Casas R (2018) Reproductive traits and evolutionary divergence between Mediterranean crops and their wild relatives. Plant Biol 20:78–88. https://doi.org/10.1111/plb.12640

Jiang L, Wang L, Baskin CC, Tian CY, Huang ZY (2019) Maternal effects on seed heteromorphism: a dual dynamic bet hedging strategy. Seed Sci Res 29:149–153. https://doi.org/10.1017/S0960258519000114

Johnson JS, Cantrell RS, Cosner C, Hartig F, Hastings A, Rogers HS, Schupp EW, Shea K, Teller BJ, Yu X, Zurell D, Pufal G (2019) Rapid changes in seed dispersal traits may modify plant responses to global change. AoBP 11: plz020 https://doi.org/10.1093/aobpla/plz020.

Johnston IG, Bassel GW (2018) Identification of a bet-hedging network motif generating noise in hormone concentrations and germination propensity in Arabidopsis. J R Soc Interface 15:20180042. https://doi.org/10.1098/rsif.2018.0042

Khanday I, Skinner D, Yang B, Mercier R, Sundaresan V (2019) A male-expressed rice embryogenic trigger redirected for asexual propagation through seeds. Nature 565:91–95

Kumar S (2017) Epigenetic control of apomixis: a new perspective of an old enigma. Adv Plants Agric Res 7:227-233. https://doi.org/10.15406/apar.2017.07.00243

Lamont BB, Pausas JG, He T, Witkowski ETF, Hanley ME (2020) Fire as a selective agent for both serotiny and nonserotiny over space and time. Crit Rev Plant Sci 39:140–172. https://doi.org/10.1080/07352689.2020.1768465

Lenser T, Tarkowská D, Novák O, Wilhelmsson PKI, Bennett T, Rensing SA, Strnad M, Theißen G (2018) When the BRANCHED network bears fruit: how carpic dominance causes fruit dimorphism in *Aethionema*. Plant J 94:352–371. https://doi.org/10.1111/tpj.13861

Lepiniec L, Debeaujon I, Routaboul JM, Baudry A, Pourcel L, Nesi N, Caboche M (2006) Genetics and biochemistry of seed flavonoids. Annu Rev Plant Biol 57:405–430. https://doi.org/10.1146/annurev.arplant.57.032905.105252

Leverett LD, Jolls CL (2013) Cryptic seed heteromorphism in *Packera tomentosa* (Asteraceae): Differences in mass and germination. Plant Spec Biol 29:169–180. https://doi.org/10.1111/1442-1984.12011

Li W, Liu X, Hanada A, Khan MA (2011) Effect of cold stratification, scarification and hormones on germination of dimorphic seeds of *Atriplex centralasiatica* under saline conditions. Seed Sci Technol 1: 82–92 https://doi.org/10.15258/sst.2011.39.1.08

Liu S, Bradford KJ, Huang Z, Venable DL (2020) Hydrothermal sensitivities of seed populations underlie fluctuations of dormancy states in an annual plant community. Ecology 101:e02958. https://doi.org/10.1002/ecy.2958

Long RL, Gorecki MJ, Renton M, Scott JK, Colville L, Goggin DE, Commander LE, Westcott DA, Cherry H, Finch-Savage WE (2015) The ecophysiology of seed persistence: A mechanistic view of the journey to germination or demise. Biol Rev 90:31–59. https://doi.org/10.1111/brv.12095

Lu JJ, Tan DY, Baskin JM, Baskin CC (2013) Trade-offs between seed dispersal and dormancy in an amphi-basicarpic cold desert annual. Ann Bot 112:1815–1827. https://doi.org/10.1093/aob/mct240

Martínez-Berdeja A, Stitzer M, Taylor M, Okada M, Ezcurra E, Runcie DE, Schmitt J (2020) Functional variants of *DOG1* control seed chilling responses and variation in seasonal life-history strategies in *Arabidopsis thaliana*. PNAS 117:2526–2534. https://doi.org/10.1073/pnas.1912451117

Minow MAA, Colasanti J (2020) Does variable epigenetic inheritance fuel plant evolution? Genome 63:253–262. https://doi.org/10.1139/gen-2019-0190

Mitchell J, Johnston IG, Bassel GW (2017) Variability in seeds: Biological, ecological, and agricultural implications. J Exp Bot 68:809–817. https://doi.org/10.1093/jxb/erw397

Moncalvillo B, Méndez M, Iriondo JM (2019) Ecotypic differentiation reveals seed colour-related alkaloid content in a crop wild relative. Plant Biol 21:942–950. https://doi.org/10.1111/plb.12996

Mueller NG (2017) Documenting domestication in a lost crop (*Polygonum erectum* L.): evolutionary bet-hedgers under cultivation. Veg Hist Archaeobot 26:313–327. https://doi.org/10.1007/s00334-016-0592-9

Nave M, Avni R, Ben-Zvi B, Hale I, Distelfeld A (2016) QTLs for uniform grain dimensions and germination selected during wheat domestication are co-located on chromosome 4B. Theor Appl Genet 129:1303–1315. https://doi.org/10.1007/s00122-016-2704-4

Nichols BS, Leubner-Metzger G, Jansen VAA (2020) Between a rock and a hard place: adaptive sensing and site-specific dispersal. Ecol Lett 23:1370–1379. https://doi.org/10.1111/ele.13564

Niklas KJ, Cobb ED (2017) The evolutionary ecology (evo-eco) of plant asexual reproduction. Evol Ecol 31:317–332. https://doi.org/10.1007/s10682-016-9828-5

Nilsson P, Tuomi J, Åström M (1996) Bud dormancy as a bet-hedging strategy. Am Nat 147:269–281. https://doi.org/10.1086/285849

Nisar F, Gul B, Khan MA, Hameed A (2019) Heteromorphic seeds of coastal halophytes *Arthrocnemum macrostachyum* and *A. indicum* display differential patterns of hydrogen peroxide accumulation, lipid peroxidation and antioxidant activities under increasing salinity. Plant Physiol Bioch 144:58–63. https://doi.org/10.1016/j.plaphy.2019.09.031

O'dea RE, Noble DWA, Johnson SL, Hesselson D, Nakagawa S (2016) The role of non-genetic inheritance in evolutionary rescue: epigenetic buffering, heritable bet hedging and epigenetic traps. Environmental Epigenetics 2:1–12. https://doi.org/10.1093/eep/dvv014

Olofsson H, Ripa J, Jonzén N (2009) Bet-hedging as an evolutionary game: the trade-off between egg size and number. Proc R Soc Lond [Biol] 276:2963–2969. https://doi.org/10.1098/rspb.2009.0500

Peters EM, Martorell C, Ezcurra E (2011) The effects of serotiny and rainfall-cued dispersal on fitness: Bet-hedging in the threatened cactus *Mammillaria pectinifera*. Popul Ecol 53:383–392. https://doi.org/10.1007/s10144-010-0239-0

Philippi T (1993) Bet-hedging germination of desert annuals: variation among populations and maternal effects in *Lepidium lasiocarpum*. Am Nat 142:488–507. https://doi.org/10.1086/285551

Philippi T, Seger J (1989) Hedging one's evolutionary bets, revisited. Trends Ecol Evol 4:41–44. https://doi.org/10.1016/0169-5347(89)90138-9

Renzi JP, Duchoslav M, Brus J, Hradilová I, Pechanec V, Václavek T, Machalová J, Hron K, Verdier J, Smýkal P (2020) Physical dormancy release in *Medicago truncatula* seeds is related to environmental variations. Plants 9:503. https://doi.org/10.3390/plants9040503

Rodrigues JCM, Koltunow AMG (2005) Epigenetic aspects of sexual and asexual seed development. Acta Biol Cracov Bot 47:37–49

Santini BA, Martorell C (2013) Does retained-seed priming drive the evolution of serotiny in drylands? An assessment using the cactus *Mammillaria hernandezii*. Am J Bot 100:365–373. https://doi.org/10.3732/ajb.1200106

Scholl JP, Calle L, Miller N, Venable DL (2020) Offspring polymorphism and bet hedging: a large-scale, phylogenetic analysis. Ecol Lett 23:1223–1231. https://doi.org/10.1111/ele.13522

Seger J, Brockmann HJ (1987) What is Bet-Hedging. In: Harvey PH, Partridge L (eds) Oxford Surveys in Evolutionary Biology: 182–211. Oxford University Press, Oxford, UK

Simons AM (2009) Fluctuating natural selection accounts for the evolution of diversification bet hedging. Proc R Soc Lond [Biol] 276:1987–1992. https://doi.org/10.1098/rspb.2008.1920

Simons AM, Johnston MO (2006) Environmental and genetic sources of diversification in the timing of seed germination: implication for the evolution of bet hedging. Evolution 60:2280–2292. https://doi.org/10.1111/j.0014-3820.2006.tb01865.x

Slatkin M (1974) Hedging one's evolutionary bets. Nature 250:704–705. https://doi.org/10.1038/250704b0

Soliman MI (2003) Genetic diversity of achene heteromorphism in Egyptian *Calendula micrantha* Tineo et Guss. Asian J Plant Sci 2:782–789. https://doi.org/10.3923/ajps.2003.782.789

Springthorpe V, Penfield S (2015) Flowering time and seed dormancy control use external coincidence to generate life history strategy. ELife 4:e05557. https://doi.org/10.7554/eLife.05557

Stadler MR, Eisen MB (2017) Atlas…t, patterns from every cell. Science 358:172–173. https://doi.org/10.1126/science.aap8493

Starrfelt J, Kokko H (2012) Bet-hedging-a triple trade-off between means, variances and correlations. Biol Rev 87:742–755. https://doi.org/10.1111/j.1469-185X.2012.00225.x

Still DW, Bradford KJ (1997) Endo-beta-mannanase activity from individual tomato endosperm caps and radicle tips in relation to germination rates. Plant Physiol 113:21–29

Suter L, Widmer A (2013) Phenotypic effects of salt and heat stress over three generations in *Arabidopsis thaliana*. PLoS ONE 8:e80819. https://doi.org/10.1371/journal.pone.0080819

Tellier A (2018) Persistent seed banking as eco-evolutionary determinant of plant nucleotide diversity: novel population genetics insights. New Phytol 221:725–730. https://doi.org/10.1111/nph.15424

ten Brink H, Gremer JR, Kokko H (2020) Optimal germination timing in unpredictable environments: the importance of dormancy for

both among- and within-season variation. Ecol Lett 23:620–630. https://doi.org/10.1111/ele.1346

Thurlby KAG, Wilson PG, Sherwin WB, Connelly C, Rossetto M (2012) Reproductive bet-hedging in a rare yet widespread rainforest tree, *Syzygium paniculatum* (Myrtaceae). Austral Ecol 37:936–944. https://doi.org/10.1111/j.1442-9993.2011.02353.x

Topham AT, Taylor RE, Yan D, Nambara E, Johnston IG, Bassel GW (2017) Temperature variability is integrated by a spatially embedded decision-making center to break dormancy in Arabidopsis seeds. Proc Natl Acad Sci USA 114:6629–6634. https://doi.org/10.1073/pnas.1704745114

Vaerbak S, Andersen SB (2004) Genetic control of seed set linked and unlinked to flower heteromorphism in inbred lines of *Primula vulgaris* Hudson. Euphytica 93:55–62

van Dooren TJM, Silveira AB, Gilbault E, Jiménez-Gómez JM, Martin A, Bach L, Tisné S, Quadrana L, Loudet O, Colot V (2020) Mild drought in the vegetative stage induces phenotypic, gene expression, and DNA methylation plasticity in Arabidopsis but no transgenerational effects. J Exp Bot 71:3588–3602. https://doi.org/10.1093/jxb/eraa132

Venable DL (1985) The evolutionary ecology of seed heteromorphism. Am Nat 126:577–595. https://doi.org/10.1086/284440

Venable DL (2007) Bet hedging in a guild of desert annuals. Ecology 88:1086–1090. https://doi.org/10.1890/06-1495

Verin M, Tellier A (2018) Host-parasite coevolution can promote the evolution of seed banking as a bet-hedging strategy. Evolution 72:362–1372. https://doi.org/10.1111/evo.13483

Volis S (2014) Dormancy-related seed positional effect in two populations of an annual grass from locations of contrasting aridity. PLoS ONE 9:e93061. https://doi.org/10.1371/journal.pone.0093061

Volis S, Bohrer G (2013) Joint evolution of seed traits along an aridity gradient: seed size and dormancy are not two substitutable evolutionary traits in temporally heterogeneous environment. New Phytol 197:655–667. https://doi.org/10.1111/nph.12024

Wang L, Dong M, Huang Z (2010) Review of research on seed heteromorphism and its ecological significance. Chinese J Plant Ecol 34:578–590. https://doi.org/10.3773/j.issn.1005-264x.2010.05.012

Wilhelmsson PKI, Chandler JO, Fernandez-Pozo N, Graeber K, Ullrich KK, Arshad W, Khan S, Hofberger JA, Buchta K, Edger PP, Pires JC, Schranz ME, Leubner-Metzger G, Rensing SA (2019) Usability of reference-free transcriptome assemblies for detection of differential expression: a case study on *Aethionema arabicum* dimorphic seeds. BMC Genom 20:95. https://doi.org/10.1186/s12864-019-5452-4

Xu Y, Liu R, Sui N, Shi W, Wang L, Tian C, Song J (2016) Changes in endogenous hormones and seed-coat phenolics during seed storage of two *Suaeda salsa* populations. Aust J Bot 64:325. https://doi.org/10.1071/BT16014

Xu Y, Zhao Y, Duan H, Sui N, Yuan F, Song J (2017) Transcriptomic profiling of genes in matured dimorphic seeds of euhalophyte *Suaeda salsa*. BMC Genomics 18:1–14. https://doi.org/10.1186/s12864-017-4104-9

Zhao Y, Ma Y, Li Q, Yang Y, Guo J, Song J (2018) Utilisation of stored lipids during germination in dimorphic seeds of euhalophyte *Suaeda salsa*. Funct Plant Biol 45:1009–1016. https://doi.org/10.1071/FP17309

Zinsmeister J, Leprince O, Buitink J (2020) Molecular and environmental factors regulating seed longevity. Biochem J 477:305–323

7.6 Book chapter

Gianella M, Pagano A, Forti C, Guzzon F, Mondoni A, de Sousa Araújo S, Macovei A, Balestrazzi A. Molecular aspects of seed priming as means of progress in crop improvement. In: Tuteja N. (Ed.) '*Advancement in crop improvement techniques'*, Woodhead Publishing (Elsevier). 2020; chapter 6, 89-100. https://doi.org/10.1016/B978-0-12-818581-0.00006-1.

Chapter 6

Molecular aspects of seed priming as a means of progress in crop improvement

Maraeva Gianella[a], Andrea Pagano[a], Chiara Forti[a], Filippo Guzzon[b,c], Andrea Mondoni[b], Susana de Sousa Araújo[d], Anca Macovei[a] and Alma Balestrazzi[a]

[a]Department of Biology and Biotechnology "L. Spallanzani", University of Pavia, Pavia, Italy, [b]Department of Environment and Earth Sciences, University of Pavia, Pavia, Italy, [c]International Maize and Wheat Improvement Center (CIMMYT), Texcoco, Mexico, [d]Institute of Chemical and Biological Technology António Xavier, New University of Lisbon (ITQB-NOVA), Oeiras, Portugal

1 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, 2019). It is quantified by parameters such as germination speed and rates, long-term and after-storage viability, seed lot homogeneity and purity, resistance to mechanical damage, and the lack of biological and chemical contaminants (Finch-Savage and Bassel, 2016). The notion that seed germination could be enhanced through specific treatments was historically attested by Theophrastus (371–287 BCE) and Gaius Plinius Secundus (23–79 CE), then later 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, herbs, and endangered species (Di Girolamo and Barbanti, 2012; Momin, 2013; Paparella et al., 2015). Different priming protocols have been optimized that vary temperature, aeration, oxygenation, and integrating protocols with additional treatments to maximize and homogenize the priming effect and improve stress resistance (Paparella et al., 2015).

Because seed viability and vigor are strongly influenced by deterioration during storage, aging is an issue in terms of seed lot quality, field establishment, and crop productivity. Different priming methods have been used to overcome aging-associated damage by improving the germination rate, uniformity, and percentage. The promising availability of multiple kinds of "omics" provides researchers with global views of their experimental systems at the genome, epigenome, transcriptome, proteome, metabolome, ionome, and lipidome levels. This is also true in the field of seed biology, where extensive studies are currently carried out to define the molecular profiles of seed priming.

2 Seed priming in the context of current challenges facing agriculture and crop production

The development of techniques to improve germination rates, speed, consistency, and viability under stress conditions has evolved in the modern concept of "seed priming," which 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 the seed pregerminative 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).

2.1 Priming agents and treatments: An overview

From a historical perspective, hydropriming was the first protocol that was developed. It consists of controlled preimbibition 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,

Advancement in Crop Improvement Techniques. https://doi.org/10.1016/B978-0-12-818581-0.00006-1

considering that water uptake depends mainly on 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 the seed due to its large molecular size (6000–8000 Da), avoiding any cytotoxic effects (Michel and Kauffmann, 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); this is known as "halo-priming" (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 in cytotoxic effects and nutritional imbalance within the seed (Bradford, 1995; Balestrazzi et al., 2011a,b).

Physical treatments can be used to improve germination and stress resistance. Thermopriming consists of presowing at specific temperatures to reduce the thermoinhibition of germination. 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 radiation (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., 2016a,b).

Chemopriming involves the administration of disinfectants (e.g., sodium hypochlorite or hydrochloric acid), fungicides, or pesticides to avoid growth impairments due to contamination (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 postpriming dehydration and under suboptimal storage conditions still represents a common critical point.

3 Seed priming versus seed aging in the context of seed bank storage

The term "seed bank" usually refers to a facility endowed with a system of collecting, cleaning, packing, storing, testing, and distributing seeds. Conventional seed banking exploits conditions of low temperature and moisture content to slow the seed aging 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 the 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. Because orthodox seeds tolerate a higher degree of water loss than recalcitrant seeds (0.2 g H_2O g_{DW}^{-1} versus less than 0.07), they can be stored for longer periods under seed bank conventional conditions, that is, 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 are extended (Ballesteros and Walters, 2011). Conversely, recalcitrant seeds cannot be preserved under conventional storage and cryopreservation is the safest approach, which is 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 because 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 aging (AA) or controlled deterioration (CD) tests. These tests exploit high temperatures (40–60°C) and RH (75%–100%) to accelerate the natural processes of aging. 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 the leakage of solutes caused by damage to lipidic membranes, which can be measured through electrodes. It is used to assess damage during aging, 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 aging 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).

"*Aging in all organisms is the sum total of the deteriorative processes that eventually lead to death*" (Matthews, 1985). The aging 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 aging 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 a sigmoidal shape (Bernal-Iugo and Leopold, 1998). Apart from the decline in germination rate, other phenotypical signs of aging can also be: (i) production within aged seed lots of smaller seedlings when compared with those produced by unaged seed lots; and (ii) even if viable, that is, seeds able to produce a radical protrusion, seedlings show abnormal phenotypes (Matthews, 1985). The primary processes of seed deterioration during aging are thought to be oxidative and peroxidative reactions. Free radicals form spontaneously, and because water tends to quench these reactions and maintain antioxidant activity, they have 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 byproducts that can damage other macromolecules such as proteins and nucleic acids (Bewley et al., 2013).

Because seed viability and vigor are strongly influenced by deterioration during storage, thus aging is an issue in terms of seed lot quality, field establishment, and crop productivity. Identifying effective aging hallmarks is therefore necessary to predict seed longevity and evaluate seed lots. Besides 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 aging hallmarks and processes (Marcos-Filho, 2015). Several markers of quantitative trait loci (QTLs), detected after AA tests, are associated with stress response and aging. 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., the role of DNA repair during aging has been underlined with crucial players such as DNA ligase 4 and 6 (Waterworth et al., 2010). In maize (*Zea mays* L.), some QTLs identified after AA are linked to the energy metabolism, stress response, signal transduction, and protein degradation pathways (Wang et al., 2016).

One of the main processes occurring during aging is lipid peroxidation. It can be assessed with an EC test and biochemical assays that measure its byproducts, such as malondialdehyde (MDA) and proline, or tocopherols, which help prevent lipid peroxidation and decrease during aging. Oxidative reactions can also be indirectly measured through gene expression analysis: the upregulation of genes encoding antioxidant enzymes can be viewed as a stress hallmark. Other genes related to stress response are involved in aging processes, such as 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., 2018a,b). Seed deterioration is also marked by the reduction of α-amylase activity and total soluble sugar content because the reduction in the starch metabolism observed in aged seeds can impair germination (Wang et al., 2018a,b; Pandey et al., 2017).

3.1 Seed priming as a tool to limit aging-associated damage

Different priming methods have been used to overcome aging-associated damage by improving the germination rate, uniformity, and percentage. Seeds of different species have been submitted to several priming techniques. It has been suggested that the effectiveness of these treatments depends on repair mechanisms activated during the hydration phase, namely nucleic acids, lipids, protein repair, and the reactivation of transcription and antioxidant enzyme scavenging activity (Probert et al., 1991; Kibinza et al., 2006; 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 the seed leachate measured through EC tests is reduced after the treatment (Pandey et al., 2017). However, it is not clear whether the damage repaired with priming is already present in aged seeds or the priming plays a preventive role by recovering antioxidant and repair enzymes before the onset of germination and subsequent

damage 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 seed priming treatments with positive effects both on germination and the 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 the 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., 2018a,b). The viability of primed stored seeds can be partially restored with poststorage treatments, namely repriming and heat shock, thereby maintaining the priming treatment benefits and allowing longer storage (Rao et al., 1987; Bruggink et al., 1999).

4 The molecular know-how of seed priming and its implications in promoting new advances in the sectors of seed biology and technology

The dynamics of water uptake and metabolic reactivation during seed imbibition have 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 the 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, 1997; Kranner et al., 2010). Subsequently, water absorption slows down and the 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,b). 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).

4.1 The redox context of the pregerminative metabolism and the harmful oxidative damage

The 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 damage to biological macromolecules but playing fundamental roles in several physiological processes. The recent scientific literature annotated the specific roles of the most abundant ROS and NRS, including hydroxyl radicals (OH·), hydrogen peroxide (H_2O_2), superoxide radicals (O_2^{-}), nitric oxide (NO), and other reactive molecules involved in the seed and seedling metabolism (Bailly et al., 2002, 2008; Bailly, 2004; Morohashi, 2002; Sarath et al., 2007). Additional ROS sources are found in seeds, such as oxidative reactions occurring in peroxisomes and glyoxysomes during the mobilization of seed reservoirs. ROS production, particularly at the mitochondrial level, is prominent in seed-specific developmental stages. Besides the physiological routes of ROS production, the occurrence of stress conditions can impair ROS homeostasis and elicit different ROS production and distribution patterns (stress-associated ROS signatures) (Choudhury et al., 2017; 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 Río 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

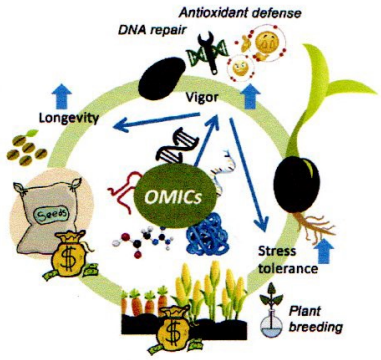


FIG. 1 Seed vigor and the underlying molecular mechanisms, particularly DNA repair and antioxidant defense, play crucial roles in maintaining high-standard seeds for both market and conservation purposes. The beneficial effects of seed priming range from enhanced/synchronized germination to increased 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.

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., 2011a,b; Confalonieri et al., 2013; Donà et al., 2013; 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., 2011b, 2014), processes major DNA damage that implies covalent adducts or UV-photoproducts. It is specifically elicited during transcription (NER-TCR, NER-transcription coupled repair) and in nontranscribed 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) is involved in the abiotic stress response in *M. truncatula* (Macovei et al., 2011b) 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 intricate network of molecular events has been envisaged as a source of novel hallmarks of seed vigor (Fig. 1) (Balestrazzi et al., 2011a,b; Paparella et al., 2015; Pagano et al., 2017, 2018).

5 Technology advancement

5.1 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 led to the identification of QTLs related to seed quality (Wang et al., 2018a,b). 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 overall, the increased expression of auxin-related genes was required for cell division and elongation in the subsequent phases (Holdsworth et al., 2008b). However, substantial differences have been found between the embryo and endosperm in pathways related to reserve mobilization and energy utilization (Penfield et al., 2006). The role of posttranscriptional 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* (Fu et al., 2019; Wei et al., 2018).

Through proteomic approaches, a global understanding of seed metabolism associated with improved seed vigor has been achieved for model plants such as *Arabidopsis* (Gallardo et al., 2001; Rajjou et al., 2006) and crop plants such 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 has highlighted the overall upregulation of genes involved in water uptake (e.g., genes encoding aquaporins), cell cycle and division, the 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 with different cultivars of *T. aestivum* (Das et al., 2017), *Zea mays* (Feenstra et al., 2017), and *H. vulgare* (Gorzolka et al., 2016), highlighting the differential accumulation and distribution of specific classes of amino acids, lipids, and carbohydrates. Metabolomic approaches have also been used to unveil the metabolic footprint of stress conditions in *A. thaliana* (Cohen and Amir, 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 and Chapman, 2014). Other high-throughput techniques are aimed at obtaining 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 tracing 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).

6 Conclusion and future perspectives

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 systems 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 interesting output for plant biotechnology and the seed industry. The identification of such "molecular markers of seed quality" will allow 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 more attention. The advanced avenues of genome editing are now open to explore the multifaceted aspects of seed vigor. CRISPR (clustered regularly interspaced short palindromic repeat)-Cas9 technology appears to be a promising tool to improve this complex trait.

Acknowledgments

This work was supported by the 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 M.G., C.F., A.P., A.M., A.B.). C.F. and A.P. have been awarded research fellowships from the CARIPLO Foundation in the frame of the WAKE-APT project (Code 2016-0723). S.S.A. acknowledges the financial support from Fundação para a Ciência e a Tecnologia (Lisbon, Portugal) through the research unit "GREEN-IT -Bioresources for Sustainability" (UID/Multi/04551/2019) and her Ph.D. holder contract (DL57).

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., 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, A.C., 1998. The dynamics of seed mortality. J. Exp. Bot. 49, 1455–1461.
Bewley, J.D., 1997. Seed germination and dormancy. Plant Cell 9, 1055–1066.
Bewley, J.D., Bradford, K.J., Hilhorst, H.W.M., Nonogaki, H., 2013. Seeds: Physiology of Development, Germination and Dormancy, third ed. Springer Science+Business Media, LLC, New York, NY.
Biedermann, S., Mooney, S., Hellmann, H., 2011. Recognition and repair pathways of damaged DNA in higher plants. In: Chen, C. (Ed.), Selected Topics in DNA Repair. InTechOpen, London, pp. 201–236.
Black, M., Pritchard, H.W., 2003. Desiccation and survival in plants. Drying without dying. Ann. Bot. 91, 105.
Bradford, K.J., 1995. Water relations in seed germination. In: Kigel, J., Galili, G. (Eds.), Seed Development and Germination. Marcel Dekker, Inc., New York, pp. 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 Phytol. 168, 511–528.
Bruggink, G.T., Ooms, J.J.J., Van Der Toorn, P., 1999. Induction of longevity in primed seeds. Seed Sci. Res. 9, 49–53.
Buchanan, B.B., Balmer, Y., 2005. Redox regulation: a broadening horizon. Annu. Rev. Plant Biol. 56, 187–220.
Buitink, J., Leprince, O., 2008. Intracellular glasses and seed survival in the dry state. C R Biol. 331, 788–795.
Buitink, J., Leprince, O., Hoekstra, F.A., 2000. Dehydration-induced redistribution of amphiphilic molecules between cytoplasm and lipids is associated with desiccation tolerance in seeds. Plant Physiol. 124, 1413–1426.
Butler, L.H., Hay, F.R., Ellis, R.H., Smith, R.D., Murray, T.B., 2009. Priming and re-drying improve the survival of mature seeds of *Digitalis purpurea* during storage. Ann. Bot. 103, 1261–1270.
Charles, S.A., Halliwell, B., 1980. Effect of hydrogen peroxide on spinach (*Spinacia oleracea*) chloroplast fructose bisphosphatase. Biochem. J. 189, 373–376.
Chiu, K.Y., Chen, C.L., Sung, J.M., 2002. Effect of priming temperature on storability of primed sh-2 sweet corn seed. Crop Sci. 42, 1996–2003.
Choudhury, F.K., Rivero, R.M., 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, V.A., Smerdon, M.J., 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, R.R., 2009. Single-nucleotide and long-patch base excision repair of DNA damage in plants. Plant J. 60, 716–728.
Corpas, F.J., Barroso, J.B., del Río, L.A., 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, D.W., Khadka, P., Rakwal, R., Rohila, J.S., 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, P.A., Drew, R.L.K., 1986. Effects of osmotic priming and aging on onion seed germination. Ann. Appl. Biol. 108, 639–648.
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. J. Exp. Bot. 53, 1255–1272.
Delouche, J.C., Baskin, C.C., 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.
Donà, M., Confalonieri, M., Minio, A., Biggiogera, M., Buttafava, A., Raimondi, E., Delledonne, M., Ventura, L., Sabatini, M.E., 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, S.F., Saifi, G.M., Weinfeld, M., Johansson, F., Helleday, T., Lupski, J.R., Caldecott, K.W., 2005. Defective DNA single-strand break repair in spinocerebellar ataxia with axonal neuropathy-1. Nature 434 (7029), 108–113.
Ellis, R.H., Roberts, E.H., 1980. Improved equations for the prediction of seed longevity. Ann. Bot. 45, 13–30.

Ellis, R.H., Hong, T.D., Roberts, E.H., 1990. An intermediate category of seed storage. J. Exp. Bot. 41, 1167–1174.
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.
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, S.S., Siddique, K.H.M., 2018. Impact of abiotic stresses on grain composition and quality in food legumes. J. Agric. Food Chem. 66, 8887–8897.
Feenstra, A.D., Alexander, L.E., Song, Z., Korte, A.R., Yandeau-Nelson, M.D., Nikolau, B.J., Lee, Y.J., 2017. Spatial mapping and profiling of metabolite distributions during germination. Plant Physiol. 174, 2532–2548.
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. J. Proteome 91, 486–499.
Fercha, A., Capriotti, A.L., 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. Proteome 108, 238–257.
Finch-Savage, W.E., Bassel, G.W., 2016. Seed vigour and crop establishment: extending performance beyond adaptation. J. Exp. Bot. 67, 567–591.
Fu, Y., Mason, A.S., Zhang, Y., Lin, B., Xiao, M., Fu, D., Yu, H., 2019. MicroRNA-mRNA expression profiles and their potential role in cadmium stress response in *Brassica napus*. BMC Plant Biol. 19, 570.
Gallardo, K., Job, C., Groot, S.P., 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. Biotechnol. 18, 21–26.
Gorzolka, K., Kölling, J., Natikemper, T.W., Niehaus, K., 2016. Spatio-temporal metabolite profiling of the barley germination process by MALDI MS imaging. PLoS One 11, e0150208.
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. J. Mol. Biol. 386, 598–611.
Groot, S.P., Surki, A.A., de Vos, R.C., Kodde, J., 2012. Seed storage at elevated partial pressure of oxygen, a fast method for analysing seed aging under dry conditions. Ann. Bot. 110, 1149–1159.
Hamayun, M., Khan, S.A., Khan, A.L., Tang, D.S., Hussain, J., Ahmad, B., Anwar, Y., Lee, I.J., 2010. Growth promotion of cucumber by pure cultures of gibberellin-producing Phoma sp.GAH7. World J. Microbiol. Biotechnol. 26, 889–894.
Hay, F.R., 2017. Seed banks. In: Thomas, B., Murray, B.G., Murphy, D.J. (Eds.), Encyclopedia of Applied Plant Sciences, 2nd ed. Elsevier B.V., pp. 327–333.
Heydecker, W., Coolbear, P., 1977. Seed treatments for improved performance-survey and attempted prognosis. Seed Sci. Technol. 5, 353–425.
Hill, H.J., Cunningham, J.D., Bradford, K.J., 2007. Primed lettuce seeds exhibit increased sensitivity to moisture content during controlled deterioration. Hort. Sci. 42, 1436–1439.
Holdsworth, M.J., Bentsink, L., Soppe, W.J., 2008a. Molecular networks regulating *Arabidopsis* seed maturation, after-ripening, dormancy and germination. New Phytol. 179, 33–54.
Holdsworth, M.J., Finch-Savage, W.E., Grappin, P., Job, D., 2008b. Post-genomics dissection of seed dormancy and germination. Trends Plant Sci. 13, 7–13.
Hong, T.D., Ellis, R.H., 1996. A protocol to determine seed storage behaviour. IPGRI Tech. Bull. 1, 1–62.
Horn, P.J., Chapman, K.D., 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. Biochemist 44, 724–731.
Huang, Z., Boubriak, I., Osborne, D.J., 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.
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.
ISTA (International Seed Testing Association), 2019. International rules for seed testing. https://www.seedtest.org/.
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.
Kibinza, S., Vinel, D., Côme, D., Bailly, C., Corbineau, F., 2006. Sunflower seed deterioration as related to moisture content during aging, energy metabolism and active oxygen species scavenging. Physiol. Plant. 128, 496–506.
Kranner, I., Birtić, S., Anderson, K.M., Pritchard, H.W., 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, F.V., Beckett, R.P., Seal, C.E., 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, B.A., Anderson, H.J., Osmond, M.J., Vonarx, E.J., 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, J.S., 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.

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., Faé, 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, J.F.J., Gill, S.S., 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., Faè, M., Biggiogera, M., de Sousa, A.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, A.K., 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 aging. Outlook Agric. 14, 89–94.

Michel, B.E., Kauffmann, M.R., 1973. The osmotic potential of polyethylene glycol 6000. Plant Physiol. 51, 914–916.

Momin, K.C., 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, U.M., Sun, W.Q., 2000. Protein modification by Amadori and Maillard reactions during seed storage: roles of sugar hydrolysis and lipid per-oxidation. 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, M.A.R., 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, C.E., Colville, L., Fernández-Marín, B., Börner, A., 2015. Genome-wide association mapping and biochemical markers reveal that seed aging 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. In: Technical Information Sheet_01. Royal Botanic Gardens Kew, London.

Nguyen, T.P., Cueff, G., Hegedus, D.D., Rajjou, L., Bentsink, L., 2015. A role for seed storage proteins in *Arabidopsis* seed longevity. J. Exp. Bot. 66, 6399–6413.

Niedzielski, M., Walters, C., Luczak, W., Hill, L.M., Wheeler, L.J., 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, N.P., Shetty, H.S., 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.

Oracz, K., El-Maarouf Bouteau, H., Farrant, J.M., 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, D.J., 1994. DNA and desiccation tolerance. Seed Sci. Res. 4, 175–185.

Osborne, D.J., Dell'Aquila, A., Elder, R.H., 1984. DNA repair in plant cells. An essential event of early embryo germination in seeds. Folia Biol. (Praha). 30, 155–169.

Osburn, R.M., Schroth, M.N., 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, A.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. 42, 259–269. https://doi.org/10.1111/pce.13342.

Pandey, P., Bhanuprakash, K., Umesha, 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, S.S., 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, C.A., Cantliffe, D.J., 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, A.D., Graham, S., Graham, I.A., 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, B.B., Murai, J., Marchand, C., 2014. Tyrosyl-DNA-phosphodiesterases (TDP1 and TDP2). DNA Repair 19, 114–129.
Powell, A.A., 1986. Cell membranes and seed leachate coductivity in relation to the quality of seed for sowing. J. Seed Tech. 10, 81–100.
Powell, A.A., Matthews, S., 1981. Evaluation of controlled deterioration, a new vigour test for small seeded vegetables. Seed Sci. Technol. 9, 633–640.
Pritchard, H.W., 2004. Classification of seed storage 'types' for *ex situ* conservation in relation to temperature and moisture. In: Guerrant, E.O., Havens, K., Maunder, M. (Eds.), *Ex situ* Plant Conservation: Supporting Species Survival in the Wild. Island Press, Washington, DC, USA, pp. 139–161.
Probert, R.J., Bogh, S.V., Smith, A.J., Wechsberg, G.E., 1991. The effects of priming on seed longevity in *Ranunculus sceleratus* L. Seed Sci. Res. 1, 243–249.
Probert, R.J., Daws, M.I., Hay, F.R., 2009. Ecological correlates of *ex situ* seed longevity: a comparative study on 195 species. Ann. Bot. 104, 57–69.
Radhakrishnan, R., Khan, A.L., Lee, I.-J., 2013. Endophytic fungal pre-treatments of seeds alleviates salinity stress effects in soybean plants. J. Microbiol. 51, 850–857.
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., 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, S.P., 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., Duval, M., Gallardo, K., Catusse, J., Bally, J., Job, C., Job, D., 2012. Seed germination and vigor. Annu. Rev. Plant Biol. 63, 507–533.
Rao, N.K., Roberts, E.H., Ellis, R.H., 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, E.H., 1973. Predicting the storage life of seeds. Seed Sci. Technol. 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, J.C., Colombié, S., Dieuaide-Noubhani, M., Gallardo, K., Jeudy, C., Ourry, A., Prudent, M., Voisin, A.S., Rolin, D., 2017. Fluxomics links cellular functional analyses to whole-plant phenotyping. J. Exp. Bot. 68, 2083–2098.
Sarath, G., Hou, G., Baird, L.M., Mitchell, R., 2007. Reactive oxygen species, ABA and nitric oxide interactions on the germination of warm-season C4-grasses. Planta 226, 697–708.
Sattler, S.E., Gilliland, L.U., 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, S.A., 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, W.Q., Leopold, A.C., 1995. The Maillard reaction and oxidative stress during aging of soybean seeds. Physiol. Plant. 94, 94–104.
Taylor, A.G., Allen, P.S., Bennet, M.A., Bradford, K.J., Burris, J.S., Misra, M.K., 1998. Seed enhancements. Seed Sci. Res. 8, 245–256.
Tsai, Y.C., Wang, Y.H., Liu, Y.C., 2017. Overexpression of PCNA attenuates oxidative stress-caused delay of gap-filling during repair of UV-induced DNA damage. J. Nucleic Acids 2017, 8154646.
van Zanten, M., Koini, M.A., Geyer, R., Liu, Y., Brambilla, V., Bartels, D., Koornneef, M., Fransz, P., Soppe, W.J., 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.
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. Natl. Acad. Sci. U. S. A. 102, 13386–13391.
Walters, C., 1998. Understanding the mechanisms and kinetics of seed aging. Seed Sci. Res. 8, 223–244.
Walters, C., Wheeler, L., Stanwood, P.C., 2004. Longevity of cryogenically stored seeds. Cryobiology 48, 229–244.
Walters, C., Wheeler, L.M., Grotenhuis, J., 2005. Longevity of seeds stored in a genebank: species characteristics. Seed Sci. Res. 15, 1–20.
Walters, C., Berjak, P., Pammenter, N.W., 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 aging 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 aging. Mol. Gen. Genomics. 29, 1421–1435.

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. Plant J. 63, 848–860.

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, 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, C.L., Burgers, P.M., Lieber, M.R., 1996. Processing of branched DNA intermediates by a complex of human FEN-1 and PCNA. Nucleic Acids Res. 24, 2036–2043.

Zhang, F., Yu, J., Johnston, C.R., 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.

Further Reading

Bailly, C., Benamar, A., Corbineau, F., Come, D., 1998. Free radical scavenging as affected by accelerated aging and subsequent priming in sunflower seeds. Physiol. Plant. 104, 646–652.

Diaz-Vivancos, P., Barba-Espín, G., Hernández, J.A., 2013. Elucidating hormonal/ROS networks during seed germination: insights and perspectives. Plant Cell Rep. 32, 1491–1502.

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, 669–688.

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.

7.5 Short Communication

Müller J V, Cockel C P, **Gianella M**, Guzzon F. Treasuring crop wild relative diversity: analysis of success from the seed collecting phase of the 'Adapting Agriculture to Climate Change' project. *Genet Resour Crop Evol*. 2021. https://doi.org/10.1007/s10722-021-01229-x

Genet Resour Crop Evol
https://doi.org/10.1007/s10722-021-01229-x

SHORT COMMUNICATION

Treasuring crop wild relative diversity: analysis of success from the seed collecting phase of the 'Adapting Agriculture to Climate Change' project

Jonas V. Müller · Christopher P. Cockel · Maraeva Gianella · Filippo Guzzon

Received: 1 March 2021 / Accepted: 30 May 2021

Abstract The seed collecting phase of the 'Adapting Agriculture to Climate Change' project was, to the best of our knowledge, the most comprehensive crop wild relatives (CWR) collecting and conservation mission to-date and provides priceless genetic diversity for ongoing and future crop breeding efforts. The seed collecting started in 2013 and was concluded in 2019, it was carried out in 22 countries in Africa, Asia, the Americas and Europe, involving CWR taxa of 28 different crop genepools. 3002 target seed accessions of 242 taxa were collected and are currently stored long-term in the countries of collection and, in most of the cases, backed up at the Millennium Seed Bank (UK). Considering also non-target species, 3854 seed accessions were collected. For the genepools of bambara groundnut (*Vigna subterranea* (L.) Verdc.), barley (*Hordeum* L.), grass pea (*Lathyrus sativus* L.), sorghum (*Sorghum bicolor* (L.) Moench), and wheat (*Triticum* L.), the collecting phase was highly successful in terms of diversity of both, species and populations. Despite the overall success of the project, in our analysis we discovered several issues that were encountered in the seed collecting. In particular, comparing the initial collecting targets with the seed accessions effectively collected it emerges that: (1) some important crop genepools were characterized by a low collecting success (e.g. banana/plantain (*Musa* L.), potato (*Solanum tuberosum* L.), rice (*Oryza* L.), (2) genepool 1 (the most important for breeding efforts) of some crop genepools was under-collected (e.g. eggplant (*Solanum* L.) and sorghum), (3) some important centres of plant biodiversity (especially the Indian Subcontinent) were underrepresented in the seed collecting. This analysis can guide further collecting missions in order to fill gaps in the long-term conservation of CWR of great importance for crop improvement.

Keywords Crop wild relatives · Seed collecting · Seed conservation · Seed bank · Plant breeding

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10722-021-01229-x.

J. V. Müller · C. P. Cockel
Millennium Seed Bank, Conservation Science Department, Royal Botanic Gardens Kew, Wakehurst Place, Ardingly, UK

M. Gianella (✉)
Department of Biology and Biotechnology "L. Spallanzani", University of Pavia, Pavia, Italy
e-mail: maraeva.gianella01@universitadipavia.it

F. Guzzon
International Maize and Wheat Improvement Center (CIMMYT), Texcoco, Mexico State, Mexico

Published online: 15 June 2021

Introduction

A growing human population will increase the demand on food systems in the coming decades (Dwivedi et al. 2017). Food production, however, is limited by the availability of natural resources and the need to decrease its detrimental impact on ecosystems (Godfray et al. 2010; Asseng et al. 2015). For these reasons, both the productivity as well as the sustainability of current agricultural systems clearly need to increase. To meet these aims, the eroded genetic basis of modern agricultural cultivars needs to be enlarged to select for the productive and resilient cultivars of the future (Dempewolf et al. 2017). The exploitation of genetic diversity in crop improvement in order to produce crop varieties with traits such as increased drought and heat tolerance, and input use efficiency will be key to that process (Prohens et al. 2017). The bulk of genetic diversity in crop genepools is found in crop wild relatives (CWR = wild, undomesticated plant taxa that have an indirect use derived from their relatively close genetic relationship to a crop; Maxted et al. 2006). CWR are important sources of useful alleles for plant breeding and crop improvement (Kishii 2019). They possess essential traits that contribute to resilient and high-yielding agricultural production. The main benefit of CWR is the introgression of genes into crop varieties to overcome biotic (e.g. pest resistance) and abiotic (e.g. drought tolerance) stresses, also in relation to climate change (Dempewolf et al. 2014).

In order to facilitate the use of CWR for plant breeding, they need to be readily accessible in ex situ conservation facilities such as germplasm banks (Guzzon and Ardenghi 2018). One of the most effective strategies to ensure the long-term conservation and availability of plant genetic resources (PGR) with orthodox seeds is through storage of dry seeds at sub-zero temperatures (Davies and Allender 2017; Riviere and Müller 2018). It was shown that over 95% of all crop wild relatives were either absent or insufficiently represented in gene banks, and therefore mostly inaccessible for plant breeding and crop improvement (Castañeda-Álvarez et al. 2016).

To address this issue, the Global Crop Diversity Trust and the Millennium Seed Bank of the Royal Botanic Gardens, Kew embarked in 2011 on a global, long-term effort to collect, conserve, and initiate the use of CWR (Dempewolf et al. 2014). This 10-year project, funded by the Government of Norway, was called ''Adapting Agriculture to Climate Change'' and focused on wild relatives of 29 crops of major importance for food security (i.e., alfalfa (*Medicago sativa* L.), apple (*Malus* Mill.), Asian/African rice (*Oryza* L.), bambara groundnut (*Vigna subterranea* (L.) Verdc.), banana/plantain (*Musa* L.), barley (*Hordeum* L.), bean (*Phaseolus* L.), carrot (*Daucus carota* L.), chickpea (*Cicer arietinum* L.), cowpea (*Vigna unguiculata* (L.) Walp.), durum/bread wheat (*Triticum* L.), eggplant (*Solanum* L.), faba bean (*Vicia faba* L.), finger millet (*Eleusine coracana* (L.) Gaertn.), grasspea (*Lathyrus sativus* L.), lentil (*Lens culinaris* Medik.), oat (*Avena sativa* L.), pea (*Pisum sativum* L.), pearl millet (*Pennisetum glaucum* (L.) R.Br.), pigeon pea (*Cajanus cajan* (L.) Millsp.), potato (*Solanum tuberosum* L.), rye (*Secale cereale* L.), sorghum (*Sorghum bicolor* (L.) Moench), sunflower (*Helianthus annuus* L.), sweet potato (*Ipomoea batatas* (L.) Lam.) and vetch (*Vicia sativa* L.)). The seed collecting phase of this project was carried out in close collaboration with universities, national agricultural and natural resources research centres and was concluded in July 2019. To our knowledge, the collecting phase of this project was the most comprehensive targeted CWR collecting mission to-date and provides priceless genetic diversity for ongoing and future breeding efforts to improve crop cultivars and to help them adapt to climate change-driven stresses (Dempewolf et al. 2014). The collected seed material is available under the terms and conditions of the International Treaty on Plant Genetic Resources for Food and Agriculture (FAO 2004).

In this paper we present a full geographical analysis of the main results of this worldwide collecting mission. The analysis is based on crop genepools. It is fundamental to identify remaining issues and gaps encountered in the seed collecting phase also in order to guide further CWR collecting and conservation projects (Guzzon and Müller 2016). In particular, by analysing the results of the seed collecting phase of the "Adapting Agriculture to Climate Change" project, we aim to identify, (1) crop genepools that were particularly difficult to collect; and (2) regional agrobiodiversity hotspots which remain underrepresented in ex situ collections.

Material and methods

In each country identified for seed collecting after a prioritisation exercise, the project first signed collecting agreements with in-country partners. These collecting agreements, generally one per country, defined in each country and for each of the 28 crop genepools (sunflower was excluded from the seed collecting as its centre of origin is in the USA, which a priori had been excluded as a priority country for collecting) the individual target CWR taxa to collect and the number of populations per target CWR taxa. Seeds were collected for each target CWR taxa, including for wild relatives of clonally propagated crops (e.g. banana/plantain and potato). In some cases, institutions collected seeds of other species in addition to the target taxa as identified in the collecting agreements. We define these additional taxa as "non-target species".

Collections data were accessed on the 2nd of July 2020 from the Seed Bank Database of the Millennium Seed Bank (Royal Botanic Gardens, Kew).

In the following analysis, we consider the CWR seed collections from 22 countries: Armenia, Azerbaijan, Chile, Costa Rica, Cyprus, Ecuador, Ethiopia, Georgia, Ghana, Guatemala, Italy, Kenya, Lebanon, Malaysia, Nepal, Nigeria, Pakistan, Portugal, Spain, Sudan, Uganda, and Vietnam. Two additional countries in South America (Brazil and Peru) participated in the project but have not yet sent data of their collections to the Royal Botanic Gardens, Kew. Therefore, the collections from these two countries were not included in our analyses. A duplicate of all the seed samples conserved in the countries of collection were sent to the Millennium Seed Bank (MSB) for long-term storage, except for Ethiopian and Guatemalan collections that are currently stored only in the countries of origin. In a regional approach, the individual countries were grouped into geographical regions partly inspired by centres of crop origin and diversity proposed by Vavilov (1926) and further updates (Meyer et al. 2012). The following geographical areas were defined and considered: Caucasus (Armenia, Azerbaijan and Georgia); Central America (Costa Rica and Guatemala); East Africa (Ethiopia, Kenya, Sudan and Uganda); Indian Subcontinent (Nepal and Pakistan); Mediterranean (Cyprus, Italy, Lebanon, Spain, Portugal); South America (Chile and Ecuador); Southeast Asia (Malaysia and Vietnam); West Africa (Ghana and Nigeria).

Overall, in these 22 countries, 372 target CWR taxa (including subspecies and botanical varieties), belonging to 314 different species, with a total number of 4658 target populations were identified by the project for collecting, using the 'gap analysis' method described in Ramírez-Villegas et al. (2010). Within these 372 target taxa, the project identified a subset of high priority species (HPS) of particular importance for collecting. Their identification was based on their relatedness to the crop and to their pre-project accessibility in germplasm banks. A total number of 104 target HPS, corresponding to 1420 populations were defined as high priority targets for collection. In addition, following the genepool concept developed by Harlan and De Wet (1971), the project categorized for each genepool the target CWR taxa into different "genepool levels" (i.e. GP1: genepool level 1, GP2: genepool level 2, GP3: genepool level 3), based on formal taxonomy and genetic relatedness, determined by the crossing ability between the CWR species and the related crop. Genepool levels were extracted from the Harlan and de Wet CWR Inventory (CWR Diversity 2020). For a few CWR taxa no information about their genepool level was available, therefore they were marked as "not assigned".

Statistical analysis

Data analysis and graphic representation were carried out in R software environment for statistical computing and graphics (v. 4.0.2), using the following packages: *dplyr*, *ggplot2*, *lsmeans* and *rgdal*. A generalized linear model (GLM) with binomial distribution and logit as link function was used to determine the effect of geographical area, crop genepool, genepool level and genus on the collection success, expressed as collection success of the target populations. The scripts used for the analyses were filed in a Github repository: (https://github.com/MaraevaGianella/Treasuring-CWR-diversity).

Results and discussion

In the framework of the project "Adapting Agriculture to Climate Change" 3002 target CWR populations belonging to 242 taxa and 222 species of the 28 crops

genepools were collected in 22 different countries in Africa, the Americas, Asia and Europe. This incredible wealth of genetic diversity is already being used in pre-breeding projects across the world (Kilian et al. 2021). Seeds of the collected accessions are long-term stored, following international standards (FAO 2014) in genebanks in the country of collection. Moreover, 2809 target accessions are stored at the Millennium Seed Bank, of the Royal Botanic Gardens, Kew (UK), with regenerated material ultimately to be deposited at the Svalbard Global Seed Vault, located on the Arctic island of Spitsbergen (Crop Trust 2021).

Considering the percentage of achieved CWR collections compared to the targets set by the project, 65% and 64.4% of the CWR target taxa and of the total target populations respectively were successfully collected. Considering only the high priority species (HPS) among the target taxa, 658 populations belonging to 65 HPS were collected, representing 62% and 46.3% of target HPS and populations, respectively. The relatively high percentage of target taxa and populations (especially considering the HPS) that could not be collected by the project points towards certain issues during the collecting of some of the target species (e.g. outdated information on the distribution of some species, habitat changes occurred in the last years, phenology of the species).

Regarding the factors that influenced the percentages of collecting success, the number of achieved collections (i.e. target populations that were successfully collected at the end of the seed collecting phase of the project) was significantly different among crop genepools (d.f. 24, Resid. Dev. 4184.42, $p < 0.001$) genepool levels (d.f. 3, Resid. Dev. 4567.11, $p < 0.001$) and geographical areas (d.f. 7, Resid. Dev. 3875.38, $p < 0.001$). It is important to consider not only the percentage of target species that were successfully collected but also the number of collected populations in order to define the success of CWR collecting missions, since not only the availability of different species but also the within-taxon genetic diversity are fundamental for the successful use of CWR in crop improvement (Fig. 1, Smýkal et al. 2017). For some genepools, a very high percentage of target taxa and populations (> 75%, Fig. 1) was collected i.e. bambara groundnut, barley, grass pea, sorghum, and wheat. For these genepools we can assert that the collecting phase was highly successful in terms of diversity of both, species and populations.

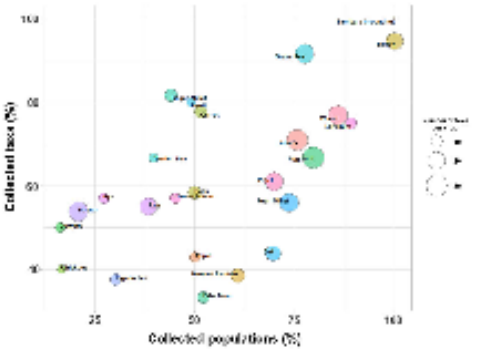


Fig. 1 Percentages of collected target taxa (vertical axis) and populations (horizontal axis). The number of target taxa within a crop genepool is represented by the diameter of the circle representing the crop genepool

For other genepools i.e. carrot, finger millet and lentil, a large number of target taxa was achieved (> 75%) but with lower success in terms of number of populations (< 55%). For these taxa, additional populations should be collected to better represent the genetic diversity within-taxa and therefore the possible presence of useful traits for crop improvement. Conversely, considering the results of the collecting of CWR of oat, the collecting success in terms of populations (70%) was higher than in terms of taxa (44%), in this case future collecting efforts should aim at filling this gap in terms of specific and sub-specific diversity. On the other hand, for some genepools (i.e. cowpea, chickpea, pigeon pea), we could observe a low collecting success rate (< 50%) both in terms of taxa and populations. For these genepools, urgent further collecting is needed to make the valuable genetic resources represented by these CWR available to breeders (Fig. 1).

Unfortunately, the seed collecting success of some fundamental crop genepools for food security such as banana/plantain, potato and rice was low (Fig. 1), even though these crops have quite large genepools in terms of target taxa. Further collecting efforts are needed to fill those gaps in seed colleting of the wild relatives of these crop species in which CWR diversity is already playing a great role as a source of useful traits for crop breeding (see e.g. Li et al. 2015; Tin et al. 2021).

The collecting success varied also among genepool levels within the same crop genepool (Fig. 2). As an

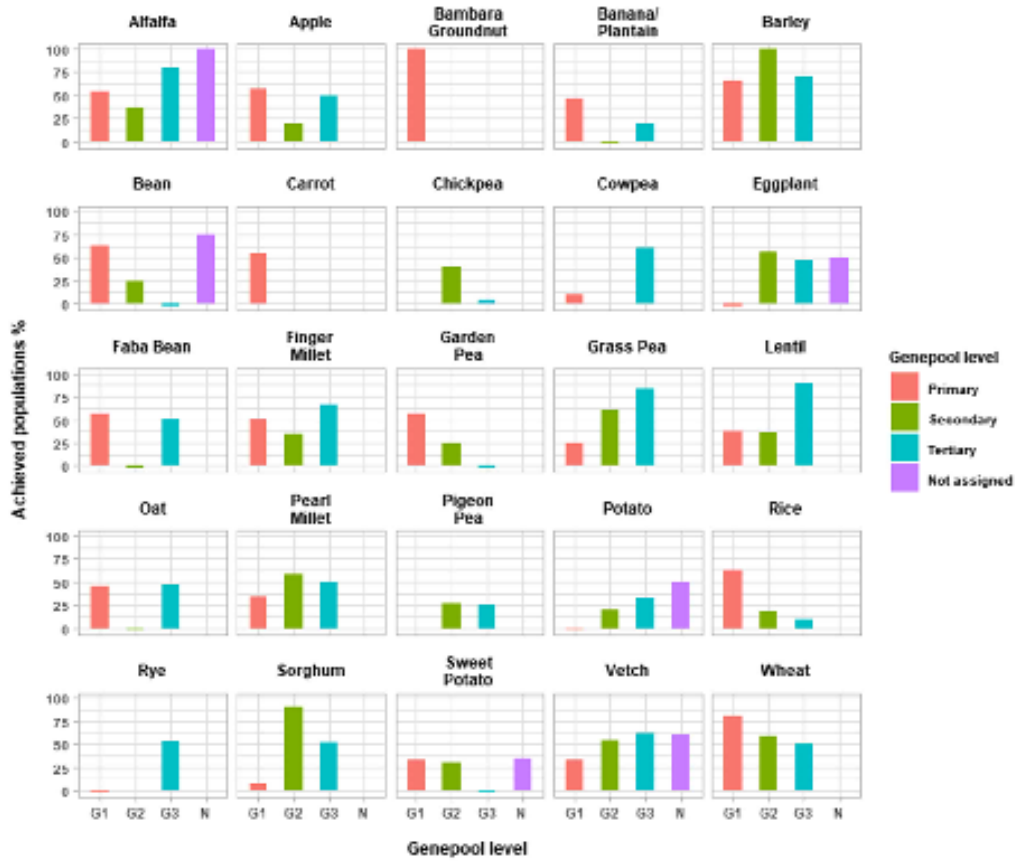


Fig. 2 Percentage of collected target populations by genepool, considering different genepool levels

example, barley, grass pea and sorghum were the crop genepools with the highest success in terms of both populations and taxa collected when compared to the targets (Fig. 1). At the same time genepool 1 (GP1) in these three crops was the one with the lowest percentage of success when compared to the other genepool levels (Fig. 2). GP1 is considered the most important one for breeding among the genepool levels, because it groups together the direct ancestors and closest relatives of the domesticated crop (and so the most directly usable species for crop improvement, Harlan and De Wet 1971). Further collecting missions are needed to complement the diversity, in terms of populations, of the GP1 for several target crops (i.e. alfalfa, cowpea, eggplant, grass pea, lentil, pearl millet, sorghum, sweet potato and vetch, Fig. 2).

Significant differences between the levels of collecting success were detected among the different geographical areas. The area in which the highest median number of seed accessions were collected were the Mediterranean (231) and the Caucasus (159), while the lowest median number of accessions were collected in East Africa (80) and on the Indian Subcontinent (71, Fig. 3). Comparing the percentages of successful target populations collected, the highest percentage of success was achieved in the Caucasus (71.9%) followed by West Africa (58.0%) while the lowest percentage was achieved in East Africa (35.3%) and on the Indian Subcontinent (26.5%,

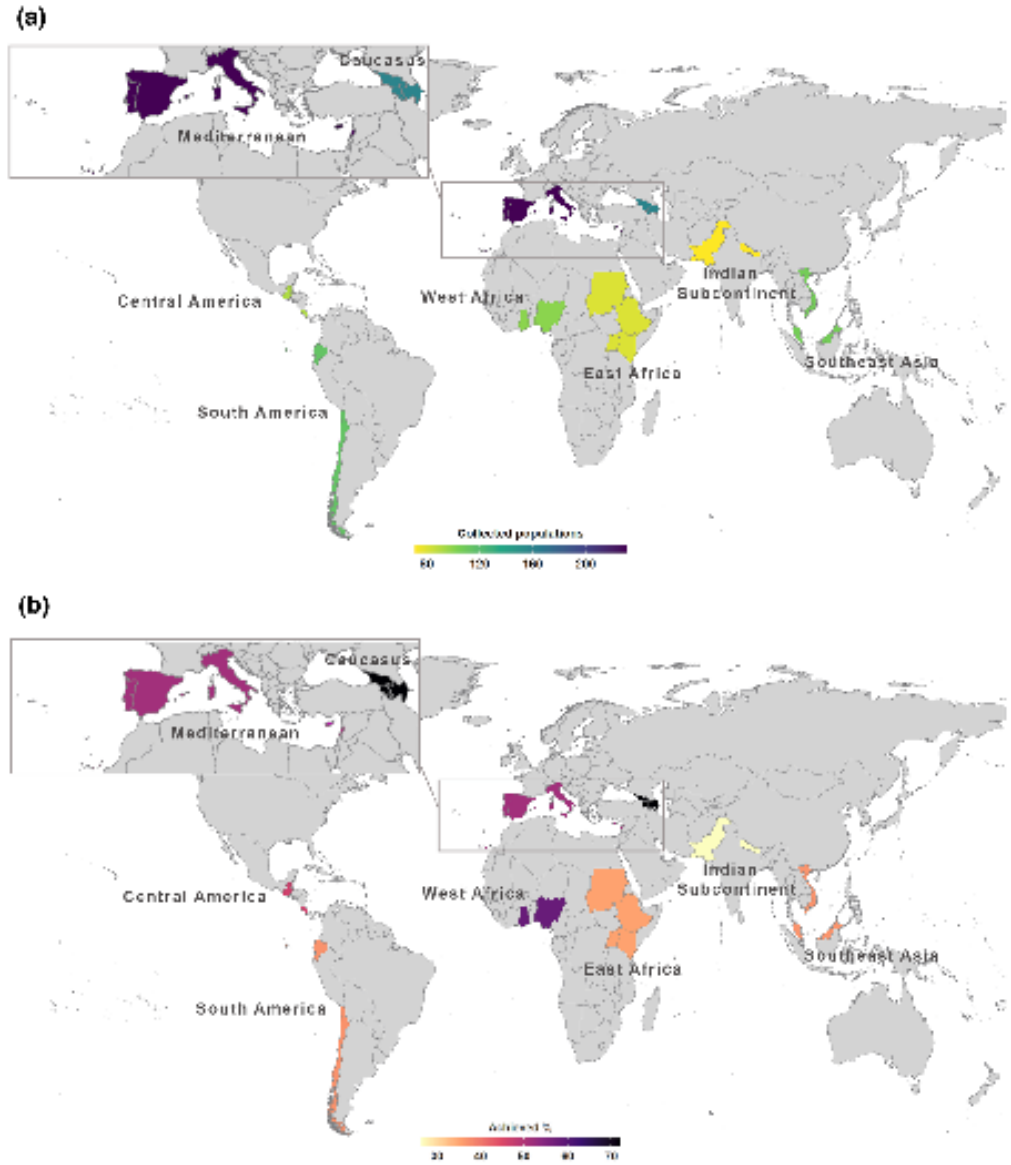


Fig. 3 a Median number of collected populations by geographical area; b Percentage (Median) of target populations collected by geographical area

Fig. 3). It emerges that further collection efforts are needed to better represent the genetic diversity of many CWR to be found in underrepresented centres of crop origin and diversity. This is particularly relevant for the Indian Subcontinent which is considered one of the main centres of origin and diversity of many crops

(e.g. chickpea, pigeon pea, cowpea, eggplant) and a geographical area in which several plant biodiversity hotspots are located (Myers et al. 2000).

It is also important to note that a total of 852 non-target populations, belonging to 152 plant taxa and 137 species, were also collected which had not been part of the individual country lists of target taxa. If these taxa are included, the total number of seed accessions collected and conserved long-term increases from 3002 to 3854. Of these additional collections, 743 populations of 90 taxa are CWR that are part of the 28 target genepools of this project. However, these CWR taxa, although they belong to the target genepools, had not been part of the initial in-country agreements with the collecting institutions; 47 populations of 14 taxa are CWR that belong to 6 additional crop genepools on top of the 28 genepools targeted in this project (i.e. adzuki bean (*Vigna angularis* (Willd.) Ohwi & H.Ohashi), barrel medic (*Medicago truncatula* Gaertn.), Hungarian vetch (*Vicia pannonica* Crantz), melon (*Cucumis melo* L.), sesame (*Sesamum indicum* L.), white millet/Siberian millet (*Echinochloa frumentacea* Link)); 62 populations of 48 taxa do not fall into the definition of a CWR following the Harlan and de Wet Inventory (CWR Diversity 2020). The pro-activity of the institutions involved in this project in collecting non-target taxa and populations increases the accessibility for breeding and research to genetic resources that had not been part of the initial collecting agreements and leads to an increased diversity within the target taxa.

Information on the collected species, the genepool to which they belong, the number of collected accessions and the geographical areas where they were collected can be found in the Supplementary Information 1. For additional information about the species, the individual accessions or how to order individual seeds, we recommend to contact the data administrator of the Millennium Seed Bank (http://apps.kew.org/seedlist/SeedlistServlet and http://brahmsonline.kew.org/msbp/SeedData/DW).

In conclusion we can assert that,

- The CWR collecting phase of the "Adapting Agriculture to Climate Change" project has guaranteed the long-term conservation and accessibility of almost four thousand CWR populations of 28 crop genepools, collected in 22 different countries.
- Despite the overall success of the project, the collecting success for some important crop genepools for food security was poor (e.g. rice and potato). For some crops (e.g. eggplant and sorghum), there was low collecting success for populations of genepool 1. We advocate for further collecting missions in order to fill these gaps.
- Some centres of crop origin and diversity came out underrepresented in the final collections; this is in particular the case for the Indian subcontinent. Further collecting efforts should be dedicated to more completely represent the CWR diversity of these areas.

Acknowledgements The authors acknowledge the great effort and commitment of the collecting institutions in the 22 collecting countries. We thank Dr John Dickie and Dr Udayangani Liu for their help with data queries and access to the Millennium Seed Bank's Seed Bank Database.

Funding No funding was received to assist with the preparation of this manuscript.

Declarations

Conflict of interest The authors do not have any conflicts of interest.

References

Asseng S, Ewert F, Martre P et al (2015) Rising temperatures reduce global wheat production. Nat Clim Change 5:143–147. https://doi.org/10.1038/nclimate2470

Castañeda-Álvarez NP, Khoury CK, Achicanoy HA et al (2016) Global conservation priorities for crop wild relatives. Nat Plants 2:1–6. https://doi.org/10.1038/NPLANTS.2016.22

Crop Trust (2021) Svalbard global seed vault. https://www.croptrust.org/our-work/svalbard-global-seed-vault/. Accessed 1 Feb 2021

CWR Diversity (2020) The Harland and de Wet CWR Inventory. https://www.cwrdiversity.org/checklist/. Accessed 14 Aug 2020

Davies LR, Allender CJ (2017) Who is sowing our seeds? A systematic review of the use of plant genetic resources in research. Genet Resour Crop Evol 64(8):1999–2008. https://doi.org/10.1007/s10722-017-0491-7

Dempewolf H, Baute G, Anderson J et al (2017) Past and future use of wild relatives in crop breeding. Crop Sci 57:1070–1082. https://doi.org/10.2135/cropsci2016.10.0885

Dempewolf H, Eastwood RJ, Guarino L et al (2014) Adapting agriculture to climate change: a global initiative to collect, conserve, and use crop wild relatives. Agroecol Sustain

Food Syst 38:369–377. https://doi.org/10.1080/21683565.2013.870629

Dwivedi SL, Lammerts van Bueren ET, Ceccarelli S etal (2017) Diversifying food systems in the pursuit of sustainable food production and healthy diets. Trends Plant Sci 22:842–856. https://doi.org/10.1016/j.tplants.2017.06.011

FAO (2004) International treaty on plant genetic resources for food and agriculture (ITPGRFA), http://www.fao.org/plant-treaty/en/

FAO (2014) Genebank standards for plant genetic resources for food and agriculture. Italy, Rome

Godfray HCJ, Crute IR, Haddad L et al (2010) The future of the global food system. Philos Trans R Soc B Biol Sci 365:2769–2777. https://doi.org/10.1098/rstb.2010.0180

Guzzon F, Ardenghi NMG (2018) Could taxonomic misnaming threaten the ex situ conservation and the usage of plant genetic resources? Biodivers Conserv 27:1157–1172. https://doi.org/10.1007/s10531-017-1485-7

Guzzon F, Müller JV (2016) Current availability of seed material of enset (*Ensete ventricosum*, Musaceae) and its Sub-Saharan wild relatives. Genet Resour Crop Evol 63:185–191. https://doi.org/10.1007/s10722-015-0355-y

Harlan, J.; De Wet J (1971) Toward a rational classification of cultivated plants. Taxon 40:509–517

Kilian B, Dempewolf H, Guarino L et al (2021) Crop Science special issue: adapting agriculture to climate change: a walk on the wild side. Crop Sci 61:32–36. https://doi.org/10.1002/csc2.20418

Kishii M (2019) An update of recent use of *Aegilops* species in wheat breeding. Front Plant Sci. https://doi.org/10.3389/fpls.2019.00585

Li WM, Dita M, Wu W et al (2015) Resistance sources to *Fusarium oxysporum* f. sp. cubense tropical race 4 in banana wild relatives. Plant Pathol 64:1061–1067. https://doi.org/10.1111/ppa.12340

Maxted N, Ford-Lloyd BV, Jury S et al (2006) Towards a definition of a crop wild relative. Biodivers Conserv 15:2673–2685. https://doi.org/10.1007/s10531-005-5409-6

Meyer RS, Duval AE, Jensen HR (2012) Patterns and processes in crop domestication: an historical review and quantitative analysis of 203 global food crops. New Phytol 196:29–48. https://doi.org/10.1111/j.1469-8137.2012.04253.x

Myers N, Mittermeier RA, Mittermeier CG et al (2000) Biodiversity hotspots for conservation priorities. Nature 403:853–858

Prohens J, Gramazio P, Plazas M et al (2017) Introgressiomics: a new approach for using crop wild relatives in breeding for adaptation to climate change. Euphytica. https://doi.org/10.1007/s10681-017-1938-9

Ramírez-Villegas J, Khoury C, Jarvis A, Debouck DG, Guarino L (2010) A gap analysis methodology for collecting crop genepools: a case study with phaseolus beans. PLoS ONE 5(10):e13497. https://doi.org/10.1371/journal.pone.0013497

Rivière S, Müller JV (2018) Contribution of seed banks across Europe towards the 2020 Global Strategy for Plant Conservation targets, assessed through the ENSCONET database. Oryx 52(3):464–470. https://doi.org/10.1017/S0030605316001496

Smýkal P, Hradilová I, Trněný O et al (2017) Genomic diversity and macroecology of the crop wild relatives of domesticated pea. Sci Rep 7:1–12. https://doi.org/10.1038/s41598-017-17623-4

Tin HQ, Loi NH, Bjornstad Å, Kilian B (2021) Participatory selection of CWR-derived salt-tolerant rice lines adapted to the coastal zone of the Mekong Delta. Crop Sci 61:277–288. https://doi.org/10.1002/csc2.20405

Vavilov NI (1926) Origin and geography and cultivated plants. Bull Appl Bot Plant Breed 14:1–245

Acknowledgements

I would like to express my gratitude to the University of Pavia and the doctoral program for the opportunity and the scholarship that made this project possible.

I would like to thank my primary supervisor, Prof. Alma Balestrazzi, who assisted and guided me throughout this project, and all the people I had the fortune to work with, in Pavia and outside Italy, during these three exciting years.

I am also grateful for the big sacrifices made by my mother Maria Grazia, that allowed me to attend university, for her constant support and that of my father Vladimir and my wonderful siblings, Hidalgo, Seida and Frieda.

Last but not least, Dr. Filippo Guzzon, friend, mentor and great scientist. Without him, none of this would have been possible, and I will always be grateful for everything he taught me.