UNIVERSITÀ
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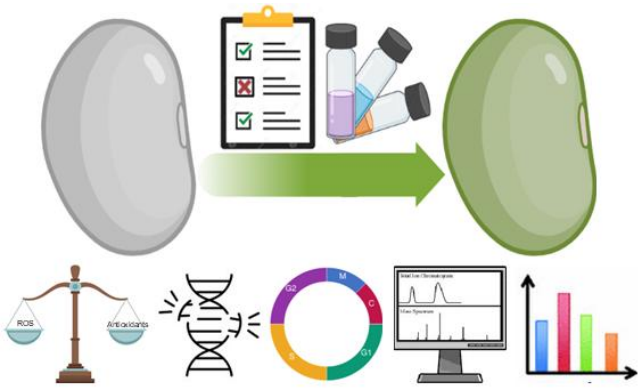
Dipartimento di Biologia e Biotecnologie "L. Spallanzani"

Exploring the molecular mechanisms of seed deterioration in the model species *Medicago truncatula* L. and *Populus alba* L.: designing anti-aging treatments to protect seeds in storage



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Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXVII – A.A. 2021-2024

Acknowledgements

I am grateful to my supervisor Prof. Alma Balestrazzi for her constant support and guidance during my PhD program. Thank you for continuously encouraging me and being patient when I needed it the most.

I would also like to thank Prof. Anca Macovei for her insightful advice. Her guidance has been essential in shaping my PhD journey and motivating me to work hard toward my goals.

From the bottom of my heart, I would like to say big thank you to Dr. Andrea Pagano for his mentorship which has been instrumental in my development as a young researcher. You've been a steady source of support throughout my PhD, offering calm and clarity when things got overwhelming

My appreciation also goes to all the people in Seed Molecular Physiology lab and Plant Biotechnology lab for their camaraderie and the stimulating discussions especially to Dr. Conrado Dueñas Jr. for his invaluable help with my experiments and unwavering support as a friend throughout this journey.

My heartfelt thanks to Dr. Fabrizio Araniti for teaching me how to analyze metabolomics data and his constructive feedback

All my love to Amma, Mama and my little brother Shri, it is their love and support which they have provided to me helped me finish this PhD. Thank you for always believing in me.

A big shoutout to all my friends for always being there for me giving me the perfect distractions to take a break. You have made this journey so much more enjoyable.

And finally, I would like to thank my Appa even though he is not around anymore he is one of the most inspiring people in my life. Thank you for inspiring me to pursue Science and teaching me the importance of hard work. The unconditional love you showed me during our time together has helped me to keep going all this time.

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Abstract of the research

Seed longevity is a complex trait influenced by numerous factors. It is especially significant in the context of climate change, since it is essential for advancing sustainable agriculture, germplasm conservation, and ensuring high seed quality. Seed priming is a technique employed to improve seed quality by stimulating pre-germinative metabolism, encompassing DNA damage response and antioxidant response, which facilitate superior germination performance. Despite the numerous benefits, the longevity of primed seeds is compromised. Developing optimal priming techniques that protect primed seeds against aging is an efficient strategy to expand the application of seed priming. This study examines the ability of primed *Medicago truncatula* seeds to withstand deterioration during storage by designing and using an experimental system to screen compounds that may protect primed seeds in storage. This research evaluated the efficacy of quercetin, rutin, and spermidine as seed priming agents to improve the response of *M. truncatula* seeds exposed to artificial aging. The addition of quercetin or rutin effectively mitigated the adverse effects of post-priming aging, in comparison with hydropriming, resulting in enhanced seed germination and viability. These positive effects were consistently linked to decrease in ROS levels and the concomitant enhancement in antioxidant capacity. The reported data showed that quercetin and rutin can alleviate the effects of post-priming aging by improving the seed antioxidant profile. When spermidine was applied to *M. truncatula* aged seeds, enhanced viability was observed. Nonetheless, improved germination percentage was associated with a higher incidence of aberrant seedlings, indicating that the protective effects of spermidine were decreased during seedling establishment. To what extent might cumulative DNA damage in seeds hinder the subsequent developmental stages? Effective DNA repair requires the inhibition of cell cycle progression, which is essential for plant development. Spermidine significantly lowered DNA damage and modified the expression of key DDR (DNA damage response) genes at different developmental stages. Results from non-targeted metabolomics highlighted that exogenous spermidine treatment triggered accumulation of endogenous polyamines, and affected antioxidants and nucleotide metabolites that could improve seed viability. These findings will improve our understanding of the deterioration mechanisms impacting the quality of primed seeds during storage and assist in the development of more mitigation techniques. Spermidine was also evaluated in a parallel study on long-term stored *Populus alba* clone ‘Villafranca’ seeds. Poplar seeds exhibit low viability and are categorized as intermediate seeds. Given that poplars are frequently propagated clonally, it is essential to consider the potential reduction of genetic variability within the existing germplasm. To address this issue, new populations should be developed from seeds. Soaking with a solution containing spermidine significantly enhanced seed germination. Decreased ROS levels were observed in spermidine-treated seeds relative to water-soaked seeds. Increased expression of genes related to desiccation tolerance acquisition, polyamine synthesis, and antioxidant defense was observed only in dry seeds. We report the successful development of a treatment that improves the germination of long-term stored, deteriorated ‘Villafranca’ seeds, addressing current seed quality issues in poplars.

Investigating the mechanisms of seed deterioration and the maintenance of seed viability improves production and storage efficiency, while also guiding suitable management and conservation techniques for seeds in long-term storage, which possess significant biological and economic value.

Abbreviations

AA (Accelerated aging)
aa (ascorbic acid)
ABI3 (ABA INSENSITIVE 3)
Abr (Aberrant)
ANOVA (two-way analysis of variance)
APX (Ascorbate Peroxidase)
Ar (aerial)
ATM (Ataxia Telangiectasia Mutated)
ATR (ATM and Rad3-related)
BER (Base excision repair)
CA (Citric acid)
CAT (Catalase)
CD (controlled deterioration)
CDK (cyclin dependent kinase)
CYC (Cyclin)
DB (dry-back)
DCFH-DA (2′,7′-dichlorofluorescin diacetate)
DDR (DNA damage response)
DHA (dehydroascorbic acid)
DHAR (Dehydroascorbate Reductase)
DPPH (1,1-Diphenyl-2-picrylhydrazyl)
DSB (double strand breaks)
DT (desiccation tolerance)
DW (Dry weight)
FUS3 (FUSCA3)
GA (gibberellins)
GABA (γ-aminobutyric acid)
GR (Glutathione Reductase)
GST (Glutathione-S-Transferase)
HP (hydropriming)
HR (homologous recombination)
IAA (indole-3-acetic acid)
ICA (isocitric acid)
LEA (late embryogenesis abundant)
LEC1 (Leafy Cotyledon 1)
LEC2 (Leafy Cotyledon 2)
LigIV (DNA LIGASE IV)
LOX (lipoxygenase)
MC (Moisture content)
MDA (Malondialdehyde)
MRE11 (Meiotic recombination 11)
NBS (nature-based solutions)
NBS1 (Nijmegen Breakage Syndrome 1)
NER (Nucleotide excision repair)
NHEJ (non-homologous end joining)
Nor (Normal)

NP (nanoparticles)
OGG1 (8-Oxoguanine Glycosylase/Lyase 1)
PA (Polyamines)
PCA (Principal Component Analysis)
PCD (programmed cell death)
Put (Putrescine)
QP (quercetin priming)
R (Root)
Rad50 (Radiation sensitive 50)
RBR (Retinoblastoma Related 1)
RD (Radicle protrusion)
RFC (RAD17-replication factor C)
RFO (raffinose family oligosaccharides)
RH (Relative humidity)
Rh (Rehydration)
ROS (Reactive oxygen species)
RPA (replication protein A)
RP (rutin priming)
SA (Salicylic acid)
SMR5 (Siamese Related 5)
SMR7 (Siamese Related 7)
SP (Spermidine priming)
Spd (Spermidine)
Spd50 (50μM spermidine)
Spd100 (100μM spermidine)
Spermidine synthase (SPDS)
Spermine synthase (SPMS)
Spm (Spermine)
SOD (superoxide dismutase)
SOG1 (Suppressor of Gamma response 1)
SSB (single strand breaks)
TF (transcription factors)
TTC (2,3,5-triphenyl tetrazolium chloride)
UA (unaged)
UP (unprimed)
UT (Untreated)

Chapter 1: General introduction

1.1 Relevance of seeds in the context of agroindustry and climate change

Seeds have a vital role in our lives, since they are integrated into our cultures, religions, and medicines. Wild species were domesticated by humans several centuries ago. The seeds were collected, sown, and subsequently harvested from the plants exhibiting favorable traits. Following that, we have gained comprehensive knowledge regarding the complex biological processes of seeds. The global seed market is expected to grow up to 105.3 billion U.S.D. by the year 2031 (http://www.worldseed.org, www.euroseeds.eu/). This is closely correlated with the fact that the population has increased over the past century, resulting in a considerable increase in food demand. According to the United Nations, the global population is projected to reach 9.7 billion by 2050, 10.8 billion by 2080, and 11.2 billion by 2100. This rapid expansion, that requires increased production of food and other necessary goods, has led to the progressive environmental deterioration, further exacerbated by climate change. The negative effects of climate change on crop yields are expected to intensify more in the latter part of this century. Over the course of time, it is predicted that these consequences will become increasingly prominent (FAO, 2018). In light of this circumstance, it is crucial to prioritize strategies for improving agricultural productivity and implementing more sustainable agricultural practices. High-quality seeds are crucial to achieve sustainable agricultural production, as recognized by both the seed industries and farmers (ISTA, 2018). The complexity and variability of the seed physiological responses require dedicated specialized actions to boost quality together with a deeper understanding of the seed complex morphological, physiological and molecular features.

1.1.1 Classification of seeds

The ability to withstand dehydration and survive desiccation emerged as a crucial evolutionary step, enabling ancestral plants to inhabit terrestrial environments. While desiccation tolerance is mostly absent in the vegetative tissues of modern plants, the majority of seeds possess the ability to withstand desiccation (Smolikova et al., 2020). Desiccation sensitivity is still an important issue for operators involved in *ex situ* conservation in seed banks, an essential activity that contributes to the preservation of biodiversity and protects species that are experiencing threats from changes in the environment. Thus, it is extremely important to deeply understand of the ability of the seed to withstand desiccation (Wyse and Dickie, 2017).

Seeds can be classified into three distinct categories, based on their ability to withstand desiccation: (1) *Orthodox seeds*: seeds that are able to withstand drying to low moisture levels (water contents that range between 0.03 and 0.07 g H_2O g^{-1} DW that is ~7%) and subsequent rehydration without experiencing a major loss of viability. These seeds are also the most appropriate for storage in

seed bank (Roberts, 1973; Walters, 2015). (2) *Recalcitrant seeds*: seeds that are susceptible to desiccation as it is performed in conventional seed bank facilities because they quickly lose their viability when they are dried. These seeds typically do not go through maturation drying or they can afford only very minimal drying. As a result, they have high moisture contents and remain metabolically active. The seed moisture content beyond which viability is lost, it is typically 0.20 and 0.3 g H_2O g^{-1} DW (> 20% RH), although there is strong variation between recalcitrant species (Kermode and Finch-Savage, 2002; Walters, 2015). (3) *Intermediate seeds*: these seeds exhibit seed storage traits that are intermediate, falling between those of orthodox and recalcitrant classes. They show desiccation tolerance to quite low levels, viability diminishes when the moisture content drops below approximately 10-12% RH (Tweddle et al., 2003) (**Figure 1.1**). The molecular networks underlying the different physiological responses of orthodox, recalcitrant and intermediate seeds to desiccation stress still deserve extensive investigation.

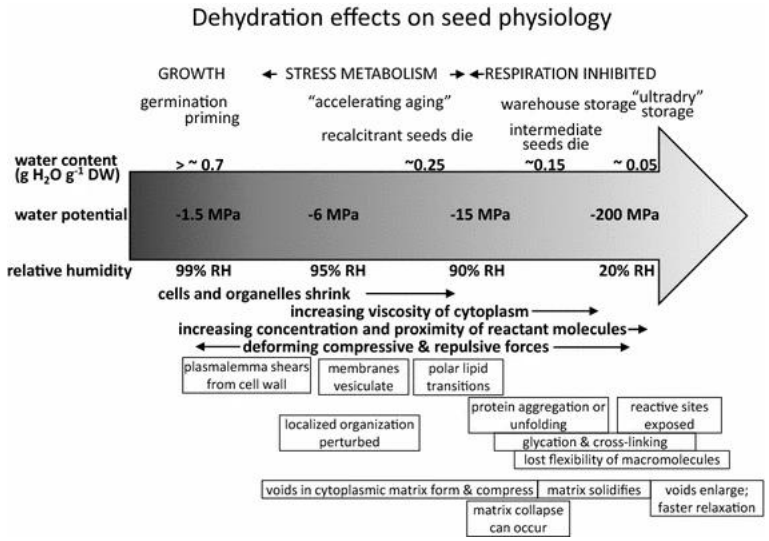

Figure 1.1 A schematic diagram of the cell response to water stress in seeds. Water stress ranges from 0 MPa (pure fluid water) to −∞ (no water). At ψw > −10 MPa, large changes in water content occur with small changes in water potential (Walters 2015). DW, dry weight. RH, relative humidity. Mpa, Mega Pascal (Walters, 2015).

1.1.2 Mechanisms behind acquisition and loss of desiccation tolerance

The ability of an organism, particularly seeds, to withstand and recover from excessive dehydration or drying out is referred to as desiccation tolerance (DT). It is quite evident that DT is important for the survival of seeds and germplasm preservation. A better understanding of the physiological and molecular mechanisms underlying DT could be beneficial for upgrading agricultural practices and environmental protection (Smolikova et al., 2020). Orthodox seeds undergo desiccation, which causes a water loss that ranges from 5-10% w/w. This feature enables them to withstand adverse environmental circumstances, including high temperatures and drought. On the other hand, recalcitrant seeds

do not undergo water loss during maturation. These seeds are susceptible to dryness like the vegetative tissues of fully developed vascular plants, thus they can be irreversibly damaged by severe desiccation. The survival of recalcitrant seeds is influenced by environmental factors. As a result, their range is limited to tropical woodlands. It has been hypothesized that recalcitrant seeds could have developed from ancestors having orthodox seeds, as a consequence of their adaptation to a wet climate characterized by constant rainfall. In such an environment, fast germination upon separation from the parental plant would provide an evolutionary advantage (Marques et al., 2018).

The mechanisms behind DT acquisition in orthodox seeds are activated during the late phases of maturation. The ability to withstand desiccation is no longer present during the process of germination when the radicle protrusion occurs. The exact molecular pathways responsible for DT acquisition and loss during seed maturation and germination, respectively, are mostly unknown (Leprince et al., 2017; Matilla, 2021). DT is a complex trait resulting from the interplay between many genes and factors, and it includes both structural and macromolecular changes in the compartments involved, particularly at the cell wall, an extremely dynamic cellular component (Zhang et al., 2021). When water is lost during desiccation the cell undergoes shrinkage, with detrimental effects on macromolecule structure and function. In order to withstand this stress, both the cell wall and plasmalemma fold, allowing protection of the membrane and cell wall surface area. This is crucial for cells to endure rehydration without experiencing any damage (Ballesteros et al., 2020). Membranes are regarded as the primary target by dehydration, and their stability is a crucial process for developing the ability to withstand dehydration (Matila et al., 2021). During seed maturation, the gradual reduction in water content also leads to the formation of "cytoplasmic glass". This structure is an amorphous and dense substance that resembles a solid state, causing a significant decrease in the movement and relaxation rates of molecules. The transition towards the glassy state, which is thermodynamically unstable with an exceptionally high viscosity, is facilitated by a reduced cellular water content and it is linked to enhanced storage stability (Sun, 1997; Buitink et al.,1998). The presence of a glassy state is essential to protect the cellular components against oxidative damage and preserve the original structure of macromolecules and membranes.

At the molecular level, DT is acquired through the accumulation of various protective molecules like late embryogenesis abundant (LEA) proteins, small heat shock proteins (sHSP), non-reducing oligosaccharides of the raffinose group (RFO) (Lima et al., 2017; Marques et al., 2018) and low molecular weight antioxidants -glutathione (Cairns et al., 2006), tocopherols (Chen et al., 2016) and carotenoids (Smolikova and Medvedev, 2015). Several gene regulatory networks significantly influence the response to unfavorable environments, resulting in desiccation tolerance or sensitivity. The primary regulation of the response to desiccation is ruled by abscisic acid (ABA)-mediated signaling, but there are also other mechanisms that operate independently of ABA (Kijak and Ratajczak, 2020).

The LAFL [LEAFY COTYLEDON 2 (LEC2), ABA INSENSITIVE 3 (ABI3), FUSCA3 (FUS3), and LEAFY COTYLEDON 1 (LEC1)] subfamily of B3 transcription factors (TFs) plays an important role in ABA signaling during seed development and maturation (**Figure 1.2**) (Baud et al., 2016; Carbonero et al., 2017).

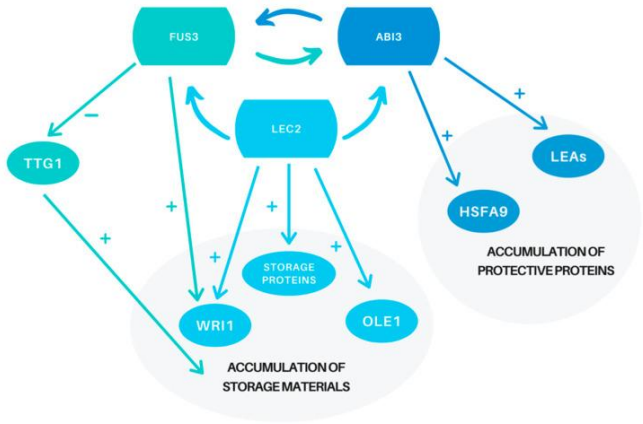

Figure 1.2 Representation of B3 transcription factors effect on seed maturation. **a.** FUS3 indirectly increases storage reserve accumulation by repressing TRANSPARENT TESTA GLABRA 1 (TTG1) transcription factor (TF), a negative regulator of fatty acid and storage protein biosynthesis genes, and positively regulating the inducer factor WRINKLED 1 (WRI1). ABI3 is likewise regulated by FUS3 in lateral cotyledons. **b.** LEC2 modulates other B3 transcription factors - FUS3 and ABI3, inhibiting anthocyanin and chlorophyll accumulation, and by favorably regulating WRI1 and OLE1, intensifies fatty acid production and storage. It also positively regulates 2S and 12S storage proteins. **c.** ABI3, a master regulator of late embryogenesis abundant (LEA) protective proteins, promotes FUS3 expression in the embryo axis and cotyledons, and indirectly accumulates heat shock protective proteins by positively regulating HSFA9 TF (Kijak and Ratajczak, 2020).

The LAFL-type TFs contain a B3 DNA-binding domain, that has a seven-stranded β-sheet arranged in an open barrel structure. This barrel is bounded by two short α-helices positioned at its ends (Yamasaki et al., 2012). The VIVIPAROUS-1 (VP1) B3 TF, first identified in maize (*Zea mays* L.), is an ortholog of the *Arabidopsis thaliana* ABI3 transcription factor. The maize *vp1* mutant seeds exhibit ABA insensitivity and are unable to enter the quiescent state. Indeed, *vp1* mutant seeds start to germinate when they are still enclosed within the corn cob (Grimault et al., 2015). Using systems biology approach in *Medicago truncatula*, a stable co-expression network was built using 104 transcriptomes from seed developmental time courses obtained in five different growth environments. Comparative analysis of the maturation processes in both *M. truncatula* and *A. thaliana* seeds and mining Arabidopsis interaction databases revealed highest number of correlations of DT-related genes with ABI3 (Righetti et al., 2015).

LAFL TFs carry out highly specific functions (**Figure 1.2**). In Arabidopsis *lec1*, *abi3*, or *fus3* mutants, DT is drastically affected but this response was not observed in the *lec2* Arabidopsis mutants. ABI3 act both an autoregulator and a regulator of FUS3 (To et al., 2006). Ectopic expression of *LEC1*, *FUS3*, or *ABI3* genes in single- or double-mutant backgrounds of the other two TFs activated

processes related to seed maturation, such as lipid and seed storage protein accumulation. However, DT was not triggered, indicating that all three regulators are necessary to activate this process (Roscoe et al., 2015). *LEC2* was expressed during the earlier stages of seed development as well as until mid-phase of maturation, inducing *ABI3* and *FUS3* gene expression. LEC2 is also involved in seed maturation and the accumulation of lipids and seed storage proteins (SSPs) (Braybrook et al., 2006). The LAFL functions in different seed subregions turned out to be very complex and tightly regulated to ensure progression and success of seed development and maturation. Despite the extensive recent studies, there is still a gap of knowledge about the majority of the targets of LAFL TFs. Overall, these studies highlight that LAFL TFs are master regulators involved in many aspects of seed development, including longevity and germination (Gazzarini and Song, 2024).

1.2 Seed Longevity

The ability of plants to generate desiccation tolerant seeds represents an effective survival strategy, it extends the lifespan of embryos, enabling survival under severe environmental conditions such as prolonged drought and extreme temperatures. Seed longevity refers to a time period in which a seed population maintains viability under certain environmental conditions. This complex trait, impacted by the interplay of various genetic and environmental factors, can differ even within closely related ecotypes (Ellis, 1991; Colville and Pritchard, 2019). Seeds need to be stored throughout the processing, distribution, and marketing processes, the amount of time that seeds are in storage can range from a few months to several years. Storage parameters are regarded as one of the most relevant determinants of seed quality. Even under optimal conditions, seeds progressively deteriorate during prolonged storage (Chhabra and Singh, 2019). As seeds age in storage, most commonly observed changes are membrane and organelle damage, an increase in the loss of seed leachate, decrease in respiratory rates and ATP production, and a loss of enzymatic activity (Zhang et al., 2021).

1.2.1 Factor affecting Seed longevity

There are numerous factors that participate in seed deterioration in storage (**Figure 1.3**). The quality of seeds is influenced by genetic factors. Even under optimum storage conditions different genera, species, cultivars, or individual plants demonstrate different storage behavior. Seeds of some species, e.g., pea (*Pisum sativum* L.) and wheat (*Triticum* spp.) show a longer life in storage compared to seeds of other species, e.g., onion (*Allium cepa* L.) and lettuce (*Lactuca sativa* L.) (Zhang et al., 2021). There are numerous biochemical reactions that participate in seed deterioration. Reactive Oxygen Species (ROS) accumulation, resulting from aerobic respiration in seeds, is increased under unfavorable or long storage conditions. Viability loss during ageing is caused by high production of ROS ($O_2^{-\bullet}$, H_2O_2 and $^{\bullet}OH$) along with reduced antioxidant potential. In imbibed seeds, ROS production occurs in mitochondria, glyoxysomes, and by NADPH oxidases in plasma membrane. In case of dry

seeds, ROS accumulate through non-enzymatic reactions (Kurek et al., 2019). Two wheat wild varieties, *Aegilops tauschii* and *Triticum monococcum subsp. Aegilopoides* showed high ROS levels in aged seed lots (Gianella et al., 2022). The effect of different storage conditions was tested on the onion cultivars Horand, Kazerun, and Zarghan. The germination percentage of onion seeds decreased as the aging period increased. Kazerun landrace was the most susceptible to storage conditions, showing a decrease in antioxidant activity (Kamaei et al., 2024). *Allium fistulosum* seeds stored for 22 months under six different conditions of temperature (25, 10, and 7.5 °C) and relative humidity (25% and 45%) showed a decrease in germination and genotype-specific ROS accumulation. The Maillard reaction is a non-enzymatic attack on amino groups of proteins and nucleic acid/protein complexes, facilitated by reducing sugars or aldehydes. In *Vigna radiata* L. seeds under different moisture content and storage temperatures, loss of viability was associated with the progressive accumulation of the Maillard products during storage (Murthy et al., 2003). In a study, investigating seed protein stability and seed deterioration in storage in the Ollagüe and Baer II quinoa (*Chenopodium quinoa* Willd.) cultivars, significant levels of advanced glycation end-products (AGEs) was present in stored seeds with decreased viability, thus showing a correlation between seed aging and accumulation of Maillard products (Castellión et al., 2010). Lipid peroxidation is triggered by increased ROS levels in the seed. The by-products of lipid peroxidation are aldehydes, ketones, alkanes, carboxylic acids, and polymerization products which are very reactive and easily penetrate biological membranes, thus compromising their stability and function. It has been demonstrated that the overexpression of three rice lipoxygenase (LOX)-related genes caused loss of membrane integrity whereas decreased expression of the same genes enhanced seed vigor and longevity (Gayen et al., 2015; Oenel et al., 2017). Gianella et al. (2022b) assessed within-species variations in seed longevity of eight pea accessions in long-term (approximately 20 years) storage. The stored seeds were tested for their biochemical and physical status, ROS profiles, lipid peroxidation, tocopherols, free proline and reducing sugars. Those accessions able to survive long-term storage revealed low lipid peroxidation levels (Gianella et al., 2022b).

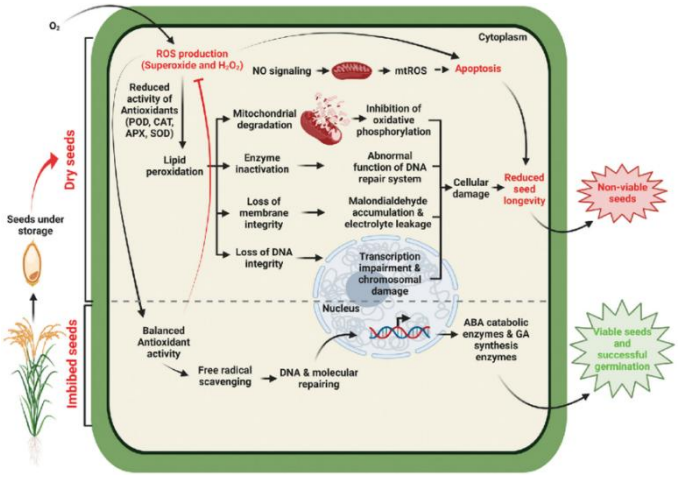

Figure 1.3 Pathways involved in seed aging during storage that affect the viability of seeds and factors playing roles in maintaining the viability (Choudhary et al., 2022).

1.2.2 Impact of storage on seed quality, germination and seedling establishment

Seed longevity and vigor decline over time in storage, which tends to directly impact germination and seedling establishment. Seed longevity is a crucial agronomic trait which assures high germination percentage after long-term storage (Colville and Pritchard, 2019). Seed longevity is not only determined by factors such as germinability but also other traits that prevent the degradation of flavor, nutritional content, and aroma. Henceforth, increased resistance to deterioration is a desirable trait to prolong germinability and keeping acceptable palatability, which ultimately results in increased global food security (Rehmani et al., 2023). Seed germination begins with water uptake by dry mature seeds and terminates with the occurrence of radicle protrusion. When germination starts, the embryo is dependent on storage reserves established during maturation until the seedling will be capable of autotrophic growth (Bewley, 1997). High quality seeds exhibit fast, synchronous germination, enhanced tolerance to abiotic stress and establish robust seedlings. Low seed vigor is evidenced by a decrease in germination speed and synchronization that possibly leads to seed viability loss. All these unfavorable characteristics are enhanced under long-term storage due to seed aging (Powell, 2022). Three high oil content canola (*Brassica napus* L.) varieties (45H29, Invigor 5440 and Nex4 105) and one low oil content canola variety (5525 Clearfield) with 8, 10, 12 and 14% initial moisture contents were stored at four different temperatures (10, 20, 30 and 40 °C) for 20 weeks. Increase of moisture content and temperature were correlated with germination loss and mold infection (Sun et al., 2014). To establish the safe storage protocol for industrial hemp (*Cannabis sativa* L.), seeds were stored in three dockage levels (0, 5, and 15%) at four temperatures (20, 25, 30 and 35°C) and four RH conditions (50, 63, 75, and 92%) for 26 weeks. Based on the results of germination tests and seed free fatty acid value (FAV), the best storage temperature was 25 °C with the safe storage time of 20.8 and 2.8 weeks for hemp seeds without dockage and 8% and 14% RH contents, respectively (Jian, 2019).

Seed vigor was tested on einkorn (*Triticum monococcum* L.) seeds for different storage durations (0, 60, 120, 180 days) in air-conditioning cabinets set to increasing temperatures (4, 10, 20 °C) as well as at room temperature. After seven days of storage, germination tests were performed. The seeds stored at room temperature rapidly lost their germinability whereas the seeds stored at 4°C were able to maintain a stable germination (Kibar and Yücesan, 2021). The innate capacity of seeds to withstand deterioration is vital for long-term seed storage (germplasm conservation) and crop improvement. However, a rational approach should be undertaken to anticipate reduced seedling establishment as a result of seed aging, leading to undesired depletion of seed reserves. Based on the current literature, it appears that the majority of longevity studies have been focused on germination performance whereas the aspects related to the impact on seedling establishment have been highly overlooked (Rehmani et al., 2023). Seed longevity becomes a critical issue when primed seeds are stored for a long period.

1.3 Seed priming

Seed priming is a pre-sowing technique in which seeds are subjected to controlled imbibition with water or other various priming agents. For successful imbibition, the seed coat must be permeable to water whereas seeds having a not permeable coat need to be scarified to facilitate imbibition. Following the controlled imbibition step, seeds are brought back to their original moisture content (dry-back). This step must be started before radicle protrusion occurs, to prevent DT loss (Paparella et al., 2015; Pedrini et al., 2020; Pagano et al., 2023) (**Figure 1.4**). The ideal duration of priming might vary based on several aspects, such as the priming agent, species/genotype, seed size, the dormancy status, and the speed of germination.

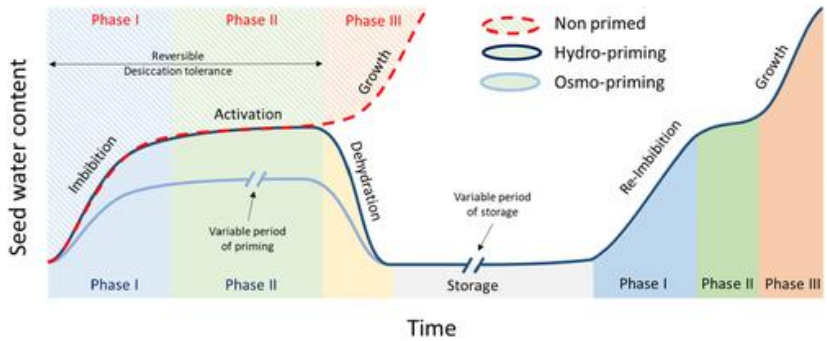

Figure 1.4 Comparison between the seed hydration curves and germination phases in primed and unprimed seeds. Red dotted line: non-primed seeds. Dark blue: hydroprimed seeds. Light blue line: osmoprimed seeds (Pedrini et al., 2020).

1.3.1 Types of Seed priming

There are many priming approaches, based on different types of priming agents. Some of the main seed priming techniques include hydropriming, osmopriming, chemopriming, hormopriming and thermopriming, however more recent priming

approaches are gaining attention, such as nanopriming, biostimulant priming and hybrid priming. The types of priming are briefly described below.

Hydropriming. This technique, inexpensive and easy to apply, uses only water to imbibe the seeds under controlled conditions. The beneficial effects of hydropriming have been reported in several crops. Uncontrolled water uptake by the seeds is one of the disadvantages of hydropriming. The effect of hydropriming was tested on 27 different genotypes of rice (*Oryza sativa* L.) under dry to wet soil moisture conditions. Hydropriming enhanced growth under the moderate dry conditions of 10% and 15% soil moisture. However, hydropriming was not effective in water stress conditions of 5% and 20% soil moisture (Nakao et al., 2023). Hydropriming demonstrated beneficial effects on the canola varieties Punjab and Faisal by improving germination and plant height (Bibi et al., 2024).

Osmopriming. This type of priming is based on the use of osmotic agents like polyethylene glycol (PEG), mannitol, and sorbitol, as well as inorganic salts (NaCl, KCl, KNO_3, K_3PO_4, KH_2PO_4, $MgSO_4$ and $CaCl_2$). Osmotic agents control the amount of water that enters the seeds, sufficient to activate the pre-germinative metabolism (Lutts et al., 2016). Maize is highly susceptible to drought. El-Sanatawy et al. (2021) tested the effects of NaCl (4000 and 8000 ppm), in field trials under conditions of water deficiency. Salt priming was beneficial towards water deficit stress, particularly seeds treated with of 4000 ppm NaCl resulted in significant benefits such as increased grain yield and improved water use efficiency, compared to unprimed seeds. *Coronilla varia* L. (purple crown-vetch) seeds primed with 10% PEG showed improved germination performance under water stress. Osmopriming significantly enhanced soluble proteins content, proline content, and SOD (superoxide dismutase) activity in *Coronilla varia* L. seedlings compared to unprimed seeds (Ma et al., 2024).

Chemopriming. Exogenous and plant-derived chemicals have been used as priming agents as well as molecules like hydrogen sulfide (H_2S) and nitric oxide (NO), natural compounds, e.g. chitosan, melatonin, ascorbic acid, alpha-tocopherol, trehalose, and polyamines (Zulfiqar et al., 2022). Mahmood et al. (2024) examined the impact of melatonin-priming on antioxidant defense systems and its impact on leaf ultrastructure and stomatal characteristics in maize seedlings subjected to osmotic stress induced by PEG6000. Melatonin-priming managed to increase seed germination, root and shoot length, fresh seedling weight, proline, total soluble protein, sugar, and chlorophyll content, as well as stomatal aperture size under drought stress compared to unprimed seeds (Mahmood et al., 2024). The beneficial effects of seed priming with 100 µM NO and/or 0.01 mM spermine in ameliorating the chromium-induced toxic effects in rice plants were reported by Basit et al. (2023). Combined applications of NO and spermine significantly reversed the chromium-induced toxic effects by reducing metal accumulation, maintaining the nutrient balance, improving germination and levels of photosynthetic pigments (Basit et al., 2023).

Hormopriming. Seed soaking in solutions containing phytohormones like salicylic acid (SA), ABA, auxins, gibberellins (GAs), cytokinins, and ethylene has proven

beneficial in promoting plant stress tolerance (Sytar et al., 2018). SA (138 mg/L) and gibberellic acid (300 mg/L) were tested on two rapeseed cultivars, tolerant (ZY15) and sensitive (HY49), respectively, to low temperature and drought. Germination was carried out at 15°C and -0.15 MPa ($T_{15}W_{15}$) and at 25°C and 0 MPa ($T_{25}W_0$). For ZY15 seeds under $T_{15}W_{15}$, SA priming, shortened the average germination time. For HY49 seeds under $T_{25}W_0$, GA priming reduced the average germination time (Zhu et al., 2021). Treating radish (*Raphanus sativus* L.) seeds with 1 mM indole-3-acetic acid (IAA) resulted into seedlings with increased concentration of photosynthetic pigments, total soluble leaf proteins, phenolic compounds and flavonoids, compared to seedlings developed from unprimed seeds. These results indicate the potential use of IAA in commercial seed priming protocols (Kanjevac et al., 2022).

Thermopriming. Seeds are exposed to high temperature prior to sowing which results in enhanced plant abiotic stress response and reduces thermoinhibition. The potential of thermopriming should be more effectively utilized, in light of the limitations to agriculture caused by global warming. In Arabidopsis, following seed thermopriming, the resulting plants performed better under heat stress when compared to plants developed from unprimed seeds (Serrano et al., 2019). Electrospray ionization mass spectrometry, used to study the dynamics of the response to heat stress in Arabidopsis plants derived from thermoprimed seeds, highlighted the increased production of branched-chain amino acids, raffinose family oligosaccharides (RFOs), lipolysis products, and tocopherols which act as osmolytes, growth precursors, antioxidants able to support the plant recovery from heat stress (Serrano et al., 2019). The long-term effects of thermopriming on the growth, development, and fruit yield of tomato plants were assessed by Körner et al. (2024). After 6 weeks of thermopriming, the plants were subjected to subsequent salt stress. Tomato plants developed from thermoprimed seeds exhibited temporarily increased stress tolerance by accumulation of protective phenols and flavonoids in the leaves (Körner et al., 2024).

Nanopriming. This is one of the most recently introduced type of priming, in which seeds are soaked in solutions containing nanoparticles (NPs). In spite of the concerns regarding the biosafety and toxic side effects, an increasing number of reports demonstrate that nanoprimed seeds show improved germination and stress response (Nile et al., 2022). When canola seeds were primed with cerium oxide nanoparticles (CeO_2 NPs), increased shoot and root length, and seedling dry weight was observed, compared to unprimed seeds. The nanoprimed seeds exposed to salt stress showed increased SA biosynthesis in shoots and roots compared to unprimed seeds (Khan et al., 2022). Rice seeds treated with silver nanoparticles (AgNPs) at a concentration of 80 mg/L enhanced root elongation and chlorophyll content. With the transcriptomics approach, genes associated with root growth and development like glucosyltransferases, glutathione pathway genes, calcium-ion binding pathway genes, peroxidase precursor and nitrilase-associated protein were upregulated (Santhoshkumar et al., 2024).

Biostimulant priming. Biostimulants are made from diverse source materials, such as waste from the food and paper industries. They are utilized to enhance

seed germination. These mixes are environmentally safe, making them reliable alternatives for chemical fertilizers and exhibit a wide range of biological effects (Wazeer et al., 2024). Hydrochar processed from the hydrocarbonization process of by-products from the sugarcane industry was shown to increase the germination rate of corn seeds especially with polar hydrochar (Bento et al., 2021). Two concentrations (10 and 20%) of extracts from the green alga *Cladophora glomerata* were applied to lupin (*Lupinus angustifolius* L.) seeds and the 20% extract resulted in enhanced germination and seedling growth of all lupin cultivars, compared to the 10% application (Lewandowska et al., 2023).

1.3.2 Pre-germinative metabolism of the seed

Seed germination can be divided into three phases. Phase I is associated with rapid water uptake by dry seeds under favourable environmental conditions. During phase II (lag phase) there is no further water uptake. One of the important features of phase III is radicle protrusion which is facilitated by endosperm rupture. During phase I and phase II, there is re-activation of the seed metabolism in order to accomplish the germination process until radicle protrusion (Bewley, 1997). The initial metabolic processes include ATP biosynthesis, redistribution of reserves, activation of the translation mechanism, and protection from antioxidant stress through antioxidant mechanisms and DNA repair pathways. This part confined within the early germination phase has been termed 'pre-germinative metabolism' (Macovei et al., 2016) (**Figure1.5**).

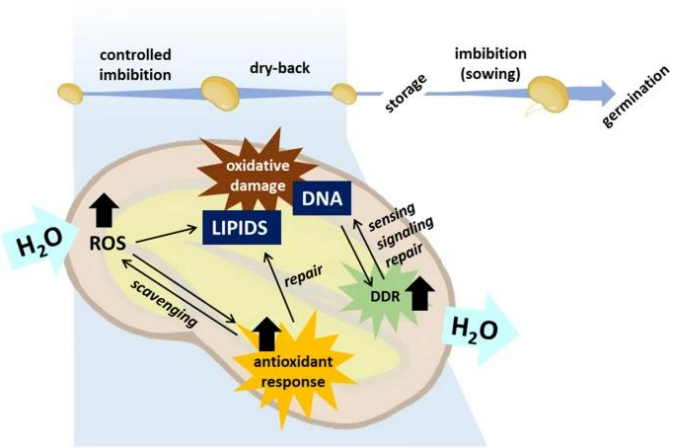

Figure 1.5 Representation of the rehydration-dehydration cycle applied in standard seed priming protocols. Primed seeds undergo post-priming germination. The different steps of the imbibition and dry-back can be regarded as potential sources of novel seed quality hallmarks which could genes, proteins, metabolites (Pagano et al., 2023).

During seed imbibition, when cellular respiration is re-initiated, the mitochondrial electron transport chain is reactivated, leading to higher ROS production. Other secondary ROS sources are glyoxysomes, peroxisomes, chloroplasts, and the plasma membrane. ATP synthesis and oxygen consumption take place upon rehydration and ATP is required for high germination rates (Bewley et al., 2013; Domergue et al., 2019). Sunflower (*Helianthus annuus* L.) seed germination

significantly dropped at 45°C, and this response was associated with an increase in K^+ leakage and total electrolytes as well as with malondialdehyde accumulation in the embryonic axis and cotyledons, suggesting a loss of membrane integrity. ATP and ADP levels increased during the first few hours of imbibition at 45°C but the levels decreased after 48 hours, indicating a role of ATP in successful germination (Corbineau et al., 2002). Extracellular ATP is a signal molecule required for regulating growth, development, and for the responses to external environments. Treatment with extracellular ATP delayed Arabidopsis seed germination whereas in the Arabidopsis *dorn 1-3* (*does not respond to nucleotides 1*) carrying mutations in the extracellular ATP (exATP) receptor showed higher germination percentage compared to wild type seeds. The *dorn 1–3* mutant seeds also had higher GA levels and lower ABA content (Wang et al., 2022).

Germination is influenced by environmental factors like temperature, photoperiod, light intensity, water and nutrient availability. For the seeds to germinate, integration of these environmental factors is crucial. These environmental cues, perceived by plants, are translated to internal cues mediated by different phytohormones (Yan and Chen, 2020). In this context, the most important phytohormones are ABA, which has an inhibitory effect on seed germination, and GA which has a stimulatory effect on the process. An intense cross-talk exists between the two regulatory pathways in order to tightly control germination. ABI4 (ABSCISIC ACID-INSENSITIVE4) is a TF that promotes the ABA signal transduction pathway leading to enhanced ABA biosynthesis while inhibiting GA biosynthesis (Shu et al., 2017). ABA gradually accumulates in the seed throughout seed development. Loss-of-function mutation of *ABI3* (*ABSCISIC ACID INSENSITIVE 3*) gene, a positive regulator of ABA signalling, causes premature germination in Arabidopsis (Raz et al., 2001). The key GA biosynthesis genes *GA3ox1* (*GIBBERELLIN 3-OXIDASE 1*) and *GA3ox2* (*GIBBERELLIN 3-OXIDASE 2*) were strongly upregulated in cortex and endodermis of the embryo axis of germinating seeds, contributing to the *de novo* synthesis of GA in the embryo (Mitchum et al., 2006; Holdsworth et al., 2008). The Arabidopsis *ga1-3* and *ga2* mutants, defective in GA biosynthesis, fail to germinate without exogenous GA application (Mitchum et al., 2006; Shu et al., 2013). On the other hand, mutation in the GA catabolic gene *GA2ox2* leads to elevated GA4 levels, enhancing germination (Yamauchi et al., 2007). The timing of seed germination is influenced by the interplay between endogenous ABA and GA levels, rather than being solely determined by either ABA or GA. In the Arabidopsis ABA-deficient mutant *aba2*, GA synthesis was drastically enhanced whereas transcription of *GA3ox1* and *GA3ox2* genes increased in germinating seeds. In the *cyp707a2-1* mutant, characterised by ABA over-accumulation mutant, *GA3ox1* and *GA3ox2* gene expression is inhibited. These results suggest that GA biosynthesis is negatively regulated by ABA (Seo et al., 2006; Seo et al., 2008). Together, these observations demonstrate the mutual regulation of ABA and GA pathways during seed germination. The intricate interaction between ABA and GA serves as internal factors that determine the shift towards germination (Yan and Chen, 2020).

Seeds accumulate mRNAs during maturation and these mRNAs are translated during early seed imbibition (Rajjou et al., 2004). Stored mRNAs, loaded into polysomes, include those involved in redox processes, glycolysis, and protein synthesis that are essential for germination. The recruitment of ribosomal subunits directly onto *cis*-acting elements, known as internal ribosome entry sites (IRES), is facilitated by certain *trans*-acting factors (ITAFs). This process is crucial for initiating m7G cap-independent translation of mRNAs, particularly in the context of seed germination (Sano et al., 2020). *De novo* protein synthesis in Arabidopsis resulted in increased production of antioxidant enzymes involved in ROS scavenging, suggesting a prominent role of antioxidant mechanisms in seed germination (Galland et al., 2014).

1.3.2.1 ROS homeostasis in seed germination

Along with activation of the pre-germinative metabolism, there is increased ROS production in the seeds. ROS levels should be stringently regulated in order to avoid the deleterious oxidative damage (Tommasi et al., 2001). On the other hand, ROS have also a prominent role as signaling molecules in seed germination. Bailly et al. (2008) defined the term “oxidative window” as the crucial threshold of ROS accumulation that allows these reactive species to act as signaling molecules, without causing oxidative stress.

The antioxidant system plays a crucial role in regulating redox balance and cellular homeostasis by removing ROS and preventing molecular damage (Noctor et al., 2018). The antioxidant system includes both enzymatic and non-enzymatic ROS scavengers (**Figure 1.6**). Some of the enzymatic scavengers include Ascorbate Peroxidase (APX), Catalase (CAT), Glutathione Reductase (GR), Superoxide Dismutase (SOD), Dehydroascorbate Reductase (DHAR), Glutathione-S-Transferase (GSTs) and Glutathione Peroxidase (GPX). The non-enzymatic scavenger system includes glutathione, α-tocopherol, flavonoids, carotenoids and proline (Choudhary et al., 2020).

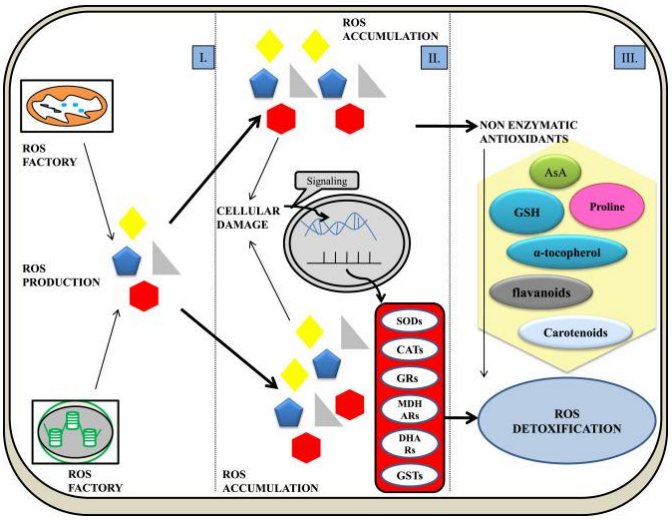

Figure 1.6 Fate of ROS in the plant cell. Ⅰ. ROS production Ⅱ. Oxidative stress: ROS accumulation-induced cellular damage triggers the production of antioxidant components Ⅲ. Detoxification: Enzymatic and non-enzymatic antioxidants (Choudhary et al., 2020).

The impact of different seed priming protocols, in terms of enhanced antioxidant response, has been assessed in a wide range of species. Jasmonic acid (JA)-mediated priming was tested on tomato (*Solanum lycopersicum* L.) seedlings under nematode infestation. JA-based priming helped in reducing oxidative damage by decreasing $O_2^{\bullet-}$ content, nuclear and membrane damage in presence of nematodes, by triggering the expression of *SOD*, *POD*, *CAT*, and *GPX* genes (Bali et al., 2020). Nanopriming with titanium dioxide nanoparticles (TiO_2) applied to maize seeds under salinity stress induced by 200 mM NaCl was able to enhance germination percentage and seedling vigor index. Nanopriming also enhanced the antioxidant response by increasing the activity of SOD and CAT enzymes (Shah et al., 2021). Seeds from two sorghum *(Sorghum bicolor L.)* cultivars (CFSH-30 and '1230') primed with various salt priming agents were able to withstand the adverse effects of salt stress by decreasing malondialdehyde content and enhancing antioxidant enzymes (CAT, POD and SOD) activities as well as proline content (Guo et al., 2022). ROS can breakdown deoxyribose in DNA molecules mainly by releasing a hydrogen atom causing various types of DNA damage (Sharma et al., 2012).

1.3.2.2 DNA Damage Response in the context of seed germination

An important aspect of the pre-germinative metabolism is the molecular network that detects, signals, and repairs DNA damage. This network is known as the DNA damage response (DDR) and is crucial for maintaining genome integrity. Genome maintenance in seeds is crucial for both growth, development and for preserving the long-term stability of plant germplasm at the population and species level. Henceforth, it is necessary to repair DNA damage early during the process of imbibition, before cell division begins, in order to maintain germination potential and minimize the occurrence of mutations during subsequent seedling

development (Waterworth et al., 2016). The delay in germination in low vigor seeds is caused by the need for DNA damage repair. Seed aging is specifically linked to the gradual buildup of DNA damage in the embryo. This includes generation of abasic sites, base modification, single strand DNA breaks (SSBs) and DNA double strand breaks (DSBs) (Waterworth et al., 2010). DNA damage can be caused by various endogenous factors and environmental stresses. This includes ultraviolet (UV) radiation, various pollutants, ROS-induced DNA modifications and errors occurring during replication (Sancar et al., 2004). Survival depends on the synergistic action of various DNA repair pathways present in plants. Nucleotide excision repair (NER) removes the damage on a single strand, namely bulky adducts that could block RNA polymerase. Base excision repair (BER) resolves damaged bases and repairs single strand breaks (SSBs). For DSBs, NHEJ (non-homologous end joining), alt-NHEJ (alternative NHEJ) and homologous recombination (HR) are involved. In plants, particularly model organism Arabidopsis and rice, these pathways have been well studied. These crucial pathways are conserved across all eukaryotic organisms (Bray and West, 2005) (**Figure 1.7**).

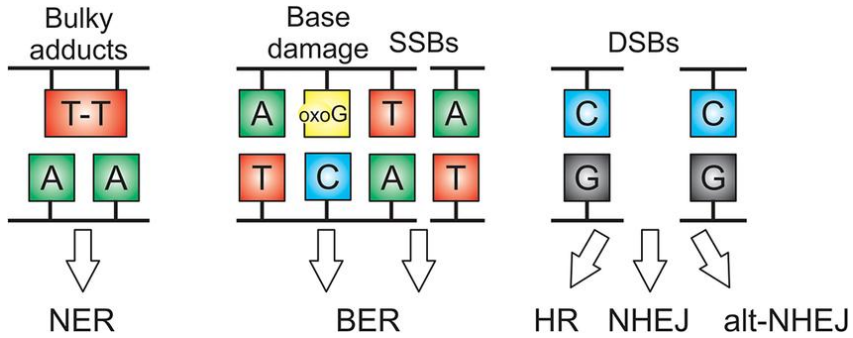

Figure 1.7 DNA damage lesions and their DNA repair pathways in seeds. Nucleotide excision repair (NER), Base excision repair (BER), single strand breaks (SSBs), DNA double strand breaks (DSBs), homologous recombination (HR), non-homologous end joining (NHEJ), or alternative NHEJ. Oxo G (8-oxoguanine) (Waterworth et al., 2019).

DNA damage accumulated in the embryo and the repair ability of the seed to remove DNA lesions are the major factors that determine seed vigor and viability. DDR plays an important role in regulating germination. It causes a delay in germination in aged seeds to minimize the harmful effects of DNA damage that occurs while the seed is in a dry and quiescent state. It is very crucial to repair damage before the initiation of cell division to have increased germination performance and evade mutagenesis during seedling development. In situations where the damage is not too severe, the DDR factors will activate the repair mechanisms by blocking the progression of the cell cycle, which will allow time for repair. However, under severe DNA damage, PCD (programmed cell death) will be initiated (Fulcher and Sablowski, 2009; Nisa et al., 2019). When controlled imbibition is performed during seed priming treatments, DDR mechanisms are triggered. In *Medicago truncatula*, occurrence of oxidative DNA damage was associated with increased levels of 7,8-dihydro-8-oxoguanine (8-oxo-dG), the most common type of oxidative base lesion. These changes were observed in *M. truncatula* seeds along the course of imbibition (Balestrazzi et al., 2011; Macovei

et al., 2011) and similar results were observed in eggplant (*Solanum melongena* L.) seeds (Kiran et al., 2020).

A detailed understanding of the molecular mechanisms involved in DDR will be foundation for crop enhancement and improved conservation of plant genetic resources in seed banks (Manova and Gruszka, 2015; Waterworth et al., 2019). (**Figure 1.8**).

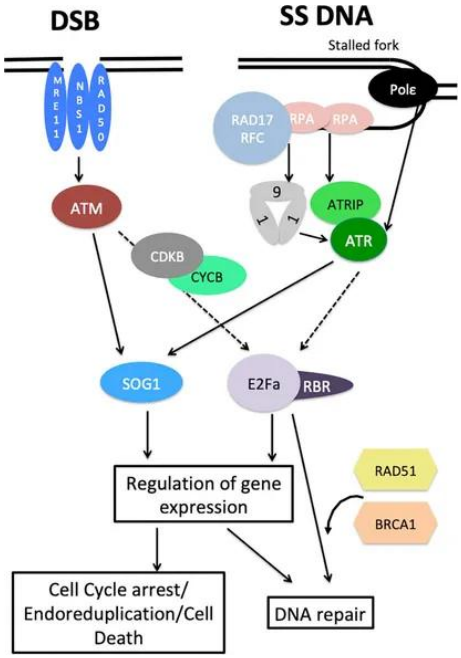

Figure 1.8 DDR signaling in plants (Nisa et al., 2019).

The MRN complex is a conserved complex consisting of three proteins: Meiotic recombination 11 (MRE11), Radiation sensitive 50 (RAD50), and Nijmegen Breakage Syndrome 1 (NBS1). The MRN complex detects and binds to DSBs sites, unwinding DNA and processing DNA ends. The interaction with NBS1 activates Ataxia Telangiectasia Mutated (ATM) kinase activity (Amiard et al., 2010; Lamarche et al., 2010). ATM is one of the major DDR regulators. In Arabidopsis seeds, DSBs accumulation triggers the ATM kinase which mediates the activation of a crucial DNA damage checkpoint and delays germination (Maréchal and Zou, 2013). In case of SSBs, RPA (replication protein A) coats itself on SSBs and recruits DNA polymerase α, RFC (RAD17-replication factor C), and the 9-1-1 complex (RAD9, RAD1, and HUS1). ATR (ATM and Rad3-related) kinase is then activated by the 9-1-1 complex that is loaded on damaged DNA by the RFC subunits 2-5. As soon as ATM and ATR kinases are activated, they set off a cascade that phosphorylates various DDR proteins downstream (Amiard et al., 2010; Saldivar et al., 2017). Both the ATR and ATM pathways cause an increase of γH2AX (a histone variation that has been phosphorylated) at the DNA damaged sites, a crucial step for recruiting and activating DDR signaling and repair components (Kinner et al., 2008).

Both ATM and ATR have unique and complementary functions in DDR. Both Arabidopsis *atm* and *atr* mutants are sensitive to DSBs caused by γ-irradiation, but only ATR is necessary for responding to replicative stress (Culligan et al., 2006). The *atm* mutants were able to germinate but they showed significant chromosomal defects, and this finding allowed to establish a connection between genome stability and successful germination (Waterworth et al., 2016). SOG1 (Suppressor of Gamma response 1) is the DDR master regulator in plants, the functional equivalent of the p53 protein that controls DDR and cell cycle progression in animals, activated by ATM and ATR. The Arabidopsis SOG1 transcription factor belongs to the NAC (NAM, ATAF1/2, and CUC2) family (Yoshiyama et al., 2009). Upregulation of *SOG1* gene was observed along dry-back in hydroprimed *M. truncatula* seeds, indicating an active role of DNA damage-dependent signaling in this context (Pagano et al., 2022).

Even though DDR is a highly conserved mechanism among eukaryotes, plants have evolved a unique set of cell cycle checkpoint regulators, due to their sessile nature (Hu et al., 2016). The cell cycle consists of four phases: G1 (gap 1), S (DNA synthesis), G2 (gap 2 and M (mitosis). These phases are stringently regulated by protein complex of SOG1 (Suppressor of Gamma response 1) (CDKs) which are activated upon phosphorylation and when in complex with the appropriate regulatory cyclin subunit. CDK-cyclin complexes phosphorylate various substrates to promote cell cycle progression. In case of unfavorable conditions, cell cycle progression can be halted at the checkpoints. The main checkpoints are present at the G1/S, intra-S, and G2/M transitions. Depending on what point of cell cycle occurs, cell cycle can be arrested either at S and G phase and replication stress can activate intra-S phase (Hochegger et al., 2008; Gentric et al., 2021) (**Figure 1.9**).

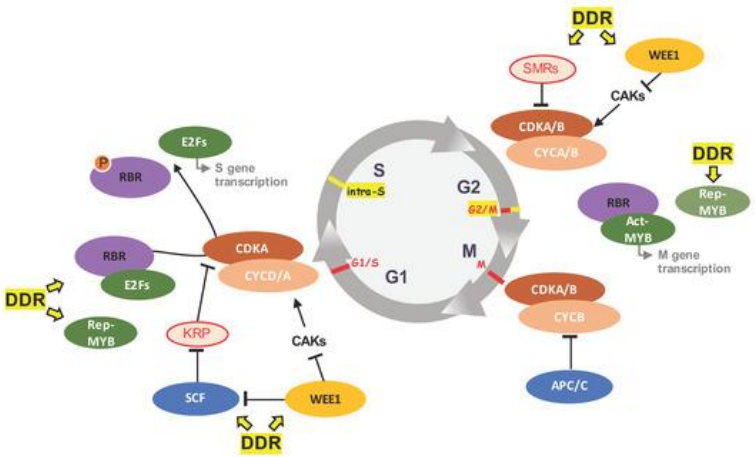

Figure 1.9. Schematic overview of the cell cycle and its link with the DDR. Canonical checkpoints, in red, and those activated by the DDR, in yellow, are placed on the cell cycle (Gentric et al., 2021).

SOG1 activates inhibitors of CDKs such as the SMR5 and SMR7 (Siamese Related 5/7) proteins. Arabidopsis *smr5* and *smr7* knockout mutant showed a defective checkpoint in leaf cells upon treatment with hydroxyurea (replication inhibitory drug). *SMR* genes can also be upregulated through ROS-dependent

transcriptional activation (Yi et al., 2014). SOG1 also suppresses the activity of CDKB2s that are core mitotic regulators, activating the G2/M checkpoint and blocking cell cycle. On the other hand, SOG1 stimulates CYCB1 which enhances the activity of CDKB1. CDKB1-CYCB1 were demonstrated to be involved in HR, where they recruit the core HR regulator RAD51 (Radiation sensitive 51) (Weimer et al., 2016). SOG1 also activates the *Rep-MYB3R* genes which are primary repressors of genes required for M-phase onset. In the absence of DNA damage, CDK complexes phosphorylate Rep-MYBR for proteasomal degradation, allowing G2/M progression. SOG1 activates SMR5/7 in case of DNA damage, leading to Rep-MYB3R accumulation. The latter blocks the G2/M progression (Bourbousse et al., 2018). One of the possible SOG1-independent DDR response could involve E2F-RBR1 (RetinoBlastoma Related 1) transcription regulators that control S-phase entry. RBR1 blocks expression of S-phase genes by repressing E2F transcription factors (Berckmans and De Veylder, 2009). RBR has been known to accumulate at DNA damage sites along with E2Fa, possibly with the help of CYCB1/CDKB and ATM/ATR activity. E2F-RBR1 complex recruit repair proteins like RAD51 and BRCA1 at the DNA damage site (Biedermann et al., 2017; Horvath et al., 2017).

Loss of seed vigor and viability is concomitant with elevated levels of DSBs mis repair. Cell cycle activation is linked to the detection of genome integrity by ATM that localizes to the damage site. The activation of ATM by DSBs leads to the phosphorylation of various substrates involved in DNA repair, cell-cycle arrest, apoptosis, and other downstream processes. This results in delay of germination process as seed vigor declines. However, the way seeds mitigate the toxic effects of high DNA damage levels, accumulated during the quiescent stage, allowing germination and seedling development, still needs to be fully elucidated (Maréchal et al., 2013; Waterworth et al., 2016). Imbibed Arabidopsis seeds exhibit remarkable resilience to DNA damage compared to seedlings. This resilience is accompanied by low cell cycle activity and DDR activation during early imbibition. However, as the seeds progress towards germination, this resilience is lost. Indeed, changes in the activity of the plant DDR machinery are dependent on the developmental stages, as seen by unique transcriptional DDR profiles in both Arabidopsis seeds and seedlings. If DNA damage-induced cell cycle checkpoints are not activated, this could lead to accumulation of growth inhibiting lesions which could affect species fitness (Pedroza-Garcia et al., 2021; Waterworth et al., 2022). Despite the presence of intricate DDR processes in plants, there are instances where permanent DNA damage is unavoidable. In this case, plants exhibit either endoreduplication, a process in which cells reproduce DNA without undergoing mitosis, resulting in cell expansion and differentiation, or PCD which removes damaged cells. The interconnection between cell cycle progression and DDR is highly evident, as they play a crucial role in determining the outcome of damaged cells. Plants that experience DNA damage exhibit a notable decrease in production and yield. Therefore, understanding the pathway could be highly advantageous and result in a substantial economic influence (Szurman-Zubrzycka et al., 2023b).

1.4 Drawbacks of seed priming

Priming has numerous benefits in relation to germination, seedling establishment, and response to abiotic stress. One intriguing topic to explore is under what circumstances can seed priming be unsuccessful, and what are the drawbacks of this technique. The seed response to priming can be strongly genotype- and seed lot-dependent, and this requires intense efforts to design tailored protocols. However, there are specific drawbacks that further impair the benefits of seed priming, such as overpriming and the poor storability of primed seeds (Pagano et al., 2023).

1.4.1 Overpriming

One crucial aspect to consider in priming is optimizing the rehydration time. Controlled imbibition must be blocked before radicle protrusion occurs or seeds will lose DT and their survival under dry-back step will be compromised. This undesired response, that can compromise the effectiveness of seed priming protocols, is known as overpriming. Interestingly, DT can be restored during the very early stage of seedling development if seeds are exposed to osmotic stress (Peng et al., 2017). In *M. truncatula*, this crucial window exists when the radicle length is between 1 and 3 mm (Buitink et al., 2003; Maia et al., 2011). In order to better explore the dynamics of DT loss and gain, an experimental system has been established in *M. truncatula* by Pagano et al. (2022a, b). In this system both primed and overprimed *M. truncatula* seeds were investigated and compared in terms of antioxidant response and DNA repair pathways as well as other molecular events (e.g. ribogenesis) associated with the progressive loss of desiccation during dry-back (Pagano et al., 2022a, 2022b). Both spectrophotometric assays and DAB staining revealed ROS accumulation in *M. truncatula* embryos during the drying process after priming, and similar patterns were also observed in a commercial variety of alfalfa (*Medicago sativa* L.). These results suggested that ROS accumulation can be used as a proper indicator of loss of desiccation tolerance (Pagano et al., 2022a, 2022b).

1.4.2 Loss of storability in primed seeds

Several reports highlight that seed priming can alter storability of primed seeds in various species. The increased vulnerability of primed seeds to storability is due to various factors associated with the more advanced physiological state of primed seeds. These factors gradually subject cellular structures to oxidative damage as seeds shift from their dormant dry state to the metabolic pre-germinative phase triggered by priming (Varier et al., 2010). Primed seeds are at a more advanced physiological state with regard to the germination process, compared to unprimed seeds, consequently rendering them more vulnerable to deterioration. Primed tomato seeds were stored at 6% MC at either 4 °C or 30 °C for 1 year. Viability and germination rate were unaffected at 4°C whereas viability and germination rate were reduced after 6 months of storage at 30°C in primed seed, compared to untreated seeds (Argerich et al., 1989). Lettuce seeds treated with osmopriming (-1.5 MPa PEG8000) for various imbibition times were tested

for longevity during storage. Increasing time of imbibition reduced the mean germination time up to 61%, compared to untreated seeds, but also reduced seed longevity by 84% (Tarquis and Bradford, 1992). A series of storage conditions were tested on primed rice seeds. Long-term storage of primed seeds at 25°C significantly reduced germination by 90% and growth parameters like shoot length, root length, and their fresh weights compared to primed seeds that were not in long-term storage. The beneficial effects of osmopriming observed at 25°C only lasted for 15 days. The deterioration of primed rice seeds in storage was associated with reduced starch metabolism (Hussain et al., 2015). Naturally aged Chinese cabbage (*Brassica rapa* subsp. pekinensis) seeds were hydroprimed at 20 °C in the dark for 10 h and subsequently stored at 4, 20 or 30 °C. The seeds were tested for deterioration at regular intervals of 1, 3, 6 and 9 months. Germination and seedling vigor of seeds stored at 30 °C for 9 months significantly decreased compared with primed seeds not subjected to storage. However, such negative effects were not observed in primed seeds stored at 4°C and 20 °C for 9 months, indicating that it would be preferable to store seeds at lower temperature (Yan, 2017). The deterioration of seed in storage was associated with decreased activities of POD and CAT enzymes, soluble sugar and soluble protein content, with increased MDA levels in primed Chinese cabbage seeds (Yan, 2017). The effect of temperature, RH and oxygen were tested on the longevity of primed rice seeds in a range of 60 days-storage (Wang et al., 2018). The following seed storage conditions were applied: low temperature (-4°C)-vacuum, room temperature (30°C)-vacuum, room temperature-aerobic-low RH and room temperature-aerobic- high RH up to 60 days. The viability of primed rice seeds significantly reduced under room temperature-aerobic-high RH. High RH was identified as the primary factor reducing the longevity of primed seeds. Reduced starch metabolism, consumption of starch reserves, MDA accumulation and decrease in antioxidant enzyme activities were associated with poor storability of rice primed seeds (Wang et al., 2018).

1.5 Mitigation strategies for loss of longevity in primed seeds

The connection between priming and aging needs to be understood in order to gain knowledge concerning the mechanisms underlying the loss of storability that occurs after priming. There is an established literature that focuses on seed aging and longevity. It is important to investigate molecular and physiological dynamics underlying the loss of longevity in primed seeds, this will help us design mitigation strategies to protect primed seeds from deterioration in storage (Pagano et al., 2023).

1.5.1 Artificial aging as a tool to study seed longevity

Many seed lots do not maintain their initial germination rate or germination ability when they are in storage. Seed storability is difficult to be determined, based on germination parameters measured prior to storage. Development of tests able to predict the storage potential of seeds began when Crocker and Groves (1915) postulated that subjecting seeds to higher temperatures, accelerates

deterioration due to protein coagulation. A substantial amount of research was carried out for several decades, until James Delouche developed the current accelerated aging technique, which has been widely used in order to study seed quality and storability (Delouche and Baskin, 1973). The protocol was based on the principle that, during accelerated aging (AA), seed deterioration would be similar to the process occurring under normal conditions but the deterioration is dramatically increased by high temperature and RH. Seed lots that maintain high germination profiles following AA are regarded as seeds with high longevity (Delouche and Baskin, 1973). One of the modifications of the AA protocol is saturated salt accelerated aging (SSAA) where the water is replaced by salt solutions to maintain the proper RH in the aging environment (McDonald, 1997). Another variant of the AA technique developed by Powell and Matthews (1981), allowed to accelerate the deterioration process of small sized seeds by increasing temperature and seed moisture content. Controlled Deterioration (CD) was considered to be more precise, compared to AA. CD is based on precise MC value, thus all the seeds in the lot will have the same MC. In order to identify the efficiency of AA tests, six seed lots of onion seeds (cultivars Aurora and Petroline) were subjected to SSAA (41°C for 48 and 72 h, respectively) and CD (24% of seed MC at 45ºC for 24 h). Based on the seedling emergence test, it was concluded that both AA and CD tests represent effective tools for determining the physiological potential of onion seeds and that they could be incorporated into the seed quality control programs (Rodo and Filho, 2003). Several studies have been carried out to explore the storability of seeds using AA techniques. Four canola cultivars (Okapi, Orient, Fornax and SLM046) were subjected to artificial ageing (40 °C, 100% RH for 0, 24, 48, 72 and 96 h) for storability assessment. Germination was reduced along time and sensitivity to aging was different, depending on the cultivar (Janmohammadi et al., 2008). Seeds from 175 barley (*Hordeum vulgare*) genotypes were subjected to two AA protocols. Genome-wide association mapping revealed 107 marker trait associations related to seed aging (Nagel et al., 2014). In order to study seed storability, transcriptome analyses were performed on rice thermo-sensitive genic male sterile lines, S1146S (storage-tolerant) and SD26S (storage-susceptible) lines. When 0 and 7 days of seed aging treatment was performed, genes associated with seed storability and seed vitality genes were identified, providing novel perspectives into molecular response and different-storability related genes under AA conditions (Li et al., 2024).

The molecular pathways involved in the deterioration of seeds triggered by natural aging and AA treatments have been an intriguing subject of discussion. Soybean was used to investigate seed longevity either during natural aging or AA (36°C and 75% RH). Maillard reactions were observed in both naturally aged and accelerated aged seeds, suggesting an important role in seed deterioration during storage (Sun et al., 1995). CD (85% RH at 40°C for up to 7 days) applied to Arabidopsis seeds with different treatment periods was studied and the changes were compared to the Arabidopsis seeds that were naturally aged (up to 11 years at 5 °C). Seeds showed similar changes like protein oxidation and carbonylation with both types of aging, suggesting that AA techniques could possibly mimic natural aging (Rajjou et al., 2008). Correlation analysis between

naturally aged coffee (*Coffea arabica* L.) seeds and artificially aged coffee seeds revealed a strong link between the period of artificial aging of 4 days, and the natural storage for 2 months for the Catuaí Amarelo cultivar (Fantazzini et al., 2018). In order to identify the seed-specific long-lived mRNAs involved in seed longevity in rice, aging experiments with 14 rice varieties were performed (Wang et al., 2022b). This helped to categorize higher- and lower-longevity rice varieties. RNA-Seq analysis performed on these varieties highlighted genes with similar expression patterns during natural and artificial aging, which means that the effects of these two aging methods on gene transcription are similar (Wang et al., 2022b). For research purposes, the development of artificial and accelerated aging protocols has enabled to replicate the effects of natural aging in a shorter time period and with more precise control over the experimental conditions. These conditions typically consist of high temperatures and relative humidity applied for several days or weeks. The utilization of cross-validation methods across different natural and artificial aging methods has facilitated the identification of common underlying mechanisms and the integration of seed longevity tests into seed quality assessment (Rajjou et al., 2008; Schwember and Bradford, 2010; Fantazzini et al., 2018).

1.5.2 How can the priming benefits of stored seeds be preserved?

Farmers should be informed about the seed shelf life in order to properly maintain the seed lot post-harvest, seed marketing and distribution, and seed companies are usually able to provide this essential information (Pagano et al., 2023). Small molecules, used commercially as drugs, herbicides, have been widely studied in basic research as they interact with biological macromolecules. Phytohormones, metabolites, activators and inhibitors of various essential pathways, are investigated by screening for candidate targets (Dejonghe & Russinova, 2017).

Exploring approaches to reduce the effects of priming-related aging involves using specialized experimental systems, in order to develop focused solutions to increase the seed lifespan after priming. Tomato seeds were subjected to controlled deterioration to study the effects of hydropriming and heat shock as a treatment to prevent deterioration of stored primed seeds, by stimulating the expression of heat shock proteins. Primed seeds subjected to heat shock showed enhanced longevity compared to hydroprimed seeds when subjected to controlled deterioration (Gurusinghe et al., 2002). One of the successful strategies to prevent deterioration of primed seeds is vacuum packaging which leads to low moisture content and limited respiration of seeds. Vacuum packaging has been successful in various species like corn (Chiu et al., 2003), bitter gourd (*Momordica charantia*) (Yeh et al., 2005) and rice (Wang et al., 2018). Primed Arabidopsis seeds treated with the cell cycle inhibitor mimosine showed higher survival rate compared to untreated seeds, suggesting a strong role of cell cycle checkpoints in storability of primed seeds. Other molecules with similar function like aphidicolin, hydroxyurea, and oryzalin were also able to improve storability in primed Arabidopsis seeds (Sano and Seo, 2019). Priming with antioxidants, like ascorbic acid and glutathione, carried out on aged oat (*Avena sativa* L.) seeds demonstrated positive affects in alleviating the damage caused by ageing.

Seed germination, antioxidant enzymes, cytochrome c oxidase and mitochondrial malate dehydrogenase activities, as well as the mitochondrial ultra structures of the embryonic root cells were markedly improved in aged oat seeds after post-priming (Xia et al., 2020). To study the effects of different priming techniques on aged sunflower seeds, hydropriming, ascorbic acid priming and sodium nitroprusside priming were tested. Priming with ascorbic acid and sodium nitroprusside allowed an improved performance of stored sunflower seeds, as the antioxidant defense was boosted (Pereira et al., 2022). These findings suggest that the longevity of primed seeds can be improved by selecting optimal priming parameters, storage, and post-storage conditions. A deeper understanding of the molecular and cellular mechanisms involved in the reduced storability of primed seeds is required in order to speed up the design of innovative and smart assays to detect (or prevent) deterioration of primed seeds (Fabrissin et al., 2021; Pagano et al., 2023).

1.5.3 Polyamines

Polyamines (PAs) are nitrogenous bases with several amino groups, known for their many biological functions. They have been observed in nearly all eukaryotic and prokaryotic species. Putrescine (Put), spermidine (Spd), and spermine (Spm) are the primary polyamines (PAs) found in plants. They play crucial roles in several processes such as floral development, embryogenesis, organogenesis, senescence, and fruit maturation and development. Furthermore, they participate in alleviating both abiotic and biotic stressors. (**Figure 1.10**) (Chen et al., 2019).

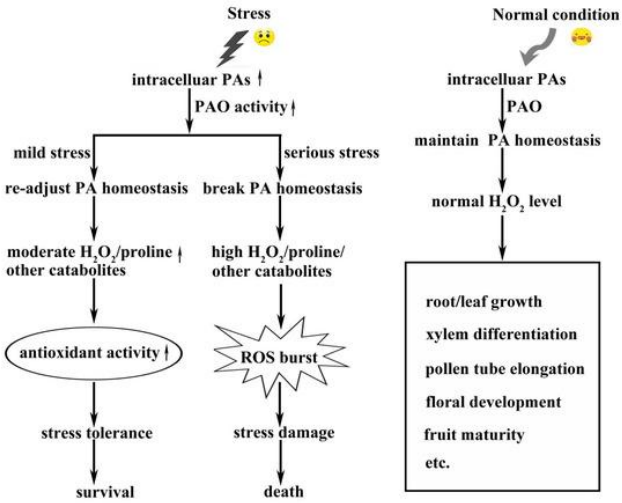

Figure 1.10 Diagrammatic representation of the roles of polyamines involved in developmental growth and environmental stress response in plants. PAO: Polyamine oxidase, ROS: reactive oxygen species (Zhang et al., 2019).

In higher organisms PAs mainly exist in free forms, covalently conjugated or non-covalently conjugated forms. In plants, PAs are primarily in free forms that bind to macromolecules like proteins, nucleic acids, uronic acid and lignins. The

conjugated forms of PAs are known to mediate crucial functions like DNA replication, transcription, cell division, plant development and abiotic stress response (Gholami et al., 2013; Igarashi and Kashiwagi, 2015; Chen et al., 2019). PAs biosynthesis begins with arginine being converted to Put through subsequent reactions. In the next step, Put is converted to Spd by spermidine synthase (SPDS). Spd is further converted to Spm by spermine synthase (SPMS) (Zhang et al., 2019) (**Figure 1.11**).

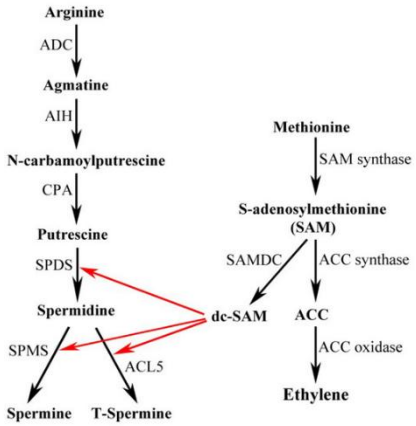

Figure 1.11 The intracellular polyamine pathway. ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; CPA, N-carbamoylputrescine amidohydrolase; SPDS, Spd synthase; SPMS, Spm synthase; ACL5, ACAULIS5, T-Spm synthase; SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase; dcSAM, decarboxylated S-adenosylmethionine; ACC, 1-amino-cyclopropane-1-carboxylic-acid (Zhang et al., 2019).

1.5.3.1 Spermidine

The beneficial effects of Spd on seed germination under stress have been reported by numerous studies. White clover (*Trifolium repens* L.) seeds challenged with PEG6000 showed improved germination when primed with spermidine. Improved starch metabolism as well as low lipid peroxidation levels were associated with seed invigoration (Li et al., 2014). The salt-sensitive and salt-tolerant rice varieties IR-64 and Nonabokra, respectively, were primed with Spm and Spd and exposed to salt stress (Paul and Roychoudhury, 2017). Transcriptomic profiles showed that polyamine-mediated priming enhanced the expression of antioxidant genes, osmolyte biosynthetic genes, ABA biosynthesis genes in the shoots and roots compared to the untreated shoots and roots. These results demonstrate the complex molecular networks in which PAs are involved (Paul and Roychoudhury, 2017). Sweet corn (*Zea mays* L.) seeds soaked with Spd revealed enhanced vigor. When seeds were treated with the Spd biosynthesis inhibitor CHA (cyclohexylamine), germination was impaired. Decrease of Spd and Spm levels in CHA-treated seed embryos mainly because CHA significantly decreased *ZmSPDS* gene expression (Huang et al., 2017). Spd significantly increased GA levels by increasing the expression of GA biosynthesis genes and on the other hand decreased ABA and ethylene in Spd treated seeds. H_2O_2 accumulation was observed in Spd-treated seeds whereas CHA decreased

H_2O_2 production. H_2O_2 accumulation was linked to increased PAO activity in Spd-treated seeds as H_2O_2 is produced during polyamine oxidation. CHA also compromised the membrane integrity of the seeds indicating the role of Spd in membrane activity (Huang et al., 2017). Spd alleviated osmotic stress caused by alkalinity of soil in wild rye (*L. chinensis* Trin. Tzvel.) (Hongna et al., 2021). This could be due to various mechanisms: accumulation of proline and soluble sugar which maintain osmotic potential in plants, enhanced expression of *SOD* gene was associated with low levels of superoxide ions and low MDA content and increased H_2O_2 production could be due to the increase redox processes and cellular respiration in germinating seeds as seen in transcriptomic profile (Hongna et al., 2021). Spd-treated aged sorghum seeds (*Sorghum bicolor* L.) showed an improvement in the germination performance as well as the root and shoot length. Further analyses highlighted that Spd-mediated priming increased the activity of enzymes involved in glycolysis, tricarboxylic acid cycle, and pentose phosphate pathway. Spermidine also protected the mitochondrial structure of the aged seeds, leading to increased respiratory activity and ATP levels during germination (Zhang et al., 2022). In silver maple (*Acer saccharinum* L.), characterized by desiccation-sensitive seeds, Spd-mediated priming successfully increased germination capacity of seeds subjected to mild or severe desiccation. On the other hand, spermidine had no effect on silver maple seeds that were aged for 6 months in storage (Fuchs et al., 2023).

1.5.4 Flavonoids

Flavonoids are low molecular weight polyphenolic compounds that play important biological functions in plants and critical in human diet. The number of flavonoids that have been discovered is approximately 8000. Flavonoids are divided into seven subgroups and they are flavonols, flavones, isoflavanoids, flavanones, isoflavones, catechins, and anthocyanidins (Nakabayashi et al., 2013; Singh et al., 2021). Flavonoids are secondary metabolites that are produced in specific locations in plant cells and regulate several physiological processes, including spore and seed germination, the formation of floral scent and color, seedling growth, and the attraction of pollinators to pollen for dispersal (Panche et al., 2016). Flavonoids have antioxidant properties and thus they are involved in ROS scavenging under biotic and abiotic stresses. Flavonoids limit the metabolic activity of enzymes involved in pathways that generate ROS, therefore promoting the antioxidant defense mechanism. The structural diversity of flavonoids allows them to simultaneously interact with an array of biomolecules (Baskar et al., 2018).

1.5.4.1 Quercetin and Rutin

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) is a polyphenolic flavonoid that can be found in all plants, including vegetables and fruits for human consumption (Nkpaa et al., 2019). A transcriptional analysis was conducted on tobacco (*Nicotiana tabacum* L.) seedlings treated with quercetin. The analysis revealed that quercetin regulates the activity of antioxidant enzymes, including glutathione reductase (GR), GPX, glutathione-S-transferase

(GST), APX, SOD, and POX enzymes (Mahajan and Yadav, 2012). Quercetin is known for its antimicrobial activity. It inhibits the synthesis of nucleic acids by repressing enzymes like DNA gyrase which is necessary for DNA replication by binding to the beta subunit of gyrase enzyme (Plaper et al., 2003; Górniak et al., 2018). Quercetin is essential for activating several plant physiological processes, including seed germination, growth, photosynthesis, and yield in both favorable and stress conditions. This compound is important for maintaining redox balance and reduce lipid peroxidation in seeds making it important in the context of stress response (Singh et al., 2021). Alleles of flavonoid biosynthesis genes and differential accumulation of flavonoids have been reported in rice as contributors to seed longevity. Metabolite profiling was conducted on seeds of the rice accessions WAS170 (short-lived) and IR65483 (long-lived) stored at 10.9% MC and 45 °C and tested on 0 and 20 days of storage. Quercetin-3-arabinoside was not detected in short lived accession after storage (Lee and Hay, 2020). *Apocynum pictum* and *Apocynum venetum* seeds subjected to osmotic stress (400 mmol L^{-1} mannitol) and supplemented with 5 µmol L^{-1} quercetin showed improved germination and seedling development under osmotic stress. It also decreased ROS production, cell membrane damage and increased the expression profiles of antioxidant genes (Yang et al., 2021). Quercetin primed *Trigonella corniculata* L. seeds resulted in enhanced growth and physiochemical properties of seedlings grown in both normal and Cr-contaminated soil. The increase in intercellular CO_2 concentration due to quercetin led to an increase in stomatal conductance, transpiration rate, photosynthetic rate, and the activity of antioxidative enzymes was also enhanced (Aslam et al., 2022).

Rutin (3′,4′,5,7-tetrahydroxy-flavone-3-rutinoside) is part of the large family of flavonoids, is more specifically classified as a flavonol, and it is widely distributed in fruits, vegetables and other plant food sources. The strong antioxidant properties of this molecule have been largely documented (Gullón et al., 2017). Rutin consist of aglycone quercetin and the disaccharide rutinose and is synthesized by 3-*0*-glycosylation of quercetin and also by rhamnosylation of isoquercitrin. In various plant species, glycosylation of flavonoids is catalyzed by 3-o-glycosyltransferases and rhamnosylation is catalyzed by rhamnosyltransferase (Suzuki et al., 2015). The effect of rutin (50 µg mL^{-1}) was investigated on rice seeds. Rutin treated seedling had better growth compared to untreated seedlings. Microscopic analysis confirmed that the exogenous application of rutin led to a decrease in the overall levels of free radicals such as H_2O_2 and O^{2-} (Singh et al., 2017). Cui et al. (2019) investigated the changes of phenolic compounds in artificially aged soybean seeds and gene expression profiles of key enzymes of phenolic metabolism. Rutin levels were decreased in aged soybean seeds suggesting a role in seed longevity. Rutin has been reported to have prominent role in biotic stress response in plants. The role of rutin (2 mM) in the induction of resistance was tested in rice, tobacco and Arabidopsis. Rutin enhanced resistance against *Xanthomonas oryzae* pv. oryzae, *Ralstonia solanacearum*, and *Pseudomonas syringae* pv. tomato strain DC3000 in all the tested species. SA-dependent pathways were modulated by rutin (Yang et al., 2016). Tomato seeds were primed with rutin (1, 2, 5, 10, and 20 mM) to test its role in resistance against whitefly (*Bemisia tabaci*). Rutin had no impact on shoot

growth but rutin priming reduced the developmental rate of nymphs and feeding efficiency of adult females on plants grown from primed seeds. Callose deposition and expression of jasmonic acid-responsive genes in tomato plants grown from rutin-treated seeds significantly increased upon *B. tabaci* infestation (Tang et al., 2023).

1.6 Model organisms for studying seed quality, priming, and longevity

1.6.1 The model legume *Medicago truncatula*

Medicago truncatula (**Figure 1.12**) belongs to the Fabaceae family, which is the third biggest family of plants and consists of around 20.000 species. The Fabaceae family include economically important plants, including soybean, pea, and alfalfa. Due to its phylogenetic relationship and genetic similarities with other legume species, it is considered an appropriate model system for molecular genetics. *M. truncatula* is characterized by having a compact, diploid, fully sequenced genome. Additional significant characteristics of *M. truncatula* include autogamy, a brief generation time of around three months, and the ability to regenerate *in vitro*. These qualities collectively provide practical benefits for cultivating *M. truncatula* in both field/greenhouse and *in vitro* settings (Frugoli and Harris, 2001; Rose, 2008). *M. truncatula* was introduced as one of the model organisms by Barker et al. (1990). *M. truncatula* seeds represent a successful model system for studying DDR during imbibition, under normal and stressful conditions. Indeed, various molecular aspects of DDR have been evaluated in *M. truncatula* seeds (Balestrazzi et al., 2011; Macovei et al., 2011). Furthermore, this particular species, characterized by orthodox seeds, has been utilized in elucidating factors related to desiccation tolerance and an experimental system has been established to study overpriming, a drawback of seed priming (Pagano et al., 2022a, 2022b).

Figure 1.12 *Medicago truncatula* seeds (up left corner), fruits (up right corner), flower (bottom left corner) and plant leaves (bottom right corner). https://legumeinfo.org/organism/Medicago/truncatula

1.6.2 The model woody plant: poplar

The genus *Populus* spp. includes over 30 species, among which are poplars, aspens, and cottonwoods (Eckenwalder, 1996). The rapid growth of poplars makes them appealing options for the production of environmentally friendly biomass. Poplars also have the potential to grow in poor soils and reduce soil erosion due to the intricate root networks they possess (Pellegrino et al., 2011; Cantamessa et al., 2022). Not only is poplar advantageous from an ecological and economic standpoint, but it is also useful as a model for basic research in woody plants (Shi et al., 2023). *Populus alba*, more commonly referred to as white poplar, is native to the central and southern regions of Europe. Prevention of soil erosion and the generation of biomass are the primary applications of white poplars. A large number of clones have been produced as there is a need to acclimate the species to harsh climatic conditions and to meet the requirements of the demanding industry. One of the most relevant clones, *Populus alba* var "Villafranca", was selected by the Forestry and Wood Research Centre of Casale Monferrato (Italy) in 1957. This clone produces high-quality wood with a basal density of 0.33 g/cm^3 (Cadullo et al., 2016; Corona et al., 2016). Due to its economic importance, monocultures of poplar are very common. There is a lack of biodiversity in monocultures, which poses serious threats in the context of climate change. This is because the plantation is susceptible to changes in yield, as a result of extreme weather conditions due to climate change (Altieri et al., 2015). Knowledge of the conditions that are necessary for optimal germination and seedling establishment is essential for the restoration of ecological systems and biodiversity preservation (Dalziell et al., 2022). Due to the ease with which poplars can be vegetatively propagated, there has been less research on seed germination in this species. As a result, studies focusing on seed germination are scanty. Furthermore, because of the intermediate nature of poplar seeds, they have a relatively shorter lifespan, ranging from a few days to a few weeks, which significantly decreases the likelihood of surviving in the natural environment. One possible explanation for the increased emphasis placed on vegetative propagation could be the poor seed viability. There has been a substantial body of research performed on the physiological, molecular, and morphological aspects of germination and post-germination processes model organisms and significant crop species whereas there is still a major gap of knowledge in the case of woody species (Qu et al., 2020).

Chapter 2: Aim of the research

Given the impact of climate change and the rapid demographic growth, it is essential to give emphasis to strategies that enhance agricultural production and promote the implementation of sustainable farming practices. High quality seeds are very crucial for establishing sustainable agriculture. To enhance seed quality and gain a better understanding of the related intricate morphological, physiological, and molecular characteristics, specialized approaches are needed due to the diverse nature of seed physiological responses. Seed priming is a well-established seed quality improvement technique where seeds are subjected to controlled imbibition with various priming agents. Priming offers several advantages in terms of germination, seedling establishment, and response to abiotic stress. Despite the various advantages of priming, one of the major drawbacks is the decreased longevity of primed seeds. The decline in storability of primed seeds is a complex process. In order to acquire knowledge regarding the mechanisms that underlie the loss of longevity in primed seeds, it is essential to understand the relationship between priming and aging in storage. There is a well-established body of literature that emphasizes on the relationship between seed longevity and aging. However, the mitigation strategies for protecting primed seeds from deterioration during storage are scanty. It is crucial to examine the molecular and physiological dynamics that contribute to the reduction in longevity of primed seeds. This will enable us to develop mitigation strategies that will protect primed seeds from deterioration in storage.

Based on this premise, the main goal of this work is to investigate the molecular and cellular mechanisms that contribute to the deterioration of primed seeds during storage. This will be accomplished by establishing an experimental system that can assess the ability of the primed seeds to tolerate the effects of aging. Such dedicated experimental system will be developed in the model legume *M. truncatula*. This system will be designed to explore the molecular dynamics of the pre-germinative metabolism challenged with oxidative stress and genotoxic injury in primed seeds subjected to accelerated aging that mimics storage, as well as to screen candidate molecules/treatments that could possibly protect the primed seeds from deterioration. By assessing the seed response to treatments, using molecular and cellular tools, it will be possible to understand the molecular mechanisms of the most promising treatment. This will aid in designing protocols that could improve primed seeds longevity in storage.

The established experimental system allowed to assess the beneficial impact of quercetin, rutin, and spermidine, that were able to protect primed *M. truncatula* seeds against deterioration. The seed response to these compounds was extensively investigated. In case of quercetin and rutin, polyphenols with strong antioxidant activity, attention was focused on the analysis of ROS accumulation and antioxidant mechanisms in *M. truncatula* seeds subjected to accelerated aging. Spermidine was also able to rescue seed viability after storage in the same experimental system. However, the increase in germination percentage

correlated with enhanced frequency of aberrant seedlings, suggesting that the beneficial effect of spermidine was lost during seedling establishment. This led us to explore the effect of aging on seedling establishment which has been poorly explored in the context of longevity. It has been reported that seeds are more resistant to DNA damage than seedlings, due to a highly effective DNA damage response (DDR) machinery. To what extent does the accumulation of DNA damage in seeds impair seedling establishment is a major gap of knowledge. Efficient DNA repair requires the inhibition of cell cycle progression, which is essential for plant growth and development. In order to explore the underlying mechanisms behind the gain and loss of spermidine-mediated protective effects during germination and seedling development, the following approaches were selected: Comet assay, DDR/cell cycle gene expression profiles, and non-targeted metabolomics. Non-targeted metabolomics was performed in collaboration with Prof. Fabrizio Araniti, University of Milan (Italy). The output of this investigation has provided significant insights into spermidine-related dynamic changes at different developmental stages (seed germination *vs* seedling development) in terms of DDR and cell cycle regulation, and the consequent impact on seed longevity.

Another significant challenge is the storage of intermediate/ recalcitrant seeds, which are highly vulnerable to desiccation. This holds significant importance within the field of germplasm and biodiversity preservation. *Populus alba* is a model woody plant with seeds classified as intermediate. Poplars have a significant impact on both the economy and the environment. The clone *Populus alba* var "Villafranca" was selected by the Forestry and Wood Research Centre of Casale Monferrato (Italy) in 1957. Due to the intermediate nature of poplar seeds, their lifespan and longevity are quite short. The objective of this work was to develop seed priming strategies that would enhance the germination performance of long-term stored *P. alba* clone Villafranca seeds provided by Dr. Pier Mario Chiarabaglio, Forestry and Wood Research Centre of Casale Monferrato (Italy). To do this, seed viability and the dynamics of rehydration-dehydration cycles were first monitored and spermidine was used as priming agent. To assess the efficiency of the treatments and understand the dynamic changes during imbibition and dehydration, germination parameters, ROS analysis and gene expression profiles related to desiccation tolerance acquisition, polyamine biosynthesis, antioxidants and cell cycle regulation genes analysis were investigated.

Chapter 3. Quercetin and rutin enhance longevity of primed seeds by enhancing the antioxidant response

This article was published on 9th May 2024, on the journal *Agriculture MDPI* with the title "Quercetin and Rutin as Tools to Enhance Antioxidant Profiles and Post-Priming Seed Storability in *Medicago truncatula*"

My contribution to this article includes *i)* seed germination and phenotypic analyses of unaged and aged *M. truncatula* seeds subjected to hydropriming, quercetin- and rutin-mediated priming, *ii)* TTC viability assay, *iii)* DCFH-DA and DPPH assays performed to assess ROS profiles and seed antioxidant potential, respectively, *iv)* Folin-Ciocalteu assay conducted to assess the seed phenolic content. I also performed statistical analysis of the resulting data and actively participated in their critical assessment and scientific discussion.

Abstract

Seed priming is commonly used to enhance germination and seedling establishment. However, the reduced longevity of primed seeds is a significant disadvantage that compromises their long-term storability. Developing optimized priming techniques able to protect primed seeds from aging is an effective way to broaden the application of seed priming. This study investigates the potential of quercetin- and rutin-supplemented seed priming to enhance the response to artificial aging in the model legume *M. truncatula*. The addition of quercetin or rutin was effective in reducing the negative effects of post-priming aging, compared to hydropriming, leading to improved seed germination and viability. These positive outcomes were consistently associated with a decrease in ROS levels and an increase in antioxidant capacity, demonstrating that quercetin and rutin have the ability to mitigate the impacts of post-priming aging by enhancing the seed antioxidant profiles. The present study offers new insights into the physiological changes that occur during seed priming and aging, and the potential impact of these compounds on the development of customized priming protocols that overcome storage limitations caused by post-priming aging.

3.1 Introduction

Seed priming refers to a variety of well-established methods used to improve the germination, seedling growth, and ability to withstand stress in plants (Paparella et al., 2015; Macovei et al., 2016; Farooq et al., 2019; Srivastava et al., 2021; Pagano et al., 2023). Seed priming can be performed in a basic way by using water without any additional substances (hydropriming). However, more advanced methods involve using chemical compounds, osmotic agents, physical treatments, phytohormones, beneficial microorganisms, and other substances to induce specific seed responses. These methods offer a range of benefits, such

as increased stress tolerance and memory (Balmer et al., 2015; Marthandan et al., 2020; Srivastava et al., 2021; Pagano et al., 2023). Although seed priming is practical and versatile, it has several drawbacks. These include the requirement for empirical optimization for different species, cultivars, and seed lots, the gradual decrease in desiccation tolerance as the seed moves towards germination, and the increased vulnerability of primed seeds in storage. The decreased longevity of seeds leads to a decrease in their ability to be stored, as they have lower germination rates and impaired seedling establishment. This limits the effectiveness of seed priming in current agricultural practices, reduces the marketability of primed seeds, and hinders the use of seed conservation strategies. This phenomenon has been recorded in various plant species, such as tomato (*Solanum lycopersicum*) (Argerich et al., 1989), lettuce (*Lactuca sativa*) (Tarquis and Bradford, 1992), corn (*Zea mays*) (Chiu et al., 2002), wheat (*Triticum aestivum*) (Abnavi and Ghobadi, 2012), rice (*Oryza sativa*) (Hussain et al., 2015; Wang et al., 2018), Chinese cabbage (*Brassica rapa* subsp. Pekinensis) (Yan, 2017), and *Arabidopsis thaliana* (Sano and Seo, 2020). These studies attributed the higher vulnerability to aging after priming to various factors associated with the more advanced physiological condition of primed seeds. These factors gradually expose cellular structures to oxidative damage as seeds transition from their dormant dry state to the metabolic pre-activation caused by priming (Varier et al., 2010).

In order to understand the storability of primed seeds, it is necessary to examine the relationship between priming and aging. The scientific literature on seed aging and longevity is extensive and well-established. It covers several aspects such as molecular and physiological changes, ecological and agricultural impacts and measures to mitigate the effects of aging (Waterworth et al., 2019; Zhou et al., 2019; Nadarajan et al., 2023; Pirredda et al., 2023; Waterworth et al., 2024). Seeds gradually degrade over time when stored for a long period, and their ability to be stored successfully depends on the specific species, ecotypes, and storage techniques that are most suited for orthodox and recalcitrant seeds (Donà et al., 2013; Chhabra et al., 2019). Storability variations result from a combination of genetic, biochemical, and physiological factors, even when storage conditions are optimal. However, high temperatures and relative humidity are external factors that significantly contribute to seed deterioration during storage. These factors affect moisture content, oxidative processes, and the stability of macromolecules. ROS accumulation is a significant factor in the aging process, leading to the peroxidation of membrane lipids, loss of protein function, and DNA damage, ultimately decreasing seed viability (Kurek et al., 2019; Zinsmeister et al., 2020; Zhang et al., 2021). For research purposes, the development of artificial ageing protocols has enabled us to replicate the effects of natural aging in a shorter time period and under more controlled experimental conditions. These conditions often involve elevated temperatures and humidity. The utilization of cross-validation across various natural and artificial aging methods has facilitated the identification of shared mechanisms and the integration of longevity testing into the assessment of seed quality (Rajjou et al., 2008; Schwember and Bradford, 2010; Fantazzini et al., 2018). The investigation of methods to reduce

the effects of priming-associated aging involves the use of specific experimental setups and has led to the development of focused approaches to increase longevity after priming. Examples of these targeted solutions include the use of vacuum packaging for bitter gourd (*Momordica charantia*) seeds (Yeh et al., 2005), priming with KNO_3 for hot pepper (*Capsicum frutescens*) seeds (Tu et al., 2022), heat treatment for tomato seeds (Gurusinghe et al., 2002), and spermidine priming for rice seeds (Xu et al., 2020). However, there is scanty knowledge concerning the relationship between seed priming and aging, particularly in terms of developing treatments to enhance storage quality after priming. Considering the significant impact of oxidative damage on seed aging, antioxidant chemicals are the primary choice in addressing this issue. Flavonoids are abundant, with approximately 8.000 compounds identified in plants. They fall into six major classes, found throughout the plant kingdom. Flavonoids have various tissue localizations and biological functions, including antioxidant properties and protection against different biotic and abiotic stressors (Singh et al., 2021; Tang et al., 2023). Quercetin is a polyphenolic flavonoid that is commonly present in vegetables and fruits. Quercetin is primarily found in a glycoside form known as 3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside, or quercetin-3-rutinoside, rutoside, sophorin, phytomelin, rutin, and other names. It is a flavonol compound that can be found in *Passiflora* spp., buckwheat (*Fagopyrum esculentum*) seeds, citrus (*Citrus* spp.) fruits, vegetables, and tea (Nkpaa et al., 2019; Huang et al., 2020; Ożarowski & Karpiński, 2021). The role of naturally occurring flavonoids in the longevity and storability of seeds is well-documented. This includes their antioxidant effects, which are linked to their presence in the seed coat and embryo (Rajjou & Debeaujon, 2008). Genes involved in flavonoid biosynthesis and their alleles have been identified as factors that affect seed longevity in rice and soybean (Cui et al., 2019; Lee & Hay, 2020). Studies have shown that quercetin, rutin, and other flavonoids can provide protection when applied externally to seeds, seedlings, or adult plants. This can be done through direct application or priming treatments. In various experimental setups, these flavonoids have been found to decrease ROS accumulation and prevent damage to cell membranes (Singh et al., 2017; Singh et al., 2021; Yang et al., 2021; Tang et al., 2023).

In line with the cited research, this study explores the potential of quercetin and rutin to counteract the negative impact of inadequate or extended storage on primed seeds. This concept is investigated in the model legume *Medicago truncatula* by subjecting quercetin- and rutin-primed seeds to artificial aging, using also unprimed and hydroprimed control treatments. Biometric evaluation, that included germination rates, seed viability, and seedling morphology, was combined with the analysis of oxidative stress indicator (ROS profiles, antioxidant potential, and phenolic compounds content). This allowed to gather evidence on how quercetin and rutin can enhance the seed antioxidant response and improve post-priming longevity. The current work utilized *M. truncatula* as a model legume, building upon prior research that examined the impacts and limitations of seed priming in this species (Pagano et al., 2022a, 2022b). The findings of this work provide a new foundation for examining and alleviating the effects of post-

priming aging in legumes. The reported treatments own the potential to improve seed priming techniques that can be used alongside seed storage methods.

3.2 Materials and Methods

Plant Material, Treatments, and Germination Tests

Medicago truncatula Gaertn. seeds (commercial genotype, kindly provided by Continental Semences S.p.A., Traversetolo, Parma, Italy) were treated with four priming conditions: unprimed control condition (UP), hydropriming (HP), quercetin-supplemented priming (QP), and rutin-supplemented priming (RP). Each priming condition was followed by two accelerated aging conditions: unaged control conditions (UA) or accelerated aging (AA). In all the priming conditions the seeds were imbibed for 4 h in sealed Petri dishes (diameter 90 mm) containing a layer of filter paper with 2 mL of water (HP), 2 mM quercetin (QP) or 1 mM rutin (RP). Quercetin and rutin concentrations were selected based on preliminary screening in the ranges of 0.05 to 2 mM, compatible with the ranges reported for other species (Yang et al., 2016; Tang et al.,2023). Priming was followed by dry-back, which was carried out by distributing the seeds into open Petri dishes and incubating them for 4 h. Priming protocols and dry-back were carried out at 25 °C. Artificial aging was performed using a Memmert Universal Oven U55 (Memmert, Schwabach, Germany), exposing the seeds to 45 °C and 95% relative humidity for 24 h, adapting the protocol by Colombo et al. (2023). Relative humidity was calculated using a TA298 Digital thermo-hygrometer (JZK, Shenzhen JinZhiKu Electronic Co., Ltd., Shenzhen, China). The effective temperature for accelerated aging, avoiding complete loss of seed viability, was selected based on preliminary screening in the temperature range of 40 to 60 °C. Germination tests were carried out in sealed Petri dishes (diameter 90 mm) containing a layer of filter paper moistened with 2 mL of distilled water. For the duration of the germination tests, Petri dishes were kept in in a growth chamber at 25 °C under light conditions, with a photon flux density of 150 µmol m^{-2} s^{-1}, and a photoperiod of 16 h. For each experimental condition, five independent replications (Petri dishes), each containing 20 seeds, were monitored every 2 h for 4 days, sufficient for all the treatments to reach germination plateau. Seeds displaying a protrusion of the primary radicle were considered germinated. Germination parameters were calculated according to Ranal and Garcia de Santana (2006). Seedling morphology was assessed at the end of the germination test and aberrant seedlings were distinguished from normal seedlings by an impaired growth, especially visible at the level of the radicle (Pagano et al., 2022a). An overview of the experimental system is provided in **Figure 3.1**.

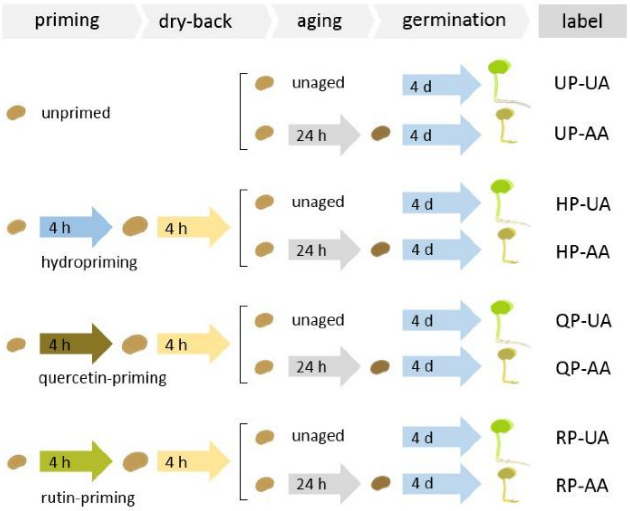

Figure 3.1. Overview of the experimental system designed to compare the effects of accelerated aging on *Medicago truncatula* seeds after hydropriming, quercetin-priming or rutin-priming. UP, unprimed control conditions; HP, hydropriming; QP, quercetin priming; RP, rutin priming; UA, unaged control conditions; AA, artificial aging.

Viability Assay Using 2,3,5-Triphenyl Tetrazolium Chloride

The 2,3,5-triphenyl tetrazolium chloride (TTC) assay was carried out on the four priming conditions before and after accelerated aging in order to provide further indication of seed viability together with direct germinability assessment and to provide tissue-specific evidence of viability loss in *M. truncatula* seeds. The positivity to TTC staining is an established technique in seed viability testing (De Barros França-Neto and Krzyzanowski, 2019). Specifically, TTC is a white compound that is converted by dehydrogenases into TPF (1,3,5-triphenylformazan), a red and stable compound only in metabolically active tissues. Seeds were imbibed in distilled water for 1 h to allow the removal of seed coat and an easier visualization of the tissue staining. De-coated seeds were subsequently incubated in a 1% (*w*/*v*) solution of TTC (Merck, Darmstadt, Germany), at 20 °C for 18 h in the dark. For each experimental condition, 50 seeds were screened and classified as viable or dead/aberrant according to their staining pattern. Particularly, seeds whose embryo axis was positive to TTC staining were classified as viable and results were expressed as percentage of viable seeds on the total of screened seeds.

Assessment of ROS Levels by 2′,7′-Dichlorofluorescin Diacetate (DCFH-DA) Assay

ROS levels were assessed for the four priming conditions before and after accelerated aging. The assay was based on the fluorogenic dye 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich, Milan, Italy). The DCFH-DA molecule penetrates the cell membrane and is deacetylated by cellular esterases. Subsequently, it is oxidized by ROS into the fluorescent compound

2′,7′-dichlorofluorescein (DCF), whose fluorescence is spectroscopically detected at excitation and emission spectra of 495 nm and 529 nm, respectively. The assay was carried out as described by Pagano et al. (2022a). Seeds (5 replicates per condition, 3 seeds per replicate) were incubated in dark conditions for 1 h in 50 µL of 10 µM DCF-DA. Subsequently, the solution was mixed by pipetting and a 20 µL aliquot was transferred to new tubes. A tube containing only DCFH-DA not exposed to seed samples was used as a blank control. Fluorescent emission was measured at 517 nm using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia), setting the software for one cycle of 30 s at 25 °C. Relative fluorescence was obtained by subtracting the fluorescence detected from the blank and expressing the results as relative fluorescence units (RFU).

Assessment of the Antioxidant Potential by DPPH (1,1-Diphenyl-2-picrylhydrazyl) Assay

The seed extracts required for DPPH and Folin–Ciocalteu assay were prepared as follows. Samples (for each condition, 200 mg pooling ~100 seeds, five replicates) were homogenized with mortar and pestle to a fine powder in presence of 2 mL 80% acetone. The extracts suspended in 80% acetone were transferred to 2 mL tubes and further 80% acetone was added to standardize the extraction volumes to 2 mL. The extracts were incubated overnight at 25 °C in the dark under gentle shaking and subsequently stored at −20 °C until use. The ROS-scavenging activity (antioxidant potential) of the samples was determined by DPPH test, that relies on the reactivity of the DPPH radical with the antioxidant compounds contained in the extracts (Braca et al., 2001). A standard curve was obtained from serial dilutions (50-400 mg L^{-1}) of ascorbic acid (Sigma-Aldrich). Aliquots (0.1 mL) of the sample extracts or points of the standard curve were added to 1.5 mL of a solution of 0.1 mM DPPH (Sigma-Aldrich) dissolved in methanol. The reaction was incubated for 30 min at room temperature in the dark. A blank solution was prepared dissolving 0.1 mL 80% acetone into 1.9 mL methanol and used as a background for absorbance measurements at λ = 517 nm. The reduction in absorbance as a consequence of DPPH radical scavenging by antioxidant compounds was measured with a Biochrom WPA Biowave spectrophotometer (Biochrom Ltd., Cambridge, UK). The antioxidant potential of the extracts was calculated according to the standard curve and expressed as ascorbic acid equivalents (AAE) mg^{-1} fresh weight.

Assessment of the Content in Phenolic Compounds by Folin–Ciocalteu Assays

The content in total phenolic compounds was measured as described by Spanos and Wrolstad (1990) from the same extracts used for DPPH assay, using the Folin-Ciocalteu reagent and a standard curve obtained from serial dilutions (50–400 mg L^{-1}) of gallic acid (Sigma-Aldrich). Aliquots (20 µL) of the sample extracts or points of the standard curve were added to 1.58 mL distilled water and with 100 µL of the Folin-Ciocalteu reagent (Sigma-Aldrich). After 8 min incubation in

the dark, the reactions were neutralized with 300 µL of 7.5% (*w*/*v*) Na_2CO_3 (Sigma-Aldrich) and incubated for 120 min at 25 °C in the dark. The resulting increase in absorbance was measured at λ = 765 nm with a Biochrom WPA Biowave spectrophotometer (Biochrom Ltd., Cambridge, UK) from the background of a blank solution of 1.58 mL distilled water mixed with 300 µL of 7.5% (*w*/*v*) Na_2CO_3. The content in total phenolic compounds of the extracts was calculated according to the standard curve and expressed as gallic acid equivalents (GAE) mg^{-1} fresh weight. Five replicates were used for each condition. Using the data from DPPH and Folin-Ciocalteu assays, the specific antioxidant activity (SAA) was calculated as the ratio between the antioxidant potential and the total content in phenolic compounds, and expressed as µg AAE $µg^{-1}$ GAE, as reported (Pagano et al., 2017; Rakariyatham et al., 2019).

Statistical Analyses

Five replicates were used for each level of analysis. Data concerning germination performance (germination parameters), seedling phenotype, ROS accumulation, content in antioxidant and phenolic compounds, specific antioxidant activity, and seed viability were analyzed through two-way analysis of variance (ANOVA) and the Duncan's test, using the software Rapid Publication-Ready MS Word Tables Using Two-Way ANOVA 1.0 (Assaad et al., 2015), available online (https://houssein-assaad.shinyapps.io/TwoWayANOVA/). The comparison groups for two-way ANOVA were priming groups (UP, HP, QP, RP) and aging groups (UA, AA) with a *p*-value < 0.05 as the threshold for significance. Pearson's correlation and Principal Component Analysis (PCA) were carried out using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/docs/Publications.xhtml) (Ewald et al., 2024), normalizing the values by Z-score (mean-centered and divided by the standard deviation of each variable) and considering a *p*-value < 0.05 as the threshold for significance for correlation analyses.

3.3 Results

Germination Performance

The germination of *M. truncatula* seeds was evaluated after being subjected to hydropriming, quercetin-priming, or rutin-priming, followed by accelerated aging. The assessment focused on the germination percentage at the end of the germination test (germinability) and the speed of germination (lower T_{50}, the time required to reach 50% of the final germination). **Figure 3.2** shows that the germinability profiles of unaged seeds were consistently above 90%, and there were no significant changes caused by priming. Germinability after aging declined to less than 60% for all priming treatments, with the most pronounced decrease reported in hydroprimed seeds (30 ± 7.07%). In contrast, seeds that underwent priming with quercetin (54 ± 10.8%) and rutin (40 ± 11.72%) had considerably higher germinability after aging compared to hydroprimed seeds. These results were consistent with the germinability of unprimed seeds after aging. Hydropriming, quercetin-supplemented priming, and rutin-supplemented

priming dramatically enhanced the germination speed of unaged seeds, as indicated by a decreased T_{50}. Artificial aging caused a substantial delay in germination for all priming treatments and the unprimed control. The hydroprimed seeds experienced the largest delay (60.93 ± 2.08h), while there were no significant differences identified among the other priming treatments and the unprimed control. On a global scale, the germination parameters show a notable decline in both the percentage and speed of germination due to artificial aging. This decline is particularly evident in hydroprimed seeds compared to unprimed seeds. However, the negative effects of aging can be partially alleviated by supplementing the priming process with quercetin or rutin.

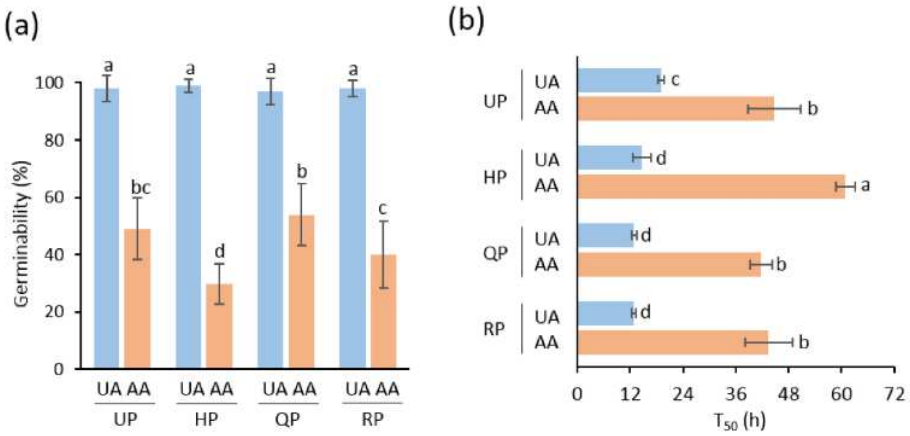

Figure 3.2. Germination performance of *M. truncatula* seeds subjected to hydropriming, quercetin- or rutin-mediated priming followed by accelerated aging. (**a**) Germinability percentage. (**b**) T_{50}. UP, unprimed control conditions; HP, hydropriming; QP, quercetin-priming; RP, rutin-priming; UA, unaged control conditions; AA, artificial aging. T_{50}; time (h) to reach 50% of final germinants. Means without a common letter are significantly (*p*-value < 0.05) different as analyzed by two-way ANOVA and Duncan test.

Seed Viability

The 2,3,5-triphenyl tetrazolium chloride (TTC) assay was carried out on the four priming conditions both before and after accelerated aging. This was done to confirm the germinability profiles and offer more evidence of the changes in seed viability when combining priming methods and artificial aging. According to **Figure 3.3a**, the viability profiles of unaged seeds, as measured by TTC-positivity, were consistently above 70% regardless of the priming protocol used, and there were no significant differences seen. Seed viability was decreased by artificial aging in both the unprimed control and all priming treatments. However, following artificial aging, the quercetin-primed seeds exhibited a higher level of viability (56 ± 11.40%) compared to the hydroprimed seeds (40 ± 13.14%). **Figure 3.3b** contains representative images of seed viability in *M. truncatula* seeds, as determined by TTC-positive/negative staining. Seeds that show positive staining on the embryo axis are differentiated from seeds that show

negative staining on the embryo axis. This characteristic is regarded significant for classifying the viability of the seeds.

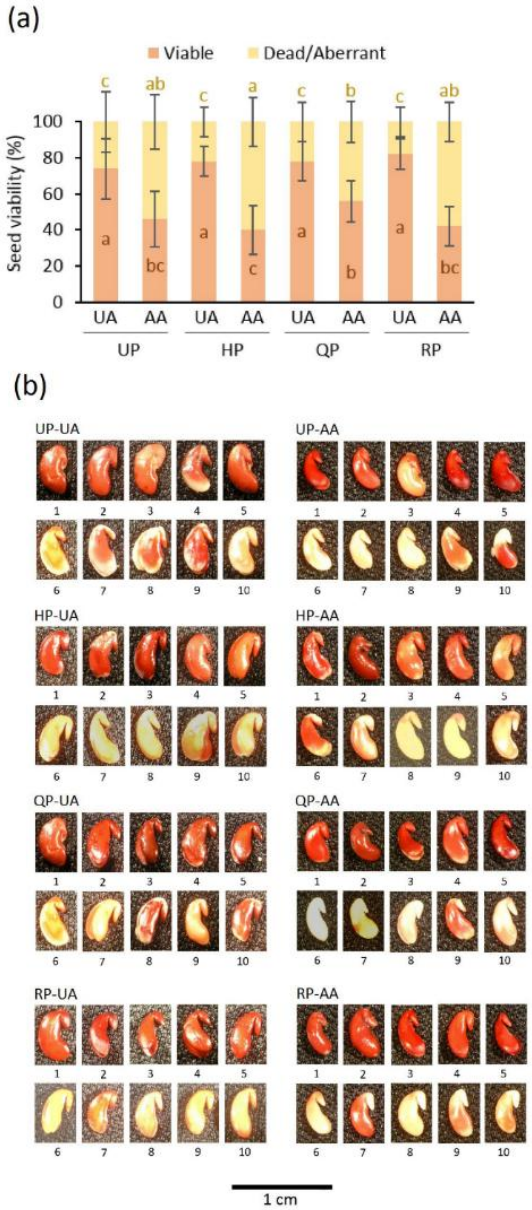

Figure 3.3 Viability of *M. truncatula* seed following hydropriming, quercetin-supplemented priming or rutin-supplemented priming combined with accelerated aging. (**a**) Seed viability percentage assessed with TTC staining. (**b**) Representative pictures of viable (row numbers 1 to 5) and non-viable (row numbers 6 to 10) seeds as assessed by TCC assay for each treatment category. UP, unprimed control conditions; HP, hydropriming; QP, quercetin-priming; RP, rutin-priming; UA, unaged control conditions; AA, artificial aging. Means without a common letter are significantly (*p*-value < 0.05) different as analyzed by two-way ANOVA and Duncan test. The letters referring to different comparison series are indicated with different colors.

Seedling Development

At the end of the germination test, the seedling morphology was evaluated by differentiating between aberrant phenotypes with impaired root growth and normal seedling development. This assessment also accounted for the fraction of seeds that did not germinate, which had already been measured using germination parameters. The data shown in **Figure 3.4a** indicates that seedlings derived from unaged seeds exhibit a similar distribution of normal (ranging from 79% to 87%) and aberrant (ranging from 7% to 15%) seedlings, with no significant variations observed across the different priming treatments. Following accelerated aging, the distribution of phenotypic classes in seedlings varied significantly according to the priming treatments. Quercetin-supplemented priming (22 ± 11.51%), and rutin-supplemented priming (19 ± 11.51%) resulted in a considerable decrease in the number of normal seedlings compared to unprimed seeds (37 ± 9.74%). Furthermore, subjecting the seeds to accelerated aging after hydropriming led to a complete absence of normal seedlings. **Figure 3.4b** contains representative images showcasing both normal and abnormal seedling morphology in *M. truncatula.*

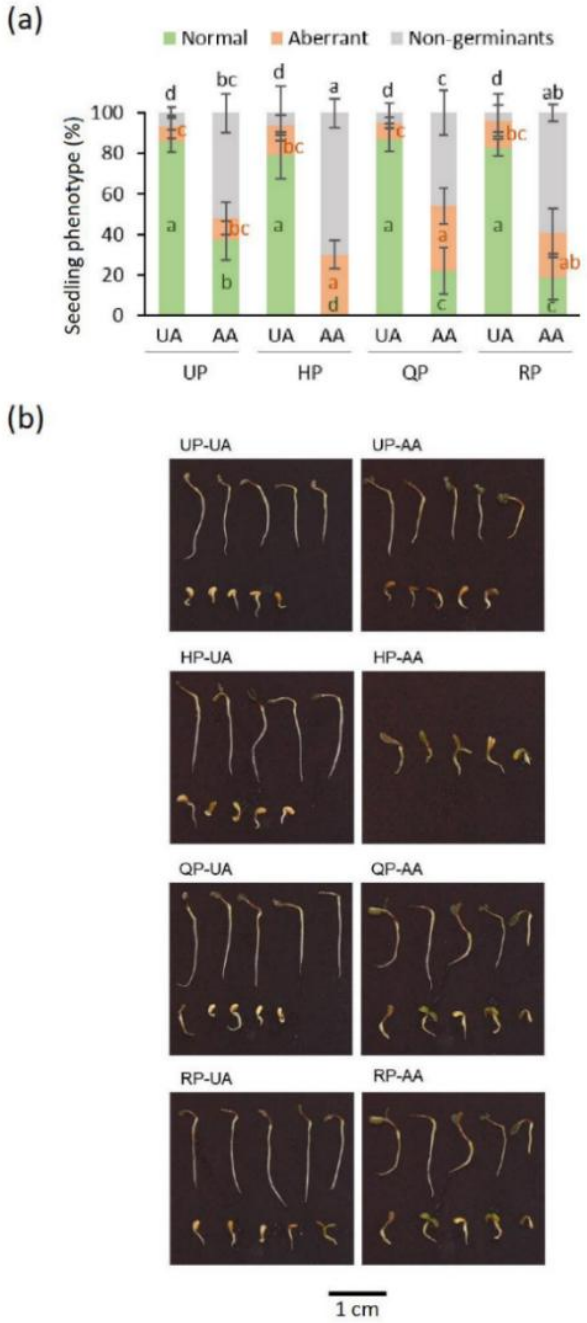

Figure 3.4. Phenotype of *M. truncatula* seedlings following hydropriming, quercetin-supplemented priming or rutin-supplemented priming combined with accelerated aging. (**a**) Seedling phenotype percentage. (**b**) Representative pictures of normal (top rows) and aberrant (bottom rows) seedling morphology for each treatment category. UP, unprimed control conditions; HP, hydropriming; QP, quercetin-priming; RP, rutin-priming; UA, unaged control conditions; AA, artificial aging. Means without a common letter are significantly (p-value < 0.05) different as analyzed by two-way ANOVA and Duncan test. The letters referring to different comparison series are indicated with different colors.

Antioxidant Parameters and ROS Accumulation

To investigate the impact of ROS and antioxidant response on seed aging and longevity, we evaluated the antioxidant potential, phenolic compound content, specific antioxidant activity, and ROS levels in *M. truncatula* seeds. These seeds were treated with hydropriming, quercetin-priming, or rutin-priming, and then subjected to accelerated aging. Based on the data presented in **Figure 3.5a**, when looking at unaged seeds, all priming techniques resulted in a decrease in antioxidant potential, as measured by the DPPH assay, compared to unprimed seeds (1.72 ± 0.06 ug AAE / mg FW). The decline was more pronounced with hydropriming (1.22 ± 0.05 ug AAE / mg FW). Following artificial aging, there was a universal decrease in antioxidant capacity observed in all primed and unprimed circumstances compared to their unaged counterparts. However, the same pattern of antioxidant capacity seen in unaged seeds was still consistent. When evaluating unaged seeds, the overall number of phenolic compounds (measured by the Folin-Ciocalteu assay) reduced as a result of hydropriming and quercetin-priming, compared to unprimed seeds. All primed and unprimed conditions showed a decrease in total phenolic compounds as a result of accelerated aging (**Figure 3.5b**). When comparing the specific antioxidant activity of unaged seeds, quercetin-primed seeds (0.87 ± 0.06 ug AAE / mg FW) showed a significantly higher specific antioxidant activity than hydroprimed seeds (0.73 ± 0.07 ug AAE / mg FW). The specific antioxidant activity is obtained by dividing the antioxidant potential by phenolic compounds content. Accelerated aging caused a significant reduction in the specific antioxidant activity of all primed seeds, but not in unprimed seeds. However, seeds that were primed with quercetin (0.44 ± 0.06 ug AAE / mg FW) and rutin (0.41 ± 0.10 ug AAE / mg FW) maintained a higher level of specific antioxidant activity compared to seeds that were hydroprimed (0.24 ± 0.07 ug AAE / mg FW), even after undergoing artificial aging (**Figure 3.5c**). The levels of ROS, measured by the DCFH-DA assay, were significantly higher in unaged seeds that underwent hydropriming (8.87 ± 4.75 RFU). compared to the other conditions that were assessed. Accelerated aging induced a significant reduction in ROS only for hydroprimed seeds (4.75 ± 1.64 RFU) compared to their unaged counterparts. After aging, quercetin primed seeds (1.84 ± 1.14 RFU) exhibited reduced levels of ROS in comparison to unprimed seeds. Similarly, rutin primed seeds (0.94 ± 0.34 RFU) revealed decreased ROS levels compared to both unprimed seeds and hydroprimed seeds.

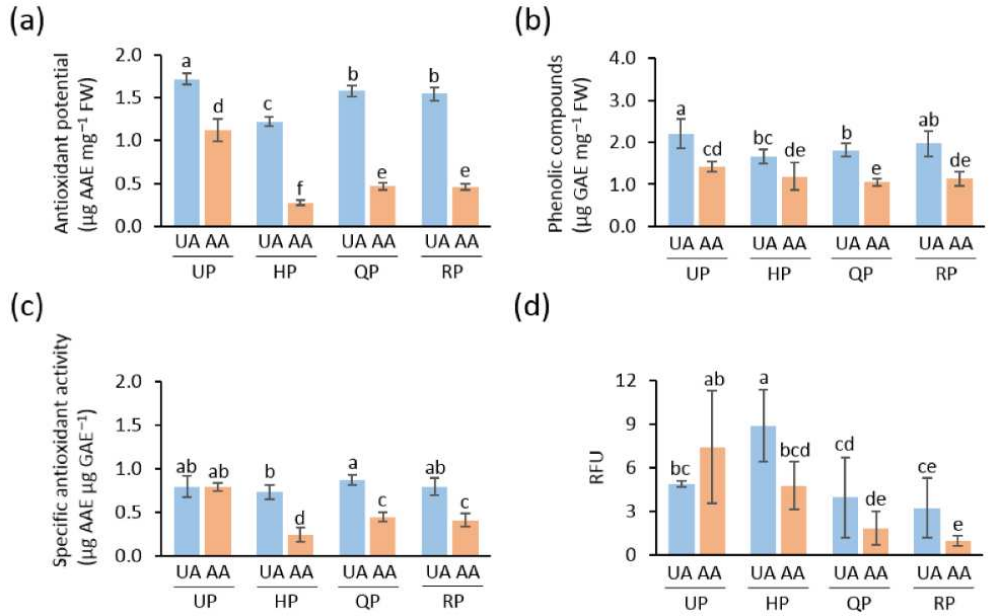

Figure 3.5. Antioxidant potential, phenolic compounds, and specific antioxidant activity of *M. truncatula* seeds subjected to hydropriming, quercetin-priming or rutin-priming followed by accelerated aging. (**a**) Antioxidant potential assessed by DPPH assay. (**b**) Content in phenolic compounds assessed by Folin–Ciocalteu assay. (**c**) Specific antioxidant activity calculated from DPPH and Folin–Ciocalteu data. (**d**) ROS detection by DCF-DA assay. UP, unprimed control conditions; HP, hydropriming; QP, quercetin priming; RP, rutin priming; UA, unaged control conditions; AA, artificial aging. Means without a common letter are significantly (p-value < 0.05) different as analyzed by two-way ANOVA and Duncan test. AAE, ascorbic acid equivalents; GAE, gallic acid equivalents; SAA, specific antioxidant activity; FW, fresh weight. RFU, relative fluorescence units.

Correlation of Germination and Seedling Growth Parameters with Antioxidant Response Indicators

Correlation analyses and principal component analysis (PCA) were conducted to gain a comprehensive understanding of the data obtained from the experimental system applying accelerated aging on unprimed, hydroprimed, quercetin-primed and rutin-primed *M. truncatula* seeds. The results are displayed in **Figure 5.6**. Pearson's correlation analysis (**Figure 5.6a**) revealed significant positive associations between antioxidant properties (including antioxidant potential, phenolic compound content, and specific antioxidant activity) and positive indicators of germination performance (germinability and peak value), seed viability (as measured by the TTC assay), and seedling establishment (percentage of seedlings with normal morphology). Furthermore, there was a significant correlation between the antioxidant potential and the quantity of phenolic compounds, indicating a synergistic interaction of various antioxidant processes. In contrast, Pearson's correlation analysis revealed significant negative correlations between antioxidant properties and negative indicators of germination speed (T_{50}, mean germination time), as well as negative indicators

of seedling establishment efficiency (percentage of abnormal and non-germinant seeds). The dataset for PCA was organized to emphasize the changes caused by the combination of priming treatments and accelerated aging (**Figure 5.6b**). The eight resulting treatment groups were treated as distinct clusters of replicates. The primary factor influencing the variations within the dataset seemed to be the impact of accelerated aging, resulting in the identification of two separate super-clusters: unaged and aged seeds. The scatter plot did not show clear grouping differences among priming groups in unaged seeds, while more distinct clusters were observed for artificially aged seeds. More precisely, the cluster associated with hydroprimed seeds was clearly separate from the cluster associated with unprimed seeds. Notably, the cluster associated with quercetin-supplemented priming partially coincided with the cluster associated with unprimed seeds. Similarly, the cluster associated with rutin-supplemented priming partially coincided with the clusters associated with unprimed controls, hydropriming, and quercetin-priming. On a global scale, when considering the parameters analyzed, Pearson's correlation analyses consistently indicated a connection between antioxidant properties, improved germination performance, and successful seedling establishment. Additionally, PCA clustering showed that the addition of quercetin and rutin could help reduce the negative effects of post-priming artificial aging, making the patterns observed for unprimed aged seeds more similar.

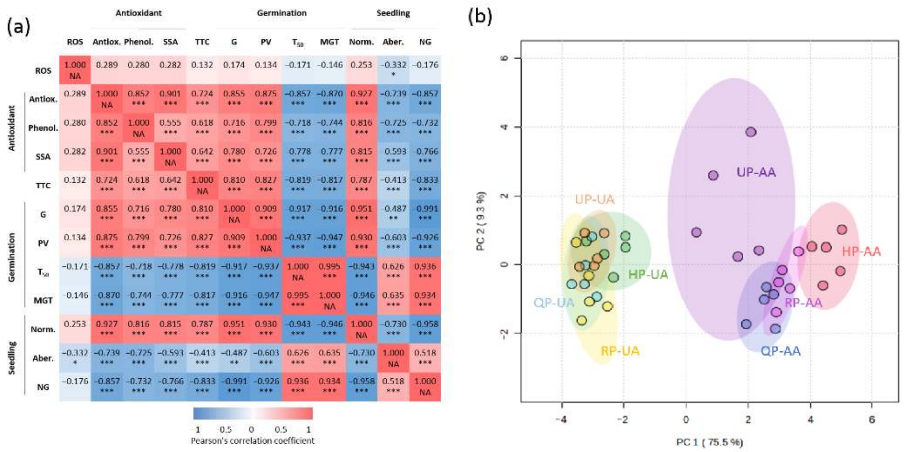

Figure 3.6. Overview of the results of Pearson's correlation analysis and principal component analysis. (**a**) Pearson's correlation analysis of the results obtained from *M. truncatula* seeds subjected to hydropriming, quercetin-priming or rutin-priming followed by accelerated aging. The correlation coefficients are indicated. The statistical significance of the Pearson's correlations is indicated by asterisks (* *p*-value < 0.05, ** *p*-value < 0.01, *** *p*-value < 0.001). NA, not applicable. ROS, reactive oxygen species as assessed by DCF-DA assay. Antiox., antioxidant potential as assessed by DPPH assay. Phenol., content in total phenolic compounds as assessed by Folin–Ciocalteu assay. SSA, specific antioxidant activity. TTC, seed viability percentage as assessed by TTC assay. G, germinability. PV, peak value. T_{50}, time required to reach 50% of final germination. MGT, mean germination time. Norm., percentage of normal seedlings. Aber., percentage of aberrant seedlings. NG, percentage of non-germinant seeds. (**b**) Two-dimensional score plot of the principal component analysis of the results obtained from *M. truncatula* seeds subjected to hydropriming, quercetin-priming or rutin-priming followed by accelerated aging. UP, unprimed control conditions; HP, hydropriming; QP, quercetin priming; RP, rutin priming; UA, unaged control conditions; AA, artificial aging; PC, principal component.

3.4 Discussion

This study focused on a frequently reported disadvantage of seed priming, which is the reduction in longevity of primed seeds (Argerich et al.,1989; Tarquis, and Bradford 1992; Chiu et al., 2002; Abnavi et al., 2012; Hussain et al., 2015; Yan et al., 2017 Wang et al., 2018; Chhabra and Singh, 2019; Sano and Seo, 2020). Seeds are regularly stored during the processing, distributing, and trading, for varying durations. Therefore, given the numerous studies that have demonstrated the advantages of seed priming and highlighted the negative effects of seed aging, it is important to investigate new methods to reduce the susceptibility of primed seeds to aging. This could expand the potential of seed priming as a means to enhance seed quality and post-harvest practices, particularly in terms of seed storage.

The choice of *M. truncatula* for this investigation was based on its established use as a model legume for seed physiology and being consistent with prior research that have examined the effects and disadvantages of seed priming (Pagano et al., 2022a, 2022b). The experimental system used in this study consisted of four priming conditions: unprimed, hydroprimed, quercetin-supplemented priming, and rutin-supplemented priming. The study combined four different priming conditions with the presence or absence of accelerated aging to assess the possible protective benefits of these two flavonoids against post-priming longevity impairment (**Figure 3.1**).

To initially characterize the experimental system, germination performance was assessed based on the percentage and speed of germination. The accelerated aging protocol used in this study involved subjecting the seeds to a temperature of 45 °C and a relative humidity of 95% for a duration of 24 h. This protocol, adapted from Colombo et al. (2023), successfully reduced the germination percentage for all priming treatments and the unprimed control, without causing complete loss of seed viability. As a result, it allowed for accurate estimation of germination and seedling growth parameters. Accelerated aging protocols have proven to be practical and accurate in simulating storability problems in various model and crop plants (Schwember et al., 2001; Rajjou et al., 2008; Fantazzini et al., 2018). It would be beneficial to further validate these protocols using natural aging, different storage methods and timeframes, and a wider range of species and cultivars. This additional validation would help confirm the findings of the current study and provide more practical applications. The findings of this study validate that primed seeds in *M. truncatula* have a shorter longevity compared to unprimed seeds. Primed seeds exhibit reduced viability, lower germinability, delayed germination, and an impaired seedling phenotype. In line with the hypothesis of this study, the addition of quercetin or rutin during seed priming reduced these effects of post-priming aging compared to hydropriming. This resulted in enhanced germination and seed viability (**Figure 3.2**). This hypothesis was formulated based on the documented benefits of administering external flavonoids to seeds, seedlings, and plants in under various biotic and abiotic stress (Yanget al., 2016; Yang et al., 2021; Tang et al., 2023), except the

specific conditions of post-priming aging that were the focus of this study. The importance of assessing seedling establishment, in addition to germination parameters, has been emphasized as a crucial factor for achieving optimal crop yields, particularly in relation to the impacts of extended storage (Rehmani et al., 2023).

The current study aimed to propose potential explanations for the observed responses to priming when combined with aging by examining indicators of the antioxidant response in seeds. ROS accumulation is a significant factor in seed aging. It is regulated by antioxidants and hormones to prevent oxidative damage. However, ROS also plays a role as signaling molecule in various seed processes such as stress response, cell wall plasticity, reservoir mobilization, hormonal modulation, dormancy release, and other physiological functions (Bailly and Kranner, 2011; Bailly, 2019). During prolonged storage, seeds remain in a dry quiescent state with low moisture levels, which restricts the oxidative processes linked to an active metabolism. This state also inhibits the ability of enzymes to repair damage and detoxify ROS (Powell and Matthews, 2012; Bailly and Kranner, 2011; Bailly, 2019; Sano and Seo 2020). ROS production is often seen as a result of a functioning metabolism. However, it has been observed that ROS can also accumulate in dry seeds that have undergone natural or artificial aging. This accumulation is linked to damage membrane lipids, hinder germination, and the activation of both enzymatic and non-enzymatic antioxidant responses (Diaz-Vivancos et al., 2013; Bailly, 2019; Choudhary et al., 2020; Zhang et al., 2021). Given the intricate nature of this phenomenon, comprehending the dynamics and consequences of ROS accumulation and elimination might provide challenges in interpretation of the results. The current study examined dry seeds, it was thought that the metabolic activities in these seeds were limited, and the oxidative state was primarily influenced by ROS non-enzymatic sources and scavenging processes. The findings of this study demonstrate that induced aging has a substantial impact on decreasing the antioxidant capability and levels of phenolic compounds (**Figure 3.5 a,b**). This decline might be attributed to a loss of antioxidant and phenolic chemicals during aging, in line with earlier reports. An experiment was conducted using oat (*Avena sativa*) to study the effects of various intensities of artificial aging. The aging process lasted from 20 to 42 days, with a temperature of 45 °C and a moisture content of 10%. Additionally, there was a decrease in the levels of various antioxidants, both enzymatic and non-enzymatic, including ascorbic acid and glutathione. This decline in antioxidant levels was accompanied by a loss in germinability of the oat seeds (Sun et al., 2022). Exposing *G. max* seeds to accelerated aging conditions (0 to 8 days, 45 °C, 100% relative humidity) resulted in a drop in the levels of several phenolic compounds, such as protocatechuic acid, morin, and rutin. Additionally, the expression of genes responsible for producing these phenolic compounds was downregulated (Cui et al., 2019). Furthermore, a decline in the antioxidant activity was observed in canola (*Brassica napus*) seeds that were stored underground for several months. This decline was accompanied by a decrease in seed vigor, a drop in soluble sugars, and hormonal changes (Wang et al., 2023). Conversely, the process of artificially aging canola seeds increased their ability to produce

enzymes and non-enzymatic antioxidants, such as phenolic chemicals, flavonoids, and flavonols (Naghisharifi et al., 2024). When *Fagus sylvatica* seeds are stored for a long time, their ability to germinate is influenced by the levels of ascorbic acid and α-tocopherol. Higher levels of these compounds are associated with better germination capacity. On the other hand, the accumulation of superoxide radical, hydrogen peroxide, and lipid hydroxyperoxides has a negative effect on germination (Pukacka and Ratajczak, 2007). These contrasting data validate the species-specific differences in the enzymatic and non-enzymatic antioxidant processes, which may be related to different strategies for dealing with oxidative stress during aging. While this study examined global antioxidant indicators, it is important to emphasize the unique importance of enzymatic antioxidant mechanisms in seed longevity and stress response. This should be considered as a potential area of focus for future research on the interaction between seed aging and seed priming. For instance, prolonged storage led to a reduction in enzymatic antioxidant activity and germination in *Trifolium* spp. (Cakmak et al., 2010), while the use of quercetin (15 to 40 μM) enhanced the enzymatic antioxidant response and the ability to withstand soil contamination in *Trigonella corniculata* (Aslam et al., 2022). Furthermore, catalase is found in the same location as hydrogen peroxide and seems to have a specific role in the rejuvenation process of *Helianthus annuus* by preparing it for subsequent aging, together with the help of other antioxidant enzymes (Kibinza et al., 2011).

In the current study, primed seeds exhibited a reduced antioxidant potential in comparison to unprimed seeds, both before and after aging. This finding may indicate that a higher demand for antioxidants is present in response to priming and subsequent dry-back (**Figure 3.5a**). This could be attributed to the reported more advanced metabolic state that is induced by priming (Pagano et al., 2023; Tian et al., 2023). The observed decrease in germination rates and seedling establishment is consistent with the premise of the work, which is that primed seeds are subject to an increased amount of aging in comparison to unprimed seeds. Priming was reported to result in a reduction in an enzymatic and/or non-enzymatic antioxidant response. For example, the expression of genes implicated in the antioxidant response and antioxidant activity in sorghum (*Sorghum bicolor*) seeds were reduced by calcium chloride priming in the presence of salt stress (Chen et al., 2022). An impaired antioxidant apparatus, which includes enzymes (catalase, superoxide dismutase) and antioxidant compounds (glutathione, ascorbic acid), has been attributed to the decreased post-priming longevity of *Zea mays* seeds (Chiu et al., 2002). Osmoprimed *Spinacia oleracea* seeds (Chen and Arora, 2011), cysteine-primed *Hordeum vulgare* seeds (Genisel et al., 2014), and hydroprimed *Brassica rapa* subsp. pekinensis seeds (Yan et al., 2017) also exhibited reductions in antioxidant activity following priming. In contrast, seed priming results in an increase in stress response and antioxidant activities in other experimental systems that do not assume post-priming senescence or that evaluate antioxidant parameters at later growth stages (Farooq et al., 2017). Priming was found to increase the total antioxidant activity and the content of phenolic compounds (including flavonoids)

in *Triticum aestivum* seedlings, while previous accounts in *G. max* reported a decrease in ROS levels and an increase in the expression of antioxidant response genes (catalase, ascorbate peroxidase, superoxide dismutase) following priming (Griffo et al., 2023). These patterns in the antioxidant response to priming underscore the necessity of examining seed priming and dry-back in the context of a controlled stress, potentially investigating the aspects of priming-associated stress memory (Balmer et al., 2015; Srivastava et al., 2021). Consequently, it is possible that the application of aging to primed seeds serves as a bottleneck in priming effectiveness, which is mediated by stress memory mechanisms and/or the pre-activation of antioxidant responses leading reversal of priming benefits and the subsequent reduction in longevity when in long-term storage.

The main experimental question in the current study was the efficacy of quercetin- and rutin-supplementation in mitigating the effects of artificial aging in *M. truncatula* seeds in comparison to hydropriming. The observed enhancements were interpreted in terms of oxidative/antioxidant status. Rutin-primed seeds presented lower ROS levels than unprimed and hydroprimed seeds after artificial aging. Additionally, both quercetin- and rutin-supplemented priming exhibited enhanced antioxidant profiles in comparison to hydroprimed seeds. The enhanced post-aging germination and growth performance of quercetin- and rutin-primed seeds in comparison to hydroprimed seeds is consistent with this observation. Collectively, these findings indicate that quercetin or rutin priming may have potential protective effects in terms of improved antioxidant profiles. This antioxidant effect of quercetin and rutin is consistent with numerous reports from other experimental systems, such as *Apocynum pictum* and *Apocynum venetum* seeds under osmotic stress (Yang et al., 2017), *G. max* artificially aged seeds (Cui et al., 2019), *O. sativa* plants (Singh et al., 2017). The data collected are insufficient to differentiate between the direct and indirect effects of quercetin or rutin on antioxidant profiles, germination rates, and seedling growth, nor to determine the relative contribution of other factors that are purported to contribute to seed longevity, despite the significant correlations. These comprise compounds (e.g., heat shock proteins, late embryogenesis abundant proteins, raffinose family oligosaccharides that stabilize macromolecules, membranes, and cellular structures (Salvi et al., 2022; Tu et al., 2022) or mechanisms that maintain DNA integrity (Waterworth et al., 2010, 2019). The longevity of seeds is significantly influenced by phytohormones, which modulate seed maturation, germination, and stress responses above these layers (Pirredda et al., 2023). In order to develop a comprehensive model of post-priming seed senescence, it is essential to conduct additional research that concentrates on the role of these factors in various experimental systems and model species.

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Chapter 3. Quercetin and rutin enhances longevity in primed seeds

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Chapter 4. Spermidine-mediated enhancement of longevity in primed *Medicago truncatula* seeds: interplay between DNA damage response and cell cycle regulation and a focus on metabolomic changes

This article is in preparation to be published.

My contribution to this article includes i) seed germination and phenotypic analyses of unaged and aged *M. truncatula* seeds subjected to hydropriming, spermidine priming, ii) Comet assay to assess DNA damage accumulation, iii) qRT-PCR to assess the gene expression profiles of DNA damage response, cell cycle regulation and polyamine biosynthesis genes iv) Sample preparation for GC-MS analysis and statistical analysis of the data acquired from GC-MS analysis. I also performed statistical analysis of the resulting data and actively participated in their critical assessment and scientific discussion.

Abstract

Seed longevity is a multifaceted trait that is affected by a variety of factors. It is particularly important in the context of climate change, being crucial for promoting sustainable agriculture, preserving germplasm, and maintaining excellent seed quality. However, longevity is compromised in primed seeds. Seed priming is a method used to enhance seed quality by activating the pre-germinative metabolism, including DNA damage response and antioxidant response that promote better germination performance. This study investigates the ability of primed *Medicago truncatula* seeds to endure deterioration during storage by developing an experimental system that allows to screen compounds that would protect primed seeds in storage. When spermidine priming was tested on primed, aged *M. truncatula* seeds, viability was improved in aged seeds. However, increased germination percentage corresponded with increased frequency of aberrant seedlings, showing that the beneficial effects of spermidine were lost during seedling establishment. To which extent DNA damage accumulated in seeds can impair subsequent developmental stages? Proper DNA repair requires the block of cell cycle progression which, in turn is required for plant development. Spermidine effectively reduced DNA damage and altered the expression of different DNA damage response genes at distinct developing stages. Non-targeted metabolomics results demonstrated that exogenous spermidine treatment induced an accumulation of endogenous polyamines. It impacted antioxidants and nucleotide metabolites that may contribute to enhancing seed viability. These findings will improve our understanding of the deterioration mechanisms affecting primed seeds during storage and contribute to the development of more mitigation strategies.

4.1 Introduction

High quality seeds are essential players in the fight against climate change. Enhancing seed quality results in better seedling establishment and stress tolerance, hence increasing the level of resilience needed for profitable and sustainable farming in changing environmental conditions (Bailly and Gomez Roldan, 2023; Pagano et al., 2023). Seed quality is determined by longevity and the ability to germinate, which are greatly affected by the seed genetic profile and the surrounding environment (Rajjou et al., 2012; Zinmeister et al., 2020). Conventional breeding and genetic engineering are used to enhance seed-related traits relevant for increasing yield. In contrast, seed priming technology involves treating seeds before sowing to enhance germination and stress tolerance. This is done by soaking the seeds in water or other priming agents to activate specific metabolic processes that improve seed quality (Pagano et al., 2023). Oxidative damage occurs in the lipid membranes, proteins, and nucleic acids throughout the entire seed life cycle, from maturation to imbibition, as a consequence of unfavorable environmental conditions. The primary cause of oxidative damage are ROS, which can be effectively scavenged by the antioxidant apparatus. Simultaneously, it is essential to restore the ROS-induced genotoxic damage by activating the DNA damage response (DDR), as failure to do so will impair germination (Waterworth et al., 2019).

Priming allows for an extension of the temporal window during which the seed repair activity is activated; however, the treatment must be terminated prior to the occurrence of radicle protrusion. At this point, seeds lose their ability to tolerate desiccation and to survive the dry-back process (Pagano et al., 2022a, 2022b). Seed priming benefits include the ability to germinate in a broader temperature range and a decreased susceptibility to oxygen deprivation. These treatments are also employed to improve the germination performance of aged seeds (Devika et al., 2021; Corbineau et al., 2023). Nevertheless, primed seeds deteriorate at a greater rate than unprimed seeds during storage (Wang et al., 2018; Tu et al., 2022; Corbineau et al., 2023; Pagano et al., 2023). Despite the attention that has been devoted to this issue, there are still numerous unanswered questions regarding the relationship between seed longevity and priming. This is a result of the broad variety of parameters (e.g., priming agents/conditions, storage protocol, genotype, and seed lot) that contribute to the intricate cellular and molecular network responsible for the observed seed response (Fabrissin et al., 2021). Osmoprimed seeds outperformed hydroprimed seeds in *Brassica napus* L. and *Allium porrum* L. (Corbineau et al., 1994; Basra et al., 2003), while *Allium cepa* L. exhibited the opposite profile (Dorna et al., 2013). The seed response under storage may be influenced by changes in the metabolic pathways that are induced by various priming agents, as documented by high-throughput analyses (Gallardo et al., 2001; Farooq et al., 2019; Pagano et al., 2023). The temporal window when priming treatments are applied is a critical element, as prolonged imbibition can result in detrimental effects on seed quality (Powell et al., 2000). Conversely, priming conducted at low temperatures can prevent oxidative damage and seed deterioration (Hsu et al., 2003; Chiu et

al., 2005; Lutts et al., 2016). Priming can either shorten or extend longevity when it is applied to high-quality or low-quality seeds, according to the current evidence (Powell et al., 2000; Butler et al., 2009; Fabrissin et al., 2021). Seed quality can also be influenced by the dry-back conditions, and it has been hypothesized that rapid drying causes oxidative injury to the cellular and molecular structures, including lipid membranes (Chiu et al., 2005; Butler et al., 2009). In contrast, conflicting reports indicate that gradual drying may not always be effective in safeguarding primed seeds from deterioration (Bruggink et al., 1999; Soeda et al., 2005; de Melo et al., 2021). Is there a method for improving the low germinability of primed seeds? Chemicals targeting specific molecular players that are responsible for the degradation of cellular structures have been employed (Dejonghe and Russinova, 2017; Sano and Seo, 2019). Among these, cell cycle inhibitors, such as mimosine, were found to be effective in preventing the deterioration of Arabidopsis primed seeds (Sano and Seo, 2019). Additionally, the response of primed seeds may be influenced by changes in chromatin organization (van Zanten et al., 2011). In order to speed up the development of innovative and sophisticated assays to detect (or prevent) deterioration, it is necessary to have a deeper comprehension of the molecular and cellular mechanisms that contribute to the reduced storability of primed seeds (Pagano et al., 2023). Aged oat (*Avena sativa* L.) seeds were primed with antioxidant molecules, such as glutathione and ascorbic acid, which exhibited beneficial effects against injury (Xia et al., 2020). Polyamines are frequently employed as seed priming agents and are recognized for their antioxidant properties (Li et al., 2014; Huang et al., 2017; Hongna et al., 2021). The expression of antioxidant genes, osmolyte biosynthetic genes, and ABA biosynthesis genes in shoots and roots was increased by polyamine-mediated priming, as demonstrated by transcriptomic profiles of rice seeds primed with spermine and spermidine and exposed to salt stress (Paul and Roychoudhury, 2017). The activity of enzymes involved in glycolysis, tricarboxylic acid cycle, and pentose phosphate pathway was increased in aged sorghum (*Sorghum bicolor* L.) seeds treated with spermidine. Additionally, mitochondrial structures were protected, which resulted in increased respiratory activity and ATP levels (Zhang et al., 2022). The germination of silver maple (*Acer saccharinum* L.) seeds was effectively enhanced by spermidine-mediated priming; however, this effect was absent after six months of storage (Fuchs et al., 2023). Hofer et al. (2022) conducted a recent review that summarizes the diverse functions of spermidine, particularly the anti-ageing functions that have been observed in fungi, nematodes, insects, and rodents, as well as its role in epigenetic regulation. In the present work, the cellular and molecular mechanisms underlying the deterioration of primed *Medicago truncatula* seed in storage have been investigated using an experimental system able to provide information on the ability of primed seeds to withstand the impact of ageing and allow to test treatments designed to improve viability of primed seeds. We are currently exploring such issues in the spermidine-primed, aged *M. truncatula* seeds using Comet assay, DDR/cell cycle gene expression profiles and metabolomics. This will give us significant insights into spermidine-related dynamic changes at different developmental stages

(seed germination *vs* seedling development) in terms of DDR and cell cycle regulation, and the consequent impact on seed longevity.

4.2 Materials and methods

Plant material, treatments and germination tests

Medicago truncatula seeds (commercial genotype, kindly provided by Continental Semences S.p.A., Traversetolo, Parma, Italy) were treated as follows. Hydropriming was carried out by imbibing seeds for 4 h in Petri dishes (diameter 90 mm) containing two filter papers moistened with 2.5 ml H_2O, sealed and kept in a growth chamber at 22°C under light conditions with photon flux density of 150 μmol m^{-2} s^{-1}, photoperiod of 16/8 h and 70-80% relative humidity. Imbibition was followed by dry-back (DB): primed seeds were transferred into glass tubes, placed between two cotton disks, covered with silica beads (disidry® Orange Silica Gel, The Aerodyne, Florence, Italy) with a seed:silica ratio of 1:10, and kept at 24-25°C. For spermidine treatment, seeds were imbibed as previously described in presence of 10 mM spermidine (SP; Sigma-Aldrich, Milan, Italy). For each treatment, five independent replications (20 seeds per replication) were analysed. For germination tests, seeds were transferred to Petri dishes (diameter 90 mm; 20 seeds/Petri dish) containing two filter papers moistened with 2.5 ml H_2O, sealed and kept in a growth chamber as previously described. Seeds with protrusion of the primary radicle were considered germinated. Germination parameters were calculated according to Ranal and Garcia de Santana (2006). Samples were collected at the indicated timepoints, frozen using liquid N_2 and stored at -80°C for subsequent molecular analyses.

Artificial aging

The artificial aging (AA) protocol was performed as described by Colombo et al. (2023) and Gaonkar et al., (2024), with the following modifications. *M. truncatula* seeds were placed in open Petri dishes (diameter 90 mm; 20 seeds per dish) that were transferred to a rectangle glass container (1.4 l; Bormioli Rocco, Fidenza, Italy). dH_2O (100 mL) was added to the container which was closed and incubated for 24 h at the indicated temperature in a Memmert Universal Oven U55 (Memmert, Schwabach, Germany) for 24 h. Relative humidity was measured using a TA298 Digital thermohygrometer (JZK, Shenzhen JinZhiKu Electronic Co., Ltd, Shenzhen, China).

Comet Assay

The alkaline version of the comet assay was used to quantify single strand breaks (SSBs) formed from alkali-labile sites as well as DNA-DNA or DNA-protein crosslinks (Collins, 2004). Seeds were harvested at the indicated time points, embryo axes, embryos with radicle protrusion and aerial part and root tip of 4-

day seedlings were isolated from the cotyledons and seed coat using a razor blade as reported by Pagano et al. (2022). Nuclei were extracted as previously described (Ventura et al., 2014). The resulting suspension containing purified nuclei was mixed in equal volume with a solution containing 1% low melting point (Sigma-Aldrich) agarose in phosphate-buffered saline buffer (PBS: 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) maintained at 38°C. Two drops of the resulting suspension were then pipetted onto agarose precoated slides and solidified on ice. Slides were incubated for 30 min at 4°C in alkaline buffer (1 mM Na_2 EDTA, 300 mM NaOH, pH 13.0) and then electrophoresed in the same buffer for 25 min at 0.72 V cm^{-1} in a cold chamber under dark conditions. After electrophoresis, slides were washed in 0.4 M Tris HCl pH 7.5 three times for 5 min, rinsed in 70% ethanol (v/v) three times for 5 min at 4°C, and dried overnight at room temperature. Slides were stained with 20 µL 4′,6-diamidino-2-phenylindole (DAPI, 1 mg mL^{-1}, Sigma-Aldrich). For each slide, 100 nucleoids were scored, visualized using an Olympus BX51 fluorescence microscope with an excitation filter of 340-380 nm and a barrier filter of 400 nm. Images were captured using an Olympus MagnaFire camera equipped with Olympus Cell-F software. Nucleoids were classified as previously described by Collins (2004), where each type of nuclei morphology belongs to a class from 0 to 4. The results were expressed in arbitrary units (a.u.), calculated using the following formula: a.u. = $[\Sigma(Nc \times c) \times 100]/N_{tot}$, where N_c is the number of nuclei of each class, c is the class number (e.g., 0, 1, 2, 3, 4), and N_{tot} is the total number of counted nuclei (Collins, 2004).

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

RNA isolation was carried out using the TRIZOL® Reagent (Fisher Molecular Biology, Trevose, U.S.A.) according to the supplier's indications. cDNAs were obtained using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Milan, Italy) according to the manufacturer's suggestions. Quantitative real-time polymerase chain reaction (*q*RT-PCR) was performed with the Maxima SYBR Green qPCR Master Mix (2X) (Thermo Fisher Scientific) according to supplier's indications, using a CFX Duet Real-Time PCR System (Bio-Rad, Segrate, Milan, Italy). Amplification conditions were as follows: denaturation at 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 30 s and 72°C for 30 s. Oligonucleotide primers were designed using the Real-Time PCR Primer Design program Primer3Plus (https://primer3plus.com) from GenScript and further validated through the online software Oligo Analyzer (https://eu.idtdna.com/calc/analyzer). Quantification was carried out using as reference gene Mt18SrRNA (LOC112418413)The following genes were tested: *MtOGG1* (*8-OXOGUANINE GLYCOSYLASE/LYASE 1*) (Medtr3g088510), *MtNBS1*, (*NIJMEGEN BREAKAGE SYNDROME 1*) (Medtr5g076180), *RAD50* (*RADIATION SENSITIVE 50)* (Medtr3g084300), *RAD51* (*RADIATION SENSITIVE 51)* (Medtr4g062540), *MtLigIV* (*DNA LIGASE IV*) (Medtr2g038030), *CYCB1* (*CYCLIN B1*) (Medtr5g088980)*, CYCA3* (*CYCLIN A3*) (Medtr3g102520)*, CYCD2* (*CYCLIN D2*) (Medtr5g032550)*, CYCD3* (*CYCLIN D3*)

(Medtr3g102310), *SPDS,* (*SPERMIDINE SYNTHASE*) (Medtr5g034470), *SPMS* (*SPERMINE*/*SPERMIDINE SYNTHASE*) (Medtr3g091090).

The Thomsen method was employed for relative quantification of transcript accumulation using a standardized efficiency (E) value of 1.8 (Thomsen et al., 2010). All reactions were carried out in triplicates. Z-score was calculated on the linearized Ct values and used to generate heatmaps with the GraphPad Prism version 8.0.1 program.

Metabolite extraction and sample derivatization

Seeds and plant materials were collected at the indicated timepoints, and immediately snap frozen in liquid nitrogen to quench the endogenous metabolism. Freshly homogenized (100 mg) plant material was obtained from each biological sample (seeds, seedling) and replicates. These were transferred to 2 mL microcentrifuge round bottom screw cap tubes (Eppendorf). Extraction was done by adding 1400 µL of methanol (at −20 °C) and vortexing for 10 s after addition of 60 µL ribitol (0.2 mg/mL stock in ddH2O) as an internal quantitative standard for the polar phase. Samples were transferred in a thermomixer at 70 °C and were shaken for 10 min (950 rpm) and were then further centrifuged for 10 min at 11000 g. The supernatants were collected and transferred to glass vials where 750 µL CHCl3 (−20 °C) and 1500 µL ddH2O (4 °C) were sequentially added. All the samples were vortexed for 10 s and then centrifuged for another 15 min at 2200 g. Upper polar phase (150 µL) for each replicate was collected, transferred to a 1.5 mL tube and dried in a vacuum concentrator without heating. Before freezing and storing at −80 °C, the tubes were filled with argon and placed in a plastic bag with silica beads (to avoid moisture and hydration during short-term storage). Before derivatization, stored samples were placed in a vacuum concentrator for 30 min to eliminate any trace of humidity. Then, 40 µL methoxyamine hydrochloride (20 mg/mL in pyridine) was added to the dried samples, which were then incubated for 2 h in a Thermomixer (950 rpm) at 37 °C. Methoxyaminated samples were then silylated by adding 70 µL of MSTFA to the aliquots. Samples were further shaken for 30 min at 37°C. Derivatized samples (110 µL) were then transferred into glass vials suitable for the GC/MS autosampler for analysis.

GC-quadrupole/MS analysis

The derivatized extracts were injected into a TG-5MS capillary column (30 m x 0.25 mm x 0.25 µm) (Thermo Fisher Scientific, Waltham, MA, USA) using a gas chromatograph apparatus (Trace GC1310, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a single quadrupole mass spectrometer (ISQ LT, Thermo Fisher Scientific, Waltham, MA, USA). Injector and source were set at 250 °C and 260 °C, respectively. One µl of sample was injected in splitless mode with a helium flow of 1 mL/min using the following programmed temperature:isothermal 5 min at 70 °C followed by a 5 °C/ min ramp to 350 °C and a final 5 min heating at 330 °C. Mass spectra were recorded in electronic

impact (EI) mode at 70 eV, scanning at 40-600 m/z range, scan time 0.2s. Mass spectrometric solvent delay was settled as 9 min. Pooled samples that served as quality controls (QCs), n-alkane standards, and blank solvents (pyridine) were injected at scheduled intervals for instrumental performance, tentative identification, and monitoring of shifts in retention indices (RI).

GC/MS analysis and data acquisition: GC/MS data analysis using MS-DIAL

Raw data (.RAW) from the single quadrupole instrument was converted to. mzML format with the MSConvertGUI from ProteoWizard (http://proteowizard.sourceforge.net/tools.shtml). MS-DIAL ver. 2.90 (with open source publicly available EI spectra library), was used for raw peaks extraction,baseline filtering and calibration of the baseline, peak alignment, deconvolution, peak identification, and integration of the peak height were essentially followed as described (Tsugawa et al., 2015). An average peak width of 20 scans and a minimum peak height of 1000 amplitudes was applied for peak detection, and a sigma window value of 0.5, EI spectra cut-off of 5000 amplitudes was implemented for deconvolution. For identification, the retention time tolerance was 0.2 min, the m/z tolerance was 0.5 Da, the EI similarity cut-off was 60 %, and the identification score cut-off was 80 %. In the alignment parameters setting process, the retention time tolerance was 0.5 min, and retention time factor was 0.5. For MS-DIAL 2.90 data annotations, we used publicly available spectral libraries for compound identification, based on the mass spectral pattern and compared to EI spectral libraries such as NIST Mass Spectral Reference Library (NIST14/2014; National Institute of Standards and Technology, USA; with EI- MS data of 242,466 compounds), the MSRI spectral libraries from Golm Metabolome Database (Kopka et al., 2004) available from Max-Planck-Institute for Plant Physiology, Golm, Germany (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html), MassBank (Horai et al., 2010), and MoNA (Mass Bank of North America, (http://mona.fiehnlab.ucdavis.edu). For metabolite annotation and assignment of the EI-MS spectra, we followed the metabolomics standards initiative (MSI) guidelines for metabolite identification (Sansone et al., 2007), i.e., Level 2: identification was based on spectral database (match factor>80 %) and Level 3: only compound groups were known, e.g. specific ions and RT regions of metabolites.

Statistical analyses

Statistical analysis of germination data, phenotypical analysis, DCFH-DA assay, comet assay, gene expression profiles and concentration of metabolites was performed using two-way analysis of variance (ANOVA) and the DUNCAN test, carried out using a web-based program available online (https://houssein-assaad.shinyapps.io/TwoWayANOVA/) that provides publication-ready result tables (Assaad et al., 2015). Treatment means that are not significantly different, as reported by an all-pairwise comparison procedure, are followed by a common superscript letter. Two treatments without a common letter are statistically significant at the chosen level of

significance (α = 0.05). GC-MS data were statistically analyzed by using MetaboAnalyst version 6.0 (Pang et al., 2021). Briefly, relative Lowess-normalized abundance values from the MS-DIAL outputs were Log-transformed and Pareto-scaled before performing univariate (ANOVA), cluster analysis (using Euclidean distance and Ward as the clustering algorithms), and multivariate analysis, i.e., PCA (principal component analysis).

4.3 Results

Spermidine rescues the viability of primed, artificially aged *M. truncatula* seeds: building the experimental system

Germination of *M. truncatula* seeds subjected to hydropriming (HP) and spermidine priming (SP), followed by accelerated aging (AA), was evaluated in terms of germination percentage at the end of the germination test (germinability, %) and germination speed (T_{50}, time required to reach 50% of final germination) (**Figure 4.1A)**. The germinability of SP-AA seeds (32.00 ± 16.04 %) was significantly higher ($P < 0.05$) compared to HP-AA seeds (6.00 ± 4.10 %). The *M. truncatula* seedlings exhibited a combination of normal and aberrant phenotype, as shown in **Figure 4.1B**. The percentage of seedlings with normal morphology was 3 ± 4.47% for SP-AA, which is significantly (*P < 0.05*) lower compared to UP-AA (59 ± 6.51%) (**Figure 4.1C**). These findings emphasize the efficacy of SP treatment in restoring the viability of deteriorated HP-AA seeds. Although germinability was enhanced in SP-AA compared to HP-AA, the protective effect of SP was lost during the transition from seed germination to seedling development. This was evident as the majority of seedlings developed from SP-AA seeds displayed an aberrant phenotype (**Figure 4.1C**).

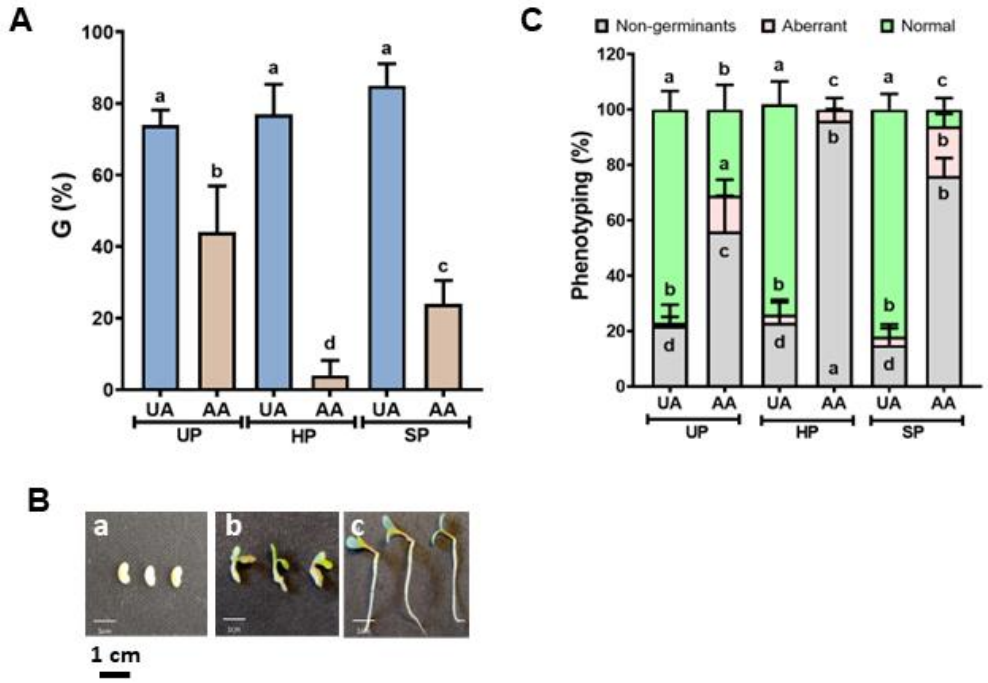

Figure 4.1. Germination performance expressed as **A.** G (germination percentage) of *M. truncatula* unprimed (UP) and hydroprimed (HP) seeds as well as seeds treated with 10 mM spermidine (SP). For each treatment (UP, HP, SP), unaged (UA) and artificially aged (AA) seeds were tested. **B.** Phenotyping: non germinating seeds (a), aberrant (b) and normal (c) phenotypes of four-day old *M. truncatula* seedlings. **C.** Percentage of non-germinating seeds, as well as aberrant and normal phenotypes in *M. truncatula* four-day old seedlings developed from unprimed (UP) and hydroprimed (HP) seeds as well as seeds treated with 10 mM spermidine (SP), both unaged (UA) and artificially aged (AA).

Based on the reported data, an experimental setup was developed to study the molecular dynamics associated with the contrasting responses observed in *M. truncatula* seeds and seedlings upon spermidine priming. **Figure 4.2** provides a description of the experimental system which includes unaged UP (unprimed control), HP, and SP seeds that undergo artificial ageing (AA). The resulting samples (UP-AA, HP-AA, SP-AA) are subsequently tested for germination and samples collected at different timepoints: i) aged dry seeds (UP-AA, HP-AA, SP-AA), ii) seeds that have been soaked in water for 4 hours after priming/aging (UP-AA-Rh, HP-AA-Rh, SP-AA-Rh), iii) seeds at radicle protrusion stage (end of germination; UP-AA-RD, HP-AA-RD, SP-AA-RD) (**Figure 4.2**). The sample collection is extended to include seedlings, based on the scheme shown in **Figure 4.2**, based on seedling phenotype (normal, aberrant) and the tissues/organs (aerial parts, roots). The experimental system provided different responses in terms of seedling phenotype, when considering the UP control, as well as the impact of HP and SP treatments.

In case of UP-AA, both normal and aberrant seedlings were observed, showing abnormal aerial parts and root organization whereas for HP-AA only aberrant

seedlings were detected (**Figure 4.2**). When considering SP-AA, both normal aberrant seedlings were observed, with irregular structures in aerial parts and roots. All the samples shown in **Figure 4.2** underwent molecular analyses.

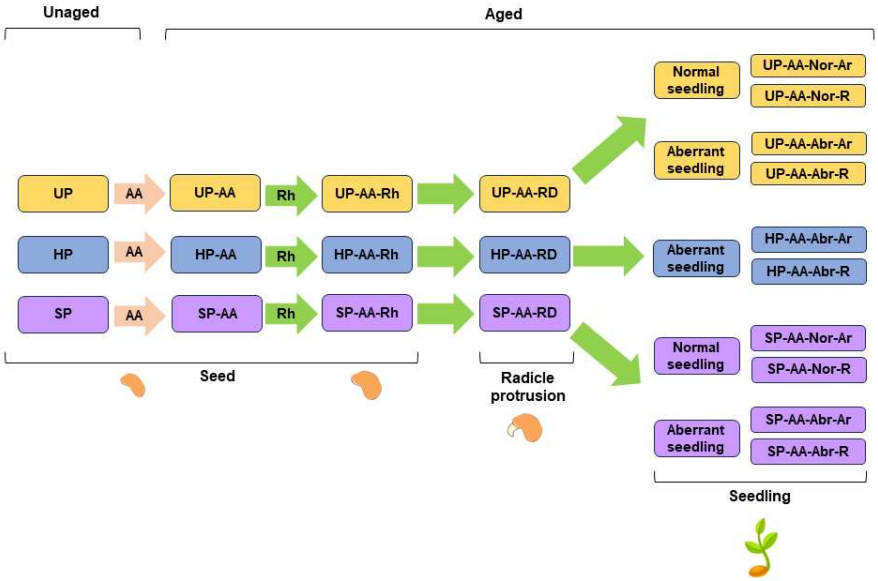

Figure 4.2 Schematic representation of the experimental system used in this study. UP, HP and SP samples, as controls. After ageing *M. truncatula* seeds undergo germination tests and samples are collected at different timepoints along germination: *i)* aged dry seeds (UP-AA, HP-AA, SP-AA), *ii)* imbibed (collected at 4 h of post-priming/ageing imbibition; UP-AA-Rh, HP-AA-Rh, SP-AA-Rh), *iii)* radicle protrusion stage (end of germination; UP-AA-RD, HP-AA-RD, SP-AA-RD). iv) Normal seedling development (UP-AA-Nor-Ar, SP-Nor-AA-Ar, UP-AA-Nor-R and SP-Nor-AA-R), aberrant seedling development (UP-AA-Abr-Ar, HP-AA-Abr-Ar, SP-Nor-AA-Ar, UP-AA-Abr-R, HP-AA-Abr-R, SP-Nor-AA-R). Means without a common letter are significantly ($P < 0.05$) different as analyzed by two-way ANOVA and Duncan test.

Spermidine mitigates DNA damage accumulation in deteriorated primed seeds

The alkaline comet assay was used to determine the DNA damage profile, in terms of DNA strand breaks (DSSs) accumulation, in the *M. truncatula* seeds and assess the possible impact of spermidine on the maintenance of genome integrity. In the case of unprimed seeds (UP), the levels of DSSs were significantly ($P < 0.05$) lower in the unaged dry seeds (UP-UA) (118.23 ± 13.15 a.u.) as compared to the levels of DSSs in aged dry seeds (UP-AA, 190.38 ± 25.28 a.u.) and aged imbibed seeds (UP-AA-Rh) (151.77 ± 10.83 a.u.) (**Figure 2A**). Hydroprimed seeds (HP) were found to be more susceptible to genotoxic damage, as indicated by the estimated DSSs values of 183.67 ± 16.78 a.u. (unaged dry seeds, HP-UA), 234.69 ± 12.78 a.u. (aged dry seeds, HP-AA), and 215.32 ± 16.78 a.u. (aged imbibed seeds, HP-Rh). Unaged seeds exposed to spermidine (SP-UA), showed DSSSs levels (142.09 ± 11.93 a.u.) within the range detected for UP-UA. No substantial increase of DSSs was detected in the SP-AA

seeds (152.37 ± 7.57 a.u.), and this trend was maintained in the aged imbibed seeds (SP-AA-Rh, 173.04 ± 7.46 a.u.) (**Figure 4.2A**). This indicates that accelerated aging did not have a major impact on the genomic integrity of SP seeds. When compared to HP seeds, the levels of DNA damage in aged primed seeds in the dry (SP-AA) and imbibed (SP-Rh) phases were reduced.

An investigation based on qRT-PCR analysis was carried out (**Figure 4.2B**) in order to assess changes in the expression of key DDR genes. With the exception of *MtLigIV*, most of the DDR genes were down-regulated in UP seeds (**Figure 4.2B**). Up-regulation of *MtNBS1*, *MtRAD50*, and *MtRAD51* genes was observed in aged hydroprimed seeds (HP-AA), as a possible response to increased DNA damage. On the other hand, the imbibed SP-treated seeds (SP-AA-Rh) showed the simultaneous up-regulation of *MtRAD50* and *MtRAD51* genes (**Figure 4.2B**). Cell cycle checkpoints, activated as part of the DDR downstream processes, represent surveillance systems that interrupt cell cycle progression under stress conditions, allowing proper DNA repair. The breakdown of cyclin and the subsequent inhibition of cyclin gene expression are the outcomes of cell cycle checkpoints activation. The expression patterns of *MtCYCB1*, *MtCYCA3*, *MtCYCD2*, and *MtCYCD3* genes, coding for cyclins CYCB1 (G2/M transition), CYCA3, CYCD2, and CYCD3 (G1/S transition), were investigated in order to identify any potential influence that spermidine treatment may have on cell cycle dynamics. In the UP seeds, the majority of the cyclin genes were down-regulated, with the exception of *MtCYCB1* (**Figure 4.2B**). *MtCYCD3* transcript was accumulated in all HP samples whereas the *MtCYCD2* gene was upregulated in the dry seeds, both unaged and aged. There was an increase in the expression of the *MtCYCB1* and *MtCYCA3* genes in HP aged dry seeds (HP-AA) and HP aged imbibed seeds (HP-AA-Rh) (**Figure 4.2B**). *MtCYCA3*, *MtCYCD2*, and *MtCYCD3* genes were upregulated in the aged imbibed seeds treated with spermidine. The overall decrease of cyclin gene expression in the UP seeds may be indicative of a decreased cell cycle activity and, thus, a lower rate of cell proliferation. On the other hand, the dynamics that were found in HP seeds may be indicative of an advanced physiological stage that was boosted by hydropriming. Such a response was more prominent approximately four hours after imbibition for the spermidine-treated aged seeds (**Figure 4.2B**). Finally, the expression profiles of the *MtSPDS* and *MtSPMS* genes involved in polyamine biosynthesis revealed a general downregulation, except for the UP unaged dry seeds, which showed accumulation of *MtSPMS* transcript, and the SP-treated imbibed aged seeds, which showed accumulation of the *MtSPDS* mRNA (**Figure 4.2B**).

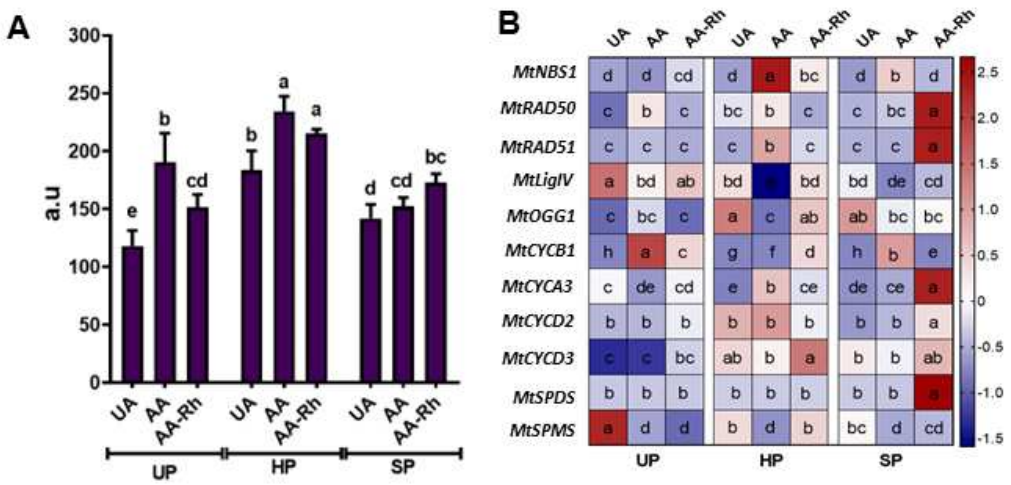

Figure 4.3 A. Alkaline comet assay was used to measure DNA strand breaks (DSSs) accumulation in *M. truncatula* seeds. **B.** Heat map representing the expression profiles of DDR, cyclin, and polyamines biosynthesis genes evaluated by qRT-PCR. UP, unprimed. HP, hydroprimed. SP, spermidine (10 mM) treated seeds. UA, unaged dry seed. AA, artificially aged dry seed. Rh, aged imbibed seed. a.u., arbitrary units. Mt, *Medicago truncatula*. OGG1, 8-oxoguanine glycolsylase/lyase. NBS, Nijmegen Breakage Syndrome. RAD, radiation sensitive. LigIV, DNA Ligase IV. CYC, Cyclin. SPDS, spermidine synthase. SPMS, spermine/spermidine synthase. Means without a common letter are significantly ($P < 0.05$) different as analyzed by two-way ANOVA and Duncan test.

Metabolome changes are distinctive features of UP, HP and SP seeds

A non-targeted metabolomics approach was conducted using gas chromatography-mass spectrometry (GC-MS) to assess the metabolic changes induced by spermidine in *M. truncatula* seeds. The univariate analysis of variance (ANOVA) showed that 112 out of 120 metabolites, mainly belonging to chemical classes such as carbohydrates, amino acids, organic acids, sugar alcohols, nucleic acids, and alkaloids, exhibited significant alterations across the three treatments. Significant differences in metabolite accumulation were observed in unaged dry seeds, as well as in both aged dry and imbibed seeds. Principal component analysis (PCA) was utilized to provide a complete overview of the metabolomics results obtained from untreated (UP) and treated (HP, SP) seeds. The dataset for PCA was structured to highlight the variations induced by HP and SP treatments in unaged, aged and aged imbibed seeds (**Figure 4.4A**). The main determinant of the variation in the dataset was the impact of aging and imbibition, leading to the formation of three distinct super-clusters: unaged seeds, aged seeds, and aged imbibed seeds. The scatter plot, utilizing the PC1 and PC2 axes, did not exhibit distinct clustering variations between the primed groups (HP and SP) in unaged seeds. Nevertheless, noticeable clusters were identified after aging, specifically in the UP-AA, HP-AA, and SP-AA groups (**Figure 4.4A**).

Interestingly, the clusters lost their distinguishability once more during post-aging imbibition, as the HP-AA-Rh and SP-AA-Rh clusters merged. These findings suggest that the process of aging has an impact on the metabolic alterations linked to HP and SP priming. The top 50 metabolites that were reported to be significantly accumulated by ANOVA were represented as a heatmap (**Figure 4.4B**). A general downregulation of amino acids like threonine, isoleucine and glycine is observe din unaged seeds. An overall accumulation of sugars (ribose, glucose, arabinose, fructose, glyceric acid, and galactitol) was observed in aged seeds, and this pattern appears to be consistent in aged imbibed seeds compared to unaged seeds. A general downregulation of organic acids like p-Coumaric acid, citric acid, isocitric acid, malic acid and succinic acid is also observed in aged imbibed seeds

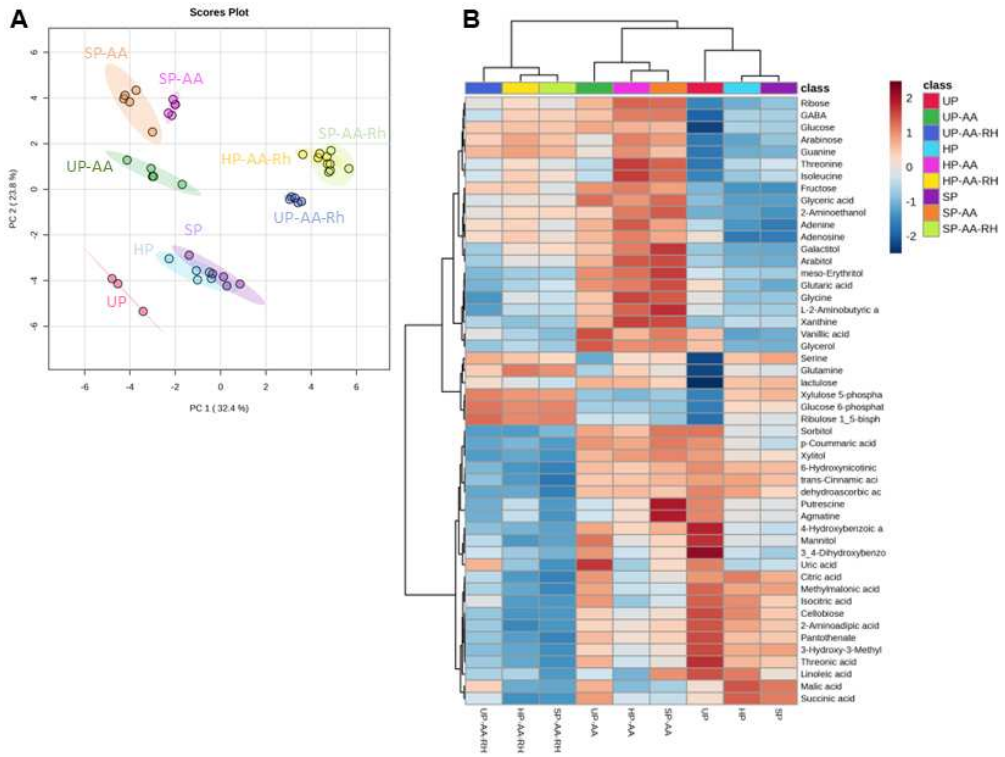

Figure 4.4. (A) Two-dimensional score plot of the principal component analysis of the results obtained from *M. truncatula* seeds **(B)** The clustering result is shown as a heatmap (distance measure using Euclidean, and clustering algorithm using Ward D) reporting the top 50 out of 120 metabolites resulting from the ANOVA (LSD test with $P \leq 0.05$ and FDR ≤ 0.05) in *M. truncatula* seeds. UA, unaged dry seed. AA, artificially aged dry seed. Rh, aged imbibed seed. a.u., arbitrary units. Mt, *Medicago truncatula.*

Spermidine protective effects are maintained at the radicle protrusion stage

The radicle protrusion stage (RD) indicates the end of the germination process and the transition to the seedling development program. When radicle protrusion was assessed, no aberrant morphologies were observed in any of the tested samples. In comparison to the previous observation conducted on dry and imbibed seeds (**Figure 4.5A**), the alkaline comet assay was implemented to evaluate potential modifications in DNA damage profiles during this physiological stage. The UP samples exhibited a substantially lower DSSs value (143.32 ± 8.64 a.u.) than the HP samples (205.30 ± 7.58 a.u.) at RD stage. The treatment with spermidine was associated with a decrease in DSSs (165.26 ± 7.58 a.u.), indicating that it may have a protective effect against genotoxic stress (**Figure 4.5A**). UP seeds exhibited a general downregulation of DDR, cyclin, and polyamine biosynthesis genes, with the exception of *MtCYCA3*. It is possible that the active DDR process requested to reduce DNA damage, which was reflected in the up-regulation of all DDR genes in the HP sample (**Figure 4.5B**). At the same time, *MtCYCA3* and *MtCYCD3* transcripts were accumulated together with *MtSPDS* and *MtSPMS* mRNAs. The SP sample exhibited upregulation of the *MtRAD50*, *MtCYCB1*, and *MtCYCD3* genes

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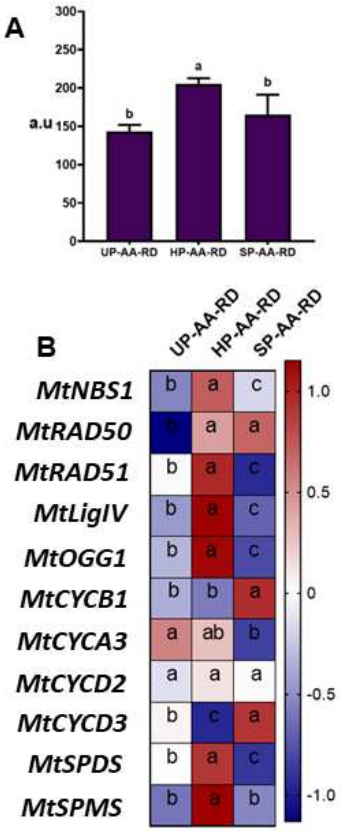

Figure 4.5 A. Alkaline comet assay was used to measure DNA strand breaks (DSSs) accumulation in *M. truncatula* seeds at the radicle protrusion stage. The assay was performed on radicles excised from germinating seed. **B.** Heat map representing the expression profiles of DDR, cyclin, and polyamines biosynthesis genes evaluated by qRT-PCR. Means without a common letter are significantly ($P < 0.05$) different as analyzed by two-way ANOVA and Duncan test. UP, unprimed. HP, hydroprimed. SP, spermidine (10 mM)-treated seeds. All seeds were artificially aged. a.u., arbitrary units. Mt, *Medicago truncatula*. OGG1, 8-oxoguanine glycolsylase/lyase. NBS, Nijmegen Breakage Syndrome. RAD, radiation sensitive. LigIV, DNA Ligase IV. CYC, Cyclin. SPDS, spermidine synthase. SPMS, spermine/spermidine synthase.

Metabolome changes at the radicle protrusion stage

GC-MS analysis was employed to evaluate the metabolic alterations at the radicle protrusion stage of *M. truncatula* seeds that were induced by spermidine. PCA was implemented to offer a thorough analysis of the metabolomics results UP seeds, HP seeds, and at SP seeds at the radicle protrusion stage. The results indicated three distinct clusters, which correspond to UP, HP, and SP seeds at the radicle protrusion stage (**Figure 4.6A**). These findings imply that the

metabolites identified at the radicle protrusion stage were significantly different. ANOVA revealed that the three treatments significantly altered 100 out of 120 metabolites. A heatmap (**Figure 4.6B**) is used to illustrate the top 50 metabolites that were identified as significant by ANOVA. The radicle protrusion stage reveals that all three treatments exhibit distinct differences in metabolites. The accumulation of metabolites in HP-AA-RD is generally reduced compared to UP-AA-RD and SP-AA-RD indicating metabolite depletion. At the radicle protrusion stage, some carbohydrates, including ribulose 5-phosphate and xylonic acid, exhibited an increase in SP seeds. Conversely, carbohydrates such as maltose, trehalose, sucrose, and xylulose 5-phosphate were upregulated in both UP and SP seeds. In response to spermidine treatment, an accumulation of succinic acid, 4-hydroxybenzoic acid, beta-manno glycerate, 3-hydroxy-3-methylglutaric acid, O-phosphoethanolamine, and nucleic acid components such as adenine and guanine was observed. At the radicle protrusion stage, both HP and SP seeds exhibited downregulation of amino acids such as tyrosine, glutamine, tryptophan, and glutamic acid. In contrast, beta-alanine, serine, methionine, citrulline, valine, isoleucine, phenylalanine, 2-aminoadipic acid, aspartic acid, and pantothenate were only found in reduced levels in HP seeds.

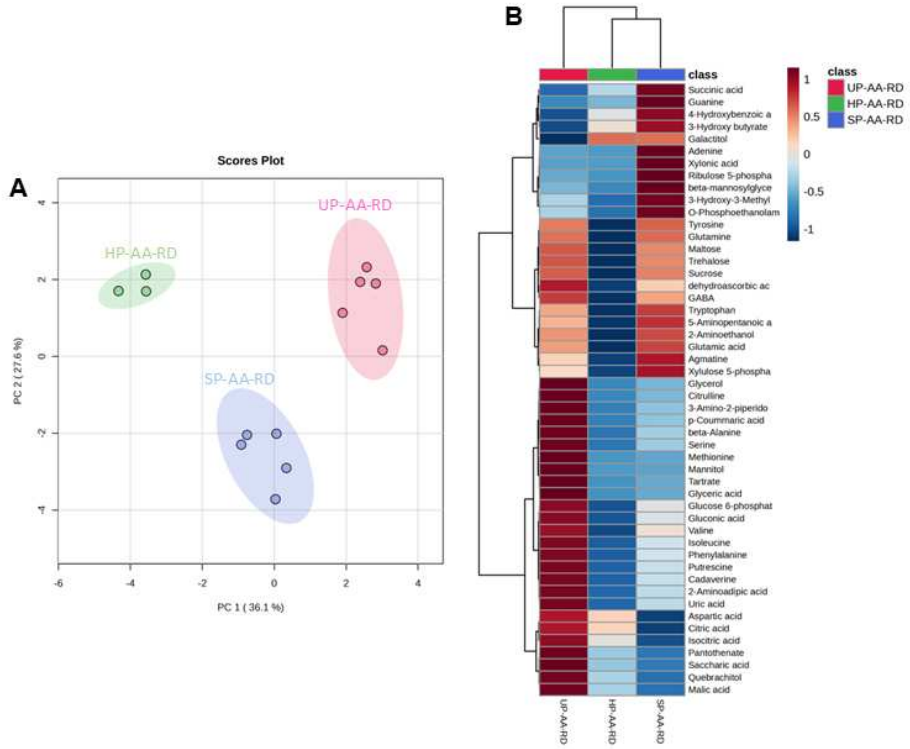

Figure 4.6 (A) Two-dimensional score plot of the principal component analysis of the results obtained *M. truncatula* seeds at the radicle protrusion stage **(B)** The clustering result is shown as a heatmap (distance measure using Euclidean, and clustering algorithm using Ward D) reporting the top 50 out of 120 metabolites resulting from the ANOVA (LSD test with $P \leq 0.05$ and FDR ≤ 0.05) at radicle protrusion stage. Means without a common letter are significantly ($P < 0.05$) different as analyzed by two-way ANOVA and Duncan test. UP, unprimed. HP, hydroprimed. SP, spermidine (10 mM)-treated seeds. AA, All seeds were artificially aged. RD, Radicle protrusion.

Deciphering the link between spermidine exposure and occurrence of abnormal seedling

The 4-day-old seedlings developed from UP, HP, and SP aged seeds were analyzed to evaluate the impact of spermidine on the phenotype of the resulting seedlings. The analysis was conducted on isolated aerial parts and roots of seedlings with both normal and aberrant phenotypes. The occurrence and extent of DNA damage in normal and aberrant seedlings were among the intriguing questions. The DSSs level was considerably higher (185.92 ± 9.15 a.u.) in the aberrant aerial parts of seedlings developed from UP-aged seeds compared to normal tissues (147.21 ± 2.06 a.u.), as demonstrated in **Figure 4.6A**. A similar trend was observed when comparing the normal and aberrant aerial parts of the SP samples (169.01 ± 7.71 and 213.37 ± 2.94 a.u., respectively). The estimated DSSs value in the aerial portions of all seedlings developed from HP-aged seeds was further increased (255.06 ± 9.15 a.u.)

(**Figure 4.6A**). Additionally, the seedlings exhibited an aberrant phenotype. The same scenario was evident when the analysis was conducted on the normal and aberrant roots of the same seedlings.

The qRT-PCR results indicated a general decrease in the expression of the DDR, cyclin, and polyamine biosynthesis genes in both the normal and abnormal aerial parts of the UP samples (**Figure 4.6B**). The aberrant aerial parts of HP seedlings exhibited up-regulation of all the tested genes, whereas the normal and aberrant aerial parts of SP seedlings exhibited distinct patterns. The molecular landscape of the aberrant aerial portions of *M. truncatula* seedlings developed from UP, HP, and SP aged seeds was characterized by the distinct involvement of DDR, cyclin, and polyamine biosynthesis genes, as demonstrated by the reported data. These unique profiles may be indicative of the varying effects of AA-induced deterioration on UP, HP, and SP seeds, as well as the necessity of managing varying degrees of genotoxic damage in the aerial portions. The roots appear to be deficient in this requirement, as both normal and aberrant roots of UP, HP, and SP seeds exhibited overall gene downregulation (**Figure 4.6B**).

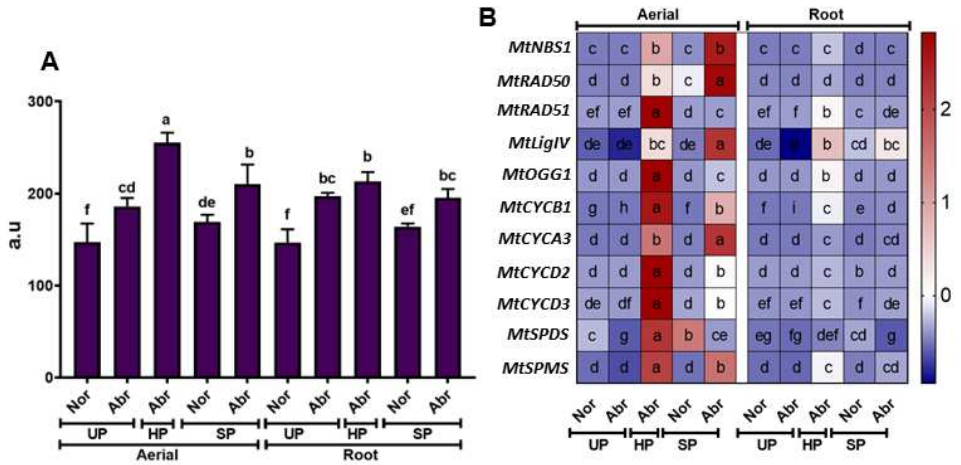

Figure 4.6 A. Alkaline comet assay was used to measure DNA strand breaks (DSSs) accumulation in *M. truncatula* four-day old seedlings developed from unprimed (UP), hydroprimed (HP) and spermidine (10 mM) treated (SP) seeds. The assay was performed on both normal (Nor) and aberrant (Abr) aerial parts (Ar) as well as on normal and aberrant roots (R). **B.** Heat map representing the expression profiles of DDR, cyclin, and polyamines biosynthesis genes evaluated by qRT-PCR. Mt, *Medicago truncatula*. OGG1, 8-oxoguanine glycolsylase/lyase. NBS, Nijmegen Breakage Syndrome. RAD, radiation sensitive. LigIV, DNA Ligase IV. CYC, Cyclin. SPDS, spermidine synthase. SPMS, spermine/spermidine synthase. a.u., arbitrary units. Means without a common letter are significantly ($P < 0.05$) different as analyzed by two-way ANOVA and Duncan test.

Contrastive metabolome profiles as molecular feature of normal and aberrant seedlings

The phenotype of *M. truncatula* seedlings was examined by conducting a metabolomic analysis on 4-day-old seedlings that were derived from UP, HP, and SP seeds. The impact of SP treatment was investigated. Seedlings that demonstrated both normal and aberrant phenotypes were evaluated. The seedlings' aerial roots were examined separately in the course of the investigation. PCA was conducted on these seedlings. The dataset for PCA was organized to emphasize the impact of priming on the development of seedlings. This was accomplished by assessing both normal and aberrant seedlings and by distinguishing between the aerial and root parts (**Figure 4.7A**). The primary factor contributing to the dataset's differences was the distinct components of the seedling, which resulted in the formation of two distinct super-clusters: the aerial and root portions. The aberrant seedlings that resulted from all three treatments were categorized into distinct clusters with respect to the aerial part. However, the seedlings that were obtained from UP and SP-treated seeds were grouped together in the case of normal seedlings. Interestingly, the normal roots that were developed from UP and SP seeds formed a cluster with the aberrant roots that originated from HP seeds (**Figure 4.7A**). The aberrant seedlings that were obtained from UP and SP seeds were categorized separately (**Figure 4.7A**).

A heatmap (**Figure 4.7B**) displays the top 50 metabolites that were found to be statistically significant by ANOVA. ANOVA revealed that 114 out of 120 metabolites exhibited significant changes in both normal and aberrant seedlings resulting from UP, HP, and SP treatment. As expected, the aerial and root sections of the seedlings have unique profiles of metabolites. Distinct variations in treatments, normal and aberrant phenotype were noted, in relation to metabolites. Amino acids were the primary metabolites accumulated in aerial part of the seedlings, particularly tyrosine, isoleucine, valine, tryptophan, citrulline, phenylalanine, methionine, homoserine, lysine and aspartic acid (**Figure 4.7B**). A general accumulation of methylmalonic acid, vanillic acid, fructose, 5-aminopentanoic acid was observed only in roots (**Figure 4.7B**). The accumulation of metabolites in the aerial parts of the seedlings developed from HP seeds is one of the interesting aspects to take into consideration. Adenosine accumulated only in aberrant roots from SP seeds, similarly to what observed in normal seedlings an such a pattern was also observed in aerial parts of the aberrant seedlings derived from HP and SP seeds (**Figure 4.7B**).

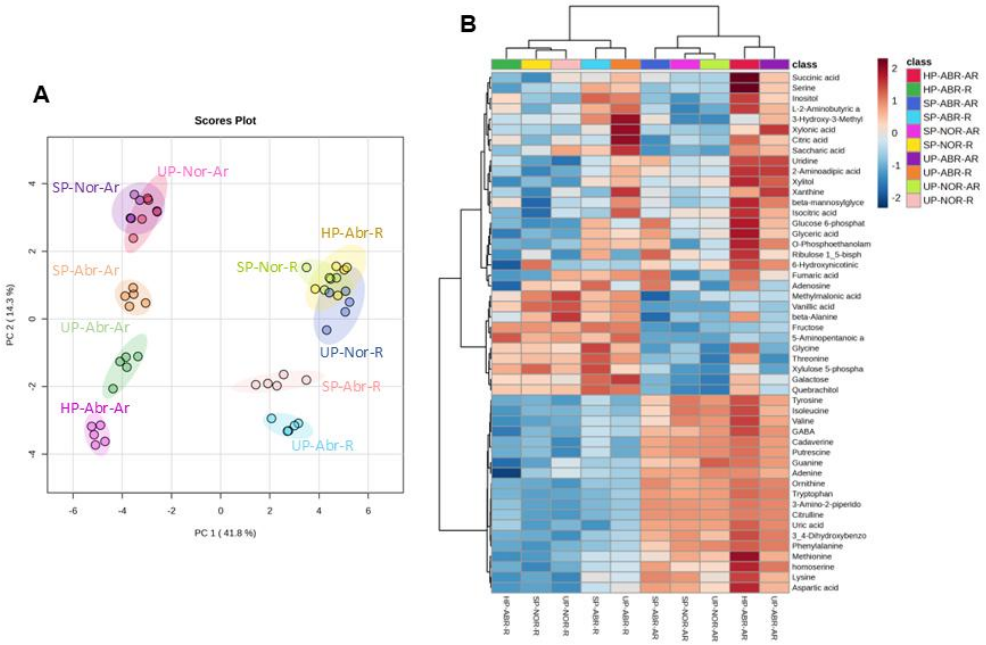

Figure 4.7 A) Two-dimensional score plot of the principal component analysis of the results obtained *from M. truncatula* four-day old seedlings developed from unprimed (UP), hydroprimed (HP) and spermidine (10 mM) treated (SP) seeds. The assay was performed on both normal (Nor) and aberrant (Abr) aerial parts (Ar) as well as on normal and aberrant roots **(B)** The clustering result is shown as a heatmap (distance measure using Euclidean, and clustering algorithm using Ward D) reporting the top 50 out of 120 metabolites resulting from the ANOVA (LSD test with $P \leq 0.05$ and FDR ≤ 0.05) at radicle protrusion stage. Means without a common letter are significantly ($P < 0.05$) different as analyzed by two-way ANOVA and Duncan test.

Impact of exogenous spermidine on the profiles of endogenous polyamines

GC-MS analysis highlighted the effect of spermidine treatment on the profiles of endogenous polyamines. A significant increase in agmatine, cadaverine, and putrescine was detected in SP-AA seeds compared to UP-AA and HP-AA seeds indicating that exogenous spermidine may stimulate polyamine biosynthesis in reaction to aging (**Figure 4.8A, D, G**). During radicle protrusion, a significant increase in agmatine levels was seen exclusively in spermidine-treated seeds (SP-AA-RD), in contrast to UP-AA-RD and HP-AA-RD, while cadaverine and putrescine concentrations were higher when compared to HP-AA-RD (**Figure 4.8B, E, H**). Polyamines predominantly accumulated in the aerial part of seedlings, in contrast to the roots (**Figure 4.8C, F, I**). There was no significant difference in the levels of agmatine when comparing seedling phenotype and treatments. Cadaverine and putrescine exhibit similar patterns across all seedlings derived from the three treatments. The significantly highest concentrations of these polyamines are found in the aerial portions of seedlings derived from HP-AA seeds, while the root parts of seedlings from SP-AA seeds displayed the highest accumulation of cadaverine and putrescine.

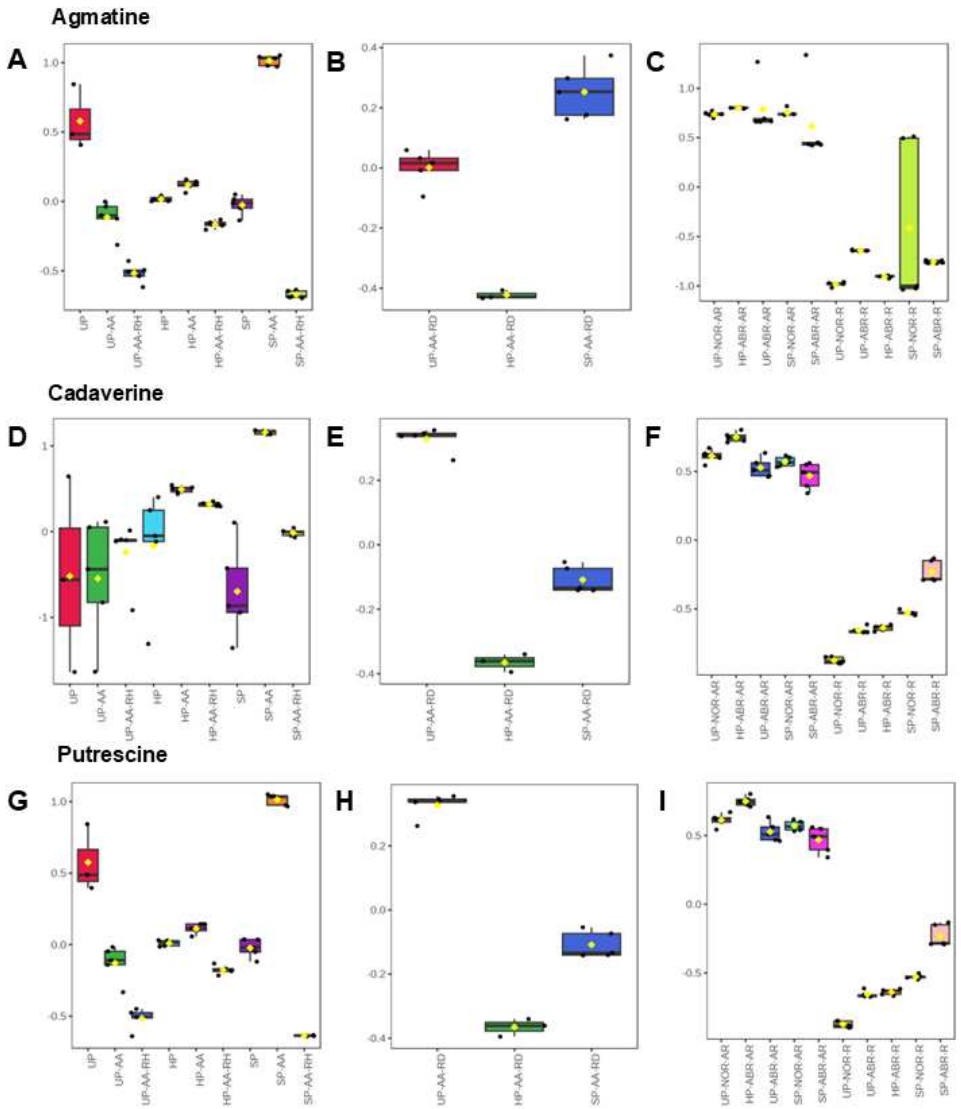

Figure 4.8 Impact of exogenous spermidine on the profiles of endogenous polyamines in *M. truncatula* seeds and seedlings. Relative concentrations of agmatine, cadaverine and putrescine **A, B, C.** Agmatine levels detected in aged seeds, seeds at the radicle protrusion stage, and seedlings. **D, E, F.** Cadaverine levels detected in aged seeds, seeds at the radicle protrusion stage, and seedlings. **G, H, I.** Putrescine levels detected in aged seeds, seeds at the radicle protrusion stage, and seedlings. Data were analyzed through one-way ANOVA (LSD test with $p \leq 0.05$ and FDR ≤ 0.05). UP, unprimed. HP, hydroprimed. SP, spermidine. AA, artificially aged. UP-AA, HP-AA, SP-AA, aged dry seeds. UP-AA-Rh, HP-AA-Rh, SP-AA-Rh, artificially aged seeds at 4 h of post-priming/ageing imbibition. UP-AA-RD, HP-AA-RD, SP-AA-RD, radicle protrusion stage. The assay was performed on both normal (Nor) and aberrant (Abr) aerial parts (Ar) as well as on normal and aberrant roots (R).

Putrescine can be converted into γ-aminobutyric acid (GABA), a non-proteinogenic amino acid that functions as a signaling molecule in several stress response pathways, through the action of monoamine oxidases. **Figure 4.9** depicts the GABA levels identified in seeds and seedlings. Similar GABA patterns

were detected in both HP-AA and SP-AA seeds (**Figure 4.9A**), indicating that spermidine likely did not influence GABA levels at the seed level during aging. SP-AA-RD at the radicle protrusion stage demonstrated significantly higher accumulation of GABA in comparison to the HP-AA-RD (**Figure 4.9B**). During the seedling stage, no significant differences in GABA levels were found in UP-AA seedlings, both normal and aberrant (**Figure 4.9C**), but a significant increase was noted in the aberrant aerial parts derived from HP-AA seeds. Both normal and aberrant aerial parts of seedlings originating from SP-AA seeds exhibited a significant reduction in GABA levels compared to UP-AA and HP-AA. In the roots, we see significantly reduced levels of GABA in all seedlings compared to the aerial parts. Elevated levels of GABA are seen in aberrant roots derived from UP-AA and SP-AA seeds, while aberrant seedlings from HP-AA seeds exhibit significantly reduced levels of GABA **(Figure 4.9C**).

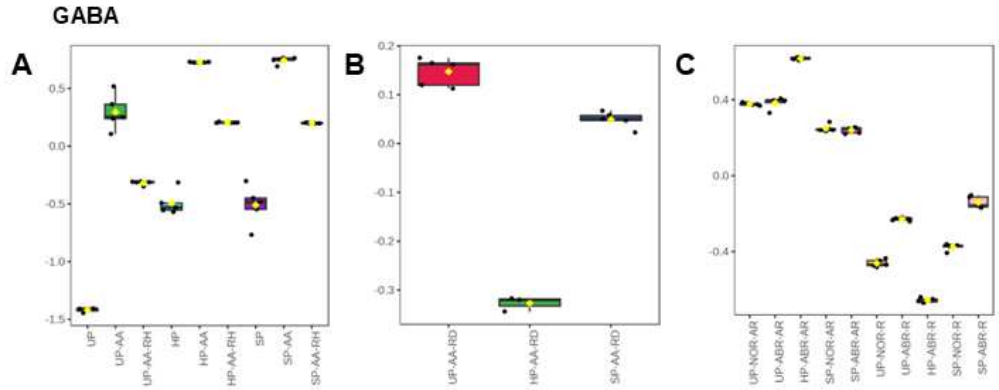

Figure 4.9 Impact of exogenous spermidine on the profiles of g-aminobutyric acid (GABA) in *M. truncatula* seeds and seedlings. Relative concentrations of GABA are reported GABA levels detected in aged seeds (**A**), radicle protrusion stage (**B**), and seedlings (**C**). Data were analyzed through one-way ANOVA (LSD test with $p \leq 0.05$ and FDR ≤ 0.05). UP, unprimed. HP, hydroprimed. SP, spermidine. AA, artificially aged. UP-AA, HP-AA, SP-AA, aged dry seeds. UP-AA-Rh, HP-AA-Rh, SP-AA-Rh, artificially aged seeds at 4 h of post-priming/ageing imbibition. UP-AA-RD, HP-AA-RD, SP-AA-RD, radicle protrusion stage. The assay was performed on both normal (Nor) and aberrant (Abr) aerial parts (Ar) as well as on normal and aberrant roots (R).

Changes of antioxidant levels in response to exogenous spermidine

Considering the function of polyamines, especially spermidine, in the antioxidant response, the profiles of antioxidant compounds were assessed. GC-MS analysis revealed significant changes in ascorbic acid (aa), dehydroascorbic acid (DHA), citric acid (CA), and isocitric acid (ICA) (**Figure 4.10**). AA facilitates germination and development and is a well-known antioxidant (Terzaghi & De Tullio, 2023). DHA, an oxidized variant of aa, is actively transported into the endoplasmic reticulum, where it is reduced to ascorbic acid by glutathione and other thiols (Dučić et al., 2003). CA, an efficient H_2O_2 scavenger, inhibits oxidation by chelating metal ions and has been utilized to enhance germination ICA, a key component of TCA, has established antioxidant effects (Sh and Orabi, 2015).

Significant aa accumulation was observed in SP-AA seeds, in contrast to HP-AA (**Figure 4.10A**), as well as throughout the radicle protrusion phase (**Figure 4.10B**). In case of DHA, spermidine appeared to demonstrate no major impact on seeds (**Figure 4.10D)**. During radicle protrusion SP-AA-RD had significantly higher accumulation of DHA compared to HP-AA-RD (**Figure 4.10E**). However, during seedling development, a significant DHA accumulation occurred in the aerial part of aberrant seedlings derived from HP-AA seeds (**Figure 4.10F**). Exogenous spermidine did not influence the levels of both CA (**Figure 4.10G,H,I**) and ICA (**Figure 4.10J,K,L**).

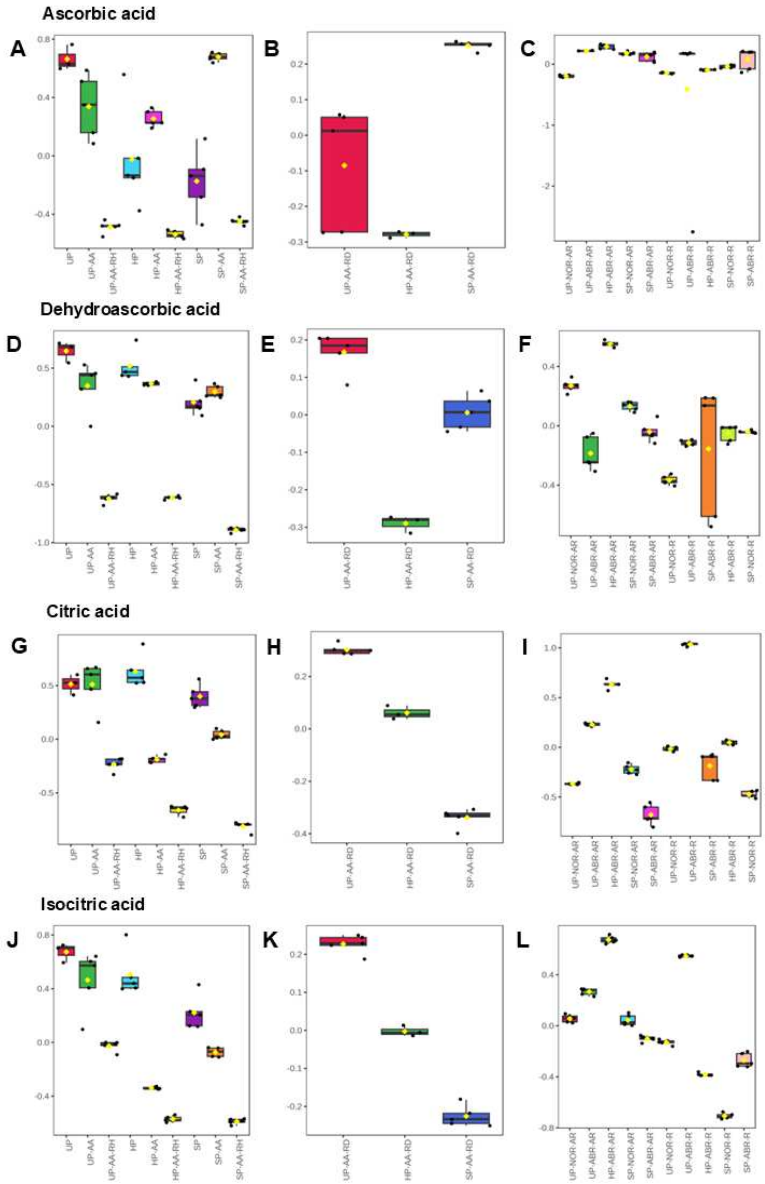

Figure 4.10 Impact of exogenous spermidine on the profiles of antioxidant compounds in *M. truncatula* seeds and seedlings. Relative concentration of ascorbic acid (AA), dehydroascorbic acid (DHA), citric acid (CA), and isocitric acid (ICA) concentrations are reported **A, B, C.** AA levels detected in aged seeds, seeds at the radicle protrusion stage, and seedlings. **D, E, F.** DHA levels detected in aged seeds, seeds at the radicle protrusion stage, and seedlings. **G, H, I.** CA levels detected in aged seeds, seeds at the radicle protrusion stage, and seedlings. **J, K, L.** ICA levels detected in aged seeds, seeds at the radicle protrusion stage, and seedlings. Data were analyzed through one-way ANOVA (LSD test with $p \leq 0.05$ and FDR ≤ 0.05). UP, unprimed. HP, hydroprimed. SP, spermidine. AA, artificially aged. UP-AA, HP-AA, SP-AA, aged dry seeds. UP-AA-Rh, HP-AA-Rh, SP-AA-Rh, artificially aged seeds at 4 h of post-priming/ageing imbibition. UP-AA-RD, HP-AA-RD, SP-AA-RD, radicle protrusion stage. The assay was performed on both normal (Nor) and aberrant (Abr) aerial parts (Ar) as well as on normal and aberrant roots (R).

Exogenous spermidine triggers changes in nucleic acid metabolites

The levels of xanthine, uric acid (the final product of purine metabolism), and allantoin (the oxidation product of uric acid) exhibited changes in aged seeds, during the radicle protrusion phase, and in seedlings (**Figure 4.11**). The degradation product of the purine nucleotide xanthine, facilitated by xanthine oxidase, yields uric acid, which is subsequently transformed into allantoin by the enzyme uricase (Lee et al., 2013). **Figure 4.11A** shows that xanthine accumulates in UP-AA, HP-AA, and SP-AA seeds due to aging, with the highest accumulation observed in HP- and SP-treated seeds. During radicle protrusion, only exogenous spermidine induced xanthine accumulation (**Figure 4.11B**). Xanthine was accumulated in the aberrant aerial parts of seedlings originating from UP-AA and HP-AA seeds, as well as in the aberrant roots of seedlings generated from UP-AA seeds (**Figure 4.11C**). The highest accumulation of uric acid was observed in UP seeds both before and after aging. A similar trend was observed during radicle protrusion (**Figure 4.11D, E**). Uric acid concentrations elevated in the aberrant aerial part of seedlings originating from UP-AA and HP-AA seeds (**Figure 4.10F**). The data indicate a role of uric acid in UP seeds throughout the imbibition and radicle protrusion stages, as well as in aberrant aerial parts. UP-AA and HP-AA seeds accumulated allantoin, while exogenous spermidine (SP-AA) did not trigger this response (**Figure 4.11G**). During radicle protrusion, allantoin concentration was elevated in both UP-AA-RD and SP-AA-RD seeds (**Figure 4.11H**). The highest concentrations of allantoin were seen in the aberrant aerial parts of seedlings originating from UP-AA and HP-AA seeds. Conversely, allantoin concentrations were lower in all analyzed roots, with the most significant reduction in both normal and aberrant roots of seedlings originating from SP-AA seeds (**Figure 4.11I**).

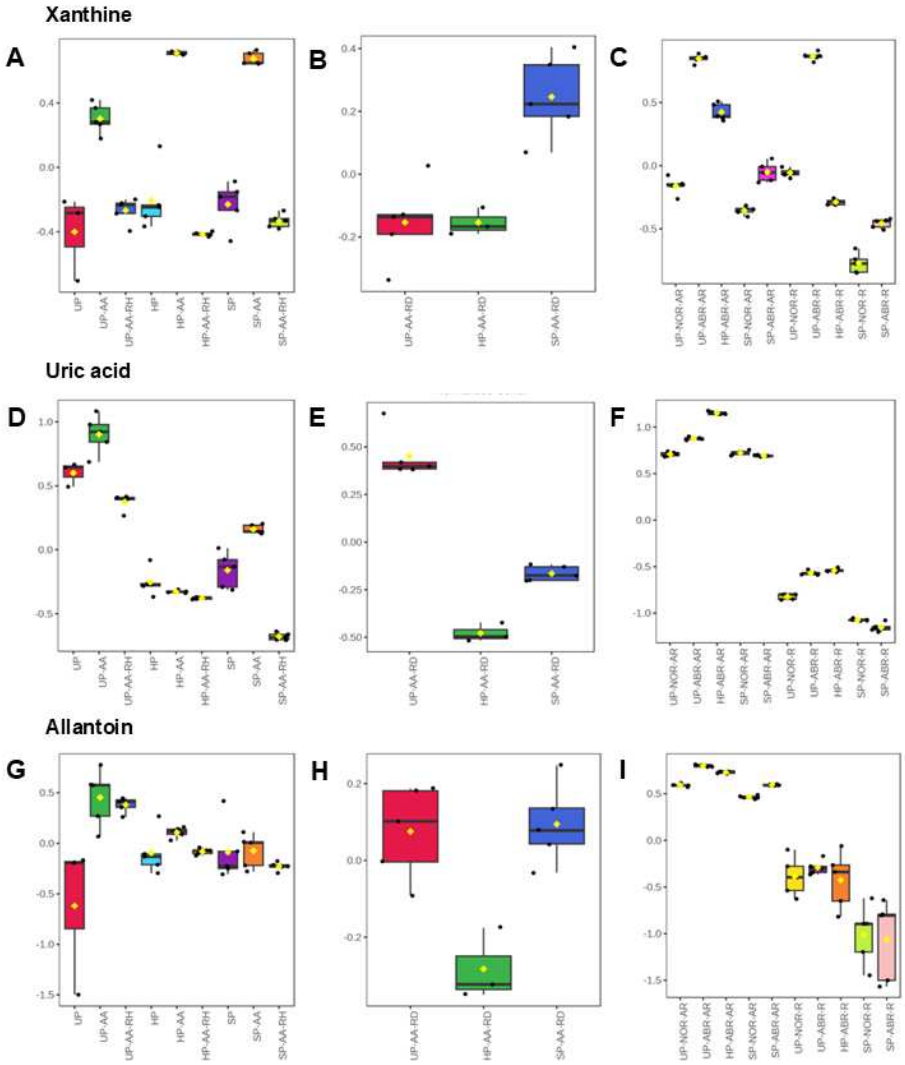

Figure 4.10 Impact of exogenous spermidine on the profiles of nucleic acid metabolites in *M. truncatula* seeds and seedlings. Relative concentration of xanthine, uric acid, and allantoin concentrations are reported **A, B, C.** Xanthine levels detected in aged seeds, seeds at the radicle protrusion stage, and seedlings. **D, E, F.** Uric acid levels detected in aged seeds, seeds at the radicle protrusion stage, and seedlings. **G, H, I.** Allantoin levels detected in aged seeds, seeds at the radicle protrusion stage, and seedlings. Data were analyzed through one-way ANOVA (LSD test with $p \leq 0.05$ and FDR ≤ 0.05). UP, unprimed. HP, hydroprimed. SP, spermidine. AA, artificially aged. UP-AA, HP-AA, SP-AA, aged dry seeds. UP-AA-Rh, HP-AA-Rh, SP-AA-Rh, artificially aged seeds at 4 h of post-priming/ageing imbibition. UP-AA-RD, HP-AA-RD, SP-AA-RD, radicle protrusion stage. The assay was performed on both normal (Nor) and aberrant (Abr) aerial parts (Ar) as well as on normal and aberrant roots (R).

4.4 Discussion

The poor longevity of primed seeds hinders the advancement of novel, 'next-generation' seed priming techniques necessary for promoting the adoption of sustainable, climate-resilient agroecosystems. The current initiatives concentrate on developing customized treatments to alleviate the often-encountered genotype- and seed lot-dependent variability that illustrates the intricacy of molecular networks governing the seed repair response (Pagano et al., 2023). The antioxidant response and DDR-mediated genome maintenance mechanisms are essential for seed viability, successful germination, and enhanced stress tolerance in seedlings. Numerous studies have emphasized the significance of ROS scavenging activities in contrast to regulated ROS production, as well as the role of lesion-specific DNA repair mechanisms during the temporal phase when pre-germinative metabolism is initiated (Waterworth et al., 2019; Kiran et al., 2020). This context is essential for defining the successful seed response to priming, achieved by identifying optimal conditions to enhance seed metabolism while mitigating adverse effects, such as oxidative stress, which can ultimately result in DNA damage (Waterworth et al., 2019; Gupta et al., 2020; Griffo et al., 2023; Pagano et al., 2023). The most successful seed priming treatments could enhance the competitiveness by ensuring prolonged survival of treated seeds during long-term storage. This challenge can be tackled by enhancing the existing understanding of the seed repair response by specialized experimental systems that not only replicate the impacts of long-term storage (e.g., through artificial aging) in primed seeds but also deliver an optimal environment for evaluating innovative 'anti-aging' solutions (Sano and Seo, 2019; Fabrissin et al., 2021; Gaonkar et al., 2024). This is the methodology developed and evaluated in the current study, utilizing the model legume *M. truncatula*. The performance of unprimed aged seeds was compared to that of hydroprimed seeds and spermidine-primed seeds to assess the efficacy of spermidine as an anti-aging agent. According to the reported data, spermidine successfully restored the viability of compromised hydroprimed seeds. However, these protective effects diminished during seedling development, resulting in a high incidence of abnormal phenotypes. This study highlights the intricacy of molecular events and regulatory mechanisms at the intersection of the two separate genetic programs: germination and seedling development (Waterworth et al., 2022; Rehmani et al., 2023).

M. truncatula was utilized to investigate the longevity of primed seeds, serving as a model legume suitable for translational research in seed biology (Bandyopadhyay et al., 2019). Its response to seed priming has been previously characterized, focusing on genome maintenance and antioxidant response (Macovei et al., 2011; Macovei et al., 2016; Pagano et al., 2017; Pagano et al., 2018; Pagano et al., 2022a, 2022b; Pagano et al., 2023). To characterize the experimental system, germination performance was assessed based on germination percentage and speed. The method used for

accelerated aging protocol (48 °C and 95% relative humidity for 24 h) was adapted from Gaonkar et al. (2024) which successfully reduced the germination percentage for all priming treatments and the unprimed control, without completely compromising seed viability, allowing the assessment of germination and seedling growth parameters. It is worth noting that the germinability of spermidine-treated, aged (SP-AA) seeds was significantly higher than that of hydroprimed, aged (HP-AA) seeds.

Several reports highlight the beneficial role of spermidine in mitigating the detrimental effects of aging on seed viability. Hu et al. (2020) investigated the effects of exogenous spermidine on rice seeds subjected to accelerated aging, demonstrating that the treatment enhanced gibberellic acid content and increased the activity of the CAT and APX antioxidant enzymes. Exogenous spermidine facilitated the germination of aged sorghum seeds by elevating soluble sugar levels through the enhancement of amylase and sucrose invertase activities, while also improving the activities of critical enzymes in glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway (Zhang et al., 2022). Xu et al. (2021) underlined an intriguing aspect related to the ability of spermidine in enhancing the viability of aged primed seeds. These authors administered spermidine to high- and low-quality rice seeds to evaluate the efficacy and the effect of priming on seed longevity. According to the germination profiles, extended seed longevity was noted only in low-quality seeds treated with spermidine, while the viability of high-quality seeds was significantly compromised by aging. A recent study utilized *M. truncatula* seeds to evaluate the efficacy of quercetin- and rutin-mediated seed priming in enhancing viability under artificial aging. Both compounds effectively alleviated the effects of post-priming aging on seed viability, germination performance, and seedling phenotype by enhancing the seed antioxidant response (Gaonkar et al., 2024).

DDR genes are essential in seed aging and can influence seed vigor and germination (Waterworth et al., 2019). Delayed germination is associated with an extended duration of pre-germinative DNA repair triggered in the initial phases of imbibition (Elder and Osborne, 1993). In *Arabidopsis thaliana*, cytological analyses have demonstrated a relationship between decreased vigor and viability in seed aging and the DNA damage accumulation, leading to enhanced occurrence of aberrant seedlings (Waterworth et al., 2015). Similar results have been observed in this study performed in *Medicago truncatula* where the concomitant increase of aberrant seedlings and DNA damage were observed. Particularly, seedlings developed from HP seeds, exhibited a total absence of normal seedlings. This may result from significant DNA damage accumulation, as demonstrated by comet assay, alongside the high DDR gene expression. The role of spermidine in the context of DDR remains poorly explored. Spermidine has been documented to reduce DNA damage in adipose stem cells (Minguzzi et al., 2019), mouse fibroblasts (Rider et al., 2007) and protect from DNA mutations in bacteria (Pillai and Shankel, 1997). In *M. truncatula* seeds, spermidine treatment significantly decreased

DNA damage following priming and aging. Similar results were observed in maple seeds where treatment with spermidine resulted in reduced levels of DNA damage accumulation, as assessed through comet assay and 8-oxoG levels (Fuchs et al., 2023). Exogenous spermidine may trigger DNA repair mechanisms that remove oxidative damage but at the same time the antioxidant properties of this polyamine may contribute to protect DNA from ROS (Fuchs et al., 2023). Spermidine was found to mitigate DNA oxidative damage in wheat (Taie et al., 2019).

As the aged *M. truncatula* seeds approached germination, the protective effect of spermidine in terms of protection against DNA slightly decreased. This protective effect persisted during the radicle protrusion stage (RD) whereas a progressive decrease was observed in seedling development. Although the DNA damage levels in seedlings developed from spermidine-treated seeds were significantly lower compared to those observed in seedlings developed from HP seeds, it seems that this protective effect was not sufficient to guarantee normal seedling development. The reported data are in agreement with the results by Waterworth et al. (2022), who demonstrated that Arabidopsis seeds exhibit high tolerance to genotoxic stress whereas this feature diminishes upon the initiation of the seedling developmental program, resulting in the occurrence of aberrant phenotypes.

The observed gain and loss in spermidine-mediated protective effects associated with the two distinct genetic programs, namely seed germination and seedling development, raise compelling research questions: is spermidine exclusively active during the germination phase? Which are the factors that contribute to the failure of spermidine protective function during seedling development? What are the molecular targets or cellular processes implicated in these responses? What is the relationship between spermidine and DDR in seeds?

The MRN complex, consisting of the MRE11, NBS1, and Rad50 subunits, plays a pivotal role in the detection of DSBs and homologous recombination as well as the recruitment of ATM protein kinase at the damaged sites (Harper and Elledge, 2007; Manova and Gruszka, 2015). The *MtNBS1* and *MtRad50* genes, coding for the NBS1 and Rad50 subunits of the DSBs sensor complex MRN, were significantly up-regulated in HP and SP *M. truncatula* seeds in response to aging and imbibition. At the radicle protrusion stage, both genes were still significantly up-regulated in HP seeds whereas only the *MtRAD50* transcript was accumulated in SP seeds. During seedling development, The *MtNBS1* and *MtRad50* genes showed a significant upregulation in the aerial parts of aberrant seedlings developed from HP and SP seeds. The *MtOGG1* gene encodes the 8-oxoguanine glycosylase/lyase enzyme, essential for the base excision repair (BER) pathway that facilitates the removal of oxidative DNA damages (Zharkov et al., 2000). *MtOGG1* gene upregulation was observed in both HP and SP aged seeds. However, at the radicle protrusion stage and during seedling development the upregulation of *MtOGG1* gene

was detected only in HP seeds and seedlings, possibly suggesting for a repair response associated with enhanced oxidative DNA damage accumulation in HP seeds. It has been reported that DNA ligase IV plays distinct roles in the NHEJ pathway for DSBs repair (Critchlow et al., 1997). Various DNA ligases facilitate the rejoining of single-strand breaks (SSBs) and DSBs, a crucial process in DNA repair, however, DNA ligase IV is a primary predictor of seed quality and longevity (Waterworth et al., 2010). The expression profiles of *MtLigIV* gene closely resembled those of *MtOGG1* gene in aged *M. truncatula* seeds during imbibition and at the radicle protrusion stage. Upregulation of *MtLigIV* gene was identified in aberrant seedlings developed from HP and SP seeds. It is worth noting that the previous report by Pagano et al. (2017) has showed the upregulation of *MtOGG1* and *MtLigIV* genes in response to DNA damage accumulation, in *M. truncatula* seeds exposed to TSA-mediated genotoxic stress. The RAD51 protein, essential for HR-mediated DNA repair, facilitates the essential strand-invasion phase, wherein the resected 3′ single-stranded DNA end aligns with a homologous template, ensuring the accurate positioning of broken DNA strand overhangs (Shinohara et al., 1992; Yu et al., 2023). Significant upregulation of *MtRAD51* gene was observed in HP and SP seeds in response to aging and post-aging imbibition, respectively. The expression of *MtRAD51* gene was exclusively detected in HP seeds during radicle protrusion, with a similar pattern observed in seedlings. DNA damage must be repaired immediately during imbibition before cell division starts, to safeguard genome stability, germination potential and reduce mutagenesis in later seedling development (Waterworth et al., 2016). Even though DDR is a highly conserved mechanism among eukaryotes, plants evolved to have a unique set of cell cycle checkpoint regulators (Hu et al., 2016). The progression of cell cycle through the different phases is stringently regulated by cyclin dependent kinases (CDKs) which are activated upon phosphorylation and when in complex with the appropriate cyclins (Gentric et al., 2021). CYCB1 plays a role in the G2/M transition, it is often used as a marker for cell proliferation and it has been identified as a major HR regulator. The *CYCB1* gene is significantly upregulated in cells with DNA damage. The RAD51 protein, a fundamental HR mediator, is a substrate of CDKB1-CYCB1 complexes (Weimer et al., 2016; Schnittger and De Veylder, 2018). Additional measured cyclins are *CYCA3*, *CYCD2*, and *CYCD3*. All cyclins in seeds were exclusively increased in primed seeds. *CYCA3* was increased in UP and HP seeds during radicle protrusion. All cyclins were elevated in the aberrant seedlings derived from HP and SP seeds during seedling development.

Spermidine and spermine are produced through the sequential insertion of aminopropyl groups, facilitated by the enzymes Spd synthase (SPDS) and Spm synthase (SPMS), respectively (Marco et al., 2011). Drought stimulates the expression of *SPDS1* and *SPMS* genes in Arabidopsis (Alcázar et al., 2006). Similar patterns were observed in *M. truncatula* HP seeds at the radicle protrusion stage and in the aerial part of aberrant seedlings developed from HP seeds. Results from untargeted metabolomics highlighted the dynamic alterations of three polyamines: cadaverine, agmatine and putrescine in

response to the applied treatment and depending on the developmental phase. During germination, a decrease in cadaverine, agmatine and putrescine content was detected in both UP-AA and HP-AA seeds, consistent with the findings of Hofer et al. (2022) and earlier research conducted on wheat (Anguillesi et al., 1990), barley (Christiansen and Gregersen, 2014), and *Cariniana legalis* (Lerin et al., 2022). These findings highlight the influence of exogenous spermidine application on the endogenous polyamine metabolism of *M. truncatula* seeds and seedlings. The results hereby obtained in *M. truncatula* align with studies conducted on rice (Roychoudhury et al., 2011; Chen et al., 2021), tomato (Hu et al., 2012), and *Agrostis stolonifera* (Li et al., 2014), indicating that application of exogenous spermidine triggers accumulation of endogenous polyamines. An interesting finding is related to the GABA profiles observed in *M. truncatula* seeds and seedlings. The tetra-carbon non-proteinogenic amino acid GABA functions as a signaling molecule, influencing different aspects of plant growth and development, as well as responses to biotic and abiotic stress in several plant species (Xu et al., 2022). GABA is mostly metabolized by the GABA shunt pathway, however under stressful conditions, it can be produced through a non-enzymatic mechanism using polyamines such spermidine and putrescine, in conjunction with pyrolline (Signorelli et al., 2015; Ansari et al., 2021). Putrescine can subsequently be converted into GABA through a reaction catalyzed by diamine oxidases. Thus, GABA levels are somewhat contingent upon alterations in polyamine metabolism (Xu et al., 2022). The reported data showed that exogenous spermidine did not influence the GABA content in *M. truncatula* seeds, while spermidine induced GABA accumulation during radicle protrusion and in roots. GABA has been linked to aging in humans (Chamberlain et al., 2021; Zuppichini et al., 2024) and in plants (Khan et al., 2021). Based on these studies, the potential role of GABA as mitigating agent against the deterioration of primed seeds should be better investigated.

The beneficial effects of spermidine on antioxidant activities have been thoroughly investigated in multiple species, including tomato (Zhang et al., 2014), alfalfa (Lou et al., 2018), rice (Hu et al., 2020), mung bean (Zhou et al., 2020), and cucumber (Korbas et al., 2022). The antioxidant metabolites exhibiting dynamic changes in *M. truncatula* seeds and seedlings include ascorbic acid and dehydroascorbic acid (the oxidized form of ascorbic acid and its stable degradation product) (Dewhirst et al., 2017). Zechmann (2018) demonstrated that ascorbate and its redox state play a significant role in the regulation of plant growth, development, and defense. Ascorbic acid specifically affects stress responses in plants (Akram et al., 2017; Kakan et al., 2021). The ascorbic acid-dehydroascorbic acid cycle is essential for plant growth and development (Suekawa et al., 2018). On the contrary, the accumulation of ascorbic acid and dehydroascorbic acid led to significant physiological damage, primarily due to excessive ROS production caused by an imbalance between excess energy accumulation in photosystems and low CO_2 absorption due to reduced stomatal conductance (Castro et al., 2018). In the *M. truncatula* experimental system, Ascorbic acid accumulated in *M.*

truncatula spermidine-treated, aged seeds during imbibition and at the radicle protrusion stage. A similar response was documented in lettuce (Li et al., 2020) and white clover (Li et al., 2024). Dehydroascorbic acid was detected in *M. truncatula* seeds at the radicle protrusion phase and in seedlings. Dučić et al. (2002) demonstrated that ascorbic acid was present in detectable amounts solely in *Chenopodium rubrum* seeds prior to radicle emergence, whereas dehydroascorbic acid was identified throughout the germination process. Further work is required to determine the involvement of dehydroascorbic acid in germination, especially in the context of pre-germinative metabolism. The increase of ascorbic acid levels in SP-AA seeds suggests a potential connection between spermidine and ascorbic acid in alleviating the adverse effects of seed aging. Ascorbic acid-mediated seed priming of oat and *Elymus sibiricus* seeds, followed by artificial aging, was able to mitigate the effects of aging (Xia et al., 2020; Yan et al., 2015), underscoring the role of ascorbic acid as a viable candidate for strategies aimed at protecting primed seeds from deterioration. Citric acid is a 6-carbon tricarboxylic acid synthesized through the condensation of oxaloacetate and acetyl-CoA, catalysed by the enzyme citrate synthase. Isocitric acid is produced from citric acid by the enzyme aconitase. Both metabolites are components of the mitochondrial tricarboxylic acid cycle (Goldberg and Rokem, 2009; Tahjib-Ul-Arif et al., 2021). The administration of exogenous citric acid safeguarded plants by enhancing the activity of antioxidant defense mechanisms, as evidenced in *Solanum nigrum* (Gao et al., 2010), *Brassica napus* (Ehsan et al., 2014), soybean (Imran et al., 2023), and *Capsicum annuum* (Ferdous and Akter, 2023). The metabolomics analysis performed on *M. truncatula* seeds and seedlings in the present work showed that application of exogenous spermidine significantly enhanced antioxidant activity in the ascorbate-glutathione pathway more than the processes involving citric acid and isocitric acid.

The identification of metabolite that could be used as potential markers of genotoxic stress during seed germination is still a complex issue. Nevertheless, metabolites associated with the nucleotide super-pathway linked to DNA damage caused by sodium butyrate were identified in *M. truncatula* seeds (Pagano et al., 2018). Purine catabolism may have a role in the plant stress response and adaptability to environmental stress. A study in Arabidopsis demonstrates that impairment of purine catabolism, due to the downregulation of the essential enzyme xanthine dehydrogenase, reduces the lifespan of Arabidopsis under drought conditions and reduces tolerance to oxidative stress (Watanabe et al., 2014). Xanthine is an intermediate in purine metabolism and its overaccumulation is used as a hallmark of DNA damage in irradiated animal cells (Menon et al., 2016). According to the reported data, xanthine accumulation increased in *M. truncatula* seeds with aging, particularly in primed seeds. This accumulation was observed in SP-treated seeds at radicle protrusion stage. However, during seedling development, reduced xanthine levels were observed in seedlings developed from primed seeds. The observed changes in xanthine levels may be associated with

genotoxic damage, however comprehensive investigations are required to substantiate this hypothesis. Uric acid, generated by xanthine oxidoreductase, show both antioxidant and pro-oxidant characteristics *in vitro* by both ROS scavenging and production (Ames et al., 1981; Sautin et al., 2007; Kang and Ha, 2014). Intracellular uric acid typically exerts harmful effects as a pro-oxidant in cultured cells and animal models of hyperuricemia. Conversely, uric acid functions as an antioxidant only in a hydrophilic environment (Kang & Ha, 2014). Uric has been reported to reduce chloroplastic H_2O_2 in Arabidopsis leaves (Ma et al., 2016). In animal cells, DNA damage induces the activation of Transforming growth factor-β activated kinase 1(TAK1), NF-κB, and the MAP kinase pathways (Mukhopadhyay and Lee, 2019), and recent reports indicate that uric acid can stabilize TAK1 in an active-state conformation, leading to prolonged TAK1 kinase activation (Singh et al., 2019). Allantoin, formed by the oxidative degradation of uric acid in the late stages of purine catabolism (Kim et al., 2007), acts as a signaling molecule to activate stress-responsive genes and ROS scavenging enzymes (Kaur et al., 2023). Exogenous application of allantoin enhanced stress tolerance of Arabidopsis seedlings exposed to NaCl, and this effect was linked to reduced production of superoxide and hydrogen peroxide, as well as modified expression of several antioxidant genes (Irani and Todd, 2017). Exogenous allantoin pretreatment in *Beta vulgaris* seedlings exposed to salt stress increased putrescine levels in response to increased exogenous doses. The allantoin pretreatment also enhanced leaf, root, and overall plant biomass (Liu et al., 2020). In *M. truncatula* seeds, allantoin accumulation was detected in response to spermidine treatment only at the radicle protrusion stage.

Overall, the reported data provide an original picture of the seed response to spermidine treatment in the model legume *M. truncatula*, contributing to define the role of key players of the pre-germinative metabolism, namely the antioxidant response and the DNA damage response. Such crucial determinants of seed quality were investigated in the context of seed longevity, specifically focusing on the response of primed seeds to ageing. Molecular responses analyzed at the level of gene expression and DNA damage profiles were integrated with seed and seedling metabolome analysis, adding relevant information on the role played in this context by specific metabolites. Farmers must be informed of the seed shelf life to effectively manage the seed lot after harvest; seed marketing and distribution organizations typically possess this essential information. Research on the regulation of seed deterioration and the preservation of seed vitality enhances both production and storage efficacy, while also informing effective management and conservation strategies for seeds in long-term storage, which hold substantial biological and economic importance.

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Chapter 5. Spermidine treatments enhance germination of naturally aged *Populus alba* clone 'Villafranca' seeds

This article has submitted to the journal *Annals of Forest Science* with the title "Spermidine treatments improve germination of long-term stored seeds of *Populus alba* clone 'Villafranca'"
My contribution to this article includes *i)* seed germination and phenotypic analyses of aged *P. alba* clone 'Villafranca' seeds subjected to hydropriming and presoaking treatments *ii)* TTC viability assay, *iii)* DCFH-DA assays performed to assess ROS profiles iv) qRT-PCR to assess gene expression profiles. I also performed statistical analysis of the resulting data and actively participated in their critical assessment and scientific discussion.

Abstract

Populus alba clone 'Villafranca' (white poplar), which is extremely suitable for biomass production and ecosystem restoration, has served as a model system in previous molecular and physiological research in forest species; nevertheless, findings addressing seed quality issues are still scanty. While clonal propagation of poplars is the preferred method for commercial applications, it is imperative to address the potential loss of genetic variability within the current germplasm. To tackle this issue, new populations should be cultivated starting from seeds. Poplar seeds have low germinability and viability when stored. This work aims to develop customized treatments to enhance the germination of long-term stored, deteriorated white poplar seeds, serving as a model to examine seed quality issues in forest tree species. Protocols for priming and soaking, utilizing water or spermidine (Spd, 50 and 100 µM), were evaluated on white poplar seeds. The efficiency of treatments was evaluated based on germination performance, reactive oxygen species (ROS) profiles, and gene expression patterns. The soaking for 4 h with 100 µM Spd significantly improved germination percentage and speed. Reduced ROS levels were observed in Spd-treated seeds in comparison to water-soaked seeds. Enhanced expression of genes associated with desiccation tolerance acquisition, polyamine production, and antioxidant defense was noted exclusively in dry seeds. We report the successful development of a treatment that enhances the germination of long-term stored, deteriorated 'Villafranca' seeds, as a solution to mitigate present seed quality challenges in poplars.

5.1 Introduction

The genus *Populus* spp. has approximately thirty species, including poplars, aspens, and cottonwoods (Eckenwalder, 1996). Poplars have an important impact on both the economy and the environment. Among the wooden species,

they are distinguished by their rapid growth, useful for sustainable biomass production. Their adaptability enables growth on infertile soils, hence reducing soil erosion through complex root systems (Pellegrino et al., 2011; Cantamessa et al., 2022). Additional characteristics relate to the removal of airborne particulate matter and the sequestration of soil metals by phytoremediation (Nissim et al., 2023). In Europe, poplar plantations cover almost one million hectares, with France, Turkey, Italy, Spain, and Hungary as the largest producers. Hybrid poplar plantations in Italy provide the principal source of industrial timber production, accounting for almost 50% of the domestic supply of industrial hardwood (Pra and Pettnella, 2019).

Populus alba, commonly known as white poplar, is indigenous to central and southern Europe (Cadullo and de Rigo, 2016) and primarily utilized for soil erosion control and biomass production. The need to acclimate the species to harsh climatic conditions and meet the demands of the industry has resulted in the development of numerous clones. Among these, *Populus alba* clone 'Villafranca' was developed by the Forest and Wood Research Center of Casale Monferrato (Italy) in 1957. This clone is characterized by good quality wood with a basal density of 0.33 g/cm^3, optimal vegetative propagation and the ability to quickly root in soil (Cadullo and de Rigo, 2016; Corona et al., 2016).

Clonal propagation is crucial for commercial timber production, resulting in enhanced productivity and uniformity in plantations (Mushtaq et al., 2017). Despite this advantage, clonal propagation has markedly diminished genetic variability, which has become essential for maintaining crop yields in the context of climate change. Global environmental initiatives promote extensive ecological restoration as nature-based solutions (NBS) to combat land degradation and biodiversity loss. In this context, seeds are crucial as they are the main source for producing plants for extensive restoration initiatives. Understanding the ideal circumstances for seed germination and seedling establishment is essential for ecological restoration and biodiversity conservation (Dalziell et al., 2022). Due to the preference for vegetative propagation in poplars, research on seed germination has been limited. Additionally, poplar seeds possess a rather short lifespan (Kim, 2018). Extensive studies have been conducted on the physiological, molecular and morphological aspects of germination and post-germination processes in model organisms and crops but for woody species a major gap of knowledge is underlined (Qu et al., 2020).

Long-term seed storage is the most efficient method for preserving the genetic variability of plants. Storage parameters affecting seed quality including temperature, moisture content, duration, and oxygen pressure. However, seed desiccation tolerance (DT) is a vital endogenous factor (Kijak & Ratajczak, 2020). According to DT, seeds are classified into three storage categories: (1) orthodox seeds, which can withstand low moisture levels and are suitable for long-term storage; (2) recalcitrant seeds, which are highly vulnerable to desiccation and freezing, resulting in a swift decline in viability; (3) intermediate seeds, which possess viability characteristics that are intermediate between orthodox and recalcitrant seeds (Tweddle et al., 2003). In the past, poplar seeds were categorized as either recalcitrant due to their brief lifespan (Gosling, 2007) or as

sub-orthodox due to their ability to retain viability for prolonged storage at low temperatures (Bonner, 2008). According to a recent categorization, numerous poplar species are classified as intermediate seeds (Michalak, 2014).
Seed priming, a pre-sowing technique that entails soaking seeds in water or other priming agents followed by drying to restore original moisture content, represents a promising solution to mitigate seed quality issues in poplars. The benefits of seed priming, including higher germination and increased tolerance to both abiotic and biotic stresses, are extensively demonstrated across various species (Bento et al., 2021; Zulfiqar et al., 2022; Bibi et al., 2024; Dueñas et al., 2024; Gaonkar et al., 2024). Priming techniques are typically regarded as empirical and are dependent on species, genotype, and even seed lot (Paparella et al., 2015; Pagano et al., 2023). When developing priming protocols, the seed response concerning DT should also be taken into account (Smolikova et al., 2020; Pagano et al., 2022a, 2022b). Polyamines (PAs) function as priming agents (Kusano et al., 2008; Lechowska et al., 2021; Shao et al., 2022). PAs are small nitrogenous bases with several amino groups, present in nearly all eukaryotic and prokaryotic species. Putrescine (Put), spermidine (Spd), and spermine (Spm) are the primary polyamines identified in plants. They are integral to various biological processes, including flower and fruit development, embryogenesis, organogenesis, senescence, and responses to abiotic and biotic stress (Li et al., 2014; Huang et al., 2017; Paul and Roychoudhury, 2017; Chen et al., 2019; Hu et al., 2020; Zhang et al., 2022; Fuchs et al., 2023). Recent research has evidenced the beneficial impacts of Spd on seed germination under stress in white clover (Li et al., 2014), rice (Paul and Roychoudhury, 2017), corn (Huang et al., 2017), wild rye (Hongna et al., 2021), sorghum (Zhang et al., 2022), and maple (Fuchs et al., 2023). Polyamines, especially spermidine, have a significant role in the antioxidant response, which is crucial for seed viability (Zhang et al., 2014; Lou et al., 2018; Hu et al., 2020; Zhou et al., 2020; Korbas et al., 2022). The increase of reactive oxygen species (ROS) leads to seed deterioration during storage (Li et al., 2022), however, ROS can function as signaling molecules that facilitate germination. Bailly et al. (2008) characterized the phase known as 'oxidative window' as the pivotal threshold of reactive oxygen species (ROS) buildup that permits their role as signaling molecules, while preventing oxidative stress.

Poplar seeds have a short lifespan and have been previously categorized as either recalcitrant or intermediate, due to their limiting dehydration tolerance, elevated water content at maturity, and heightened metabolic activity at dehiscence, which results in a reduced survival duration in storage (Gosling, 2007; Michalak et al., 2014; Lefebvre et al., 2021). This reduced seed viability substantially hinders breeding and genetic conservation initiatives. The objective of this study was to develop customized seed priming techniques to enhance the germination efficacy of long-term stored, deteriorated *Populus alba* clone ‘Villafranca’ seeds, utilized as a model system. Seed viability and the dynamics of rehydration-dehydration cycles were assessed using spermidine as a priming agent, in parallel with water imbibition and untreated controls. Phenotypical analysis (germination % and speed) and molecular studies (ROS detection, gene expression profiles) were utilized to evaluate the efficacy of the treatments.

5.2 Material and methods

Seed material and storage conditions

Seeds of *Populus alba* clone 'Villafranca' were obtained from the Forestry and Wood Department of CREA (Council for Agricultural Research and Analysis of Agricultural Economics), Casale Monferrato, Italy. The seeds were collected in April 2008 at the Institute's facility. The seeds were dried in thermostatic chambers at controlled temperature, and then vacuum-packed at -30°C. The seed samples were then stored at 4°C.

Germination tests

Poplar seeds were transferred to Petri dishes (diameter 90 mm) containing two filter papers moistened with 2.5 ml H_2O, sealed with parafilm and kept in a growth chamber at 22°C under light conditions with photon flux density of 150 µmol m^{-2} s^{-1}, photoperiod of 16 h/8 h and 70-80% relative humidity (RH) inside the containers. Seeds with protrusion of the primary radicle were considered germinated. Germination tests were performed in triplicates of 30 seeds each. Germination was monitored for four days and germination parameters were calculated (Pagano et al., 2022a). For the evaluation of germination performance, the following parameters were used: germinability (G%) and time to reach 50% of final germinants (T_{50}). G% is defined as the percentage of germinated seeds at the end of the germination test. T50 was calculated using the following formula: $T_{50} = Ti + (N/2 - Ni) (Tj - Ti) / (Nj - Ni)$, where N is the final number of germinated seeds, N/2 is half of final number of germinated seed, Ni and Nj are the total number of seeds germinated in adjacent counts at time Ti and Tj.

Viability assay

To measure seed viability, poplar seeds were first imbibed in H_2O for 6 h to remove seed coat. The de-coated seeds were then incubated in a 1% (w/v) solution of 2,3,5-triphenyl tetrazolium chloride (TTC; Merck, Darmstadt, Germany), and maintained at 20°C for 18 h in the dark (Faria et al., 2005). The TTC test relies on the activity of dehydrogenase enzymes in mitochondria. TTC penetrates into the seed tissues where it interferes with the reduction processes of the living cells by accepting a hydrogen ion. In the reduced form, the TTC-salt is a red-colored, stable, non-diffusible substance called triphenylformazan or formazan (De Barros França-Neto and Krzyzanowski, 2019). Stained tissues are considered viable, and unstained white tissues are considered dead. The TTC test was carried out using five replicates of 10 seeds per treatment.

Treatments

The seed response along the rehydration-dehydration cycle was monitored by measuring the seed weight, thus producing imbibition and dehydration curves. Water uptake and loss (in presence/absence of Spd) were evaluated along multiple imbibition (1, 2, 3, 4 and 5 h) and dehydration (1, 2 and 3 h) timepoints

by measuring the weight of 100 seeds at each time point. All measurements were performed in triplicates.

Seed treatments were devised as follows: seed priming in water (HP, hydropriming), seed presoaking in the presence/absence of Spd. For the HP treatments, seeds were imbibed in water for 2, 4, 6, and 8 h (HP2, HP4, HP6, HP8). For each imbibition treatment seeds were then dried-back (DB) at 25 °C for 1, 2, 3 and 4 h (DB1, DB2, DB3, DB4). For the presoaking treatments, seeds were imbibed in H2O for 1, 2, 4, and 6 h, without the DB step. For the Spd treatments, two Spd concentrations (50 µM, Spd50; 100 µM, Spd100) were applied for 1 h and 4 h, in parallel with untreated controls (UT). After each treatment, germination tests were conducted and monitored as described in paragraph 2.2.

ROS quantification

ROS levels were quantified in dry and imbibed seeds collected at the indicated timepoints, using the fluorogenic dye 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, Milan, Italy). The dye is converted to a non-fluorescent molecule following deacetylation mediated by cellular esterases, and it is subsequently oxidized by ROS into the fluorescent compound 2',7'-dichlorofluorescein. DFC can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm, respectively (LeBel et al., 1992). The assay was carried out as described by Griffo et al. (2023), with the following modifications. Samples of 40 seeds per time point were incubated for 60 min with 50 µl of 10 µM DCFH-DA and subsequently fluorescence was determined at 517 nm using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia), setting the program for one cycle of 30 s at 25°C. As negative control, a sample containing only DCFH-DA was used to subtract the baseline fluorescence. The fluorescence was calculated by normalizing samples to controls and are expressed as Relative Fluorescence Units (R.F.U.).

RNA extraction and cDNA synthesis

Seeds imbibed with water and Spd were collected at different imbibition time (0, 1, 4 h) and DB timepoints (1, 2 h) for molecular analyses. Seed aliquots (80-90 mg) were grinded in liquid N_2 and collected into 1.5 ml Eppendorf tubes. RNA was extracted as described by Oñate-Sánchez and Vicente-Carbajosa (2008) with the following modifications. A volume of 550 µl extraction buffer (0.4 M LiCl, 0.2 M Tris pH 8, 25 mM EDTA, 1% SDS) and 550 µl chloroform, were added to each Eppendorf tube containing approx. 50 mg of seed powder and vortexed for 10 s. The samples were centrifuged at 10.000 rpm for 3 min at 4°C. The upper phase was collected and transferred to new 1.5 ml Eppendorf tubes, 500 µl of water-saturated acidic phenol was added and vortexed for 10 s. A volume of 200 µl chloroform was added to the sample, gently mixed, and centrifuged at 10.000 rpm for 3 min at 4°C. The upper phase was collected and transferred to new 1.5 ml Eppendorf tubes and a 1/3 volume of lithium chloride was added. The samples were incubated at 4°C for 60 min then centrifuged at 10.000 rpm for 30 min at

4°C. Supernatant was discarded, the RNA pellet was washed with 100 µl ice-cold 70% ethanol and centrifuged at 10.000 rpm for 1 min at 4°C, followed by one additional wash with 100 µl ice-cold 100% ethanol. The RNA pellet was air dried and resuspended in nuclease-free H_2O. The concentration of each RNA sample was measured using a Biowave spectrophotometer (Biochrom Ltd., England), and RNA integrity was assessed using agarose gel electrophoresis. For cDNAs synthesis, the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Monza, Italy) was used, according to the manufacturer's recommendations.

Quantitative Real-time PCR (qRT-PCR)

The qRT-PCR reactions were carried out using a CFX Duet Real-time PCR system machine (Bio-Rad Laboratories Inc., Milan, Italy) and the Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Monza, Italy), as indicated by the manufacturer. The machine is operated using the Bio-ad CFX maestro software (Bio-Rad Laboratories Inc.) and the following amplification protocol was applied: denaturation at 95°C, 10 min, and 40 cycles of 95°C, 15 s and 60°C, 30 s, final extension at 72°C, 30 s.

Oligonucleotide primers were designed using the Real-Time PCR Primer Design program Primer3Plus (https://primer3plus.com) and further validated using Oligo Analyzer (https://eu.idtdna.com/calc/analyzer). Target specificity was assessed through Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Relative quantification was carried out using PaTub (tubulin beta-4 chain, LOC118055616) as reference gene. The following genes were tested: *PaABI3* (*B3 DOMAIN-CONTAINING TRANSCRIPTION FACTOR ABI3*, LOC118049976), *PaFUS3* (*B3 DOMAIN-CONTAINING TRANSCRIPTION FACTOR FUS3-LIKE*, LOC118038131), *PaSPDS* (*SPERMIDINE SYNTHASE 1*, LOC118055381), *PaSPMS* (*SPERMINE SYNTHASE*, LOC118033446), *PaAPX* (*ASCORBATE PEROXIDASE*, cytosolic-like, LOC118045884), *PaCycB1* (*CYCLIN B1-2*, LOC118029686), *PaCDKA1* (*CYCLIN-DEPENDENT KINASE A1*, LOC118050756), and *PaCycD2* (*CYCLIN-D2-LIKE* LOC118031573). Oligonucleotide sequences are provided in Appendix Table 1. The Thomsen method was employed for relative quantification of transcript accumulation using a standardized efficiency (E) value of 1.8 (Thomsen et al., 2010). All reactions were carried out in triplicates. Z-score (mean-centered and divided by the standard deviation of each variable) was calculated on the linearized Ct values and used to generate heatmaps with the GraphPad Prism version 8.0.1 program.

Statistical analysis

For the germination tests and viability assay data, statistical analyses were performed using Student's *t*-test. Asterisks indicate statistically significant differences determined using Student's t-test (*, $p \leq 0.05$). ROS quantification and gene expression analyses were analyzed through two-way analysis of variance (ANOVA) and the Duncan's test, using the software Rapid Publication-Ready MS Word Tables Using Two-Way ANOVA 1.0 (Assaad et al., 2015), available online (https://houssein-assaad.shinyapps.io/TwoWayANOVA/).

Pearson's correlation and Principal Component Analysis (PCA) were carried out using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/docs/Publications.xhtml), normalizing the values by Z-score and considering a *p*-value ≤ 0.05 as the threshold for significance (Pang et al., 2021).

5.3 Results

Reduced seed viability in long-term stored *P. alba* seeds

The germination percentage (G%) of long-term stored 'Villafranca' seeds was evaluated to establish the baseline of the utilized materials. The germinability was observed during 48 h, yielding an estimated ultimate germination percentage of 19.00 ± 4.47% (**Figure 5.1A**). The TTC assay conducted to measure seed viability indicated that the proportion of non-viable seeds was significantly ($P < 0.05$) higher (68 ± 13.03%) than that of viable seeds (32 ± 13.03%) (**Figure 5.1B**). Collectively, these data suggest that the decreased germination observed in 'Villafranca' seeds resulted from a significant loss of viability during storage.

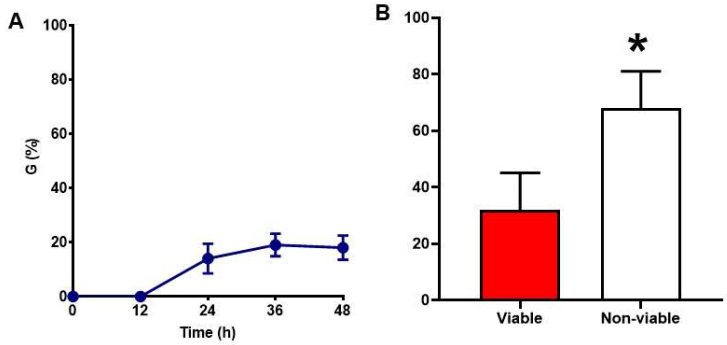

Figure 5.1. Baseline characterization of long-term stored *Populus alba* clone 'Villafranca' seeds. **(A)** Germination percentage (G%). **(B)** Viability test performed through the TTC method. Statistically significant differences calculated using Student's t-test are indicated with an asterisk (*, $P \leq 0.05$).

Imbibition curves denote rapid water loss by 'Villafranca' seeds during dehydration

The dynamics of water absorption and loss (rehydration-dehydration cycle) were assessed in naturally aged *P. alba* seeds at various time points during imbibition (1, 2, 3, 4, 5 h) using water (H_2O), Spd50 (50 µM spermidine), and Spd100 (100 µM spermidine), as well as during dry-back (DB) (1, 2 h) (**Figure 5.2**). A rapid water absorption was noted during the initial two hours across all treatments, however the process slowed down at later timepoints. No substantial changes in the absorption of water or Spd solutions were observed. Significant water loss was seen within the first hour of the dry-back process (**Figure 5.2**, 1 h DB). Furthermore, the seed weight after 2 hours post-dry back (0.31 ± 0.03 mg) was

lower to that of the original dry state of the seeds (0.47 ± 0.01 mg). No significant differences were seen between the treatments with water or Spd (**Figure 5.2**).

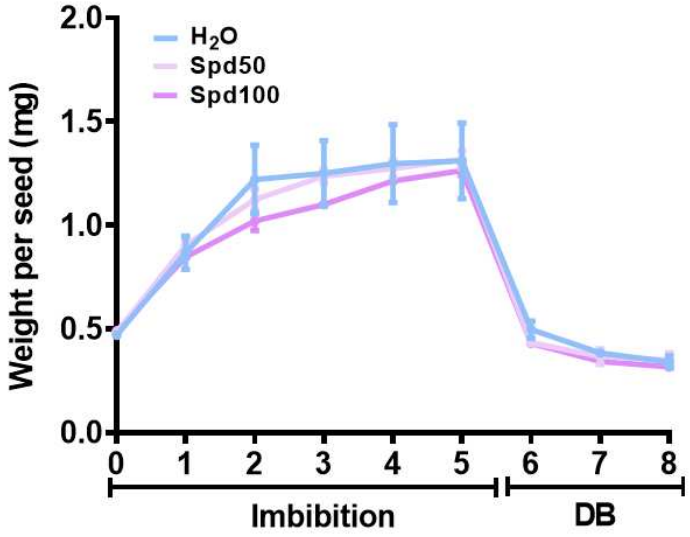

Figure 5.2. Imbibition curves represented as seed weight gain and loss in long-term stored *P. alba* clone 'Villafranca' seeds subjected to different intervals of rehydration (1, 2, 3, 4, 5 h) and dehydration (1, 2 h). Statistically significant differences calculated using Student's *t*-test are indicated with an asterisk (*, $P \leq 0.05$). DB, dry-back; Spd50, spermidine 50 µM; Spd100, spermidine 100 µM.

Seed priming does not improve germination of naturally aged 'Villafranca' seeds

The investigation examined the response of long-term stored seeds to priming treatments by testing various hydropriming treatments, considering varied imbibition and DB timepoints. The seeds underwent progressively extended imbibition durations of 2, 4, 6, and 8 h, corresponding to the respective DB durations of 1, 2, 3, and 4 h. Germination tests were conducted to evaluate the efficacy of the treatments, with untreated (UT) seeds as the control group. As illustrated in **Figure 5.3**, none of the HP treatments succeeded in improving G%. Furthermore, a significant decline in germinability was recorded following the 8-h HP treatment (3.33 ± 5.77%) in comparison to the UT control (28 ± 2.88%). This suggests that HP was perceived as stress. We propose that this may result from the fast water loss previously observed during the DB process (**Figure 5.2**).

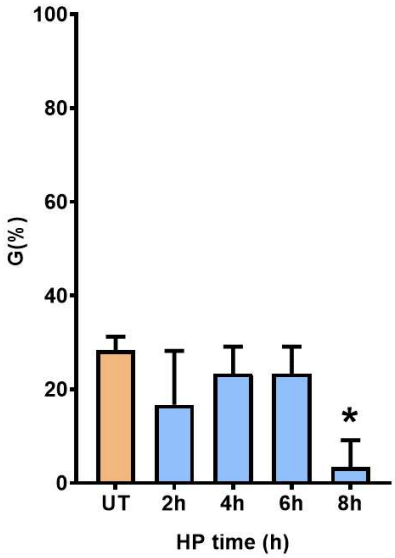

Figure 5.3. Germination percentage (G%) of long-term stored *P. alba* clone 'Villafranca' seeds treated/untreated with hydropriming (HP) at different imbibition timepoints (2, 4, 6, 8 h) followed by dry-back (DB). Statistically significant differences were calculated compared to untreated (UT) controls using Student's *t*-test and are indicated with an asterisk (*, $P \leq 0.05$).

Seed soaking with spermidine enhances germination performance

Due to the inefficiency of HP protocol, the subsequent action involved implementing only seed soaking treatments, hence eliminating the DB phase. In this experiment, seeds were immersed in water or Spd solutions (50 µM, Spd50; 100 µM, Spd100) for varying durations of 1, 2, 4, and 6 h. Of the evaluated water soaking durations, only 1 h (43.33 ± 5.77%) and 4 h (46.66 ± 5.77%) shown a significant enhancement in germination percentage (G%) relative to the untreated seeds (26.66 ± 5.77%) (**Figure 5.4A**). Consequently, these timepoints were chosen to conduct imbibition with the Spd solutions as well. The subsequent germination tests demonstrated that soaking in 100 µM spermidine for 4 h was the most effective treatment, with a considerable increase in germination percentage (G%) to 36.66 ± 5.77%, compared to the untreated group (UT) at 15 ± 5% (**Figure 5.4B**).

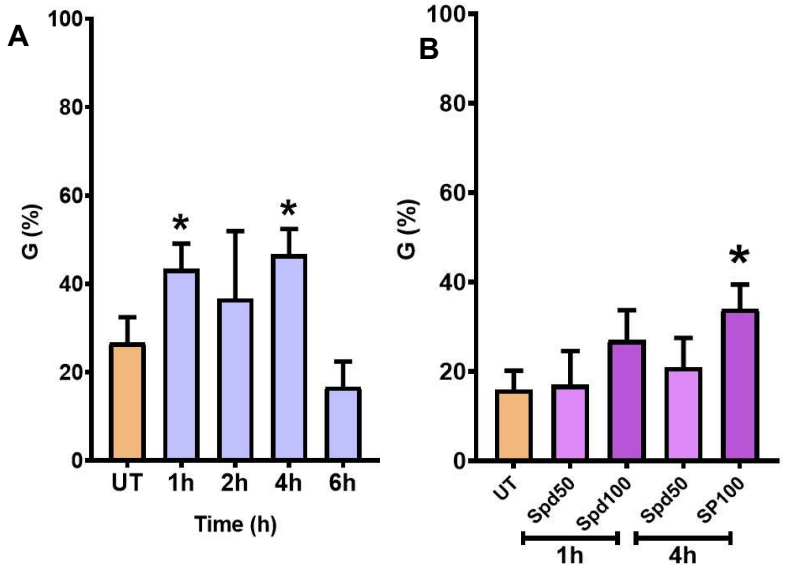

Figure 5.4. Germination percentage (G%) of long-term stored *P. alba* clone 'Villafranca' seeds **(A)** Seeds were subjected to different soaking time intervals, namely 1, 2, 4 and 6 h. **(B)** Seeds were subjected to different soaking time intervals, namely 1 and 4 h. Statistically significant differences were calculated compared to untreated (UT) controls using Student's *t*-test and are indicated with an asterisk (*, $P \leq 0.05$).

To validate these findings, an additional series of germination tests was performed utilizing the optimal timepoint (4 h) and Spd concentration (100 µM) (Fig. 4). In this instance, both germination percentage (G%) and germination rate (T_{50}) were evaluated. Concerning G% (**Figure 5.5A**), only seeds treated with 100 µM Spd (Spd100) shown a statistically significant increase in germination (35.00 ± 5.00%) relative to UT controls (20.00 ± 5.00%). This result was strongly supported by a notable decrease in the T_{50} values, found exclusively for the Spd100 treatment (**Figure 5.5B**). These results demonstrate that seed soaking with Spd is an effective method to enhance the germination potential of long-term stored *P. alba* seeds.

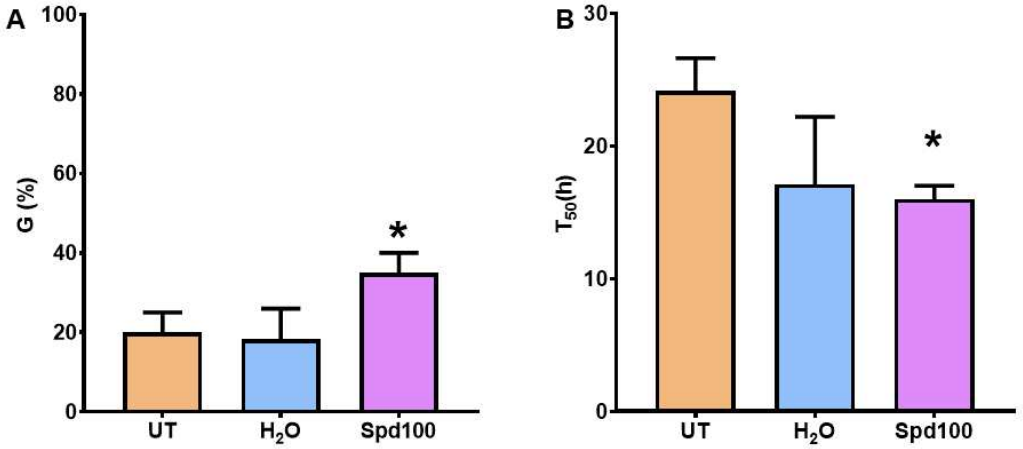

Figure 5.5. Germination performance of long-term stored *P. alba* clone 'Villafranca' seeds soaked for 4 h in water or spermidine. **(A)** Germination percentage (G%). **(B)** Germination speed calculated in terms of T_{50}. Statistically significant differences were calculated compared to untreated (UT) controls using Student's *t*-test and are indicated with an asterisk (*, $P \leq 0.05$). UT, untreated; H_2O, water soaking; Spd100, 100 µM spermidine soaking; DB, dry-back.

Molecular characterization of pre-germinative metabolism in 'Villafranca' poplar seeds

Molecular characterization was performed to better understand the molecular mechanisms behind the efficacy or inefficacy of the imposed treatments on long-term stored 'Villafranca' seeds germination (**Figure 5.5**). In addition to detecting ROS generation with the DCFH-DA test, qRT-PCR was used to evaluate the expression levels of chosen genes involved in early seed germination. The experimental system included dry seeds (DS, 0 h), H_2O or 100 µM Spd-imbibed seeds for 1 and 4 h, and dry-back at 1 and 2 h DB (**Figure 5.5A**).
The levels of ROS were evaluated due to its crucial role in seed aging and stress response. A significant elevation in ROS levels was seen in seeds treated with H_2O after 4 h of imbibition and at 1 and 2 h of DB, with the peak accumulation recorded in the 1DB samples (3.17 ± 0.62 R.F. U) (**Figure 5.5B**). Notably, in the seeds subjected to Spd100 treatment, ROS levels remained stable (showing no significant variations compared to the control) until the 1DB stage, with only a marginally significant rise observed in the 2DB samples. Comparing the effects of H_2O and Spd100 treatments during seed imbibition (4 h) and dry-back (1DB) reveals a notable reduction in ROS levels in seeds treated with spermidine (**Figure 5.5B**). This indicates that Spd may function as an antioxidant mechanism to mitigate ROS generation during both water absorption and loss.
To further explore this aspect, we analyzed the expression patterns of genes associated with DT acquisition (*PaABI3*, *PaFUS3*), polyamine biosynthesis (*PaSPDS1*, *PaSPMS*), antioxidant response (*PaAPX*), and cell cycle regulation (*PaCycB1*, *PaCDKA1*, *PaCycD2*). The outcomes are presented as a heatmap, where blue signifies low expression and red denotes high gene expression (**Figure 5.5C**). Changes in gene expression profiles were noted during the

rehydration and dehydration phases. All examined genes exhibited elevated expression levels in dry seeds (0 h). In contrast, both H_2O and Spd100 treatments led to a significant decrease in the expression of the *PaABI3*, *PaSPMS*, and *PaCDKA1* genes at 1 h of imbibition, and this lower expression continued at successive time periods, including DB. The Spd100 treatment appeared to stimulate the expression of the *PaFU3*, *PaAPX*, and *PaCycB1* genes in response to 2 h of dehydration (2DB), a phenomenon not observed in H_2O-treated seeds. In this instance, the only significantly elevated genes relative to the control (0 h) were *PaSPDS1* at 1 h imbibition and *PaCycD2* at 2 h DB.

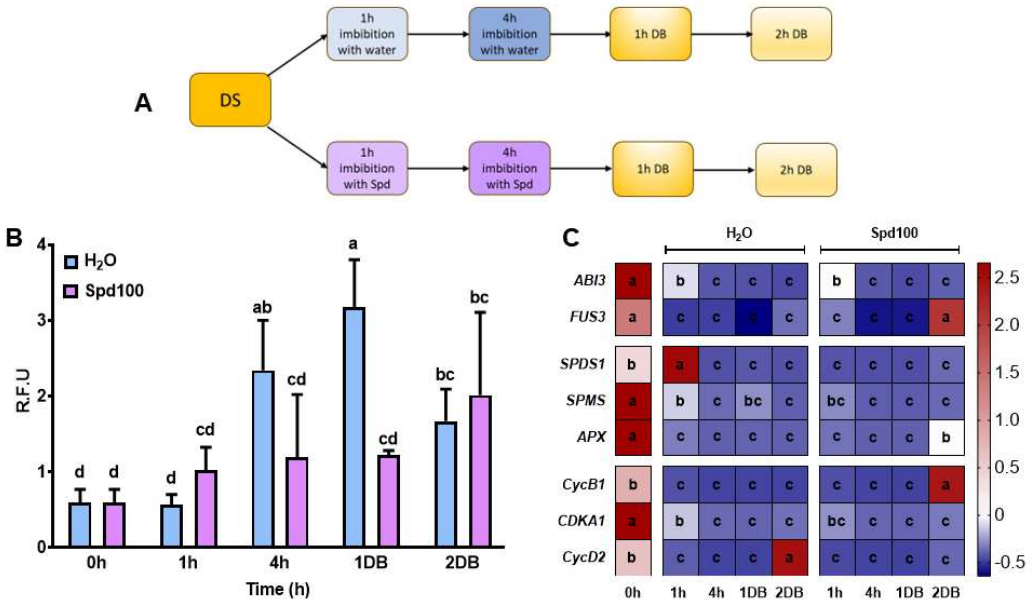

Figure 5.6. Molecular profiling of long-term stored *P. alba* clone 'Villafranca' seeds along the rehydration-dehydration cycle. **(A)** Schematic representation of the experimental design including the selected rehydration (1 and 4 h) and dehydration (1 and 2 h) time points. The imposed treatments included imbibition in H_20 or a spermidine (Spd) solution at a concentration of 100 µM alongside dry seeds (0 h) used as untreated controls. **(B)** Quantification of reactive oxygen species (ROS) through the DCFH-DA assay. **(C)** Heatmaps representing the expression patterns of indicated genes obtained through qRT-PCR analyses. Blue color indicates low gene expression while red color indicates high expression. Samples followed by different letters indicate statistically significant differences ($P \leq 0.05$) as per Duncan test. R.F.U, relative fluorescence units; H_2O, water soaking; Spd100, 100 µM spermidine soaking; DB, dry-back; *ABI3*, B3 Domain-Containing Transcription Factor ABI3; *FUS3*, B3 Domain-Containing Transcription Factor FUS3-like; *SPDS1*, Spermidine synthase 1; *SPMS*, Spermine synthase; *APX*, Ascorbate peroxidase, cytosolic like; *CYCB1*, Cyclin B1-2; *CDKA1*; Cyclin dependent kinase A; *CYCD2*, Cyclin D2-1 like.

Principal Component Analysis (PCA) was conducted to combine the data from the molecular and phenotyping analyses, thereby illustrating the overall effects of the applied treatments. The PCA clustering revealed the emergence of three unique groups (UT, H_2O, Spd100) corresponding to each of the applied treatments (**Figure 5.7**).

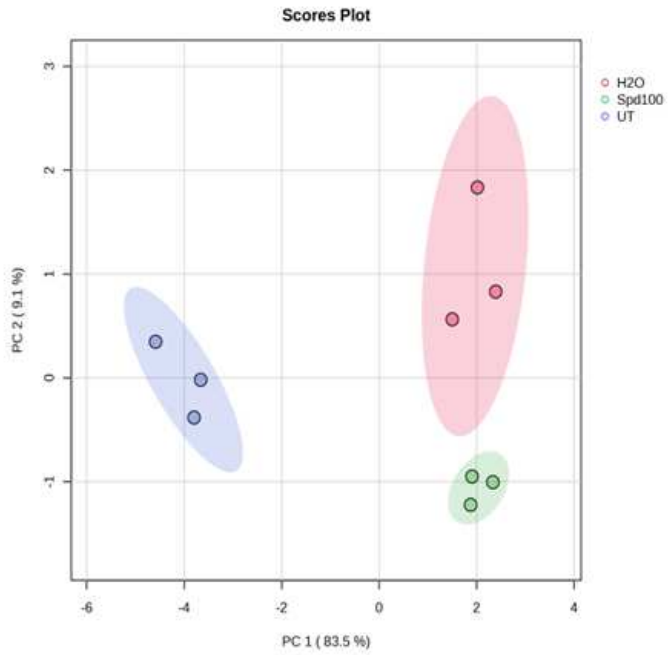

Figure 5.7. Principal Component Analysis (PCA) plots using data gathered for the treatments (UT, H_2O, Spd100) imposed to long-term stored *P. alba* clone 'Villafranca' seeds.

The biplot analysis indicated that the differentiation of the UT samples was primarily attributed to gene expression data. The primary variable influencing the differentiation of H_2O samples was the DCFH-DA assay, whereas the germination parameters predominantly facilitated the unique separation of the Spd100 samples (**Figure 5.8**).

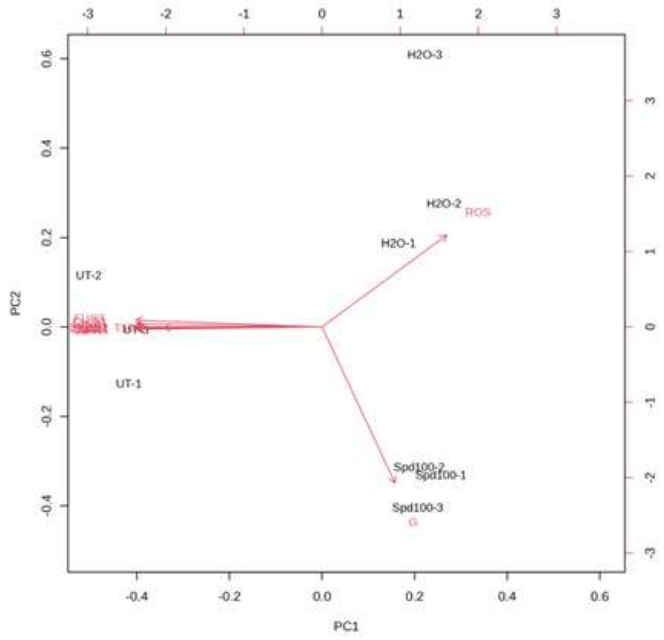

Figure 5.8. Biplot obtained with data from germination tests (G and T_{50}), ROS measurements (DCHF-DA) and gene expression data (*ABI3, FUS3, SPDS1, SPMS, APX, CycB1, CDKA1, CycD2*). UT, untreated; H_2O, water soaking; Spd100, 100 µM spermidine soaking.

5.4 Discussion

The present work aimed to develop seed treatments to improve the germination performance of long-term stored *P. alba* clone 'Villafranca' seeds. The research concentrated on a collection of seeds preserved under vacuum for 16 years at -30°C. Due to the short viability of poplar seeds during post-harvest (Wyckoff and Zasada 2005; Gosling, 2007), rapid preparation for their storage is essential. Furthermore, as they are classified as intermediate, desiccation-sensitive seeds (Michalak, 2014) the storage conditions constitute a limiting factor that reduces seed viability.

The initial evaluation of the long-term stored 'Villafranca' seeds revealed limited viability (about 20%) (**Figure 5.1**). Numerous studies have documented a decline in viability during storage in various tree species, including *Salix caprea* and *Salix gracilistyla* (Popova et al., 2012), as well as several poplar species such as *Populus nigra* (Suszka et al., 2014), *Populus davidiana*, *Populus koreana* (Kim et al., 2018), and the hybrid *Populus alba* × *Populus glandulosa* (Popova et al., 2013). The storage duration and temperature, as well as the seed moisture content, are critical and vary by species. Based on the literature, *P. nigra* seeds may be effectively maintained up to two years at temperatures of -10 °C or -20 °C, while preserving high viability (Suszka et al., 2014). Significant differences in

seed viability based on seed water content was reported for *P. davidiana* (orthodox seeds) and *P. koreana* (intermediate seeds) (Kim, 2018). Cryopreservation was identified as an effective storage technique for seeds with limited lifespans (Pritchard, 2007). The hybrid *P. alba* x *P. glandulosa* demonstrated a germination rate comparable to the controls when cryopreserved at a water content of 0.07-0.10 g g^{-1} for a duration of 2 weeks (Popova et al., 2013). Seed priming has recently been promoted as a component of integrated resource management initiatives for vulnerable ecosystems (Siva Devika et al., 2021). Hydropriming (HP), regarded as one of the most straightforward, sustainable, and economical seed priming techniques (Forti et al., 2020; Sushma et al., 2023), was first chosen for the treatment of 'Villafranca' seeds in this study.

Several imbibition and dry-back (DB) timepoints were tested but none positively influenced the germination of long-term stored 'Villafranca' seeds. Furthermore, upon 8 h imbibition followed by 4 h DB led to a significant decrease in germination percentage (**Figure 5.3**). This detrimental effect may result from the rapid water loss experienced during the dry-back phase of the treatment (**Figure 5.2**), a response commonly observed in desiccation-sensitive seeds, including both intermediate and recalcitrant types (Lah et al., 2023; Zhang et al., 2023). HP proved to be ineffective also for other species with recalcitrant seeds like *Cupania glabra* and *Cymbopetalum baillonii* (Becerra-Vázquez et al., 2020), or it had limited success after a mild dry back treatment, as in the case of *Quercus rugosa* (Castro-Colina et al., 2011).

Due to the failure of HP treatments and the potential damaging effects of dry back, soaking treatments were subsequently evaluated, hence omitting the DB step. Soaking seeds in Spd50 and Spd100 solutions (50 and 100 μM spermidine, respectively), the Spd100 treatment for 4 h proved to be the most promising one, resulting in a two-fold increase in germinability and enhanced germination speed compared to untreated (UT) seeds (**Figure 5.4**). These findings corroborate earlier reports indicating that soaking treatments effectively enhance germination in various species with intermediate and recalcitrant seeds, such as *Osyris lanceolata* (Mwang'Ingo et al., 2004), *Psidium guajava* (Bhanuprakash et al., 2008), *Pinus roxburghii* (Ghildiyal et al., 2009), *Litchi chinensis* (Zhang et al., 2015), and *Pouteria campachiana* (Amoakoh et al., 2017). In the present work, germinability of long-term stored 'Villafranca' seeds significantly enhanced only with spermidine addition to the soaking solution (**Figure 5.5**). This crucial polyamine has been thoroughly investigated for its advantageous impact on seed germination under stress conditions (Li et al., 2014; Huang et al., 2017; Paul and Roychoudhury, 2017; Chen et al., 2019; Hu et al., 2020; Zhang et al., 2022; Fuchs et al., 2023). A recent study indicated that recalcitrant *Acer saccharinum* seeds treated with spermidine exhibited enhanced germination during mild and severe desiccation (Fuchs et al., 2023).

The beneficial impact of spermidine on the germination performance of aged seeds may also be attributed to its antioxidant characteristics (Fuchs et al., 2023; Cao et al., 2023; He et al., 2024), hence aiding in ROS homeostasis during germination. ROS serve as crucial signaling molecules in various seed

physiological processes (Bailly, 2019), whereas excessive ROS accumulation results in adverse effects and lower seed viability (Kurek et al., 2019). The DCFH-DA assay conducted to assess ROS levels in 'Villafranca' seeds demonstrated a significant reduction in ROS levels following spermidine-mediated treatment (**Figure 5.6B**). This aligns with findings from earlier research in which spermidine was administered to *Solanum lycopersicum* (Zhang et al., 2014), *Medicago sativa* (Lou et al., 2018), *Oryza sativa* (Hu et al., 2020), *Vigna radiata* (Zhou et al., 2020), and *Cucumis sativus* (Korbas et al., 2022).

To thoroughly examine the molecular mechanisms responsible for the beneficial effects of spermidine and the detrimental influence of the DB step on the germination of long-term stored 'Villafranca' seeds, a gene expression analysis was performed, concentrating on genes associated with desiccation tolerance acquisition, polyamine biosynthesis, antioxidant response, and cell cycle regulation (**Figure 5.6C**). The *ABI3* and *FUS3* genes are pivotal in seed maturity and desiccation tolerance acquisition (Lepiniec et al., 2018). ABI3 serves as both an autoregulator and regulator of FUS3 (To et al., 2006). During germination, and therefore DT loss, the functions of the *ABI3* and *FUS3* genes are inhibited, as they play a significant role in embryo development and seed maturation (Lepiniec et al., 2018). This aligns with the results from qRT-PCR analysis conducted in this study, demonstrating consistent downregulation of these two genes during imbibition with both water and spermidine in comparison to dry seeds. In the context of polyamine biosynthesis, the *SPDS* gene encodes the spermidine synthase enzyme that catalyzes the conversion of putrescine into spermidine, which is then converted into spermine by the spermine synthase enzyme encoded by the *SPMS* gene (Smirnova et al., 2018). In this experimental setup, the *PaSPDS* gene exhibited upregulation after 1 h of imbibition in H_2O, whereas the *PaSPMS* gene was mostly expressed in desiccated seeds. Similar results were reported in the literature, indicating that *SPDS* gene expression was enhanced after imbibition in maize seeds (Huang et al., 2017), whereas the *SPMS* gene expression was predominantly confined to desiccated seeds in *Medicago truncatula* (Pagano et al., 2022b). The findings suggest that the overexpression of polyamines biosynthesis genes in mature seeds and during initial imbibition is essential for sustaining the antioxidant system to maintain an appropriate ROS balance. In agreement with this statement is also the high expression of the *APX* gene, involved in H_2O_2 scavenging (Pandey et al., 2017), in dry seed and its subsequent downregulation. The onset of the G1 phase and the activation of the G1-to-S transition are critical processes for proper germination, as germination entails the resumption of cell cycle progression (Barrôco et al., 2005). Expression profiles of cell cycle genes were assessed during the initial phase of 'Villafranca' seed germination to obtain further insights. The *CDKA1* gene encodes an A-type cyclin-dependent kinase, whereas the *CycD2* gene encodes a D-type cyclin, both of which are involved in the G1-to-S phase transition (Mironov et al., 1999; Sanz et al., 2011). The *CycB1* gene encodes a protein that facilitates cell cycle progression during M-phase (Menges et al., 2003) and participates in the homologous recombination (HR) repair pathway (Weimer et al., 2016). In our experimental setup, the *CycB1* gene

exhibited elevated expression during DB following spermidine administration, whereas the *CycD2* gene expression peaked at the same timepoint with water treatment. DNA damage can accumulate during desiccation, necessitating efficient repair prior to germination (Waterworth et al., 2010). The detected overexpression of the *CycB1* and *CycD2* genes may suggest that cell cycle progression is halted to facilitate more effective repair. Additional research has demonstrated that spermidine and spermine treatments enhanced protection against DNA oxidative damage in wheat plants subjected to heavy metal stress (Taie et al., 2019). Overall, the results of this investigation represent an original contribution to the complex issues of poor viability and longevity in the desiccation sensitive seeds of forest species, paving the way to more in-depth molecular studies that might help improving the efforts of operators and breeding working in the forest sector.

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Chapter 6. Comprehensive discussion

The research topic explored in the present work addresses key open questions related to one of the main drawbacks of the current seed priming technology, namely the decreased longevity of primed seeds (Argerich et al., 1989; Tarquis and Bradford, 1992; Chiu et al., 2002; Abnavi et al., 2012; Hussain et al., 2015; Yan et al., 2017; Wang et al., 2018; Chhabra and Singh, 2019; Sano and Seo, 2020). Seeds must be effectively preserved during the different steps from harvest to processing and distribution. Consequently, in light of the extensive research illustrating the benefits of seed priming and the detrimental impacts of seed aging, it is essential to explore novel techniques to mitigate the vulnerability of primed seeds to aging. This may improve the efficacy of seed priming as a method to improve seed quality and post-harvest procedures, especially regarding seed storage (Gaonkar et al., 2024). The choice of *M. truncatula* for this study depended on its role as a model legume for seed physiology and its alignment with previous studies investigating the effects and drawbacks of seed priming (Pagano et al., 2022a, 2022b).

The experimental system reported was carefully designed to address such issues. The accelerated aging protocol was employed in this investigation to mimic the effects of long-term storage, and the protocol was optimized based on the previous experimental work carried out in both maize (Colombo et al., 2023) and *M. truncatula* (Gaonkar et al., 2024) seeds. The tested conditions were able to effectively lower the germination percentage of unprimed and hydroprimed *M. truncatula* seeds. Accelerated aging protocols have demonstrated efficacy and precision in replicating storability issues in diverse model and crop plants (Schwember et al., 2001; Rajjou et al., 2008; Fantazzini et al., 2018). The importance of assessing seedling establishment, in addition to germination parameters, has been emphasized as a crucial factor for achieving optimal crop yields, particularly in relation to the impacts of extended storage (Rehmani et al., 2023).

The main achievement of this study was the demonstrated efficacy of quercetin, rutin, and spermidine supplementation in alleviating the impacts of accelerated aging in *M. truncatula* seeds treated with these compounds, compared to hydroprimed seeds. All three treatments had beneficial effects on aged *M. truncatula* seeds. Considering that the protective compounds tested in the present study are plant secondary metabolites, they can be found the different types of plant tissues and species. This opens to the possibility of using these compounds starting from agricultural plant waste, a strategy that fully aligns with the concept of circular bioeconomy (Chiocchio et al., 2021). The choice of quercetin and rutin was based on the reported advantages of applying external flavonoids to seeds, seedlings, and plants under diverse biotic and abiotic stress conditions (Yang et al., 2016; Yang et al., 2021; Tang et al., 2023). Rutin-primed seeds exhibited reduced ROS levels compared to unprimed and hydroprimed seeds during accelerated aging. Furthermore, priming with quercetin and rutin resulted into enhanced antioxidant profiles relative to hydroprimed seeds. The higher post-aging germination and growth performance of quercetin- and rutin-

primed seeds, relative to hydroprimed seeds, corroborates this observation. These findings suggest that quercetin or rutin priming may provide protective benefits to the treated seeds subjected to ageing by enhancing their antioxidant profiles.

Spermidine successfully improved the germination of aged seeds compared to hydroprimed seeds, however, these protective effects decreased during seedling development, resulting in a high occurrence of aberrant phenotypes. We explored the intricacy of molecular events and regulatory mechanisms at the intersection of the two separate genetic programs: germination and seedling development (Waterworth et al., 2022; Rehmani et al., 2023). Several reports have underlined the beneficial role of spermidine in mitigating the detrimental effects of aging on seed viability (Li et al., 2014; Huang et al., 2017; Paul and Roychoudhury, 2017; Chen et al., 2019; Hu et al., 2020; Zhang et al., 2022; Fuchs et al., 2023). In *Arabidopsis thaliana*, it has been shown that DDR (DNA damage response) genes are crucial players for seed aging, contributing to seed vigor and germination performance (Waterworth et al., 2019). Cytological investigations have revealed a correlation between decreased vigor and viability in seed aging and DNA damage accumulation and the resulting aberrant cytogenetic structures may increase the likelihood of abnormal seedlings (Waterworth et al., 2015). Similar results were reported in this study since the response of *M. truncatula* seeds to spermidine-mediated treatment resulted into enhanced frequency of aberrant seedlings and progressive DNA damage accumulation with aging. When the expression profiles of DDR and cell cycle regulation genes were assessed in *M. truncatula* seeds subjected to aging, spermidine appeared to influence the upregulation of several DDR genes across different developmental stages. Non-targeted metabolomics was also used to investigate spermidine-associated metabolic alterations at the transition between seed germination and seedling development. Spermidine induced a range of metabolic changes in *M. truncatula* but the main focus was on the patterns of endogenous polyamine levels, antioxidants, and nucleotide metabolites that may contribute to enhance the germination efficacy of aged *M. truncatula* seeds. The decline in polyamine levels, due to aging, has been described in multiple organisms (Christiansen and Gregersen, 2014; Hofer et al., 2022; Lerin et al., 2022). The molecular mechanisms underlying the beneficial effects of spermidine have been extensively investigated, particularly in relation to the antioxidant activities, in multiple species (Zhang et al., 2014; Lou et al., 2018; Hu et al., 2020; Zhou et al., 2020; Korbas et al., 2022).

Based on the current literature, highlighting the positive effects of spermidine in improving seed longevity, spermidine was tested, in a parallel work, on long-term stored *P. alba* clone ‘Villafranca’ seeds preserved under vacuum for 16 years at -30°C. Poplar seeds, characterized by poor viability, are classified as intermediate seeds (Wyckoff and Zasada 2005; Michalak, 2014; Gosling, 2007), and are therefore desiccation-sensitive. In these seeds, storage conditions constitute a limiting factor that reduces viability. The initial assessment of germination performance of the long-term stored 'Villafranca' seeds indicated reduced germination and viability. Seed priming was perceived as a stress

treatment by polar seeds, likely due to the rapid water loss faced during the dry-back phase of the treatment, a response frequently observed in desiccation-sensitive seeds, encompassing both intermediate and recalcitrant varieties (Lah et al., 2023; Zhang et al., 2023). Following that, soaking treatments were assessed by excluding the dry-back step. The 100 µM Spd (Spd100) treatment for four hours proved to be the most promising, yielding a two-fold increase in germinability and an accelerated germination rate, compared to untreated seeds. The beneficial effect of spermidine on the germination of aged poplar seeds may be ascribed to its antioxidant properties (Fuchs et al., 2023; Cao et al., 2023; He et al., 2024), hence facilitating ROS homeostasis during germination. Gene expression analysis was conducted to investigate the molecular mechanisms underlying the advantageous effects of spermidine and the adverse impact of the dry-back step on the germination of long-term stored 'Villafranca' seeds, focusing on genes related to desiccation tolerance acquisition, polyamine biosynthesis, antioxidant response, and cell cycle regulation. Increased expression of genes related to desiccation tolerance, polyamine synthesis, and antioxidant defense was observed only in dry seeds. The successful development of a spermidine-based treatment that enhances the germination of long-term stored, deteriorated 'Villafranca' seeds, hereby reported, represents the starting point for future studies aimed at expanding such approach to a wider range of desiccation sensitive seeds.

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Quercetin and Rutin as Tools to Enhance Antioxidant Profiles and Post-Priming Seed Storability in *Medicago truncatula*

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Abstract: Seed priming is routinely applied to improve germination rates and seedling establishment, but the decrease in longevity observed in primed seeds constitutes a major drawback that compromises long-term storability. The optimization of priming protocols able to preserve primed seeds from aging processes represents a promising route to expand the scope of seed priming. The present work explores this possibility in the model legume *Medicago truncatula* by testing the effectiveness of quercetin- and rutin-supplemented seed priming at improving the response to subsequent artificial aging. In comparison with a non-supplemented hydropriming protocol, supplementation with quercetin or rutin was able to mitigate the effects of post-priming aging by increasing germination percentage and speed, improving seed viability and seedling phenotype, with consistent correlations with a decrease in the levels of reactive oxygen species and an increase in antioxidant potential. The results suggest that quercetin and rutin can reduce the effects of post-priming aging by improving the seed antioxidant profiles. The present work provides novel information to explore the physiological changes associated with seed priming and aging, with possible outcomes for the development of tailored vigorization protocols able to overcome the storability constrains associated with post-priming aging processes.

Keywords: barrel medic; seed priming; artificial aging; quercetin; rutin; antioxidant profiles; reactive oxygen species; phenolic compounds

Citation: Gaonkar, S.S.; Sincinelli, F.; Balestrazzi, A.; Pagano, A. Quercetin and Rutin as Tools to Enhance Antioxidant Profiles and Post-Priming Seed Storability in *Medicago truncatula*. *Agriculture* **2024**, *14*, 738. https://doi.org/10.3390/agriculture14050738

Academic Editor: Bernard Hauser

Received: 15 April 2024
Revised: 3 May 2024
Accepted: 8 May 2024
Published: 9 May 2024

1. Introduction

Seed priming encompasses a range of established techniques to enhance germination performances, seedling establishment and stress tolerance. The effectiveness of seed priming relies on an incomplete imbibition step that induces the activation of pre-germinative metabolism, followed by a dehydration (dry-back) step that leaves the seed in the primed state that allows for a faster and more uniform germination [1–5]. The simplest form of seed priming relies on a rehydration–dehydration cycle mediated by non-supplemented water (hydropriming), whereas the administration of chemical compounds, osmotic agents, physical treatments, phytohormones, beneficial microorganisms, etc. can induce targeted responses, thus allowing for a customizable array of benefits, including stress tolerance and stress memory [4–7]. Despite its practicality and versatility, the main drawbacks of seed priming include the need of empirical optimization for different species, cultivars and seed lots, the progressive loss of desiccation tolerance as the seed transitions toward germination [8,9], and the increased exposure of primed seeds to aging processes. Such reduced longevity results in a loss of storability in the form of impaired germination rates and suboptimal seedling establishment, limiting the scope of seed priming within the current agricultural practices, the marketability of primed seeds, and the applicability of seed conservation strategies. This tendency has been documented in several crops, including tomato (*Solanum lycopersicum*) [10], lettuce (*Lactuca sativa*) [11], corn (*Zea mays*) [12], wheat (*Triticum*

aestivum) [13], rice (*Oryza sativa*), [14,15] Chinese cabbage (*Brassica rapa* subsp. *Pekinensis*) [16], and other species, including *Arabidopsis thaliana* [17]. These studies ascribed the increased susceptibility to post-priming aging to multiple factors linked to the more advanced physiological state of primed seeds, that progressively expose cellular structures to oxidative damage as seeds transition from their dry quiescent state to the metabolic pre-activation induced by priming [18].

To understand post-priming storability, the interaction between priming and aging needs to be investigated. The scientific literature focused on seed aging and longevity is abundant and established, exploring molecular and physiological dynamics, ecological and agricultural implications, diagnostic methods and mitigation strategies [19–23]. Seeds progressively deteriorate during prolonged storage, with storability varying depending on the species, ecotypes and storage practices optimized for orthodox and recalcitrant seeds [24,25]. Differences in storability are due to various genetic, biochemical and physiological factors even under optimal storage conditions, whereas high temperatures and relative humidity are major exogenous causes of seed deterioration during storage by influencing moisture content, oxidative processes and macromolecule stability. Particularly, accumulation of ROS (reactive oxygen species) is a major contributor to aging processes, causing the peroxidation of membrane lipids, protein loss-of-function and DNA damage, impairing seed viability [26–28]. Relevantly for research purposes, the development of artificial and accelerated aging protocols has allowed us to simulate the effects of natural aging with reduced timeframes and stricter control over the experimental conditions, that typically imply high temperatures and relative humidity applied for days or weeks. Cross-validation among different natural and artificial aging approaches has allowed the identification of common mechanisms and the implementation of longevity testing as part of seed quality screening [29–31].

The exploration of mitigation strategies for priming-associated aging implies dedicated experimental systems and has resulted in targeted solutions to extend post-priming longevity, such as vacuum packaging for bitter gourd (*Momordica charantia*) seeds [32], priming with KNO_3 for hot pepper (*Capsicum frutescens*) seeds [33], heat treatment for tomato seeds [34] and spermidine priming for rice seeds [35]. Despite these examples, the literature investigating the interaction between seed priming and aging is limited, especially concerning the formulation of treatments to improve post-priming storability. Given the major role of oxidative damage in seed aging, antioxidant compounds represent a first option in this sense. Flavonoids are numerous (around 8000 compounds identified in plants), variegate (six major classes) and widely distributed across the plant kingdom, with a variety of tissue localizations and biological functions, including antioxidant properties and protection against different biotic and abiotic stressors [36,37]. Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) is a polyphenolic flavonoid ubiquitously found in plants, including vegetables and fruits for human consumption [38]. Quercetin is mainly present in a glycoside form chemically named 3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside and also known as quercetin-3-rutinoside, rutoside, sophorin, phytomelin, rutin (etc.), a flavonol compound found in *Passiflora* spp., buckwheat (*Fagopyrum esculentum*) seeds, citrus (*Citrus* spp.) fruits, vegetables, and tea [38–40]. The contribution of endogenous flavonoids in seed longevity and storability is documented, including their antioxidant properties associated with their accumulation in seed coat and embryo [41]. Alleles of flavonoid genes and differential accumulation of flavonoids have been indicated as contributors to seed longevity in rice and soybean, respectively [42,43]. Protective effects are reported following the exogenous administration of quercetin, rutin or other flavonoids to seeds, seedlings or adult plants in the form of direct administration or priming treatments, with examples in numerous experimental systems where they reduce ROS accumulation and membrane damage [36,37,44,45].

Consistently with the cited research, the present work considers the possibility that the antioxidant and anti-aging effects reported for quercetin and rutin mitigate the effects

of poor or prolonged storage applied to primed seeds. This hypothesis is explored in the model legume *Medicago truncatula* by applying artificial aging on quercetin- and rutin-primed seeds, alongside unprimed and hydroprimed control treatments. The responses to the experimental conditions were evaluated at the biometrical level (germination rates, seed viability, and seedling morphology) in correlation with indicators of the seed oxidative state (ROS accumulation, antioxidant potential, and content in phenolic compounds) in order to provide evidence on how quercetin and rutin can improve post-priming longevity by enhancing the seed antioxidant indicators. The present study employed *M. truncatula* based on previous research on the effects and drawbacks of seed priming in this species as a model legume [8,9]. The results of this study provide a novel background to investigate and alleviate post-priming aging in legumes, with potential outcomes for the optimization of seed priming protocols compatible with seed storage practices.

2. Materials and Methods

2.1. Plant Material, Treatment Administration, and Germination Tests

Medicago truncatula Gaertn. Seeds (commercial genotype, kindly provided by Continental Semences S.p.A., Traversetolo, Parma, Italy) were treated with four priming conditions: the unprimed control condition (UP), hydropriming (HP), quercetin-supplemented priming (QP), and rutin-supplemented priming (RP). Each priming condition was followed by two accelerated aging conditions: unaged control conditions (UA) or accelerated aging (AA). All priming conditions were applied by imbibing the seeds for 4 h in sealed Petri dishes (diameter 90 mm) containing a layer of filter paper moistened with 2 mL of water (HP), 2 mM quercetin (QP) or 1 mM rutin (RP). Quercetin and rutin concentrations were selected based on preliminary screening in the ranges of 0.05 to 2 mM, compatible with the ranges reported for other species [37,46]. Priming was followed by dry-back, which was carried out by distributing the seeds into open Petri dishes and incubating them for 4 h. Priming protocols and dry-back were carried out at 25 °C. Artificial aging was carried out in an oven (Memmert Universal Oven U55, Memmert, Schwabach, Germany), exposing the seeds to 45 °C and 95% relative humidity for 24 h, adapting the protocol by Colombo et al. [47]. Relative humidity was calculated from humidity measurements using a TA298 Digital thermohygrometer (JZK, Shenzhen JinZhiKu Electronic Co., Ltd., Shenzhen, China). The effective temperature for accelerated aging without complete loss of seed viability was selected based on preliminary screening in the temperature range of 40 to 60 °C. Germination tests were carried out in sealed Petri dishes (diameter 90 mm) containing a layer of filter paper moistened with 2 mL of distilled water. For the duration of the germination tests, Petri dishes were kept in in a growth chamber at 25 °C under light conditions, with a photon flux density of 150 µmol m^{-2} s^{-1}, and a photoperiod of 16 h. For each experimental condition, five independent replications (Petri dishes), each containing 20 seeds, were monitored every 2 h for 4 days, sufficient for all the treatments to reach germination plateau. Seeds displaying a protrusion of the primary radicle were considered germinated. Germination parameters were calculated according to Ranal and Garcia de Santana [48]. Seedling morphology was assessed at the end of the germination test and aberrant seedlings were distinguished from normal seedlings by an impaired growth, especially visible at the level of the radicle [8]. An overview of the experimental system is provided in Figure 1.

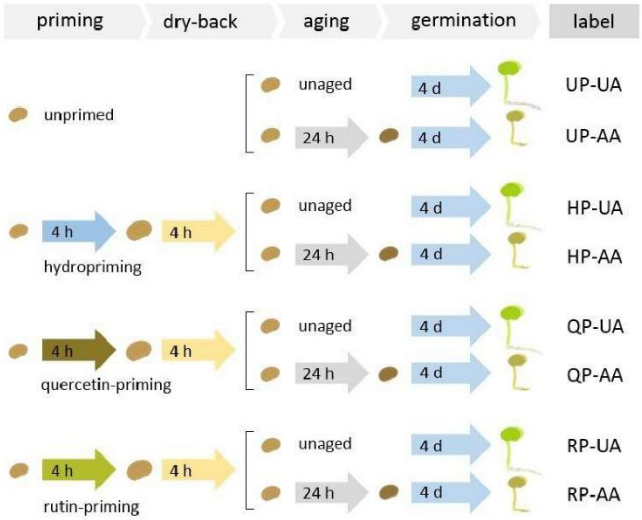

Figure 1. Overview of the experimental system to compare the effects of accelerated aging on *Medicago truncatula* seeds after hydropriming, quercetin-priming or rutin-priming. UP, unprimed control conditions; HP, hydropriming; QP, quercetin priming; RP, rutin priming; UA, unaged control conditions; AA, artificial aging.

2.2. Viability Assay Using 2,3,5-Triphenyl Tetrazolium Chloride

2,3,5-triphenyl tetrazolium chloride (TTC) assay was performed on the four priming conditions before and after accelerated aging in order to provide further indication of seed viability together with direct germinability assessment and to provide tissue-specific evidence of viability loss in *M. truncatula* seeds. The positivity to TTC staining is an established technique in seed viability testing [49,50]. Specifically, TTC is a white compound that is converted by dehydrogenases into TPF (1,3,5-triphenylformazan), a red and stable compound that differentiates metabolically active tissues from metabolically inactive ones. Seeds were imbibed in distilled water for 1 h to allow the removal of seed coat and an easier visualization of the tissue staining. De-coated seeds were subsequently incubated in a 1% (*w*/*v*) solution of TTC (Merck, Darmstadt, Germany), at 20 °C for 18 h in the dark. For each experimental condition, 50 seeds were screened and classified as viable or dead/aberrant according to their staining pattern. Particularly, seeds whose embryo axis was positive to TTC staining were classified as viable and results were expressed as percentage of viable seeds on the total of screened seeds.

2.3. Assessment of ROS Levels by 2′,7′-Dichlorofluorescin Diacetate (DCF-DA) Assay

Given the prominent role of oxidative stress in seed aging, ROS (reactive oxygen species) levels were assessed for the four priming conditions before and after accelerated aging. The assay was based on the fluorogenic dye 2′,7′-dichlorofluorescin diacetate (DCF-DA; Sigma-Aldrich, Milan, Italy). The DCF-DA molecule penetrates the cell membrane and is deacetylated by cellular esterases. Subsequently, it is oxidized by ROS into the fluorescent compound 2′,7′-dichlorofluorescein (DCF), whose fluorescence is spectroscopically detected at excitation and emission spectra of 495 nm and 529 nm, respectively. The assay was carried out as described by Pagano et al. [8]. Seeds (5 replicates per condition,

3 seeds per replicate) were incubated in dark conditions for 1 h in 50 µL of 10 µM DCF-DA. Subsequently, the solution was mixed by pipetting and a 20 µL aliquot was transferred to new tubes. A tube containing only DCF-DA not exposed to seed samples was also prepared and used as a blank control to assess the baseline fluorescence of the DCF-DA solution. Fluorescent emission was measured at 517 nm using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia), setting the software for one cycle of 30 s at 25 °C. Relative fluorescence was obtained by subtracting the fluorescence detected from the blank and expressing the results as relative fluorescence units (RFU).

2.4. Assessment of the Antioxidant Potential by DPPH (1,1-Diphenyl-2-picrylhydrazyl) Assay

The seed extracts necessary for DPPH and Folin–Ciocalteu assay were prepared as follows. Samples (for each condition, 200 mg pooling ~100 seeds, five replicates) were homogenized with mortar and pestle to a fine powder in presence of 2 mL 80% acetone. The extracts suspended in 80% acetone were transferred to 2 mL tubes and further 80% acetone was added to standardize the extraction volumes to 2 mL. The extracts were incubated overnight at 25 °C in the dark under gentle shaking and subsequently stored at −20 °C until use. The ROS-scavenging activity (antioxidant potential) of the samples was determined by DPPH test, that relies on the reactivity of the DPPH radical with the antioxidant compounds contained in the extracts [51]. A standard curve was obtained from serial dilutions (50–400 mg L^{-1}) of ascorbic acid (Sigma-Aldrich). Aliquots (0.1 mL) of the sample extracts or points of the standard curve were added to 1.5 mL of a solution of 0.1 mM DPPH (Sigma-Aldrich–Merck) dissolved in methanol. The reaction was incubated for 30 min at room temperature in the dark. A blank solution was prepared dissolving 0.1 mL 80% acetone into 1.9 mL methanol and used as a background for absorbance measurements at λ = 517 nm. The reduction in absorbance as a consequence of DPPH radical scavenging by antioxidant compounds was measured with a Biochrom WPA Biowave spectrophotometer (Biochrom Ltd., Cambridge, UK). The antioxidant potential of the extracts was calculated according to the standard curve and expressed as ascorbic acid equivalents (AAE) mg^{-1} fresh weight.

2.5. Assessment of the Content in Phenolic Compounds by Folin–Ciocalteu Assays

The content in total phenolic compounds was measured as described by Spanos and Wrolstad [52] from the same extracts used for DPPH assay, using the Folin–Ciocalteu reagent and a standard curve obtained from serial dilutions (50–400 mg L^{-1}) of gallic acid (Sigma-Aldrich–Merck). Aliquots (20 µL) of the sample extracts or points of the standard curve were added to 1.58 mL distilled water and with 100 µL of the Folin–Ciocalteu reagent (Sigma-Aldrich). After 8 min incubation in the dark, the reactions were neutralized with 300 µL of 7.5% (*w*/*v*) Na_2CO_3 (Sigma-Aldrich-Merck) and incubated for 120 min at 25 °C in the dark. The resulting increase in absorbance was measured at λ = 765 nm with a Biochrom WPA Biowave spectrophotometer (Biochrom Ltd., Cambridge, UK) from the background of a blank solution of 1.58 mL distilled water mixed with 300 µL of 7.5% (*w*/*v*) Na_2CO_3. The content in total phenolic compounds of the extracts was calculated according to the standard curve and expressed as gallic acid equivalents (GAE) mg^{-1} fresh weight. Five replicates were used for each condition. Using the data from DPPH and Folin–Ciocalteu assays, the specific antioxidant activity (SAA) was calculated as the ratio between the antioxidant potential and the total content in phenolic compounds, and expressed as µg AAE μg^{-1} GAE, as reported [53,54].

2.6. Statistical Analyses

Five replicates were used for each level of analysis. Data concerning germination performance (germination parameters), seedling phenotype, ROS accumulation, content in antioxidant and phenolic compounds, specific antioxidant activity, and seed viability were analyzed through two-way analysis of variance (ANOVA) and the Duncan's test, using the software Rapid Publication-Ready MS Word Tables Using Two-Way ANOVA 1.0 [55],

available online (https://houssein-assaad.shinyapps.io/TwoWayANOVA/, accessed on 28 February 2024). The comparison groups for two-way ANOVA were priming groups (UP, HP, QP, RP) and aging groups (UA, AA) with a p-value < 0.05 as the threshold for significance. Pearson's correlation and Principal Component Analysis (PCA) were carried out using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/docs/Publications.xhtml, accessed on 26 March 2024) [56], normalizing the values by Z-score (mean-centered and divided by the standard deviation of each variable) and considering a p-value < 0.05 as the threshold for significance for correlation analyses.

3. Results

3.1. Germination Performance

Germination of *Medicago truncatula* seeds subjected to hydropriming, quercetin-priming or rutin-priming followed by accelerated aging was assessed in terms of germination percentage at the end of the germination test (germinability) and in terms of germination speed (lower T_{50}, time to reach 50% of final germination). As shown in Figure 2 and Table S1, the underlying germinability profiles of unaged seeds were above 90% without differences induced by priming protocols. Post-aging germinability decreased below 60% for all priming treatments, with the strongest significant reduction observed in hydroprimed seeds. Conversely, seeds subjected to quercetin- and rutin-supplemented priming displayed significantly higher post-aging germinability profiles compared to hydroprimed seeds, and in line with the post-aging germinability profiles of unprimed seeds. The germination speed (assessed through a lower T_{50}) of unaged seeds was significantly accelerated in response to hydropriming, quercetin-supplemented priming and rutin-supplemented priming. Artificial aging significantly delayed germination for all priming treatments and unprimed control, with the highest delay recorded for hydroprimed seeds and no significant differences observed among the other priming treatments and the unprimed control. Globally, germination parameters indicate a significant decrease in germination percentage and speed in response to artificial aging, especially for hydroprimed seeds compared to unprimed seeds, with possible mitigating effects when priming is supplemented with quercetin or rutin.

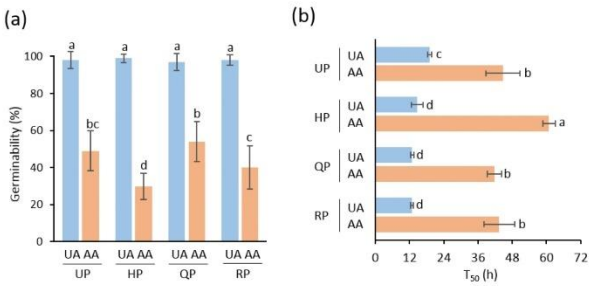

Figure 2. Germination performance of *Medicago truncatula* seeds subjected to hydropriming, quercetin-priming or rutin-priming followed by accelerated aging. (**a**) Germinability percentage. (**b**) T_{50}. UP, unprimed control conditions; HP, hydropriming; QP, quercetin-priming; RP, rutin-priming; UA, unaged control conditions; AA, artificial aging. T_{50}; time (h) to reach 50% of final germinants. Means without a common letter are significantly (p-value < 0.05) different as analyzed by two-way ANOVA and Duncan test.

3.2. Seed Viability

2,3,5-triphenyl tetrazolium chloride (TTC) assay was performed on the four priming conditions before and after accelerated aging in order to corroborate germinability profiles and provide further evidence of the gain/loss in seed viability when combining

priming protocols and artificial aging. As shown in Figure 3a and Table S1, the underlying viability profiles (in terms of TTC-positivity) of unaged seeds were above 70% without significant differences based on priming protocol. Artificial aging reduced seed viability for the unprimed control and all priming treatments. Nonetheless, after aging, the viability of quercetin-primed seeds was higher than the viability of hydroprimed seeds. Representative pictures of seed viability as assessed by TTC-positive/negative staining on *Medicago truncatula* seeds are provided in Figure 3b. Specifically, seeds with positive embryo axis staining are distinguished from seeds with negative embryo axis staining as this feature was considered contrastive for viability classification purposes.

3.3. Seedling Development

Seedling morphology was assessed at the end of the germination test by distinguishing aberrant phenotype with impaired root development from the normal seedling development, as well as from the fraction of non-germinant seeds already quantified with germination parameters. As shown in Figure 4 and Table S1, seedlings developing from unaged seeds display a comparable distribution of normal (between 79% and 87%) and aberrant (between 7% and 15%) seedlings, without significant differences among priming treatments. After accelerated aging, the distribution of seedling phenotypic classes became contrastive among priming treatments, with a significant reduction in normal seedlings in response to hydropriming, quercetin-supplemented priming and rutin-supplemented priming compared to unprimed seeds. Notably, artificial aging after hydropriming resulted in a total absence of normal seedling phenotypes, whereas quercetin- and rutin-supplemented priming preserved a subpopulation of normal seedlings after artificial aging, despite being lesser than the unprimed control. Representative pictures of normal and aberrant seedling morphology in *Medicago truncatula* are provided in Figure 4b.

3.4. Antioxidant Parameters and ROS Accumulation

Given the relevance of ROS and antioxidant response as drivers of seed aging and longevity, the antioxidant potential, content of phenolic compounds, specific antioxidant activity and ROS levels were assessed in *Medicago truncatula* seeds subjected to hydropriming, quercetin-priming or rutin-priming followed by accelerated aging. As shown in Figure 5 and Table S1, considering unaged seeds, the antioxidant potential (assessed though the DPPH assay) was slightly but significantly decreased by all priming protocols in comparison to unprimed controls, with a stronger decrease in response to hydropriming. After artificial aging, a global reduction in antioxidant potential was observed for all priming and unpriming conditions compared to their unaged counterparts but the same pattern of unaged seeds was maintained. Specifically, a strong reduction was observed for all priming conditions compared to unprimed controls, with a significantly stronger reduction in response to hydropriming compared to quercetin- and rutin-priming. Considering unaged seeds, the total content of phenolic compounds (assessed through the Folin–Ciocalteu assay) decreased in response to hydropriming and quercetin-priming compared to unprimed controls. In response to artificial aging, a reduction in total phenolic compounds was observed for all primed and unprimed conditions. Considering the specific antioxidant activity (calculated as the ratio between the antioxidant potential and content in phenolic compounds) in unaged seeds, quercetin-primed seeds displayed a slightly higher specific antioxidant activity compared to hydroprimed seeds. Accelerated aging induced a strong decrease in specific antioxidant activity for all priming conditions but not in unprimed seeds. Nonetheless, quercetin- and rutin-primed seeds maintained a higher specific antioxidant activity than hydroprimed seeds after artificial aging. The ROS levels (assessed through the DCF-DA assay) of unaged seeds were significantly higher in hydroprimed seeds compared to the other tested conditions. Artificial aging induced a significant reduction in ROS only for hydroprimed seeds compared to their unaged counterparts. After aging, quercetin-primed seeds displayed lower ROS levels compared to unprimed seeds, whereas rutin-primed seeds displayed lower ROS levels compared to unprimed and also compared to hydroprimed seeds.

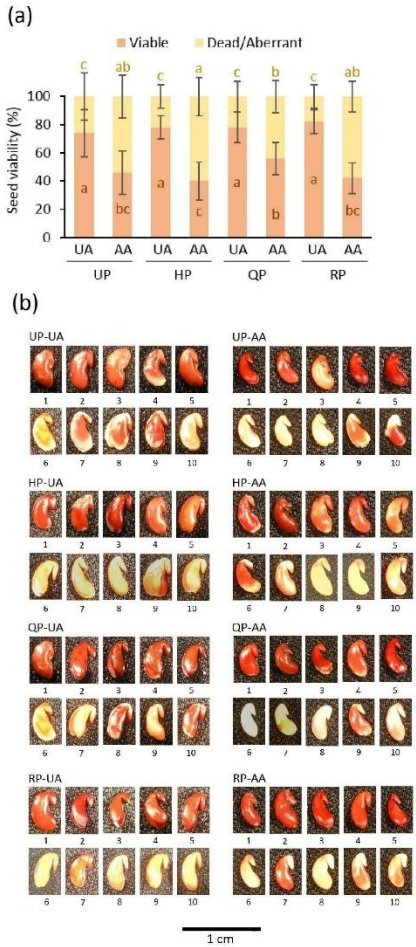

Figure 3. Viability of *Medicago truncatula* seed following hydropriming, quercetin-supplemented priming or rutin-supplemented priming combined with accelerated aging. (**a**) Seed viability percentage assessed with TTC staining. (**b**) Representative pictures of viable (row numbers 1 to 5) and non-viable (row numbers 6 to 10) seeds as assessed by TCC assay for each treatment category. UP, unprimed control conditions; HP, hydropriming; QP, quercetin-priming; RP, rutin-priming; UA, unaged control conditions; AA, artificial aging. Means without a common letter are significantly (p-value < 0.05) different as analyzed by two-way ANOVA and Duncan test. The letters referring to different comparison series are indicated with different colors.

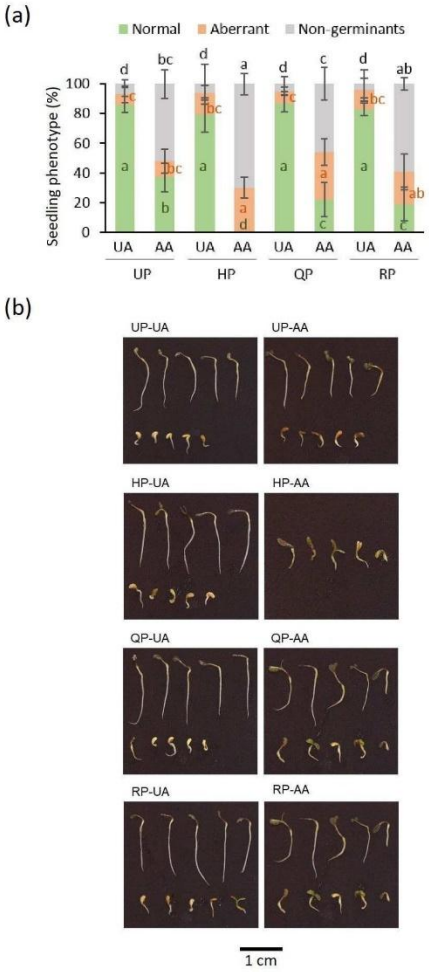

Figure 4. Phenotype of *Medicago truncatula* seedlings following hydropriming, quercetin-supplemented priming or rutin-supplemented priming combined with accelerated aging. (**a**) Seedling phenotype percentage. (**b**) Representative pictures of normal (top rows) and aberrant (bottom rows) seedling morphology for each treatment category. UP, unprimed control conditions; HP, hydropriming; QP, quercetin-priming; RP, rutin-priming; UA, unaged control conditions; AA, artificial aging. Means without a common letter are significantly (p-value < 0.05) different as analyzed by two-way ANOVA and Duncan test. The letters referring to different comparison series are indicated with different colors.

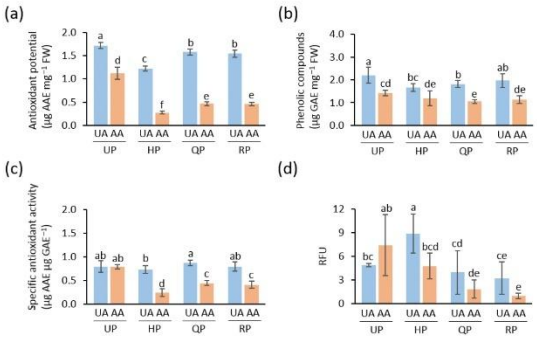

Figure 5. Antioxidant potential, phenolic compounds, and specific antioxidant activity of *Medicago truncatula* seeds subjected to hydropriming, quercetin-priming or rutin-priming followed by accelerated aging. (**a**) Antioxidant potential assessed by DPPH assay. (**b**) Content in phenolic compounds assessed by Folin–Ciocalteu assay. (**c**) Specific antioxidant activity calculated from DPPH and Folin–Ciocalteu data. (**d**) ROS detection by DCF-DA assay. UP, unprimed control conditions; HP, hydropriming; QP, quercetin priming; RP, rutin priming; UA, unaged control conditions; AA, artificial aging. Means without a common letter are significantly (p-value < 0.05) different as analyzed by two-way ANOVA and Duncan test. AAE, ascorbic acid equivalents; GAE, gallic acid equivalents; SAA, specific antioxidant activity; FW, fresh weight. RFU, relative fluorescence units.

3.5. Correlation of Germination and Seedling Growth Parameters with Antioxidant Response Indicators

Correlation analyses and principal component analysis (PCA) were performed in order to obtain an overview of the results obtained by the presented experimental system applying artificial aging on unprimed, hydroprimed, quercetin-primed and rutin-primed *Medicago truncatula* seeds. Results are shown in Figure 6. Pearson's correlation analysis indicated significant positive correlations of antioxidant properties (antioxidant potential, content in phenolic compounds and specific antioxidant activity) with positive indicators of germination performance (germinability, peak value), seed viability (TTC assay) and seedling establishment (percentage of seedlings with normal morphology). Moreover, the antioxidant potential and content in phenolic compounds were also positively correlated with each other, suggesting a synergistic response of different antioxidant response mechanisms. Conversely, Pearson's correlation analysis indicated significant negative correlations of antioxidant properties with negative indicators of germination speed (T_{50}, mean germination time), and with negative indicators of the efficiency of seedling establishment (percentage of abnormal and non-germinant seeds). The dataset for PCA was arranged in order to highlight the variations induced by priming treatments in combination with artificial aging, treating the eight resulting treatment groups as eight different clusters of replicates. The main driver of variability within the dataset along Principal Component (PC) 1 appeared to be the effect of artificial aging, determining two distinct super-clusters as unaged and artificially aged seeds. The two-dimensional plot along the PC1 and PC2 axes did not highlight contrastive clustering among priming groups in unaged seeds, whereas more distinct clusters were evident for artificially aged seeds. Specifically, the cluster referring to hydroprimed seeds was distinct from the cluster referring to unprimed seeds. Interestingly, the cluster referring to quercetin-supplemented priming partially overlapped with the cluster referring to unprimed seeds, whereas the cluster referring to rutin-supplemented priming partially overlapped with the clusters referring to unprimed controls, hydropriming and quercetin-priming. Globally, at the level of the analyzed parameters, Pearson's correlation analyses consistently suggested a relation between antioxidant properties, increased germination performance and successful seedling establishment, whereas PCA clustering indicated

that quercetin and rutin supplementation could mitigate the effects of post-priming artificial aging toward the patterns observed for unprimed aged seeds.

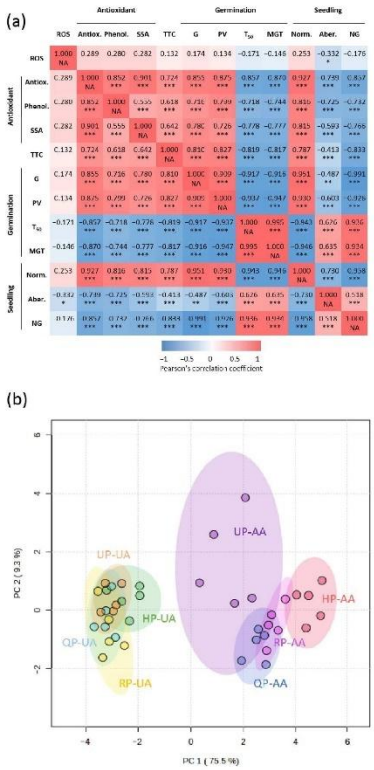

Figure 6. Overview of the results of Pearson's correlation analysis and principal component analysis. (**a**) Pearson's correlation analysis of the results obtained from *Medicago truncatula* seeds subjected to hydropriming, quercetin-priming or rutin-priming followed by accelerated aging. The correlation coefficients are indicated. The statistical significance of the Pearson's correlations is indicated by asterisks (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001). NA, not applicable. ROS, reactive oxygen species as assessed by DCF-DA assay. Antiox., antioxidant potential as assessed by DPPH assay. Phenol., content in total phenolic compounds as assessed by Folin–Ciocalteu assay. SSA, specific antioxidant activity. TTC, seed viability percentage as assessed by TTC assay. G, germinability. PV, peak value. T_{50}, time required to reach 50% of final germination. MGT, mean germination time. Norm., percentage of normal seedlings. Aber., percentage of aberrant seedlings. NG, percentage of non-germinant seeds. (**b**) Two-dimensional score plot of the principal component analysis of the results obtained from *M. truncatula* seeds subjected to hydropriming, quercetin-priming or rutin-priming followed by accelerated aging. UP, unprimed control conditions; HP, hydropriming; QP, quercetin priming; RP, rutin priming; UA, unaged control conditions; AA, artificial aging; PC, principal component.

4. Discussion

The present work focused on a frequently reported drawback of seed priming, that is the decrease in longevity of primed seeds [10–17,24]. Seeds are routinely stored during processing, distribution and marketing, for periods ranging from a few months to years. Consequently, considering the abundance of studies exposing the benefits of seed priming and detailing the damages of seed aging, the exploration of novel mitigation strategies for priming-related sensitivity to aging could broaden the scope of seed priming as an option to improve seed quality and post-harvest practices in terms of seed storability.

The choice of *Medicago truncatula* to investigate this issue was driven by its use as model legume for seed physiology and by consistency with previous studies focusing on effects and drawbacks of seed priming [8,9]. The experimental system devised for the present work included four priming conditions: unprimed seeds, hydroprimed seeds, quercetin-supplemented priming and rutin-supplemented priming. These four priming conditions were combined with presence/absence of subsequent artificial aging to estimate potential protective effects of these two flavonoids against post-priming longevity impairment.

As a first step to characterize the experimental system, germination performance was evaluated in terms of germination percentage and speed. The utilized artificial aging protocol (45 °C and 95% relative humidity for 24 h) was adapted from Colombo et al. [47] and was effective at decreasing the germination percentage for all priming treatments and the unprimed control without a total impairment of seed viability, thus facilitating the estimation of germination and seedling growth parameters. However, despite the practicality and accuracy of artificial aging protocols at simulating storability issues in an array of model and crop plants [29–31], further validation with natural aging, different storage practices and durations, and more species/cultivars would be useful to corroborate the results of the present study toward applicative directions. The results of the present work confirm the decrease in the longevity of primed seeds compared to unprimed seeds in *M. truncatula*, with decreased viability, lower germinability, delayed germination and an impaired seedling phenotype. Consistently with the hypothesis of the present work, the supplementation with quercetin or rutin during seed priming mitigated these effects compared to non-supplemented hydropriming, with increased germinability and seed viability, accelerated germination and an improved seedling phenotype. This hypothesis was based on the reported protective effects of the administration of exogenous flavonoids to seeds, seedlings and plants under various biotic and abiotic stressors [37,44,46], other than the conditions of post-priming aging considered for the present work. The relevance of evaluating seedling establishment in addition to germination parameters has been underlined as a key factor for optimal crop yields, also concerning the effects of prolonged storage [57].

In order to suggest explanations for the observed responses to priming combined with aging, the present work focused on indicators of the seed antioxidant status. ROS accumulation is considered a major driver of seed aging processes, with physiological ranges that need to be maintained by antioxidant compounds under layers of hormonal regulation to prevent oxidative damage while also allowing ROS to function as signal molecules in the stress response, cell wall plasticity, reservoir mobilization, hormonal modulation, dormancy release, and various processes of seed physiology [58,59]. During prolonged storage, seeds are in a dry quiescent state that limits the oxidative processes associated with an active metabolism, while also limiting enzymatic repair and ROS detoxification [17,58–60]. Although ROS production can be interpreted as a consequence of an active metabolism, ROS accumulation has been widely reported also in dry seeds under natural or artificial aging, in association with damage to membrane lipids, impaired germination and elicitation of enzymatic and non-enzymatic antioxidant response [28,59,61,62]. Considering this complexity, the dynamics and implications of ROS accumulation and scavenging can be difficult to interpret. By experimental design, the present work analyzed dry seeds, in which metabolic activities were assumed to be limited and the oxidative state driven mostly by non-enzymatic ROS production sources and scavenging mechanisms. The results of

the present work indicate that artificial aging significantly reduces antioxidant potential and content in phenolic compounds. This decrease can be interpreted as a scavenging of antioxidant and phenolic compounds during aging, consistently with previous accounts. For example, different intensities of artificial aging (20 to 42 days, 45 °C, and 10% moisture content) in oat (*Avena sativa*), resulting in a mild to total loss in germinability, were associated with the progressive accumulation of ROS and methylglyoxal along with a decrease in an array of enzymatic and non-enzymatic antioxidant indicators, including ascorbic acid and glutathione [63]. Artificial aging (0 to 8 days, 45 °C, 100% relative humidity) applied on *G. max* seeds decreased the content of many phenolic compounds, including protocatechuic acid, morin and rutin and downregulated the expression of genes encoding key enzymes for the biosynthesis of phenolic compounds [43]. Decreased antioxidant activity was also detected in canola (*Brassica napus*) seeds stored underground for up to several months in association with a loss of seed vigor, a reduction in soluble sugars and hormonal alterations [64]. On the other hand, artificial aging in canola seeds enhanced enzymatic and non-enzymatic antioxidant capacity, including the content of phenolic compounds, flavonoids and flavonols [65]. Considering *Fagus sylvatica* seeds under prolonged storage, germination capacity positively correlates with ascorbic acid and α-tocopherol content, and negatively with the accumulation of superoxide radical, hydrogen peroxide and lipid hydroxyperoxides, whereas glutathione did not appear to be correlated with germination performance [66]. These diverging observations confirm the species-specific variability of the enzymatic and non-enzymatic antioxidant mechanisms, possibly in terms of contrastive strategies to cope with oxidative stress during aging. Although the present work evaluated global antioxidant indicators, the specific relevance of enzymatic antioxidant mechanisms in seed longevity and stress response should be underlined as a direction for future studies focusing on seed aging interacting with seed priming. For example, long term storage decreased enzymatic antioxidant activity and germination in *Trifolium* spp. [67], and application of quercetin (15 to 40 µM) improved enzymatic antioxidant response and the response to soil contamination in *Trigonella corniculata* [68]. Moreover, catalase co-localizes with hydrogen peroxide and appears to be specifically involved in the recovery from aging through subsequent priming in *Helianthus annuus*, with the contribution of other antioxidant enzymes [69].

In the present work, primed seeds displayed a decreased antioxidant potential compared to unprimed seeds, both before and after aging, possibly suggesting an increased requirement of antioxidant compounds in response to priming and subsequent dry-back. This might be due to the reported more advanced metabolic state induced by priming [5,70]. This observation is consistent with the premises of the work, that attributes to primed seeds an increased exposure to aging compared to unprimed seeds, as evidenced by the observed decrease in germination rates and seedling establishment. The decrease in an enzymatic and/or non-enzymatic antioxidant response following priming was reported. For example, calcium chloride priming reduced antioxidant activity and the expression of genes involved in the antioxidant response in sorghum (*Sorghum bicolor*) seeds in presence of salt stress [71]. Decreased post-priming longevity in *Zea mays* has been explained with an impaired antioxidant machinery, including enzymes (catalase, superoxide dismutase) and antioxidant compounds (glutathione, ascorbic acid) [12]. Reductions in antioxidant activity after priming were detected also in osmoprimed *Spinacia oleracea* seeds [72], cysteine-primed *Hordeum vulgare* seeds [73] and in hydroprimed *Brassica rapa* subsp. *pekinensis* seeds [16]. Differently, seed priming induces an increase in antioxidant properties and stress responsiveness in other experimental systems that do not imply post-priming aging or that assess antioxidant parameters at later growth stages [74]. Previous accounts in *G. max* reported a decrease in ROS levels and an increased expression of antioxidant response genes (catalase, ascorbate peroxidase, superoxide dismutase) following priming [75], and priming enhanced the total antioxidant activity and the content of phenolic compounds (including flavonoids) in *Triticum aestivum* seedlings [76]. These patterns in the antioxidant response to priming highlight the need to consider seed priming and dry-back also in terms of a controlled

stress, possibly exploring the aspects of priming-associated stress memory [4,6]. Subsequently, it can be hypothesized that the application of aging to primed seeds represents a bottleneck in priming effectiveness mediated by stress memory mechanisms and/or by the pre-activation of antioxidant responses, reverting the advantages of priming and impairing longevity when post-priming storage is prolonged.

The major experimental question on the present work regarded the effectiveness of quercetin- and rutin-supplementation at alleviating the effects of artificial aging in *M. truncatula* seeds compared to hydropriming, and the observed improvements were interpreted in terms of oxidative/antioxidant status. After artificial aging, rutin-primed seeds presented lower ROS levels than unprimed and hydroprimed seeds, and both quercetin- and rutin-supplemented priming presented enhanced antioxidant profiles compared to hydroprimed seeds. This observation is coherent with the improved post-aging germination and growth performance of quercetin- and rutin-primed seeds compared to non-supplemented hydroprimed seeds. Considered together, these results suggest potential protective effects by quercetin or rutin supplementation with priming in terms of enhanced antioxidant profiles. This antioxidant effect of quercetin and rutin is in line with several accounts in other experimental systems, including *Apocynum pictum* and *venetum* seeds under osmotic stress [44], *G. max* artificially aged seeds [43], *O. sativa* plants [45] and aging seeds [42], and others [41]. Despite the significant correlations, the collected data are not sufficient to distinguish the direct and indirect effects of quercetin or rutin on antioxidant profiles, germination rates and seedling growth, nor the relative contribution of other factors that reportedly contribute to seed longevity. These include compounds (e.g., heat shock proteins, late embryogenesis abundant proteins, raffinose family oligosaccharides, etc.) that stabilize macromolecules, membranes and cellular structures [34,77] or processes that preserve DNA integrity [19,78]. Above these layers, phytohormones regulate seed maturation, germination and stress responses, with determinant effects on seed longevity [23]. Further research focusing on the contribution of these factors in different model species and experimental systems is necessary to outline a comprehensive model of post-priming seed aging.

5. Conclusions

The present work considered the tradeoff between the advantages of seed priming and the reduction in post-priming longevity, confirming also in the model legume *Medicago truncatula* the increased exposure of primed seeds to aging processes and suggesting a possible mitigation strategy in the form of quercetin and rutin supplementation during priming. The proposed explanation of the results relies on the positive correlations between biometrical indicators of seed quality (seed viability, germination rates, and seedling morphology) and indicators of antioxidant potential and content in phenolic compounds, highlighting potential contributors to the longevity of primed *M. truncatula* seeds. These results indicate possible directions to interpret and improve the interaction between seed priming and storability.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14050738/s1, Table S1: Comprehensive dataset of the results obtained from *Medicago truncatula* seeds subjected to hydropriming, quercetin-priming or rutin-priming followed by accelerated aging. UP, unprimed control conditions; HP, hydropriming; QP, quercetin-priming; RP, rutin-priming; UA, unaged control conditions; AA, artificial aging. ROS, reactive oxygen species as assessed by DCF-DA assay. Antiox., antioxidant potential as assessed by DPPH assay. Phenol., content in total phenolic compounds as assessed by Folin-Ciocalteu assay. SSA, specific antioxidant activity. TTC, seed viability percentage as assessed by TTC assay. G, germinability. PV, peak value. T_{50}, time required to reach 50% of final germination. MGT, mean germination time. Norm., percentage of normal seedlings. Aber., percentage of aberrant seedlings. NG, percentage of non-germinant seeds. Data are expressed as mean ± standard error of the mean. Means without a common letter are significantly (p-value < 0.05) different as analyzed by two-way ANOVA and Duncan test.

Author Contributions: Conceptualization, S.S.G., A.B. and A.P.; validation, S.S.G.; formal analysis, S.S.G. and A.P.; investigation, S.S.G. and F.S.; resources, A.B.; data curation, S.S.G. and A.P.; writing original draft preparation, A.P.; writing review and editing, A.P.; visualization, S.S.G. and A.P.; supervision, A.B. and A.P.; project administration, A.B. and A.P.; funding acquisition, A.B. All authors have read and agreed to the published version of the manuscript.

Funding: The work was supported from funds provided by the National Recovery and Resilience-Plan (NRRP) NextGenerationEU "Node 4-Ecosystems functions, services and solutions", Activity 5 (Conceptual framework and methodological tools of Nature Based Solution and Restoration Ecology), in the context of the National Biodiversity Future Center (to A.B.).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The entirety of the data shown in the present work are presented in the main manuscript as figures or as numerical values ± standard error in the Supplementary Table S1.

Acknowledgments: The authors acknowledge Anca Macovei (Department of Biology and Biotechnology "L. Spallanzani", University of Pavia) for valuable suggestions.

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

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Annals of Forest Science

Spermidine treatments improve germination of long-term stored seeds of Populus alba clone 'Villafranca'

--Manuscript Draft--

Manuscript Number:	
Full Title:	Spermidine treatments improve germination of long-term stored seeds of Populus alba clone 'Villafranca'
Article Type:	Research Paper
Funding Information:	
Abstract:	Populus alba clone 'Villafranca' (white poplar), highly suitable for biomass production and ecosystem restoration, is a model system for molecular and physiological studies, but no reports are available concerning seed quality. Although clonal propagation is the preferred approach for commercial purposes, attention should be given to face genetic variability losses in the existing germplasm. To address this challenge, new populations should be developed starting from seeds, although these have low germinability and viability during storage. Aims : This study proposes to develop tailored treatments to improve the germination of long-term stored white poplar seeds. Methods: Priming and soaking protocols, based on water or spermidine (Spd, 50 and 100 µM), were tested on white poplar seeds. Treatment efficacy was assessed based on germination performance, reactive oxygen species (ROS) profiles, and gene expression analysis. Results : The soaking solution containing 100 µM Spd applied for 4 h, significantly enhanced germination percentage and speed. Low ROS levels were evidenced in the Spd-treated seeds, compared to water-soaked seeds. High expression of genes involved in desiccation tolerance acquisition, polyamine biosynthesis, and antioxidant defense was observed only in dry seeds. Conclusion: We report the successful development of a treatment able to improve germination of long-term stored 'Villafranca'.
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Spermidine treatments improve germination of long-term stored seeds of *Populus alba* clone 'Villafranca'

Key message

Poplar seeds are short-lived and desiccation-sensitive. Tailored treatments for improving germination performance are necessary to recover the existing genetic diversity and support restoration programs. Application of spermidine as a seed soaking agent doubled the germination percentage of poplar seeds stored for more than 15 years.

Abstract

Context: *Populus alba* clone 'Villafranca' (white poplar), highly suitable for biomass production and ecosystem restoration, is a model system for molecular and physiological studies, but no reports are available concerning seed quality. Although clonal propagation is the preferred approach for commercial purposes, attention should be given to face genetic variability losses in the existing germplasm. To address this challenge, new populations should be developed starting from seeds, although these have low germinability and viability during storage.

Aims: This study proposes to develop tailored treatments to improve the germination of long-term stored white poplar seeds.

Methods: Priming and soaking protocols, based on water or spermidine (Spd, 50 and 100 µM), were tested on white poplar seeds. Treatment efficacy was assessed based on germination performance, reactive oxygen species (ROS) profiles, and gene expression analysis.

Results: The soaking solution containing 100 µM Spd applied for 4 h, significantly enhanced germination percentage and speed. Low ROS levels were evidenced in the Spd-treated seeds, compared to water-soaked seeds. High expression of genes involved in desiccation tolerance acquisition, polyamine biosynthesis, and antioxidant defense was observed only in dry seeds.

Conclusion: We report the successful development of a treatment able to improve germination of long-term stored 'Villafranca'.

Keywords: Poplar, Seed deterioration, Seed soaking, Priming, Spermidine

1. Introduction

The genus *Populus* consists of approximately 30 species including poplars, aspens and cottonwoods (Eckenwalder, 1996). Among these, poplars have a significant impact on both the economy and the environment. Among the wooden species, poplars are characterized by a fast growth which makes them promising candidates for sustainable biomass production. Additionally, their adaptability allows them to grow on poor soils, thus mitigating soil erosion due to the elaborate root networks (Pellegrino et al., 2011; Cantamessa et al., 2022). Other characteristics are related to the elimination of airborne particulate matter and soil metal sequestration through phytoremediation (Nissim et al., 2023). In Europe, poplar plantations cover almost one million hectares, with France, Turkey, Italy, Spain, and Hungary being the largest producers. In Italy, hybrid poplar plantations are the primary source of industrial timber production, contributing with more than 50% to the domestic supply of industrial hardwood (Pra and Pettenella et al., 2019).

Populus alba, commonly known as white poplar, is indigenous to central and southern Europe (Cadullo and de Rigo et al., 2016) and primarily utilized for soil erosion control, restoring rivers and floodplains and biomass production. The need to acclimate the species to harsh climatic conditions and meet the demands of the industry has resulted in the development of numerous clones. Among these, *Populus*

alba clone 'Villafranca' was developed by the Forest and Wood Research Center of Casale Monferrato (Italy) in 1957. This clone is characterized by good quality wood with a basal density of 0.33 g/cm^3, optimal vegetative propagation and the ability to quickly root in soil (Cadullo and de Rigo et al., 2016; Corona et al., 2016).

Clonal propagation is important for commercial wood production, leading to increased productivity and homogeneity in plantations (Mushtaq et al., 2017). Despite this benefit, clonal propagation has significantly reduced genetic diversity, which is detrimental for ensuring crop yields in the face of climate change and diseases. In this context, the breeding programs could benefit from a compromise strategy balancing the greater genetic gain from vegetative propagation (capturing non-additive genetic variance) and the maintenance of genetic diversity in the seed orchard. Seeds are of utmost importance as they serve as a primary source for generating plants for large-scale restoration efforts. Understanding the optimal conditions for seed germination and seedling establishment is crucial for ecological restoration and biodiversity preservation (Dalziell et al., 2022). Given the preference for vegetative propagation in poplars, studies regarding seed germination are scanty. Furthermore, poplar seeds have a relatively short lifespan (Kim, 2018). While extensive studies have been conducted on the physiological, molecular and morphological aspects of germination and post-germination processes in model organisms and crops, a major gap of knowledge is underlined for woody species (Qu et al., 2020).

Long-term seed storage is the most effective approach for safeguarding the genetic diversity of plant materials. Storage parameters that influence seed quality include temperature, moisture content, time, and oxygen pressure, however seed desiccation tolerance (DT) is a crucial endogenous factor (Kijak & Ratajczak, 2020). Based on DT, seeds are categorized into three storage categories: (1) orthodox seeds, capable to endure low moisture levels, can be stored for extended periods of time; (2) recalcitrant seeds, highly susceptible to drying out and freezing, leading to rapid loss of viability; (3) intermediate seeds, falling between orthodox and recalcitrant in terms of viability (Tweddle et al., 2003). In the past, poplar seeds were classified either as recalcitrant, since the seeds have a short lifespan (Gosling, 2007), or as sub-orthodox given their capacity to maintain viability for extended periods at low temperatures (Bonner, 2008). However, according to a more recent classification, many poplar species are categorized as having intermediate seeds (Michalak, 2014).

Seed priming, a pre-sowing method that involves imbibing seeds in water or other priming agents and subsequent drying to the original moisture content, represents a promising option to address seed quality issues in poplars. The advantages of seed priming, ranging from improved germination to enhanced resistance to biotic/abiotic stresses, are well documented in multiple species (Bento et al., 2021; Zulfiqar et al., 2022; Bibi et al.,2024; Dueñas et al., 2024; Gaonkar et al., 2024). Generally, priming protocols are considered as empiric and known to be species/genotype- and even seed lot-dependent (Paparella et al., 2015; Pagano et al., 2023). The seed response in terms of DT should be also considered when designing priming protocols (Smolikova et al., 2020; Pagano et al., 2022a, b). Polyamines (PAs) are used as priming agents (Kusano et al., 2008; Lechowska et al., 2021; Shao et al., 2022). PAs are small nitrogenous bases with several amino groups, present in nearly all eukaryotic and prokaryotic species. Putrescine (Put), spermidine (Spd), and spermine (Spm) are the primary PAs found in plants. They play crucial roles in several biological processes such as flower and fruit development, embryogenesis, organogenesis, senescence, as well as abiotic and biotic stress responses (Li et al., 2014; Huang et al., 2017; Paul and Roychoudhury, 2017; Chen et al. 2019; Hu et al., 2020; Zhang et al., 2022; Fuchs et al., 2023). Recently, numerous studies have demonstrated the positive effects of Spd on seed germination under stress in white clover (Li et al., 2014), rice (Paul and Roychoudhury, 2017), corn (Huang er al., 2017), wild rye (Hongna et al., 2021), sorghum (Zhang et al., 2022) and maple (Fuchs et al., 2023). PAs, particularly Spd, contribute to the antioxidant response, essential for seed viability (Zhang et al., 2014; Lou et al., 2018; Hu et al.,2020; Zhou et al., 2020; Korbas et al., 2022). ROS (reactive oxygen species) accumulation causes seed deterioration during storage (Li et al., 2022), however ROS also act as

signaling molecules, promoting germination. Bailly et al. (2008) defined the term 'oxidative window' as the critical limit of ROS accumulation that enables them to function as signaling molecules, without inducing oxidative stress.

Poplar seeds have a very short life span and they have been previously classified both as recalcitrant or intermediated, due to their low dehydration tolerance, high water content at maturity, and high metabolic activity at dehiscence which makes their survival time in storage very short (Gosling, 2007, Michalak et al., 2014; Lefebvre et al., 2021). This reduced seed viability significantly impairs breeding and genetic conservation programs. The aim of the current study was to design tailored seed priming protocols to improve germination performance of long-term stored, deteriorated *Populus alba* clone 'Villafranca' seeds, used as model system. To do this, seed viability and the dynamics of rehydration-dehydration cycles were monitored using Spd as a priming agent alongside water imbibition and untreated controls. To assess the efficiency of the treatments, phenotypical parameters (germination percentage and speed) and molecular analyses (ROS detection, gene expression profiles) were employed.

2. Material and methods

2.1. Seed material and storage conditions

Seeds of *Populus alba* clone 'Villafranca' were obtained from open pollination at the Forestry and Wood Department of CREA (Council for Agricultural Research and Analysis of Agricultural Economics), Casale Monferrato, Italy. The seeds were collected in April 2008 at the Institute's facility. The seeds were dried in thermostatic chambers at controlled temperature, and then vacuum-packed at -30°C.

2.2. Germination tests

Poplar seeds were transferred to Petri dishes (diameter 90 mm) containing two filter papers moistened with 2.5 ml H_2O, sealed with parafilm and kept in a growth chamber at 22°C under light conditions with photon flux density of 150 µmol m^{-2} s^{-1}, photoperiod of 16 h/8 h and 70-80% relative humidity (RH) inside the containers. Seeds with protrusion of the primary radicle were considered germinated. Germination tests were performed in triplicates of 30 seeds each. Germination was monitored for four days and germination parameters were calculated (Pagano et al., 2022a). For the evaluation of germination performance, the following parameters were used: germinability (G%) and time to reach 50% of final germinants (T_{50}). G% is defined as the percentage of germinated seeds at the end of the germination test. T_{50} was calculated using the following formula: $T_{50} = T_i + (N/2 - N_i)\ (T_j - T_i)/(N_j - N_i)$, where N is the final number of germinated seeds, N/2 is half of final number of germinated seed, N_i and N_j are the total number of seeds germinated in adjacent counts at time T_i and T_j.

2.3. Viability assay

To measure seed viability, poplar seeds were first imbibed in H_2O for 6 h to remove seed coat. The de-coated seeds were then incubated in a 1% (w/v) solution of 2,3,5-triphenyl tetrazolium chloride (TTC; Merck, Darmstadt, Germany), and maintained at 20°C for 18 h in the dark (Faria et al., 2005). The TTC test relies on the activity of dehydrogenase enzymes in mitochondria. TTC penetrates into the seed tissues where it interferes with the reduction processes of the living cells by accepting a hydrogen ion. In the reduced form, the TTC-salt is a red-colored, stable, non-diffusible substance called triphenylformazan or formazan (De Barros França-Neto and Krzyzanowski, 2019). Stained tissues are considered viable, and unstained white tissues are considered dead. The TTC test was carried out using five replicates of 10 seeds per treatment.

2.4. Treatments

The seed response along the rehydration-dehydration cycle was monitored by measuring the seed weight, thus producing imbibition and dehydration curves. Water uptake and loss (in presence/absence of Spd) were evaluated along multiple imbibition (1, 2, 3, 4 and 5 h) and dehydration (1, 2 and 3 h) timepoints by measuring the weight of 100 seeds at each time point. All measurements were performed in triplicates.

Seed treatments were devised as follows: seed priming in water (HP, hydropriming), seed presoaking in the presence/absence of Spd. For the HP treatments, seeds were imbibed in water for 2, 4, 6, and 8 h (HP2, HP4, HP6, HP8). For each imbibition treatment seeds were then dried-back (DB) at 25 °C for 1, 2, 3 and 4 h (DB1, DB2, DB3, DB4). For the presoaking treatments, seeds were imbibed in H_2O for 1, 2, 4, and 6 h, without the DB step. For the Spd treatments, two Spd concentrations (50 µM, Spd50; 100 µM, Spd100) were applied for 1 h and 4 h, Untreated controls (UT) were used. After each treatment, germination tests were conducted and monitored as described in paragraph 2.2.

2.5. ROS quantification

ROS levels were quantified in dry and imbibed seeds collected at the indicated timepoints, using the fluorogenic dye 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, Milan, Italy). The dye is converted to a non-fluorescent molecule following deacetylation mediated by cellular esterases, and it is subsequently oxidized by ROS into the fluorescent compound 2',7'-dichlorofluorescein. DFC can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm, respectively (LeBel et al., 1992). The assay was carried out as described by Griffo et al. (2023), with the following modifications. Samples of 40 seeds per time point were incubated for 60 min with 50 µl of 10 µM DCFH-DA and subsequently fluorescence was determined at 517 nm using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia), setting the program for one cycle of 30 s at 25°C. As negative control, a sample containing only DCFH-DA was used to subtract the baseline fluorescence. The fluorescence was calculated by normalizing samples to controls and are expressed as Relative Fluorescence Units (R.F.U.).

2.6. RNA extraction and cDNA synthesis

Seeds imbibed with water and Spd were collected at different imbibition time (0, 1, 4 h) and DB timepoints (1, 2 h) for molecular analyses. Seed aliquots (80-90 mg) were grinded in liquid N_2 and collected into 1.5 ml Eppendorf tubes. RNA was extracted as described by Oñate-Sánchez and Vicente-Carbajosa (2008), with the following modifications. A volume of 550 µl extraction buffer (0.4M LiCl, 0.2M Tris pH 8, 25 mM EDTA, 1% SDS) and 550 µl chloroform, were added to each Eppendorf tube containing approx. 50 mg of seed powder and vortexed for 10 s. The samples were centrifuged at 10.000 rpm for 3 min at 4°C. The upper phase was collected and transferred to new 1.5 ml Eppendorf tubes, 500 µl of water-saturated acidic phenol was added and vortexed for 10 s. A volume of 200 µl chloroform was added to the sample, gently mixed, and centrifuged at 10.000 rpm for 3 min at 4°C. The upper phase was collected and transferred to new 1.5 ml Eppendorf tubes and a 1/3 volume of lithium chloride was added. The samples were incubated at 4°C for 60 min then centrifuged at 10.000 rpm for 30 min at 4°C. Supernatant was discarded, the RNA pellet was washed with 100 µl ice-cold 70% ethanol and centrifuged at 10.000 rpm for 1 min at 4°C, followed by one additional wash with 100 µl ice-cold 100% ethanol. The RNA pellet was air dried and resuspended in nuclease-free H_2O. The concentration of each RNA sample was measured using a Biowave spectrophotometer (Biochrom Ltd., England), and RNA integrity was assessed using agarose gel electrophoresis. For cDNAs synthesis, the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Monza, Italy) was used, according to the manufacturer's recommendations.

2.7. Quantitative Real-time PCR (qRT-PCR)

The qRT-PCR reactions were carried out using a CFX Duet Real-time PCR system machine (Bio-Rad Laboratories Inc., Milan, Italy) and the Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Monza, Italy), as indicated by the manufacturer. The machine is operated using the Bio-ad CFX maestro software (Bio-Rad Laboratories Inc.) and the following amplification protocol was applied: denaturation at 95°C, 10 min, and 40 cycles of 95°C, 15 s and 60°C, 30 s, final extension at 72°C, 30 s.

Oligonucleotide primers were designed using the Real-Time PCR Primer Design program Primer3Plus (https://primer3plus.com) and further validated using Oligo Analyzer (https://eu.idtdna.com/calc/analyzer). Target specificity was assessed through Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Relative quantification was carried out using *Tub* (tubulin beta-4 chain, LOC118055616) as reference gene, given its stable expression in the used samples (**Appendix Fig. 1**). The following genes were tested: *ABI3* (B3 domain-containing transcription factor ABI3, LOC118049976), *FUS3* (B3 domain-containing transcription factor FUS3-LIKE, LOC118038131), *SPDS* (spermidine synthase, LOC118055381), *SPMS* (spermine syntase, LOC118033446), *APX* (ascorbate peroxidase, cytosolic-like, LOC118045884), *CycB1* (cyclin B1-2, LOC118029686), *CDKA1* (cyclin-dependent kinase A1, LOC118050756), and *CycD2* (cyclin-D2-like, LOC118031573). Oligonucleotide sequences are provided in **Appendix Table 1**. The Thomsen method was employed for relative quantification of transcript accumulation using a standardized efficiency (E) value of 1.8 (Thomsen et al., 2010). All reactions were carried out in triplicates. Z-score (mean-centered and divided by the standard deviation of each variable) was calculated on the linearized Ct values and used to generate heatmaps with the GraphPad Prism version 8.0.1 program.

2.8. Statistical analysis

For the germination tests and viability assay data, statistical analyses were performed using the Student's *t*-test. Asterisks indicate statistically significant differences determined using Student's t-test (*, $p \leq 0.05$). ROS quantification and gene expression analyses were analyzed through two-way analysis of variance (ANOVA) and the Duncan's test, using the software Rapid Publication-Ready MS Word Tables Using Two-Way ANOVA 1.0 (Assaad et al., 2015), available online (https://housseinassaad.shinyapps.io/TwoWayANOVA/). Pearson's correlation and Principal Component Analysis (PCA) were carried out using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/docs/Publications.xhtml), normalizing the values by Z-score and considering a p-value ≤ 0.05 as the threshold for significance (Pang et al., 2021).

3. Results

3.1. Reduced seed viability in long-term stored *P. alba* seeds

The germination percentage (G%) of long-term stored 'Villafranca' seeds was assessed to define the baseline of the used materials. The germinability was monitored for 48 h, with an estimated final G% of 19.00 ± 4.47 %) (**Fig. 1A**). TTC assay, performed to quantify the levels of seed viability showed that the percentage of non-viable seeds was significantly higher (68 ± 13.03%) compared to the viable ones (32 ± 13.03%) (**Fig. 1B**). Taken together, these data indicate that the low germination observed in 'Villafranca' seeds was due to a substantial loss of viability during storage.

3.2. Imbibition curves denote rapid water loss during dehydration

The dynamics of water uptake and loss (rehydration-dehydration cycle) were evaluated in naturally aged *P. alba* seeds at different time points during imbibition (1, 2, 3, 4, 5 h) with water (H_2O), Spd50 (50 µM spermidine) and Spd100 (100 µM spermidine) as well as during dry-back (DB) (1, 2 h) (**Fig. 2**). Across all treatments, a rapid water uptake was observed during the first two hours while at the

subsequent timepoints the process was slower. No significant differences between the absorption of water or Spd solutions was evidenced. A very rapid water loss was evidenced already from the first hour of the dry-back process (**Fig. 2**, 1 h DB). Additionally, the seed weight at 2 h DB (0.31 ± 0.03 mg) was lower than that of the initial dry state of the seeds (0.47 ± 0.01 mg). Also in this case, no significant differences were observed between the treatment with water or Spd (**Fig. 2**).

3.3. Seed priming does not improve germination of naturally aged seeds

In investigate if long-term stored seeds were responsive to priming treatments, different HP protocols were tested taking into consideration various imbibition and DB timepoints. The seeds were subjected to an increasing imbibition time of 2, 4, 6 and 8 h, associated with the corresponding DB time of 1, 2, 3 and 4 h. To test the efficiency of the treatments, germination tests were carried out alongside untreated (UT) seeds used as control. As shown in **Fig. 3**, none of the HP treatments applied was able to enhance G%. Moreover, a significant reduction in germinability was observed after the 8 h HP treatment (3.33 ± 5.77%) compare with the UT control (28 ± 2.88%). This indicates that HP was perceived as a stress treatment rather than a beneficial one. We hypothesize that this may be due to the rapid water loss evidenced previously during the DB process (**Fig. 2**).

3.4. Seed soaking with spermidine enhances germination performance

Given the inefficiency of standard HP protocols, the next step consisted in applying only seed soaking treatments, thus avoiding the DB step. In this experiment, seeds were soaked in water or Spd solutions (50 µM, Spd50; 100 µM, Spd100) for different time intervals, namely 1, 2, 4, and 6 h. Among the tested water soaking times, only 1 h (43.33 ± 5.77%) and 4 h (46.66 ± 5.77%) proved to be efficient in significantly increasing G% compared to the UT seeds (26.66 ± 5.77%) (**Appendix Fig. 2**). Therefore, these timepoints were selected to carry out imbibition also with the Spd solutions. The germination tests conducted subsequently evidenced that soaking with 100 µM spermidine for 4 h was the most efficient treatment resulting in a significant rise of G% (36.66 ± 5.77%), compared to UT (15 ± 5%) (**Appendix Fig. 3**).

To confirm these results, another set of germination tests was conducted using the optimized timepoint (4 h) and Spd concentration (100 µM) (**Fig. 4**). In this case, both G% and germination speed(T_{50}) was assessed. Regarding G% **(Fig. 4A)**, only seeds treated with 100 µM Spd (Spd100) exhibited significantly enhanced germination (35.00 ± 5.00%) compared to UT controls (20.00 ± 5.00%). This finding was well corroborated by a significant reduction in the T_{50} values, observed only for the Spd100 treatment **(Fig. 4B)**. Overall, these results indicate that seed soaking with Spd represents an efficient treatment to improve the germination potential of long-time stored *P. alba* seeds.

3.5. Molecular characterization of pre-germinative metabolism in 'Villafranca' poplar seeds

To better understand the molecular mechanisms behind the efficacy or inefficacy of the imposed treatments on the germination performance of long-term stored 'Villafranca' seeds, a molecular characterization was carried out (**Fig. 5**). This included the evaluation of the seed oxidative status, by measuring the production of ROS through the DCFH-DA assay, along with qRT-PCR analyses performed to quantify the expression levels of selected genes with relevant roles during early seed germination. The experimental system used was composed of dry seeds (DS, 0 h), seeds imbibed with H_2O or 100 µM Spd for 1 h and 4 h, as well as following the dry-back process at 1 and 2 h DB (**Fig. 5A**).

ROS quantification was assessed given its significant role in relation to seed aging and stress response. In the case of the seeds treated with H_2O, a significant increase in ROS levels was observed at 4 h imbibition and 1 and 2 h DB, with the highest accumulation registered for the 1DB samples (3.17 ± 0.62 R.F.U) (**Fig. 5B**). Interestingly, in the seeds treated with Spd100, ROS levels remained constant

(with no significant differences compared to control) up to the 1DB step, and only a slightly significant increase was evidenced in the case of 2DB samples. When comparing the differences between H_2O and Spd100 treatments during seed imbibition (4 h) and dry-back (1DB), it is possible to observe a significant decrease in ROS levels when the seeds are treated with spermidine (**Fig. 5B**). This suggests that Spd may act as an antioxidant mechanism to limit ROS production both during water uptake and loss.

To further investigate this aspect, we examined the expression patterns of genes involved in DT acquisition (*ABI3*, *FUS3*), polyamine biosynthesis (*SPDS1*, *SPMS*), antioxidant response (*APX*), and cell cycle regulation (*CycB1*, *CDKA1*, *CycD2*). The results are displayed as a heatmap, with blue color indicating low expression and red color indicating high gene expression (**Fig. 5C**). Changes in gene expression profiles were observed during the rehydration and dehydration steps. An intriguing finding was that all the investigated genes were highly expressed in dry seeds (0h). Compared to this, both H_2O and Spd100 treatments resulted into a significant reduction in the expression of *ABI3*, *SPMS*, and *CDKA1* genes at 1 h imbibition, and this low expression persisted at the subsequent time points, including DB. The Spd100 treatment seemed to induce the expression *FU3*, *APX* and *CycB1* genes in response to 2 h dehydration (2DB) and this upregulation was not observed in H_2O-treated seeds. In this case, the only significantly upregulated genes compared to control (0 h) were *SPDS1* at 1 h imbibition and *CycD2* at 2 h DB.

To integrate all the data obtained from the molecular and phenotyping analyses, a PCA (Principal Component Analysis) was carried out to illustrate the overall behavior of the applied treatments (**Fig. 6**). The PCA clustering evidenced the formation of three distinct groups (UT, H_2O, Spd100) corresponding to each of the imposed treatments (**Fig. 6A**). Based on the biplot analysis, the separation of the UT samples was mainly due to gene expression data. The key variable contributing to the separation of H_2O samples was the DCFH-DA data, while the germination parameters mostly contributed to the distinct separation of the Spd100 samples (**Fig. 6B**).

4. Discussion

The current study aimed at developing seed treatments to enhance the germination performance of long-term stored *P. alba* clone 'Villafranca' seeds. The study focused on a batch of seeds stored under vacuum for 16 years at -30°C. Given that poplar seeds have a short lifespan following harvest (Wyckoff and Zasada, 2005; Gosling, 2007), they need to be prepared for storage immediately. Also, because they are defined as intermediate seeds (Michalak, 2014), hence desiccation-sensitive, the storage conditions represent a limiting factor hampering seed viability.

The baseline characterization of the long-term stored 'Villafranca' seeds evidenced low viability (approximately 20%). Loss of viability during storage has been reported in many trees, including *Salix caprea* and *S. gracilistyla* (Popova et al., 2012), as well as different poplar species like, *Populus nigra* (Suszka et al., 2014) *P. davidiana*, *P. koreana* (Kim et al., 2018) and the *P. alba* × *P. glandulosa* hybrid (Popova et al., 2013). When considering poplars, the storage time and temperature, along with the seed water content, are essential and species-dependent. For instance, it was shown that *P. nigra* seeds were successfully stored for only two years at temperatures of -10 °C or -20 °C, while maintaining high levels of viability (Suszka et al., 2014). Significant differences in seed viability based on seed water content were reported for *P. davidiana* (orthodox seeds) and *P. koreana* (intermediate seeds) (Kim, 2018). Cryopreservation was indicated as a more suitable storage method for short lifespan seeds (Pritchard, 2007). *P. alba* x *P. glandulosa* hybrids exhibited an equivalent germination rate as the controls when cryopreserved at a water content of 0.07-0.10 g g^{-1} for 2 weeks (Popova et al., 2013). Recently, seed priming has been promoted as part of the integrated resource management programs for fragile ecosystems (Siva Devika et al., 2021). HP, considered among the most simple, sustainable, and cost-

effective seed priming methods (Forti et al., 2020; Sushma et al., 2023), was first selected for 'Villafranca' seed treatments in this work.

In our study, multiple imbibition and DB timepoints were tested but none had positive effects on the germination of long-term stored 'Villafranca' seeds. Moreover, imbibition for 8 h followed by 4 h DB resulted in a significant reduction of G%. Such a deleterious effect might be due to the rapid water loss occurring during the dry-back phase of the treatment. This is also a typical feature of desiccation-sensitive seeds, both intermediate and recalcitrant (Lah et al., 2023; Zhang et al., 2023). HP proved to be ineffective also for other species with recalcitrant seeds like *Cupania glabra* and *Cymbopetalum baillonii* (Becerra-Vázquez et al., 2020), or it had limited success after a mild dry-back treatment in the case of *Quercus rugosa* (Castro-Colina et al., 2011).

Given that HP was not successful, and the dry-back might be potentially harmful to seed viability, soaking treatments were then tested, thus eliminating the dehydration step. Soaking either in water of Spd50 and Spd100 solutions at 50 and 100 µM showed that the Spd100 applied for 4 h was the most effective treatment, doubling G% and increasing germination speed, compared to untreated seeds. These results are in agreement with previous reports showing that soaking treatments were efficient in improving germination of various species with intermediate and recalcitrant seeds, like *Osyris lanceolata* (Mwang'Ingo et al., 2004), *Psidium guajava* (Bhanuprakash et al., 2008), *Pinus roxburghii* (Ghildiyal et al., 2009), *Litchi chinensis* (Zhang et al., 2015), and *Pouteria campachiana* (Amoakoh et al., 2017). In our study, the germinability of long-term stored 'Villafranca' seeds was significantly enhanced only when Spd was added in the soaking solution. This essential polyamine has been extensively studied for its beneficial effect on seed germination under stress (Li et al., 2014; Huang et al., 2017; Paul and Roychoudhury, 2017; Chen et al. 2019; Hu et al., 2020; Zhang et al., 2022; Fuchs et al., 2023). In a recent study, recalcitrant *Acer saccharinum* seeds treated with Spd showed improved germination after exposure to mild and severe desiccation (Fuchs et al., 2023).

The positive effect of Spd on the germination of aged seeds could be explained also through its antioxidant properties (Fuchs et al., 2023; Cao et al., 2023; He et al., 2024), thus contributing to maintaining the ROS balance during germination. ROS are important players given that they act as signaling molecules in many seed physiological processes (Bailly, 2019) while high ROS accumulation leads to detrimental effects and loss of seed viability (Kurek et al., 2019). The DCFH-DA assay carried out in this study to monitor ROS production in 'Villafranca' imbibed seeds evidenced a significant decrease in ROS levels when seeds were treated with Spd. This is in agreement with results from other studies where Spd was applied to *Solanum lycopersicum* (Zhang et al., 2014), *Medicago sativa* (Lou et al., 2018), *Oryza sativa* (Hu et al., 2020), *Vigna radiata* (Zhou et al., 2020) and *Cucumis sativus* (Korbas et al., 2022).

To investigate more in-depth the molecular events underlying the positive effect of Spd as well as the negative impact of the DB step on the germination of long-term stored 'Villafranca' seeds, gene expression analysis was conducted, focusing on genes related to DT acquisition, PA biosynthesis, antioxidant response, and cell cycle regulation. *ABI3* and *FUS3* genes play major roles in seed maturation and DT acquisition (Lepiniec et al., 2018). ABI3 functions as both an autoregulator and a regulator of FUS3 (To et al., 2006). During germination, and therefore DT loss, the function of *ABI3* and *FUS3* genes are suppressed as they have a prominent role during embryo development and seed maturation (Lepiniec et al., 2018). This is in agreement with the qRT-PCR analyses carried out in this study, showing consistent downregulation of these two genes during imbibition with both water and Spd when compared to dry seeds. Considering PA biosynthesis, *SPDS* encodes for the enzyme that converts putrescine into Spd which is subsequently transformed into spermine by the SPMS enzyme (Smirnova et al., 2018). In our experimental system, the *SPDS1* gene was upregulated after 1 h of imbibition in H_2O while *SPMS* was highly expressed only in dry seeds. Similar results were obtained in other studies where *SPDS* gene expression increased during imbibition in maize seeds (Huang et al., 2017) and the

activity of the *SPMS* gene was mostly limited to dry seeds in *Medicago truncatula* (Pagano et al., 2022b). These results indicate that the upregulation of these genes in mature seeds and during early imbibition could be associated with the activation of the antioxidant system in maintaining a proper ROS balance. In agreement with this statement is also the high expression of the *APX* gene, involved in H_2O_2 scavenging (Pandey et al., 2017), in dry seed and subsequent downregulation afterwards. Considering that germination involves the restart of cell cycle progression, the initiation of G1 phase and activation of the G1-to-S transition are crucial steps for a successful germination (Barrôco et al., 2005). The expression profiles of cell cycle genes were evaluated during the early stages of 'Villafranca' seed germination to gain additional information. *CDKA1* encodes for an A type cyclin-dependent kinase while *CycD2* encodes a D-type cyclin, both playing roles in the transition from G1-to-S phase (Mironov et al., 1999; Sanz et al., 2011). On the other hand, *CycB1* encodes for a protein that promotes the progression of the cell cycle during M-phase (Menges et al., 2003) and it is also involved in the homologous recombination (HR) repair pathway (Weimer et al., 2016). In our experimental system, the *CycB1* gene was highly expressed during DB after the Spd treatment while the *CycD2* gene was most expressed at the same timepoint but when water was used. During the desiccation process, DNA damage can accumulate and this damage must be effectively repaired before the start of germination (Waterworth et al., 2010). The observed upregulation of the *CycB1* and *CycD2* genes may indicate that the cell cycle progression could be stalled to allow more time for an efficient repair. Other studies have also indicated that spermidine and spermine treatments promoted protection against DNA oxidative damage in wheat plants exposed to heavy metal stress (Taie et al., 2019).

5. Conclusion

To conclude, the current study reports the development of an optimized seed treatment able to improve germination of long-term stored *P. alba* clone 'Villafranca' seeds. The use of a spermidine solution at a concentration of 100 µM applied as seed soaking for 4 h, doubled G% and improved germination speed. HP treatments were ineffective in the case of poplar seeds because of the rapid water loss during the DB step. Molecular analyses revealed that the positive effect of the Spd treatment can be attributed to lower ROS production when the pre-germinative metabolism is resumed. Additionally, gene expression analyses indicate the upregulation of genes involved in DT acquisition, PA biosynthesis, and antioxidant defense in dry seeds. To our knowledge, this is the first reports addressing the issues of seed quality in white poplar. The tailored protocol hereby developed will be validated in other poplar clones and species, and further optimized to become a useful tool supporting seed bank operators, breeders and seed technologists specialized in the agroforestry.

Acknowledgments

The authors received funding from National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4—Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union–NextGenerationEU; Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP B83C22002930006 and CUP F13C22000720007, Project title "National Biodiversity Future Center—NBFC".

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Figures captions

Figure 1. Baseline characterization of long-term stored *Populus alba* clone 'Villafranca' seeds. **(A)** Germination percentage (G%). **(B)** Viability test performed through the TTC method. Statistically significant differences calculated using the Student's *t*-test are indicated with an asterisk (*, $P \leq 0.05$).

Figure 2. Imbibition curves represented as seed weight gain and loss in long-term stored *Populus alba* clone 'Villafranca' seeds subjected to different intervals of rehydration (1, 2, 3, 4, 5 h) and dehydration (1, 2 h). Statistically significant differences calculated using the Student's *t*-test are indicated with an asterisk (*, $P \leq 0.05$). DB, dry-back; Spd50, spermidine 50 µM; Spd100, spermidine 100 µM.

Figure 3. Germination percentage (G%) of long-term stored *Populus alba* clone 'Villafranca' seeds treated/untreated with hydropriming (HP) at different imbibition timepoints (2, 4, 6, 8 h) followed by dry-back (DB). Statistically significant differences were calculated compared to untreated (UT) controls using the Student's *t*-test and are indicated with an asterisk (*, $P \leq 0.05$).

Figure 4. Germination performance of long-term stored *Populus alba* clone 'Villafranca' seeds soaked for 4 h in water or spermidine. **(A)** Germination percentage (G%). **(B)** Germination speed calculated in terms of T_{50}. Statistically significant differences were calculated compared to untreated (UT) controls using the Student's *t*-test and are indicated with an asterisk (*, $P \leq 0.05$). UT, untreated; H_20, water soaking; Spd100, 100 µM spermidine soaking; DB, dry-back.

Figure 5. Molecular profiling of long-term stored *Populus alba* clone 'Villafranca' seeds along the rehydration-dehydration cycle. **(A)** Schematic representation of the experimental design including the selected rehydration (1 and 4 h) and dehydration (1 and 2 h) time points. The imposed treatments included imbibition in H_20 or a spermidine (Spd) solution at a concentration of 100 µM alongside dry seeds (0 h) used as untreated controls. **(B)** Quantification of reactive oxygen species (ROS) through the DCFH-DA assay. **(C)** Heatmaps representing the expression patterns of indicated genes obtained through qRT-PCR analyses. Blue color indicates low gene expression while red color indicates high expression. Samples followed by different letters indicate statistically significant differences ($P \leq 0.05$) as per Duncan test. R.F.U, relative fluorescence units; H_20, water soaking; Spd100, 100 µM spermidine soaking; DB, dry-back; *ABI3*, B3 Domain-Containing Transcription Factor ABI3; *FUS3*, B3 Domain-Containing Transcription Factor FUS3-like; *SPDS1*, Spermidine synthase 1; *SPMS*, Spermine synthase; *APX*, Ascrobate peroxidase, cytosolic like; *CYCB1*, Cyclin B1-2; *CDKA1*; Cyclin dependent kinase A; *CYCD2*, Cyclin D2-1 like.

Figure 6. Principal Component Analysis (PCA) plots using data gathered for the treatments (UT, H_20, Spd100) imposed to long-term stored *Populus alba* clone 'Villafranca' seeds. **(A)** Score plot clustering. **(B)** Biplot obtained with data from germination tests (G and T_{50}), ROS measurements (DCHF-DA) and gene expression data (*ABI3, FUS3, SPDS1, SPMS, APX, CycB1, CDKA1, CycD2*). UT, untreated; H_20, water soaking; Spd100, 100 µM spermidine soaking.

Figures

Fig.1

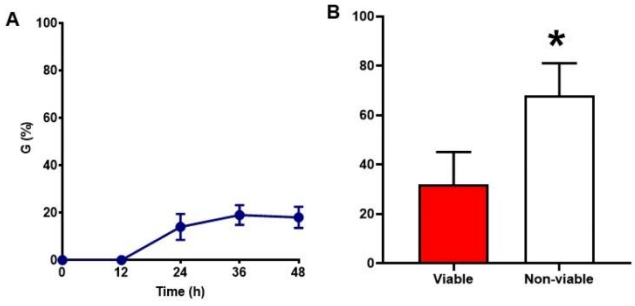


Fig.2

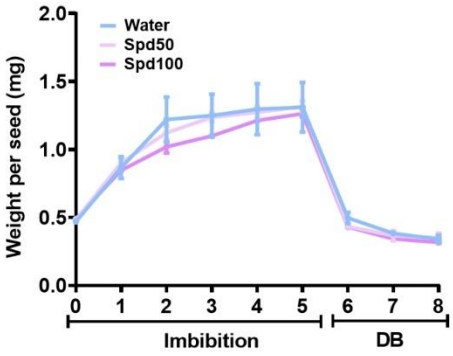


Fig. 3

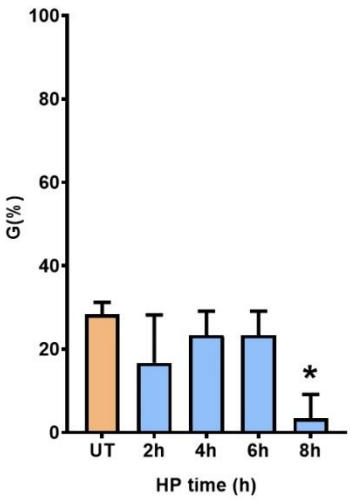

100
80
60
40
20
0
G(%)
*
UT
2h
4h
6h
8h
HP time (h)

Fig.4

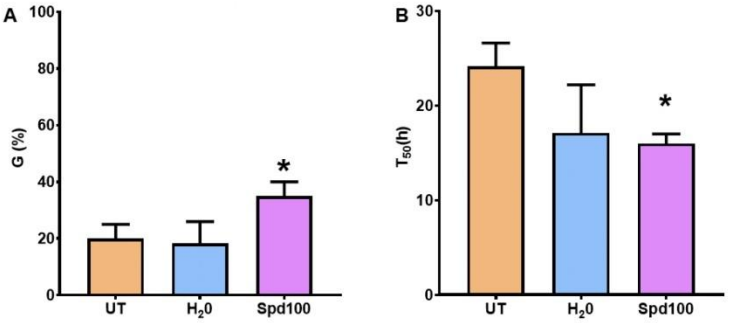

A
100
80
60
40
20
0
G (%)
*
UT
H20
Spd100
B
30
20
10
0
T50(h)
*
UT
H20
Spd100

Fig.5

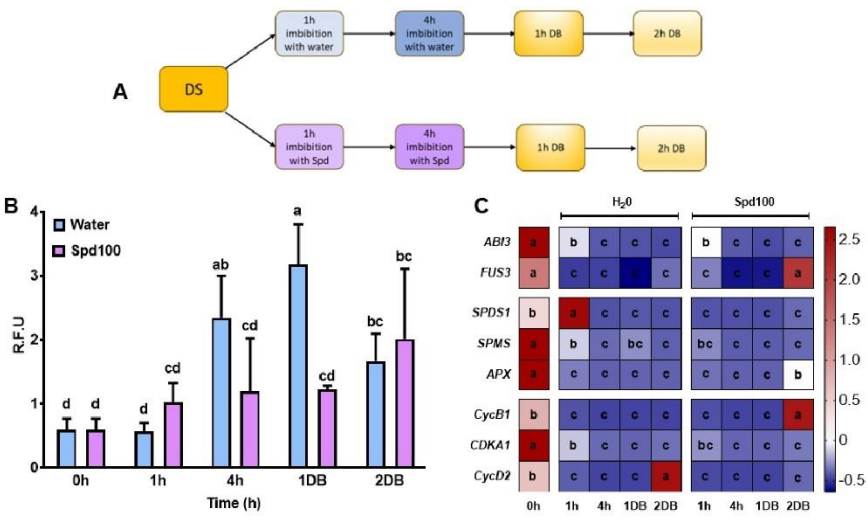
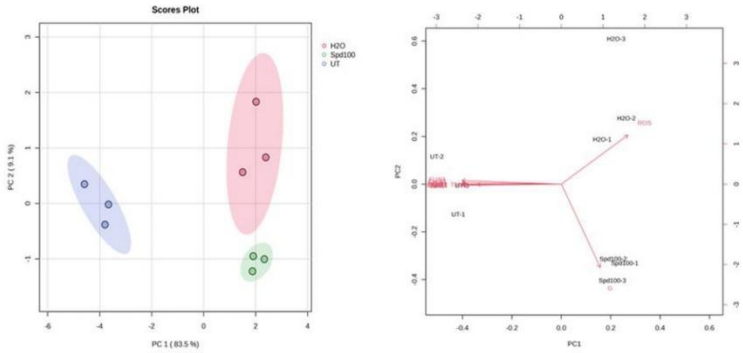

A
DS
1h imbibition with water
4h imbibition with water
1h DB
2h DB
1h imbibition with Spd
4h imbibition with Spd
1h DB
2h DB
B
Water
Spd100
R.F.U
Time (h)
0h
1h
4h
1DB
2DB
C
H2O
Spd100
ABI3
FUS3
SPDS1
SPMS
APX
CycB1
CDKA1
CycD2

Fig. 6

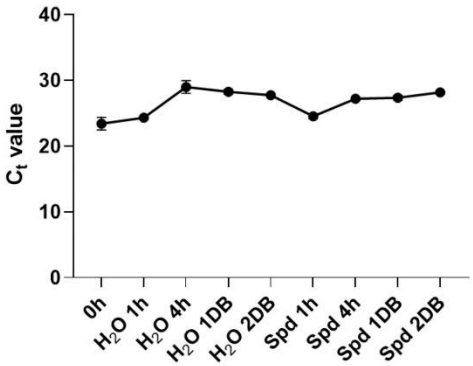

Scores Plot
H2O
Spd100
UT
PC 1 (83.5 %)
PC 2 (9.1 %)
PC1
PC2

Appendix

Appendix Table 1. List of oligonucleotide primers used for qRT-PCR analyses. *TUB,* Tubulin beta-4 chain; *ABI3*, B3 Domain-Containing Transcription Factor ABI3; *FUS3*, B3 Domain-Containing Transcription Factor FUS3-like; *SPDS1*, Spermidine synthase 1; *SPMS*, Spermine synthase; *APX*, Ascrobate peroxidase, cytosolic like; *CYCB1*, Cyclin B1-2; *CDKA1*; Cyclin dependent kinase A; *CYCD2*, Cyclin D2-1 like.

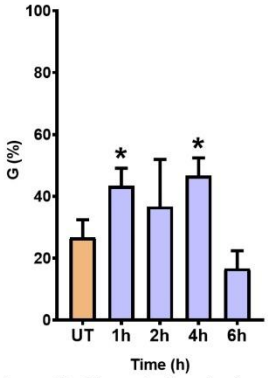
Gene	Oligonucleotide forward sequences	Oligonucleotide reverse sequences
Tub	5' TTTGCTCCTCTTACATCCCG 3'	5' GCAGCACACATCATGTTTT 3'
ABI3	5' CGC AGA TGA TCC CAA TGG TG 3'	5' CCA AGA CTC CCC ACA TCA CT 3'
FUS3	5' GGATGCCCAGACAGAGGA 3'	5' AGG TGT GTC TCG GCT GCT 3'
SPDS1	5' GTGCTCCTCCTCCTCTTTC 3'	5' AATGCAACTCCATCACCGAC 3'
SPMS	5' GTGGTGATGGCGGTGTTC 3'	5' CCC ACA TGA AGT TGG ACCC 3'
APX	5' AGC GTT CTG GAT TTG AGG GA 3'	5' GCA AAC CCA AGC TCT GAG AG 3'
CycB1	5' GCTGCTGGTGTTGATGGAGT 3'	5' ACCTCTTCACTATCGGGGCT 3'
CDKA1	5' AGGGGATTCGGAGATTGAT 3'	5' TCTTGAAGTCGGGCAAAGAA 3'
CycD2	5'AACCAGGCAGCCAAAACCAA 3'	5' GCCACAGCCCACGAATCATT 3'

Appendix Figure 1



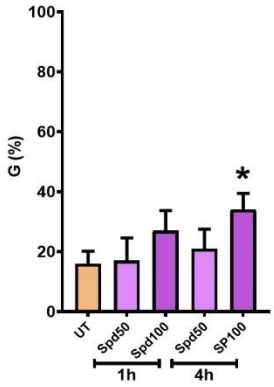
Appendix Figure 1. C_t values of *Tub* gene utilized as reference in the experimental system, including the selected rehydration (1 and 4 h) and dehydration (1 and 2 h) time points.

Appendix Figure 2



Appendix Figure 2. Germination percentage (G%) of long-term stored *P. alba* clone 'Villafranca' seeds following water soaking. Seeds were subjected to different soaking time intervals, namely 1, 2, 4 and 6 h. Statistically significant differences were calculated compared to untreated (UT) controls using the Student's *t*-test and are indicated with an asterisk (*, $P \leq 0.05$).

Appendix Figure 3



Appendix Figure 3. Germination percentage (G%) of long-term stored *P. alba* clone 'Villafranca' seeds following soaking in spermidine (Spd) solutions (50 µM and 100 µM). Seeds were subjected to different soaking time intervals, namely 1 and 4 h. Statistically significant differences were calculated compared to untreated (UT) controls using the Student's *t*-test and are indicated with an asterisk (*, $P \leq 0.05$).

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Review

Plant-Based Biostimulants for Seeds in the Context of Circular Economy and Sustainability

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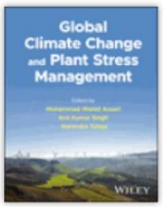
https://doi.org/10.3390/plants13071004



Global Climate Change and Plant Stress Management

Editor(s): Mohammad Wahid Ansari, Anil Kumar Singh, Narendra Tuteja

First published: 14 August 2023

Print ISBN: 9781119858522 | Online ISBN: 9781119858553 | DOI: 10.1002/9781119858553

Chapter 22

Seed Quality Assessment and Improvement Between Advancing Agriculture and Changing Environments

Andrea Pagano, Paola Pagano, Conrado Dueñas, Adriano Griffo, Shraddha Shridhar Gaonkar, Francesca Messina, Alma Balestrazzi, Anca Macovei

Book Editor(s):Mohammad Wahid Ansari, Anil Kumar Singh, Narendra Tuteja

First published: 07 July 2023 | https://doi.org/10.1002/9781119858553.ch22