agriculture

MDPI

Article

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

Shraddha Shridhar Gaonkar, Federico Sincinelli, Alma Balestrazzi and Andrea Pagano *

Department of Biology and Biotechnology "L. Spallanzani", University of Pavia, Via Ferrata 9, 27100 Pavia, Italy; shraddha.shridhargaonka01@universitadipavia.it (S.S.G.); federico.sincinelli01@universitadipavia.it (F.S.); alma.balestrazzi@unipv.it (A.B.)

* Correspondence: andrea.pagano01@universitadipavia.it

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.

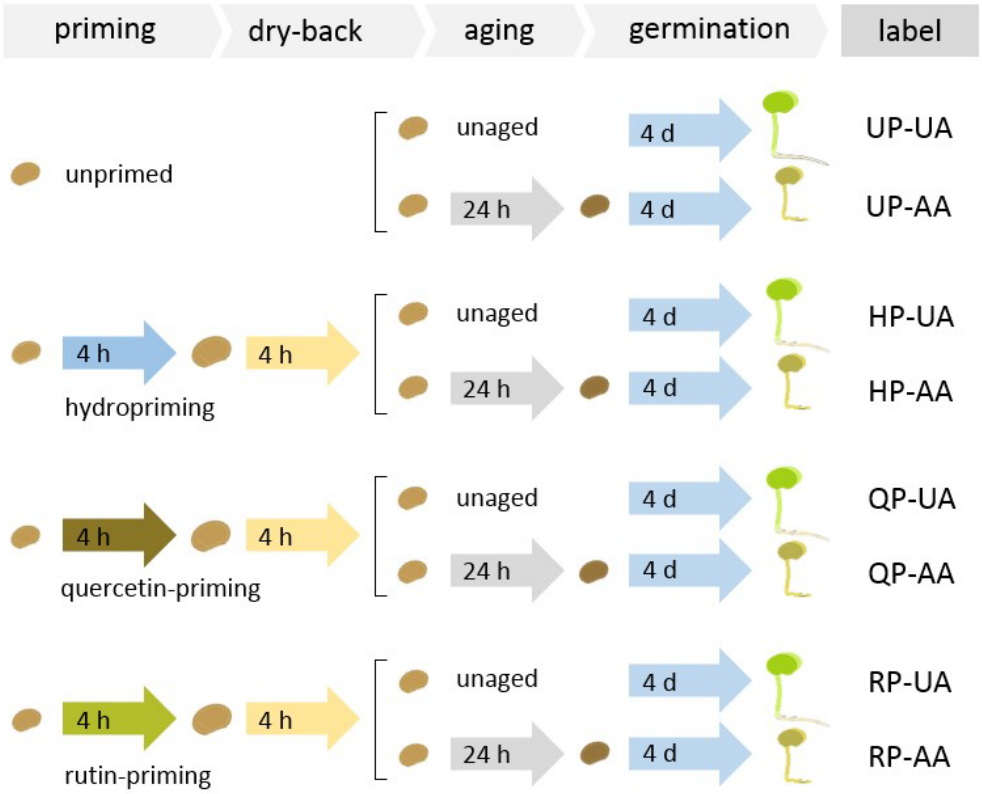

Figure 1. Overview of the experimental system to compare the effects of accelerated aging on *Medicago truncatula* seeds after hydropriming, quercetin-priming or rutin-priming. UP, unprimed control conditions; HP, hydropriming; QP, quercetin priming; RP, rutin priming; UA, unaged control conditions;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

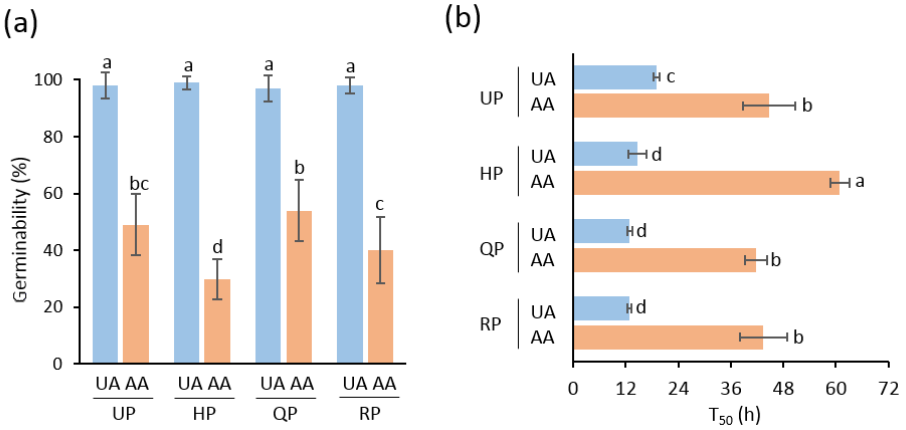

Figure 2. Germination performance of *Medicago truncatula* seeds subjected to hydropriming, quercetin-priming or rutin-priming followed by accelerated aging. (**a**) Germinability percentage. (**b**) T_{50}. UP, unprimed control conditions; HP, hydropriming; QP, quercetin-priming; RP, rutin-priming; 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.

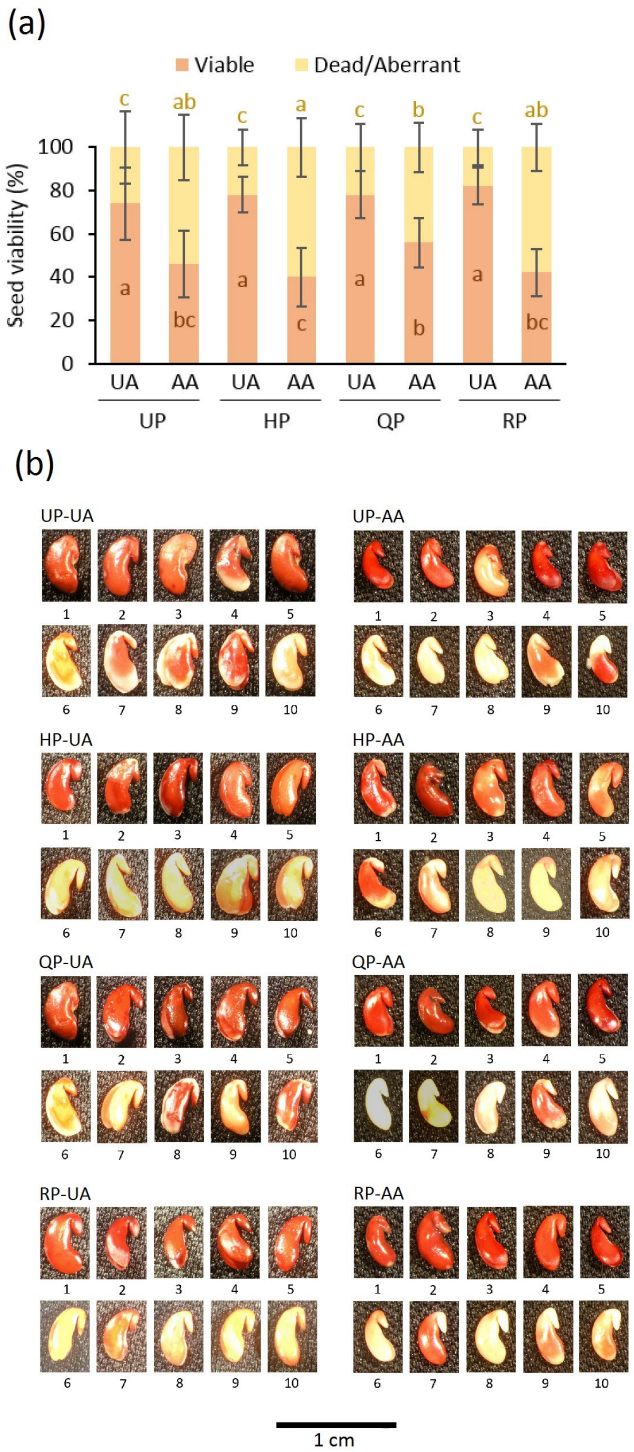

Figure 3. Viability of *Medicago truncatula* seed following hydropriming, quercetin-supplemented priming or rutin-supplemented priming combined with accelerated aging. (**a**) Seed viability percentage assessed with TTC staining. (**b**) Representative pictures of viable (row numbers 1 to 5) and non-viable (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.

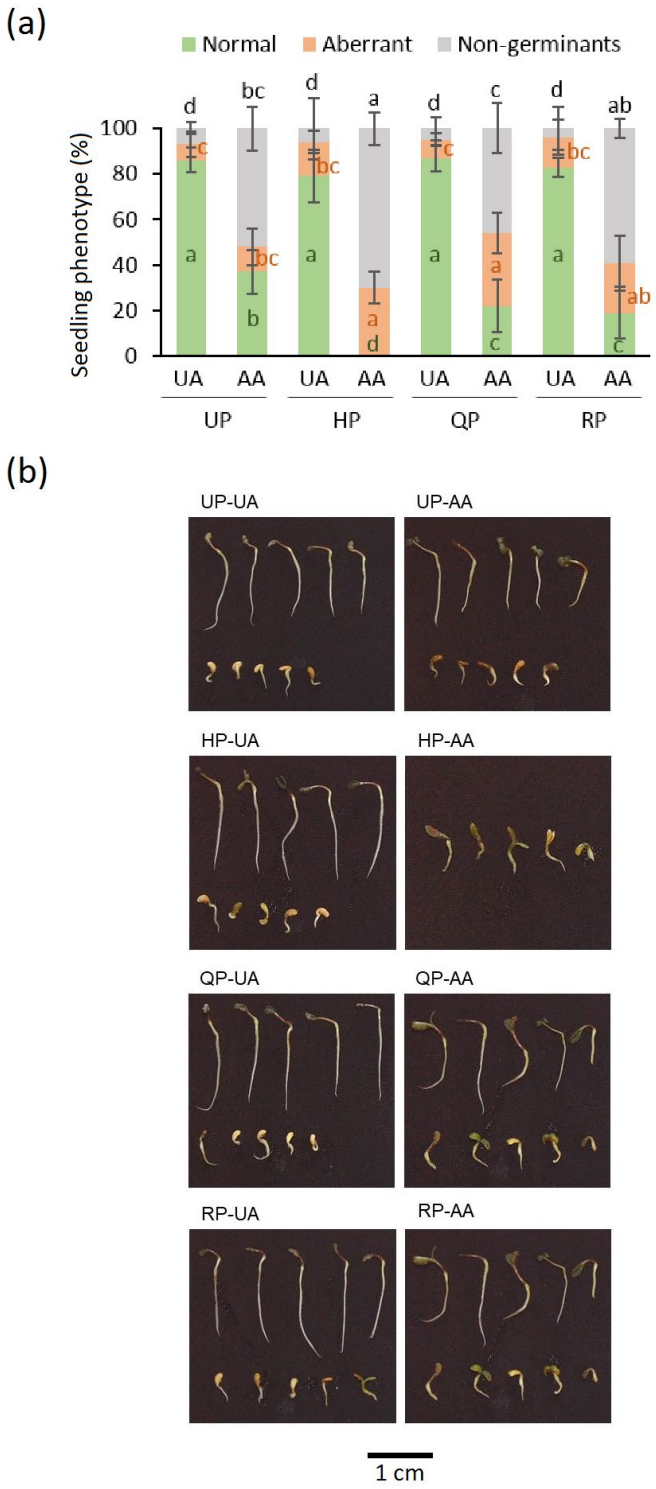

Figure 4. Phenotype of *Medicago truncatula* seedlings following hydropriming, quercetin-supplemented priming or rutin-supplemented priming combined with accelerated aging. (**a**) Seedling phenotype percentage. (**b**) Representative pictures of normal (top rows) and aberrant (bottom rows) seedling 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.

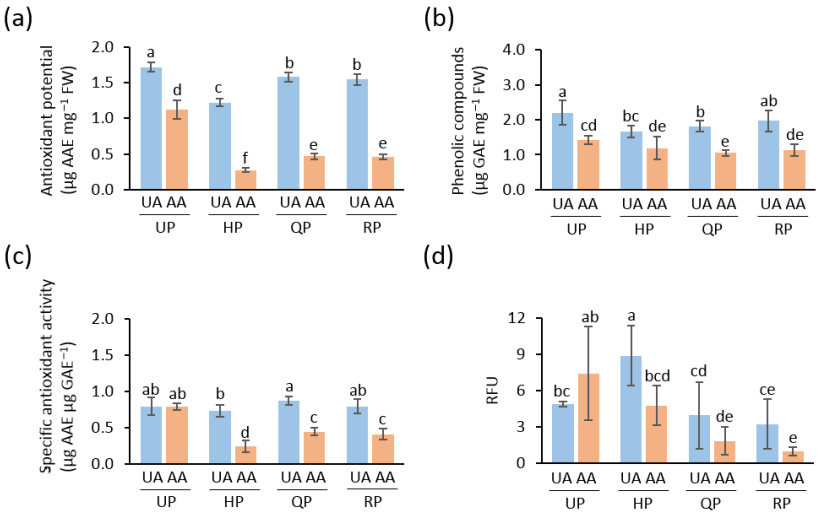

Figure 5. Antioxidant potential, phenolic compounds, and specific antioxidant activity of *Medicago truncatula* seeds subjected to hydropriming, quercetin-priming or rutin-priming followed by accelerated aging. (**a**) Antioxidant potential assessed by DPPH assay. (**b**) Content in phenolic compounds 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.

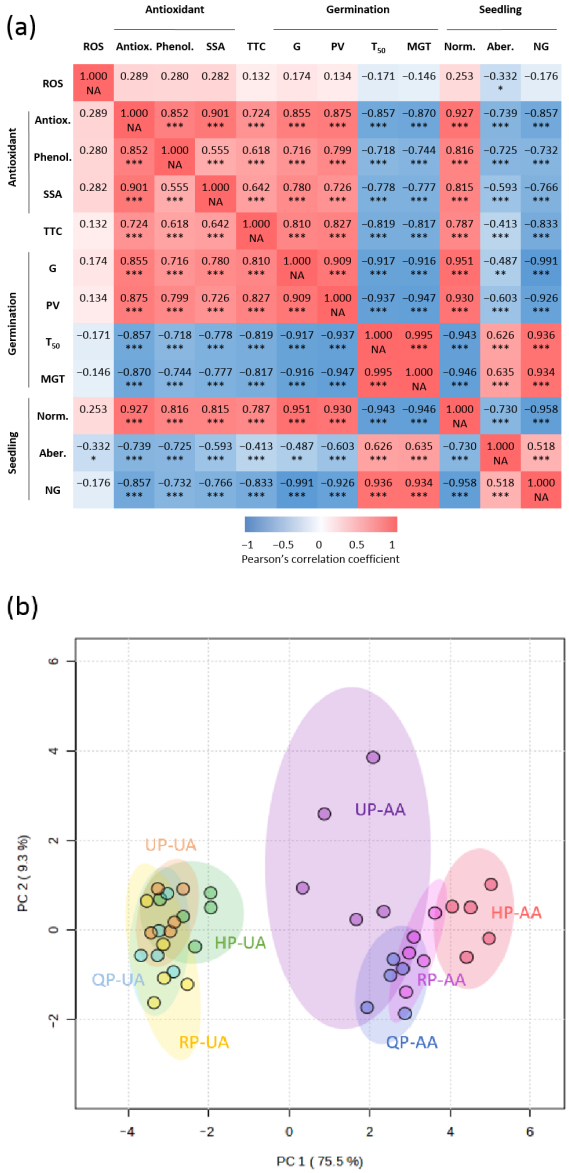

Figure 6. Overview of the results of Pearson's correlation analysis and principal component analysis. (**a**) Pearson's correlation analysis of the results obtained from *Medicago truncatula* seeds subjected to hydropriming, quercetin-priming or rutin-priming followed by accelerated aging. The correlation 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.

References

1. Paparella, S.; Araújo, S.S.; Rossi, G.; Wijayasinghe, M.; Carbonera, D.; Balestrazzi, A. Seed priming: State of the art and new perspectives. *Plant Cell Rep.* **2015**, *34*, 1281–1293. [CrossRef] [PubMed]
2. Macovei, A.; Pagano, A.; Leonetti, P.; Carbonera, D.; Balestrazzi, A.; Araújo, S.S. Systems biology and genome-wide approaches to unveil the molecular players involved in the pre-germinative metabolism: Implications on seed technology traits. *Plant Cell Rep.* **2017**, *36*, 669–688. [CrossRef] [PubMed]
3. Farooq, M.; Usman, M.; Nadeem, F.; Rehman, H.U.; Wahid, A.; Basra, S.M.A.; Siddique, K.H.M. Seed priming in field crops: Potential benefits, adoption and challenges. *Crop Pasture Sci.* **2019**, *70*, 731–771. [CrossRef]
4. Srivastava, A.K.; Kumar, J.S.; Suprasanna, P. Seed 'primeomics': Plants memorize their germination under stress. *Biol. Rev. Camb. Philos. Soc.* **2021**, *96*, 1723–1743. [CrossRef] [PubMed]
5. Pagano, A.; Macovei, A.; Balestrazzi, A. Molecular dynamics of seed priming at the crossroads between basic and applied research. *Plant Cell Rep.* **2023**, *42*, 657–688. [CrossRef] [PubMed]
6. Balmer, A.; Pastor, V.; Gamir, J.; Flors, V.; Mauch-Mani, B. The 'prime-ome': Towards a holistic approach to priming. *Trends Plant Sci.* **2015**, *20*, 443–452. [CrossRef] [PubMed]
7. Marthandan, V.; Geetha, R.; Kumutha, K.; Renganathan, V.G.; Karthikeyan, A.; Ramalingam, J. Seed Priming: A Feasible Strategy to Enhance Drought Tolerance in Crop Plants. *Int. J. Mol. Sci.* **2020**, *21*, 8258. [CrossRef] [PubMed]
8. Pagano, A.; Folini, G.; Pagano, P.; Sincinelli, F.; Rossetto, A.; Macovei, A.; Balestrazzi, A. ROS Accumulation as a Hallmark of Dehydration Stress in Primed and Overprimed *Medicago truncatula* Seeds. *Agronomy* **2022**, *12*, 268. [CrossRef]
9. Pagano, A.; Zannino, L.; Pagano, P.; Doria, E.; Dondi, D.; Macovei, A.; Biggiogera, M.; de Sousa, A.S.; Balestrazzi, A. Changes in genotoxic stress response, ribogenesis and PAP (3′-phosphoadenosine 5′-phosphate) levels are associated with loss of desiccation tolerance in overprimed *Medicago truncatula* seeds. *Plant Cell Environ.* **2022**, *45*, 1457–1473. [CrossRef]
10. Argerich, C.A.; Bradford, K.J.; Tarquis, A.M. The Effects of Priming and Ageing on Resistance to Deterioration of Tomato Seeds. *J. Exp. Bot.* **1989**, *40*, 593–598. [CrossRef]
11. Tarquis, A.M.; Bradford, K.J. Prehydration and Priming Treatments that Advance Germination also Increase the Rate of Deterioration of Lettuce Seeds. *J. Exp. Bot.* **1992**, *43*, 307–317. [CrossRef]
12. Chiu, K.Y.; Chen, C.L.; Sung, J.M. Effect of Priming Temperature on Storability of Primedsh-2Sweet Corn Seed. *Crop Sci.* **2002**, *42*, 1996–2003. [CrossRef]
13. Abnavi, M.S.; Ghobadi, M.E. The effects of source of priming and post-priming storage duration on seed germination and seedling growth characteristics in wheat (*Triticum aestivum* L.). *J. Agric. Sci.* **2012**, *4*, 256–268.
14. Hussain, S.; Zheng, M.; Khan, F.; Khaliq, A.; Fahad, S.; Peng, S.; Huang, J.; Cui, K.; Nie, L. Benefits of rice seed priming are offset permanently by prolonged storage and the storage conditions. *Sci. Rep.* **2015**, *5*, 8101. [CrossRef] [PubMed]
15. Wang, W.; He, A.; Peng, S.; Huang, J.; Cui, K.; Nie, L. The Effect of Storage Condition and Duration on the Deterioration of Primed Rice Seeds. *Front. Plant Sci.* **2018**, *9*, 172. [CrossRef]
16. Yan, M. Prolonged storage reduced the positive effect of hydropriming in Chinese cabbage seeds stored at different temperatures. *S. Afr. J. Bot.* **2017**, *111*, 313–315. [CrossRef]
17. Sano, N.; Seo, M. Correction to: Cell cycle inhibitors improve seed storability after priming treatments. *J. Plant Res.* **2020**, *133*, 605. [CrossRef] [PubMed]
18. Varierl, A.; Vari, A.K.; Dadlani, M. The subcellular basis of seed priming. *Curr. Sci.* **2010**, *99*, 450–456.
19. Waterworth, W.M.; Bray, C.M.; West, C.E. Seeds and the Art of Genome Maintenance. *Front. Plant Sci.* **2019**, *31*, 475–488. [CrossRef]

20. Waterworth, W.; Balobaid, A.; West, C. Seed longevity and genome damage. *Biosci. Rep.* **2024**, *44*, BSR20230809. [CrossRef]
21. Zhou, W.; Chen, F.; Luo, X.; Dai, Y.; Yang, Y.; Zheng, C.; Yang, W.; Shu, K. A matter of life and death: Molecular, physiological, and environmental regulation of seed longevity. *Plant Cell Environ.* **2020**, *43*, 293–302. [CrossRef]
22. Nadarajan, J.; Walters, C.; Pritchard, H.W.; Ballesteros, D.; Colville, L. Seed Longevity-The Evolution of Knowledge and a Conceptual Framework. *Plants* **2023**, *12*, 471. [CrossRef] [PubMed]
23. Pirredda, M.; Fañanás-Pueyo, I.; Oñate-Sánchez, L.; Mira, S. Seed Longevity and Ageing: A Review on Physiological and Genetic Factors with an Emphasis on Hormonal Regulation. *Plant* **2023**, *13*, 41. [CrossRef] [PubMed]
24. Chhabra, R.S.; Singh, T. Seed Aging, Storage and Deterioration: An Irresistible Physiological Phenomenon. *Agric. Rev.* **2019**, *40*, 234–238. [CrossRef]
25. Donà, M.; Balestrazzi, A.; Mondoni, A.; Rossi, G.; Ventura, L.; Buttafava, A.; Macovei, A.; Sabatini, M.E.; Valassi, A.; Carbonera, D. DNA profiling, telomere analysis and antioxidant properties as tools for monitoring ex situ seed longevity. *Ann. Bot.* **2013**, *111*, 987–998. [CrossRef] [PubMed]
26. Kurek, K.; Plitta-Michalak, B.; Ratajczak, E. Reactive Oxygen Species as Potential Drivers of the Seed Aging Process. *Plants* **2019**, *8*, 174. [CrossRef] [PubMed]
27. Zinsmeister, J.; Leprince, O.; Buitink, J. Molecular and environmental factors regulating seed longevity. *Biochem. J.* **2020**, *477*, 205–323. [CrossRef] [PubMed]
28. Zhang, K.; Zhang, Y.; Sun, J.; Meng, J.; Tao, J. Deterioration of orthodox seeds during ageing: Influencing factors, physiological alterations and the role of reactive oxygen species. *Plant Physiol. Biochem.* **2021**, *158*, 475–485. [CrossRef] [PubMed]
29. Rajjou, L.; Lovigny, Y.; Groot, S.P.; Belghazi, M.; Job, C.; Job, D. Proteome-wide characterization of seed aging in Arabidopsis: A comparison between artificial and natural aging protocols. *Plant Physiol.* **2008**, *148*, 620–641. [CrossRef]
30. Schwember, A.R.; Bradford, K.J. Quantitative trait loci associated with longevity of lettuce seeds under conventional and controlled deterioration storage conditions. *J. Exp. Bot.* **2010**, *61*, 4423. [CrossRef]
31. Fantazzini, T.B.; da Rosa, S.D.V.F.; Pereira, C.C.; de Sousa Pereira, D.; Cirillo, M.Â.; Ossani, P.C. Association between the artificial aging test and the natural storage of coffee seeds. *J. Seed Sci.* **2018**, *40*, 164–172. [CrossRef]
32. Yeh, Y.M.; Chiu, K.Y.; Chen, C.L.; Sung, J.M. Partial vacuum extends the longevity of primed bitter gourd seeds by enhancing their anti-oxidative activities during storage. *Sci. Hortic.* **2005**, *104*, 101–112. [CrossRef]
33. Tu, K.; Cheng, Y.; Pan, T.; Wang, J.; Sun, Q. Effects of Seed Priming on Vitality and Preservation of Pepper Seeds. *Agriculture* **2022**, *12*, 603. [CrossRef]
34. Gurusinghe, S.; Powell, A.L.T.; Bradford, K.J. Enhanced Expression of BiP Is Associated with Treatments that Extend Storage Longevity of Primed Tomato Seeds. *J. Am. Soc. Hortic. Sci.* **2002**, *127*, 528–534. [CrossRef]
35. Xu, L.; Xin, X.; Yin, G.; Zhou, J.; Zhou, Y.; Lu, X. Timing for antioxidant-priming against rice seed ageing: Optimal only in non-resistant stage. *Sci. Rep.* **2020**, *10*, 12294. [CrossRef] [PubMed]
36. Singh, P.; Arif, Y.; Bajguz, A.; Hayat, S. The role of quercetin in plants. *Plant Physiol. Biochem.* **2021**, *166*, 10–19. [CrossRef] [PubMed]
37. Tang, J.; Shen, H.; Zhang, R.; Yang, F.; Hu, J.; Che, J.; Dai, H.; Tong, H.; Wu, Q.; Zhang, Y.; et al. Seed priming with rutin enhances tomato resistance against the whitefly *Bemisia tabaci*. *Pestic. Biochem. Physiol.* **2023**, *194*, 105470. [CrossRef] [PubMed]
38. Nkpaa, K.W.; Onyeso, G.I.; Kponee, K.Z. Rutin abrogates manganese-Induced striatal and hippocampal toxicity via inhibition of iron depletion, oxidative stress, inflammation and suppressing the NF-κB signaling pathway. *J. Trace Elem. Med. Biol.* **2019**, *53*, 8–15. [CrossRef] [PubMed]
39. Huang, F.M.; Chang, Y.C.; Su, C.H.; Wu, S.W.; Lee, S.S.; Lee, M.W.; Yeh, K.L.; Chiang, C.Y.; Tu, D.G.; Lu, Y.C.; et al. Rutin-protected BisGMA-induced cytotoxicity, genotoxicity, and apoptosis in macrophages through the reduction of the mitochondrial apoptotic pathway and induction of antioxidant enzymes. *Environ. Toxicol.* **2021**, *36*, 45–54. [CrossRef]
40. Ożarowski, M.; Karpiński, T.M. Extracts and Flavonoids of Passiflora Species as Promising Anti-inflammatory and Antioxidant Substances. *Curr. Pharm. Des.* **2021**, *27*, 2582–2604. [CrossRef]
41. Rajjou, L.; Debeaujon, I. Seed longevity: Survival and maintenance of high germination ability of dry seeds. *C. R. Biol.* **2008**, *331*, 796–805. [CrossRef] [PubMed]
42. Lee, J.S.; Hay, F.R. Variation in Seed Metabolites between Two Indica Rice Accessions Differing in Seed Longevity. *Plants* **2020**, *9*, 1237. [CrossRef] [PubMed]
43. Cui, M.; Wu, D.; Bao, K.; Wen, Z.; Hao, Y.; Luo, L. Dynamic changes of phenolic compounds during artificial aging of soybean seeds identified by high-performance liquid chromatography coupled with transcript analysis. *Anal. Bioanal. Chem.* **2019**, *411*, 3091–3101. [CrossRef] [PubMed]
44. Yang, J.; Zhang, L.; Jiang, L.; Zhan, Y.G.; Fan, G.Z. Quercetin alleviates seed germination and growth inhibition in *Apocynum venetum* and *Apocynum pictum* under mannitol-induced osmotic stress. *Plant Physiol. Biochem.* **2021**, *159*, 268–276. [CrossRef] [PubMed]
45. Singh, A.; Gupta, R.; Pandey, R. Exogenous application of rutin and gallic acid regulate antioxidants and alleviate reactive oxygen generation in *Oryza sativa* L. *Physiol. Mol. Biol. Plants* **2017**, *23*, 301–309. [CrossRef]
46. Yang, W.; Xu, X.; Li, Y.; Wang, Y.; Li, M.; Wang, Y.; Ding, X.; Chu, Z. Rutin-Mediated Priming of Plant Resistance to Three Bacterial Pathogens Initiating the Early SA Signal Pathway. *PLoS ONE* **2016**, *11*, 146910. [CrossRef]

47. Colombo, F.; Pagano, A.; Sangiorgio, S.; Macovei, A.; Balestrazzi, A.; Araniti, F.; Pilu, R. Study of Seed Ageing in lpa1-1 Maize Mutant and Two Possible Approaches to Restore Seed Germination. *Int. J. Mol. Sci.* **2023**, *24*, 732. [CrossRef] [PubMed]
48. Ranal, M.A.; Garcia de Santana, D. How and why to measure the germination process? *Braz. J. Bot.* **2006**, *29*, 1–11. [CrossRef]
49. De Franca Neto, J.; Krzyzanowski, F.C. Tetrazolium: An important test for physiological seed quality evaluation. *J. Seed Sci.* **2019**, *41*, 359–366. [CrossRef]
50. ISTA (International Seed Testing Association). Biochemical test for viability: The topographical tetrazolium test. In *International Rules for Seed Testing*; ISTA: Wallisellen, Switzerland, 2019; Chapter 6.
51. Braca, A.; Tommasi, N.D.; Bari, L.D.; Pizza, C.; Polliti, M.; Morelli, I. Antioxidant principles from *Branhinia terapotensis*. *J. Nat. Prod.* **2001**, *64*, 892–895. [CrossRef]
52. Spanos, G.A.; Wrolstad, R.E. Influence of processing and storage on the phenolic composition of Thompson seedless grape juice. *J. Agr. Food Chem.* **1990**, *38*, 1565–1571. [CrossRef]
53. Pagano, A.; Araújo, S.; Macovei, A.; Leonetti, P.; Balestrazzi, A. The Seed Repair Response during Germination: Disclosing Correlations between DNA Repair, Antioxidant Response, and Chromatin Remodeling in *Medicago truncatula*. *Front. Plant Sci.* **2017**, *8*, 1972. [CrossRef] [PubMed]
54. Rakariyatham, K.; Liu, X.; Liu, Z.; Wu, S.; Shahidi, F.; Zhou, D.; Zhu, B. Improvement of Phenolic Contents and Antioxidant Activities of Longan (*Dimocarpus longan*) Peel Extracts by Enzymatic Treatment. *Waste Biomass Valor.* **2020**, *11*, 3987–4002. [CrossRef]
55. Assaad, H.I.; Hou, Y.; Zhou, L.; Carroll, R.J.; Wu, G. Rapid publication-ready MS-Word tables for two-way ANOVA. *SpringerPlus* **2015**, *23*, 4–33. [CrossRef]
56. Ewald, J.; Zhou, G.; Lu, Y.; Kolic, J.; Ellis, C.; Johnson, J.D.; Macdonald, P.E.; Xia, J. Web-based multi-omics integration using the Analyst software suite. *Nat. Protoc.* **2024**, *19*, 1467–1497. [CrossRef]
57. Rehmani, M.S.; Xian, B.; Wei, S.; He, J.; Feng, Z.; Huang, H.; Shu, K. Seedling establishment: The neglected trait in the seed longevity field. *Plant Physiol. Biochem.* **2023**, *200*, 107765. [CrossRef]
58. Bailly, C.; Kranner, I. Analyses of reactive oxygen species and antioxidants in relation to seed longevity and germination. *Methods Mol. Biol.* **2011**, *20*, 443–452.
59. Bailly, C. The signalling role of ROS in the regulation of seed germination and dormancy. *Biochem. J.* **2019**, *476*, 3019–3032. [CrossRef] [PubMed]
60. Powell, A.; Matthews, S. Seed Aging/Repair Hypothesis Leads to New Testing Methods. *Seed Technol.* **2012**, *34*, 15–25.
61. Diaz-Vivancos, P.; Faize, M.; Barba-Espin, G.; Faize, L.; Petri, C.; Hernández, J.A.; Burgos, L. Ectopic expression of cytosolic superoxide dismutase and ascorbate peroxidase leads to salt stress tolerance in transgenic plums. *Plant Biotechnol.* **2013**, *11*, 976–985. [CrossRef]
62. Choudhary, A.; Kumar, A.; Kaur, N. ROS and oxidative burst: Roots in plant development. *Plant Divers* **2019**, *42*, 33–43. [CrossRef]
63. Sun, M.; Sun, S.; Mao, C.; Zhang, H.; Ou, C.; Jia, Z.; Wang, Y.; Ma, W.; Li, M.; Jia, S.; et al. Dynamic Responses of Antioxidant and Glyoxalase Systems to Seed Aging Based on Full-Length Transcriptome in Oat (*Avena sativa* L.). *Antioxidants* **2022**, *11*, 395. [CrossRef]
64. Wang, J.; Tan, B.; He, Y.; Liu, C.; Li, N.; Tan, X.; Lu, H. Potential Biochemical Markers Affecting Aging and "the Compensatory Effects" of Canola (*Brassica napus* L.) Seeds Stored in Deep Underground. *Agriculture* **2023**, *13*, 320. [CrossRef]
65. Naghisharifi, H.; Kolahi, M.; Javaheriyan, M.; Zargar, B. Oxidative stress is the active pathway in canola seed aging, the role of oxidative stress in the development of seedlings grown from aged canola seed. *Plant Stress* **2024**, *11*, 100313. [CrossRef]
66. Pukacka, S.; Ratajczak, E. Age-related biochemical changes during storage of beech (*Fagus sylvatica* L.) seeds. *Seed Sci. Res.* **2007**, *17*, 45–53. [CrossRef]
67. Cakmak, T.; Atici, Ö.; Agar, G. The natural aging-related biochemical changes in the seeds of two legume varieties stored for 40 years. *Acta Agric. Scand. Sect. B—Plant Soil Sci.* **2010**, *60*, 353–360. [CrossRef]
68. Aslam, M.A.; Ahmed, S.; Saleem, M.; Shah, A.A.; Shah, A.N.; Tanveer, M.; Ali, H.M.; Ghareeb, R.Y.; Hasan, M.E.; Khan, J. Quercetin ameliorates chromium toxicity through improvement in photosynthetic activity, antioxidative defense system; and suppressed oxidative stress in *Trigonella corniculata* L. *Front. Plant Sci.* **2022**, *13*, 956249. [CrossRef]
69. Kibinza, S.; Bazin, J.; Bailly, C.; Farrant, J.M.; Corbineau, F.; El-Maarouf-Bouteau, H. Catalase is a key enzyme in seed recovery from ageing during priming. *Plant Sci.* **2011**, *181*, 309–315. [CrossRef] [PubMed]
70. Tian, Y.; Gama-Arachchige, N.S.; Zhao, M. Trends in Seed Priming Research in the Past 30 Years Based on Bibliometric Analysis. *Plants* **2023**, *12*, 3483. [CrossRef]
71. Chen, X.; Zhang, R.; Li, B.; Cui, T.; Liu, C.; Liu, C.; Chen, B.; Zhou, Y. Alleviation of Oxidative Damage Induced by $CaCl_2$ Priming Is Related to Osmotic and Ion Stress Reduction rather than Enhanced Antioxidant Capacity during Germination under Salt Stress in Sorghum. *Front. Plant Sci.* **2022**, *13*, 881039. [CrossRef]
72. Chen, K.; Arora, R. Dynamics of the antioxidant system during seed osmopriming, post-priming germination, and seedling establishment in Spinach (*Spinacia oleracea*). *Plant Sci.* **2011**, *180*, 212–220. [CrossRef] [PubMed]
73. Genisel, M.; Erdal, S.; Kizilkaya, M. The mitigating effect of cysteine on growth inhibition in salt-stressed barley seeds is related to its own reducing capacity rather than its effects on antioxidant system. *Plant Growth Regul.* **2015**, *75*, 187–197. [CrossRef]

74. Farooq, M.; Gogoi, N.; Hussain, M.; Barthakur, S.; Paul, S.; Bharadwaj, N.; Migdadi, M.H.; Alghamdi, S.S.; Siddique, M.H.K. Effects, tolerance mechanisms and management of salt stress in grain legumes. *Plant Physiol. Biochem.* **2017**, *118*, 199–217. [CrossRef] [PubMed]
75. Griffo, A.; Bosco, N.; Pagano, A.; Balestrazzi, A.; Macovei, A. Noninvasive Methods to Detect Reactive Oxygen Species as a Proxy of Seed Quality. *Antioxidants* **2023**, *12*, 626. [CrossRef] [PubMed]
76. Kanjevac, M.; Bojović, B.; Ćirić, A.; Stanković, M.; Jakovljević, D. Seed Priming Improves Biochemical and Physiological Performance of Wheat Seedlings under Low-Temperature Conditions. *Agriculture* **2023**, *13*, 2. [CrossRef]
77. Salvi, P.; Varshney, V.; Majee, M. Raffinose family oligosaccharides (RFOs): Role in seed vigor and longevity. *Biosci. Rep.* **2022**, *42*, BSR20220198. [CrossRef]
78. Waterworth, W.M.; Masnavi, G.; Bhardwaj, R.M.; Jiang, Q.; Bray, C.M.; West, C.E. A plant DNA ligase is an important determinant of seed longevity. *Plant J.* **2010**, *63*, 848–860. [CrossRef]