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ROS Accumulation as a Hallmark of Dehydration Stress in Primed and Overprimed *Medicago truncatula* Seeds

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Abstract: Seed priming protocols implement incomplete imbibition phases, as well as physical, chemical or biological treatments, to activate pre-germinative metabolism and stress response, thus improving germination performances, seedling establishment and stress tolerance according to agricultural productivity requirements. The dehydration phase following priming treatments represents a critical variable, since an excessively prolonged imbibition (overpriming) impairs desiccation tolerance, compromising seed viability and seedling establishment. Priming protocols generally optimize imbibition-dehydration timing empirically to avoid overpriming. Hence, a better understanding of the dynamics underlying the loss of desiccation tolerance represents a promising route to test and develop efficient and cost-effective priming techniques. In the present work, priming and overpriming conditions were defined to explore the role of desiccation tolerance in seed priming efficiency in the model legume *Medicago truncatula*. The positive effects of hydropriming and kinetin-mediated hormopriming on germination parameters were screened in combination with conditions of short/prolonged priming and mild/severe overpriming. Biometric analyses highlighted contrasting responses in terms of germination performances and seedling development, while ROS (reactive oxygen species) levels measured during dehydration positively correlate with the loss of desiccation tolerance in early seedlings, suggesting possible applications to monitor priming progression and predict overpriming occurrence.

Keywords: *Medicago truncatula*; seed priming; overpriming; kinetin; reactive oxygen species; dichlorofluorescin diacetate assay; diaminobenzidine staining; hydropriming; hormopriming

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1. Introduction

Seed priming techniques are routinely employed to improve germination performances and stress tolerance of commercial seed lots [1,2]. Seed priming stimulates the activation of pre-germinative metabolism through an incomplete imbibition that prepares the seeds for an accelerated and coordinated germination and a more efficient stress response. Primed seeds are subsequently dehydrated (dry-back) and stored in view of sowing or commercialization [3,4].

Whereas hydropriming protocols rely on imbibition-dehydration cycles to activate seed metabolism, other priming protocols involve the administration of osmotic agents (osmopriming), chemicals (chemopriming), physical treatments (thermopriming, etc.), phytohormones (hormopriming) or microorganisms (biopriming) to regulate imbibition, coordinate germination or trigger stress memory [1,5].

Relevantly for priming protocols, orthodox seeds can withstand significant desiccation events through their endowment in osmo-protective proteins and sugars stabilizing cellular components, while antioxidant compounds protect lipids, proteins and nucleic

acids from the oxidative stress associated with dehydration [3,6–13]. The sum of these properties, known as 'desiccation tolerance', is acquired during seed maturation and progressively lost as prolonged imbibition and radicle development lead to the depletion of osmo-protectants [14]. In the context of seed priming, this loss of desiccation tolerance is known as 'overpriming' and impairs germination rates, seed storability and seedling development [15,16]. The use of multiple biometrical indicators is useful to dissect the effectiveness of seed priming [17], highlighting differential effects on post-priming viability, germination synchronization and seedling development [18,19].

The circumstances under which prolonged imbibition results in overpriming vary between and within species, cultivars and seed lots, and require diverse panels of model systems to be understood, predicted and avoided. While for certain species a decrease in desiccation tolerance and/or in priming efficacy is predictable based on imbibition time [19,20], in the model legume *Medicago truncatula*, the occurrence of overpriming appears to be empirically determinable from radicle protrusion length, with a critical range between 1 and 3 mm in which desiccation tolerance decreases substantially [21,22]. Moreover, cotyledons, hypocotyls and radicles display differential desiccation tolerance in *M. truncatula*, *A. thaliana*, *Pisum sativum* and other model systems, with cotyledons being the most desiccation-tolerant and radicles displaying the highest sensitivity [23–25]. Furthermore, desiccation tolerance can be enhanced through seed priming itself [5] and restored through the administration of osmotic agents such as polyethylene glycol (PEG) or phytohormones (e.g., abscisic acid, ABA), as shown in *M. truncatula* [21] and *P. sativum* [24].

Although imbibition timing is critical for priming efficiency, the validation and the optimization of priming protocols to avoid overpriming are still mainly empirical, especially considering the variability in germination rates within seed lots. Therefore, this work aims to characterize the effects of short/prolonged priming and mild/severe overpriming while also assessing the effectiveness of kinetin-mediated hormopriming in accelerating and coordinating germination.

The role of ROS in the establishment and the loss of desiccation tolerance has been demonstrated during seed development, storage and germination [26,27], and hydrogen peroxide accumulation has been used as an indicator of drought stress in primed Arabidopsis seeds [28], maize embryos [29], wheat plants [30] and other model and commercial plants [31–33]. In this work, the accumulation of ROS during desiccation has been evaluated as a possible indicator of post-priming seedling establishment performances in correlation with the occurrence of overpriming conditions in the model legume *M. truncatula*. The experimental questions driving this work concern: (i) how dry-back affects post-priming germination and seedling establishment, (ii) how kinetin-mediated hormopriming can improve germination rates, (iii) whether ROS emission during dehydration can act as a reliable indicator of priming-related stress.

2. Materials and Methods

2.1. Plant Material and Priming/Overpriming Treatments

M. truncatula seeds (Jemalong cultivar, provided by Dr. Ana Barradas, Fertiprado L.d.a., Vaiamonte—Monforte, Portugal) were used for this study. For imbibition, seeds were transferred to Petri dishes containing a layer of filter paper moistened with 2 mL dH_2O with/without 0.5 mM kinetin (Sigma-Aldrich-Merck, Milan, Italy). For priming and overpriming treatments, seeds were collected after 2 h and 24 h imbibition, respectively, and dehydrated as follows: 20 seeds/dish were distributed into 5 Petri dishes and covered with a layer of absorbing paper, ~10 g of silica beads (Disidry® Orange Silica Gel, The Aerodyne, Florence, Italy) were added upon the layer of absorbing paper and the Petri dishes were subsequently sealed with parafilm. The subpopulation of 24 h-imbibed seeds displaying the protrusion of the primary radicle after imbibition and before dehydration were considered overprimed. Seed relative water content (RWC) was calculated hourly during dehydration. Dehydration was carried out for 4 h (2 h-imbibed seeds) or 6 h (24 h-imbibed seeds, overprimed seeds).

Two treatments, designated as H (hydropriming with dH_2O) and K (hormopriming with kinetin), were tested in combination with five priming conditions (unprimed, 2 h-priming, 24 h-priming, overpriming with 1 mm radicle protrusions, overpriming with 2 mm radicle protrusions), for a total of ten protocols: (1) UP, water-imbibed unprimed seeds; (2) KUP, kinetin-imbibed unprimed seeds; (3) HP2h, 2 h-hydroprimed seeds; (4) KP2h, 2 h-hormoprimed seeds; (5) HP24h, 24 h-hydroprimed seeds; (6) KP24h, 24 h-hormoprimed seeds; (7) HOP1mm, hydroprimed seeds displaying 1 mm radicle protrusion; (8) KOP1mm, hormoprimed seeds displaying 1 mm radicle protrusion; (9) HOP2mm, hydroprimed seeds displaying 2 mm radicle protrusion; (10) KOP2 mm, hormoprimed seeds displaying 2 mm radicle protrusion. Imbibition, dehydration, reimbibition and germination were carried out in a growth chamber at 24 °C under light (photon flux density of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$, photoperiod of 16/8 h, and 70–80% relative humidity). An overview of the experimental system is provided in Figure 1.

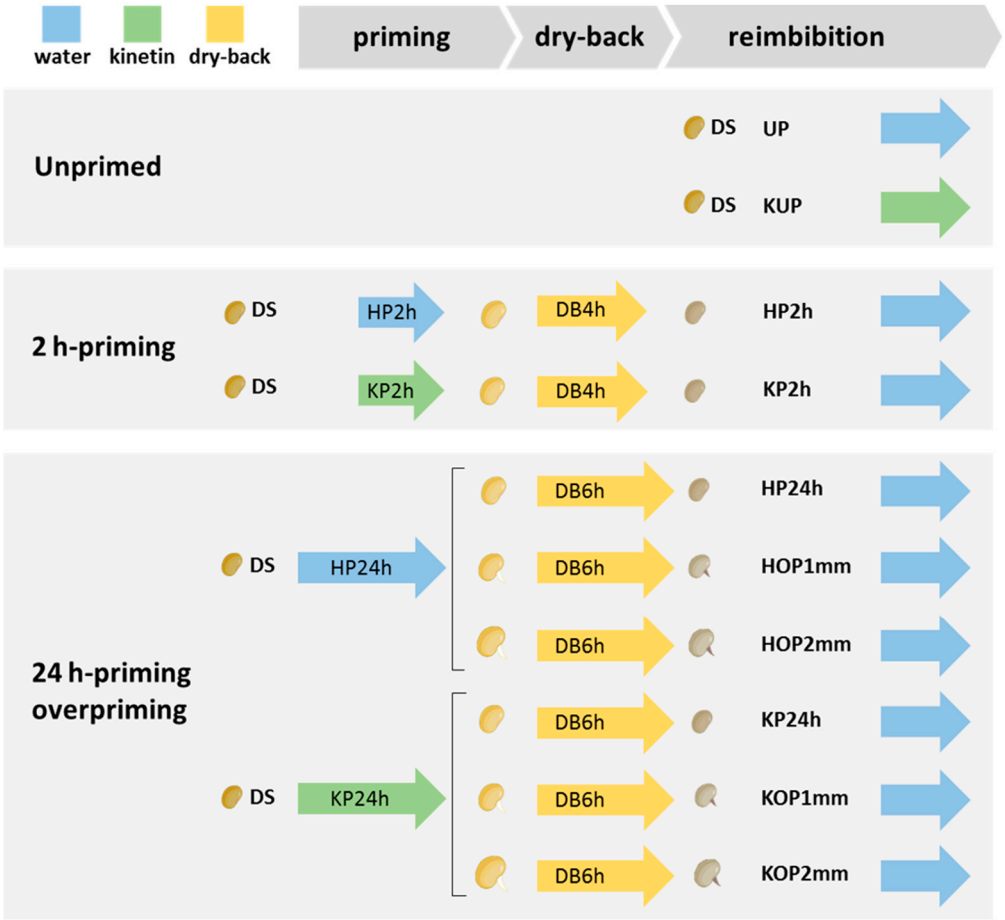

Figure 1. Overview of the experimental system aimed to compare the effects of short (2 h) and prolonged (24 h) hydropriming and kinetin-mediated hormopriming on *M. truncatula* and *M. sativa* seeds, including mild (radicle protrusion 1 mm) and severe (radicle protrusion 2 mm) overpriming conditions. Imbibition steps carried out in water are indicated in blue, imbibition steps carried out in presence of 0.5 mM kinetin are indicated in green, and dry-back steps are indicated in yellow. UP, water-imbibed unprimed seeds; KUP, kinetin-imbibed unprimed seeds; HP2h, 2 h-hydroprimed seeds; KP2h, 2 h-hormoprimed seeds; HP24h, 24 h-hydroprimed seeds; KP24h, 24 h-hormoprimed seeds; HOP1mm, hydroprimed seeds with 1 mm radicle protrusion; KOP1mm, hormoprimed seeds with 1 mm radicle protrusion; HOP2mm, hydroprimed seeds with 2 mm radicle protrusion; KOP2mm, hormoprimed seeds with 2 mm radicle protrusion; DB4h, 4 h-dry-back; DB6h, 6 h-dry-back.

As a proof-of-concept of the main conclusions of this study, *Medicago sativa* seeds (commercial genotype) underwent seed priming treatments as previously described for *M. truncatula* seeds and outlined in Figure 1.

2.2. Germination Tests and Biometrical Analyses

For germination tests, *M. truncatula* unprimed seeds (UP, KUP), primed seeds (HP2h, KP2h, HP24h, KP24h) and overprimed seeds (HOP1mm, KOP1mm, HOP2mm, KOP2mm) were imbibed in parallel. Germination was assessed hourly for 48 h and the following germination parameters were calculated: G (germinability), MGT (mean germination time), MGR (mean germination rate), CVG (coefficient of velocity of germination), U (uncertainty index), Z (synchronization index), T_{50} (time required to reach 50% of maximum germination) [17]. For each treatment, five independent replicates with 20 seeds per replicate were analyzed. Biometric data of seedling development were collected four days after the beginning of reimbibition (primed seeds) or imbibition (unprimed seeds). Radicle length was measured on millimetric paper (10 seedlings per condition), and fresh and dry weight data were retrieved using 5 replicates of 5 seedlings each. For dry weight measurements, seedlings were dried 24 h at 80 °C.

2.3. Relative Water Content (RWC) Measurement

RWC was measured for *M. truncatula* dry seeds and for primed and overprimed seeds along dry-back (0, 2, 4 and 6 h) and expressed as percentage over the seed fresh weight according to the following formula: RWC [%] = [(Fw − Dw)/Fw] × 100, where fresh weight (Fw) was measured at the indicated timepoints after removing the excess of superficial water from the seeds, when present, and dry weight (Dw) was measured after 24 h dehydration at 80 °C [34]. For these measurements, 5 replicates of 20 seeds each were used to calculate RWC.

2.4. ROS Detection by 2′,7′-Dichlorofluorescin Diacetate (DCF-DA) Assay

ROS (reactive oxygen species) levels were quantified in dry seeds and in primed/overprimed seeds before, during and at the endpoint of dehydration. ROS levels were assessed before (at 0 h), during (at 2 h) and at the endpoint of dry-back (4 h or 6 h). The assay was carried out using the fluorogenic dye 2′,7′-dichlorofluorescin diacetate (DCF-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 (DCF), that can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 nm and 529 nm, respectively. The assay was carried out as described by Macovei et al. [35], with the following modifications. Seeds were collected at the indicated timepoints and dried on filter paper. Samples (5 replicates per timepoint, 5 seeds per replicate) were incubated for 15 min with 100 µL of 10 µM DCF-DA and subsequently fluorescence at 517 nm was determined using a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia), setting the program for one cycle of 30 s at 25 °C. A sample containing only DCF-DA was used as a control to subtract the baseline fluorescence. Relative fluorescence was expressed as relative fluorescence units (RFU).

2.5. H_2O_2 Detection by 3,3′-Diaminobenzidine (DAB) Staining

The distribution of H_2O_2 within *M. truncatula* dehydrated embryos was assessed through DAB staining performed at the endpoint of dry-back according to Kiran et al. [36]. Seeds were incubated for 30 min at room temperature in 1 mL of 1 mg/mL DAB (Sigma-Aldrich, Milan Italy) prepared in 10 mM disodium hydrogen phosphate (pH 7.4). For positive control treatments 100 µL of 1 M H_2O_2 was also added. For negative controls 100 µL of 100 µM ascorbic acid was also added. The seed coat was manually removed and images of 5 seeds per treatments were acquired via stereomicroscope (Olympus SZX9;

Olympus®, Tokyo, Japan) and camera (Camedia C-5060 Wide Zoom; Olympus®, Tokyo, Japan), supported by the relative software (Olympus DP-Soft 5.0).

2.6. Statistical Analyses

Germination, biometrical and ROS level data were analyzed by two-way ANOVA and Tuckey–Kramer test, using the software developed by Assaad et al. [37] in order to compare priming conditions (UP, P2h, P24h, OP1mm, OP2mm) and priming treatment groups (hydropriming, hormopriming). The Pearson's correlation coefficient and the relative *p*-values were calculated using MetaboAnalyst 5.0 (Xia Lab, Ste. Anne de Bellevue, Quebec, Canada) [38] to assess the correlations between radicle length class (0 mm, 1 mm, 2 mm), ROS levels at 0 h, 2 h and 4/6 h of dry-back and seedling phenotype ('normal', 'aberrant', 'dead'). Statistically significant differences are indicated in the figures by the occurrence of different letters or by asterisks ('*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$).

3. Results

3.1. Hydropriming and Hormopriming Improve Germination Performances in M. truncatula

The optimal dehydration timing to be applied after short (2 h) priming protocols and prolonged (24 h) priming and overpriming protocols in presence/absence of kinetin was determined by assessing the decrease in RWC during dry-back in order to reach the RWC measured for dry seeds. As a result, 4 h dry-back was sufficient for 2 h-primed seeds, whereas 6 h dry-back was necessary for 24 h-primed and overprimed seeds. No significant differences were observed comparing hydroprimed and hormoprimed seeds (Figure 2).

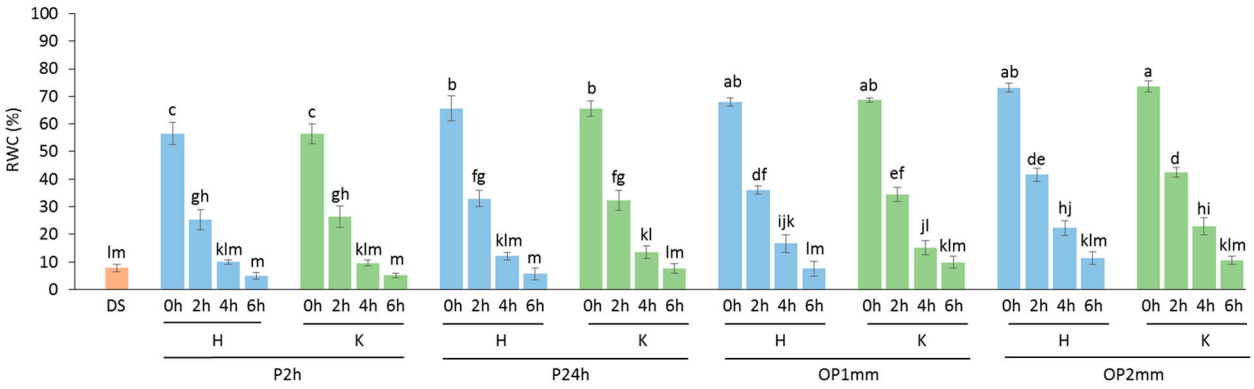

Figure 2. Relative water content (RWC) measured during dry-back of *M. truncatula* seeds subjected to short/prolonged priming and mild/severe overpriming, considering hydropriming (blue histograms) and kinetin-mediated hormopriming (green histograms). DS, dry seed; P2h, 2 h-priming; P24h, 24 h-priming; OP1mm, hydroprimed seeds with 1 mm radicle protrusion; OP2mm, hydroprimed seeds with 2 mm radicle protrusion; H, hydropriming; K, hormopriming. Means with common superscript letters are not significantly different ($p < 0.05$) as analyzed with two-way ANOVA and Tukey test.

The effects of short and prolonged hydropriming and hormopriming on *M. truncatula* germination performances were assessed through multiple germination parameters to highlight the advantages and disadvantages of the tested priming protocols. Having already undergone germination during priming, overprimed seeds were excluded from post-priming germination tests. Germination speed (expressed as T_{50}, MGT, MGR, CVG) increased in response to all priming conditions (HP2h, KP2h, HP24h, KP24h), as well as to exposure to kinetin without priming (KUP) compared to unprimed water-imbibed seeds (UP) (Table 1). Prolonged hormopriming (KP24h) further increased germination speed and decreased germination uncertainty index (U) compared to prolonged hydropriming (HP24h), while prolonged hydropriming and hormopriming (HP24h and KP24h, respectively) impaired germination percentage (G) measured after 48 h imbibition (Table 1).

Table 1. Germination parameters calculated on unprimed *M. truncatula* seeds in presence/absence of 0.5 mM kinetin, 2 h-hydropriming/hormopriming and 24 h-hydropriming/hormopriming, expressed as mean of 5 replicates (5 dishes each, 20 seeds/dish) ± standard deviation. G, germinability; T_{50}, time required to reach 50% of G; PV, peak value; MGT, mean germination time; MGR, mean germination rate; CVG, coefficient of velocity of germination; U, uncertainty index; Z, synchronization index; UP, unprimed seeds; P2h, 2 h-primed seeds; P24h, 24 h-primed seeds; H, water imbibition and hydropriming protocols; K, kinetin imbibition and hormopriming protocols. Values that are followed by common letters are not significantly different ($p < 0.05$) as analyzed with two-way ANOVA and Tukey test. *p*-values are also provided referring to the difference observed between treatment comparison groups (H vs. K), priming comparison groups (UP vs. P2h vs. P24h) and their interaction.

Priming	**Treatment**	**G (%)**	**T50 (h)**	**PV (N/h)**	**MGT (h)**
UP	H	68 ± 4.36 bc	25.9 ± 0.98 a	0.327 ± 0.038 c	30.5 ± 0.78 a
UP	K	81 ± 3.67 ab	19.4 ± 0.54 d	0.642 ± 0.026 a	21.5 ± 0.59 d
P2h	H	87 ± 5.15 a	22.4 ± 1.04 bc	0.511 ± 0.041 ab	25.8 ± 1.26 bc
P2h	K	77 ± 4.64 ab	18.9 ± 0.36 d	0.533 ± 0.048 ab	22.6 ± 0.74 cd
P24h	H	65 ± 1.58 bc	22.5 ± 0.27 b	0.363 ± 0.022 c	26.4 ± 0.42 b
P24h	K	55 ± 4.74 c	19.6 ± 0.32 cd	0.414 ± 0.015 bc	20.9 ± 0.36 d
p-value treatment		0.502	<0.001	<0.001	<0.001
p-value priming		<0.001	0.015	0.002	0.011
p-value interaction		0.015	0.028	<0.001	0.003
Priming	**Treatment**	**MGR (1/h)**	**CVG (%)**	**U (bit)**	**Z (unit)**
UP	H	0.0328 ± 0.0008 d	3.28 ± 0.081 d	2.71 ± 0.130 ab	0.102 ± 0.029 b
UP	K	0.0466 ± 0.0013 a	4.66 ± 0.13 a	2.06 ± 0.150 c	0.234 ± 0.027 a
P2h	H	0.0392 ± 0.0021 bc	3.92 ± 0.208 bc	2.77 ± 0.125 a	0.115 ± 0.018 b
P2h	K	0.0445 ± 0.0015 ab	4.45 ± 0.152 ab	2.97 ± 0.053 a	0.084 ± 0.005 b
P24h	H	0.0380 ± 0.0006 cd	3.80 ± 0.060 cd	2.88 ± 0.080 a	0.080 ± 0.011 b
P24h	K	0.0478 ± 0.0008 a	4.78 ± 0.083 a	2.17 ± 0.193 bc	0.164 ± 0.030 ab
p-value treatment		<0.001	<0.001	0.001	0.002
p-value priming		0.061	0.061	0.003	0.015
p-value interaction		0.012	0.012	0.003	0.004

3.2. Radicle Emergence Impairs Seed Desiccation Tolerance and Post-Priming Seedling Development

The effects of the different priming conditions on seedling development, in terms of seedling morphology, radicle growth, fresh and dry biomass, were assessed 4 days after reimbibition. The 4-day-old seedlings displayed three main contrastive phenotypes: (i) 'normal', (ii) 'aberrant', (iii) 'dead'. 'Normal' seedlings displayed the same morphology as the seedlings developing from unprimed controls (UP or KUP), 'aberrant' seedlings were characterized by a substantially impaired radicle growth and by green cotyledons, whereas 'dead' seedlings did not display radicle growth after reimbibition nor cotyledon greening (Figure 3b). The occurrence of the three phenotypes varied according to the priming condition. The seedlings developing from unprimed seeds (UP, KUP), short priming (HP2h, KP2h) and prolonged priming without radicle protrusion (HP24h, KP24h) displayed 100% 'normal' morphology, whereas the percentage of 'normal' seedlings was reduced to 62–68% in response to mild overpriming (HOP1mm, KOP1mm) and to 5–8% in response to severe overpriming (HOP2mm, KOP2mm) (Figure 3a).

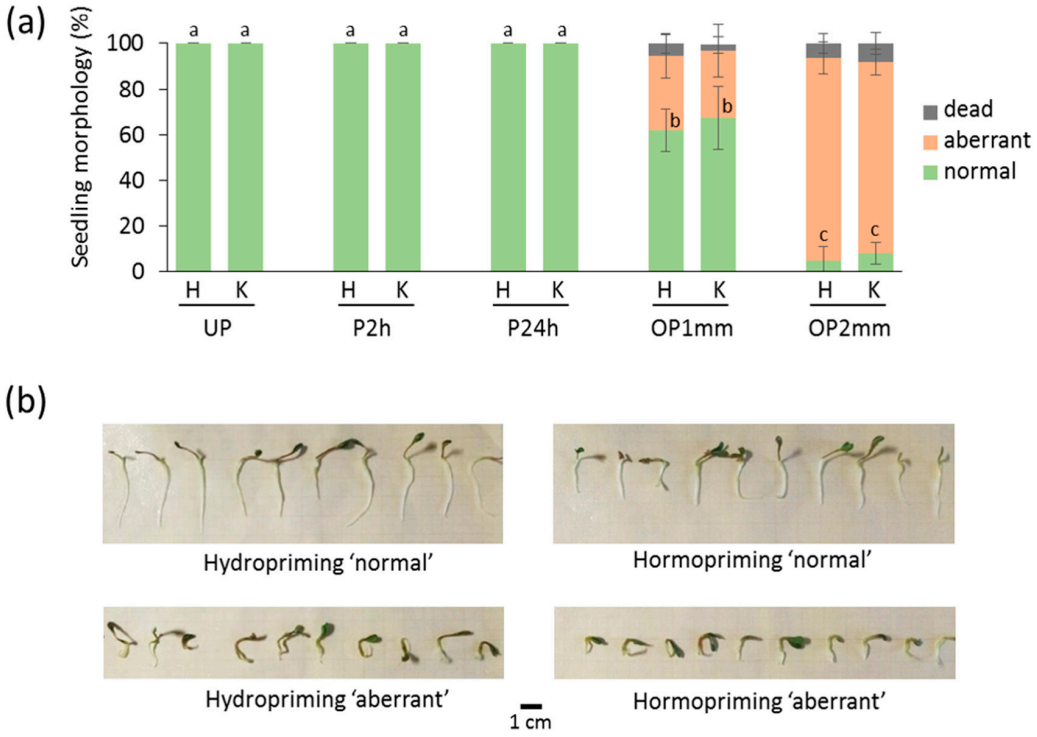

Figure 3. Biometrical analyses of 4-day-old *M. truncatula* seedlings as a result of imbibition and germination in presence/absence of kinetin and as a result of different hydropriming/hormopriming protocols and overpriming conditions. (**a**) Percentage of the occurrence of 'normal', 'aberrant' and 'dead' seedling morphology. UP, unprimed; P2h, 2 h-priming; P24h, 24 h-priming; OP1mm, overpriming with 1 mm radicle protrusion; OP2mm, overpriming with 2 mm radicle protrusion; H, water; K, kinetin. Means with common superscript letters are not significantly different ($p < 0.05$) as analyzed with two-way ANOVA and Tukey test comparing the occurrence of 'normal' seedling morphology. (**b**) Morphology of 'normal' and 'aberrant' 4-day-old seedlings developing from prolonged hydropriming and hormopriming.

Hormopriming treatments (KP2h, KP24h, KOP1mm, KOP2mm), as well as prolonged exposure to kinetin (KUP), consistently led to a 26–51% reduction in radicle length in 'normal' 4-day-old seedlings (Figure 4a). Fresh and dry biomass of 'normal' seedlings did not appear to be influenced by exposure to kinetin or priming condition, and fresh biomass appeared to be consistently reduced (20–30% lower) only in 'aberrant' seedlings, compared to 'normal' seedlings (Figure 4b).

3.3. Overpriming Results into ROS Accumulation after Dry-Back

Given the critical importance of dehydration phases in seed priming protocols and the risks connected to the loss of desiccation tolerance on seedling establishment, this work focused on the identification of stress signatures during dry-back. The fluorogenic dye DCF-DA was used to quantify ROS levels in dry unprimed seeds and in primed/overprimed seeds before dry-back (DB0h, i.e., at the end of the imbibition), after 2 h dry-back (DB2h) and at the end of dry-back. Based on the dehydration curves (Figure 2), the endpoint of dry-back was established at 4 h (DB4h) for 2 h-primed seeds (HP2h, KP2h), and at 6h for prolonged priming/overpriming conditions (HP24h, HOP1mm, HOP2mm, KP24h, KOP1mm, KOP2mm), when the RWC of DS was restored. The results of DCF-DA assay are shown in Figure 5a. For all priming/overpriming conditions, ROS emission before dry-back was not significantly different from the ROS emission of dry seeds and did not display a significant increase at 2 h dry-back. Short priming treatments (HP2h, KP2h) did not result in significant changes in ROS levels during and after desiccation, despite a general increasing trend. The highest ROS emission was detected at 6 h dehydration of severely

overprimed seeds (HOP2mm and KOP2mm). Considering the effects of kinetin, ROS emission appeared to be higher at 6 h desiccation following prolonged kinetin-mediated priming and overpriming (KP24h, KOP1mm, KOP2mm) compared to the respective hydropriming treatments (HP24h, HOP1mm, HOP2mm) at the same timepoint.

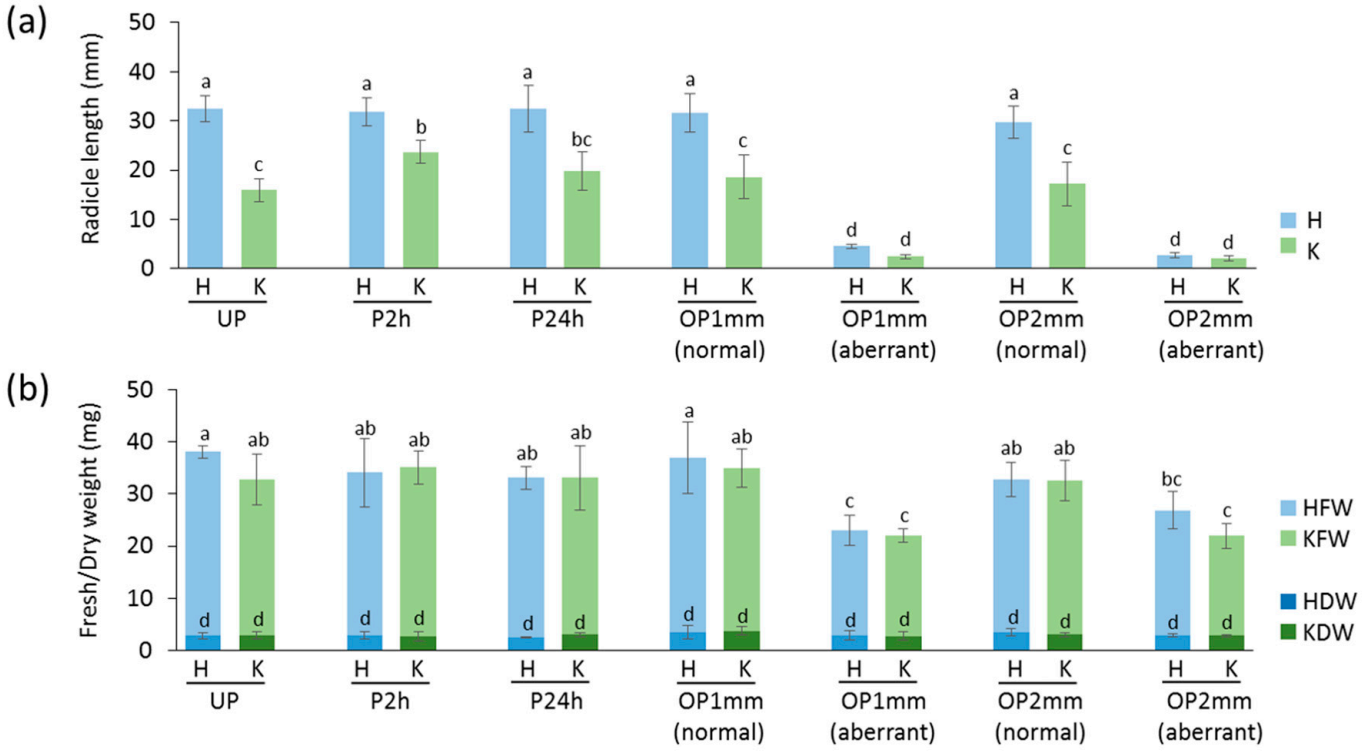

Figure 4. Biometrical analyses of 4-day-old *M. truncatula* seedlings as a result of imbibition and germination in presence/absence of kinetin and as a result of different hydropriming/hormopriming protocols and overpriming conditions. (**a**) Radicle length of 'normal' and 'aberrant' seedlings. (**b**) Fresh and dry biomass of 'normal' and 'aberrant' seedlings. UP, unprimed; P2h, 2 h-priming; P24h, 24 h-priming; OP1mm, overpriming with 1 mm radicle protrusion; OP2mm, overpriming with 2 mm radicle protrusion; H, water; K, kinetin; FW, fresh weight; DW, dry weight. Means with common superscript letters are not significantly different ($p < 0.05$) as analyzed with two-way ANOVA and Tukey test.

Since the DCF-DA assay evidenced more contrastive patterns of ROS accumulation at the endpoint of dry-back, this timepoint was selected for DAB staining to qualitatively assess the distribution of H_2O_2 within *M. truncatula* dehydrated embryos. Although no significant differences were observed in response to hormopriming compared to hydropriming, the DAB-positive coloration was observed only in the radicle tips of all mildly (OP1mm) and severely (OP2mm) overprimed seeds (Figure 5b), consistent with the higher ROS levels detected through DCF-DA assay at the endpoint of dry-back in overprimed seeds (Figure 5a).

3.4. The Loss of Desiccation Tolerance Correlates with Radicle Protrusion Length and with ROS Levels during Dry-Back

The analysis of ROS levels during dry-back (Figure 5a) provided useful hints to monitor oxidative stress in primed and overprimed seeds and suggested possible correlations with the occurrence of 'aberrant' seedling phenotypes in overprimed seeds (Figure 3a). To statistically assess this hypothesis, a correlation analysis was performed between the length of radicle protrusion before dry-back (0 mm, 1 mm or 2 mm), seedling phenotype (expressed as percentage of 'normal', 'aberrant' and 'dead' seedlings), and ROS levels

(expressed as relative fluorescence units) before, during and after dry-back. The results are shown in Figure 6. Radicle protrusion length displayed strong positive correlations with ROS emission at the end of dry-back (0.837) and with the occurrence of 'aberrant' and 'dead' seedling phenotypes (0.977 and 0.673, respectively), while displaying strong negative correlation with the percentage of 'normal' phenotypes (−0.981). ROS levels at the endpoint of dry-back (DB4/6h) showed strong positive correlation with the percentage of 'aberrant' and 'dead' phenotypes (0.796 and 0.629, respectively) and a strong negative correlation (−0.804) with the percentage of 'normal' seedlings. Weaker significant correlations were also highlighted. For example, ROS levels at 2 h dry-back (DB2h) correlated with radicle protrusion length (0.503), ROS levels at 4/6 h (0.469), percentage of 'aberrant' seedlings (0.537), percentage of 'dead' seedlings (0.330) and percentage of 'normal' seedlings (−0.527).

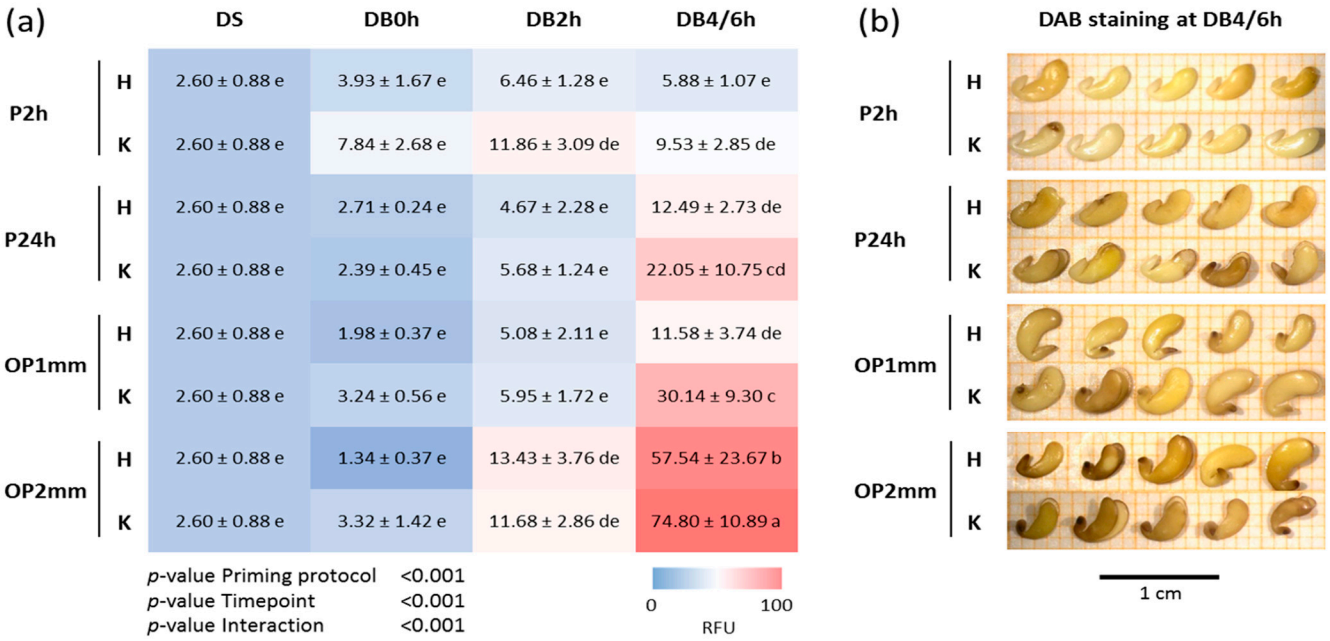

Figure 5. Accumulation and distribution of ROS in *M. truncatula* seeds during post-priming dehydration. (**a**) ROS levels in dry seeds and during dry-back (at 0, 2 and 4/6 h) assessed through DCF-DA assay. Mean values of relative fluorescence units (RFU) ± standard deviation are shown. *p*-values are also provided referring to the differences between priming protocols, timepoints of dry-back, and their interaction, as analyzed with two-way ANOVA and Tukey test. Within the dataset, values that are followed by common letters are not significantly different (*p*-value < 0.05). (**b**) Distribution of H_2O_2 within embryos assessed through DAB staining at the endpoint of dry-back (4/6 h). DS, dry seed; P2h, 2 h-priming; P24h, 24 h-priming; OP1mm, hydroprimed seeds with 1 mm radicle protrusion; OP2mm, hydroprimed seeds with 2 mm radicle protrusion; H, hydropriming protocols; K, hormopriming protocols; DB2h, ROS levels after 2 h dry-back; DB4/6h, ROS levels at the endpoint of dry-back; RFU, relative fluorescence units.

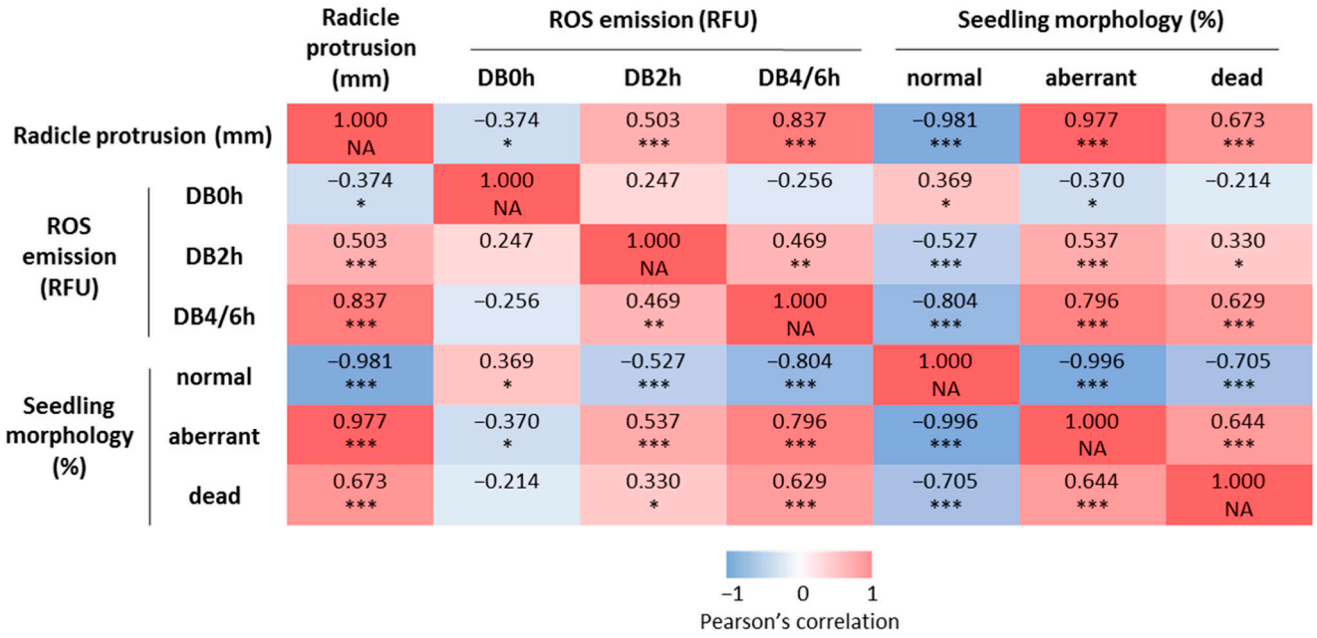

Figure 6. Correlation analysis carried out between the radicle protrusion length before dry-back, the ROS emission during dry-back and seedling morphology. DB0h, ROS levels before dry-back; DB2h, ROS levels after 2 h dry-back; DB4/6h, ROS levels at the endpoint of dry-back. The Pearson's correlation coefficients are indicated. The significance of the Pearson's correlation is shown by asterisks ('*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$). NA, not applicable.

3.5. The Correlation between ROS Accumulation and Loss of Desiccation Tolerance Is Reproducible in Medicago sativa

The strong significant correlations between ROS levels at the endpoint of dry-back (DB4/6h) and the occurrence of 'aberrant' seedling phenotype observed in *M. truncatula* seeds (Figure 6) indicated a possible route to predict the loss of desiccation tolerance in early seedlings. In order to test this hypothesis on a related species, a proof-of-concept was carried out on a commercial genotype of *Medicago sativa*, by measuring ROS levels through DCF-DA assay at the endpoint of dry-back and the occurrence of 'normal', 'aberrant' and 'dead' seedling morphology after 4 days of germination. The endpoint of dry-back (DB4/6h) was selected because it was the timepoint that consistently displayed the highest level of ROS accumulation for all the priming conditions tested on *M. truncatula* seeds. The same priming and overpriming conditions and the same morphological criteria previously described for *M. truncatula* were applied to *M. sativa*. The results are shown in Figure 7a. The pattern of ROS accumulation in *M. sativa* seeds at the endpoint of dry-back was comparable to the results obtained in *M. truncatula*. Specifically, the highest ROS emission was detected in severely overprimed seeds (HOP2mm and KOP2mm) in association with the highest percentage of 'aberrant' seedlings. 'Dead' seedling morphology was present in response to all mild and severe overpriming conditions (HOP1mm, KOP1mm, HOP2mm, KOP2mm). Considering the effects of kinetin, ROS emission appeared to be higher in response to kinetin-mediated severe overpriming (KOP2mm) compared to the respective hydropriming treatment (HOP2mm). To statistically assess the correlation between ROS levels after dry-back and the loss of desiccation tolerance in *M. sativa* seedlings, a Pearson's correlation analysis was carried out (Figure 7b). Significant positive correlations were obtained between ROS levels and the percentage of 'aberrant' and 'dead' seedlings (0.827 and 0.472, respectively), whereas the correlation with the percentage of 'normal' seedlings was significantly negative (−0.797). These correlation trends are comparable to those to those obtained for *M. truncatula* seeds (Figure 6).

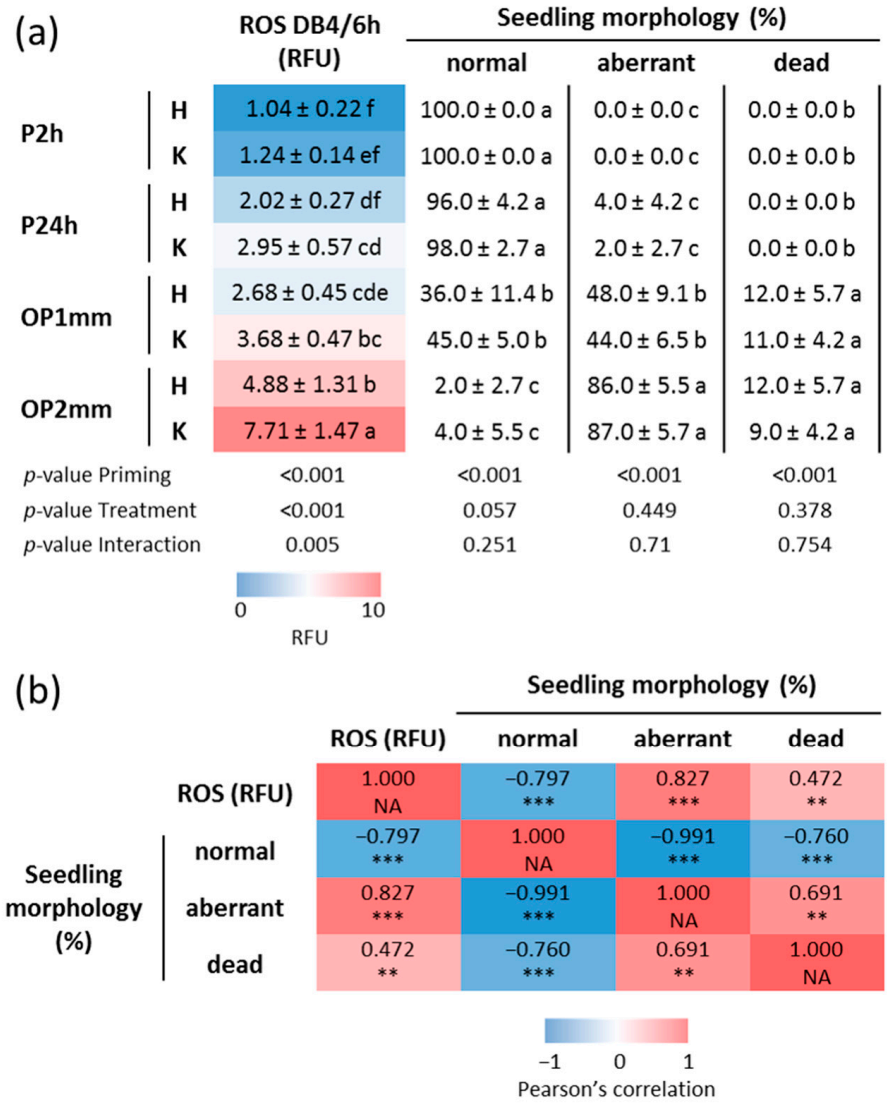

Figure 7. Proof-of-concept to verify the positive correlation between ROS levels and the loss of desiccation tolerance in *M. sativa*. (**a**) ROS levels measured by DCF-DA assay at the endpoint of dry-back (DB4/6h) of *M. sativa* seeds and occurrence of 'normal', 'aberrant' and 'dead' seedling morphology in response to short/prolonged priming and mild/severe overpriming. RFU (relative fluorescence units) and the percentage of seedling morphology occurrence are expressed as mean values ± standard deviation. *p*-values are also provided referring to the comparisons between priming protocols (P2h vs. P24 vs. OP1mm vs. OP2mm), treatment groups (H vs. K), and their interaction, as analyzed with two-way ANOVA and Tukey test. Within each column, values that are followed by common letters are not significantly different (p-value < 0.05). P2h, 2 h-priming; P24h, 24 h-priming; OP1mm, hydroprimed seeds with 1 mm radicle protrusion; OP2mm, hydroprimed seeds with 2 mm radicle protrusion; H, hydropriming; K, hormopriming; RFU, relative fluorescence units. (**b**) Correlation analysis carried out between the ROS emission at the endpoint of dry-back and the seedling morphology in *M. sativa*. The Pearson's correlation coefficients are indicated. The significance of the Pearson's correlation is shown by asterisks ('**' $p < 0.01$, '***' $p < 0.001$). NA, not applicable.

4. Discussion

In the experimental system considered in this work, the combined effects of short and prolonged hydropriming and kinetin-mediated hormopriming were tested on a commercial *M. truncatula* seed lot. Germination tests confirmed the effectiveness of short and prolonged

hydropriming and hormopriming in improving germination performances in *M. truncatula* seeds as previously observed [12,39], with further improvements in germination speed in response to hormopriming and an increase in germination consistency (expressed as a decrease in the uncertainty index and as an increase in the synchronization index) in response to prolonged hormopriming.

Although increases in germination speed and consistency represents an added value in agricultural contexts [40], the requirements of prolonged priming protocols to improve germination parameters may lead them to apply imbibition-dehydration timing patterns that operate at the limits of desiccation tolerance windows. Avoiding the occurrence of overpriming is crucial to optimize seed priming protocols but needs to account for the variability of different subpopulations within a seed lot in their response to priming and dry-back. In the experimental system considered in this work, mild and severe overpriming conditions were also defined for *M. truncatula* primed seeds.

A significant increase in the proportion of 'aberrant' seedlings was observed with increasing length of the radicle protrusions before dry-back. This observation was corroborated by a positive correlation index and is in agreement with previous literature, where it is explained as the result of the higher sensitivity of radicle protrusion to desiccation stress compared to the other embryonal tissues [21,25]. The exposure to osmotic agents has been proven effective to improve desiccation tolerance of primed seeds and early seedlings [6,21]. Conversely, in the present work, kinetin-mediated hormopriming per se does not appear to alter overpriming occurrence compared to the same physiological stage (1 mm and 2 mm radicle protrusion) of hydroprimed seeds. Nonetheless, treatments that are specifically able to synchronize germination may represent an alternative route to decrease the exposure to desiccation stress within the defined frame of a priming protocol, whereas the underlying variability of a seed population would represent an added value under less controllable natural environments [41,42]. This might be an applicative outcome of kinetin as a priming agent.

Although kinetin treatments did not alter the incidence of overpriming in the present work, they were effective in accelerating and synchronizing germination. The positive effects of cytokinins, including kinetin, on dormancy release and germination performances have been observed in *A. thaliana*, *T. aestivum*, *M. truncatula* and other crop and model species. These properties have been attributed to the capacity of cytokinins to promote cell division and antagonize the signaling pathways of auxins and abscisic acid, which are the key hormones in dormancy induction and maintenance [39,43–45]. On the other hand, as evidenced in the present work, prolonged exposure to kinetin results in reduced radicle growth. This observation agrees with previous studies [45], where the inhibitory effects of kinetin on radicle development in *M. truncatula* have been correlated with global metabolomic depletion and accumulation of DNA double strand breaks. Subsequently, prolonged exposure to kinetin to accelerate and synchronize germination needs to be considered along with its side effects.

Considering the observed relation between radicle protrusion length before dry-back and the occurrence of 'aberrant' seedling phenotypes, *M. truncatula* represents an unambiguous model system for the study of overpriming conditions in species in which such correlations are less explicit and predictable [20]. In the context of the tested priming protocols, the occurrence of overpriming has provided a model to identify possible hallmarks of desiccation stress in *M. truncatula*, specifically in terms of accumulation and distribution of ROS along the dry-back phase.

ROS accumulation, either as a byproduct of the reactivation of pre-germinative metabolism or as a consequence of biotic/abiotic stress, causes oxidative damage to lipids, proteins and nucleic acids, potentially compromising cellular structures and seed viability [3,6,46]. Nonetheless, within controlled ranges, ROS also have physiological functions in the context of germinative metabolism, promoting cell wall plasticity, endosperm weakening, stress response and reservoir mobilization, either directly or by modulating hormonal signaling [3,46,47]. As a consequence of their dual role, ROS levels in seeds are controlled

by an endowment of antioxidant compound accumulated during seed maturation and by the reactivation of antioxidant response pathways during imbibition [48]. The DCF-DA assay carried out in this work highlighted contrastive responses in terms of ROS accumulation during the dehydration phase following short/prolonged imbibition. Direct correlations were evidenced between radicle protrusion length before dehydration, ROS levels at the endpoint of dehydration and the occurrence of 'aberrant' seedling morphology. DAB staining allowed the assessment of the distribution of H_2O_2 within *M. truncatula* embryos at the endpoint of dehydration, indicating a localized accumulation in the tips of the radicle protrusions of overprimed seeds. The correlation between radicle elongation and the progressive decrease of desiccation tolerance has been reported in *Pisum sativum*, *Fagus sylvatica* and other model systems, and it has been linked to the progressive depletion of compounds, including LEA (Late Embryogenesis Abundant) proteins, stabilizing proteins and cellular structures. In these contexts, the accumulation of H_2O_2 observed in early seedlings exposed to dehydration stress was explained in terms of ROS leakage through damaged membranes [32,49–51].

Following this direct route of ROS accumulation under drought stress, ROS can subsequently modulate hormonal signaling (specifically ABA) to promote the expression of genes involved in desiccation tolerance, antioxidant response and DNA repair to restore redox homeostasis and preserve seed viability [33,52,53]. The maintenance of desiccation tolerance in germinating seeds implies transcriptomic and metabolomic changes that partially overlap those involved in the acquisition of desiccation tolerance during seed maturation. These include ROS detoxification and synthesis of osmo-protectants, but also lipid, starch and oligosaccharide reservoir mobilization, as highlighted comparing desiccation-sensitive and desiccation-tolerant *M. truncatula* early seedlings [54]. From a molecular standpoint, these dynamics likely underlie the transition from a prolonged and reversible priming into a condition of irreversible overpriming, as observed in the present work.

To support molecular interpretations, ROS accumulation is measured through multiple detection techniques, including nitroblue tetrazolium chloride staining, fluorescent dyes, confocal microscopy, and electronic spin resonance spectroscopy, with different degrees of sensitivity and specificity [33,55], also allowing the use of ROS as reliable sensors to monitor the occurrence of stress conditions in a variety of experimental systems [56]. In the present work, DCF-DA assay and DAB staining were used to quantify and localize, respectively, the emission of ROS in *M. truncatula* embryos during post-priming dry-back. The results obtained from DCF-DA assay confirmed ROS accumulation with dry-back progression and increasing radicle length, displaying significant correlations with the loss of desiccation tolerance in both *M. truncatula* and *M. sativa*.

5. Conclusions

This work reported the beneficial effects of hydropriming and kinetin-mediated hormopriming on germination performances in the model legume *Medicago truncatula*. The contrastive effects of short/prolonged priming protocols and mild/severe overpriming conditions supported the concept of 'overpriming' in terms of loss of desiccation tolerance during the radicle protrusion phase, resulting in impaired seedling development. ROS accumulation during dry-back in response to overpriming suggests the potentialities of ROS as hallmarks to monitor seed priming progression and assess dehydration stress with reliable and resource-effective approaches. These conclusions were confirmed with a proof-of-concept on a commercial variety of *Medicago sativa*, defining a reproducible experimental system to study and test seed priming protocols in model and crop species.

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