DENDROBIOLOGY

2022, vol. 87, 137–148

https://doi.org/10.12657/denbio.087.010

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Effects of spermidine on germination of *Salix* spp. after storage under controlled conditions

Received: 27 February 2022; Accepted: 04 May 2022

Abstract: Willows produce fast germinating and short-lived seeds, difficult to store in the long-term under controlled conditions. The aim of this study was to examine the feasibility of storage of three *Salix* spp. at controlled temperatures (3°, −10°, −196 °C). We also analyzed the effect of spermidine (Spd) as an antioxidant factor in desiccated seeds.

Collected seeds were either desiccated or hydrated to obtain 10 levels of moisture content (between app. 4% and 2%) and subjected to storage at temperatures 3°, −10°, or −196 °C (liquid nitrogen; LN). After two months, seeds were germinated on the light at 20 °C. Seeds desiccated below a safe range of moisture content were further tested and germinated on filter paper with additions of 0.25 mM Spd solution. After 7 days seedlings were examined for hydrogen peroxide content (H_2O_2) and total antioxidant capacity (TAC). Fresh seeds of three *Salix* species: Persian willow (*S. aegyptiaca* L.), heartleaf willow (*S. cordata* Michx.) and crack willow (*S.* ×*fragilis* L.) were successfully stored at temperature −10° and −196 °C for two months. After cryopreservation seed of *S. aegyptiaca*, *S. cordata*, and *S.* ×*fragilis* germinated without viability loss in moisture content ranging from 4.4–15.9%, 6.4–18.5%, and 7.1–11.5% respectively. The addition of Spd during germination of desiccated seed did not affect germination capacity. However, seedlings of *S. aegyptiaca* had lower hydrogen peroxide content in comparison with control (germination on water). Seedlings of *S. cordata* showed an increase in hydrogen peroxide content in control after storing in LN. In seedlings of Crack willow Spd increased hydrogen peroxide content.

Seeds of tested species differ in response to storage conditions. *Salix* seeds can be stored successfully for two months at −10° or −196 °C without losing viability in the safe range of moisture content. Storing at 3 °C can be used for storage in the narrower range of seeds' moisture content, however, seedlings stored at this temperature produce a higher level of reactive oxygen species. Germinating seeds in Spd did not increase their germination, however in *S. aegyptiaca* and *S. cordata* decreased hydrogen peroxide content.

Keywords: hydrogen peroxide, cryopreservation, spermidine, Salix, antioxidant, seed storage

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Introduction

The genus *Salix* comprises 330–500 species of tree and shrubs mostly distributed in temperate and arctic climate zones. Willows occur predominantly in the northern hemisphere, often being only woody species in tundras and areas lying above latitudinal tree lines. However, many species grow in riparian or alluvial areas as well as upland habitats (roadside ditches, abandoned agricultural fields, railroads, forest edges) where they serve as pioneer species (Dickmann et al., 2014). A major factor controlling the distribution and abundance of this species is moisture availability. Willow species are widely used for soil stabilization, basketry, or biomass production (Towill & Widrlechner, 2004).

All willows are dioecious and usually flower and set seed in spring. Seeds are very small (0.8–3 mm) and embedded in white down which allows seeds to be dispersed by wind (Steyn et al., 2004). Seeds contain chlorophyll but no endosperm, are short-lived, and germinate almost immediately after being released (Niiyama, 2008). Due to relatively high desiccation tolerance, most of the willow seeds are classified as *orthodox*, however, willow seeds are short-lived in dry storage, rapidly losing viability within a few days at room temperature (Hong & Ellis, 1996; Maroder et al., 2000; Liu et al., 2019). In consequence willows, seeds are often treated as *reclacitrant* or *suborthodox* species. However, storing seeds at subzero temperatures can improve their longevity (Densmore & Zasada, 1983; Simpson & Daigle, 2007). Especially cryopreservation proves to be a safe and effective method for storing short-lived seeds like those produced by willows or poplars (Popova et al., 2012; Michalak et al., 2014; Ballesteros & Pence, 2017). The most critical factor affecting the cryopreservation of seeds is their moisture content (Pritchard, 2007). For effective storage, seeds have to be dried to a safe range of moisture content before storage. Seeds with moisture content between their critical points showed increased survival and high viability (Chmielarz, 2010; Wawrzyniak et al., 2020).

However, during every storage seeds deteriorate with advancing time as their life span is finite. Lowering the temperature and moisture content can extend the shelf-life of stored *orthodox* seeds, approximately doubling its time with every 5.6 °C decrease of storage temperature and every 1% of seeds moisture content (Bewley et al., 2012). Although storability below a certain threshold of moisture content and temperature can differ from expectation (Walters, 2007; Wawrzyniak et al., 2020). The main factor controlling the deterioration of seeds during storage is an accumulation of reactive oxygen species (ROS) i.e. hydrogen peroxide (H_2O_2) as their production and metabolism depends on the physiological fitness of stored seeds (Kranner et al., 2010; Ratajczak et al., 2015; Foyer et al., 2017). Hydrogen peroxide, the most stable ROS, easily migrates through membranes over relatively long distances, even in dry seeds (Ratajczak et al., 2015; Wojtyla et al., 2016). H_2O_2 is regarded as a signal molecule, which participates in the regulation of seed dormancy and germination. A balanced H_2O_2 level is beneficial, as it promotes germination, whereas excessive H_2O_2 content induces oxidative damage, which prevents or delays germination (Bailly et al., 2008; El-Maarouf-Bouteau & Bailly, 2008).

An effective antioxidative system can swiftly decrease ROS levels and increase seeds' survival. Especially the presence of chloroplasts with the chlorophyll in mature seeds contribute to their deterioration via ROS-mediated photo-oxidation (Roqueiro et al., 2010). Seed priming is a seed performance improvement method. Priming seeds involve the partial hydration of seeds by maintaining water content at values that enable to start the initial stages of germination, but hinder the cell elongation (Roqueiro et al., 2010). However, priming fast germinating seeds, like *Salix,* is difficult as they germinate within a few hours after imbibition. Primed seeds have more uniform and rapid germination with vigorous and high-quality seedlings (Sheteiwy et al., 2017). Polyamines (PAs) such as putrescine (Put), spermidine (Spd), and spermine (Spm) play important role in plant response to abiotic stresses, scavenging ROS, and synthesizing osmolytes (Alcázar et al., 2006; Kusano et al., 2007). Furthermore, it has been reported that priming seeds in exogenous Spd increased seeds germination, vigor, and subsequent seedling growth (Huang et al., 2017). Exogenous Spd used in priming white clover (*Trifolium repens* L.) seeds enhanced antioxidant enzyme activities (superoxide dismutase, peroxidase, catalase, and ascorbate peroxidase), as well as an ascorbate-glutathione cycle (ASC-GSH cycle) and transcript level of genes encoding antioxidant enzymes (Li et al., 2014).

The main aim of this study was to assess the storage feasibility of three different *Salix* species: *S. aegyptiaca*, *S. cordata,* and *S.* ×*fragilis*. The secondary objective was to examine the antioxidative effects of spermidine on the germination of stored seeds. We hypothesized that (i) there is a safe hydration window for cryopreservation of *Salix* seeds; (ii) spermidine decreases oxidative stress effects of seeds storage conditions (temperature and moisture content).

Material and methods

Plant material

Persian willow or musk willow (*Salix aegyptiaca* L.) is a small tree, 4–8 m tall distributed in the

Caucasus on territories of Turkey, Armenia, Iran, and Turkmenistan. It grows on the slopes and banks of streams, as well as forest edges and meadows (Dickmann et al., 2014). Heartleaf willow or dune willow (*Salix cordata* Michx.) is a multi-trunked small tree 2–4 m tall. Distributed in the northeast parts of North America grows on sandy dunes of lakeshores, edges of wetlands, meadows, and fields (Seneta et al., 2021). Crack willow (*Salix* ×*fragilis* L.) is a tall tree (20–25 m), commonly distributed across Europe, North Africa, and West Asia. Crack willow is a very variable natural hybrid between *S. alba* (L.) and *S. euxinia* (I.V. Belyaeva). Natural occurrences include riparian areas, along rivers, streams, and road ditches, as well as meadows and marshes (Seneta et al., 2021).

Selected trees of *S. aegyptiaca* and *S. cordata* were cultivated in Arboretum Kórnik (Kórnik, Poland) or grow near drainage ditch (*S.* ×*fragilis*; Table 1; Fig. 1). Branches of these three willow species with closed catkins were collected in May-June from female trees at the moment when catkins started turning from

Fig. 1 Dry and imbibed seeds of *S. aegyptiaca* (A, D), *S. cordata* (B, E), and *S.* ×*fragilis* (C, F)

Table 1. Seed collection site and seed characteristic after collection of tested species. Mean±SD

Species	Seed collection	Collection date	Initial moisture content, %	Initial germination, %	1000 seed mass, g
Salix aegyptiaca	52°14'43.4"N 17°06'10.0"E	May 2021	12.6±0.22	75.5±4.12	0.0102±0.00037
Salix cordata	52°14'38.1"N 17°05'35.4"E	May 2021	12.6±0.51	93.0±3.46	0.0097±0.00055
Salix ×fragilis	52°20'57.8"N 17°02'59.9"E	June 2021	9.2±0.64	84.0±0.00	0.0135±0.00063

green to yellow. For each species, we collected scions from 3 different individuals. Branches of every species were put into a vase with water and left for 2 days in ambient conditions (ap. 22 °C) until the catkins were fully open and released seeds embedded in white down. Seeds with surrounding down were collected using fine mesh and vacuum cleaner and separated from down using sieves. Subsequently, seeds were examined for their initial moisture content (3 replication per 200 seeds), initial germination (3 replication per 50 seeds), and one thousand seed mass (5 replication per 100 seeds; Table 1). The remaining seeds were stored at 3 °C in sealed vials, until the start of the experiment (10 days).

Moisture content determination, desiccation, and hydration

Seed moisture content (MC) was determined by drying in the oven at 103±2 °C for 17 h (3 replications per ap. 20 mg of seeds; ca. 1800 seeds). The MC was calculated based on fresh mass (%) using the following formula:

$$MC_1 = \frac{(FM_1 - DM) \times 100}{FM_1}$$

where MC_1 is the moisture content, FM_1 is the initial fresh mass, and *DM* is the dry mass after drying. Before the experiment, the *MC* of the seeds was adjusted (either by desiccating or moisturizing) to obtain 10 levels of *MC* ranging from 2% to 4% to 22% with increments of app. 2%. Seeds based on their initial *MC* were either desiccated under silica gel or moisturized above distilled water in closed containers. Adjusting the *MC* of the seeds was based on the *FM* of seeds according to the formula:

$$FM_1 = \frac{FM_1 \times (100 - MC_1)}{100 - MC_1}$$

where FM_1 is the desired fresh mass and MC_1 is the desired moisture content. After obtaining the desired *MC*, seeds were left in tightly closed vials for 3 days at 3 °C to even out the *MC*. After reaching the mass of desired *MC* level, the exact *MC* was determined as described above.

Seeds storage and germination

After adjusting the desired MC levels of seeds, seeds were stored at temperatures of 3°, −10°, or −196 °C (liquid nitrogen; LN) for 2 months in tightly closed vials. Not stored seeds served as a control treatment. The material stored in LN was frozen by direct immersion in LN. Subsequently, vials containing seeds were thawed at 42 °C in the water bath for 5 min (Chmielarz, 2010).

After the storage, seeds were germinated in Petri dishes with filter papers moisturized with ap. 2 ml of distilled water at constant temperature 25 °C in light (60 μmol m^{-2} s^{-1} for 12 h per day; 4 replication per 50 seeds). Germination was counted after the 3rd and 7th days after sowing. For further analyses, we used counted number of germinated seeds 7th day after sowing and included only fully develop seedlings (without abnormalities). Additionally, seeds desiccated below the safe range of MC (app. ≤10%), were germinated in Petri dishes with filter papers moisturized with the solution of 0.25 mM spermidine (Spd; Sigma-Aldrich, ≥99% GC) in the same controlled conditions.

Biochemical assays

Seedlings (after 7 days, with fully emerged cotyledons and radicle) both on distilled water or Spd solution were used for examination of oxidative stress.

Hydrogen peroxide content was determined using the ferrithiocyanate method described by Sagisaka (1976). The sample at each collection was finely ground in liquid nitrogen and homogenized with 5 mL of 5% (w/v) trichloroacetic acid (TCA) containing 10 mM EDTA. The homogenate was centrifuged at 4 °C at 26,000 × g for 20 min. The total volume of supernatant was analyzed.

Total antioxidant capacity (TAC) was determined by the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Molyneux, 2004). Tissues (20 mg FW) in 3 replications were homogenized in 0.5 ml of 100% (v/v) methanol. Homogenates were centrifuged at 7 000 × g for 10 min at 4 °C and then, 20 μl of the extract was added to 180 μl of 120-μM DPPH dissolved in methanol on 96 well plates. The reaction mixture was incubated for 15 min in darkness at room temperature. The concentration of reduced DPPH was

measured at 517 nm using a microplate reader. Antioxidant capacity was expressed as reduction of DPPH defined as $(A_0 - A)_s / A_0 \times 100\%$, where A_0 is the absorbance of a blank, and A_s is the absorbance of the sample.

Statistical analyses

Data were analyzed using R statistical computing software (R Core Team, 2021). The safe range of moisture content for each temperature was modeled using polynomial regression. The model was selected based on R^2 and diagnostic plots to avoid overfitting. Assumptions of the model were checked using diagnostic plots. Percentage data were transformed using arcsine transformation to ensure normal distributions of the model's residuals. Germination percentage of desiccated seeds (on water or Spd) was assessed using a generalized linear model (GLM) with the binomial distribution. Model assumptions for GLM were checked using the DHARMa package (Hartig, 2022). Biochemical analysis was analyzed using two-way ANOVA. Assumptions of the model were checked using diagnostic plots. Post-hoc analysis was performed using Tukey's test using the emmeans package (Lenth, 2022).

Results

Storage feasibility of *Salix* spp.

Seeds of *S. eagyptiaca* recorded no significant differences in germination after two months of storage regardless of storage temperature (Fig. 2). In general, there were no significant differences in the germination of seeds stored in −10° and −196 °C in all tested species, where seeds germinated between 70–96% depending on the species. Seeds stored at 3 °C recorded the lowest germination in all tested species, 84% in *S. aegyptiaca*, 90% in *S. cordata,* and 51% in *S. ×fragilis* (Fig. 2).

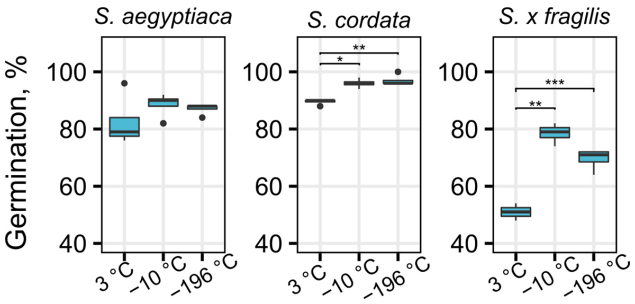

Fig. 2. Seed germination after 2 month storage at different temperatures (3, −10 or −196 °C) for seed *S. aegyptiaca* (9.3% MC), *S. cordata* (7.9% MC) and *S. ×fragilis* (9.4% MC). * – p≤ 0.05; ** – p≤ 0.01; *** – p≤ 0.001

Table 2. The estimated safe range of moisture content of tested species is stored at different temperatures and control. Safe moisture range for seeds to germinate ≥80% in *S. aegyptiaca* and *S. cordata* and ≥50% in case of *S. ×fragilis*

Species	Non-stored	3 °C	–10 °C	–196 °C
S. aegyptiaca	8.5–14.4%	4.4–9.7%	5.0–16.0%	4.4–15.9%
S. cordata	8.5–19.4%	6.5–11.6%	6.3–18.8%	6.4–18.5%
S. ×fragilis	8.1–12.4%	5.4–9.4%	6.1–12.8%	7.1–11.5%

Seeds of all tested species were feasible for storage in controlled conditions in all tested temperatures. Non-stored seeds of *S. aegyptiaca* had germination above 80% between 8.9 and 14.4%, with similar results observed for *S. cordata* and *S. ×fragilis* in seeds with MC 8.5–19.4% and 8.1–12.4% respectively (Fig. 3; Table 2). Storing partially desiccated seeds for two months in 3°, −10°, or −196 °C (liquid nitrogen; LN) helped maintain germination on a high level. Though, for *S. ×fragilis* there was a considerable decrease compared with initial germination. Seeds above 10–12% of MC stored at temperature 3 °C rapidly lost viability and ability to germinate in all tested species. Non-stored seeds partially desiccated below 8% of MC also decreased in germination capacity regardless of tested species. Seeds stored either in −10° or −196 °C germinated in the widest moisture content range. *S. aegyptiaca* between 5–16% after storage at −10 °C and between 4.4–15.9% after cryopreservation in LN. Similarly, *S. cordata* seeds germinated between 6.3–18.8% after storage at −10 °C and between 6.4–18.5% after storage in LN. *S. cordata* characterized with the highest moisture content freezing limit in all tested species. The most narrow range of safe moisture content was observed for *S. ×fragilis*. Seeds germinated between 6.1–12.8% after storage at −10 °C and 7.1–11.5% after storage in LN.

Germination on Spd

There was no substantial difference in seeds germinated on filter paper with water and 0.25 mM Spd solution in all tested species. Germination of *S. aegyptiaca* seeds was significantly lower after the addition of 0.25 mM of Spd in comparison with control in seeds desiccated to 6.7% after storage in 3 °C (78 and 87% respectively; Fig. 4). Seeds of *S. ×fragilis* germinated significantly better after the addition of Spd when desiccated to 7.6%, after storage in LN (51 and 59% respectively). Germination of seeds desiccated below or near their critical moisture content (3.7–4.4%) decrease in comparison with control throughout all tested species. Only seeds of *S. aegyptiaca* stored at 3 °C recorded no significant difference in germination regardless of seed MC and germinated between 77 and 84% (Fig. 4).

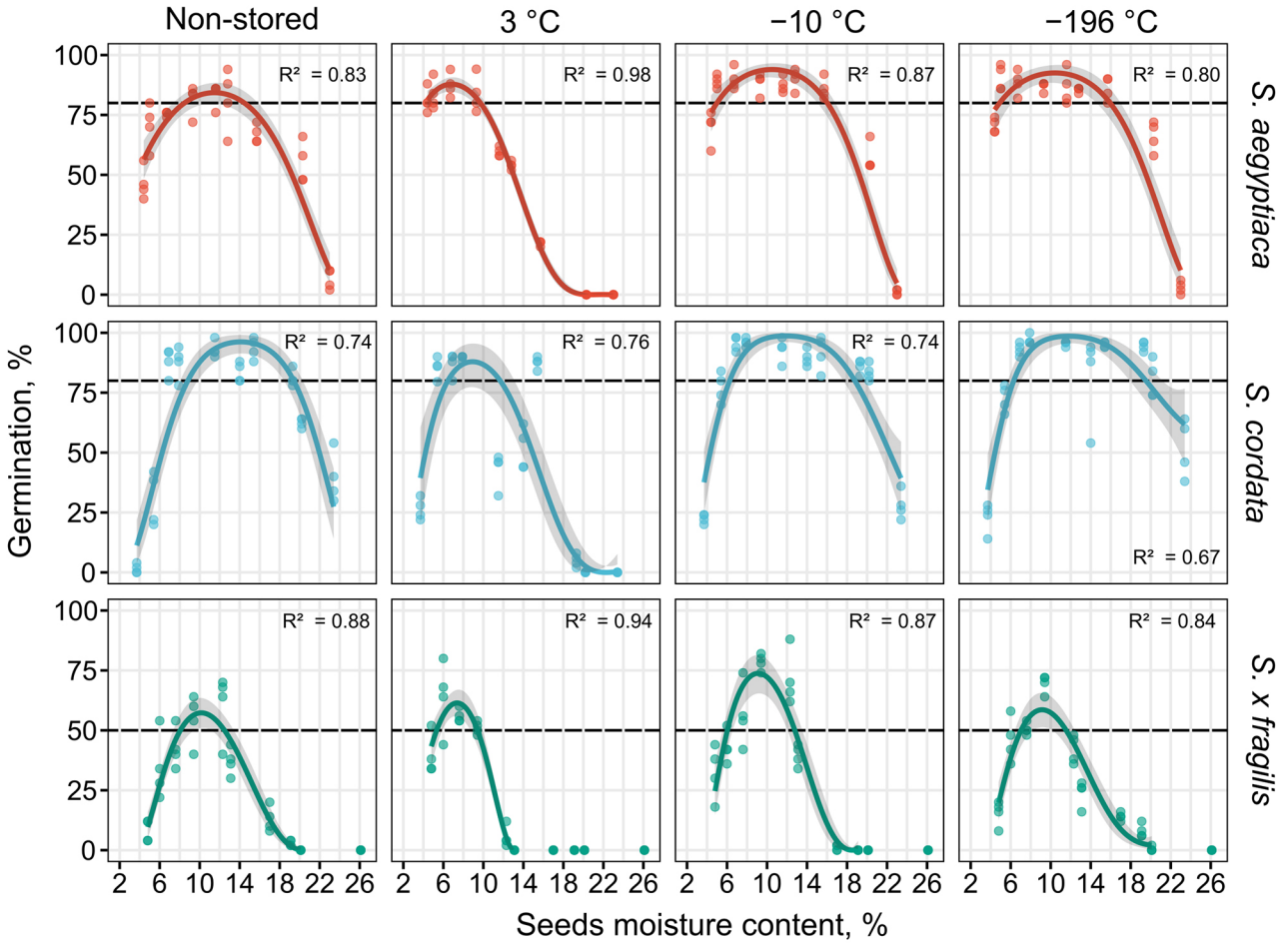

Fig. 3. Seeds germination of *S. aegyptiaca*, *S. cordata*, and *S. ×fragilis* after 2 months storage in controlled conditions. Seeds were stored at different temperatures (3°, −10°, and −196 °C) after adjusting their moisture content (between 3.7–26 %). Dashed lines show a safe moisture range for seeds to germinate ≥80% in *S. aegyptiaca* and *S. cordata* and ≥50% in the case of *S. ×fragilis*. The safe range of moisture content for each temperature was modeled using polynomial regression $y = \beta_0 + \beta_1 \times + \beta_2 \times^2 + \beta_3 \times^3$

Biochemical analysis

S. aegyptiaca seedling had significantly higher H_2O_2 content in control than in Spd solution in all tested temperatures and moisture content levels (Fig. 5A). In both treatments, H_2O_2 increased with decreasing moisture content, especially at 3 °C. It was not so evident in lower temperatures, however, the lowest MC is significantly higher than two other MC. The highest value was recorded for 4.4% after storing in 3 °C, and the lowest for control (9.3%) seeds stored at 3 °C (Fig. 5A). Germinating in Spd tends to give a lower response of TAC, decreasing even further with increasing MC. However those differences it is not observed in germination capacity directly (Fig. 5B).

Seeds of *S. cordata* seeds seem to be more robust and had doubled overall H_2O_2 concentration after storage at −196 °C (Fig. 5A). However, there were no significant differences in H_2O_2 concentration regardless of seeds MC in temperatures 3 and −10 °C. Addition Spd during germination lowered H_2O_2 concentration in seeds stored at −196 °C. There was no such effect observed at −10 °C. In 3 °C seeds with MC 5.4, had a significantly lower concentration of H_2O_2 in received seedlings in comparison with other tested MC. TAC significantly decreased in seeds stored at −10 and −196 °C with increasing MC of seeds. TAC values after germinating in water were significantly higher than in seeds germinated in Spd (Fig. 5B).

S. ×fragilis seedlings had 10 times lower concentrations of H_2O_2 in comparison with other tested species (Fig. 5A). Seedling had a higher concentration of H_2O_2 after exposure to Spd. At 3 °C concentration of H_2O_2 was significantly higher in 9.4% than other tested MC. H_2O_2 concentration was similar in water, being the lowest in seeds desiccated to 7.6% (0.37 nmol/g). Seeds stored at −10° showed an increase in H_2O_2 after Spd treatment for control (9.4%) and seed desiccated to 7.6%. Seedling tested from temperature −196 °C showed no significant interactions and 9.4% concentration was the lowest after desiccating

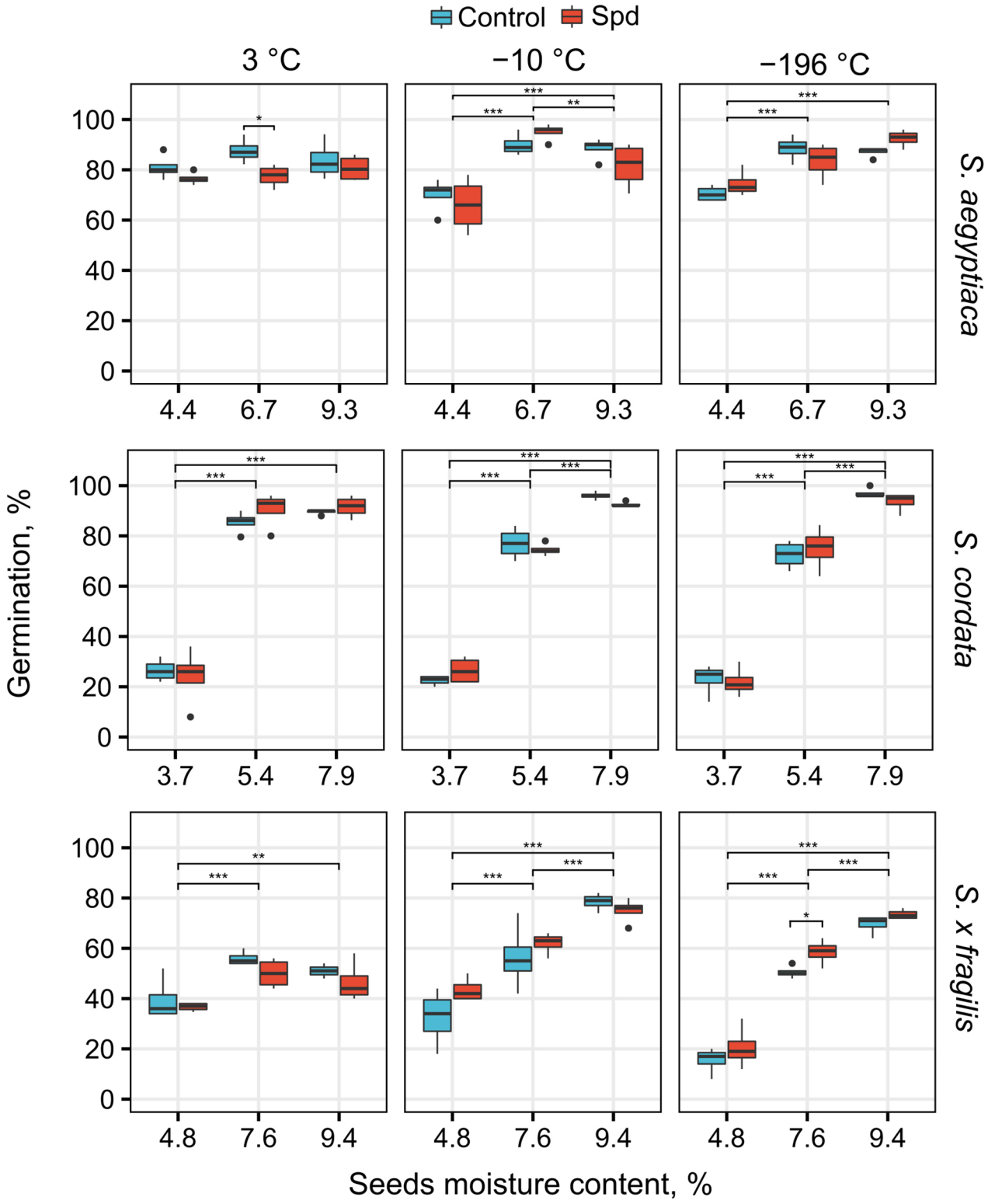

Fig. 4 Seeds germination after storage (at temperature 3°, −10° or −196 °C; A) and seeds partially desiccated below the safe range of moisture content and germinating either on water (control) or 0.25 mM spermidine (Spd; B). Two-way ANOVA analysis was presented for each temperature separately. * – p≤ 0.05; ** – p≤ 0.01; *** – p≤ 0.001

to 4.8% (Fig. 5A). No evident pattern was observed in TAC. TAC was significantly higher in seedlings received from seeds desiccated to 4.8% at temperatures 3°, −196°, and −10 °C (Fig. 5B).

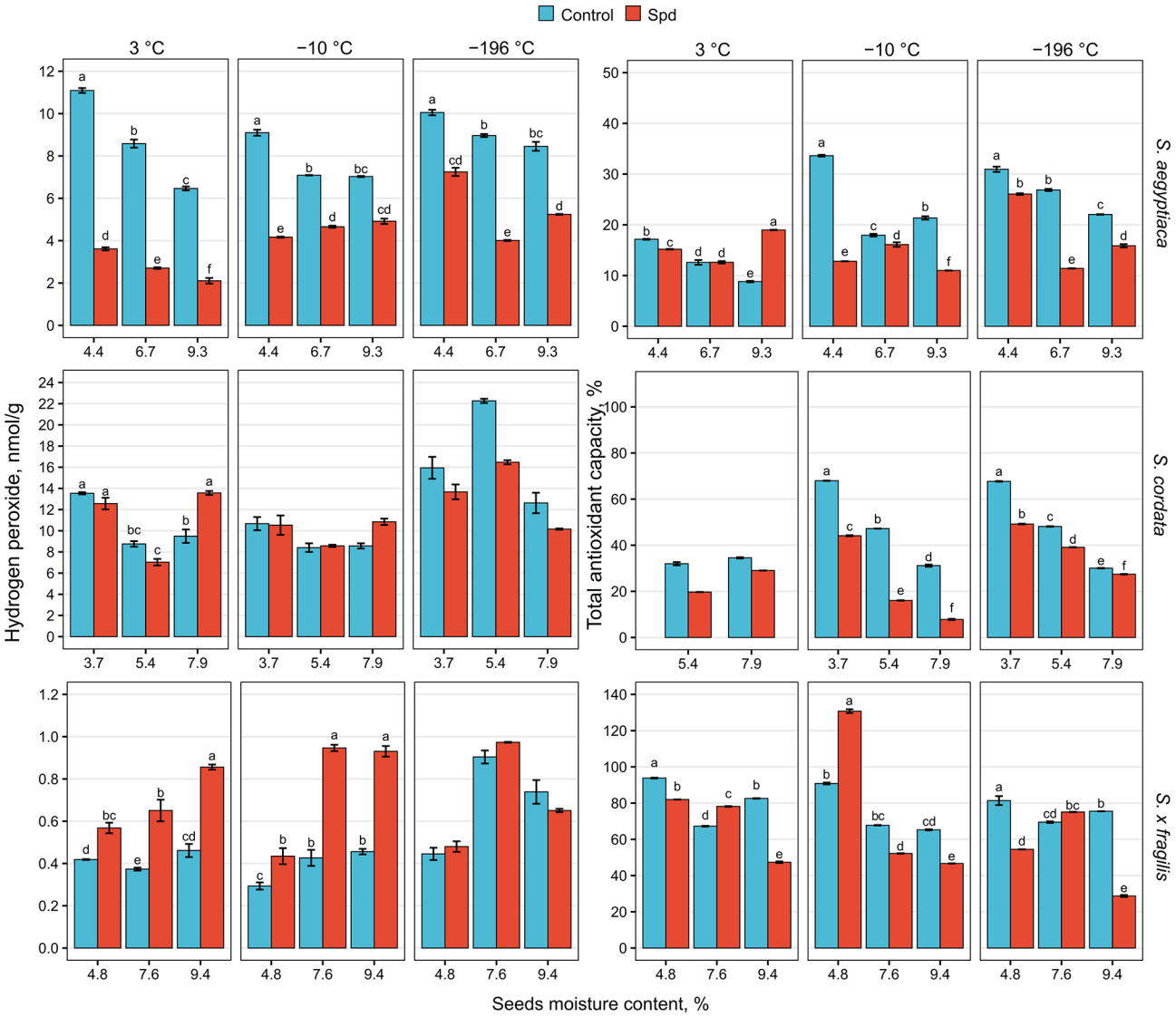

Fig. 5 Hydrogen peroxide content (H_2O_2; A), and total antioxidant capacity (TAC; B) of seedlings received from seeds germinating on water (control) or 0.25 mM Spd after storage at different conditions (for *S. aegyptiaca*, *S. cordata*, and *S.* ×*fragilis*). Due to the small amount of material received from seeds 3.7% stored in 3 °C of *S. cordata* were excluded from TAC analysis. Two-way ANOVA for each temperature separately. Post-hoc Tukey at p ≤0.05. Groups with no significant differences are marked with the same letter. Mean±SE

Discussion

Seeds of *Salix* are known to be desiccation-tolerant, however short-lived in storage (Densmore & Zasada, 1983; Maroder et al., 2000; Popova et al., 2012), with only a few examples of seeds classified as *intermediate* or *recalcitrant* (Liu et al., 2019). Seeds of *S. aegyptiaca*, *S. cordata,* and *S.* ×*fragilis*, all showed a decline in germination after 60 days in storage at 3 °C depending on their moisture content during storage. Desiccation below 12 % had beneficial effects on storage at 3 °C, as germination within this moisture content range was similar to control (non-stored seeds). Seeds stored at −10° and −196 °C germinated in a much wider range of moisture content. With exception of *S.* ×*fragilis* which safe range at −10 °C was between 6.1–12.8% of MC. Those results are consistent with studies on other *Salix* species seeds storage, where increased storability at sub-zero temperatures was observed and reached up to 6 or 12 years, however, their viability after the storage is low (Simak, 1982; Wang, 1982; Walters, 2015). Seeds which like *Salix* spp. requires the application of methods not always available in conventional seed banks are defined as exceptional species (Pence et al., 2022). Storing seeds at liquid nitrogen temperature (–196 °C) is expected to prolong seeds viability for hundreds of years, minimizing any metabolic activities in cells (Walters et al., 2004; Ballesteros et al., 2020). As recently (Ballesteros & Pence, 2017) showed short-lived species of *Salix* and *Populus* stored in LN maintained viability after 20 years,

however, the deterioration process was not completely stopped and seed viability was lower than expected. The seed of the tested species was viable after two months of storage in LN and germinated similarly to the control. The safe seed MC range for *S. aegyptiaca* was between 4.4 and 15.9%. Similar results were obtained for *S. cordata* with a safe range of MC between 6.4 and 18.5%, whereas *S.* ×*fragilis* had a very narrow safe MC range of 7.1 and 11.5%. The highest desiccation tolerance was obtained for *S. aegyptiaca* (4.4%) with slightly higher values in *S. cordata* (6.3%) and *S.* ×*fragilis* (5.4%). However, the high moisture freezing limit (HMFL) during storage in LN was the highest in *S. cordata* (18.5%) and *S. eagyptiaca* (15.9%) and the lowest in *S.* ×*fragilis* (11.5%). Similar, relatively low HMFL was reported in Black poplar (*Populus nigra* L.; 15%) (Michalak et al., 2014) and Easter cottonwood (*Populus deltoides* Bartr.; 15%) (Pence, 1996). *S. aegyptiaca* and *S. cordata* high hydration window for cryopreservation are similar to those obtained for goat willow (*S. caprea* L.) where it was between 5 and 22% and varied depending on initial seeds viability (Popova et al., 2012). Other riparian species such as Common alder (*Alnus glutinosa* L.) were successfully stored in LN when seeds were partially desiccated to MC between 3 and 15% (Chmielarz, 2010).

Even during storage in LN aging of seeds is not stopped completely (Ballesteros & Pence, 2017). Also, theoretical benefits for storability of severe desiccated seeds (≤5% MC) and extremely low temperatures (≤−160 °C) can be potentially damaging for their viability (Walters, 2007). Seeds of Crab apple (*Malus sylvestris* L.), although categorized as *orthodox*, decrease their viability when desiccated to 5% of MC (Wawrzyniak et al., 2020). Deterioration of seeds is associated with the accumulation of reactive oxygen species (ROS) e.g. hydrogen peroxide (H_2O_2). The amount of their production depends on the metabolic and physiological state of seeds during storage. Seed priming with polyamines (PAs), such as spermidine (Spd), can be a potentially efficient method of balancing the antioxidative response of stored seeds and promoting germination (Li et al., 2014; Hongna et al., 2021). Spd regulates antioxidant defense in white clover under desiccation conditions and can promote seed germination (Li et al., 2014, 2015). Also, it has been reported that Spd is closely associated with anti-aging properties (Hu et al., 2020). *Salix* seeds were not subjected to priming *per se* as they germinate within a few hours after imbibition and are impossible to store after. In our study seeds were primed and germinated at the same time. We did not observe any significant differences in germination between seeds primed in Spd and control in any tested *Salix* species, regardless of seeds' MC content and storage temperature. However, in *T. repens* seeds primed in exogenous Spd increased germination percentage, vigor index, and seedling growth under different water stress conditions (Li et al., 2014). Similarly, other polyamines, spermine (Spm), showed beneficial effects on rice seeds, where priming alleviated salt stress injury by lowering H_2O_2 level and avoiding chlorophyll degradation in seedling (Paul & Roychoudhury, 2016). That result seems to be consistent with *S. aegyptiaca* whose seeds had a lower concentration of H_2O_2 after germinating in Spd. H_2O_2 was increasing with decreasing seed MC indicating higher oxidation in seeds desiccated below or near minimum safe moisture content value (Fig. 1). On the other hand, the Spd effect on H_2O_2 was not so evident in *S. cordata* seeds. However, those seeds showed the highest viability and the widest range of safe moisture content (Table 1, Fig. 1). On the opposite seeds of *S.* ×*fragilis* were seeds that lost viability very rapidly and had overall the lowest viability. Storage showed a significant decrease in germination and the addition of Spd increased H_2O_2 in seedlings received form seeds stored at 3 and −10 °C. It may be a result of both the storage conditions, which could reduce the antioxidant activity and the relatively high concentration of Spd causing a stress reaction of the tested seedlings.

TAC was increasing with decreasing seed MC (Fig. 3). This observation is compatible with an investigation of physiological responses of *Oudneya africana* R.Br. to drought, which showed total antioxidant capacity increase close to 2−3-fold, with increasing drought stress severity (Talbi et al., 2020). TAC differed analogous to H_2O_2 levels in the case of *S. aegyptiaca* and *S. cordata*, increasing due to lower MC. It indicates the intensified activity of low-molecular-weight elements of the antioxidant system in response to drought stress due to desiccation (Pisoschi et al., 2016). To prevent oxidative damage to the structure and functions of cell membranes as the result of excessive ROS accumulation, plants trigger defense mechanisms, such as accumulation of polyphenols, glutathione, ascorbic acid, carotenoids, or flavonoids, which have a strong antioxidant effect (Halliwell & Gutteridge, 2015). However, in *S.* ×*fragilis* this effect was the opposite, which may be due to the specific response of this species. Spd treatment resulted in the decrease of TAC, which suggests that exogenous Spd prevents oxidative stress during germination and thus the level of antioxidant system activity is lower in comparison to seeds germinated in water.

Conclusions

Seeds of *Salix* although short-lived, if rapidly desiccated and processed after collection are feasible for

storage at sub-zero temperatures −10° and −196 °C (LN). However, the safe range of seeds' moisture content can differ depending on species characteristics and initial seed viability. Storage of *S. aegyptiaca* in LN should be between 4.4 and 15.9%, *S. cordata* between 6.4 and 18.5%, and *S.* ×*fragilis* between 7.1 and 11.5%. For the first time effect of Spd was examined in seeds of *Salix* spp. Concentration of 0.25 mM of Spd did not enhance germination of seeds, however, decreased H_2O_2 content in *S. aegyptiaca* seedlings. The reverse effect was observed in *S.* ×*fragilis* indicating different metabolic responses from these species. Treating short-lived seeds after storage with polyamines shows a possible reduction of ROS produced during storage. However, further investigation on the right concentration of Spd and the mechanism in which it operates in seeds is needed.

Contribution

Conceptualization: MKW and ER; Conducting the experiment: MKW, JMLL, JKO; writing – original draft preparation: MKW, JKO; writing – review and editing: MKW, ER, JMLL, PC; Data analysis: MKW; JKO; supervision: ER, PC; All authors have read and agreed to the published version of the manuscript.

Funding

This work was supported by statutory research of the Institute of Dendrology, Polish Academy of Sciences.

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