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*Joanna Kijowska-Oberc*, Mikołaj Krzysztof Wawrzyniak, Aleksandra Maria Staszak, Ewelina Ratajczak*

# Exogenous seed treatment with proline and its consequences to Norway spruce (*Picea abies* (L.) H. Karst) seedling establishment

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**Abstract:** Accumulation of proline is a defense mechanism against external stress conditions, preventing damage to the structure and function of cells and improving plant development processes, such as germination.

The purpose of this study was to investigate proline treatment as a means of improving the germination and development of Norway spruce seedlings. The effect of exogenous proline has been studied in three stages of initial plant development.

The collected seeds were soaked in water or 8 mM proline solution and placed on the germinators. The germination capacity and the mean germination time were determined. Seedlings with radicles >10 mm were transferred to the sand-peat substrate at a constant temperature of 20 °C. Seedlings at 3 subsequent developmental stages (S1 – germinated seeds with radicles > 3 mm; S2 – seedlings with radicles >10 mm; S3 – established seedlings grown for 90 days) were examined for the oxygen consumption rate, total antioxidant capacity, hydrogen peroxide level, malondialdehyde level and intracellular proline content.

Proline treatment was conducive to lowering the levels of hydrogen peroxide and malondialdehyde at stage S1. At the subsequent stages of development, the levels of hydrogen peroxide and malondialdehyde increased, and at the S3 stage, there was also a marked increase in total antioxidant capacity. At stage S3, the seedlings of the proline treatment were characterized by a lower total mass, and the response to exogenous proline was stronger in the root tissues than in the leaves. The oxygen consumption rate was higher for the proline treatment at all stages of development.

Seedlings at the analyzed stages of establishment differed in response to proline treatment. Exogenous proline had some beneficial effects during the first phase of germination by reducing the level of hydrogen peroxide and improving the condition of lipid membranes. In the subsequent stages of seedling development, in response to the same concentration of proline solution, undesirable effects, such as an increase in hydrogen peroxide levels and damage to cytoplasmic membranes, were observed. Optimal concentrations of exogenous proline should be determined prior to commercial use of proline treatment to improve plant stress tolerance.

**Keywords:** proline, seed treatment, Norway spruce, seedlings development, tree seeds, oxidative stress

**Addresses:** J. Kijowska-Oberc, M. K. Wawrzyniak, E. Ratajczak, Institute of Dendrology, Polish Academy of Sciences, Parkowa 5, 62-035 Kórnik, Poland, e-mail: joberc@man.poznan.pl;
JK-O https://orcid.org/0000-0001-6053-482X; MKW https://orcid.org/0000-0002-4297-5741;
ER https://orcid.org/0000-0003-2710-4638
A.M. Staszak, Laboratory of Plant Physiology, Department of Plant Biology and Ecology, Faculty of Biology, University of Bialystok, Ciolkowskiego 1J, 15-245 Bialystok, Poland;
https://orcid.org/0000-0002-7913-3716
*Corresponding author

## Introduction

The long-term effects of climate change are especially severe in tree species because they are long-lived organisms (Krejza et al., 2021). With climate change, drought frequency is expected to increase over the globe. Drought-induced stress causes an increase in tree mortality on an unprecedented scale. The adaptation of tree species to climate change and its consequences is one of the most important challenges facing ecosystems and forest management (Locatelli et al., 2010; Kijowska-Oberc et al.,, 2020; Malhi et al., 2020). The probability of seedling establishment and survival in unfavorable environmental conditions starts to shape from the beginning – development and maturation of the seed (Fang et al., 2017). However, according to global warming, we observe a modification in seed metabolism, e.g., in broadleaf species as the *Acer* level of proline increases (Kijowska-Oberc et al., 2020). Seed viability relies on the level of metabolic activity of cells that ensure the correct course of physiological processes in the seeds and can be expressed in the germination capacity. External factors may modulate germination and initial seedling growth and have a significant impact on the natural range limits of species and the establishment of populations (Solarik et al., 2018).

In seed physiology, reactive oxygen species (ROS) may play a role as signaling agents for different developmental processes (Wojtyla et al., 2016). Numerous metabolic processes in cells, such as photosynthetic and respiratory metabolism, as well as environmental responses and signaling, induce oxidation and reduction (redox) reactions; thus, the redox signaling network is believed to be a key player in controlling all biological responses. The redox signaling network detects every metabolic imbalance and forms an adaptation response to changing environmental conditions (Mittler et al., 2011). ROS are key molecules in redox signaling. In seeds, ROS are involved in cell growth regulation; therefore, they play important roles in seed germination and seedling growth (El-Maarouf-Bouteau & Bailly, 2008). However, unfavorable external conditions, such as extreme temperatures, water deficit, high concentrations of toxic metals, or high salinity, disturb redox homeostasis in cells, which results in the excessive accumulation of ROS and thus the initiation of oxidative stress. Excess ROS cause damage to the structure and functions of cell membranes by lipid peroxidation, which disturbs the permeability of membranes and decreases the osmotic potential of cells. A product of lipid peroxidation is malondialdehyde (MDA), often referred to as a marker of oxidative damage to cell membranes. Hydrogen peroxide ($H_2O_2$) is a stable, reactive molecule that easily diffuses through membranes and can be transported over long distances in the cell; therefore, $H_2O_2$ is believed to play a dual role in the physiological and developmental processes of the plant. The mutual relationship between the positive and negative roles of $H_2O_2$ in cells depends on the level of activity of the antioxidant system and the activity of the processes affected by this molecule (El-Maarouf-Bouteau & Bailly, 2008; Wojtyla et al., 2016). An increase in the level of $H_2O_2$ and other ROS is observed during seed hydration in the early stages of germination (Kranner et al., 2010; Kubala et al., 2015). $H_2O_2$ is also produced in the dry seed but acts as a signal or toxic molecule primarily when the seed is hydrated (Bailly, El-Maarouf-Bouteau & Corbineau, 2008). In hydrated seeds, there is an intensive increase in the production of superoxide anions and then $H_2O_2$ (El-Maarouf-Bouteau & Bailly, 2008).

To prevent stress conditions, plants have developed a mechanism of proline accumulation. This cyclic amino acid is an interesting research subject because it plays a significant role in the regulation of the antioxidant system, improving the stability and integrity of the proteins involved. Proline is an ROS scavenger that retains the redox balance of cells via ROS removal, which reduces oxidative damage (Kaul et al., 2008; Kaur et al., 2011; Liang et al., 2013). Moreover, proline acts as an osmolyte, preventing water loss from cells. Our preliminary research showed that proline content changes with thermal and precipitation conditions during seed maturation (Kijowska-Oberc et al., 2020); therefore, proline has been indicated as a promising biochemical indicator to examine oxidative changes that occur due to seed development and affect seed viability. Proline-primed seeds were observed to improve the germination capacity and increase resistance to stress in the seedlings developing from them (Karalija & Selović, 2018; Shafiq et al., 2018). The proline biosynthesis and catabolism cycle are involved in balancing the redox potential not only under stressful conditions but also during the process of plant development under normal conditions, i.e., during germination and initial growth of seedlings (Hare and Cress, 1997). Despite this information, the regulation of proline levels in response to ROS production during plant initial establishment is still not fully explained (Chaum et al.,, 2019; Bailly & Merendino, 2021).

Seed germination is a multiphase process that leads to changes in metabolism, membrane composition, mitochondrial activity, respiration, and chloroplast activity. It begins with the uptake of water and ends with the appearance of roots. Seeds of Norway spruce belong to the orthodox category (Roberts, 1973), because they can be desiccated to a low level of moisture content (3–5%) and stored at low temperature without damage (Suszka et al., 2005). In orthodox seeds in a dry state, cellular metabolism and respiration are significantly reduced. As a result,

the dry seeds retain a low level of metabolic activity, which allows them to remain viable for years (Buitink & Leprince, 2008; Leprince et al., 2017). Estimating the oxygen consumption rate (OCR) allows conclusions to be drawn about the ability to synthesize adenosine triphosphate (ATP) and about the function of mitochondria; thus, the OCR provides important insight into the metabolic activity and physiological state of plant tissues (Sew et al., 2013).

The proline metabolism mechanism and scavenging abilities are well known, and they have been studied in herbaceous species, such as *Arabidopsis* (Hare et al, 2003), wheat (*Triticum aestivum* L.) (Shafiq et al., 2018; Ambreen et al., 2021), mung bean (*Vigna radiata* (L.) R. Wilczek) (Posmyk & Janas, 2007), sweet corn (Wen et al., 2013), maize (*Zea mays* L.) (Karalija & Selović, 2018) or rice (*Oryza sativa* L.) (Singh et al., 2018), as is the case with proline seed priming (Kamran et al., 2009; Karalija and Selović, 2018). Meanwhile, the results of research on short-lived species do not reflect the mechanisms occurring in seeds and seedlings of long-lived orgasms, such as trees (Duangpan et al., 2018; Sigala et al., 2020). The complexity of the regulation of proline metabolism and numerous functions of this amino acid is the reason for the difficulties in improving plants of agronomic and forestry importance. Improvement of drought or salt tolerance of crop plants by engineering proline metabolism is an existing possibility and should be explored more extensively. Proline acting as a signaling molecule and influencing defense pathways and regulating complex metabolic and developmental processes offers additional opportunities for plant improvement.

Using species distribution modeling based on climate variables, a significant decrease in suitable habitat area was predicted for numerous forest-forming tree species, including Norway spruce (*Picea abies* (L.) H. Karst) (Dyderski et al., 2018). Recent studies of the adaptation of tree species to the climate in Europe have shown that Norway spruce is among the group of forest tree species that are more sensitive to climate change than others, e.g., silver fir (*Abies alba* Mill.), European beech (*Fagus sylvatica* L.) and Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) (Zang et al., 2014; Vitali, Büntgen & Bauhus, 2018; Buras & Menzel, 2019). Norway spruce contributes to the large-scale disturbances of forest ecosystems and severe economic losses for forest stands in this region. Despite these problems, Norway spruce is still considered one of the key forest tree species in Europe (Hlásny et al., 2017). Climate change, which affects the loss of seed viability, may have long-term consequences by reducing tree species dispersal and causing the acquisition of new habitats (Dyderski et al., 2018). Minimization of the risk of tree stand stability loss by identification of populations characterized by higher seed viability or by increasing seed viability, e.g., by exogenous seed treatment with proline, may be crucial for the continuity of forest ecosystems.

The present study concerns the effect of exogenous proline on seed quality, germination, and seedling development. For the first time, proline treatment for seeds of forest tree species was applied. To gain insight into the mechanism of seedling response to proline treatment during growth, this study investigated changes in the state of cytoplasmic membranes, oxygen consumption rate, total antioxidant capacity, and the levels of hydrogen peroxide and intracellular proline at different stages of seedling development.

# Methods

## Plant material

Seeds of Norway spruce were harvested in December 2020 from The "Zwierzyniec" Experimental Forest (near Kórnik, Seed Plantation of the 2nd Generation of the Istebna population) and were stored under controlled conditions (at −3 °C) until the start of the experiment. The seeds were divided into two experimental treatments: proline-treated, soaked in

Fig. 1 Seedlings placed on filter paper on the germinator

proline solution at a concentration of 8 mM; and water-treated, soaked in water. They were placed on filter paper soaked corresponding substances and placed in germinators at a cyclically alternating temperature 20~30 °C in intervals 16/8 h and in light-dark 12/12 h cycles (Fig. 1).

To determinate germination capacity and mean germination time, germinated seeds were counted every day; as germinated seeds were considered those whose radicles reached a length >3 mm (Bewley, Bradford and Hilhorst, 2012). For germination test, we used four replicates of 50 seeds in each experimental treatment. Germinating seeds were transferred to the sand-peat substrate in seedling pallets and placed at a constant temperature of 20 °C. Seedlings grown for 90 days under controlled conditions were considered as established.

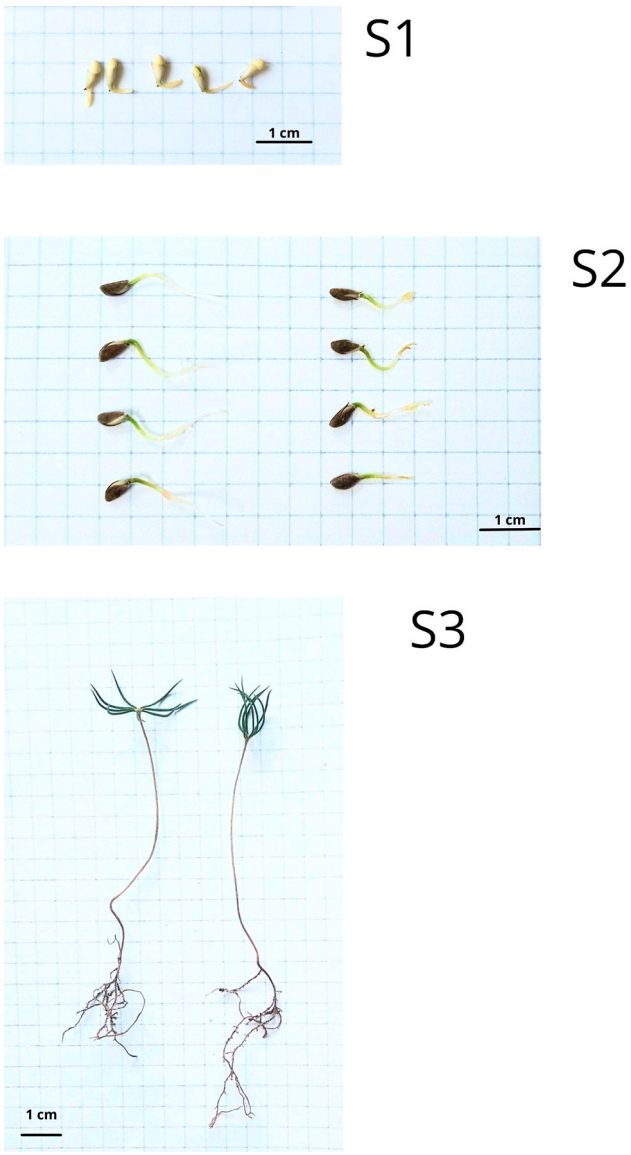


Fig. 2 Seedlings in subsequent stage of establishment

In order to analyze the successive stages of seedling development, we analyzed three development stages: (1) germinated seeds (radicle > 3 mm) – S1; (2) seedlings (radicle >10 mm) – S2; (3) established seedlings – S3 (Fig. 2).

For biochemical and morphological analysis, we used (1) ten pieces of S1 per sample in three biological replicates per treatment, (2) ten pieces of S2 per sample in three biological replicates per treatment, and (3) for S3 we used samples consisted separately needles and root systems from 3 seedlings, in three biological replicates per treatment.

## Determination of proline

Determination of proline concentrations was made according to a modified method of (Carillo & Gibon, 2011). Proline was extracted using a cold extraction procedure by mixing 10–50 mg fresh weight of tissue aliquots separately with 1–2 mL of ethanol: water (40:60 v/v). The resulting mixture was left overnight a 4 °C and then centrifuged at 14,000 × g for 5 minutes. Standards were prepared by diluting proline solutions ranging from 0.04 to 1 mM in the same medium as the one used for extraction. The reaction mix (1% (w/v) ninhydrin in 60% (v/v) acetic acid and 20% (v/v) ethanol was added to the sample aliquots and the standards and measurements were performed at 520 nm. Proline concentration was determined based on the standard curve.

## Determination of the state of cytoplasmic membranes

Determination of lipid peroxidation was carried out using Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich, MAK085) by measurements of malondialdehyde (MDA – the product of lipid peroxidation process) concentrations according to the manufacturer's instructions. Tissues (10–50 mg) were homogenized on ice in 300 $\mu$L of the MDA Lysis Buffer containing 3 $\mu$L of 3,5-Di-tert-4-butylhydroxytoluene (BHT) (100×). Samples were centrifuged at 13,000 × g for 10 minutes to remove insoluble material. Standards were prepared by diluting MDA Standard Solution in 0 (blank), 4, 8, 12, 16, and 20 nmole concentrations. Afterward, 600 $\mu$L of the thiobarbituric acid (TBA) solution were added into each vial containing 200 $\mu$L of standard and sample. All mixtures were incubated at 95 °C for 60 minutes and then cooled to room temperature in an ice bath for 10 minutes. 200 $\mu$L from each sample and standard were pipetted into a 96 well plate and measurements were performed at 532 nm microplate reader. The levels of MDA were determined based on a standard curve.

## Determination of hydrogen peroxide

Hydrogen peroxide ($H_2O_2$) levels were determined according to (Alexieva et al., 2001). The homogenate was centrifuged at 12 000 × g for 15 min and then 0.5 ml of the supernatant was added to 0.5 ml 10 mM potassium phosphate buffer (pH 7.0) and 1 ml 1 M KI. The absorbance of the supernatant was read at 390 nm. The levels of $H_2O_2$ were given on a standard curve prepared with known concentrations of $H_2O_2$.

## Measurement of total antioxidant capacity

Total antioxidant capacity (TAC) was determined by the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Molyneux, 2004). Tissues (20 mg) 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 $\mu$l of the extract was added to 180 $\mu$l of 120-$\mu$M 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 $[(A_0\text{-}A_s)/A_0] \times 100\%$, where $A_0$ is the absorbance of a blank, and $A_s$ is the absorbance of the sample.

## Measurement of oxygen consumption rate

Oxygen consumption rate (OCR) was determined using Agilent Seahorse XFp Analyzer. The assay ran for about 15 min. Intact seeds were placed in a plate, then wells were filled with 200 $\mu$l of seed respiration medium (5 mM $KH_2PO_4$, 10 mM TES, 10 mM NaCl, 2 mM $MgSO_4$, pH 7.2) and loaded into the plate reader Agilent Seahorse XFp Analyzer, after the calibration steps using the Bravo liquid handling station (Agilent Technologies). OCR was determined by 3 cycles of mixing (3 min), waiting (4 min), and measurement (5 min). The results were recorded by Seahorse XF Acquisition and Analysis software (Version 1.3; Seahorse Bioscience), and each well was normalized by the milligram weight of seeds used.

## Morphological traits of seedlings

To determine morphological traits of developed seedlings, plants of both experimental treatments were collected. Needles and roots were dried separately at 60 °C for 48 h. Subsequently, dry biomass was determined. Total plant mass was defined as the sum of dry matter of roots and needles. Leaf Mass Fraction (LMF) was defined as the ratio of needle dry mass to total dry mass. Root Mass Fraction (RMF) was defined as the ratio of root dry mass to total dry mass.

## Statistical analysis

Data were analyzed using R statistical computing software (R Core Team: The R Project for Statistical Computing, no date). Differences between treatments were measured using Student's t-test, each developmental stage separately. Test assumptions were checked using Shapiro-Wilk test (to assess normal distribution) and Levene's test (to assess the equality of variances).

# Results

There was no significant difference in germination capacity between the treatments of the experiment, and the germination capacity remained at the level of approx. 99%. The seeds germinated on average in 4.3 days (Table 1).

The level of proline content was higher in the proline treatment at all the stages of seeds (Fig. 3). The differences between experimental treatments were significant at stages S2 and S3, where an almost 2-fold increase in the proline content was observed. Interestingly, there was no significant difference in S1, which was the stage at which the treatment was applied.

Significant differences in $H_2O_2$ between treatments were observed at stages S1 and S3; however, at stage S1, the $H_2O_2$ level was higher with water treatment, in contrast to S3, where the $H_2O_2$ level was higher with proline treatment. At stage S2, the $H_2O_2$ level was higher with water treatment, but the difference was not significant.

The levels of MDA differed significantly between treatments at stages S1 and S2. At stage S1, the level of MDA was higher with proline treatment in contrast to stage S2. The MDA content generally increased during seedling development and was noticeably higher at stage S3 (reaching an average value of 17.5 nmol/mL) than at stages S1 and S2 (reaching values of 0.8 nmol/mL and 3.3 nmol/mL, respectively).

Table 1. Germination capacity and mean germination time of each applied treatment (water and proline). Mean±-standard deviation (SD)

| | Germination, % | Mean Germination Time, days |
|---|---|---|
| Water | 99.5±0.50 a | 4.1±0.03 a |
| Proline | 98.0±0.82 a | 4.3±0.07 a |

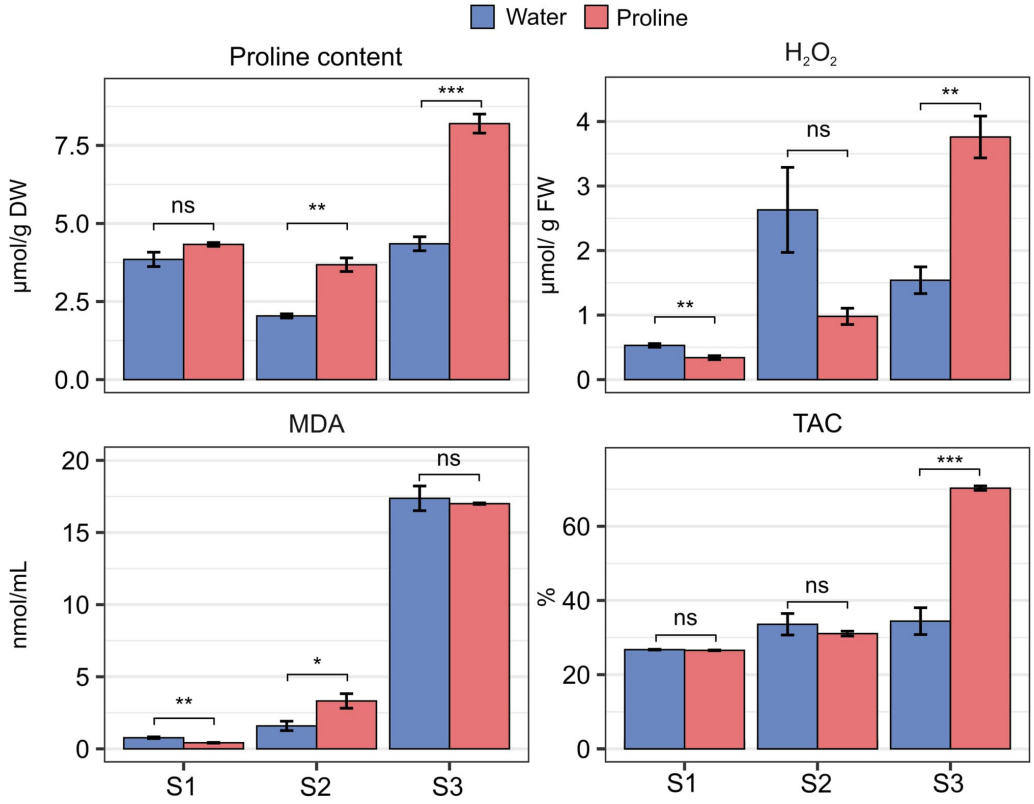


Fig. 3 Proline, $H_2O_2$ (hydrogen peroxide), MDA (malondialdehyde) content and TAC (total antioxidant capacity) of seedlings for each applied treatment (water and proline) in subsequent stages of development (S1, S2, S3). The results represent the means of three samples ± standard error of the mean (SE). Two-sample *t*-test; ns $p>0.05$; * $p\leq 0.05$; **: $p\leq 0.01$; *** $p\leq 0.001$

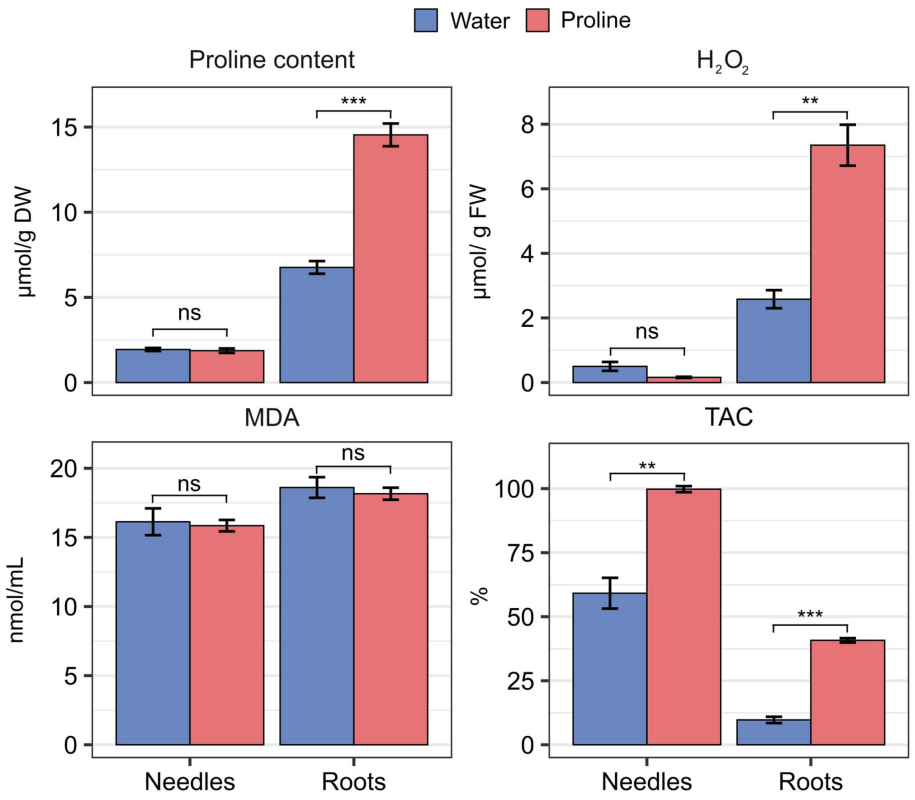


Fig. 4 Proline, $H_2O_2$ (hydrogen peroxide), MDA (malondialdehyde) content and TAC (total antioxidant capacity) of seedlings (S3) under water and proline treatment. The results represent the means of three samples ± SE two-sample *t*-test; ns $p>0.05$; *: $p\leq 0.05$; **: $p\leq 0.01$; ***: $p\leq 0.001$

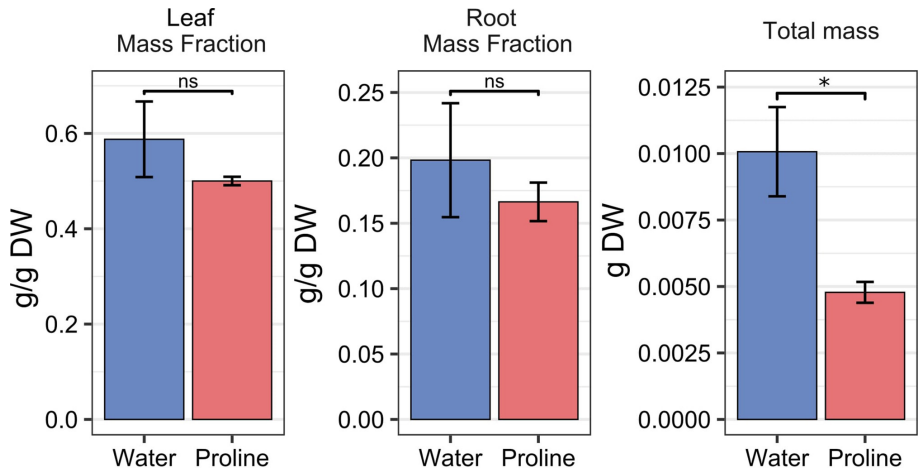


Fig. 5 Leaf mass fraction (LMF), root mass fraction (RMF) and total mass of established seedlings (S3) under the water and proline treatments. The results represent the means of five samples ± SE. Two-sample *t*-test; ns $p>0.05$; * $p\leq 0.05$; **: $p\leq 0.01$; *** $p\leq 0.001$

TAC (total antioxidant capacity) slightly increased during seedling development and did not significantly differ between experimental treatments, excluding stage S3, at which it was close to 2-fold higher (reaching 70%) with proline treatment.

At stage S3, all the biochemical analyses were performed separately on the needles and the roots of seedlings (Fig. 4). In the needles, the proline level was comparable between treatments, but in roots, the proline level was significantly higher with proline treatment than with water treatment, similar to the results of the $H_2O_2$ measurement. The levels of MDA did not differ significantly either between treatments or between parts of the seedlings, and they were only slightly higher in the roots. However, TAC was significantly higher with proline treatment in both needles and roots, and moreover, TAC was generally higher in needles.

The values of all the determined morphological traits of developed seedlings were higher with water treatment than with proline treatment (Fig. 5). Both the leaf mass fraction (LMF) and root mass fraction (RMF) did not significantly differ between the experimental treatments. The total plant mass was significantly higher in the case of seedlings in the water treatment (average 10 mg of DW) than in the proline treatment (average 4.8 mg of DW).

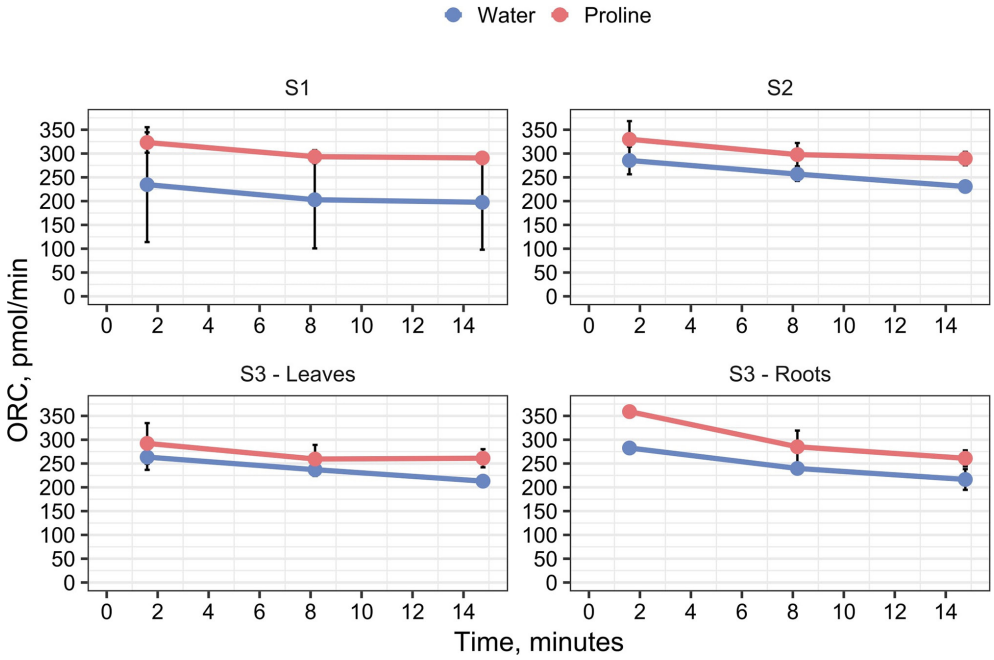


Fig. 6 The oxygen consumption rate levels of germinated seeds (S1) of water and proline treatment during the assay. Mean±SE

The OCR levels were higher with proline treatment than with water treatment and remained stable throughout the real-time analysis (Fig. 6). The OCR level was slightly higher in S2 than in S3 in the case of both treatments. Additionally, the OCR was also higher in root tissue of S3 than in leaf tissue, especially in the case of proline treatment.

## Discussion

Proline treatment did not affect germination. Both germination capacity and mean germination time were similar in both experimental treatments (Table 1). Analogously, proline content did not significantly differ between treatments at the first analyzed stage of plant development, S1 (Fig. 3, Fig. 4), interesting because S1 is the stage at which treatment with proline solution was applied, and despite this, proline treatment seems to have consequences on the other analyzed biochemical parameters and in the subsequent stages. Already after the start of visible germination (stage S1), differences in the levels of $H_2O_2$ and lipid peroxidation were noticeable depending on the applied treatment. Both MDA and $H_2O_2$ levels at this developmental stage were lower in the proline treatment than in the water treatment (Fig. 3). Such results indicate a proline stabilizing role in the protection of lipid structures that build the cell membrane or mitochondria. Proline scavenges free radicals, such as $H_2O_2$, that oxidize the components of the cell membranes, and thus, proline enables the maintenance of the proper functioning of cells under osmotic stress conditions. Seed hydration has been reported to induce a membrane leakage decrease; thus, the cell membrane is repaired after imbibition (Corbineau et al., 2002; El-Maarouf-Bouteau, 2022). By removing these strong reactive molecules, proline prevents the lipid peroxidation process, resulting in MDA formation, thus justifying the simultaneous decrease in the content of $H_2O_2$ and MDA in S1 with proline treatment. However, at stage S2, the MDA level increased during the proline level increase in proline-treated seedlings, which may indicate oxidative changes in the membranes. The increase in the level of $H_2O_2$ in S2 with water treatment may indicate more of the signal role of this molecule in germinating seeds (Finch-Savage & Leubner-Metzger, 2006; Xia et al., 2015; Wojtyla et al., 2016). Then, in S3, both $H_2O_2$ and MDA levels increased and were higher in proline-treated than in water-treated seedlings. The high level of MDA in stage S3 may be due to the high activity of lipids in young seedlings. Lipids, stored in seeds mainly as triacylglycerols (TAGs), are the most energetic form of reserves in seeds, and these reserves then support the development and growth of seedlings (Yang & Benning, 2018). Lipids are mobilized and oxidized in developing seedlings (Rajjou et al., 2012). Thus, numerous oxidation and reduction reactions take place, hence the probable increase in the MDA lipid peroxidation product. In addition, gray hair adapts to the prevailing environmental conditions by activating numerous signaling pathways and the molecules involved in them, including phospholipids. As signaling molecules, phospholipids modify the physicochemical properties of the membrane and activate the transport of many proteins necessary for plant growth (Cai et al., 2020); this modification may also influence oxidative changes in membranes and the growth of MDA. Thus, higher proline levels were no longer conducive to a decrease in the levels of $H_2O_2$ and MDA. High proline levels were observed in proline-treated S3; however, the higher proline levels no longer favored a decrease in $H_2O_2$ and MDA levels in seedlings. These observations show the changing characteristics of the optimum exogenous proline concentration with plant development.

Proline plays an important role in the reduction of oxidative stress by ROS removal, improving the stability and integrity of lipid membranes and proteins, which facilitates the capacity to withstand abiotic stress factors (Kumar et al., 2015; Peppino Margutti et al., 2017). The present study shows that exogenous proline performed this role at stage S1. Despite the beneficial impact of proline treatment, this amino acid has toxic effects, such as inhibition of growth and cellular metabolism, if applied at excessive concentrations (Ashraf & Foolad, 2007). The external supply of exogenous proline was found to be deleterious under certain conditions, causing growth retardation (Maggio et al., 2002; Nanjo et al., 2003; Yamada et al., 2005) or inhibiting germination (Hare et al., 2003). Exogenous proline has been shown to increase mitochondrial ROS levels in *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh.) (Hellmann et al., 2000; Miller et al., 2009). The adverse effect of exogenous proline has been postulated to be caused by the accumulation of pyrroline-5-carboxylate (P5C), the precursor of proline biosynthesis and an immediate degradation product (Verbruggen & Hermans, 2008; Szabados & Savoure, 2010). Proline toxicity may also be the result of gene repression related to plant metabolism functions, e.g., photosynthesis or synthesis of proteins responsible for building cell walls (Verbruggen & Hermans, 2008; Cha-um et al., 2019). The intracellular concentration of this amino acid is related to the balance of activity of the enzymes responsible for its metabolism, which are regulated by both stress and exogenous application of proline (Hare et al, 1999; Hayat et al., 2012).

The present study shows that proline treatment during seed germination and initial seedling growth initiates oxidative stress in subsequent phases of

seedling development, while a more evident reaction is seen in the roots than in the leaves of S3 (Fig. 4). Both proline and $H_2O_2$ levels were significantly higher in the roots of seedlings with proline treatment than with water treatment. This difference also applied to TAC, interestingly both in the roots and in the leaves. Additionally, TAC was higher in S3 than in the previous stages (Fig. 3), which indicates the intensified activity of nonprotein antioxidant system elements in response to increased $H_2O_2$ levels due to drought stress (Pisoschi et al., 2016). Proline has been reported to activate defense mechanisms by activating the stress-induced synthesis of polyphenols, glutathione, carotenoids, or flavonoids, characterized by a strong antioxidant effect (Shetty, 1997; Halliwell & Gutteridge, 2015). Analogous to TAC, MDA content showed no differences, but MDA content was significantly higher than in the previous developmental stages, which can be interpreted as an accumulation of oxidative damage to lipid membranes during development. The high level of $H_2O_2$ in proline-treated S3 indicates reduced activity of the enzymatic antioxidant system, especially peroxidase-type enzymes, which have a higher affinity for $H_2O_2$ reduction than low-molecular-weight glutathione or ascorbic acid antioxidants (Smirnoff & Arnaud, 2019). Although S3 was irrigated with distilled water only, its development, such as biochemical markers, was marked by the consequences of proline treatment in S1 and S2. Seedlings of the proline treatment were characterized by significantly lower total dry mass than the seedlings of the water treatment; however, the seedlings of the proline treatment did not differ in the LMF and RMF (Fig. 5). Moreover, the root tissues that were treated with the proline solution in earlier stages of the experiment proved to be more sensitive to exogenous proline activity than the leaf tissues. Exogenous proline application is used in studies, mainly on the protective role of proline in various environmental stresses, e.g., salt, drought, or heat (Liang et al., 2013). In addition to stressful conditions, proline is exploited by plant cells in developmental programs involving embryogenesis, cell division, elongation (Spollen et al., 2008), flowering, and germination (Thakur & Sharma, 2005; Mattioli et al., 2008). Proline is upregulated in seeds because it provides the plant with energy to sustain metabolically demanding plant propagation programs (Székely et al., 2008; Mattioli et al., 2009). Therefore, exogenous application of proline has been postulated to be able to effectively stimulate growth attributes (Trovato et al., 2019). Previously, proline short-term treatment has been tested to alleviate the effects of salt or drought stress and to stimulate the growth of herbaceous plants (Kamran et al., 2009; Nawaz et al., 2010). As further effects of proline short-term treatment increase in leaf length, sugar, and proline content, changes in antioxidant enzyme activities, and improvements in photosynthetic pigments were recorded (Posmyk & Janas, 2007; Karalija & Selović, 2018; Shafiq et al., 2018; Ambreen et al., 2021). In this study, proline seed treatment was applied for the first time to a tree species. Moreover, this is the first study to use long-term exposure of seeds to proline solution, and the results show that optimal concentrations of exogenous proline used for seed treatment may vary during plant growth. Proline solution at a concentration of 8 mM reduces lipid peroxidation in visible-germinating seeds (S1) and then becomes toxic, adversely affecting seedling tissues, especially the roots (S3). Some developmental phases of the plant are assumed to be more demanding for proline than the conditions of mild environmental stress (Trovato et al., 2019). Optimal concentrations of exogenously applied proline are dependent on species or genotype and developmental phase (Zouari et al., 2019). The proline biosynthesis and catabolism cycle is required for balancing redox potential in the face of stress factors, as well as for normal plant development (Hare & Cress, 1997). The toxic effect of exogenous proline is assumed to be linked to the activity of proline dehydrogenase (ProDH), the enzyme involved in proline catabolism, by excessive P5C accumulation (Verbruggen & Hermans, 2008; Miller et al., 2009; Szabados & Savoure, 2010). A noteworthy finding is the hypersensitivity of ProDH1 mutants of *Arabidopsis* to exogenous proline under nonstressed conditions (Nanjo et al., 2003b; Funck et al., 2010); however, this mechanism is still not fully explained.

The rate of water absorption by the seeds corresponds to three phases during which controlled physiological processes take place (El-Maarouf-Bouteau, 2022). Mitochondria in dry seeds are underdeveloped with low cristae numbers and low protein content. After seed imbition/hydration, i.e., under conditions favorable for germination, the activity of the mitochondrion increases, and thus the production of energy in the form of ATP occurs (Paszkiewicz et al., 2017). Activation of the mitochondrion enables not only the production of energy as ATP needed for elongation and growth but also the activation of reductors that determine redox regulation (Koch et al., 2021). The determination of the cellular respiration rate allows the assessment of the metabolic activity of plant tissues (Sew et al., 2013). A higher level of OCR in the proline treatment than in the water treatment at all stages of establishment indicates a higher energy demand of cells (Fig. 6). The increase in $H_2O_2$ levels observed in samples treated with proline may indicate that the transport of electrons in the respiratory chain may be abnormal, which may be associated with an increase in ROS accumulation (Eubel et al., 2004). The OCR level increased in S2 compared to S1 in the case of both applied treatments, which may be

a result of changes related to the growth processes of the seedling. Activation of the mitochondrion enables not only the increased action of reductors that determine redox regulation but also the production of energy as ATP needed for elongation and growth (Koch et al., 2021). The slightly higher OCR in S3 roots than in leaves is probably consistent with a higher energy demand in root tips, which is required for elongation (Sew et al., 2013). Additionally, exposure of root tissue to unfavorable conditions leads to an increased rate of root respiration and enhanced generation of ROS (Shugaeva et al., 2007; Farooq et al., 2012). We can therefore conclude that this effect is a consequence of treating the seedling with a proline solution having toxic properties, even though the treatment took place at an earlier stage of development.

ROS are naturally synthesized by plants as products of cellular oxidative metabolism, and they play key signaling roles in seed biological processes such as germination and seedling establishment (Oracz et al., 2009; Barba-Espín et al., 2011; Leymarie et al., 2012). These molecules play an important role in endosperm weakening, mobilization of seed reserves, protection against pathogens, and transmission of environmental signals during seed germination (Bailly, El-Maarouf-Bouteau & Corbineau, 2008). Better attention must be paid to the regulation of ROS production during plant establishment because in the context of seed germination and subsequent seedling development, ROS signaling specificity has not been considered (Bailly & Merendino, 2021). In reproductive tissues of *Arabidopsis*, such as florets, pollen, and seeds, proline represents up to 26% of the total amino acid pool, and in vegetative tissues, proline is only 1–3%. The noticeable increase in proline content observed in the plant reproductive tissues is similar to the increase recorded after many different types of stress, raising the question of whether proline function may be similar during both developmental processes and in response to stress.

## Conclusions

Exogenous application of proline to Norway spruce seeds and seedlings has the potential to improve the condition of lipid membranes, supporting the proper course of metabolic processes, such as respiration, and thus can effectively stimulate preliminary establishment attributes. Notwithstanding, the response to a certain concentration of proline changes with the growth of the plant. The treatment with proline solution of seeds and seedlings allowed us to show that although in the first days of visible germination proline treatment brought about beneficial effects in the form of lowering the level of $H_2O_2$ and improving the condition of lipid membranes, in the subsequent stages of seedling development, in response to exogenous proline, undesirable effects, such as an increase in the level of highly oxidizing $H_2O_2$ molecules and damage to cytoplasmic membranes, was observed. The use of seeds with viability improved by proline treatment for the production of seedlings intended for forestation or reforestation may reduce economic losses in maintained forests in the face of climate change and mitigate rapid changes in species diversity. However, optimal concentrations for exogenous proline treatment change during seedling development, and the concentration and timing of administrtion should be determined prior to commercial use of proline treatment to improve plant stress tolerance.

### Contribution

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

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