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Norway Spruce (*Picea abies* L.) Provenances Use Different Physiological Strategies to Cope with Water Deficit

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Abstract: *Research Highlights:* In this study, we found different regulatory mechanisms in two contrasting provenances of Norway spruce responding to progressive drought stress. *Background and Objectives:* In the context of climate and environmental changes, the following question of high importance in scientific studies is: How will Norway spruce, which forms a dominant component in European mountain and boreal forests, be able to cope with the increasing frequencies and intensities of drought periods? The aim of the study was to investigate the physiological responses of eight-month-old seedlings, as a most vulnerable phase of forest tree life, for different spruce provenances, to find out variable strategies in relation to controlled drought stress. *Materials and Methods:* We performed an experiment under controlled conditions with spruce seedlings from a climatologically warmer stand of 410 m above sea level (a.s.l.), and a moderately cool stand of 931 m a.s.l. The soil water content, leaf predawn water potential, the osmotic potential of needles, the relative water content of needles, and the photosynthetic performance and the contents of primary metabolites (proline and abscisic acid) were investigated as indicators of the spruce seedlings' responses to water stress. The status of the indicators was analyzed at different temporal intervals, using repeated measures analysis of variance (ANOVA), reflecting the seedlings' water conditions (early drought, drought, or recovery). On a daily basis, the differences among the indicators were tested with one-way ANOVA. A principal component analysis (PCA) was used to identify the provenance-specific responses of tree drought indicators in a multi-dimensional data set. *Results:* The responses to drought stress differed between the provenances. Whereas seedlings of 'warm' provenance from a low altitude performed a conservation strategy, with high amounts of accumulated abscisic acid and closing the stomata faster, the reactions of 'cool' provenance seedlings from a higher altitude were not so sensitive and the plants' water supply and photosynthetic performance remained significantly higher. These findings indicate that a higher drought resistance in 'cool' provenance could be related to greater amounts of proline amino-acids, which are accumulated from the beginning of the drought simulation. Furthermore, proline accumulation resulting in increased stress tolerance is controlled through another mechanism than osmotic adjustment. *Conclusions:* The observed variations in the regulatory mechanisms used to develop adaptive strategies in different provenances are an important factor for seedling survival under a changing climate.

Keywords: *Picea abies*; spruce provenances; water deficit; drought; physiological strategies; proline; ABA; photosynthetic performance; intraspecific variation; adaptive potential

1. Introduction

In terms of ongoing climate change, an increased frequency and intensity of drought periods has been predicted. Drought stress negatively influences a variety of physiological processes in trees. These processes are essential for plant growth and biomass production. Mountain forest ecosystems are considered to be extremely vulnerable [1–3]. Changes in temperature and precipitation result in changes in snow cover, the course and nature of winter rainfall, timing onset and end of growth, and dormancy. The increased winter temperatures lead to winter drying, especially in coniferous trees. Moreover, climate change threatens important mountain ecosystem services, including providing fresh water to lowland areas and supporting rich biodiversity heritage.

Increasing frequency, duration, and intensity of drought and heat stress may substantially affect the composition, structure, production and biogeography of forests in many regions, especially through a potential increase of tree mortality associated with climate-induced physiological stress and interactions with the other processes, such as disturbances (wind, wildfires) and insect-pest gradations [2].

Genetic resources of forest trees are considered a key factor for the survival and persistence of forest ecosystems. Therefore, the utilization of the available genetic variations in climate responses and the testing of alternative provenances suitable for future climatic conditions are considered important adaptation measures in forestry. Adaptation mechanisms have a major impact on the ability to adapt to ongoing changes in the environment throughout the whole life of the tree. Adaptive genetic variants are defined as phenotypic features of a tree species that have developed through local adaptation and are aimed at survival or an increase in fitness under a particular environment [4,5]. A high degree of variation in adaptive features and their strong associations with ecological variables indicate their primary importance for the development of adaptive strategies for forests under climate change, which is considered to be one of the major challenges for the 21st century [6].

Picea abies (L.) H. Karst is a dominant species in the boreal forest belt ranging from Scandinavia to the Ural Mountains, as well as in the montane regions of the temperate zone, which are characterized by relatively low temperatures and high amounts of precipitation [7]. In association with postglacial recolonization, its natural range can be subdivided into three main regions, as follows: Nordic–Baltic–Russian, Hercynian–Carpathian, and Alpine [8]. As the species is a valuable timber source, it has been planted in the warmer and drier regions throughout Europe, frequently far from its natural range, and it has become the most commercially important conifer in Europe. As a consequence of its shallow root system, spruce is poorly adapted to environments outside of its natural range and its productivity is limited by water accessibility [9]. The changes and developments of hydro-pedological conditions, which interfere with the forests outside their natural range, represent the increasing danger of the drought for the spruce [10].

However, whereas the physiological response of spruce to drought stress has been researched [11–13], only a few studies addressed biochemical responses in the functional traits of spruce provenances. After the exposure of plants to drought and heat stress, a number of biochemical and physiological changes are induced. A variety of protective mechanisms for the efficient use of available water have evolved in plants and these allow them to acclimate to these unfavorable environmental conditions for survival and growth [14,15].

One of the basic regulatory mechanisms that enables woody species to improve their response capacity and the adaptive potential to drought stress is the processes associated with the accumulation of certain specific substances. Here, abscisic acid (ABA) plays a specific key role that allows plants to respond sensitively to environmental changes. When water is scarce, plants produce the stress hormone ABA, which activates ion channels, thereby initiating the closing of the stomata to prevent the plant from withering within a matter of minutes [16]. The synthesis and accumulation of low-molecular-weight metabolites, known as compatible solutes, is a ubiquitous mechanism used for protection and osmotic adjustment in plants. Their main role is to increase the ability of the cells to retain water without affecting their normal metabolism. Proline is among the most common nitrogen-containing compatible compounds. It has been reported to be involved in the stabilization of proteins and cell structures, in redox-buffering and reactive oxygen scavenging, and it might serve to store and transfer reductants and nitrogen [17]. Many studies have noted higher proline contents in more drought-adapted varieties of wild or cultivated plants [18–20].

In our study, results from laboratory-conditioned experiments with spruce seedlings (*Picea abies* (L.) H. Karst.) of different provenances from the Western Carpathians were analyzed and discussed. Two provenances were chosen, one from "outside the natural range of Norway spruce" and another from "spruce optimum". Provenance trials provide an excellent tool to evaluate the adaptive potential of provenances to climate changes [21]. In connection with the naturally increasing continental climate in the west–east direction, forest-tree populations from Central and Eastern Europe are becoming a promising source of drought- and frost-resistant ecotypes [22,23].

Moreover, the development phase of seedlings is known to be the most vulnerable phase of forest tree life. Therefore, understanding the stress responses of the seedlings is crucial for predictions of forest tree growth and survival [24,25]. Research on adult crops often requires a long time period, which is necessary given the complexity of the simultaneous presence of many external factors, whereas experiments with young trees produce fair-quality results in over a short span of time, which are verifiable and significant for the application of the forestry of mature crops.

In the given context, the objectives of our study were to examine the effects of a simulated drought progress on photosynthesis in relation to biochemical attributes as the proportion of proline amino acid and ABA phytohormone in Norway spruce provenances. Our specific research questions were: (1) Are there certain differing physiological strategies that correspond to the adaptive potentials of two different Western-Carpathian provenances of *Picea abies*? and (2) Which provenance is more tolerant to water scarcity? Finally, we hypothesized that, during drought stress, the provenance originating from a higher altitude (over 900 m a.s.l.) where the climatic conditions are more extreme, will show enhanced regulatory mechanisms associated with the accumulation of specific biochemical substances that help to improve its response capacity and the adaptive potential to stress factors.

2. Materials and Methods

The experiment was performed in air-conditioned chambers at 65% relative humidity, at a temperature of 23 °C, and for a 12-hour day (with four high-pressure sodium lamps, simulating a bright sunny day) and 12-hour night regime. Eight-month-old seedlings from two Norway spruce provenances were tested. The characteristics of the studied provenances are stated in Table 1. Both provenances were divided into two groups of different treatments: (1) A control under fully irrigated conditions for 'warm' provenance ('warm' PV_C) and 'cool' provenance ('cool' PV_C), and (2) drought-stressed conditions for 'warm' provenance ('warm' PV_D), and 'cool' provenance ('cool' PV_ D). Both drought and control variants were represented by two plug trays containing 35 seedlings from each provenance. Seedlings in plugs were randomly distributed. The size of spruce seedlings was about 15 cm in average.

The control variants were irrigated daily to a constant weight (approximately 4900 g). For the drought variants, the experiment was designed within the three periods: early drought (23 July, 25 July, 27 July), drought (29 July and 30 July), and recovery (31 July, 1 August, 6 August). The water supply was interrupted over eight days to achieve gradual dehydration. We set the drought periods according to the course of the soil water regime. The early drought was defined as a period from the first day without irrigation to the state when the relative soil water content decreased to 50%. The period with the relative soil water content less than 50% was defined as drought. After eight days of dehydration, the seedlings were watered and their recovery was monitored at three time-points over the next seven days. The impact of the drought progression and seedling recovery was monitored by the predawn water and the osmotic potentials of the needles, the relative water contents of the needles, gas exchange measurements, and biochemical attributes, such as the concentrations of free proline and ABA. The measurements of all parameters were performed eight times during the experiment.

Table 1. Geographical and climatic descriptions of the studied provenances of Norway spruce. The climatic data were obtained from the high-resolution interpolated climate database WorldClim and represent the mean values of descriptors within the period of 1970–2000 at a resolution of 30″ seconds [26]. Different spruce provenances are signed as 'warm' PV ('warm' provenance from 'outside the natural range of Norway spruce') and 'cool' PV ('cool' provenance from 'spruce optimum').

Descriptors	'warm' PV	'cool' PV
Locality	Dubeň, Slovakia	Čierny Váh, Slovakia
Altitude	410 m a.s.l.	931 m a.s.l.
Latitude	49°12′	49°00′
Longitude	18°41′	19°58′
Climatic region	moderately humid and warm	humid and moderately cool
Mean annual precipitation	824 mm	937 mm
Mean precipitation of growing season	469 mm	557 mm
Mean annual air temperature	7.7 °C	4.9 °C
Mean temperature of growing season	15.4 °C	12.3 °C

2.1. Soil Water Content

The course of the soil water regime was monitored using a gravimetric method over the entire experiment. Each plug was weighed on a daily basis, using KERN FBK15KO.5A scales (Kern & Sohn GmbH, Balingen, Germany), with an accuracy of ±0.5 g. The relative soil water content (SWC_{rel}; %) was presented via the percentage of water per soil mass and calculated as:

$$\text{SWCrel}(\%) = \left(\frac{W_{sat} - W_{act}}{W_{sat}}\right) \times 100, \quad (1)$$

where W_{sat} is weight of fully saturated soil in grams and W_{act} is actual weight of soil in grams.

2.2. Predawn Water Potential and Osmotic Potential of Needles

The predawn water potential of the needles (Ψ_{pd}; MPa) was measured using a portable psychrometer, a PSYPRO (Wescor, South Logan, OH, USA) equipped with a C-30 sample chamber and a thermocouple, before the start of the daily light cycle. Measurements were recorded immediately after sampling. The middle parts of approximately 10 needles of the same branch from five seedlings per variant were inserted into the chamber and equilibrated for 60 minutes before measurement. The same procedure was applied for the determination of the needle osmotic potential (Ψ_S; MPa) except for the sample preparation process. In this case, the needles were freeze-dried after sampling and stored in liquid nitrogen until measurements were recorded.

2.3. Relative Water Content of Needles

The relative water content of the needles (RWC; %) was determined gravimetrically, according to González and González-Vilar [27]. Twenty needles for five seedlings per variant were randomly sampled and the fresh weight (FW; g) was measured. Then, the needles were saturated with distilled water at 4 °C for six hours, to attain full turgidity, and then weighed to determine the turgid fresh weight (TFW; g) of the needles. Finally, the needles were dried in a dry-air oven at 70 °C for 48 hours and re-weighed to obtain their dry weights (DW; g). The RWC was calculated as follows:

$$RWC(\%) = \frac{FW - DW}{TFW - DW} \times 100 \quad (2)$$

2.4. Free Proline Content

The free proline content (proline; μmol g^{-1} FW) was determined using a ninhydrin-based colorimetric method [28]. Exactly 0.5 g of freeze-dried needles (from five seedlings per variant) was homogenized with 10 mL of 3% sulfosalicylic acid and filtrated. Then, a mixture of 2 mL filtrate, 2 mL glacial acetic acid, and 2 mL acidic ninhydrin was incubated for one hour at 100 °C in a water bath. The reaction was terminated on ice and the reagent was mixed with 4 mL of toluene for 20 minutes. The absorbance of the extracted chromophore, at 520 nm, was determined with the toluene reference using a CINTRA spectrometer (GBC Scientific Equipment, Braeside, Victoria, Australia). Finally, the proline concentration was derived from a standard concentration curve and recalculated to the fresh weight of the sample.

2.5. Concentration of Abscisic Acid

The concentration of abscisic acid (ABA; pmol g^{-1} FW) was determined using two-dimensional (2D) high-performance liquid chromatography (HPLC) [29] at an accredited facility at the Institute of Experimental Botany of Czech Academy of Science (Prague, Czech Republic). Samples were taken from five seedlings for each variant, fixed, and transported to the facility in liquid nitrogen. An ABA quantification was conducted based on ultraviolet (UV) detection using a Diode Array Detector 235C (PerkinElmer, Waltham, MA, USA) and the results were verified by a comparison with gas chromatography-mass spectrometry.

2.6. Gas Exchange

The parameters of gas exchange were monitored by using a Li-6400XT open gasometric system, equipped with a chamber fitted with a 6400-02B LED light source (LI-COR Inc., Lincoln, NE, USA), for 10 seedlings per variant. The net photosynthetic rate (P_N; μmol $CO_2 \cdot m^{-2}\ s^{-1}$) and stomatal conductance (g_S; mol $H_2O \cdot m^{-2}\ s^{-1}$) were recorded after a short adaptation period under stable conditions. The CO_2 concentration inside the chamber was 390 μmol m^{-1}, the system temperature was 23 °C, and the radiation intensity used was 1200 μmol m^{-2}.

2.7. Data Analysis

A repeated measure analysis of variance (RM ANOVA) was applied to the provenances. For control variants ('warm' PV_C and 'cool' PV_C), the one-way RM ANOVA was applied to identify the changes in mean scores of tested parameters over the whole experiment separately for both provenances. For drought variants ('warm' PV_D and 'cool' PV_D), factorial RM ANOVA was used to determine the effects of sampling time and provenance origin on the Ψ_{pd}, the Ψ_S of needles, the free proline content, the concentration of ABA, the g_S, and the P_N. The data were analyzed on four different timescales, as follows: Timescale 1 covered measurements over the whole experimental timespan (15 days), timescale 2 covered the period of early drought, timescale 3 covered the period of drought, and timescale 4 corresponded to conditions of sufficient water supply and the recovery from the induced drought. Prior to the RM ANOVA analysis, Mauchly's sphericity test was performed and, if needed, a sphericity correction was applied to the RM ANOVA F-test values. The RM ANOVA was conducted using Statgraphics Centurion 18 software (Statgraphics Technologies, Inc., The Plains, VA, USA).

To explore P_N, g_S, proline content and the ABA concentration, a one-way ANOVA was applied on a daily basis. The equality of variances for individual observation dates were checked using Levene's test. To discriminate the means among the variants, Tukey's honest significant difference (HSD) procedure was applied. The Ψ_{pd}, Ψ_S, and RWC were also tested using one-way ANOVA, with the provenance as the factor. This was focused upon to determine the differences between the provenances under the control conditions (involving the whole experiment), at the end of early drought (27 July), drought (30 July) and recovery period (6 August). Prior to the test, the normality of data distribution was tested. In some cases, data transformation was considered necessary. RWC of 'warm' PV_C did not achieve normal distribution nor after transformation, therefore nonparametric Kruskal-Wallis test was used. For the analysis and plotting, R software was applied [30].

All the estimated parameters (P_N, g_S, proline, ABA, RWC, Ψ_{pd}, Ψ_S), except for soil weight, were subjected to principal component analysis (PCA) to identify the general trends of the multi-dimensional data sets. A PCA analysis was conducted using the FactoMineR package [31] and displayed as a PCA biplot, using the fviz_pca_biplot function from the R library factoextra. To satisfy the Barlett sphericity test and the Kaiser–Meyer–Olkin measure of sample adequacy test, we aggregated the values of two or three consecutive measurement days representing early drought, drought, and recovery.

3. Results

3.1. Course of Drought Stress and Recovery

The SWC_{rel} recorded regularly throughout the experiment, provided information about the progress of water stress (Supplementary Material 1). The water supply was restored when the needles started to fall down and the gas exchange parameters dropped to zero. After six days of regular watering, we observed 90% saturation of the soil.

The control variants of both provenances did not show significant changes of tested parameters over the whole experiment, except photosynthesis rate of 'cool' PV_C (Supplementary Material 2). Therefore, all measurements of control variants were merged and further presented in graphs together as reference/background values.

According to the factorial RM ANOVA (Table 2.), the 'warm' PV_D rate of photosynthesis was lower than that of 'cool' PV_D when considering the entire observation period. There were no significant differences between 'warm' PV_D and 'cool' PV_D under increasing drought conditions (early drought), but differences occurred within the drought period and during the recovery period. The same situation was observed for g_S. In all periods, the g_S of 'cool' PV_D was significantly higher, except for early drought. In the case of the biochemical parameters, in all four time periods, the concentration of ABA was significantly higher in 'warm' PV_D. The opposite situation was observed for the free proline content, where 'cool' PV_D had significantly higher values in all four time periods. There were no statistical differences between the provenances for the Ψ_{pd} and for the Ψ_S of the assimilatory organs. However, the differences in the RWCs of the needles were significant, except for recovery period. We consider this to indicate the presence of different metabolic regulatory mechanisms to cope with water deficit between the provenances.

Table 2. Discrimination of the means using RM ANOVA between the drought variants of two provenances ('warm' PV_D and 'cool' PV_D) for photosynthetic rate (P_N), stomatal conductance (g_S), abscisic acid (ABA) content, proline (Proline) content, osmotic potential (Ψ_S), predawn water potential (Ψ_{pd}), and relative water content (RWC) on four different timescales (w: whole experiment period, e-d: early drought, d: drought, r: recovery); statistically significant differences are noted with an asterisk as follows: $p > 0.05$ ns, * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. +/− Limits is a threshold value; if absolute value of Differ. 'warm' PV_ D-'cool' PV_D is greater than +/− Limits, there is a statistical significant difference between the provenances; if the value of Differ. 'warm' PV_ D-'cool' PV_D is lower than +/− Limits, there is no statistical significant difference between two provenances.

Source	Sum of Squares	F-Ratio	*p*-Value	Differ. 'warm' PV_ D-'cool' PV_D	+/− Limits
P_N w	10.34	6.71	0.024*	−0.61	0.51
P_N e-d	0.19	0.13	0.721^{ns}	0.13	0.79
P_N d	19.67	11.83	0.005**	−1.68	1.06
P_N r	4.25	6.54	0.025*	−0.64	0.54

Table 2. *Cont.*

Source	Sum of Squares	F-Ratio	p-Value	Differ. 'warm' PV_ D-'cool' PV_D	+/− Limits
g_S w	0.01	12.9	0.004**	−0.02	0.01
g_S e-d	0.01	0.17	0.684^{ns}	0	0.02
g_S d	0.02	34.96	0.000*	−0.05	0.02
g_S r	0	5.55	0.036*	−0.02	0.02
ABA w	6.08×10^7	40.16	0.000***	1591.73	559.64
ABA e-d	7×10^5	7.08	0.024*	285.03	238.71
ABA d	6.70×10^7	42.44	0.000***	3342.49	1143.15
ABA r	2.70×10^7	37.96	0.000***	1731.25	626.11
Proline w	173.7	86.7	0.000***	−2.69	0.64
Proline e-d	133.4	26.35	0.000***	−3.85	1.67
Proline d	77.05	20.22	0.001***	−3.58	1.78
Proline r	7.87	52.82	0.000***	−0.94	0.29
Ψ_S w	2.06	0	0.967^{ns}	0	0.05
Ψ_S e-d	0.07	4.64	0.057^{ns}	0.09	0.09
Ψ_S d	0.06	2.17	0.171^{ns}	−0.10	0.15
Ψ_S r	0	1.31	0.278^{ns}	−0.02	0.04
Ψ_{pd} w	0.48	4.03	0.072^{ns}	−0.14	0.16
Ψ_{pd} e-d	0.02	1.49	0.251^{ns}	−0.05	0.08
Ψ_{pd} d	0.36	4.66	0.056^{ns}	−0.24	0.25
Ψ_{pd} r	0.26	1.18	0.303^{ns}	−0.17	0.35
RWC w	5.08	16.65	0.002**	−0.46	0.25
RWC e-d	2.81	5.17	0.046*	−0.56	0.55
RWC d	7.3	28.42	0.000***	−1.10	0.46
RWC r	0.04	0.15	0.707^{ns}	0.07	0.39

3.2. Water Status

Considering the RWC-, Ψ_S- and Ψ_{pd} values from the whole experiment, the provenances in irrigation treatment ('warm' PV_C and 'cool' PV_C) slightly differed (Figure 1). We found higher RWCs and lower Ψ_S- and Ψ_{pd} values in the 'cool' provenance. The RWCs in the needles of both provenances were significantly reduced by drought, down to 88.8% of the initial values in 'warm' PV_D and to 90.3% in 'cool' PV_D. However, on the sixth day after re-watering, the RWCs increased to values that were comparable with the control (Figure 1). ANOVA showed that the selected spruce provenances did not significantly differ in their Ψ_S and Ψ_{pd} under the drought conditions. During early drought, 'warm' PV_D showed a greater decrease in Ψ_{pd} than 'cool' PV_D. On the eighth day (drought), these values were about −1.12 MPa for 'warm' PV_D and −0.87 MPa for 'cool' PV_D. Similarly, the Ψ_S was much deeper, deepening during progressive drought in 'warm' PV_D (from −0.73 MPa to −0.93 MPa), compared to 'cool' PV_D (from −0.77 MPa to −0.79 MPa) (Figure 1).

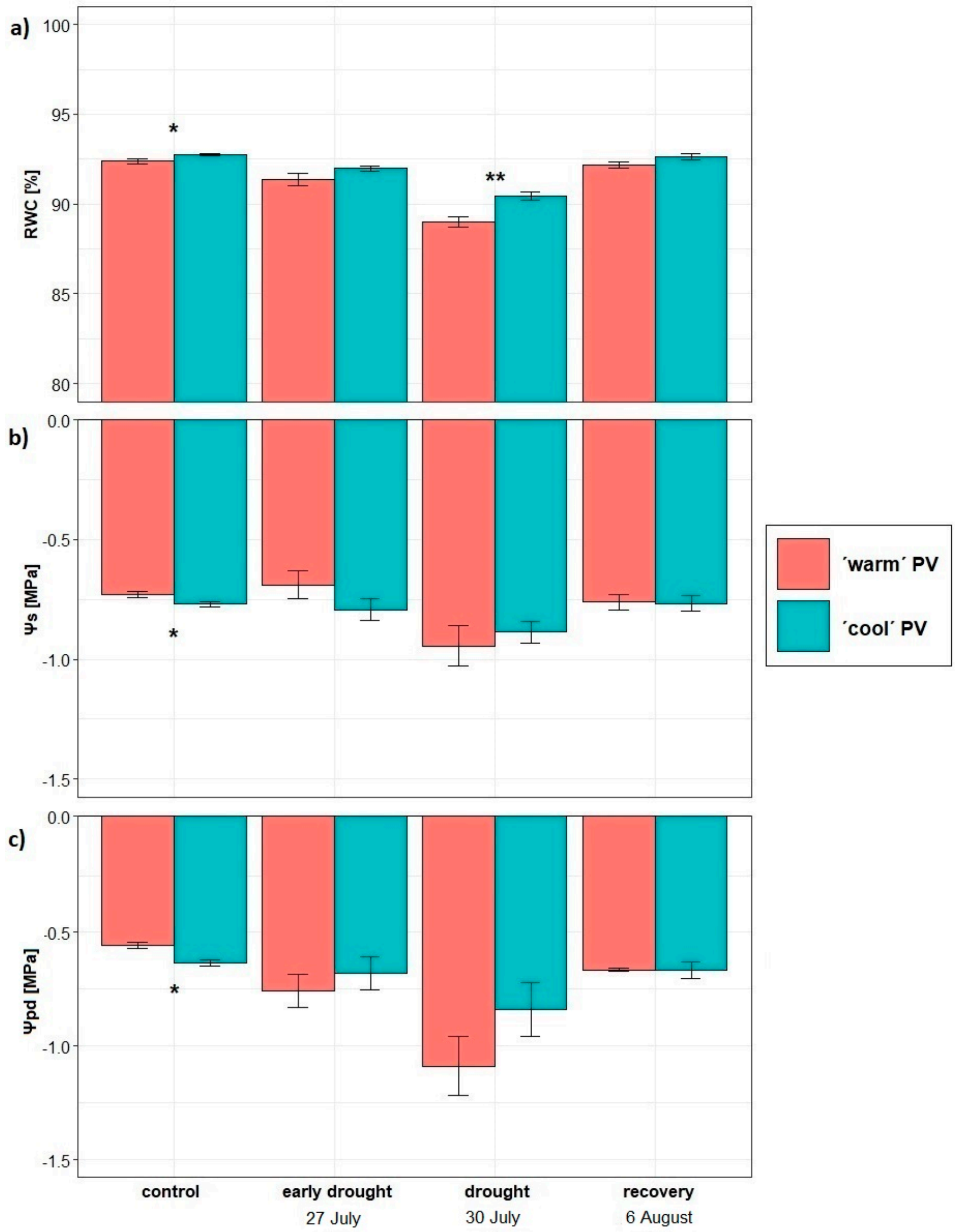

Figure 1. The provenance mean ± standard error of (**a**) RWC, (**b**) Ψ_{pd}, and (**c**) Ψ_S in the control condition, at the end of early drought (27 July), drought (30 July), and in the recovery period (6 August). Asterix marks the significant difference between provenances. Provenances are identified by different colors: 'warm' PV (red), 'cool' PV (blue).

3.3. Gas Exchange Parameters

The gas exchange parameters were similar under the control conditions in different provenances. The mean values of the P_N and the g_S fluctuated at around the 5.77 µmol CO_2 m^{-2} s^{-1} and 0.128 mol H_2O m^{-2} s^{-1}, respectively, for both provenances (Figure 2).

The dehydration course corresponded well with the course of the gas exchange traits. The P_N and the g_S decreased gradually with the deepening water deficit. A faster onset of drought was recorded in 'warm' PV_D (Figure 2). A significant decrease in the P_N and the g_S was recorded after seven days without a water supply. 'Cool' PV_D was characterized by a gradual reduction in gas exchange, whereas a significant decline occurred at the last date point of dehydration. Before re-watering, 'warm'

PV_D showed a decline in the P_N and in total g_S, by more than 82% and 83%, respectively, whereas the 'cool' PV_D showed the decrease by less than 60% for both parameters, compared to the control values.

After the restoration of the water supply, the gas exchange did not react immediately. We observed a gradual recovery. Both provenances showed values of g_S and P_N equal to the initial values after a six-day recovery.

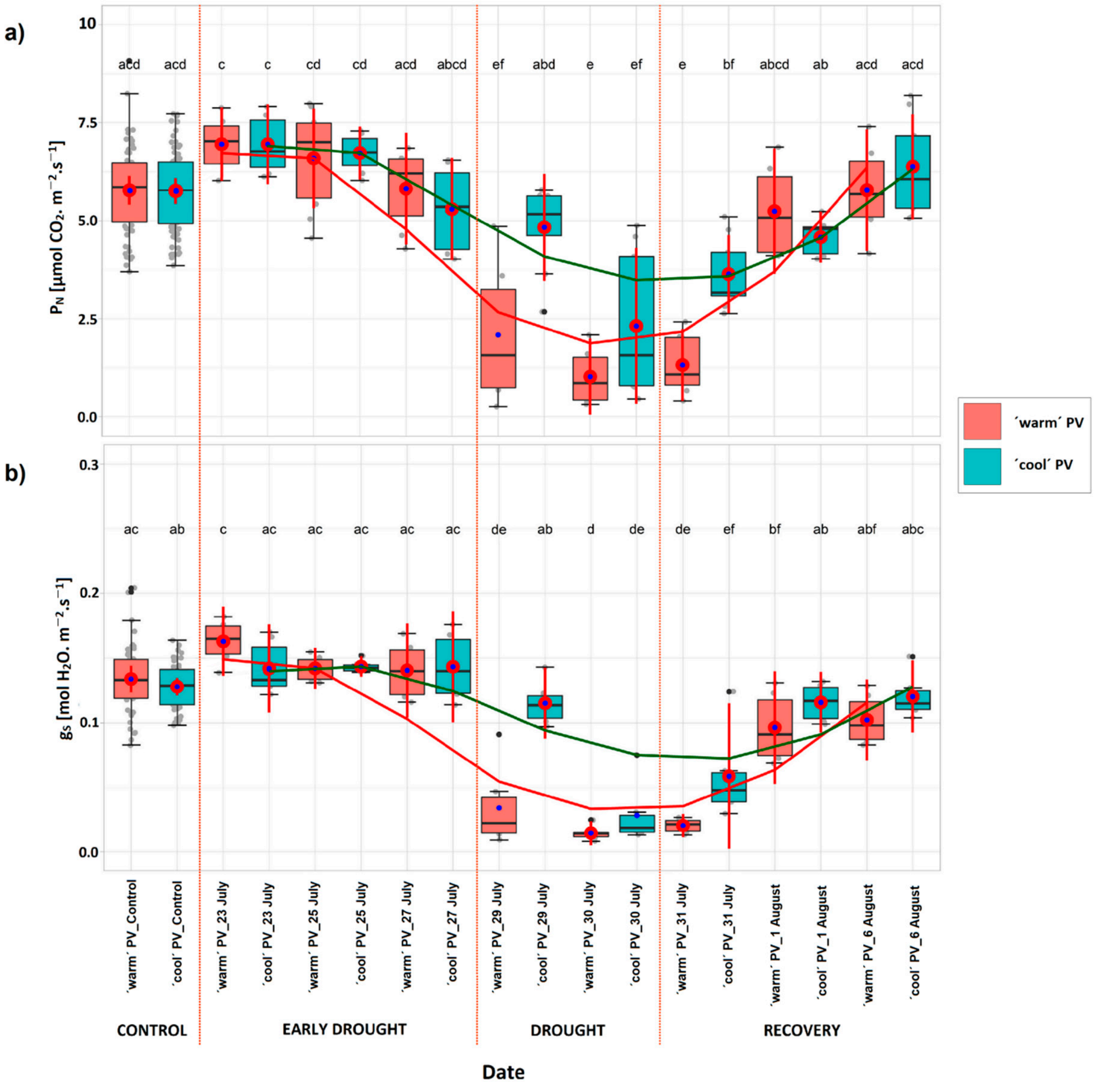

Figure 2. The courses of (**a**) the P_N, and (**b**) g_S during dehydration and recovery. Control variants represent the control values from the whole experiment. The graph's bars with provenance means ± confidence interval (±95%). Different letters indicate statistically significant differences among groups. Provenances are identified by different colors: 'warm' PV (red), 'cool' PV (blue).

3.4. Biochemical Parameters

In the evaluation of the biochemical parameters that are closely related to photosynthesis, we observed differences between the provenances under the control conditions (Figure 3). 'Warm' PV_C showed more than a 2.4-fold higher content of ABA compared to 'cool' PV_C (676.3 pmol g^{-1} and 274.2 pmol g^{-1}, respectively), whereas 'cool' PV_C showed a 3.4-fold higher content of accumulated proline compared to 'warm' PV_C (1.797 µmol g^{-1} and 0.528 µmol g^{-1}, respectively). However, these differences were not projected into the differences in gas exchange traits under optimal conditions. The synthesis of ABA and proline during drought differed significantly between the tested provenances. Whereas 'warm' PV_D supported the synthesis of ABA and the proline content increased only slightly, 'cool' PV_D accumulated proline instead of ABA. The levels of free proline began to increase earlier than ABA. The proline concentration increased three times during the first day of dehydration and it remained at the same level during the drought duration in 'cool' PV_D. Immediately after the restoration of the water supply, proline decreased to the same values as in the control variant. The proline concentration of 'warm' PV_D remained quite stable during the whole experiment.

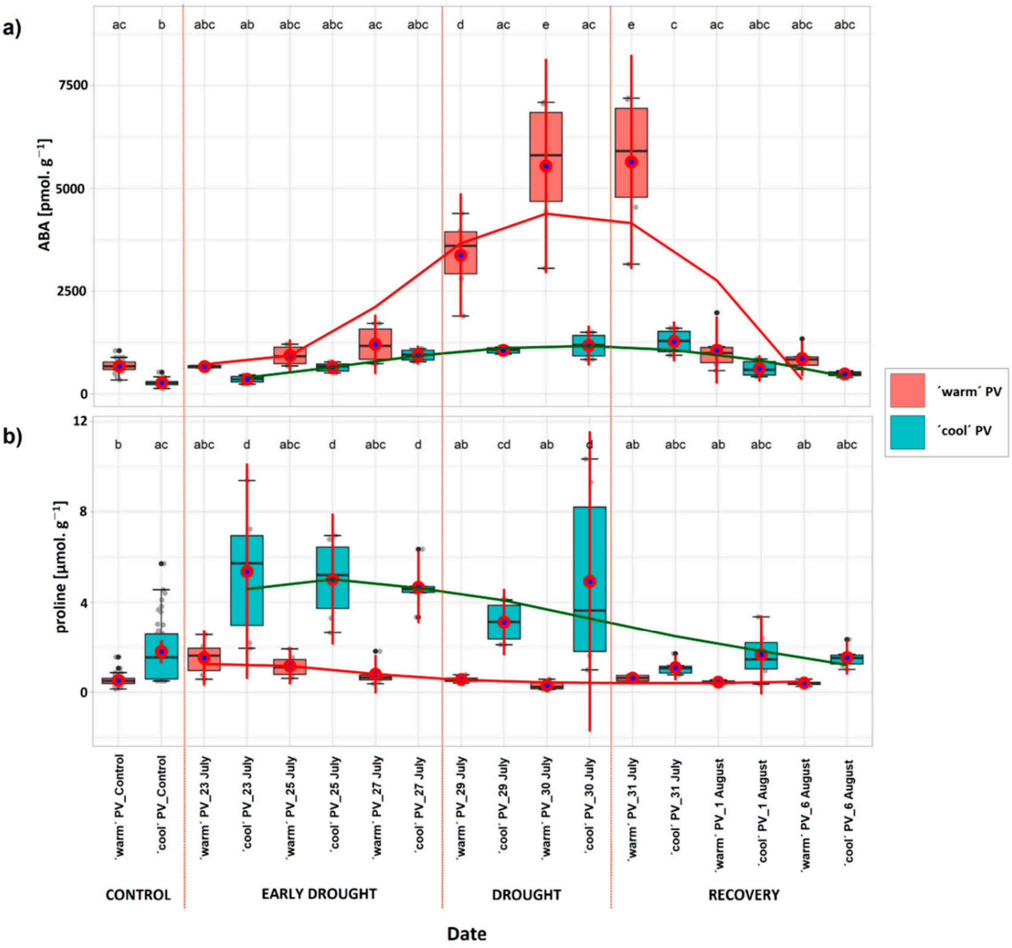

Figure 3. The courses of (**a**) the ABA concentration, and (**b**) proline concentration during dehydration and recovery. Control variants represent the control values from the whole experiment. The graph's bars with provenance means ± confidence interval (±95%). Different letters indicate statistically significant differences among groups. Provenances are identified by different color: 'warm' PV (red), 'cool' PV (blue).

The opposite behavior was recorded for ABA, which rapidly increased on the seventh day of dehydration and, after the second day of recovery, it decreased to normal values (Figure 3a). This corresponded well with the results under the control conditions, where 'warm' PV_C had already accumulated a significantly higher concentration of ABA, whereas 'cool' PV_C accumulated a higher amount of proline. This may likely allow for the more efficient synthesis of ABA or proline under future water deficits.

According to the PCAs (Figure 4), all variants largely overlapped under early-drought conditions. However, two groups were slightly distinct, as follows: 'warm' PV_D, following the ABA direction, and 'cool' PV_D, following the proline direction. With increasing drought intensity, the drought variants of both provenances started to separate further. 'Warm' PV_D followed the ABA direction and 'cool' PV_D followed the proline direction. This corresponded well with the measurements under the control conditions (Figure 3a,b), where, as mentioned above, 'warm' PV_C had already accumulated a significantly higher amount of ABA and 'cool' PV_C had accumulated a higher amount of proline. After re-watering, the variants started to overlap again, and thus, their performance started to stabilize.

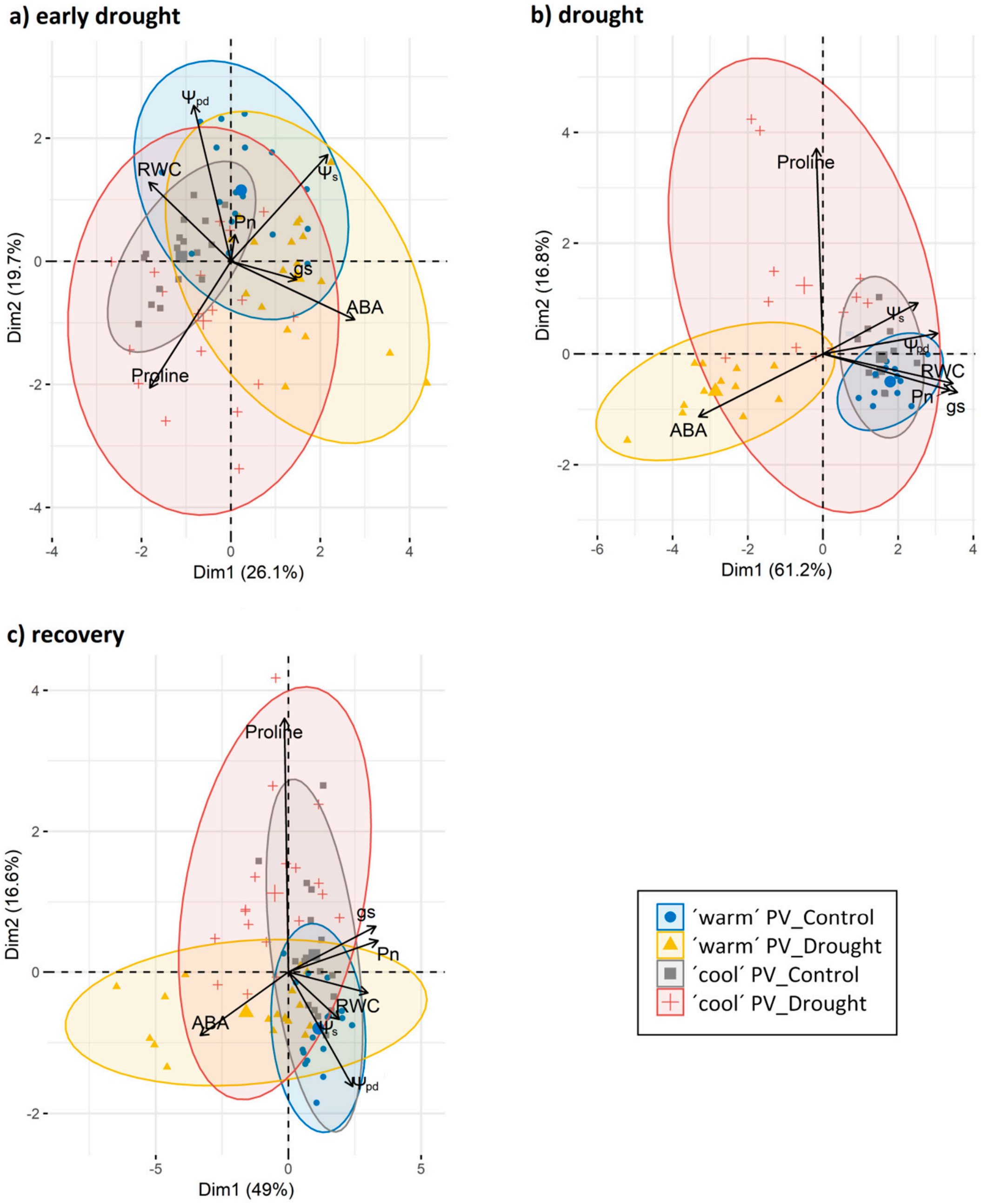

Figure 4. Principal component analyses for (**a**) early-drought conditions, (**b**) drought, and (**c**) the recovery period. The ellipses represent 95% confidence intervals around the centroid (bigger symbol) of each data cluster. The percentage on the axes indicates the proportion of the variance that is explained by the given principal component.

4. Discussion

The adaptive potential of the different spruce phenotypes (varieties) seems to be key information for nursery management [32], breeding, and development programs in forestry [33]. This experiment has proven the sensitivity of the physiological reactions of young tree seedlings to a water deficit. We required only eight days to simulate a relatively strong degree of stress for the spruce seedlings. The same studies with four-year-old spruce saplings lasted 43 days to reach the level of serious drought stress [34]. Similarly, Haas et al. [35] stated that the photosynthetic rates and stomatal conductance of spruce seedlings reached their minimums after 18 days of drought stress and, after an additional two days, deaths in seedlings occurred. Hence, spruce seedlings are extremely sensitive to drought and one day without water availability may be a critical factor determining death or survival [36]. We decided to interrupt the drought simulation when the water content per soil mass was reduced by an over 40% and the gas exchange parameters were reduced to minimal values, especially for 'warm' provenance.

Plant species have different strategies for coping with water stress, including avoidance or tolerance. A common approach classifies species as isohydric or anisohydric depending on how they regulate leaf water potential [37–39]. In contrast to mainly ABA-driven stomatal regulation in angiosperms, the regulation of gas exchange processes in gymnosperms relies on different combinations of active ABA-driven and passive hydraulic stomatal regulation [38,39]. More isohydric species use permanently high ABA levels to close stomata and maintain leaf water homeostasis during drought, while more anisohydric species rely on leaf desiccation to passively drive stomatal closure. *Picea abies* is a rather isohydric species [13,38], with a conservative-strategy in drought responses.

We have shown that the seedlings of different spruce provenances have contrasting behaviour strategies under water deficit. The similar results for divergent drought resistance mechanisms were found among the beech provenances [40,41] and among the grapevine (*Vitis vinifera*) genotypes [42]. Differences in ABA dynamics under water deficit may result in differences in adaptation strategies even within the same species [43,44]. However, differences in minimum (midday) leaf water potential and xylem cavitation among spruce provenances under drought are needed to prove if the considered intra-species isohydric/anisohydric behaviour in spruce seedlings.

Our results indicate the different regulatory mechanisms associated with the accumulation of specific biochemical substances. Similarly, studies of metabolic and physiological bases of plant tolerance and hardening against drought in *Pinus radiata* confirmed that hardening is not only species-dependent but also an intraspecific process controlled through metabolite changes [44]. Survival rate, concentration of several metabolites such as free soluble aminoacids and polyamines, and main plant hormones varied among different breeds after drought hardening, while relative growth ratio and water potential at both predawn and dawn did not.

Our results also showed that 'warm' PV from the lower altitudes, in warmer and drier regions, contained high ABA concentrations in needles, which also supported a greater and faster stomatal response, indicating that the spruce phenotypes that were located at these lower altitudes used a much faster stress signaling strategy for stomatal closure to avoid damage to the assimilation apparatus. Although the values of the Ψ_S- and Ψ_{pd} potentials did not differ between provenances, markedly lower ABA contents and the lower decline in the RWC in the needles of 'cool' PV indicated that the drought stress levels were significantly lower in the provenance originating from a higher altitude. Proline, which accumulates in the early stages of water deficit, may contribute to osmotic adjustments, reducing water losses from dehydrated cells. As the osmotic adjustment represents a whole-plant response, decreasing the water potentials of all of the key tissues from roots to shoots [45], there are also possible positive effects of enhanced proline metabolism on the maintenance of root growth and water extraction [46], which may contribute to a higher plant and leaf water content and the maintenance of g_S, which we observed in 'cool' PV from 'spruce optimum'. Proline accumulation and the expression of proline metabolism genes in plants seem to be regulated by both ABA-dependent and -independent signaling pathways [47], but in a largely ABA-independent manner, and the use of proline as a marker is likely to identify different genes, compared to approaches that are focused on ABA signaling [48]. Haas et al. [35] stated that Norway spruce reduces its stomatal conductance over a narrow range of needle water potential (between −1.1 and −1.8 MPa). However, the observed transcriptional responses of a large proportion of Norway spruce orthologs suggests the divergence of function, particularly so for genes within the ABA-dependent drought response pathway.

The accumulation of proline under stress conditions in many woody species has been correlated with stress tolerance and its concentration has been shown to be generally higher in stress-tolerant than in stress-sensitive plants [20,48,49]. Proline accumulation also occurs, to a significant extent, under mild and moderate stress treatments [50]. The question of whether or not proline accumulation is an adaptive response to abiotic stress has been widely discussed. Advances in molecular data have shown an extensive degree of proline metabolism regulation by abiotic stress, especially at the transcriptional level, and it is firmly established that stress-induced proline accumulation is an adaptive response. The challenge remains in answering the question of how proline contributes to plant stress resistance [48].

The source of glutamate for proline synthesis may vary with the nitrogen source and the stress conditions and it can be a limiting factor in proline synthesis [51]. The balance between proline biosynthesis and its degradation is suggested to be important for determining its osmoprotective and developmental roles [52]. Beech trees from a mesic climate, where drought events are rare, tolerate a moderate decline in their water status, but they activate other metabolic protection measures, the production and maintenance of which may be more costly [41].

Hare and Cress [53] proposed the mechanism in which the proline synthesized by photosynthetically active tissues may be transported via the phloem to tissues with high energy needs, especially meristem tissues, where proline degradation may contribute to the reduced nicotinamide adenine dinucleotide (NADH) production, supporting the tissues' energy needs. The Δ^1-pyrroline-5-carboxylate or glutamine products generated by proline may be translocated back to the leaves where conversion back to proline generates nicotinamide adenine dinucleotide phosphate ($NADP^+$), which contributes to the balancing of the $NADP^+$/NADPH ratio, and, hence, the restoration of the terminal electron acceptor to the photosynthetic electron transport chain. The movement of proline in the phloem was previously shown across different plant species [45,54], and proline molecular transporters are well-known [55,56]. Spolen et al. [57] indicated that proline might be generally used by the plant cell as an energy source in developmental processes involving rapid cell growth.

As the activity of growing meristems is known to be sensitive to stress, early drought may be associated with a decrease in the need for extra energy from proline degradation, leading to some accumulation of proline in the tissues of the photosynthetic apparatus in the first few days of stress. This increase was evident in 'warm' PV, but in 'cool' PV it was much more obvious. This difference may be associated with the plant altitude of origin. Beech trees originating from higher altitudes were shown to be characterized by a higher capacity for alternative photosynthetic electron pathways, compared to those from a low altitude [21]. The differences between the provenances may be associated with differences in photoprotective responses, as the plants of 'cool' PV originated from a higher altitude and a colder location in which more efficient photoprotection is needed. As proline metabolism is (via the $NADP^+$/NADPH ratio or redox signaling) tightly coupled with photosynthetic electron transport [53], an enhanced level of proline synthesis may contribute to the capacities of alternative electron acceptors, decreasing the risk of reactive oxygen species (ROS) overproduction by the photosynthetic apparatus in an adverse environment.

An increase in proline accumulation over a period of more severe drought stress was not observed in spruce in our experiment. An increase in the ABA content in 'warm' PV was not associated with a corresponding increase of proline in the samples. Hence, the ABA-mediated proline synthesis in drought stress conditions observed in many species [58,59] does not contribute significantly to osmotic adjustment in the two spruce provenances in the later phases of drought stress. Conversely, some decreases in proline content can support the possible role of proline as a source of carbon and nitrogen [60].

Similarly, the role of proline in two contrasted species of Mediterranean shrubs seems to be more closely related to a protective action in cases of severe stress conditions, rather than as an osmotic agent with an osmotic potential-depressing activity [61]. In *Sesbania sesban*, proline fully alleviated the salt stress-induced enhancement of ribulose-1,5-bisphosphate oxygenase activity, suggesting that proline accumulation in plants under stress must assist plants with maintaining photosynthetic efficiency and the overall productivity [62]. Thus, our findings suggest that the enhanced proline metabolism observed in plants of 'cool'PV originating from high altitudes contributes mostly to photoprotection and the stabilization of photosynthesis under adverse environmental conditions, which, in addition to a minor osmoprotective and osmoregulatory function of the proline molecules, may contribute to a higher level of drought stress tolerance in the seedlings.

5. Conclusions

Despite the limitation in the scale of the studied material (two Western-Carpathian provenances), this experiment showed that the different spruce provenances follow different strategies for coping with a water deficit. The synthesis and accumulation of free proline during drought seems to be more efficient for the remaining level of photosynthetic performance. This suggests that the provenances from humid regions and higher altitudes are better able to cope with the predicted climate changes and they have the capacity to plastically adjust, although some tree individuals may become more vulnerable to extreme events following this strategy. It seems that the adverse mountain environment has given rise a riskier strategy with the activation of other metabolic protection measures in this 'cool' PV. The 'warm' PV from lower altitudes, where drought events are frequent, clearly displayed a drought-avoidance strategy, with high ABA-accumulated content in the needles and a greater and faster stomatal response.

The spruce genome has only recently been described and a number of Norway spruce-specific genes have been found to be under active regulation in response to drought, though this is still uncharacterized. Eco-physiological research is an important supportive base for genetic studies, because the environment and the changes in the environment are significant factors for the expression (or non-expression) of certain genes that are responsible for adaptation.

Thus, our results can be useful for the development of forestry phenotyping tools and may serve as a guide for future studies, including the linking of physiology and genetics methods for real understanding of the mechanisms of climatic adaptation in trees.

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