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Freezing and Heating Tolerance of *Pinus nigra* Seedlings from Three South to North Balkan Provenances

Vladan Ivetić [1,*], Marianthi Tsakaldimi [2], Petros Ganatsas [2], Ivona Kerkez Janković [1] and Jovana Devetaković [1]

[1] Faculty of Forestry, University of Belgrade, 11030 Beograd, Serbia; ivonakerkez@gmail.com (I.K.J.); jovana.devetakovic@sfb.bg.ac.rs (J.D.)
[2] Lab of Silviculture, Department of Forestry and Natural Environment, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; marian@for.auth.gr (M.T.); pgana@for.auth.gr (P.G.)
* Correspondence: vladan.ivetic@sfb.bg.ac.rs; Tel.: +381-11-3053873

Abstract: To meet the restoration and reforestation goals in the changing environment, the translocation of genotypes and species northward and upward need to be considered to a great extent. *Pinus nigra* is a genetically diverse, drought sensitive species, with cold hardiness comparable to other tree species under the same climatic conditions. This study tested frost hardiness (whole plant freezing test—WPFT, and electric conductivity—EC test), and heat tolerance (heat tolerance test) of *P. nigra* seedlings from two southern Greek provenances (Kalamata and Grevena) and one northern Serbian provenance (Šargan) to better understand the potential of seed transfer from the south to the north of the species distribution in the Balkan peninsula. The results showed that, that for all studied provenances, the damage was great; the index of injury (Ii) at −18 °C was ranged from 49 to 54.5 (measured by the EC method) and the percentage of injured tissues ranged from 80–90% (measured by visual observation). For all studied provenances, a sharp increase in damages was observed with the fall of temperature from −5 and −18 °C and the time after exposure. The WPFT results showed that the highest tolerance to freezing (−18 °C) was presented by seedlings from the northern (Šargan) provenance; however, no significant differences were statistically detected among the studied provenances. The heat and drought-treated seedlings, from both provenances, presented significantly highler foliar damages than only drought-treated ones. For seedlings from both contrasting provenances (Grevena and Šargan), exposure to moderate heat (45 °C) and short drought did present damages but without significant difference between them. Considering freezing and heating tolerance, Greek provenances of *P. nigra* (i.e., Grevena region) can be successfully used in Serbian forestation and restoration programs. The present study makes a contribution towards *P. nigra* reforestation with practical implications for abiotic stress (frost, heat drought) tolerance among southern and northern provenances and could be valuable to determine the suitable provenances for reforestation programs and assisted population migration under climatic change scenarios.

Keywords: frost hardiness; global warming; drought; climate adaptive reforestation; seed source; Austrian pine

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1. Introduction

Climate adaptive forest restoration and reforestation rely, to a great extent, on the possibility of translocation of genotypes and species northward (for the northern hemisphere) and upward (i.e., assisted migration). For European areas, one of the candidate forest species for introduction from warmer and drier climates to the north, is the (sub-) Mediterranean *Pinus nigra* Arnold. Some already observed natural trends support this claim. For example, an expansion of *P. nigra* above the timberline has been recorded in the central Apennines, Italy, during the last decades, explained by reduced livestock grazing as well as due to global warming [1]. In addition, and opposite to the general opinion that *P. nigra* is a less tolerant species to cold compared to *Pinus sylvestris*, Strimbeck et al. [2]

and Climent et al. [3] reported a similar injury of the two species due to frost up to −18 °C. In addition, Kreyling et al. [4] reported that cold hardiness of the juveniles of the (sub-) Mediterranean *P. nigra* reached similar levels to the cold hardiness of tree species native to Central Europe, such as *P. sylvestris*, *Picea abies*, *Fagus sylvatica*, and *Quercus petraea*, under the same climatic conditions.

The success of migration from southern and lower provenance to colder sites depends on plant ability to survive and establish in conditions different from those they are adapted for. Among other environmental factors, tolerance to extreme temperatures will play a significant role. *P. nigra* populations are reported to have a negative reaction in both minimum and maximum temperatures [5]. Due to differences in adaptive capacity driven by the high genetic diversity of *P. nigra*, it is necessary to assess the response of different provenances to climatic extremes [6]. In Southeast European countries, foresters need to "look over the fence" in search of the best provenance to match with their local sites, especially for those species with disjunctive ranges [7]. Despite the potential of some silvicultural adaptation measures to improve the planted forest performance (e.g., selective thinning which can reduce vulnerability of *P. nigra* plantations to droughts and warming up to certain thresholds [8,9]), the initial selection of provenance(s) is critical.

An important factor for the estimation of a plant species' capacity to expand its distribution is the knowledge on that species reaction to extreme (low or high) temperatures, since temperature, especially the extreme ones, is among the most critical factor for plant species distribution [10]. A common applied method for reaction estimation is to evaluate plant (e.g., seedling) hardiness either to frost or to heat wave. For many years, plant (seedlings) hardiness either to frost or to heat (and drought) was estimated by field survival [11]. However, this requires years of observation since test frost winters or summer heat waves occur only about once in every 10 years. In the last 50 years, numerous types of freezing and heating apparatuses and procedures have been developed to obtain answers more quickly. Thus, the use of a temperature controller chamber in the laboratory has become the most popular. The minimum temperature to which seedlings are exposed will depend on the seasonal cycle of frost and heat hardiness. Generally, a range of minimum temperatures are used that bracket the seedling's frost or heat hardiness [11].

Austrian pine (*Pinus nigra*) is one of the most economically important native conifers in southern Europe and one of the most widely planted species in many southern European countries, e.g., in Greek and Serbian forestation programs [12,13]. In these countries, a large amount of degraded woodland was usually removed in the past by clear-cut harvesting, and traditionally planted with fast-growing pioneer species (usually *P. nigra*) with a goal of facilitating the introduction of late-successional hardwoods [14].

Understanding how *P. nigra* seedlings of different provenances are likely to react to changing site conditions is essential in climate adaptive forest restoration and reforestation. Thus, the aim of the study was to better understand the potential of seed transfer along the Balkan Peninsula, from southern provenances (Greece) to the northern (Serbia) and vice versa, by testing frost hardiness and heat and drought tolerance of *P. nigra* seedlings from two southern Greek provenances and one of northern Serbian provenance. Evaluation of the treatments effect on seedlings was used to determine any differences in provenances adaptation ability.

2. Materials and Methods

2.1. Plant Material

Two-year old container grown *P. nigra* seedlings from three Balkan provenances were tested. Seed provenances were selected along the axis north to south, in a distancing of 430 km from Šargan (Serbia) to Grevena (Greece) and 780 km to Kalamata (Greece), in order to cover a great part of the north-south distribution of the species in the Balkan peninsula, from 44 N parallel to 37 parallel (Figure 1). Seeds were collected from mature natural stands. The site characteristics of the seed sources are shown in Table 1.

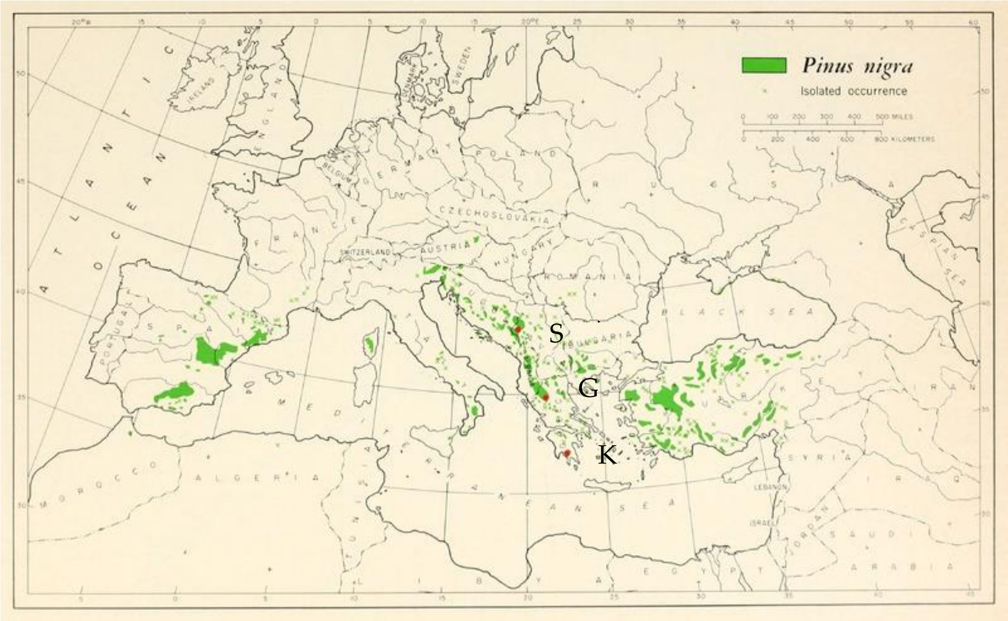

Figure 1. Map showing the sampled provenances of *Pinus nigra* seeds; S = Šargan (Serbia), G = Grevena (Greece), and K = Kalamata (Greece), as well as the species world natural distribution (*Pinus nigra* distribution map according Critchfield and Little [15].

Seedlings were grown in the nursery of the University of Belgrade (44°49′ N, 20°28′ E)—Faculty of Forestry in 2018, in Hiko 220SS containers, filled with peat:perlite 2:1 (*v:v*) ratio. Seedlings were produced by standard practice (including irrigation, fertilization, and shading, [16]) and meet the national quality standard of Serbia (as described by [17]).

Table 1. Sources of the seed material used in the study.

Provenance	Grevena (Krania)	Kalamata (Taygetos)	Šargan
Country	Greece	Greece	Serbia
Coordinates	39.951169 N; 21.210922 E	36.954413 N; 22.264140 E	43.816944 N; 19.481944 E
Altitude (m a.s.l.)	1100	1100	955
Reg. number	-	-	RS-2-2-pni-41a-089
Subspecies	*pallasiana*	*pallasiana*	*nigra*
Climate [18]			
Yearly average (°C)	10.5	11.7	7.7
Yearly average max. (°C)	15.1	16.2	12.8
Yearly average min. (°C)	3.7	8.3	3.9
Absolute maximum (°C)	35.5 (July)	32.3 (July)	35.8 (July)
Mean temperature of warmest month (°C)	21.2 (July)	20.5 (August)	17.5 (August)
Absolute minimum (°C)	−16.9 (January)	−7.6 (January)	−19.8 (January)
Mean temperature of coldest month (°C)	0.7 (January)	3.2 (January)	−2.0 (January)
Average number of frost days	96	21	116
Average number of tropical days	12	6	4
Annual precipitation (mm)	1062	885.6	1017.3

2.2. Frost Hardiness Experiments

Frost hardiness testing consists of subjecting of seedlings or parts thereof to freezing temperatures and evaluation of the treatment effects [11]. For the estimation of frost hardiness of the studied provenances of black pine, two commonly used tests were selected to be applied in the seedlings produced in the nursery: EC test, and whole-plant freeze testing (WPFT) [11,19]. Sampling for both tests took place in the first week of October 2018. The tests are analytically described below.

2.2.1. Electric Conductivity (EC) Test

This method is based on the fact that when plant tissue is injured, the site of injury is the cell membrane, which loses its selective permeability. Thus, upon injury, the electrolytes that occur in the aqueous cellular cytoplasm move more freely and diffuse (leach) out of the tissue when it is placed in water. The severity of the injury is proportionate to the amount of electrolytes that diffuse out of the tissue. By comparing the conductivity of un-injured tissue diffusive with that of injured ones, an estimate of the amount of injury can be made. However, the technique was not strictly quantitative, because of variations in total electrolytes in different samples, thus it was refined by Flint et al. [20], who proposed a scale where the unfrozen sample is given a value of zero and the totally killed sample a value of 100 and thus the release of electrolytes is expressed in percent. This index was called "Index of Injury" (Ii) and it has received considerable testing in forestry, as it was found to be an excellent technique for determining the frost hardiness of coniferous containerized seedlings [11,21]. This is the reason we selected to use it in our study.

For EC test, the following protocol was used [11,22,23]: thirty seedlings per provenance (a total of 90 samples) were selected for sampling. For each selected seedling, a sample of 2 cm long shoot segments were cut, 1 cm below the tip. Segments were randomly separated into 3 groups of 10 samples per provenance. Afterwards, three frost treatments were applied to the taken shoot segments, each of them to one subsample of the ten shoot segments per provenance: (i) control treatment (non-frozen segments at 5 °C) and two frost treatments; (ii) light to moderate frost, where shoot segments were exposed to 5 °C for 1 h and then the temperature was dropped with a rate of 5 °C per hour [18]) up to −5 °C (target temperature); (iii) high frost, where shoot segments were exposed to 5 °C for 1 h and then the temperature was dropped with a rate of 5 °C per hour up to −18 °C (target temperature). More analytically, the procedure followed was as follows.

In the control treatment, the samples after cutting were measured for mass (g), washed and rinsed (with tops up) in jars filled with 20 mL of distilled water, and then immersed in distilled water by aluminum foil, which was at the same time used to prevent sample (solution) contamination. After 24 h at room temperature (21–24 °C), jars were shaken and after 5 min of settle down the EC of control (ECc) was measured by conductivity meter (Mettler Toledo FiveEasyTM). Then, the cells were killed by placing shoot segments and capped jars in an oven at 90 °C for 2 h and left overnight at room temperature (21–24 °C). Jars were shaken and after 5 min of settle down the EC control killed (ECck) was measured by conductivity meter.

In frost treatments, twenty shoot segments per provenance were measured for mass (g), washed, and rinsed in glass jars filled with 20 mL of distilled water, which were then placed in a freezer where they were equilibrated to 5 °C for 1 h. Then, the temperature was decreased with a rate of 5 °C per hour up to the two targeted test temperatures: −5 °C and −18 °C. The samples were held at the targeted test temperature for one hour, and then removed and allowed to warm up (thaw) slowly overnight by placing in a cooler. Shoot segments were immersed in 20 mL of distilled water by aluminum foil (similarly to steps A1 and B1) and left overnight at room temperature. Then the glass jars were shaken and after 5 min of settle down the EC frozen (ECf) was measured. Frozen shoot segments cells were killed by placing shoot segments and capped jars in oven at 90 °C for 2 h. Finally, the glass jars were shaken and after 5 min of settle down the EC frozen killed (ECfk) was measured. Based on the measurements taken, we calculated:

The relative conductivity of the control samples (RC_C) − $RC_c = EC_c/EC_{ck} * 100$;

The relative conductivity of the frozen samples (RC_F) − $RC_F = EC_f/EC_{fk} * 100$;

The Index of Injury (I_i), as the expression of cold hardiness (the higher index indicate lower cold hardiness)—$I_i = (RC_f - RC_c)/(1 - (RC_c/100))$;

2.2.2. Whole-Plant Freeze Testing (WPFT)

As it as previously mentioned, the use of a freezing chamber in the laboratory is the most popular technique for plant frost hardiness testing [11]. The best way to conduct freezing tests in a tree nursery is to use a freezing chamber with a cam operated temperature controller, where the cams are cut in accordance with the temperature cycle desired. The minimum temperature to which seedlings are exposed will depend on the seasonal cycle of frost hardiness. The rate of freezing is important and should be between 2 and 6 degrees C/hr. For WPFT, a sample of 20 seedlings per provenance (total 60 seedlings) was randomly selected from the nursery. Seedlings were adequately watered prior to testing and placed in the freezing unit, with roots insulated with perlite [21]. Seedlings were placed in the freezer where they were acclimated to 5 °C for 1 h. Then, the temperature was dropped at a rate of 5 °C per hour [24] up to the two targeted test temperatures: −5 °C and −18 °C. After holding of seedlings at −5 °C for one hour, the half of the sample (10 seedlings of each provenance) was removed. For the other half, the temperature was also dropped at a rate of 5 °C per hour up to −18 °C, and after one-hour seedlings were removed from the freezing chamber.

After transferring the seedlings to the nursery, seedlings were evaluated for injuries in the 16 day period at 2–4 days intervals. The common damage assessment procedure is to determine the extent of browning 2 to 4 weeks after freezing [11]. Thus, we used a visual damage estimation of freezing injury using a 10-grade reversible scale with each increase of tolerance grade representing a 10% decrease in injury (e.g., tolerance grade 1 = 90–100% of injured tissue, tolerance grade 10 = < 10% of injured tissue). The visual grading was performed separately by three experts and their average grading for each seedling was used.

2.3. Heat and Drought Tolerance Tests

In the second half of July of the following summer (2019), 20 container seedlings each from Grevena and Šargan provenance (for the third provenance, Kalamata, there was not an adequate number of seedlings) were randomly sampled (a total of 40 seedlings) and removed from the shade and exposed to direct sun. The following day (July 14—morning temperature 20 °C and maximum day temperature 29 °C), half of the sampled seedlings was transferred to the lab and were exposed to rising temperature as follows: the seedlings were placed in the chamber and they were acclimated to 30 °C for 1 h. Then, the temperature was raised at a rate of 5 °C per hour up to the targeted test temperature of 45 °C. After staying for 4 h at 45 °C, the stressed seedlings were relocated to the nursery [25] next to the rest of the 20 non-heated seedlings. Watering was halted for both heat treated and non-heat treated seedlings. The seedlings were visually evaluated [25,26] for injuries at the 3 day interval at the one week period after the applied treatments, along with their respective controls grown under normal conditions (no heat and no drought) in the nursery. Drought and heat, and drought tolerances were evaluated by following a four grade system either for drought damages (DD) or for heat and drought damages (HDD): DD/HDD 1 = < 25% of foliar damage (with minimal leaf marginal necrosis and wilt), DD/HDD 2 = 26–50%, 3 = 51–75%, and 4 = > 76% of foliar damage. The visual grading was performed separately by three experts and their average grading for each seedling was used.

2.4. Statistical Analysis

One Way ANOVA ($p < 0.05$) was conducted on all data to determine significant differences among provenances. Tukey's Multiple Range Test was used to determine significant differences among means. The relationship between the seedlings' damages with the time after exposure to frost, for the three studied provenances, was investigated by regression analysis. Several linear and non-linear models were tested, and we selected the simplest significant ($p < 0.01$) model that best interpreted each relationship. All models were evaluated for goodness of fit by graphical analysis of residuals. The best fitting model was selected with the highest coefficient of determination (R^2) [27]. Statistica 7.0 software was used for all data analyses.

3. Results

3.1. Frost Hardiness

3.1.1. Measured by EC Test

According to the results of the EC test, the seedlings from the two southern (Greek) provenances, Grevena and Kalamata, showed higher frost hardiness to light to moderate frosts (−5 °C), since the Index of Injury (Ii) was found to besignificantly lower compared to the northern (Serbian) provenance of Šargan (Table 2). The higher frost (−18 °C) contributed to the increase in injuries (Ii) compared to the values recorded in the light frost (Table 2); however, no significant differences among the studied provenances were detected. This means that both southern and northern provenances presented low ability to withstand high frost (−18 °C). This confirms that the studied species of *P. nigra* do not belong to boreal conifers (e.g., *Pinus silvestris* and *Picea abies*), and it may also explain the natural distribution of the species along the mountainous Mediterranean region.

Table 2. Differences in *P. nigra* seedlings' frost hardiness (exposed to −5 °C and −18 °C) between the tested provenances Šargan (S), Grevena (G), and Kalamata (K), as expressed by relative conductivity (RC_C, RC_F) and Index of injury (Ii) from EC test.

	RC_c	**RC_F**	**I_i**
		−5 °C	
S	25.59 (2.47) ^{a}	41.48 (4.55) ^{a}	21.35 (1.88) ^{a}
G	20.17 (1.77) ^{b}	25.14 (5.63) ^{b}	6.22 (0.71) ^{b}
K	17.20 (1.72) ^{c}	24.91 (2.06) ^{b}	9.31 (0.94) ^{b}
F	4.92		
p	0.0257		
		−18 °C	
S	25.59 (2.47) ^{a}	65.31 (2.47)	53.37 (2.22)
G	20.17 (1.77) ^{b}	59.18 (1.78)	48.87 (2.01)
K	17.20 (1.72) c	62.34 (1.72)	54.52 (1.69)
F	0.26		
p	0.7756		

Mean values (standard error in parenthesis) followed by different lower case letters at the same column are significantly different. *F*: F value, *p*: *p* value. Mean values not followed by a low case letter are not statistically different.

3.1.2. Measured by WPFT

The damages of the seedlings' tissues due to their exposure to light-moderate frost (−5 °C) were relatively small, and they were dependent on the time (days) after exposure (Table 3). The seedlings' tolerance was high during the two days after exposure to light frost for all three provenances, and the damages increased with the time after exposure. The highest tolerance, 16 days of exposure to frost, was observed in the northern (Šargan) provenance (value 7.2, which corresponds to 20–30% damaged tissue), and the lowest in the Grevena provenance (5.4 or 40–50% damaged tissue); however, these differences were found not to be statistically significant.

The damages were more prominent in the case of high frost temperature after seedlings' exposure to −18 °C (Table 3), where the greater part of the seedlings' tissues, for all three tested provenances, were found injured, especially 16 days after exposure to frost.

For both testing temperatures, seedlings from the northern provenance (Šargan) showed the highest tolerance to freezing; yet, for exposure to −18 °C at 16 days after exposure to frost, the percentage of damaged tissues, in northern provenance, was 70–80%, while in southern provenances, it ranged from 80–90%. However, statistically significant differences between the provenances were only detected at the 6th and 13th day after exposure on −18 °C. Regression analysis showed that the best fitting model equation that better expressed the effect of exposure time to frost (−5 °C and −18 °C) on seedlings' tissue damages, in most cases, was the logarithmic equation (Figure 2), except for the Šargan provenance in the case of high frost (−18 °C), where the best fitting model equation was the linear. The most crucial period, for both frost treatments, was from the 2nd to the 6th where a sharp increase in seedlings' tissue damages was observed for all provenances.

Table 3. Tolerance of *P. nigra* seedlings to freezing temperatures of −5 °C and −18 °C determined by WPFT, between the three tested provenances Grevena (G), Kalamata (K), and Šargan (S).

			−5 °C		
Level of Factor	**D2**	**D6**	**D9**	**D13**	**D16**
S	9.40 (0.24)	7.80 (0.37)	7.20 (0.50)	7.20 (1.10)	7.20 (0.49)
G	7.40 (1.21)	6.40 (1.21)	6.20 (1.11)	5.80 (1.36)	5.40 (1.21)
K	8.40 (0.68)	7.20 (0.97)	7.20 (0.97)	7.00 (0.58)	6.80 (1.16)
Average	8.40 (0.49)	7.13 (0.52)	6.87 (0.49)	6.67 (0.58)	6.47 (0.58)
F	1.5151	0.5827	0.4132	0.5244	0.8816
p	0.2589	0.5734	0.6706	0.6049	0.4393
			−18 °C		
S	8.80 (0.97)	6.40 (0.60) [a]	3.60 (0.40)	3.00 (0.45) [a]	2.20 (0.37)
G	8.80 (0.20)	4.00 (0.00) [b]	3.00 (0.00)	3.00 (0.00) [a]	1.60 (0.24)
K	7.60 (0.68)	4.00 (0.63) [b]	2.60 (0.24)	2.00 (0.00) [b]	1.20 (0.20)
Average	8.40 (0.40)	4.80 (0.40)	3.07 (0.18)	2.67 (0.19)	1.67 (0.19)
F	1.0000	7.5789	3.4545	5.0000	3.1667
p	0.3966	0.0074	0.0653	0.0263	0.0786

Mean values (standard error in parenthesis) followed by different lower case letters at the same column (time) are significantly different. *F*: F value, *p*: *p* value. Mean values not followed by a low case letter are not statistically different.

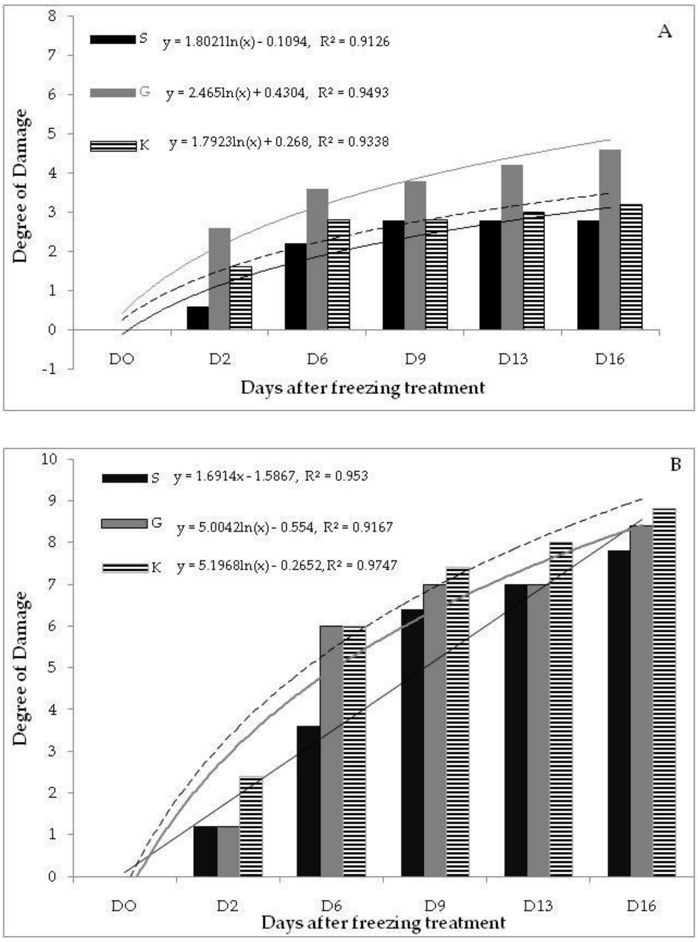

Figure 2. Relationship between seedlings' damages with the time after their exposure to frost, for the three provenances during 16 days after being subjected to freezing temperatures of −5 °C (**A**) and −18 °C (**B**). Šargan (S), Grevena (G), and Kalamata (K).

3.2. *Heat and Drought Tolerance*

The degree of foliar damages, due to the exposure to drought or to heat and drought, increased rapidly during the observation time, and was significantly greater than that of control (Table 4). Both on the fourth and the seventh day after the treatments applied, the heat & drought-treated seedlings, from both provenances, presented significantly higher foliar damages than the drought-treated ones. However, the seedlings from both provenances (northern and southern) showed similar behavior when either exposed to heat (45 °C) and drought, or only to drought; thus, no significant differences in degree of foliar damages were detected among the two contrasting provenances.

Table 4. Foliar Damages from Drought and Heat & Drought treatments of *P. nigra* seedlings from the two contrasting provenances Grevena (G) and Šargan (S).

	Control	**Drought Treatment**		**Heat and Drought Treatment**	
Provenance	Both Provenaces	Grevena	Šargan	Grevena	Šargan
Days after treatment					
1	1 (0.00)	1 (0.00)	1 (0.00)	1 (0.00)	1 (0.00)
4	1 (0.00) ^{c}	2 (0.97) ^{b}	1.9 (0.72) ^{b}	2.3 (0.88) ^{a}	2.3 (0.99) ^{a}
7	1 (0.00) ^{c}	2.8 (0.98) ^{b}	2.9 (0.81) ^{b}	3.3 (0.69) ^{a}	3.4 (0.81) ^{a}

Values are means and standard deviation (in parenthesis). Values followed by different lower case letters within a row are statistically different ($p < 0.05$).

4. Discussion

Plant species cold hardiness is an important trait reflecting species' adaptation to climate. It can be assessed by evaluating the tissue damages after artificial freezing [28] either by visual observation or electrical conductivity that are the most used methods for screening cold hardiness.

The two methods used, in the current study, to assess frost hardiness of *P. nigra* seedlings showed quite similar results, indicating that for all studied provenances the damage was great; the index of injury (Ii) at −18 °C was ranged from 49 to 54.5 (measured by EC method) and the percentage of injured tissues ranged from 80–90% (measured by visual observation). Similarly, Semerci et al. [19], who studied frost damages in *P. brutia* seedlings from various provenances of two contrasting sites (north-south), found similar results from the electrical conductivity and visual observation methods. For *P. nigra* seedlings from all studied provenances, a sharp increase in damages was observed with the fall of temperature from −5 and −18 °C, and the time exposure to it.

P. nigra is a genetically diverse pine species, and its subspecies *nigra* is considered the most frost tolerant among the other subspecies [29]. In this study, the WPFT results showed the highest tolerance to freezing (−18 °C) was presented by seedlings from the northern Šargan provenance, which is consistent with expected higher frost tolerance of *ssp. nigra* compared to *ssp. Pallasiana*; however, no significant differences were statistically detected among the studied provenances. Similarly, Savolainen et al. [24], for *P. silvestris* seedlings, found that the scores of visual damages after cold treatment (−32 and −19 °C) were approximately similar in the two contrasting populations (north-south). No differences in frost hardiness of stems at the provenance level were also found for *P. silvestris* seedlings by Repo et al. [30]; however, they also reported that the frost hardiness of needles of the northernmost provenance was higher than that of other origin. Results of the EC test indicated that seedlings from both Greek provenances showed higher frost stem hardiness compared to those of Serbian provenance Šargan, at light to moderate frost (−5 °C). Studies on *P. nigra ssp. salzmanii* showed that seedlings from the southernmost seed source were more adapted to light frost (−3.2 °C) in terms of field survival [31]. However, when stems of this study were subjected to high frost (−18 °C), both southern and northern provenances presented similar scores of damages, showing low ability to withstand high frost. Generally, plants are more sensitive to frost in the growing season, with most of the temperate zone plants killed when the air temperature drops to only a few degrees below freezing [32,33]. This was obvious for Grevena, the provenance with the highest values of yearly average minimum temperature, absolute minimum temperature, and the mean temperature of the coldest month; compared to Kalamata and Šargan (Table 1). According to Kreyling et al. [4], cold hardiness of *P. nigra* foliage is affected by local adaptation to prevailing minimum temperatures at the origin. Minimum temperature is recognized as the genetically selective parameter for *P. nigra* [4] and should be considered in provenance selection for the long-distance transfer.

Light has great influence on the water flow and physiological status of young seedlings [34,35]. Thus, growing of seedlings under shade and regular watering, and then exposing them to direct sunlight and water halting, results in high stress of the seedlings. This stress was confirmed in this study for *P. nigra* seedlings. *P. nigra* is a drought sensitive species, showing a negative tree growth trend highly correlated with increased temperature and drought in summer (in the Eastern Mediterranean [36], but in Hungary as well [37]). The results of this study showed that the exposure to moderate heat (45 °C) and short drought did increase damage to seedlings for both contrasting provenances (Grevena and Šargan), but without significant difference between them. The heat and drought-treated seedlings, from both provenances, presented significantly higher foliar damages than only drought-treated ones. Our results are in line with previous studies reporting the lack of specific local adaptation to drought [38,39] and warming [6] in *P. nigra*. Although the climate in Šargan is a little colder compared to Grevena, the similar absolute maximum temperature at both sites indicates a similar adaptation to extremely high air temperature. Also, two-year old *P. nigra* seedlings from five Balkan provenances showed high sensitivity to drought treatment regardless of origin [40].

Considering freezing and heating tolerance, Greek provenances of *P. nigra* (i.e., Grevena, northern Greece) can be successfully used in Serbian forestation and restoration programs. The reported results are based on only three provenances exposed to a small number of testing temperatures and need to be considered in this context. However, our results support the previously made recommendation that the introduction of *P. nigra* to regions outside its natural distribution range should not aim at introducing a single best-adapted provenance but at establishing populations with a high genetic diversity [39]. This can be achieved by different provenancing strategies (reviewed by [10]), e.g., by admixture [41] and climate-adjustive provenancing [42].

The present study could be valuable to address adaptive *P. nigra* seedlings transfer in reforestation and to determine the suitable provenances for reforestation programs and assisted population migration under climatic change scenarios.

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