VARIABILITY IN NEEDLE TRANSVERSE ANATOMY OF *PICEA ABIES* (L.) H. KARST.: A CASE STUDY FROM ČEMERNIK MOUNTAIN, SERBIA

PETROV, DJ.[1*] – OCOKOLJIĆ, M.[1] – GALEČIĆ, N.[1] – SKOČAJIĆ, D.[1] – VUJIČIĆ, D.[1] – ČUKANOVIĆ, J.[2] – SIMOVIĆ, I.[3]

[1]*Faculty of Forestry, University of Belgrade, Kneza Viseslava 1, Belgrade 11000, Serbia*

[2]*Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, Novi Sad 21000, Serbia*

[3]*BioSense Institute, University of Novi Sad, Dr Zorana Djindjića 1, Novi Sad 21000, Serbia*

**Corresponding author*
e-mail: djurdja.stojicic@sfb.bg.ac.rs; phone: +381-11-305-3814; fax: +381-11-254-5485

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Abstract. Norway spruce is an important forest species facing the risk of range shift from its current distributional boundaries. This study analyzes the morphological and anatomical characteristics of Norway spruce needles with the aim of determining and understanding the variability and population diversity of this species on Mt. Čemernik in Serbia, a site outside its native range. Differences between one-year-old and two-year-old needles from Norway spruce trees aged 100 and 50 years were investigated. Morpho-anatomical parameters, including needle length, width and thickness, vascular bundle diameter and area, and the number and diameter of resin canals, were analyzed. The research findings and statistical analyses confirm that the old trees possess exceptional traits and represent a valuable gene pool. Considering that ecological valence is a heritable trait, i.e., the ability to adapt is determined by the plant's genetic basis. A selected phenogroup of three old trees demonstrated successful adaptation to the Čemernik site, suggesting their potential for wider application and further introduction into other mountainous regions with similar conditions where the species is non-native.
Keywords: *Norway spruce, old conifers, needle size, multivariate analyses, adaptation, conservation*

Introduction

Norway spruce is among the most extensively cultivated conifer species, valued for its high-quality timber and applications in the wood-processing, pharmaceutical, and cosmetic industries (Skrøppa, 2003; Jansson et al., 2013). As a result, it has been widely planted beyond its native range, leading to the establishment of both natural forests and numerous plantations. This extensive cultivation has often made it difficult to distinguish between natural and artificially established Norway spruce forests.

Norway spruce belongs to the boreal-European floristic element, occurring from sea level to elevations of up to 3000 m above sea level (Cvjetićanin et al., 2016). Its natural range of distribution in Europe spans from the Balkan Peninsula at 41°27'N (southern Greece) to Siberia at 72°15'N, and longitudinally from the French Alps in the west (5°27'E) to eastern Siberia (154°E) (Caudullo et al., 2017). However, throughout history, the range of Norway spruce has shifted significantly (Huntley and Birks, 1983; Latałowa and van der Knaap, 2006). In Serbia, Norway spruce is the most widespread conifer species, with natural populations in the western and southwestern regions, while in the eastern regions, it has been artificially introduced (Ivetić, 2004; Banković et al., 2009; Cvjetićanin et al., 2016; Petrov and Ocokoljić, 2022). Nevertheless, it has not

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exhibited consistent adaptability across all planting sites, resulting in unsatisfactory reproductive material in some locations (Cvjetković, 2018).

The conservation of species and seed sources, and the preservation of both high-quality progeny and genetic diversity are of great importance in the context of climate change, as species distributions shift toward higher altitudes and the northern hemisphere. Global warming poses a significant threat to Norway spruce, a typical boreal species on the Balkan Peninsula. Bošel’a et al. (2014) report a steady decline in the health of Norway spruce populations and the species’ high sensitivity to climate change. According to Falk and Hempelmann (2013), there is a predicted range shift for populations at the edges of the species’ distribution.

Norway spruce genotypes, being adaptive specialists, are influenced by the external environment, which plays a critical role in the species’ survival and physiological functions (Frank et al., 2017). This factor is particularly important when considering species introduction and afforestation efforts. Given the wide geographic range of Norway spruce and its varied ecological and adaptive traits, considerable differences in morphological and physiological traits, as well as in the genome itself can be observed across populations. Research in gene ecology plays a vital role in understanding the adaptive capacity of individual trees and entire populations, which is essential for their survival and reproduction. Therefore, adapted Norway spruce individuals represent a valuable genetic resource for expanding the species’ range under changing environmental conditions.

The utilization of genetic variation is regarded as a crucial strategy for enhancing the adaptability of tree species that are important for both forestry and urban forestry. Conservation efforts and the sustainable use of valuable genetic resources should prioritize highly adaptive genotypes and local adaptations to warmer climates (Geburek et al., 2013; Petrov and Ocokoljić, 2023). Environmental factors, along with forestry management practices, can impact the growth potential of Norway spruce stands (Rungis et al., 2019), suggesting that better outcomes may be achieved if management practices are adjusted to accommodate changing conditions.

To preserve the valuable gene pool of Norway spruce in Serbia, especially in locations where planted individuals and plantations have successfully adapted, it is essential to conserve adaptive genotypes. This is crucial for establishing new plantations, which serve as the foundation for improving the production of selected trees. Given the morphological and anatomical differences between species within populations, this study focuses on the anatomical variability of Norway spruce needles sampled from Čemernik, a site outside the species’ natural range where Norway spruce has successfully adapted.

The primary aim of this study was to define the variability within a 100-year-old phenogroup of Norway spruce, using morphological traits and quantitative microscopic characteristics, and to compare these findings with a nearby control group, also located on Mt. Čemernik.

Materials and methods

The study was conducted in Brod, located on Mt. Čemernik in southeastern Serbia, where forest plant communities belong to the *Fagion moesiacae* Bleč. et Lak. Alliance (*Fig. 1*). The research site is located at 42°50’56” N latitude and 22°17’02” E longitude. Although Norway spruce is not native to this area, it has demonstrated remarkable

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adaptability. The three analyzed individuals of *Picea abies* (L.) H. Karst. were planted in 1925. They grow at an elevation of 856 m on two soil types: ranker (humus-silicate soil) and dystric cambisol (Škorić et al., 1985).

The climate of the research site is mountainous, belonging to the temperate continental zone. Over the past 20 years, the mean annual air temperature has been 6.7°C, and the mean annual precipitation is 887.1 mm. The climate data were obtained from the RC Vlasina meteorological station (42°44’34.48” N; 22°19’36.91” EGr) of the Republic Hydrometeorological Service of Serbia (RHMS) (accessed on 15 June 2024) [https://www.hidmet.gov.rs/index.php].

Figure 1. *(a) Location of the research area in Brod, Čemernik Mountain, Serbia, (b) Location of the research area with analyzed phenogroup (Ph) and control tree in the population of spruces (C) and (c) analyzed phenogroup of three specimens of 100-year-old spruces*

Needles were sampled from three 100-year-old Norway spruce trees growing in an isolated group on non-exposed terrain (*Fig. 1c*). This phenogroup of trees stands out for its exceptional growth habit, with crowns extending from the ground, and its excellent condition, showing no signs of diseases or damage. Needle samples were also collected from a control tree (Tree 4). Needle samples were also collected from a control tree (Tree 4). The control tree was approximately 50 years old, planted during afforestation, and located within the distance of 150 m, at 42°50’53” N latitude and 22°17’01” E longitude, at an elevation of 873 m. The sampled trees were 50 m tall, while the height of the control tree is 27.3 m (*Table 1*).

Table 1. *Dendrometric characteristics of the analyzed and control trees*

Tree	**Hight *(m)***	**Diameter at 1.30 m *(cm)***	**Trunk circumference *(m)***	**Crown diameter *(m)***	**Age *(years)***
1	53.1	118	3.71	11.98	~100
2	52.9	103	3.24	17.64	~100
3	50.94	84	2.66	11.24	~100
4	27.3	86	2.69	9.92	~50

In the studied trees, using the random sampling method at a height of 2 m, 100 one-year-old and 100 two-year-old needles were collected for morphological analysis, along

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with 10 one-year-old and 10 two-year-old needles for anatomical analysis. All samples were collected in October 2024 for the needles to mature. To avoid microclimatic influences, the needles were taken from the southern side and the outer parts of the crown (Lhotáková et al., 2007), since, according to Špunda et al. (1998), sun-exposed and shaded needles differ. According to Matějka and Krpeš (2022), quantitative microscopic analysis of the width and thickness of needle transverse sections is a sufficient indicator of variability for the study and assessment of heterogeneous populations.

Morphometric characteristics of needles were measured on the same day the samples were collected. Needle length was determined using a standard measurement procedure (the needles were scanned and measured using ImageJ software). A total of 800 needles were measured (100 one-year-old and 100 two-year-old needles from each tree).

To analyze the anatomical structure of the needles, several parameters were measured, including the thickness and width of the needles, the mean diameter of the vascular bundle, and the mean diameter of resin canals, along with a description of sclerenchyma cells. Quantitative anatomical characteristics of the needles were determined based on the transverse section of the needles. The samples were collected and fixed in 50% ethanol. Anatomical transverse sections were then made using a cryostat Leica CM 1850 at -21°C. The transverse sections were made in the mid-structure of the needle, as this part contains resin canals. The sections were bleached in Parazone, rinsed with water, and double-stained with safranin (1% w/v in 50% ethanol) and alcian blue (1% w/v, aqueous). A total of 80 samples of different needles were prepared (10 one-year-old and 10 two-year-old samples from each tree). The analysis of the anatomical structure of the needles (*Fig. 3*) included the measurement of needle thickness (Nt), needle width (Nw), and needle flatness (Fl), i.e., the ratio of needle thickness to width (Nt/Nw) calculated according to Apple et al. (2002) and Sellin (2001). Besides these parameters, the diameter and area of the vascular bundle (*Fig. 4*), as well as the number and size of resin canals were measured. The number of sclerenchyma cell layers beneath the epidermis was also determined. Microscopic analysis was conducted using a Euromex BB.4225 BioBlue Monocular 5MP microscope with an integrated camera (Digital Microscope SMP 4/10/S40 Objectives, DIN WF 10x/18 eyepiece). The magnification used was 40×. The Euromex ImageFocus Plus software was used to measure the parameters of the transverse section through the needle.

The obtained data were statistically processed using the STATISTICA 13.0 software. Descriptive statistics procedures were used to determine mean values, standard deviations, coefficients of variation, and their errors. To determine the presence of statistically significant differences in the analyzed characteristics, Duncan test, Tukey HSD, Fisher (LSD) test and ANOVA was performed. Principal Component Analysis (PCA) was carried out using the SRplot statistical program (Tang et al., 2023).

Results

Morphological characteristics of Norway spruce needles

Descriptive statistics were used to analyze the variability of needle length in the phenogroup—three mature individuals, each approximately 100 years old, and a control tree, approximately 50 years old. The range of needle lengths for the phenogroup was between 12 and 32 mm, while the control tree had needle lengths ranging from 8 to

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25 mm. Needles from the phenogroup were significantly longer compared to the control group. Additionally, the needle length in the phenogroup exceeded the values of up to 25 mm reported in the literature (Ocokoljić and Ninić-Todorović, 2003), by an average of 0.7 mm.

The results showed that the mean needle lengths for Tree 1, Tree 2, and Tree 3 were 23.51 ± 0.12 mm, 24.15 ± 0.12 mm, and 23.79 ± 0.12 mm, respectively. In comparison, the mean needle length for the control tree was 16.94 ± 0.08 mm. The difference in mean values between the phenogroup and the control tree was statistically significant (*Table 2*).

When comparing the mean values of one-year-old needles within the phenogroup, the lengths were recorded as 20.86 ± 0.07 mm, while for two-year-old needles, the mean length was 26.77 ± 0.09 mm. A significant difference of 6.16 mm in the length of one-year-old needles was observed compared to the control tree (14.7 ± 0.15 mm). The mean length of two-year-old needles on control trees was 19.17 ± 0.19 mm, with a difference of 7.59 mm.

Tree 2 stood out as the individual with the highest values for the length of both one-year-old and two-year-old needles in the phenogroup. The length of the one-year-old needles ranged from 15 to 28 mm, and the two-year-old needles from 19 to 32 mm, with mean values of 21.23 ± 0.21 and 27.07 ± 0.27, respectively.

Table 2. *Length of one-year-old and two-year-old needles in the phenogroup and control group*

Locality	Limiting value	$\bar{x} \pm S\bar{x}$	$S \pm S_S$	$V \pm S_V$
Needle length (mm)				
Ph	12-32	23.818 ± 0.040	3.923 ± 0.687	16.471 ± 0.475
Ph One-Year-Old	12-28	20.865 ± 0.069	2.303 ± 0.852	11.036 ± 0.451
Ph Two-Year-Old	18-32	26.772 ± 0.089	2.834 ± 1.093	10.585 ± 0.432
C	8-27	16.937 ± 0.084	3.880 ± 0.846	22.908 ± 1.145
C One-Year-Old	8-20	14.7 ± 0.147	3.122 ± 1.039	15.464 ± 1.093
C Two-Year-Old	14-27	19.175 ± 0.192	3.225 ± 1.356	15.973 ± 1.129

Ph – phenogroup; C – control tree; $\bar{x}$– medium values; S – standard deviations; V – coefficients of variation; $S\bar{x}$, S_S, S_V – errors

Anatomical features of Norway spruce needles

The structure of Norway spruce needles can be divided into three parts: the dermal layer (comprising the epidermis and hypodermis), the mesophyll, and the vascular components (including the endodermis, transfusion cells, and fibrovascular bundles), as outlined by Marco (1939). Transverse sections through the mid-region of the Norway spruce needle demonstrate that the needles are covered with a thick, lignified uniseriate epidermis that features a cuticle, within which sunken stomata are distinctly visible (*Fig. 2*). Beneath the epidermis, there is typically a single layer of hypodermal cells; however, in certain cases of two-year-old needles, this layer may be two-cell thick. Mesophyll cells extend from the hypodermis, while at the center of the needle lies a vascular bundle enveloped by a uniseriate endodermis. A closed vascular bundle is located within this central area (*Fig. 3*). Resin canals are situated within the mesophyll, near the dermal tissue, and measure 1-2 mm in diameter (Marco, 1939). These resin

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canals, generally numbering one or two, were observed in both one-year-old and two-year-old needles of the studied phenogroup. In contrast, the control group exhibited resin canals exclusively in the two-year-old needles. These canals are located just beneath the hypodermis (external type) along the needle margin. The dimensions of various anatomical features exhibited significant variability.

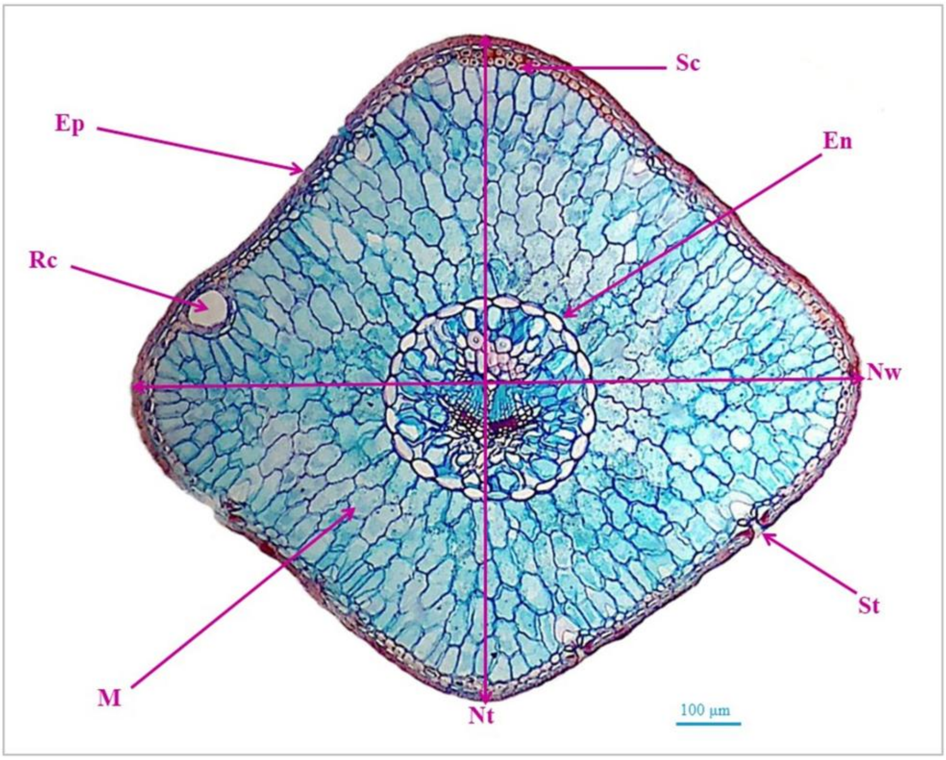

Figure 2. *Picea abies (L.) H. Karst. needle transverse section (Nt – needle thickness, Nw – needle width, Ep – epidermis with cuticle, En – endodermis, Sc – sclerenchyma (hypodermis), M – mesophyll, Rc – resin canal, St – stoma)*

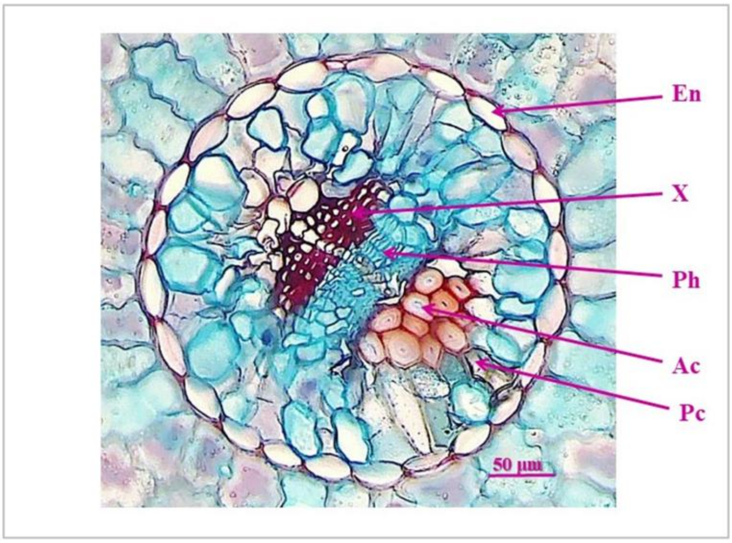

Figure 3. *Section of vascular bundle (En – endodermis; X – xylem; Ph – phloem; Ac – group of albuminous cells; Pc – pericycle)*

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It was determined that three forms of needles appear in the transverse section: rhomboid, mushroom-shaped, and elliptical (*Fig. 4*). The most frequently observed shape for individual 1 was rhomboid, for individual 2 it was mushroom-shaped, and for individual 3 it was elliptical. In contrast, the control tree exhibited all three shapes.

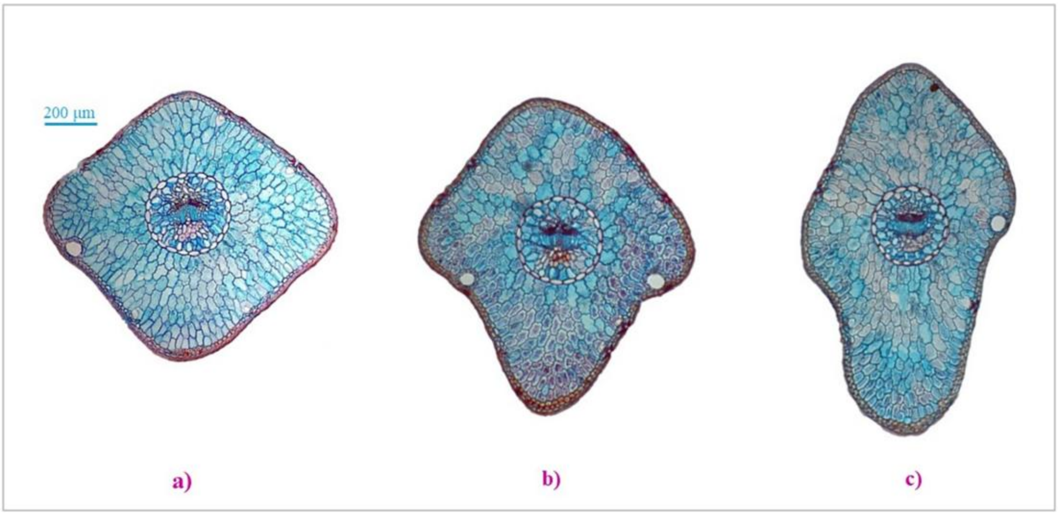

Figure 4. *Different shapes of transverse sections of Picea abies (L.) H. Karst. needles: (a) rhomboid, (b) mushroom-shaped, and (c) elliptical*

Using the analysis of the differences between the categories with a confidence interval of 95% - Duncan test (*Table 3*), statistically significant differences were confirmed between the parameters of needle thickness, needle width, resin canal diameter and area of the vascular bundle without epidermis of the phenogroup and the control group, and insignificant differences were found between the vascular bundle diameter without epidermis and flatness parameters in both one-year and two-year needles.

The thickness of all needles varied within the range of 928.06 µm to 1225.28 µm, and the width range was from 594.03 µm to 1124.36 µm. The thickness and width of one-year-old needles from the analyzed phenogroup ranged from 950.59 µm to 1181.84 µm and 648.91 µm to 1035.41 µm, while for two-year-old needles, the values were between 1000.72 µm to 1225.28 µm and 641.15 µm to 1124.36 µm (*Table 3*). Compared to the control tree, whose mean needle thickness values were 994.21 ± 37.58 µm (one-year-old) and 1031.63 ± 23.65 µm (two-year-old), the dimensions of the needles from the phenogroup differed by 66.34 µm and 57.92 µm, respectively. The mean thickness values for one-year-old and two-year-old needles in the phenogroup were 1060.55 ± 62.06 µm and 1089.55 ± 59.554 µm. The difference in needle width was 106.04 µm and 80.55 µm, respectively. The average width values for one-year-old and two-year-old needles in the phenogroup were 871.49 ± 122.04 µm and 879.99 ± 145.65 µm, while for the control tree, they were 765.45 ± 95.85 µm and 799.44 ± 76.07 µm.

Of the three trees in the phenogroup, Tree 2 stood out as having the highest values. The thickness of one-year-old needles for Tree 2 ranged from 1076.14 µm to 1181.84 µm, and for two-year-old needles from 1071.48 µm to 1225.28 µm. The width of one-year-old needles ranged from 902.35 µm to 1035.41 µm, and the width of two-year-old needles from 873.63 µm to 1124.36 µm.

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Table 3. *Anatomical characteristics of one-year-old and two-year-old needles from the phenogroup and the control tree of Picea abies (L.) H. Karst. (analysis of the differences between the categories with a confidence interval of 95% (Duncan test))*

Parameters	Mean	Min	Max	Std	SE
One-year old needles					
Ph - Nt	1060.55*a	950.59	1181.84	62.06	11.33
C - Nt	994.21*b	928.06	1030.84	37.58	11.88
Ph - Nw	871.49*a	648.91	1035.41	122.04	22.28
C - Nw	765.45*b	594.03	853.48	95.85	30.31
Ph - Rcd	52.95*a	26.90	82.55	18.54	4.63
C - Rcd	-*b	-	-	-	-
Ph - Avb	43996.62*a	32545.02	59089.14	8797.30	1606.17
C - Avb	37879.43*b	32086.24	41667.01	3707.28	1172.45
Ph - Dvb	220.81*a	115.45	274.29	47.59	8.69
C - Dvb	219.37*a	202.12	230.33	10.903	3.45
Ph - F	1.25*a	0.99	1.78	0.23	0.04
C - F	1.32*a	1.19	1.68	0.19	0.06
Two-year old needles					
Ph - Nt	1089.55*a	1000.72	1225.28	59.55	10.87
C - Nt	1031.63*b	1009.60	1073.99	23.65	7.48
Ph - Nw	879.99*a	641.15	1124.36	145.65	26.59
C - Nw	799.44*b	702.33	918.49	76.07	24.06
Ph - Rcd	65.65*a	36.05	90.66	16.43	2.99
C - Rcd	47.66*b	34.64	60.18	9.46	2.99
Ph - Avb	46108.98*a	35916.70	62604.55	7172.11	1309.45
C - Avb	38880.05*b	27125.23	44526.51	6481.03	2049.66
Ph - Dvb	226.03*a	115.45	282.33	47.14	8.61
C - Dvb	221.71*a	185.84	238.10	19.620	6.20
Ph - Fl	1.28*a	0.91	1.74	0.27	0.05
C - Fl	1.30*a	1.17	1.46	0.10	0.03

Ph – phenogroup; C – control tree; Mean – mean value; Min – minimum; Max – maximum; Std – standard deviation; SE – standard error of the mean; Nt - needle thickness (μm); Nw - needle width (μm); Rcd - resin canala diameter (μm); Avb - vascular bundle area without epidermis (μm^2); Dvb - vascular bundle diameter without epidermis (μm); Fl – flatness. *a and b indicate statistical differences between phenogroups, at the level of significance $p < 0.05$

The diameters of all resin canals varied from 26.90 μm to 90.66 μm and were located just below the adaxial side of the needle, positioned laterally to the vascular bundle. The vascular bundle is placed at the center of the needle and surrounded by a clearly defined endodermis. The diameter of the vascular bundle varied from 115.45 μm to 282.33 μm, and the area it occupied ranged from 27,125.23 μm^2 to 62,604.55 μm^2. The number of cells forming the endodermal ring varied from 16 to 23 (with a mean value of 20.25 ± 0.51). Resin canals (1-2) were observed in both the two-year-old and one-year-old needles of the studied individuals, with a mean value of 1.16. The diameter of the resin canals ranged from 26.90 μm to 90.66 μm, while in the one-year-old needles of the control group, these canals were not detected.

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Using the Tukey HSD test (*Table 4*), statistically significant differences in the parameters of one-year needles were confirmed according to: (a) needle thickness, which distinguishes genotype 2 in relation to all others, but also genotype 3 in relation to the control genotype 4; (b) needle width genotype 1 in relation to 2 and 4, as well as genotype 3 in relation to 2 and 4; (c) resin canal diameter of genotypes 1 and 3 in relation to control tree number 4, where resin canals were not identified, and it should be noted that genotypes 2 and 3 had some needles without resin canals; (d) area of vascular bundle without epidermis promotes genotype 1 compared to all others; (e) vascular bundle diameter without epidermis classifies all trees in the same group without statistically significant differences between them; (f) flatness is statistically insignificant only between genotypes 1 and 3 and g) needle length of genotypes 1, 2 and 3 in relation to control tree 4.

The results of the Tukey HSD test for two-year-old needles confirm the statistical significance according to: (a) needle thickness that separates genotypes 2 and 3 in relation to 4, as well as genotype 2 in relation to genotype 1; (b) needle width only an insignificance of genotype 4 compared to 1; (c) resin canal diameter genotype 1 compared to other genotypes, where resin canals were present in needles of all analyzed genotypes; (d) area of vascular bundle without epidermis promotes genotype 1 in relation to all others, as well as in one-year needles; (e) vascular bundle diameter without epidermis is statistically insignificant only between genotypes 2 and 4; (f) flatness is statistically insignificant only between genotypes 4 and 3 and (g) needle length, as well as with one-year-old needles, genotypes 1, 2 and 3 compared to the control genotype 4.

Table 4. *Tukey HSD test of morphoanatomical characteristics of one-year and two-year spruce needles, at the genotype level*

Parameter genotype	Nt	Nw	Rcd	Avb	Dvb	F	Nl
One-year old needles							
1	1026.20^{b}	975.15^{a}	62.61^{a}	49821.33^{a}	227.62^{a}	1.0552^{c}	20.190^{a}
2	1125.24^{a}	737.45^{b}	70.98^{a}	40296.62^{a}	225.69^{a}	1.5327^{a}	21.230^{a}
3	1030.22^{b}	901.86^{a}	41.33^{a}	41871.91^{a}	209.12^{a}	1.1478^{c}	21.175^{a}
4	994.21^{b}	765.45^{b}	0^{b}	37879.43^{b}	219.37^{a}	1.3103^{b}	14.700^{b}
Two-year old needles							
1	1053.18^{bc}	1020.05^{a}	78.14^{a}	54856.4^{a}	264.06^{a}	1.0387^{c}	26.840^{a}
2	1112.81^{a}	721.35^{c}	59.73^{b}	40874.4^{b}	227.95^{b}	1.5529^{a}	27.070^{a}
3	1102.66^{ab}	898.56^{b}	59.08^{b}	42596.1^{b}	186.09^{c}	1.2366	26.405^{a}
4	1031.63^{c}	799.44^{c}	47.66^{b}	38880.1^{b}	221.71^{b}	1.2977^{b}	19.175^{b}

Nt – needle thickness (μm), Nw – needle width (μm), Rcd - resin canal diameter (μm), Avb - area of vascular bundle without epidermis (μm2), Dvb - vascular bundle diameter without epidermis (μm), F - flatness, Nl -needle length (mm), *a, b and c are statistical differences between genotypes, at a significance level of $p < 0.05$

The results of the Fisher (LSD) test shown in *Table 5* indicate variations in the morphoanatomical characteristics of one-year and two-year-old needles for the analyzed parameters: (a) Needle thickness shows a non-significant relationship between genotypes 3 and 1, as in the Tukey test, but the relationships between

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genotypes 3 and 4, as well as 1 and 4, were also non-significant; (b) Needle width has an insignificant relationship between genotypes 4 and 2, as in the previous test, where the relationship between genotypes 1 and 3 was also insignificant; (c) Resin canal diameter has a non-significant relationship between genotypes 1 and 3, 3 and 2 and 2 and 4, as with the Tukey test, which showed non-significance between genotypes 1 and 2, noting that only needles of genotype 1 did not have needles without resin canals; (d) Area of vascular bundle without epidermis has the same insignificant relationships between genotypes 3 and 4, 4 and 2 and 2 and 4 as in the first test; (e) Vascular bundle diameter without epidermis according to the Fisher test has identical results to the Tukey test, showing no statistically significant differences in this parameter between the genotypes; (f) Flatness has an insignificant relationship between genotypes 1 and 3, as in the previous test, and (g) Needle length has no statistically significant differences between genotypes 1, 2 and 3 from the 100-year-old phenogroup, as in the Tukey (LSD) test.

The Fisher (LSD) test for two-year-old needles grouped the samples into homogeneous groups on the basis of which non-significance can be distinguished according to: (a) Needle thickness, as in the case of one-year-old needles, between genotypes 1 and 4, and 2 and 3; (b) Needle width was not recorded, unlike the Tukey test, where it was insignificant between genotypes 4 and 2; (c) Resin canal diameter only between genotypes 2 and 3, in contrast to the Tukey test, which showed insignificance between genotypes 2 and 4, 2 and 3, and 3 and 4; (d) Area of vascular bundle without epidermis between genotypes 3 and 2, 2 and 4, while the Tukey test showed insignificance between genotypes 3 and 4; (e) Vascular bundle diameter without epidermis according to the Fisher test has identical results to the Tukey test between all except for statistically significant differences in this parameter between genotypes 2 and 4; (f) Flatness between genotypes 4 and 3, as in the previous test, and g) Needle length, as in one-year-old needles, between genotypes of the phenogroup aged around 100 years, as in the Tukey (LSD) test (2-3, 2-1 and 1-3).

To assess the presence of statistically significant differences in the analyzed characteristics between the studied individuals, an ANOVA was performed. All morpho-anatomical traits were evaluated. Of the six traits tested, ANOVA revealed significant differences between the individuals for six characteristics in one-year-old and two-year-old needles (*Table 6*). Specifically, statistically significant differences were identified for two characteristics of the one-year-old needles (Rrc and Avb) and three characteristics of the two-year-old needles (Nt, Rcd, and Dvb). In contrast, no statistically significant differences were found for four traits in the one-year-old needles and three traits in the two-year-old needles.

To determine the proximity of the analyzed trees, a cluster analysis was conducted based on all the studied anatomical characteristics of the needles (length, thickness, width, resin canal diameter, vascular bundle area, vascular bundle diameter, and flatness). Multivariate cluster analysis indicates the separation of genotypes into two subclusters (*Fig. 5*). In the first subcluster, two subgroups were distinguished. The first subgroup with genotypes 2 and 3 is close in many features, especially in needle thickness and the appearance of one-year-old needles without resin canals, and the second subgroup distinguishes genotype 1 by the values of needle width, resin canal diameter and area of vascular bundle without epidermis in both one-year-old and two-year-old needles. The second subcluster isolated genotype 4, which differs significantly in terms of morphoanatomical parameters compared to the genotypes of the first

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DOI: http://dx.doi.org/10.15666/aeer/2302_37853804

subcluster, but is more similar to genotype 1 from the second subgroup of the first subcluster. Cluster analysis confirmed the separation of genotypes based on the morphoanatomical characteristics of one- and two-year-old needles, as supported by the results of the Tukey HSD and Fisher (LSD) tests.

Table 5. *Fisher (LSD) test of morphoanatomical characteristics of one- and two-year-old spruce branches, at the genotype level - analysis of the differences between the categories with a confidence interval of 95%*

Genotype	**LS means**	**Groups**				**Genotype**	**LS means**	**Groups**			
One-year old needles						**Two-year old needles**					
Needle thickness (μm)											
2	1125.24	A				2	1112.81	A			
3	1030.22		B			3	1102.66	A			
1	1026.20		B			1	1053.18		B		
4	994.21			C		4	1031.63		B		
Needle width (μm)											
1	975.15	A				1	1020.05	A			
3	901.86		B			3	898.56		B		
4	765.45			C		4	799.45			C	
2	737.45			C		2	721.35				D
Resin canal diameter (μm)											
1	37.57	A				1	78.13	A			
3	33.10	A	B			2	59.73		B		
2	14.19		B	C		3	59.08		B		
4	0.00			C		4	47.66			C	
Area of vascular bundle without epidermis (μm^2)											
1	49821.33	A				1	54856.39	A			
3	41871.91		B			3	42596.11		B		
2	40296.62		B			2	40874.41		B	C	
4	37879.43		B			4	38880.05			C	
Vascular bundle diameter without epidermis (μm)											
1	227.62	A				1	264.06	A			
2	225.69	A				2	227.95		B		
4	219.37	A				4	221.71		B		
3	209.12	A				3	186.09			C	
Flatness											
2	1.533	A				2	1.553	A			
4	1.310		B			4	1.298		B		
3	1.148			C		3	1.237		B		
1	1.055			C		1	1.039			C	
Needle length (mm)											
2	21.230	A				2	27.070	A			
3	21.175	A				1	26.840	A			
1	20.190	A				3	26.405	A			
4	14.700		B			4	19.175		B		

*A, B, C and D are statistical differences between genotypes, at a significance level of $p < 0.05$

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Table 6. *ANOVA Significance analysis for anatomical characteristics of Norway spruce on Čemernik*

	One-year-old						**Two-year-old**					
			1	**2**	**3**	**4**			**1**	**2**	**3**	**4**
Trait	*F*	*p*	*avg*	*avg*	*avg*	*avg*	*F*	*p*	*avg*	*avg*	*avg*	*avg*
Nl	155.26	ns	20190	21230	21175	14700	9.80	ns	26840	27070	26405	19175
Nt	18.68	ns	1026.20	1125.24	1030.22	994.21	6.24	0.002**	1053.18	1112.81	1102.66	1031.63
Nw	20.93	ns	975.15	737.45	901.86	765.4488	27.77	ns	1020.051	721.3544	898.5559	799.4454
Rcd	4.71	0.007**	37.57	14.19	33.06	0	9.09	0.000***	78.13	59.73	59.08	47.66
Avb	5.21	0.004**	49821.33	40296.62	41871.91	37879.43	26.38	ns	54856.42	40874.41	42596.11	38880.05
Dvb	0.39	ns	227.62	225.69	209.12	219.37	9.79	0.000***	264.06	227.95	186.09	221.71
Fl	25.70	ns	1.06	1.54	1.15	1.32	17.97	ns	1.04	1.56	1.24	1.30

F – F-value; *p* – *p*-value; avg – average; Nl – needle length; Nt – needle thickness; Nw – needle width; Rcd – resin canala diameter; Avb – vascular bundle area; Dvb – vascular bundle diameter; Fl – flatness; ns - no significance; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

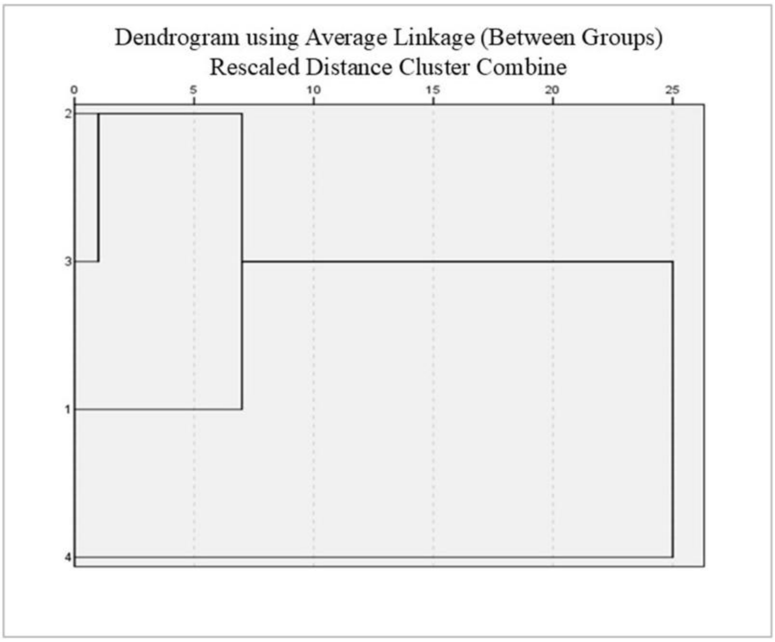

Figure 5. *Dendrogram of the cluster analysis of anatomical characteristics of Norway spruce needles*

Bearing in mind that the Tukey and Fisher tests show mostly consistent patterns of homogeneous groups, but also that certain differences were recorded that may be conditioned by the genotype, as well as the influence of the age of the trees, and to confirm the significance of the identified ecotypes, a comparison of the phenogroup with the control tree was performed using the Principal Component Analysis (PCA).

Multivariate statistical analysis involving PCA indicates an overlap between of the control tree and phenogroup of 100-year-old spruces in Brod on Mount Čemernik based on all observations from all seven morpho-anatomical characteristic of spruce needles: needle length, needle thickness, needle width, resin canal diameter, area of vascular bundle, vascular bundle diameter and flatness. For a visual presentation of the results, a scatter plot was used to show the characteristics of the needles that affect separation and

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overlap (*Fig. 6*). Although the anatomical characteristics of the groups/trees differ partially, some overlaps were observed due to the intergroup variability of the analyzed traits, namely the first two principal components explain 74.4% (47.1% and 27.3% respectively) for one-year and 73% (52.5% and 20.5% respectively) for two-year needles of the data variability (*Fig. 6a, b*). The obtained finding highlights the superiority of phenogroup trees that are about 100 years old, which is confirmation of their adequate physiological and functional properties. Significant differences (*Fig. 6c, d*) are distinguished by PCA for the morphological characteristics of the needles of the phenogroup of old trees with very high variations (PC1 70.5%) for one-year and (PC1 72.5%) for two-year spruce needles. Patterns of morphological variability of needles indicate phenotypic changes, but also adaptation and can be a starting point for a spruce conservation program according to climate change projections.

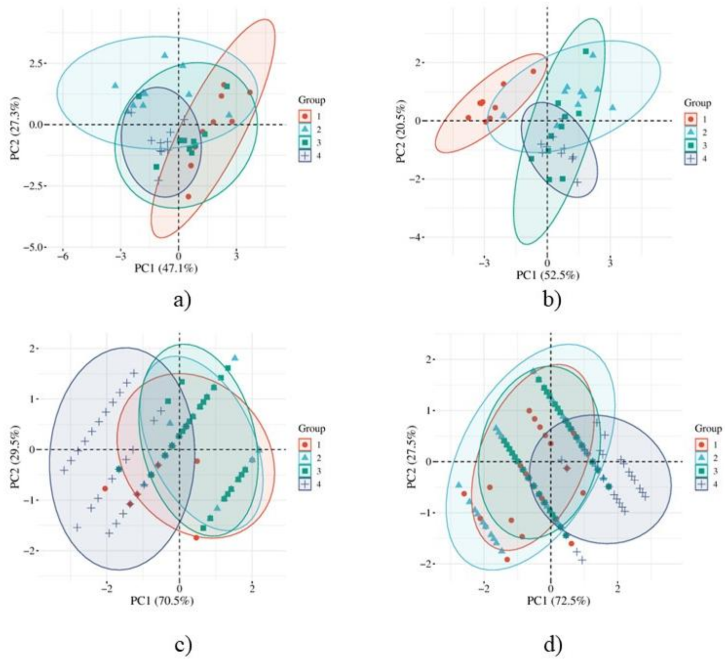

Figure 6. *Principle component analysis (PCA) of seven morpho-anatomical traits of Norway spruce needles (a) one-year anatomical components (b) two-year anatomical components (c) one-year morphological components and (d) two-year morphological components*

Discussion

Temperate zone forests are currently facing significant and challenging events due to climate change, which greatly impact forest stands and their functioning (Millar and Stephenson, 2015; Seidl et al., 2014; Seidl et al., 2017). Forest tree gene ecology, in addition to gene conservation, is crucial for predicting and adapting woody plants to climate change and for successful reforestation efforts. Due to changes in the global climate and atmospheric composition, needle morphology characteristics throughout the tree’s life cycle have become increasingly important (Pensa et al., 2004). Abiotic factors such as altitude, air temperature, atmospheric pressure, light, precipitation, wind speed, and average annual temperatures influence the physiological, anatomical, and morphological traits of needles (Kašpar et al., 2017; De La Torre et al., 2021). The geographic region and existing site conditions, along with the genetic characteristics of

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the species, largely determine the quantitative traits of needles (Živanović and Jokanović, 2023). The morphological and anatomical structure of needles reflects the adaptation of woody species to changing environmental conditions (Xing et al., 2014). Westergren et al. (2018) suggest that differentiation among populations is influenced by the location within the species' growing area. In the future, due to rapid climate changes, the adaptability of Norway spruce may diminish (Frank et al., 2017). According to Lin et al. (2001), elevated atmospheric CO_2 concentration affects the morphology and structure of plant vegetative organs by increasing needle thickness and phloem area. The study of population and individual variability of the conifer species has practical significance in genetically based researches (Ocokoljić, et al., 2022). Population variation in morphology, as well as the phenophases of needle formation in Norway spruce, are strongly correlated with air temperature and altitude (Frank et al., 2017). Furthermore, according to Zhu et al. (2022), spring air temperatures influence the morphology of Norway spruce needles. Mihai (2020) emphasizes the high adaptive genetic variability of Norway spruce, noting that genetic diversity will allow this species to better tolerate the negative effects of climate warming. A study in the Czech Republic (Matějka and Krpeš, 2022) confirmed the significant variability in Norway spruce needle traits that adapt to specific site conditions during population growth, with solar radiation having the greatest influence on crown growth. They analyzed Norway spruce needle transverse sections and processed data such as width, thickness, and vascular bundle diameter. The most distinct differences were found in artificially established stands. These artificial stands exhibited the least variability in needle traits, whereas natural populations displayed a diverse range of phenotypic variability, likely due to natural selection and plant adaptation to environmental conditions.

Research on the morphology of one-year-old needles of *Picea* A. Dietr. species has been conducted by Gebauer et al. (2011), Matějka et al. (2014), and Matějka and Krpeš (2022), while studies on two-year-old needles have been performed by Milovanović et al. (2005), Nikolić et al. (2013), and Radovanović et al. (2014). In our study, both one-year-old and two-year-old needles were analyzed, with the two-year-old needles showing higher values for all investigated characteristics. In terms of needle cross-sectional shapes, elliptical, mushroom-shaped, and rhombic shapes were recorded. Similar shapes were observed for the species *P. omorika* (Pančić) Purk., with Radovanović et al. (2014) reporting elliptical, triangular and rhombic shapes, and Nikolić et al. (2013) noting elliptical, triangular, and square shapes, which correspond to many other species within the spruce genus.

The length of one-year-old Norway spruce needles exposed to light, as reported by Gebauer et al. (2011), ranged from 8.9 to 12.4 mm, while in our study conducted on Čemernik Mountain, the lengths varied from 12.0 to 28.0 mm for one-year-old needles and 18.0 to 32.0 mm for two-year-old needles. The needle length of *Picea omorika* (Serbian spruce), according to Milovanović et al. (2005), ranged from 12.1 to 19.7 mm, while Nikolić et al. (2013) reported an average of 1.36 mm. Popović et al. (2022), studied silver fir—another genus in the same family, *Pinaceae* Spreng. ex F. Rudolphi—and reported needle lengths ranging from 20.8 to 27.5 mm.

Similar to the findings of Matějka et al. (2014) and Matějka and Krpeš (2022), the thickness of Norway spruce needles analyzed in our study was greater than the needle width, a characteristic described in the literature as an "inversive needle type." The thickness of one-year-old needles in our research (950.59–1181.84 μm) showed a coefficient of variation ranging from 1.97% to 5.70%, while the aforementioned study

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DOI: http://dx.doi.org/10.15666/aeer/2302_37853804

from the Czech Republic reported a variation of 7.6–23.9% for one-year-old needles. The coefficient of variation for two-year-old needles in our study ranged from 2.29% to 6.49%. For easier comparison millimeters (mm) from some references have been converted into micrometers (µm), as in our research. According to the research by Gebauer et al. (2011), needle thickness ranged from 1060 to 1250 µm. In our study, the maximum thickness of two-year-old needles was recorded on Tree 2 (1225.28 µm), while the minimum thickness (1009.60 µm) was observed on the control tree.

In terms of needle width, Gebauer et al. (2011) reported values of 1100–1220 µm for one-year-old needles, while in our study, values ranged from 650 to 1030 µm. According to Nikolić et al. (2013), width and thickness were the most variable traits in the studies of Serbian spruce, a species within the same genus. For *P. omorika*, Nikolić et al. reported a needle width of 1490 µm and thickness of 820 µm. In the study by Radovanović et al. (2014), needle width varied between 1115 and 1832 µm, while thickness ranged from 637.1 to 944.7 µm. For the same parameters, Milovanović et al. (2005) reported widths of 1000 to 1900 µm and thicknesses of 738.9 to 880.95 µm. Regarding silver fir, Popović et al. (2022) found that needle width ranged from 1500 to 2200 µm and thickness from 340 to 640 µm.

Needle flatness, defined as the ratio of width to thickness, was reported by Gebauer et al. (2011) to be between 0.89 and 1.14, which is comparable to the values in our study (0.99 to 1.78). Average values for individual trees in Matějka and Krpeš (2022) were significant for both width and thickness, as well as the cross-sectional area of the vascular bundle. Needle traits varied between individual trees, as well as within single individuals, which was also observed in our study where significant variability in certain characteristics was found among different individuals.

The diameter of the vascular bundle in the needles of Serbian spruce ranged from 151 to 286 µm (Radovanović et al., 2014), while in Milovanović et al. (2005) it was between 205.58 and 278.18 µm, and in our study, it ranged from 220.81 µm to 115.45 µm. The resin canals of *Picea omorika* were located directly beneath the adaxial side of the leaf, in contact with the epidermis (external type), and positioned laterally to the vascular bundle (Radovanović et al., 2014), which aligns with our findings as well. According to Marco (1939), the diameter of the resin canal in Norway spruce ranges from 70 to 440 µm, while in Serbian spruce, it ranges from 28 to 165 µm (Radovanović et al., 2014), 71 to 87 µm (Milovanović et al., 2005), and 51.82 µm (Nikolić et al., 2013). In our study, the diameter ranged from 36 to 90 µm, with an average of 65 µm.

The number of resin canals in Serbian spruce was 0.74 (Nikolić et al., 2013), while in the two-year-old needles of the Norway spruce trees in our study, the average number was 1.53.

In the study by Matějka and Krpeš (2022), all needle parameters were larger in the planted populations compared to the natural populations, indicating the influence of artificial selection. Thus, population classification results showed significant differences between populations. In our research, hundred-year-old trees were planted, and they demonstrated superiority. However, a control tree was also planted, and it did not exhibit significant differences in morpho-anatomical traits, which may be due to the influence of the genotype itself. Another possible explanation for these differences is the positioning of the trees. The phenogroup grew in an open terrain, exposed to sunlight, while the control tree was located within a dense population. This aligns with findings by Stenberg et al. (1999), Richardson et al. (2000, 2001), Sellin (2001), and Niinemets et al. (2007), which indicate that increased light exposure and canopy

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openness influence anatomical features. Additionally, based on research by Gebauer et al. (2011), the values of morphological and anatomical traits of needles were found to increase with tree height and light intensity. Needle width, cross-sectional area, surface area, and flatness differed between the upper and lower parts of the crown within the same individual. In our study, these traits were analyzed in needles collected at the same height of 2 m, but it can be concluded that dimensions were larger in hundred-year-old trees reaching heights of approximately 50 m. Additionally, in our research, the hundred-year-old planted trees exhibited larger parameter values than those of the control tree from planted population, which reached a height of 27 m. Given these findings, it is clear that the use of morpho-anatomical traits in plant species plays a critical role in studying geographic variation (Weng and Jackson, 2000) and population diversity (Nikolić et al., 2013).

Conclusions

In light of the climate scenarios projected by the WMO, a shift in the distribution range of conifer species, including Norway spruce, is anticipated. This makes it crucial to understand the morpho-anatomical traits of needles, as they can indicate adaptations to changing environmental conditions. Additionally, studying these traits is essential for understanding the geographic variability and population diversity of this key species for both forestry and urban forestry. Traits such as needle length, needle thickness, vascular bundle diameter, and the diameter of the resin canal showed more significant differences in the analyzed phenogroup of century-old trees compared to the control tree. Moreover, the analyzed individuals exhibited a closer similarity than the control tree.

Considering the results of our study, we recommend the selection of all three old Norway spruce trees, which display exceptional phenotypic characteristics and strong adaptability, with a particular emphasis on Tree 2. We further propose the protection and conservation of this phenogroup to facilitate the selection of individuals that can serve as starting material for establishing specialized plantations. A systematic approach to gene conservation is also recommended to preserve the genotypes of these outstanding Norway spruce specimens, which have shown tolerance to the adverse effects of climate warming and change. These trees have thrived for a century in isolation, in a consistently sun-exposed environment, and exhibit excellent adaptability to conditions atypical for the species.

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