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Headspace volatiles isolated from twigs of *Picea omorika* from Serbia

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Abstract: The variability of volatiles isolated from twigs by the static headspace (HS) method in seven natural populations of *Picea omorika* from Serbia was investigated for the first time. In the overall chemical profile, monoterpenes strongly dominated hydrocarbons as the most volatile compounds (95.7%). The dominant compounds were *α*-pinene (29.5 %), *β*-pinene (25.7%) and myrcene (13.0%), totaling 68.2% of the volatiles on average. The following nine volatiles were found to be present in medium-to-high amounts (0.5-10%): tricyclene, camphene, *α*-phellandrene, *δ*-3-carene, *p*-cymene, *β*-phellandrene, terpinolene, (*E*)-caryophyllene, and germacrene D. Out of the 78 volatiles detected, the six most abundant (*α*-pinene, *β*-pinene, myrcene, *δ*-3-carene, *β*-phellandrene and camphene) were selected for principle component analysis (PCA) and cluster analyses (CA). PCA revealed a high degree of similarity between populations, while CA showed a degree of separation of two populations from the others. The presented results are in agreement with previous phytochemical and molecular analyses of this species that confirm high variability in both specialized metabolites and genetic markers.

Keywords: *Picea omorika*; headspace method; PCA, cluster; volatile compounds

INTRODUCTION

Picea omorika (Panč.) Pürkyne is a relict and endemic conifer species that grows in small populations in the upper Drina River valley in Serbia and Bosnia and Herzegovina, as well as in the Mileševka River canyon in Serbia, near the city of Prijepolje, with a few small isolated populations in southeastern Bosnia and Herzegovina [1,2]. Shoots hang from long, pendulous up-curved branches [3]. Like all other conifers, *P. omorika* produces a mixture of volatile compounds, referred to as essential oils or oleoresins, that are stored in resin ducts in the trunk, branches, leaves (needles), buds and cones.

So far, several biochemical and genetic analyses of Serbian spruce have been performed [4-9], including chemical profiles of terpenes extracted from *P. omorika* needles [10-12]. There is only one study of essential oil from twigs and needles of Serbian spruce [13]. To analyze the composition of volatiles, several different techniques have been used, including hydrodistillation (HD), simultaneous distillation and extraction (SDE), pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), etc. [11,12,14-17]. The profile of the volatiles and yields varied depending on the method used, and in some cases, because of the reactivity of individual compounds, artefacts were formed during the isolation process [17].

While the supercritical fluid extraction step showed both the highest yield and the fewest artefacts, this technique is laborious and expensive for day-to-day use in the laboratory. Furthermore, for this technique, at least 1 g of plant material is needed. This could be

a significant problem in analyses where plant material is poor in volatiles or where damage to the plant from repetitive sampling would be too high, e.g. when studying the influence of stress or environmental factors on the production of volatiles. This problem can be solved with use of the headspace technique, which is both inexpensive and requires only a small amount of plant material, usually less than 0.1 g. Furthermore, since this technique is very sensitive, and individual needles or parts of a shoot can be used.

The HS method was used for studying needle volatiles of several spruces, including *Picea engelmannii* Parry ex Engelm. needles [18], *P. omorika* and *P. abies* (L.) H. Karst. [16]. Although twig volatiles were analyzed using this technique in several other conifers such as *Pinus densiflora* Siebold & Zucc.[19], to the best of our knowledge the isolation of volatiles from twigs of *P. omorika* from natural populations has never been performed using the HS technique. The resin ducts in leaves are not connected to the resin ducts in twigs, so the composition can be significantly different, and it depends on the ontological development of the organ [14]. The volatiles stored in branch resin ducts are used in a plant's defense against insects and pathogens [20,21], hence the diversity in volatile composition is paramount for the protection of natural populations from possible pest infestation. Additionally, the resin ducts in *P. omorika* leaves are close to the leaf surface, lying under just one layer of epidermal cells [22], so the influence of some environmental factors like the degree of insulation, temperature, etc. on the quantitative and qualitative profile of volatiles stored there could be much greater than the profile of volatiles in twigs.

The aim of this paper was to analyze the variability of twig volatiles collected from 100 individuals growing in natural populations in Serbia using the novel headspace method for the first time from the standpoint of this taxon's chemophenetics. The basic hypothesis is that twig volatiles are different from those in leaves and that they can be used in chemophenetic studies.

MATERIALS AND METHODS

Plant material

Small, two-year-old twigs with needles were collected at the same height from mature *Picea omorika* trees of approximate age in late summer and early autumn 2017 for researching the HS twig volatiles at the population level. Having in mind that *P. omorika* is an endangered species [23], twigs (each shorter than 10 cm) from fifteen individual trees were taken in six populations (ZVI – Zvijezda, ŠTU – Štula, BILO – Bilo, CRST – Crvene Stene, VRA – Vranjak and ZP – Zmajevački Potok). For the same reason, in one population, MIL – Mileševka River canyon, described as var. *vukomanii* [24], samples from only 10 trees out of 100 trees were taken. Fresh twigs were packed and hermetically sealed in zip bags and kept on ice until stored in the freezer. Voucher specimens were deposited at -20°C at the Institute of Forestry, Belgrade, Serbia. A map of the studied area is shown in Supplementary Fig. S1; the corresponding geographical and geological data of the seven populations are presented in Supplementary Table S1.

Extraction and isolation

The needles of freshly frozen samples were removed just before volatile extraction. A single sample was taken from each tree. For static HS experiments, about 2-cm-long, 2-mm-thick whole twigs without needles were placed into 10 mL HS vials. The samples were heated at 90°C for 20 min using the following program: incubation temperature: 90°C; incubation time: 1500 s; syringe temperature: 100°C; agitator speed: 500 rpm; fill speed: 100 μL/s; pullup delay: 1000 ms. For analysis of volatiles, 2000 μL of generated vapor was extracted from the vial and injected directly into the gas chromatograph using a heated gas-tight syringe [25,26].

Gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC/MS) analyses

GC-FID and GC/MS analyses were carried out with an Agilent 7890A apparatus equipped with a 5975C MSD, FID, and an HP-5MSI fused-silica capillary column 30 m × 0.25 mm × 0.25 μm. For the HS analyses, 2000 μL of generated vapor was extracted from the vial and injected directly into the gas chromatograph using a heated gas-tight syringe (105°C). The oven temperature was programmed linearly, rising from 60°C to 315°C for 15 min; injector: 250°C; FID detector: 300°C; carrier gas, He (1.0 mL/min at 210°C), injection volume

was 2 mL, split ratio, 10:1; EI-MS (70 eV), *m*/z range 40 to 550.

Compound Identification

Identification of all the compounds in the analyses was matched by comparison of their linear retention indices (relative to C8-C36 *n*-alkanes on the HP-5MSI column) and MS spectra with those of standards from NIST11 and homemade MS library databases. The relative contents of the identified compounds were calculated from the GC peak areas.

Statistical analyses

Standard univariate statistical tests were performed prior to multivariate tests (arithmetic means and standard deviations, frequencies, histograms, one-way analyses of variance (ANOVA)). In *P. omorika* twigs, the six most abundant terpenes, *α*-pinene, *β*-pinene, myrcene, *δ*-3-carene, *β*-phellandrene and *α*-phellandrene, were selected for the PCA. The PCA, as a descriptive multivariate method capable of suggesting the structure and tendency of the data set, as well as CA, were carried out using the software Statgraphics Plus (ver. 5.0; Statistical Graphics Corporation, USA) [27]

Table 1. Compositions of different classes of terpenes in *Picea omorika* twig volatiles. Individual compounds are presented on Table 2.

Class	Population[a)]							
	ZVI	ŠTU	BI	CRST	VRA	ZP	MIL	Average
Monoterpene hydrocarbons	96.8	95.4	96.3	96.6	95.5	94.3	92.9	95.4
Oxygenated monoterpenes	0.3	0.5	0.2	0.3	0.2	0.6	0.3	0.3
Total monoterpenes	**97.1**	**95.9**	**96.5**	**96.9**	**95.7**	**94.9**	**93.2**	**95.7**
Sesquiterpene hydrocarbons	2.8	4	2.6	2.5	4	4.2	6.6	3.8
Total sesquiterpenes	**2.8**	**4**	**2.6**	**2.5**	**4**	**4.2**	**6.6**	**3.8**
Others [b)]	0.1	0.1	0.9	0.5	0.3	0.8	0.2	0.5
Unknown	0	0	0	0.1	0	0.1	0	0
Total (%)	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

[a)]Mean values for each population, *cf.* Plant material; [b)]Mainly aliphatic aldehydes and alcohols

RESULTS

Volatile composition of *P. omorika* twigs

In the overall terpene profile of the twigs, monoterpene hydrocarbons strongly predominated, comprising 95.7% of total volatiles (Table 1). The dominant compounds were *α*-pinene (29.5% on average), *β*-pinene (25.7% on average) and myrcene (13.0% on average), comprising 68.2% of total volatiles (Table 3). The following nine volatiles were found in medium-to-high amounts (0.5-10%): tricyclene, camphene, *α*-phellandrene, *δ*-3-carene, *p*-cymene, *β*–phellandrene, terpinolene, (*E*)-caryophyllene and germacrene D (Table 2).

Table 2. Mean values and (standard deviations) of terpene compounds of seven populations of *Picea omorika* twigs.

Entry	Compound	Population*								Average (n=100)
		RI	ZVI (n=15)	ŠTU (n=15)	BI (n=15)	CRST (n=15)	VRA (n=15)	ZP (n=15)	MIL (n=10)	
1.	Hexanal	794	0.1 (0.04)	0.1 (0.06)	0.2 (0.10)	0.1 (0.08)	0.1 (0.08)	0.3 (0.25)	0.1 (0.00)	0.1 (0.04)
2.	4-methyl- Pentanol	832	0.0 (0.03)	0.0 (0.03)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.0 (0.03)	0.0 (0.00)	0.0 (0.02)
3.	*cis*-3-Heksen-1-ol	845	0.0 (0.05)	0.0 (0.04)	0.3 (0.00)	0.1 (0.08)	0.1 (0.11)	0.1 (0.08)	0.0 (0.00)	0.1 (0.02)
4.	*n*-Hexanol	856	0.0 (0.06)	0.0 (0.04)	0.4 (0.38)	0.3 (0.15)	0.3 (0.18)	0.3 (0.11)	0.1 (0.00)	0.2 (0.23)
5.	Santene	879	0.2 (0.08)	0.3 (0.16)	0.8 (0.47)	0.5 (0.43)	0.2 (0.19)	0.2 (0.11)	0.1 (0.05)	0.4 (0.35)
6.	Tricyclene	919	0.5 (0.18)	0.6 (0.26)	0.8 (0.24)	0.9 (0.30)	0.6 (0.24)	0.5 (0.24)	0.7 (0.18)	0.6 (0.27)
7.	*α*-Thujene	922	0.7 (0.09)	0.2 (0.13)	0.1 (0.04)	0.2 (0.11)	0.2 (0.16)	0.2 (0.13)	0.2 (0.09)	0.2 (0.11)
8.	***α*-Pinene**	**929**	**28.1 (10.63)**	**28.5 (7.14)**	**28.9 (5.76)**	**35.7 (9.93)**	**33.9 (4.71)**	**21.0 (5.80)**	**30.1 (7.07)**	**29.5 (8.64)**
9.	Camphene	943	4.0 (2.17)	4.1 (2.00)	5.1 (1.84)	7.4 (2.86)	3.6 (1.86)	2.7 (1.00)	6.3 (2.39)	4.7 (2.51)
10.	Thuja-2,4(10)-diene	947	0.0 (0.00)	0.0 (0.06)	0.0 (0.06)	0.0 (0.04)	0.0 (0.03)	0.1 (0.25)	0.0 (0.04)	0.0 (0.11)
11.	n.i. 1	967	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.0 (0.00)	0.1 (0.36)	0.0 (0.00)	0.0 (0.14)
12.	Sabinene	969	0.5 (0.56)	0.1 (0.28)	0.0 (0.08)	0.2 (0.22)	0.0 (0.05)	0.6 (0.59)	0.2 (0.70)	0.2 (0.40)
13.	***β*-Pinene**	**973**	**23.8(10.66)**	**27.1 (6.92)**	**27.8 (4.75)**	**17.1 (5.64)**	**35.5 (5.22)**	**23.1 (8.94)**	**26.3 (7.33)**	**25.7 (8.81)**
14.	**Myrcene**	**989**	**8.9 (5.34)**	**15.1 (5.68)**	**15.7 (8.79)**	**14.8 (5.27)**	**6.5 (2.95)**	**16.8 (5.92)**	**12.8 (3.90)**	**13.0 (6.68)**
15.	Ethyl hexanoate	993	0.0 (0.00)	0.0 (0.03)	0.0 (0.03)	0.0 (0.00)	0.0 (0.00)	0.0 (0.03)	0.0 (0.00)	0.0 (0.02)

Table 2. continued.

16.	*α*-Phellandrene	1003	1.6 (0.75)	1.5 (0.92)	2.9 (1.53)	2.2 (1.13)	3.1 (0.95)	2.5 (1.08)	1.6 (0.88)	2.2 (1.20)
17.	**δ-3-Carene**	**1008**	**18.2 (17.35)**	**7.1 (11.36)**	**3.0 (2.69)**	**8.2 (7.85)**	**1.1 (0.64)**	**16.2 (16.29)**	**5.8 (2.58)**	**8.6 (12.07)**
18.	*α*-Terpinene	1015	0.2 (0.08)	0.1 (0.09)	0.1 (0.05)	0.1 (0.08)	0.1 (0.05)	0.1 (0.10)	0.1 (0.04)	0.1 (0.08)
19.	*p*-Cymene	1021	1.2 (0.37)	0.9 (0.28)	1.1 (0.39)	0.9 (0.49)	0.5 (0.25)	1.2 (1.03)	0.6 (0.22)	0.9 (0.57)
20.	***β* -Phellandrene**	1026	**8.0 (2.60)**	**9.1 (1.92)**	**9.5 (1.36)**	**7.5 (2.04)**	**9.6 (0.95)**	**7.8 (2.41)**	**7.3 (1.53)**	**8.5 (2.07)**
21.	(*Z*)-*β*-Ocimene	1034	0.0 (0.04)	0.0 (0.04)	0.0 (0.05)	0.0 (0.05)	0.0 (0.03)	0.0 (0.04)	0.0 (0.05)	0.0 (0.05)
22.	(*E*)-*β*-Ocimene	1045	tr	tr	tr	tr	tr	tr	tr	tr
23.	*γ*-Terpinene	1056	0.2 (0.20)	0.1 (0.10)	0.1 (0.05)	0.1 (0.07)	0.0 (0.04)	0.2 (0.15)	0.0 (0.00)	0.1 (0.12)
24.	Terpinolene	1086	1.2 (0.88)	0.6 (0.53)	0.5 (0.21	0.8 (0.50)	0.5 (0.10)	1.2 (0.88)	0.7 (0.18)	0.8 (0.62)
25.	n.i. 2	1097	tr	tr	tr	tr	tr	tr	tr	tr
26.	n.i. 3	1112	tr	tr	tr	tr	tr	tr	tr	tr
27.	n.i. 4	1119	tr	tr	tr	tr	tr	0.0 (0.02)	tr	0.0 (0.01)
28.	n.i. 5	1122	tr	tr	tr	tr	tr	0.0 (0.05)	tr	0.0 (0.02)
29.	n.i. 6	1136	0.0 (0.03)	tr	tr	tr	tr	tr	tr	0.0 (0.02)
30.	*trans*-Pinocarveol	1136	tr	tr	tr	tr	tr	0.1 (0.03)	tr	0.1 (0.01)
31.	Camphor	1142	0.0 (0.05)	0.0 (0.03)	tr	0.0 (0.03)	tr	0.0 (0.03)	tr	0.0 (0.05)
32.	Borneol	1163	0.0 (0.06)	0.1 (0.06)	0.0 (0.02)	0.1 (0.07)	tr	0.1 (0.03)	0.0 (0.05)	0.0 (0.14)
33.	n.i. 7	1172	0.0 (0.04)	0.0 (0.04)	0.0 (0.03)	0.1 (0.03)	tr	tr	tr	0.0 (0.03)
34.	n.i. 8	1176	0.0 (0.03)	tr	tr	tr	tr	0.0 (0.03)	tr	0.0 (0.01)
35.	n.i. 9	1182	tr	tr	tr	tr	tr	0.0 (0.05)	tr	0.0 (0.01)
36.	n.i. 10	1185	tr	tr	tr	0.0 (0.05)	tr	tr	tr	0.0 (0.02)
37.	Myrtenal	1194	0.0 (0.03)	tr	0.0 (0.03)	tr	tr	0.1 (0.05)	tr	0.1 (0.07)
38.	Verbenone	1206	tr	tr	tr	tr	tr	0.0 (0.05)	tr	0.1 (0.02)
39.	Fenchyl acetate	1218	tr	tr	tr	tr	tr	0.0 (0.03)	tr	0.0 (0.01)
40.	Thymol, methyl ether	1232	0.1 (0.05)	0.1 (0.06)	0.1 (0.10)	0.1 (0.13)	0.1 (0.05)	0.1 (0.10)	tr	0.1 (0.09)
41.	Linalool acetate	1254	0.0 (0.05)	0.0 (0.05)	tr	0.0 (0.03)	0.0 (0.04)	0.1 (0.05)	0.1 (0.05)	0.0 (0.05)
42.	Bornyl acetate	1284	tr	tr	tr	0.0 (0.03)	0.0 (0.03)	tr	tr	0.0 (0.01)
43.	*α*-Terpinyl acetate	1348	0.2 (0.16)	0.3 (0.28)	0.1 (0.07)	0.1 (0.10)	0.1 (0.06)	0.1 (0.04)	0.2 (0.17)	0.1 (0.16)
44.	*α*-Ylangene Longicyclene	1370	0.0 (0.08)	0.0 (0.05)	0.1 (0.09)	0.0 (0.05)	0.1 (0.06)	tr	0.1 (0.07)	0.0 (0.06)
45.	*α*-Copaene	1374	0.2 (0.11)	0.2 (0.13)	0.2 (0.14)	0.1 (0.10)	0.2 (0.11)	0.2 (0.15)	0.2 (0.09)	0.2 (0.12)
46.	*β*-Cubebene	1389	0.0 (0.03)	tr	tr	0.0 (0.03)	tr	tr	0.0 (0.05)	0.0 (0.02)
47.	*β*-Elemene	1393	tr	tr	0.0 (0.03)	tr	tr	0.0 (0.06)	0.0 (0.05)	0.0 (0.03)
48.	n.i. 11	1398	0.0 (0.03)	0.0 (0.04)	tr	tr	tr	tr	tr	0.0 (0.02)
49.	Longifolene	1403	0.1 (0.12)	0.3 (0.26)	0.2 (0.38)	0.1 (0.21)	0.1 (0.07)	0.0 (0.05)	0.3 (0.36)	0.2 (0.25)
50.	n.i. 12	1415	tr	tr	tr	tr	tr	tr	tr	tr
51.	(*E*)-Caryophyllene	1418	0.4 (0.26)	0.5 (0.27)	0.3 (0.20)	0.3 (0.16)	0.6 (0.13)	0.5 (0.17)	0.8 (0.16)	0.5 (0.24)
52.	*β*- Copaene	1428	tr	0.1 (0.05)	0.1 (0.13)	0.0 (0.05)	0.1 (0.07)	0.1 (0.05)	0.2 (0.07)	0.1 (0.08)
53.	*α*- (*E*)-Bergamotene	1435	0.1 (0.09)	0.1 (0.09)	tr	0.0 (0.05)	0.1 (0.06)	0.0 (0.08)	tr	0.1 (0.07)
54.	*α*- Humulene	1453	0.1 (0.18)	0.1 (0.05)	0.0 (0.04)	0.0 (0.04)	0.1 (0.03)	0.1 (0.05)	0.1 (0.00)	0.1 (0.08)
55.	n.i. 13	1458	tr	tr	tr	tr	tr	0.0 (0.03)	tr	0.0 (0.01)
56.	(Z)-Muurola-4(14), 5-diene	1463	tr	0.0 (0.04)	0.0 (0.05)	0.0 (0.04)	0.0 (0.04)	tr	0.1 (0.04)	0.0 (0.04)
57.	4,5-di-epi-Aristolochene	1468	tr	0.0 (0.04)	0.0 (0.04)	0.0 (0.03)	0.0 (0.04)	0.0 (0.06)	0.1 (0.03)	0.0 (0.04)
58.	*γ*-Muurolene	1476	0.1 (0.03)	0.2 (0.01)	0.2 (0.28)	0.2 (0.11)	0.3 (0.18)	0.3 (0.19)	0.4 (0.15)	0.2 (0.18)
59.	Germacrene D	1481	1.4 (0.74)	1.8 (0.88)	0.8 (0.80)	1.2 (0.74)	1.7 (0.93)	1.7 (0.79)	3.3 (1.01)	1.7 (1.04)
60.	*β*-Selinene	1486	0.0 (0.03)	0.1 (0.19)	0.0 (0.03)	0.1 (0.16)	0.0 (0.04)	0.4 (0.80)	0.0 (0.05)	0.1 (0.34)
61.	*trans*- Muurola-4 (14),5-diene	1492	tr	tr	0.0 (0.02)	tr	tr	tr	0.0 (0.04)	0.0 (0.02)
62.	*α*-Selinene	1495	0.0 (0.04)	0.1 (0.18)	0.1 (0.11)	0.1 (0.12)	0.1 (0.06)	0.3 (0.64)	0.1 (0.05)	0.1 (0.27)
63.	*α*-Muurolene	1501	0.1 (0.05)	0.1 (0.09)	0.1 (0.17)	0.1 (0.08)	0.1 (0.12)	0.1 (0.06)	0.2 (0.10)	0.1 (0.11)
64.	Premnaspirodiene	1506	tr	0.0 (0.05)	tr	tr	tr	0.0 (0.04)	tr	0.0 (0.03)
65.	n.i. 14	1508	tr	tr	tr	tr	tr	tr	tr	tr
66.	*γ*-Cadinene	1515	0.1 (0.05)	0.1 (0.06)	0.1 (0.15)	0.1 (0.06)	0.1 (0.09)	0.1 (0.05)	0.2 (0.10)	0.1 (0.10)
67.	*δ*-Cadinene	1523	0.2 (0.06)	0.3 (0.06)	0.3 (0.38)	0.2 (0.14)	0.4 (0.35)	0.3 (0.14)	0.5 (0.19)	0.3 (0.23)

Table 2. continued.

68.	n.i. 15	1532	tr	tr	tr	tr	tr	tr	tr	tr
69.	α-Cadinene	1537	tr	tr	0.0 (0.03)	0.0 (0.03)	tr	tr	0.0 (0.01)	0.0 (0.01)
70.	n.i. 16	1544	tr	tr	tr	tr	tr	tr	tr	tr
71.	Germacrene B	1553	0.0 (0.06)	0.0 (0.04)	0.0 (0.04)	0.0 (0.04)	tr	0.1 (0.10)	tr	0.0 (0.06)
72.	n.i. 17		tr	tr	tr	tr	tr	tr	tr	tr
73.	n.i. 18		tr	tr	tr	tr	tr	tr	tr	tr
74.	n.i. 19		tr	tr	tr	tr	tr	tr	tr	tr
75.	n.i. 20		tr	tr	tr	tr	tr	tr	tr	tr
76.	n.i. 21		tr	tr	tr	tr	tr	tr	tr	tr
77.	n.i. 22		tr	tr	tr	tr	tr	tr	tr	tr
78.	n.i. 23		tr	tr	tr	tr	tr	tr	tr	tr

n.i. – not identified; *Mean values for group, standard deviation (SD) given in brackets; tr – trace amount (0.05<X<0.1); n – number of analyzed individuals.

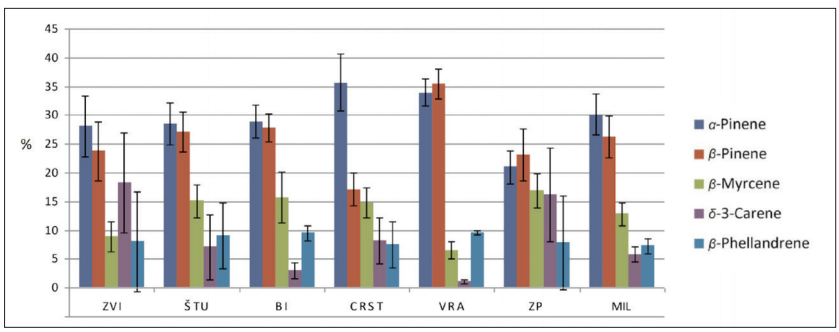

Fig. 1. Differences between twig essential volatiles of seven populations of *P. omorika* according to the content (in %) of five main volatiles in the analyzed populations: ZVI – Zvijezda, ŠTU – Štula, BILO – Bilo, CRST – Crvene Stene, Mil- Mileševka Canyon, VRA – Vranjak, and ZP – Zmajevački Potok.

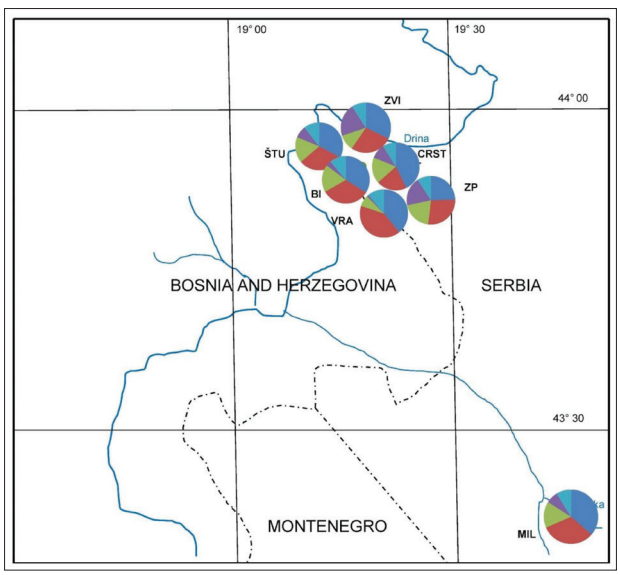

Fig. 2. Geographic distribution of twig volatile profiles characterized by dominant compounds of seven populations of *P. omorika*.

The chemical profile of the five main terpene volatiles in twigs of Serbian *Picea omorika* (presented results) was: α-pinene > β-pinene > myrcene > δ-3-carene > β–phellandrene, sensu Petrakis et al. 2001 [12]. In all seven populations, α- and β-pinene were the most abundant compounds. In addition, three groups could be distinguished: (i) ŠTU, BI, MIL and CRST with almost equal amounts of β-myrcene; (ii) ZVI and ZP with the highest amounts of δ-3-carene, (iii) VRA with a very high percentage of β-pinene and the lowest values of myrcene and δ-3-carene (Figs. 1 and 2). According to values of standard deviation, the most variable compound inside the populations was β-phellandrene (Fig. 1).

Interpopulation variability of volatile composition

Out of 78 terpenes of *P. omorika* twigs, the six most abundant (α-pinene, β-pinene, myrcene, δ-3-carene, β-phellandrene and α-phellandrene) were selected for the PCA. The PCA was performed on a correlation matrix (distances), calculated for 100 samples (Fig. 3).

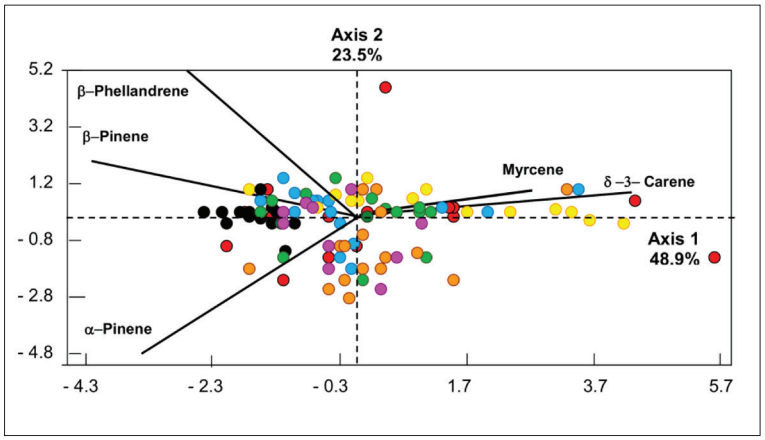

Fig. 3. PCA of six selected terpenes from 100 trees of *P. omorika* grouped in seven populations. ŠTU; ZVI; BI; CRST; VRA; ZP; MIL. The spatial distribution of the populations is presented in Supplementary Fig. S1.

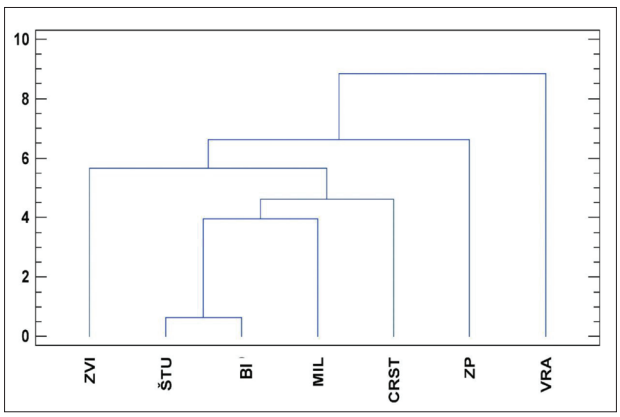

Fig. 4. Dendrogram based on the nearest-neighbor method (square Euclidian distance) of the seven populations of *P. omorika*.

The first two principal axes represent 70.9% of the total information. The PCA visualizes overlapping (similarity) between the populations. Trees from the population VRA are grouped in the plane of Axis 1, while trees of populations ZP and CRST were grouped in the plane of Axis 2, in their positive and negative parts, respectively. The CA (Fig. 4) points to a degree of separation of the southernmost populations of Mt. Tara (VR and ZP) from populations ZVI, ŠTU, BI, CRST and MIL.

DISCUSSION

The statistical analyses show a high intrapopulation variability which is congruent with both molecular and phytochemical data [8,12,28-30]. The lowest variability was found in the population VRA, where all except one individual are grouped close to each other. In all other populations, individuals showed groupings in two to four clusters based on the relative abundance of six terpenes, e.g. the population BI had three distinct clusters, and MIL and CRST had two each. The ZP population displayed a gradient in the composition of twig volatiles from those rich in δ-3-carene to those rich in β-pinene. The ZVI population had the highest variability and was spread across the entire scatter plot. While the differences between populations can also be attributed to differences in environmental factors, such as insulation, temperature, temperature seasonality, annual water precipitation, etc., such sharp differences within the same populations cannot be explained by differences in environmental conditions. Furthermore, the overall variability profile established for the leaf essential oil in a previous study of four populations [12] appears to be the same, with the populations ZP and ZVI showing the highest variability, and populations MIL, CRST and ŠTU having two distinct profiles each. Interestingly, the MIL population, which belongs to another variety (var. *vukomanii*), did not differentiate from all other individuals in the multivariate analyses.

The obtained volatile profiles differed significantly in terms of essential oil presence in twigs, as reported in [13]: isoborneol = exoborneol > β-pinene > α-pinene > β-phellandrene > bornyl acetate. The differences in the profiles of the main terpenes could be explained by the different methodologies used: (i) plant material was air-dried at room temperature, (ii) plant material was homogenized prior to isolation, (iii) essential oil was obtained by different methods. Monoterpene composition of *P. omorika* leaves revealed differences depending on the storage temperature [16], with the abundance of the monoterpene fraction getting progressively lower, which could have led to the detection of a higher abundance of sesquiterpenes. In [13], the ratio of monoterpene to sesquiterpene was more or less the same as in the presented results, i.e. 94:6 and 96:4, respectively. However, the relative abundance of monoterpene hydrocarbons and oxygenated hydrocarbons was significantly different, with oxygenated monoterpenes constituting 51.0% of the twig essential oil and oxygenated sesquiterpenes 1.9%, in contrast to

only 0.3% of oxygenated monoterpenes in the obtained results. The preparation method (homogenization vs whole-plant material) influenced the composition of HS volatiles, presumably due to differences in enzymatic reactions, but no such difference was detected for the hydrodistillation method [16,18]. When comparing *Pinus densiflora* leaf essential oil obtained by different techniques [19] (hydrodistillation vs headspace), almost all of the differences in the profiles can be attributed to the low abundance of oxygenated sesquiterpenes. Our previous comparative analyses of the HS vs traditional essential oil isolation methods [27,28] also showed a significant increase in the monoterpene fraction of the obtained volatiles, and low abundances of oxygenated sesquiterpenes. The same was true for the presented data, with 0.3% of oxygenated monoterpenes and no oxygenated sesquiterpenes detected. However, since both isoborneol and exoborneol are oxygenated monoterpenes, the difference is probably due to genetic differences in the studied material since the material was obtained from different populations. Further corroboration of this can be found in [13] where the authors also reported isoborneol and exoborneol in the leaves, although in significantly lesser abundance, while another earlier research [12] did not find any compound in the leaf essential oil.

In our previous investigation of needle terpene composition isolated by simultaneous distillation and extraction of essential oil, the chemical profile of *P. omorika* was as follows: bornyl acetate >> camphene >> α-pinene >> α-cadinol = limonene, and the MIL population was the most distant in the CA of Mt. Tara populations [12], based on the high abundance of bornyl acetate. According to previous results, the PCA showed that the composition of the less volatile compounds (e.g. sesquiterpenes) played a significant role in the separation of the analyzed populations. In [15], bornyl acetate was the most abundant, followed by α-pinene, camphene, β-phellandrene and β-pinene in the needle essential oil obtained by HD. Bornyl acetate was also dominant in other reports [10-14]. In contrast, the composition of volatiles obtained by the SFE method [16] or the HS technique [17][18] showed a strong predominance of monoterpenes, namely limonene, α-pinene and camphene, in *P. omorika* needles.

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Author contributions: NR, BN and ZM drafted the manuscript; BN and SB collected the plant material, JLJ and VT conducted essential oil isolation and analyses; SB performed statistical analyses; PM supervised the research and critically reviewed the manuscript. All authors read and approved the final manuscript.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/000ABS_Nikolic%20et%20al_5348_Supplementary%20Material.pdf