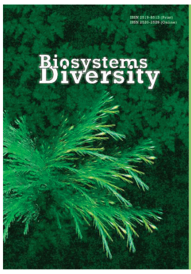

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Morphological and essential oil variations among Iranian populations of *Salvia chloroleuca* (Lamiaceae)

S. M. Talebi*, S. Behzadpour*, A. Matsyura**, ***

**Arak University, Arak, Iran*
***Altai State University, Barnaul, Russian Federation*
****Tomsk State University, Tomsk, Russian Federation*

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Arak University, Arak, 38156–8–8349, Iran. Tel.: +098-863-417-33-17. E-mail: seyedmehdi_talebi@yahoo.com

Altai State University, Lenin st., 61, Barnaul, 656049, Russian Federation. Tel.: +73-852-291-222. E-mail: amatsyura@gmail.com

Tomsk State University, Lenin st., 36, Tomsk, 634050, Russian Federation.

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Salvia chloroleuca Rech. f. & Aell. is one of the perennial species of the Lamiaceae family in Iran, which has antimicrobial and antitumoural properties. In the current research, the morphological variables and hydrodistilled essential oils of two populations, from Polor and Neyshabur, of this species were evaluated. We studied 10 and 40 individuals for morphometrics and essential oils, respectively. Morphological data were analyzed using SPSS. Furthermore, the extracted oils were analysed using GC and GC/MS. The qualitative morphological characteristics were stable between the populations, while the quantitative ones highly varied. The one-sample variance test revealed significant differences for most of the studied features. The yielded oil highly differed between these populations. The first and second main groups of compounds were the same for both populations, but with different percentages. However, the third and fourth main groups of compounds were not similar. Furthermore, the main compounds of essential oil differed between the studied populations. The major compounds of the Polor population were: β-pinene (22.7%), α-pinene (18.3%), germacrene D (7.7%) and sabinene (6.6%). However, spathulenol (19.8%), bicyclogermacrene (11.4%), p-cymene (10.8%) and β-pinene (10.4%) constituted the major compounds of the Neyshabur population. In total, the Neyshabur population had higher amounts of sesquiterpene and oxygenated compounds than the other population. The results indicated that in this species the quantitative morphological characters and essential oils were strongly affected by environmental factors.

Keywords: hydrodistilled essential oils; infraspecific variation; sesquiterpene; oxygenated compounds.

Introduction

The essential oils of aromatic plants are made of a relatively large group of plant natural products and reveal a complex mixture of several compounds such as terpenes, fatty acid degradation products, phenylpropanoids, and hydrocarbons of different biogenetic origins. These compounds are primarily involved in plants' defense against pathogens and herbivores, attraction of pollinators and plant-plant communication. In addition, volatile compounds also represent a wide range of biological effects on different organisms like bacteria, Yarmohammadi et al., (2017) have suggested that difference in essential oil compositions proved the possibility of chemical polymorphism among various populations of the same taxon. The characterization and identification of chemotypes is very important in plant materials which are used in chemical, agronomic, and pharmacological studies to produce herbal medicines. In addition, the pharmacological activity of the same plant species could differ due to variation in composition of essential oil (Potzernheim et al., 2006; Paula et al., 2011). According to Violle et al. (2012), recent evaluations have broadened in the identification of difference to incorporate the considerable morphological variation within and among populations of the same species. Moreover, several investigations (Palkovacs & Post, 2009; Govaert et al., 2016) have indicated that infraspecific difference may influence community structure and ecosystem function as much as variation among species.

Salvia is one of the largest genera of Lamiaceae and includes about 900 species, widespread throughout the world. The genus contains 61 species in Iran, with more than 15 endemic taxa (Mozaffarian, 1996; Jamzad, 2012). Some *Salvia* species are used as medicinal, aromatic and ornamental plants (Rustaiyan et al., 1999). Several species of *Salvia* have been used in the folk medicine as antibacterial (Ulubelen et al., 1997), antitumour (Topcu, 2006) and flavouring agents (Tzakou et al., 2001).

Salvia chloroleuca Rech. f. & Aell., is a perennial herb of the genus which is widely distributed in Iran. The synonym of this species is *Salvia limbata* Mey., and these species are morphologically similar (Jamzad, 2012). There are few reports on the essential oil compositions of different populations of this species in Iran (Yousefzadi et al., 2008; Yadollahi et al., 2013). Studies have confirmed the antibacterial, antifungal, antitumoural and antioxidant effects of the essential oil of *S. chloroleuca*. The antimicrobial activity was evaluated against some gram-positive and gram-negative bacteria and also three fungi. Findings showed that the oil exhibited moderate to high antimicrobial activity (Yousefzadi et al., 2008). Cytotoxic properties of total methanol extract of this species were examined on MCF-7, a breast carcinoma cell line. *S. chloroleuca* inhibited the growth of malignant cells in a dose-dependent manner (Tayarani-Najarana et al., 2013). Asghari et al. (2015) have reported several flavonoids such as luteolin 7-O-glucoside, luteolin 7-O-glucuronide, diosmetin 7-O-glucuronide and salvigenin as antioxidant agents from the aerial parts of *S. chloroleuca*.

As far as we could ascertain, no comparative study on essential oil compositions and morphological variables of this species has been made in Iran or anywhere else in the world. Therefore, in the current research, the comparative study of the morphological traits and oil compositions of two populations of *S. chloroleuca* was performed for the first time.

Material and methods

Plant material. In this study, two natural populations of *S. chloroleuca* were harvested from two parts of Iran (Table 1), and were identified according to descriptions provided in Flora of Iran (Jamzad, 2012) by the authors. Aerial parts of these populations were harvested in the flowering stage of plant development (May) during 2018. The voucher specimens were placed in the Herbarium of Arak University (AUH).

Table 1
Harvesting collection locations of the studied populations

Populations	Localities
Polor	Mazandaran province, Amol, Polor, 2100 m
Neyshabur	Khorasan Razavi province, Neyshabur, 1700 m

Morphological studies. Morphological variations were studied for reproductive organs from the collected individuals of these populations. The morphological variables of flowers were evaluated under stereo microscopes. For morphometrics, 20 plant specimens from the two populations were used. In total, 21 qualitative and quantitative features were examined for each specimen. Each variable was studied three times per each plant sample, and their average and standard deviations were determined. The morphological characteristics included: petal colour, length, width and length/width ratio, calyx width, length and calyx length/width ratio, calyx long and short teeth shape, length and width, style length, stigma length, anther length, width and length/width ratio, pedicle length, the long and short filaments length.

Isolation and GC/GC MS analysis of essential oil. We selected forty plant individuals from each population with a minimum distance of 100 m, then mixed plant materials for homogenization, and made three replications for essential oil extractions.

The aerial parts of the plant materials were dried at room temperature. Essential oils were extracted by hydrodistillation method using a Clevenger-type apparatus (Anonymous, 2011) and their yields were calculated on a dry-weight basis (V/m). The volatile compounds were identified by GC and GC-MS. For GC-MS analysis, ThermoQuest-Finnigan Trace GC-MS system equipped with a DB-1 column (30 m × 0.25 mm, 0.25 mm film thickness) was used. The volume of injection was 2 µL. Oven temperature was 60–250 °C. Helium gas with speed 1/1 mL per minute and ionizing energy 70 electron volts in the coupled mass spectrometer with gas chromatograph was used. For GC analysis, a Thermoquest gas chromatograph equipped with DB-1 column (30 m × 0.25 mm, 0.25 mm film thickness) was used. Oven temperature of 60–250 °C.

After injection of oils, using Retention Rime (RT), Retention Index (RI) and mass spectra of components and their comparison with those of standard ones or by computer matching against the library spectra, the compositions of the oils were studied qualitatively and quantitatively.

Identification of components was based on the comparison of their retention indices and mass spectra with those obtained from authentic samples and or the NIST AMDIS software, Wiley libraries, the Adams (2007) database, and available literature. The relative percentages of the identified components were computed from the peak area of GC.

The data were analyzed in Statistica 11.0 (StatSoft Inc., USA). The data in the tables are presented as x ± SD (x ± standard deviation). The differences between the values in populations were determined using the Tukey test, where the differences were considered significant at $P < 0.05$ (with taking into account the Bonferroni correction).

Results

Morphological analysis. Most important morphological characteristics differed between the studied populations (Table 2). Therefore, the percentages of variations were determined among the morphological characteristics. The highest morphological variations were reported for pedicle length (34.0%), calyx short teeth width (28.0%), stigma length (20.0%), and calyx long teeth width (17.2%), respectively. However, anther length (1.1%) and calyx width (1.2%) had the smallest variations.

However, we revealed significant variations for most of the examined traits, except for pedicle length, stigma length, calyx short teeth width, and calyx long teeth width (Table 2). The qualitative morphological characteristics, such as petal colour, petal lobe shape and calyx tooth shape, were stable between the studied populations. The petal colour was white with lower yellow parts and the calyx short and long teeth shape was lanceolate.

Table 2
Some important morphological characteristics between the studied populations (x ± SD, n = 21)

Parameter	Polor population, cm	Neyshabur population, cm	DEV, percentage deviation, %*
Anther length	4.70 ± 0.50[a]	4.80 ± 0.40[b]	1.1
Anther width	2.30 ± 0.40[a]	2.0 ± 0.00[b]	15.0
Pedicle length	3.50 ± 1.24[a]	2.30 ± 0.48[a]	34.0
Stigma length	2.20 ± 0.34[a]	2.75 ± 0.26[a]	20.0
Style length	17.10 ± 2.88[a]	19.10 ± 2.18[b]	10.5
Petal length	11.30 ± 1.05[a]	12.30 ± 1.05[b]	11.7
Petal width	4.90 ± 0.31[a]	5.30 ± 0.67[b]	7.5
Calyx short teeth length	0.86 ± 0.76[a]	1.09 ± 0.58[b]	21.0
Calyx short teeth width	0.25 ± 0.19[a]	0.18 ± 0.10[a]	28.0
Calyx long teeth length	3.40 ± 0.51[a]	3.20 ± 0.58[b]	5.9
Calyx long teeth width	0.24 ± 0.24[a]	0.29 ± 0.24[a]	17.2
Calyx length	10.10 ± 1.52[a]	11.40 ± 1.07[b]	11.4
Calyx width	8.20 ± 1.31[a]	8.30 ± 1.05[b]	1.2

Note: * – obtained by subtracting the known value from the mean, dividing the result by the known value and multiplying by 100; different letters indicate the values significantly differing one from another within a line of the Table on the results of comparison using the ANOVA ($P < 0.05$).

Essential oil analysis. The percentage of the yielded essential oil varied between the studied populations (Table 3). It was 0.2% and trace amounts for the Polor and Neyshabur populations, respectively. In the essential oils of the Polor population we identified twenty compounds and in the Neyshabur population – eighteen compounds, which represented 97.1% and 99.9% of the total essential oil, respectively.

Table 3
Chemical constituents identified in the essential oils of the *S. chloroleuca* populations

Chemical composition	Retention time	Polor, compound, %	Neyshabur, compound, %	Retention index
α-Thujene	5.76	0.55	0.82	927
α-Pinene	5.98	18.28	9.03	937
Camphene	6.39	3.67	1.83	954
Sabinene	6.86	6.61	5.42	976
β-Pinene	7.04	22.73	10.40	985
β-Myrcene	7.12	0.62	0.52	989
α-Terpinene	7.90	0.33	0.71	1020
p-Cymene	8.22	3.74	10.82	1028
Limonene	8.32	4.07	–	1032
1,8-Cineol	8.41	2.78	1.38	1037
γ-Terpinene	8.96	1.61	3.92	1060
α-Terpinolene	9.68	0.30	0.44	1088
Linalool	10.21	2.99	1.05	1107
Borneol	12.56	2.45	6.37	1181
Myrtenol	13.18	1.41	–	1195
δ–Elemene	15.17	–	6.61	1338
β -Caryophyllene	18.52	5.79	–	1424
Germacrene D	20.07	7.65	6.42	1478
Bicyclogermacrene	20.44	2.90	11.36	1500
Spathulenol	22.69	4.02	19.81	1584
Caryophyllene oxide	22.74	4.64	3.08	1590
Monoterpene hydrocarbons	–	62.51	43.91	–
Oxygenated monoterpene	–	9.63	8.80	–
Sesquiterpene hydrocarbons	–	16.34	24.39	–
Oxygenated sesquiterpene	–	8.66	22.88	–

Notes: "–" – no data; the retention index (RI) of a certain chemical compound is its retention time normalised to the retention times of adjacently eluting n-alkanes.

Monoterpene hydrocarbons were the main compounds of the oil in both populations (62.5% and 43.9%, respectively). The second group of compounds in both populations was sesquiterpene hydrocarbons (16.3% and 24.4% in the Polor and Neyshabur populations, respectively).

However, the types of third and fourth groups varied between these populations. In the Polor population, the oxygenated hydrocarbons (9.6%) and oxygenated sesquiterpenes (8.7%) were the third and fourth groups of compounds respectively, while in the Neyshabur population the reverse conditions were reported. In the latter population, the oxygenated sesquiterpenes (22.9%) formed the third group and oxygenated hydrocarbons (8.8%) comprised the fourth group.

The main compound in the Polor population was β-pinene (22.7%), the monoterpene hydrocarbon compound, while in the Neyshabur population it was the spathulenol (19.8%), the oxygenated sesquiterpenes. The second main compounds in the Polor and Neyshabur populations were α-pinene (18.3%), the monoterpene hydrocarbon compound and bicyclogermacrene (11.4%), the sesquiterpene hydrocarbons. The third and fourth major compounds of the Polor population were germacrene D (7.7%) and sabinene (6.6%), the sesquiterpene hydrocarbons and monoterpene hydrocarbon compounds, respectively. However, in the Neyshabur population, they were p-cymene (10.8%), β-pinene (10.4%), which are monoterpene hydrocarbons (Fig. 1, 2).

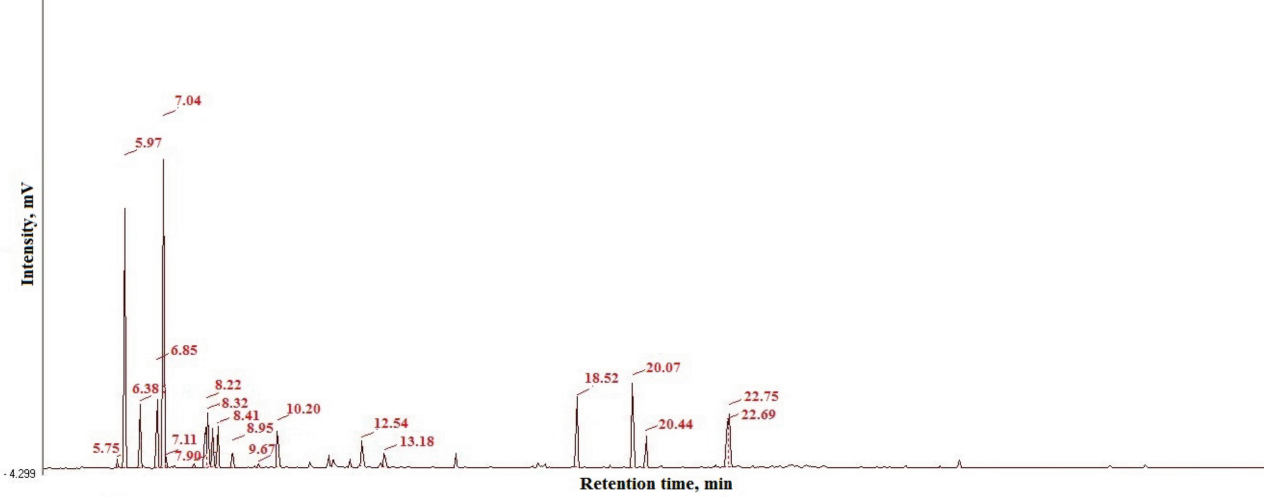

Fig. 1. Gas chromatogram of the essential oil composition of the Polor population

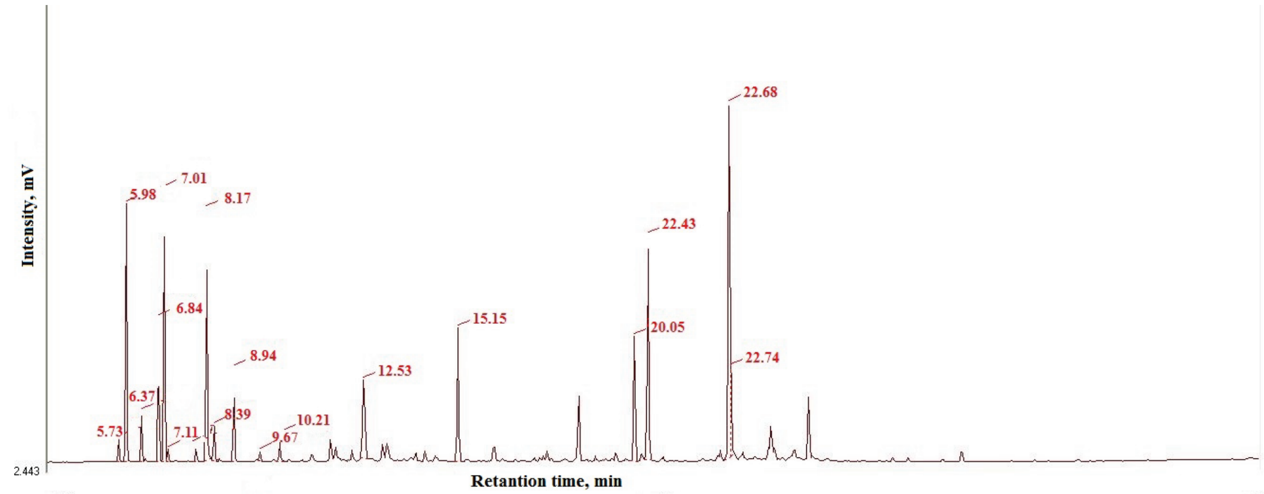

Fig. 2. Gas chromatogram of the essential oil composition of the Neyshabur population

Discussion

In the present investigation, infraspecific variations were studied in the essential oil compositions and morphological characteristics of two populations of *S. chloroleuca*. The morphological features, essential oil amounts, and contents varied between the studied populations. These populations were selected from two different parts of Iran more than 730 km apart. Furthermore, these populations belong to two different phytogeographical regions. The Neyshabur population was from the Khorasanian sub-province of Irano-Turanian region, while the Polor populations belonged to the Hyrcanian province of the Euro-Siberian region (Zohary, 1973). We evaluated reproductive morphological characteristics, because previous studies have confirmed that reproductive features are very important for plant identification and also they hardly vary among different populations of the same species (Stace, 1989). Besides, Jamzad (2012) has used variables of reproductive organs along with palynological and ITS data in her valuable work on Iranian Lamiaceae taxa, Flora of Iran.

We registered that the studied morphological characteristics varied between the studied populations, and one sampled T-test revealed significant difference between them. Infraspecific morphological variations were reported from several species of the family such as *Stachys inflata* (Talebi et al., 2014) and *Salvia multicaulis* (Talebi et al., 2017b), *S. limbata* (Talebi et al., 2019). This was also reported for the species from other plant families, such as *Linum album* (Linaceae) (Talebi et al., 2014). The identification of infraspecific phenotypic variations is very important because the morphological features used for description of species and flora identification keys were based on these characteristics. Meanwhile, the morphological characteristics have great importance in medicinal and industrial plants and any mistake in plant identification could be dangerous. Our morphological findings indicated that environmental factors have a very strong effect on difference or similarity between populations. This condition produces phenotypical polymorphism between the populations.

Previous evaluations (Yarmohammadi et al., 2017; Talebi et al., 2019) have suggested that genetic factors and physiological conditions of plant have strong effects on the yield of essential oils. However, the effects of environmental factors on the essential oil yield are not neglible (Paula et al., 2011). For example, Talebi et al. (2019) studied the essential oil yield in two populations of *Nepeta fissa* that were harvested from two different altitudes of the same region and reported variations in the amount and contents of the oil. The amount of oil in the Polor population was higher than in the Neyshabur population. According to phytogeographical region, the growth conditions of the plant in the Polor population were better than in the other population (Zohary, 1973). It seems that this variation in the amounts of essential oil largely depends on habitat conditions of the populations.

Moreover, the essential oil compounds varied between these populations. The first and second main group of compounds in both populations was the same, but with different percentages. The amount of monoterpene hydrocarbons (the first compound), in the Polor population was higher than in the other population, and we found more than 70% variation in its quanitity between the populations. Our findings agreed with Yousefzadi et al. (2008) study, who reported monoterpene hydrocarbons as the main group of compounds (37.4%) of oil in this species. The reverse pattern was registered in the second main group of compounds, sesquiterpene hydrocarbons, and its amount in the Neyshabur population was 67.0% higher than in the Polor population. The third and fourth groups of compounds were not similar between the studied populations. In total, we observed that in the Neyshabur population, the percentages of sesquiterpenes and also oxygenated compounds were higher than in the other population. This may be related to the habitat conditions of the populations. In the Neyshabur population, the oxidative stress of the habitat is greater than in the Polor population and these conditions lead to increment of oxide compounds. In the Polor population the major compound was β-pinene, as also found in the Yousefzadi et al. (2008) study, but in the Neyshabur population, spathulenol was the main compound. The amount of this compound was nearly equal that reported by Yadollahi et al. (2013), while in their study germacrene-D was the major compound. It is more important to know that spathulenol constituted less than 5% percentage of oil in the Polor population and was nearly equal in the study by Yousefzadi et al. (2008).

The percentage of α-Pinene (the second compound) in the Polor population was twice as high as in the Neyshabur population, which was similar to the results of Yousefzadi et al. (2008). The second compound of the Nyshabur population was bicyclogermacrene. The amounts of this compound in the Polor population was low, as also found by Yousefzadi et al. (2008). In both previous investigations (Yousefzadi et al., 2008; Yadollahi et al., 2013), β-caryophyllene was identified as one of the major compounds with equal amount. The amount of tuis compound was lower in the Polor population, but was not found in the Neyshabur population. p-Cymene was one of the major compounds in the Neyshabur population, while its percentage was 35% lower in the Polor population. The amount of this compound in the Polor population was equal to the amount found in the Yousefzadi et al. (2008) study. According to previous studies (Yousefzadi et al., 2008; Yadollahi et al., 2013), the essential oil of this species has antimicrobial activities. Yousefzadi et al. (2008) have indicated antimicrobial activity of essential oil in the Shahrestanak (Tehran province of Iran) population of this species against three fungi (*Candida albicans*, *Saccharomyces cerevisiae*, and *Aspergillus niger*) and some Gram-positive and Gram-negative bacteria: *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia*.

In other research, antimicrobial effects of the essential oil of the North Khorasan population (Iran) was examined against bacteria such as *Escherichia coli*, *Salmunella*, *Clostridium perfringens*, and *Staphylococcus aureus*. These results confirmed the highest antibacterial effects of essential oils on *Staphylococcus aureus* and *Clostridium perfringens* strains; however, no effect was registered against Gram-negative bacteria. Since some variations were found in the essential oil compositions and antimicrobial activity between these populations, we can predict the high difference in antimicrobial activities among our studied populations and the previous investigations. According to Yousefzadi et al. (2008), the antimicrobial activity of carvacrol was superior compared to the other major components. Moreover; α-pinene and β-caryophyllene revealed moderate antibacterial activity, except for *K. pneumoniae* and *P. aeruginosa*. We need to mention that carvacrol was absent in our studied populations, moreover, the amounts of α-pinene were not similar in our studied populations and β-caryophyllene was only registered in the Polor population. According to these results and previous evaluations (Lima et al., 2006; Potzernheim et al., 2006; Paula et al., 2011), it is extremely important to consider the chemical difference in oils caused by various factors such as genetic, physiological or environmental variables when referring to plant material used in chemical, pharmacological and agronomic investigation. This is especially valuable when we aim to obtain herbal medicines, because the pharmacological properties can definitely vary due to differences in composition of essential oil.

Conclusion

Qualitative morphological characteristics were stable between the populations, while according to T-test analysis, most of the quantitative ones significantly varied. Moreover, the amount of yielded oil varied with phytochemical evaluation. Although the first and second groups of chemical compounds were similar between populations, their percentages varied while the third and fourth groups of compounds were not similar between the populations. The major oil compounds significantly differed between the studied populations. In some cases, our findings regarding the oil compositions were similar to previous phytochemical studies on this species, although some distinct chemical differences were found. The habitats of the studied populations of this species are not characterized by identical environmental conditions, but rather by a set of various environments with different ecological conditions. Therefore, this species clearly adapts its morphological and chemical characteristics enabling it to survive under those differing environmental conditions. We suggested that this caused the emergence of morphological and chemical polymorphisms in the studied plant species, which possesses the genetic background to live in a great variety of habitats.

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