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Polymorphism of Lithuanian Scots pine (*Pinus sylvestris*) with regard to monoterpene composition in needles

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Interspecific interactions between Scots pine and other organisms depend much on tree chemistry, and monoterpenes play an important role in the interactions. Monoterpenes present in pine needles perform a defensive function against phytophagous animals (insects, molluscs, moose, deer) and the function of kairomones and synomones. A pilot study on the composition of monoterpenes in needles of 7–10-year old trees of Scots pine (*Pinus sylvestris*) from the Lithuanian population (within a relatively small area) was carried out using gas chromatography. The composition of 6 monoterpenes (α-pinene, camphene, β-pinene, myrcene, 3-carene and limonene) in needles was analysed, and the polymorphism of Scots pine was evaluated. With regard to monoterpene composition in needles, Scots pine were found to be polymorphic. The predominant monoterpenes were α-pinene and 3-carene. Their relative contents (of all the monoterpenes studied) varied from 27 to 79% and from 0 to 66%, respectively. Relative contents of α-pinene, 3-carene and β-pinene were found to be the most and those of myrcene and camphene the least variable. The relative content of limonene in needle extracts varied moderately. The variation in the relative content of 3-carene was found to be the greatest: in needles of some plants 3-carene was abundant (about 65.8% of the total amount of all the monoterpenes analysed), while in those of other plants this monoterpene was totally absent (or its content was below the level of chromatographic detection). The relative monoterpene composition in needles of Scots pine can be used for plant selection as well as for chemotype identification within the population.

Key words: *Pinus sylvestris*, population, diversity, secondary metabolites, α-pinene, β-pinene, camphene, myrcene, 3-carene, limonene, monoterpene variation

INTRODUCTION

Scots pine, *Pinus sylvestris,* is a widespread species all over Europe, its distribution area extending from the northenmost regions (the Arctic) to the Mediterranean Sea. Various animals, from tiny insects and molluscs to birds and large mammals, feed on its seeds, needles, twigs and bark. Monoterpenes present in pine needles perform a defensive function. It is known that such phytophages as omnivorous molluscs *Arion ater* prefer young pine seedlings containing less α-pinene (O'Reilly-Wapstra et al., 2007), and the larval survival of pine defoliator sawflies *Diprion pini* is lower when they feed on needles rich in 3-carene (Barre et al., 2003). Some other conifer monoterpenes are known as feeding-deterrents to moose or deer (Summerheimsjaberg, 1992, Iason et al., 1996, Vourc'h et al., 2002) as well as to insects. However, the role of monoterpenes is not merely defensive; it is much more complex. Monoterpenes also function as kairomones and synomones. Thus, they are very important for interspecific interactions between Scots pine and other organisms. Monoterpenes emitted by pine-trees attract some phytophagous insect species to feed and lay eggs. The chemicals also attract some predators of insects damaging (feeding on) pine. The following beetles are among the attracted phytophagous species: α-pinene and β-pinene attract *Ips pini* (Joseph et al., 2001), α-pinene, 3-carene and terpinolene attract *Tomicus piniperda* (Schroeder, Eidmann, 1987; Poland et al., 2004; Song et al., 2005), 3-carene attracts *Dendroctonus valens* (Sun et al., 2004), and α-pinene, β-pinene and 3-carene attract *Monochamus alternatus* (Fan et al., 2007) etc. The attracted predators are beetles as well: e. g., α-pinene attracts *Thanasimus formicarius* (Schroeder, Lindeloew, 1989). Besides, a low β-pinene level and a low β- / α-pinene ratio increased the oviposition of old house borers, *Hylotrupes bajulus* (Nerg et al., 2004). Thus, resistance and attractiveness of pine-trees to phytophagous insects as well as to predators feeding on these insects are related to the qualitative and quantitave composition of monoterpenes.

The already available data indicate that the content of α-pinene in seedlings of Scots pine is to some extent heritable (O'Reilly-Wapstra et al., 2007). This fact makes it possible to carry out artificial selection of Scots pine in nurseries.

Data indicating that Scots pine-trees vary phenotypically in their composition of monoterpenes in needles have been published (e. g., Nerg et al., 1994; Manninen et al., 2002; Semiz et al., 2007). However, these data were obtained from samples collected from vast territories, geographically distant sampling locations. It is not known whether the above-mentioned variation exists in a comparatively small territory such as Lithuania, and what is the scale of such variation. There are data on morphological and genetic polymorphism of Scots pine in Lithuania (e. g., Ozolinčius, 1998; Žvingila et al., 2002; Naugžemys et al., 2006). However, a review monograph (Skuodienė, 2005) also confirms that there are no data on variation in monoterpene composition.

The goals of this study were as follows: to perform small-scale (pilot) analysis of the composition of six monoterpenes in Scots pine needles from the Lithuanian population; to establish whether the population is polymorphic with regard to this feature, and to elucidate whether the initial material suitable for carrying out artificial selection of Scots pine is available.

MATERIALS AND METHODS

Plants. Eight trees of Scots pine were randomly chosen for analysis from different localities within Lithuania: Pasvalys, Vilnius, Labanoras (Švenčioniai district), Merkinė (Varėna district), Marcinkonys (Varėna district), Alytus, Butrimonys (Alytus district) and Palanga. The age of the trees ranged from 7 to 10 years. Several approximately 30 cm long twigs with needles were removed from each tree. For transportation to the laboratory, twigs were placed into plastic bags. Before analysis they were kept in a freezer (–18 °C) for several days. Needles of each tree were cut into 2–4 mm long segments, and the obtained material (approximately 3 g) was soaked in a solvent (6 ml of chromatographic grade hexane) for 24 h. The extract was filtered and kept in a freezer at –18 °C until analysis.

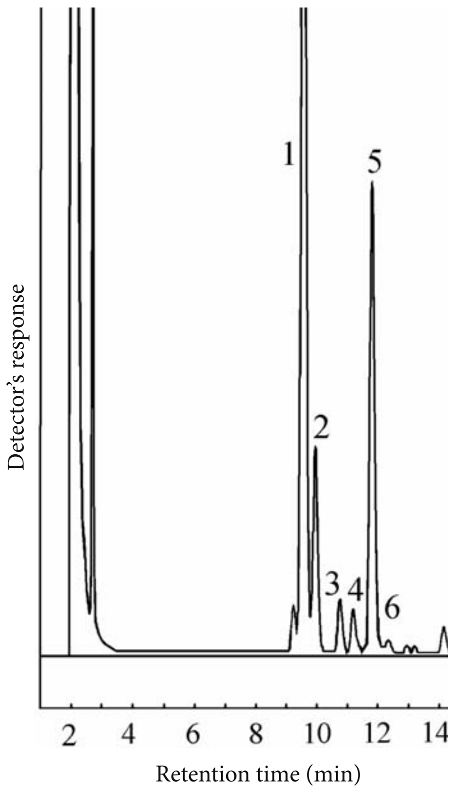

Fig. 1. Chromatogram of monoterpenes in the extract of needles derived from Scots pine, *P. sylvestris*: *1* – α-pinene; *2* – camphene; *3* – β-pinene; *4* – myrcene; *5* – 3-carene; *6* – limonene

Chromatographic analysis. Analysis was undertaken on a Chrom-5 gas chromatograph equipped with a flame ionization detector and a glass column 3 m × 3 mm. The stationary phase was 5% SE-30 on the N-AW chromaton, the termostat programme was 5 °C min^{-1} ranging from 40 °C to 250 °C, the temperature of the injector was 200 °C and of the detector 250 °C. Nitrogen was used as a carrier gas at a flow of 30 ml/min. 2 µl of the extract was injected for analysis.

Six monoterpenes were identified in a sample by comparing their chromatographic retention time with that of standards under the same chromatographic conditions. The compounds α-pinene, β-pinene, 3-carene used as standards were obtained from Roth, limonene from Fluka and camphene from Supelco. Monoterpene composition in pine needles was estimated according to their relative contents. The percentage composition of monoterpenes was computed from GC peak areas without a correction factor. Three replicates were performed for each tree; their results were averaged and a standard error was calculated.

Statistical analysis. Cluster analysis of multivariate exploratory techniques was performed using the values of six monoterpenes standardized according to the formula $z = (x_i - \bar{z}) / SD$. Simple linear correlation (Pearson's r) matrices for two lists were compiled and analysed. The analyses were performed using Statistica 6.0 software.

RESULTS AND DISCUSSION

We analysed the relative composition of six monoterpenes (α-pinene, camphene, β-pinene, myrcene, 3-carene and limonene) in extracts of needles derived from Scots pine, *P. sylvestris,* from Lithuania. Figure 1 shows a typical chromatogram. All the above-mentioned six monoterpenes were detected in all the analysed samples of Scots pine needles, except one tree in the needles of which one of the monoterpenes (3-carene) was not found. In that case, the sample could have contained 3-carene as well, but its amount could be extremely small, i.e. below the chromatographic detection level.

The relative amounts of all the six studied monoterpenes in needles of different trees of Scots pine varied significantly. The mean values of monoterpenes in needles with standard errors are presented in Table.

Two components, α-pinene and 3-carene (Table, Fig. 2) were dominant in needles of all the trees studied, except one tree in the needles of which one dominant component (3-carene) was not detected.

Comparison of the above-mentioned dominant components revealed that α-pinene was the most abundant: in 7 cases out of 8, its amount exceeded that of 3-carene 1.8- to 7.1-fold and in one case more than 40-fold (when carene was not detected, but keeping in mind the detection limit we equated its amount to 1, instead of zero, to make a comparison possible, although very approximate). It was only in one case out of 8 that the amount of 3-carene in needles exceeded that of α-pinene 2.5-fold (Table).

Figure 2 shows the distribution not only of the means (Table), but also of all the determined values of monoterpenes (three replicates for each tree). This distribution demonstrates that the variation range of α-pinene, 3-carene and β-pinene is the highest of all the monoterpenes studied, while those of

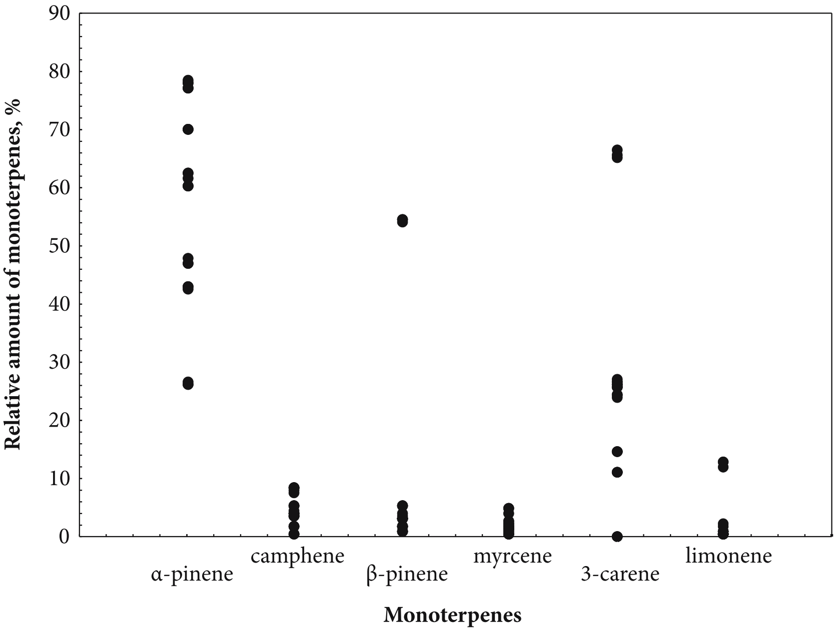

Fig. 2. Relative content (%) of monoterpenes in needles of separate individuals of *P. sylvestris* from the Lithuanian population

Table. **Relative content of monoterpenes (%, mean ± standard error) in Scots pine needles from Lithuanian population**

Monoterpene	Retention time, min	Scots pine (P)							
		P1 Vilnius	P2 Pasvalys	P3 Labanoras	P4 Merkinė	P5 Marcinkonys	P6 Butrimonys	P7 Alytus	P8 Palanga
α-pinene	8.64	47.38 ± 0.26	69.97 ± 0.05	60.16 ± 0.03	62.18 ± 0.36	78.23 ± 0.20	77.18 ± 0.05	42. 83 ± 0.16	26.44 ± 0.16
camphene	9.07	3.72 ± 0.01	1.91 ± 0.07	8.36 ± 0.003	8.01 ± 0.17	5.36 ± 0.03	4.21 ±0.03	0.57 ± 0.02	3.78 ± 0.15
β-pinene	9.90	5.28 ± 0.06	0.78 ± 0.06	2.95 ± 0.01	3.44 ± 0.22	3.18 ± 0.05	1.92 ± 0.04	54.38 ± 0.06	1.47 ± 0.21
myrcene	10.34	4.48 ± 0.26	0.76 ± 0.07	2.46 ± 0.03	1.60 ± 0.19	1.50 ± 0.16	1.42 ± 0.02	0.57 ± 0.02	1.99 ± 0.21
3-carene	10.97	26.61 ± 0.15	26.02 ± 0.02	25.65 ± 0.01	24.11 ± 0.14	11.02 ± 0.03	14.59 ± 0.01	0*	65.81 ± 0.41
limonene	11.49	12.53 ± 0.21	0.56 ± 0.03	0.43 ± 0.02	0.67 ± 0.08	0.71 ± 0.05	0.67 ± 0.05	1.65 ± 0.40	0.52 ± 0.02

* Below detection level.

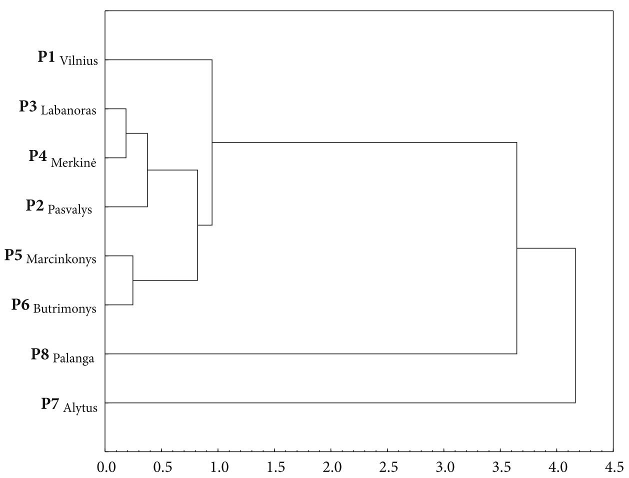

Fig. 3. Cladogram of Scots pine (*P. sylvestris*) from the Lithuanian population with regard to the composition of six monoterpenes in needles

myrcene and camphene are the least. The cladogram (Fig. 3) based on the composition of six monoterpenes shows that six trees are quite similar in the composition of terpenes. The greatest difference in monoterpene composition was recorded between two trees. Needles of one of them contained a very large amount of 3-carene, while needles of the other tree had a very small amount or no 3-carene at all (below detection level). Such trees of Scots pine with no 3-carene (or below detection level) in their needles are interesting, but not unique. Such cases were recorded in other regions as well, e. g., in Turkey (Semiz et al., 2007). Judging from the given set of samples, it is possible to assert that Scots pine from the Lithuanian population is likely to exhibit the greatest variability according to the relative content of 3-carene in needles.

Why the relative content of some monoterpenes varies more than that of others is still not clear; all we can do is to propose hypotheses. But if we take into consideration the fact that synthesis of monoterpenes is very "costly" to pine-trees (in terms of energy and material resources' consumption), we could presume that large amounts of monoterpenes are produced when a plant

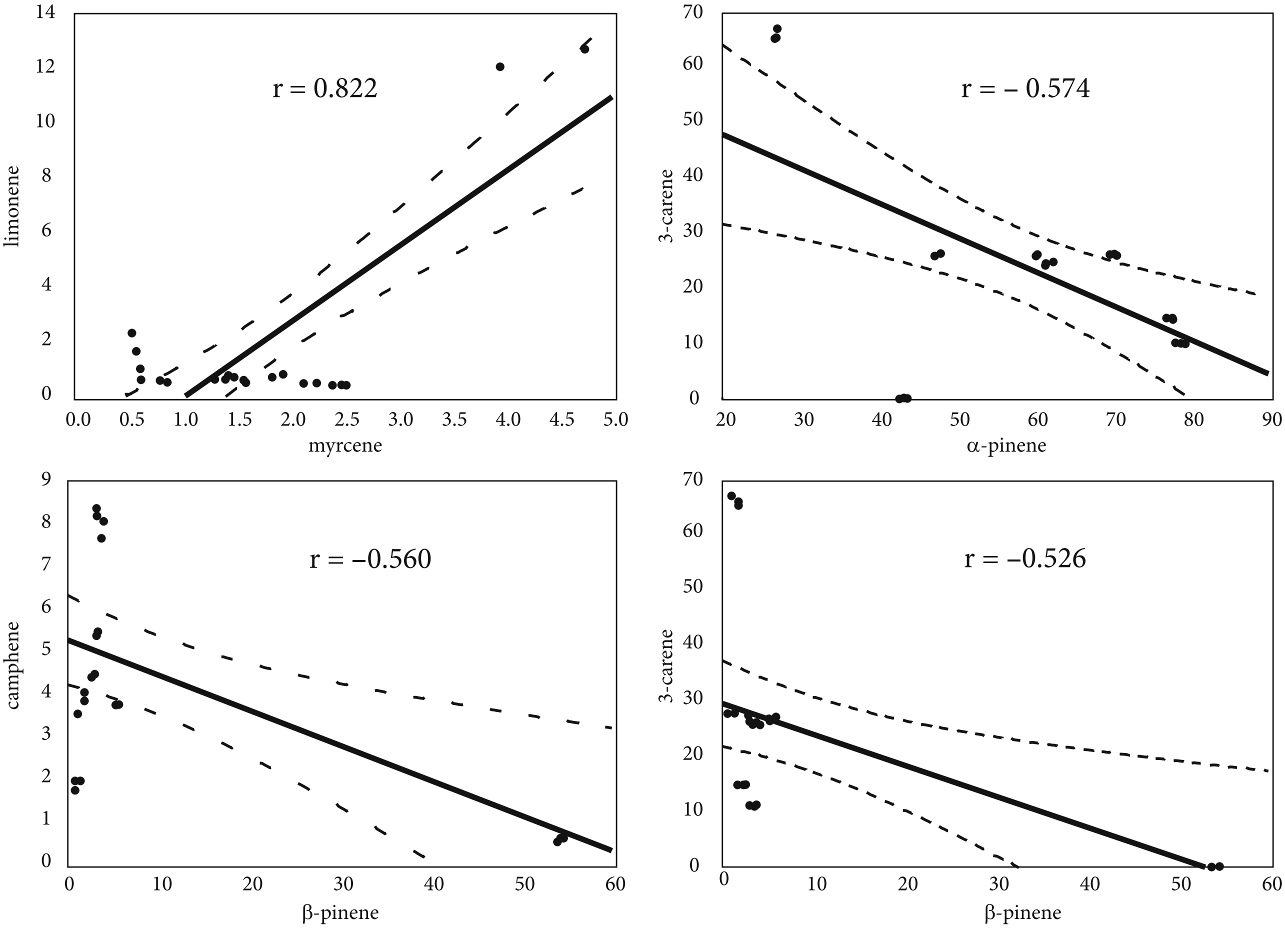

Fig. 4. Correlation between relative content (%) of monoterpenes in needles of Scots pine (*P. sylvestris*) (pairwise analysis)

is in great need of these compounds. Thus, we hypothesize that although the monoterpenes that vary in the quantitative composition most widely are "costly" to a plant, they could be more important (at least for defensive purposes) in comparison with the monoterpenes that are synthesized in small and rather stable amounts.

To answer the question as to whether all the investigated monoterpenes (judging from their relative amount) are synthesized irrespective of one another or whether some of them are interrelated, we carried out a pairwise assessment of correlations between relative amounts of monoterpenes. The highest absolute values of correlation coefficients (r) were obtained between myrcene and limonene (r = 0.822), between α-pinene and 3-carene (r = –0.574), between camphene and β-pinene (r = –0.560) and between 3-carene and β-pinene (r = –0.526) (Fig. 4). The obtained results suggest that if among the monoterpenes there are interrelated compounds whose amounts in needles are not totally independent of each other, the most probable pairs could be the following: myrcene and limonene, α-pinene and 3-carene, camphene and β-pinene, 3-carene and β-pinene. In the case of the first pair of compounds, the correlation is positive (an increase in the content of one compound is followed by an increase in the content of the other). In the other three cases, the correlation is negative (an increase in one compound is followed by a decrease in the other). To get an exhaustive answer to the question as to how the contents of monoterpenes are interrelated in needles, further investigations are needed.

Results of our investigations have demonstrated that with regard to monoterpene composition Scots pine trees from the Lithuanian population are polymorphic. What is more, analysis of the relative composition of monoterpenes can be used for the identification of chemotypes in population diversity investigations of the above-mentioned pine species in Lithuania.

CONCLUSIONS

1. Trees of Scots pine (*P. sylvestris*) from the Lithuanian population are polymorphic with regard to monoterpene composition in needles. Relative contents of α-pinene, 3-carene and β-pinene are the most variable, those of myrcene and camphene are the least, while the variation of the relative amount of limonene is moderate.

2. The most pronounced difference among Scots pine trees from the Lithuanian population was recorded in the relative amount of 3-carene: needles of some plants contain huge amounts of this monoterpene (about 65.8% of all monoterpenes), while in needles of others it was absent (or its amount was below the detection level).

3. The relative composition of monoterpenes in needles of Scots pine can be used for the selection of this plant and in population investigations.

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LIETUVOS PAPRASTŲJŲ PUŠŲ (*PINUS SYLVESTRIS*) POLIMORFIZMAS PAGAL SPYGLIŲ MONOTERPENŲ SUDĖTĮ

Santrauka

Paprastųjų pušų ir su jomis susijusių kitų organizmų sąveikos labai priklauso nuo medžių cheminės sudėties, tarp jų – nuo spyglių monoterpenų. Pušų spyglių monoterpenai atlieka apsauginę funkciją (padeda gintis nuo įvairių augalėdžių – vabzdžių, moliuskų, stambiųjų žinduolių), jie veikia ir kaip kairomonai arba sinomonai. Dujų chromatografijos metodu atlikti žvalgomieji tyrimai. Tirta 7–10 m. amžiaus paprastųjų pušų, *Pinus sylvestris,* iš Lietuvos populiacijos (santykinai nedidelės teritorijos) spyglių monoterpenų sudėtis. Analizuota 6 monoterpenų (α-pineno, kamfeno, β-pineno, mirceno, 3-kareno, limoneno) sudėtis. Nustatyta, kad pagal spyglių monoterpenų sudėtį paprastosios pušys yra polimorfiškos. Spyglių ekstraktuose vyraujantys monoterpenai – α-pinenas ir 3-karenas, jų santykiniai kiekiai (nuo visų tirtųjų monoterpenų kiekio) kito atitinkamai nuo 27 iki 79% ir nuo 0 iki 66%. Labiausiai kinta α-pineno, 3-kareno ir β-pineno, mažiausiai – mirceno ir kamfeno santykiniai kiekiai. Tarpinę padėtį užima limonenas. Labiausiai skyrėsi 3-kareno kiekis: vienų augalų spygliuose šio monoterpeno yra labai daug (apie 65,8% nuo visų monoterpenų), kitų – visiškai nėra (arba yra mažesnis nei chromatografiškai aptinkamas kiekis). Santykinę paprastosios pušies, *Pinus sylvestris,* spyglių monoterpenų sudėtį galima panaudoti selekcijai bei populiaciniuose tyrimuose, išskiriant šio augalo chemotipus.

Raktažodžiai: *Pinus sylvestris*, populiacija, įvairovė, antriniai metabolitai, α-pinenas, β-pinenas, kamfenas, mircenas, 3-karenas, limonenas, monoterpenų įvairavimas