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NOTE

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Individual variations in monoterpenes released from *Cryptomeria japonica* and *Pinus thunbergii* needles

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Abstract Variations in the compositions of low boiling point (LBP) monoterpenes in needle samples from 99 sugi (*Cryptomeria japonica*) and 100 kuromatsu (*Pinus thunbergii*) trees were investigated using a headspace technique. Considerable variations in the proportions of monoterpenes were revealed in both species. In sugi, the proportions of sabinene and α-pinene in the total LBP monoterpenes, ranging from 8.8% to 73.3% and from 14.5% to 73.3%, respectively, showed enormous variations among nine monoterpenes. The proportions of 3-carene and limonene, ranging from 0.1% to 29.5% and from 0.2% to 20.4%, respectively, also showed very specific variations. In kuromatsu, the proportions of β-pinene and α-pinene in the total LBP monoterpenes, ranging from 26.5% to 66.3% and from 18.7% to 46.9%, respectively, showed considerable variations among ten monoterpenes. The proportions of myrcene and 1,8-cineole, ranging from 0.9% to 18.5% and from 0.8% to 12.3%, respectively, also showed specific variations.

Key words *Cryptomeria japonica* · *Pinus thunbergii* · Monoterpenes · Sabinene · β-Pinene

Introduction

Monoterpenes, which encompass more than 500 naturally occurring compounds,[1] function in herbivore and pathogen defense. A number of monoterpenes are also of commercial importance as flavors and fragrances, pharmaceuticals, insecticides, and synthetic intermediates.[2,3] The constituents and proportions of monoterpenes arrange species-specific fragrances and intraspecific variations of monoterpenes in conifer species are also known as monoterpene chemotypes.[4,5] Monoterpene synthases are invariably members of gene families thought to have arisen by duplication and differentiation to permit diverse responses to multiple environmental pressures[6] (e.g., insect and microbial defenses in the case of conifers[7]). Accordingly, monoterpenes describe infraspecific characteristics; same species with different bioactive monoterpene aspects show different adaptivity to environment. The chemical ecological relationships between conifer host, beetle pest, and beetle predator are extremely complex and the variation in oleoresin monoterpenes can be seen as approaches to population resistance based on host disguise or alteration in the levels of pheromone precursors or predator attractants.[8–10]

Considerable variation in the proportions of low boiling point (LBP) monoterpenes has been observed in volatiles from hinoki (*Chamaecyparis obtusa*) needles,[5,11] The relative proportion of sabinene, which is the dominant constituent of the LBP monoterpenes, showed the biggest variation (4.9% to 78.0%) among nine monoterpenes. To investigate if such considerable variation exists in the proportions of LBP monoterpenes in Japanese representative conifers, LBP monoterpenes released from needles of sugi (*Cryptomeria japonica*) and kuromatsu (*Pinus thunbergii*) were analyzed. Aromatic components with various bioactivities such as α-pinene, camphene, β-pinene, sabinene, 3-carene, myrcene, limonene, 1,8-cineole, γ-terpinene, and terpinolene were treated as LBP monoterpenes.

Materials and methods

Plant materials

Healthy needle tips about 10 cm long were harvested from 99 sugi trees at Shimane University Pilot Forest in Matsue

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and four other sites around Matsue, Shimane, Japan and also from 100 kuromatsu trees at Katsura-jima, Shimane, Japan. Three needle tips were harvested from different parts on each of all the individual trees.

Monoterpene analysis

For monoterpene analysis, needle samples of 2–3 g were minced with scissors, and put into glass test tubes (25 × 120 mm). The test tubes were then sealed with parafilm and mixed to dissipate the LBP monoterpenes for several seconds. The volatile monoterpenes were collected with 2 ml of headspace air by a precision analytical syringe (Precision Sampling). The headspace air was injected into a gas chromatograph (Shimadzu GC-14A equipped with flame ionization detector) and analyzed. The conditions for analysis on the 0.25 mm i.d. × 25 m Shimadzu CBP 20-M25-025 column (PEG20M type) were 50°C isothermal (3 min), which was then programmed to a final temperature of 150°C at 10°C/min with helium (1.0 ml/min) as carrier. With the monoterpene composition of sugi being well defined,[12] comparison of retention times of the volatiles to those of authentic standards was sufficient to confirm identifications. For identifications of volatiles released from kuromatsu, essential oil from needles of kuromatsu was identified in a gas chromatography-mass spectrometry (GC-MS) analysis (Jeol BU20 GC mate system) by comparing retention times and mass spectra with those of library spectra or authentic standards. The conditions for analysis on the 0.25 mm i.d. × 30 m J&W Scientific DB-WAX column were 50°C isothermal (3 min), which was then programmed to a final temperature of 220°C (10 min) at 10°C/min with helium (1.0 ml/min) as carrier (ionization voltage 70 eV). The LBP monoterpenes released from three needle samples of an individual tree were analyzed on each of all the individual trees and contingency table tests indicated that there was no variation of proportions in all individual trees but one sample in sugi and two samples in kuromatsu. Average values of the LBP monoterpene proportions in these three needle samples are discussed in this article.

Results and discussion

Variation of monoterpene composition in sugi

The LBP monoterpene composition analysis revealed that considerable variations in several components were found among 99 sugi trees (Table 1). The proportions of sabinene and α-pinene in the total LBP monoterpenes, ranging from 8.8% to 73.3% and from 14.5% to 73.3%, respectively, showed enormous variations among nine monoterpenes. The proportions of 3-carene and limonene, ranging from 0.1% to 29.5% and from 0.2% to 20.4%, respectively, also showed very specific variations. The proportion of sabinene occupied 50.0%–70.0% of the total LBP monoterpenes in 46.5% of the total samples. On the other hand, the proportion of α-pinene occupied 20.0%–50.0% of the total LBP monoterpenes in 82.8% of the total samples. The 3-carene and limonene were minor constituents (0%–5.0% of the total LBP monoterpenes) in 80.8% and 73.7% of the total samples, respectively. In 5 samples (5.1% of the total samples), however, 3-carene occupied 25.0%–30.0% and limonene occupied 15.0%–25.0% of the total LBP monoterpenes.

Table 1. Compositional variations of low boiling point (LBP) monoterpenes released from needles of 99 sugi trees

Monoterpane	Compositional variation (%)		
α-Pinene	14.5	–	73.3
Camphene	0.1	–	7.3
β-Pinene	0.4	–	2.6
Sabinene	8.8	–	73.3
3-Carene	0.1	–	29.5
Myrcene	1.6	–	7.3
Limonene	0.2	–	20.4
γ-Terpinene	0.1	–	0.9
Terpinolene	0.1	–	1.0

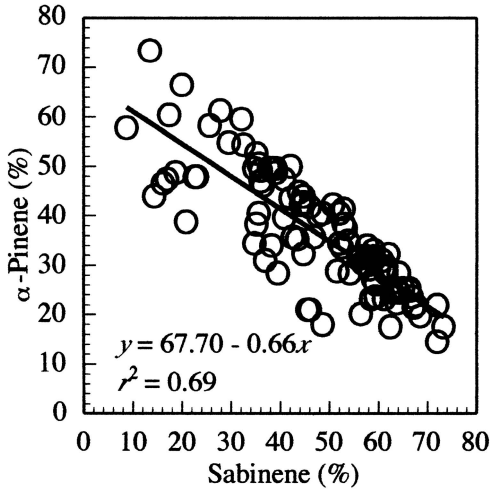

Fig. 1. Relationship between proportions of sabinene and α-pinene in low boiling point (LBP) monoterpenes released from needles of 99 sugi trees

The relationship between the proportion of sabinene and α-pinene was investigated (Fig. 1). The proportion of sabinene negatively correlated with that of α-pinene ($r = -0.83$, $P < 0.0001$). It was revealed that several monoterpene chemotypes exist in sugi, i.e., either sabinene or α-pinene, or both of them were the dominant monoterpenes released from the needles of sugi. The three monoterpene chemotypes were represented as follows; sabinene type, in which the proportion of sabinene occupies 50.0% and above, α-pinene type, in which the proportion of α-pinene occupies 50.0% and above, and intermediate type, in which the proportions of both sabinene and α-pinene are below 50.0% of the total LBP monoterpenes. In addition, samples that released high proportions of 3-carene or limonene may be treated as 3-carene chemotype or limonene chemotype. Beetle pests are attracted by specific blends of monoterpe-

Table 2. Compositional variation of LBP monoterpenes released from needles of 100 kuromatsu trees

Monoterpene	Compositional variation (%)		
α-Pinene	18.7	–	46.9
Camphene	0.7	–	3.4
β-Pinene	26.5	–	66.3
Sabinene	nd	–	1.8
3-Carene	nd	–	1.2
Myrcene	0.9	–	18.5
Limonene	nd	–	5.4
1,8-Cineole	0.8		12.3
γ-Terpinene	nd	–	0.6
Terpinolene	nd	–	1.9

nd, Not detected

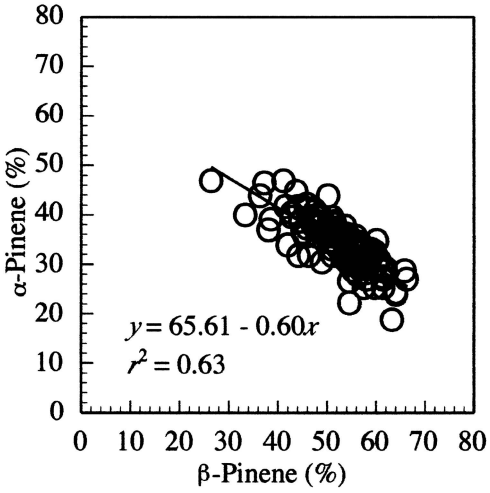

Fig. 2. Relationship between proportions of β-pinene and α-pinene in LBP monoterpenes released from needles of 100 kuromatsu trees

nes.[8,9] These monoterpene chemotypes in sugi may have different activities against the beetle pests and their fungal symbionts.

Neither 3-carene nor limonene highly correlated with any other LBP monoterpenes. Monoterpene synthases show an unusual and intriguing feature; that is, simultaneous production of multiple products from a single substrate, geranyl diphosphate.[13–16] However, it seems that 3-carene and limonene are produced independently by their own monoterpene synthases.

Variation of monoterpene composition in kuromatsu

The LBP monoterpene composition analysis also revealed that considerable variations in several components were found among 100 kuromatsu trees (Table 2). The proportions of β-pinene and α-pinene in the total LBP monoterpenes, ranging from 26.5% to 66.3% and from 18.7% to 46.9%, respectively, showed considerable variations among ten monoterpenes. The proportions of myrcene and 1,8-cineole, ranging from 0.9% to 18.5% and from 0.8% to 12.3%, respectively, also showed specific variations. The proportion of β-pinene occupied 50.0%–60.0% of the total LBP monoterpenes in 52.0% of the total samples. On the other hand, the proportion of α-pinene occupied 30.0%–40.0% of the total LBP monoterpenes in 65.0% of the total samples. The myrcene was a minor constituent (0%–10.0% of the total LBP monoterpenes) in 90.0% of the total samples. The 1,8-cineole was also a minor constituent (0%–5.0% of the total LBP monoterpenes) in 78.0% of the total samples. A few samples showed higher proportions of myrcene or 1,8-cineole.

The relationship between the proportion of β-pinene and α-pinene was investigated (Fig. 2). The proportion of β-pinene negatively correlated with that of α-pinene ($r = -0.79$, $P < 0.0001$). The variations of monoterpenes in kuromatsu were relatively small compared with those of sugi. No other high correlation was found between any combinations of the LBP monoterpenes.

The cerambycid beetle *Monochamus alternatus* is a primary vector of the destructive pinewood nematode *Bursaphelenchus xylophilus*, the causative agent of pine wilt disease in Japan.[17,18] The mature females oviposit on dying or recently killed trees of akamatsu (*Pinus densiflora*) and kuromatsu. A mixture of α-pinene and longifolene, which is released from diseased kuromatsu trees, is more attractive to the mature female beetles than a mixture of α-pinene and β-pinene, which is released from healthy kuromatsu trees.[19] The beetles soon after emerging are strongly attracted to α-pinene but not to a combination of α-pinene and β-pinene;[19] that is, β-pinene appears to have a role in reducing attractiveness of α-pinene for the beetles. The kuromatsu trees that release higher proportions of β-pinene from their needles, therefore, might be more resistant to the cerambycid beetles.

Conclusions

Both of the represented Japanese conifers, sugi and kuromatsu, had a large variation in LBP monoterpenes released from their needles. The individual variation of sugi was larger than that of kuromatsu, and almost the same as that of hinoki.[5] However, further research is needed to evaluate the degree of the individual variations among these coniferous species because the needles of kuromatsu tested in this study were collected from only one area.

The large variation in monoterpene composition among trees would appear to have important consequences for defense of the species against predation by bark beetles and their pathogenic fungal associates. Elucidating the mechanisms resulting in such variation requires biochemical and genetic understanding of the regulation of monoterpene biosynthesis. An appreciation of the chemical diversity in these species is essential for the efficient acquisition of the necessary molecular tools and for the design of appropriate experimental approaches to the genetic engineering of oleoresin content.

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