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ORIGINAL ARTICLE

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Individual variation in low boiling point monoterpenes emitted from hinoki (*Chamaecyparis obtusa*) needles

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Abstract Variations in the compositions of low-boiling-point (LBP) monoterpenes in needle samples of 50 hinoki (*Chamaecyparis obtusa*) trees were investigated using the headspace technique. Considerable compositional variations were revealed, especially in sabinene composition. The sabinene composition varied from 4.9% to 78.0% of the total LBP monoterpenes. α-Pinene, myrcene, and limonene also showed considerable variations (9.0%–32.7%, 5.5%–22.6%, 3.6%–29.0% respectively). Analysis of the monoterpene composition allowed definition of four chemotypes based on the contingency table test. No correlation was observed between tree size and LBP monoterpene composition, indicating that the compositional variation in LBP monoterpene exists genetically in this population of hinoki.

Key words *Chamaecyparis obtusa* · Hinoki · Monoterpenes · Sabinene

Introduction

Many conifer species produce monoterpenes to defend against bark beetle attack and associated fungal infection at the wound site.[1–4] The volatile monoterpenes have antiseptic properties; that is, many of them prevent fungal growth and have toxic or repellent properties toward insect pests. For example, *l*-perillaldehyde and *l*-α-terpineol inhibit the growth of the plant pathogenic fungi *Pyrenophora graminea* and *Cochlipbulus miyabeanus*.[5] Application of a mixture of α-pinene, β-pinene, limonene, and 3-carene reduces the feeding and oviposition of the carrot psyllid, *Trioza apicalis*.[6] α-Pinene attracts the pine weevils *Hylobius abietis* and *H. pinastri*, and limonene completely inhibits the attraction to α-pinene.[7] Limonene, β-pinene, α-pinene, and 3-carene are toxic to the adult female spruce spider mite *Oligonychus ununguis*.[8] Therefore, they are used as repellents or antibacterial agents.[9–11] Furthermore, the naturally occurring monoterpene *d*-limonene has chemopreventive and chemotherapeutic activity against many rodent tumors, such as mammary, liver, colon, and lung carcinomas.[12–15] The multiple antitumorigenic effects of limonene suggest that limonene and related monoterpenes may be efficacious in the chemoprevention and chemotherapy of human malignancies.[16] Monoterpenes are also important materials for the manufacture of medicines, vitamins, perfumes, and many other commodities.[17]

Individual variation in constitutive and induced monoterpene biosynthesis in grand fir (*Abies grandis*) was reported.[18] Radiochemically based in vitro assays, with [1-^{3}H]geranyl diphosphate as substrate, revealed considerable quantitative variation in constitutive and induced response levels of total monoterpene synthase activity, and the analysis of monoterpene distribution generated in the cell-free extracts allowed definition of seven distinct biosynthetic chemotypes. These individual differences are important for understanding bark beetle and associated fungal defense and the regulation of oleoresinosis. They also provide the possibility of evaluating tree activity, insect repellency, fungicidal, insect attractiveness, and genetic differences.

Low-boiling-point (LBP) monoterpenes are flavor components with the above-mentioned various bioactivities. α-Pinene, camphene, β-pinene, sabinene, myrcene, limonene, γ-terpinene, and terpinolene were treated as "LBP monoterpenes" in this study. We describe considerable individual differences of LBP monoterpene compositions emitted from hinoki (*Chamaecyparis obtusa*) needles. Four LBP

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monoterpene chemotypes were defined based on a contingency table test.

Experimental

Plant materials

Healthy needle tips about 10cm long of hinoki (*Chamaecyparis obtusa* Endl.) were harvested from 50 trees at Shimane University Pilot Forest in Matsue and four other sites around Matsue City, Shimane, Japan. Tree heights and diameters at breast height were measured with a scale.

Monoterpene analysis

For monoterpene analysis, hinoki needle samples of 2–3g were minced with scissors, and put into glass test tubes (25 × 120mm). The test tubes were then sealed with parafilm and mixed to dissipate LBP monoterpenes for several seconds. The volatile monoterpenes were collected with 2ml of air by a gas-tight syringe. The air was injected into a gas chromatograph (Shimadzu GC-14A equipped with FID (flame ionization detector)) and analyzed. Three or four replicates, which were harvested from different parts of an individual tree, were analyzed. Of the 50 samples, 20 were harvested and analyzed after 2 months from the first experiments. The conditions for analysis on the 0.25mm i.d. × 25m Shimadzu CBP 20-M25-025 column (PEG20M type) were 50°C isothermal (3min), which was then programmed to a final temperature of 100°C at 10°C/min with helium (1ml/min) as carrier. The monoterpene composition of hinoki is well defined, so the retention times of the monoterpene compounds were compared with those of authentic standards, which was sufficient to confirm the identification.[19]

Results and discussion

Monoterpenes are bioactive volatile substances that are synthesized from geranyl diphosphate by monoterpene synthases in the epithelial cells of specialized secretory structures, such as resin blisters.[20,21] Because conifer needles emit a sufficient amount of monoterpenes, the volatile monoterpenes can be easily determined using the headspace technique and gas chromatography.

Eleven LBP monoterpenes (α-pinene, camphene, β-pinene, sabinene, myrcene, α-terpinene, limonene, 1,8-cineol, γ-terpinene, p-cymene, terpinolene) in hinoki needles have been identified by gas chromatography-mass spectrometry (GC-MS).[19] Of the 11 principal monoterpenes, 8 (α-pinene, camphene, β-pinene, sabinene, myrcene, limonene, γ-terpinene, terpinolene) were determined to study individual variations of the LBP monoterpenes emitted from hinoki needles.

Table 1. Compositional variations of low-boiling-point monoterpenes emitted from needles of 50 hinoki trees

Monoterpene	Compositional variation (%)
α-Pinene	9.0–32.7
Camphene	0.4–3.7
β-Pinene	0.3–2.2
Sabinene	4.9–78.0
Myrcene	5.5–22.6
Limonene	3.6–29.0
γ-Terpinene	1.5–7.8
Terpinolene	0.6–1.2

To confirm that the variation in the LBP monoterpenes exists in an individual tree, three or four needles were harvested from different positions on each of 50 individual trees and the LBP monoterpenes were analyzed. Then, contingency table tests were carried out in all of samples. The mean χ^2 value of 50 samples was 0.49 ± 0.07 (SE), and the mean (±SE) rejection value was 26.02 ± 0.56 (α = 0.05). The contingency table tests showed that there was no variation in individual trees in this study.

The LBP monoterpenes emitted from 50 needle samples were collected using the headspace technique and were analyzed by gas chromatography. The LBP monoterpene compositions showed extraordinary variations (Table 1). Sabinene composition, ranging from 4.9% to 78.0%, showed the biggest variation in eight monoterpenes. α-Pinene, myrcene, and limonene also showed considerable variations (9.0%–32.7%, 5.5%–22.6%, and 3.6%–29.0%, respectively). These variations suggest that LBP monoterpene compositions show even intraspecific properties.

It is known that these monoterpenes prevent fungal growth and have toxic, repellent, or attractant properties toward insect pests.[9–11,22] Because these properties strongly depend on their monoterpene composition, the enormous compositional variations of hinoki needles must change the various properties. These variations were reconfirmed 2 months after the original experiments in 20 of 50 samples. Changes in LBP monoterpene compositions after 2 months were within 4.4% in sabinene composition, which showed the biggest variation (data not shown).

Variations of α-pinene, sabinene, myrcene, and limonene compositions in all of the 50 samples were determined by the contingency table test. The calculated χ^2 value was 351.8, and the $\chi^2(\phi = 147, \alpha = 0.05)$ value was 176.3; that is there was a significant difference. To determine the characteristic differences, adjusted residuals (**ds**) of four monoterpenes were calculated. The $\mathbf{d} \geq |2.0|$ means there is a characteristic difference. Based on the **d** of the sabinene composition, four distinct variants in LBP monoterpene composition were noted (Fig. 1). The **d** of sabinene of −10.8 represents a sample containing 4.9% sabinene. The mean **ds** of sabinene of −2.7, −0.3, and 3.0 represent samples containing 37.5%–47.6%, 49.6%–68.3%, and 69.8%–78.0% sabinene, respectively. These values are presumed to represent LBP monoterpene chemotypes, as each is an average of three or four replicates, and they do not change for at least 2 months. This means that these variations are not

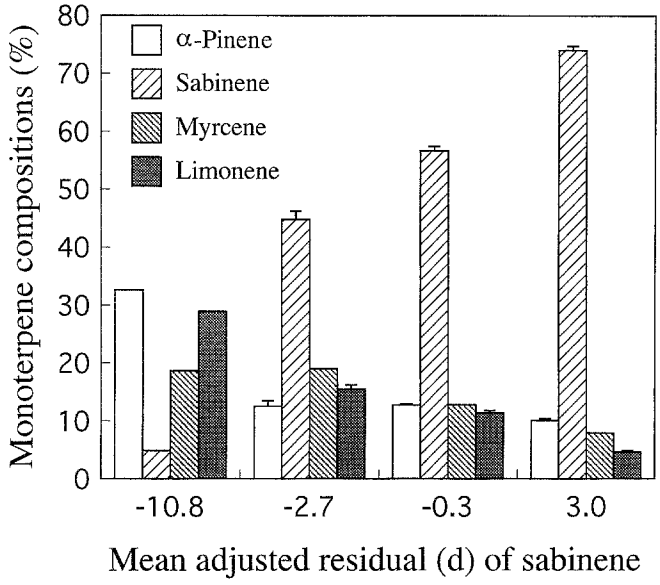

Fig. 1. Low-boiling point monoterpene chemotypes based on contingency table test. Detail of mean adjusted residual (**d**) is provided in the Results and discussion section. Error bars represent standard errors

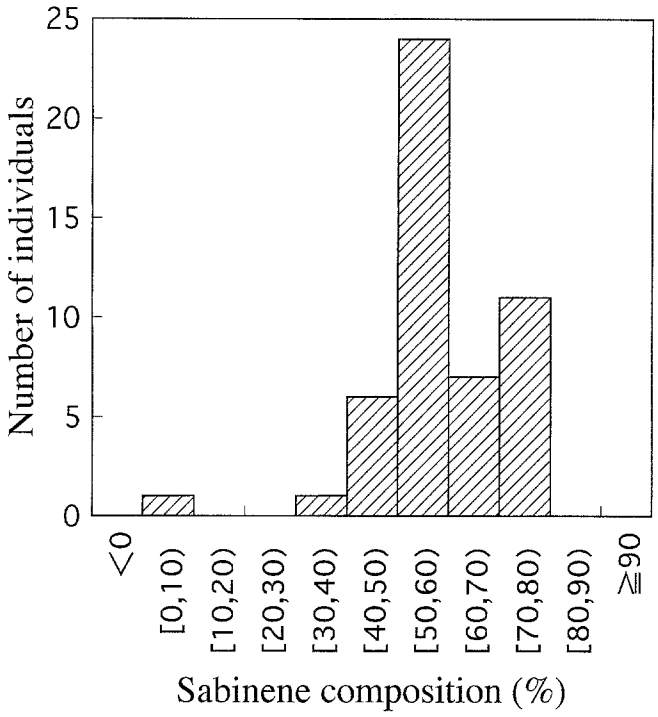

Fig. 2. Histogram of sabinene compositions emitted from needles of 50 hinoki trees

Table 2. Correlation coefficients among eight monoterpenes, tree height, and diameter at breast height

Monoterpene	α-Pinene	Camphene	β-Pinene	Sabinene	Myrcene	Limonene	γ-Terpinene	Terpinolene	Tree Height	DBH
α-Pinene	1.00									
Camphene	0.86	1.00								
β-Pinene	0.84	0.94	1.00							
Sabinene	−0.79	−0.90	−0.94	1.00						
Myrcene	0.27	0.50	0.58	−0.77	1.00					
Limonene	0.78	0.93	0.96	−0.98	0.70	1.00				
γ-Terpinene	0.73	0.86	0.91	−0.97	0.72	0.98	1.00			
Terpinolene	0.48	0.46	0.56	−0.71	0.62	0.69	0.78	1.00		
Tree Height	−0.12	−0.18	−0.22	0.39	−0.59	−0.31	−0.35	−0.32	1.00	
DBH	−0.19	−0.19	−0.28	0.33	−0.49	−0.27	−0.29	−0.19	0.93	1.00

DBH, diameter at breast height

caused by a response to wounding because the wound response of coniferous trees that do not have resin ducts is localized and short term.[4] Furthermore, these variations were found even in several trees that grew in identical environments. Seasonal variation of hinoki needle oils was reported,[19] but in this study all of the samples were collected and analyzed within a month, and monoterpene compositions hardly change in 2 months.

A histogram based on sabinene composition emitted from 50 hinoki tree needles is shown in Fig. 2. Needle samples emitted 50%–60% sabinene in LBP monoterpene compositions in 48% of 50 samples; second was 70%–80% sabinene. Needle samples emitting 0%–10% and 30%–40% sabinene accounted for only 1 each of 50 samples. A sample emitting 4.9% sabinene of the LBP monoterpene composition was special compared to the others. It is possible that this "sabinene-lacking hinoki" is a recently evolved mutant. Making clones of this sample by tissue culture and cuttage is underway to investigate the environmental influence and the genetic differences at the DNA level.

Table 2 shows correlation coefficients for eight monoterpenes, tree height, and diameter at breast height (DBH). Sabinene negatively correlated well with other monoterpenes. Myrcene and terpinolene did not correlate well with α-pinene, camphene, and β-pinene. The correlation coefficients among eight monoterpenes suggest that "sabinene synthase" in hinoki needles strongly affects other monoterpene synthases. Tree height and DBH did not correlate well any of the monoterpenes except myrcene. Myrcene showed a somewhat high negative correlation with tree height ($r = -0.59$) and DBH ($r = -0.49$). This result shows only that myrcene tends to decrease in proportion with tree age.

These results also strongly support the hypothesis that the variations in LBP monoterpene composition were genetically regulated; that is, they are chemotypes.

Variations in LBP monoterpene composition at five sites around Matsue City were studied by *t*-test using sabinene composition data. It was confirmed that combining the data showed normal distributions, and they were homoscedastic by F-test before *t*-tests were carried out. No significant difference ($\alpha = 0.05$) was observed in any combinations. Because the five sites were not far from each other, it was deduced that there is no large geographical variation. Individual variations in LBP monoterpene composition at one site were evidently greater than the geographical variation in this study.

Volatile monoterpenes are easily collected and their presence determined using the headspace technique; therefore many samples can be treated within a short-time. In this study four distinct variants in LBP monoterpene composition emitted from hinoki needles were found among 50 needle samples. Further variants must be found in other hinoki trees. By taking these variants into account, various research determinations, such as the bioactivities of monoterpenes, regulation of oleoresinosis, monoterpene synthase gene cloning, and genetic differences of hinoki, can proceed. Studies of the influence of LBP monoterpene chemotypes on tissue culture of hinoki, individual variations of LBP monoterpenes emitted from sugi (*Cryptomeria japonica* D. Don) and kuromatsu (*Pinus thunbergii* Parl.) are underway in our laboratory.

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