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ORIGINAL ARTICLE

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Tree-to-tree and clone-to-clone variations of monoterpenes emitted from needles of hinoki (*Chamaecyparis obtusa*)

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Abstract Variations in the composition of low boiling point (LBP) monoterpenes emitted from needle samples of 150 hinoki (*Chamaecyparis obtusa*) trees (30 strains, each with five clones) native to Shimane Prefecture, Japan, were investigated using a headspace technique. The assays revealed considerable proportional variations especially in the amount of sabinene, which ranged from 24% to 78% of the total LBP monoterpenes. The proportions of α-pinene, myrcene, and limonene negatively correlated with that of sabinene overall. In particular, the proportion of limonene showed clear negative correlation with that of sabinene ($r = -0.98$). Differences in the proportion of sabinene among five clones in each strain were less than 15% in 22 out of 30 strains, indicating that monoterpene composition is constitutively steady in most strains. In a few strains, however, considerable variation in the composition was observed among clones.

Key words *Chamaecyparis obtusa* · Conifer · Hinoki · Monoterpenes · Terpenoids

Introduction

Monoterpenes, a class that includes over 500 naturally occurring compounds,[1] are considered as the faces of trees, that is, the constituents and proportions of monoterpenes give species-specific fragrances. This means that monoterpene synthase (cyclase) genes and the regulatory genes have specifically diversified with speciation. Intraspecific variations of monoterpenes in conifer species, however, are also known as monoterpene-chemotypes,[2,3] indicating that the rate of monoterpene diversification is somewhat faster than that of speciation. Many monoterpene synthases are encoded by multiple-gene copies that arose by duplication and then provided the basis for diversification.[4] Accordingly, monoterpenes describe infraspecific characteristics; same species with different bioactive monoterpene aspects show different adaptivity to the 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.[5–7]

Hinoki (*Chamaecyparis obtusa*), also called Japanese cypress, is highly prized in Japan. The aromatic wood is exceedingly durable and well known for its use in Japanese luxury building materials, baths, and many other wooden products.[8]

Thirty strains of cold-wind-resistant hinoki were collected from four local areas (Nita, Yokota, Hirose, and Yoshida) of Shimane, Japan and a total of 1560 trees consisting of the clones of these strains were planted for investigation and to select further elite trees at Shimane Prefectural Greenery Center in 1978 by the Shimane Prefectural Government. This project provided an excellent opportunity to investigate the individual variation in these strains and the variation among clones in the monoterpene composition. These results and their implications are described in this article. Aromatic components with various bioactivities such as α-pinene, camphene, β-pinene, sabinene, 3-carene, myrcene, limonene, γ-terpinene, and terpinolene were treated as low boiling point (LBP) monoterpenes.

Materials and methods

Plant materials

Healthy needle tips of hinoki, about 10 cm long, were harvested from 150 trees (30 strains, each with five clones) at Shimane Greenery Center, Shimane, Japan. Main

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harvesting was carried out in May and June 2001 and reexamination for 11 strains (55 samples) was carried out in September and October 2001. Approximately 50 clones in each strain were randomly planted in a 20900-m^2 field. These strains were represented by numerals, which started from number 21 and discontinuously climbed to number 91.

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 in 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 Shimadzu CBP 20-M25-025 column (0.25 mm i.d. × 25 m, PEG20M type) were 50°C isothermal (3 min), and then rising to a final temperature of 150°C at 10°C/min with helium (1 ml/min) as carrier. Because the monoterpene composition of hinoki is well defined,[9] comparison of retention times of the volatiles to those of authentic standards was sufficient to confirm identifications.

Results

Variation in all of the needle samples

The LBP monoterpene composition analysis revealed that considerable variations in each component were found among the individuals in the strains of hinoki (Table 1). The proportion of sabinene in the total LBP monoterpenes, ranging from 23.9% to 78.0%, showed the biggest variation among nine monoterpenes. The other main constituents, α-pinene, myrcene, and limonene, also showed considerable variations (8.1%–20.5%, 6.6%–27.0%, and 2.0%–24.0%, respectively).

Proportions of four principal monoterpenes emitted from the 150 needle samples are shown as histograms in Fig. 1. α-Pinene made up 10%–15% of the total LBP monoterpenes emitted from 118 needle samples, or 78.7% of the total samples. In contrast, wide variations were observed in the emission of sabinene ranging from 20% to 80% of the total LBP monoterpene. Almost half of the needle samples showed sabinene as 50%–60% of the total LBP monoterpene. Both myrcene and limonene showed lower proportions in the total LBP monoterpene, and the

Table 1. Compositional variation of low boiling point (LBP) monoterpenes emitted from needles of 150 hinoki strains

Monoterpene	Compositional variation (%)
α-Pinene	8.1–20.5
Camphene	nd–3.8
β-Pinene	0.3–1.9
Sabinene	23.9–78.0
3-Carene	nd–1.1
Myrcene	6.6–27.0
Limonene	2.0–24.0
γ-Terpinene	0.8–6.2
Terpinolene	nd–1.0

nd, Not detected

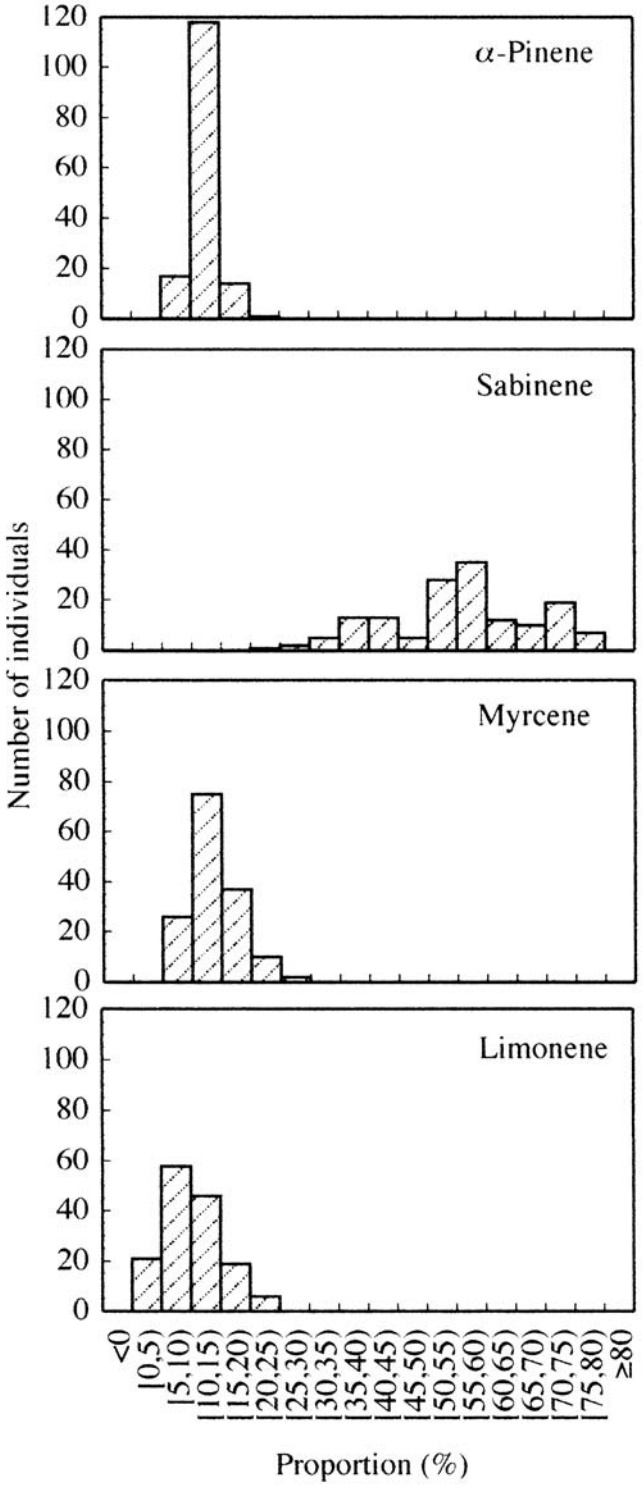

Fig. 1. Histograms showing the proportions of four principal monoterpenes emitted from the needles of 150 hinoki trees

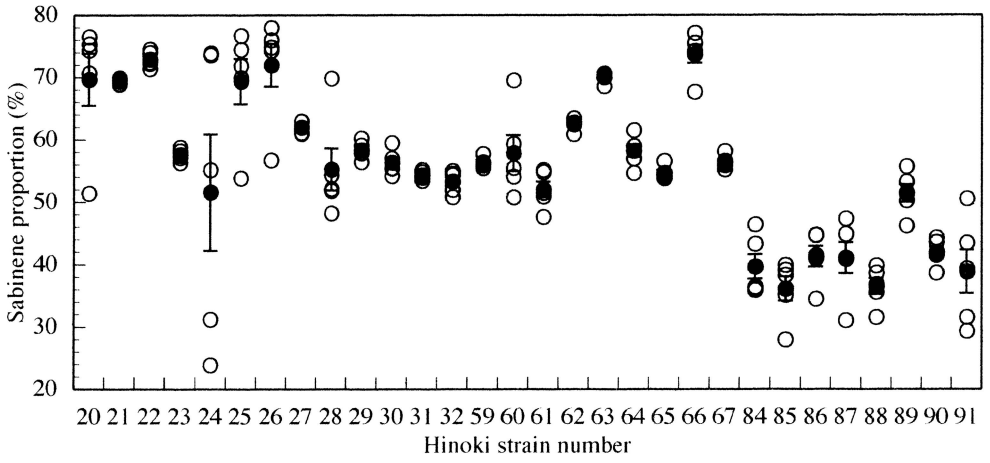

Fig. 2. Percentages of sabinene in low boiling point (LBP) monoterpenes emitted from needles of five clones in each hinoki strain. The values for each clone and the average are represented by *open circles* and *filled circles*, respectively. *Error bars* represent standard errors

proportion of myrcene was 10%–15% in 50% of the samples and that of limonene was 5%–15% in 69% of the samples.

Variation among clones

Variations among five clones in 30 strains based on the proportion of emitted sabinene, which showed the biggest variation in the LBP monoterpenes, were measured (Fig. 2). The variation in the proportion of sabinene among five clones was less than 15% in 22 out of 30 strains. Seven strains, however, showed considerable variation ranging from 16.3% to 25.1% (Nos. 20, 25, 26, 28, 60, 87, and 91). Furthermore, a difference of 50% among the five clones was observed in the proportion of sabinene in 1 strain (No. 24). The LBP monoterpene analysis was repeated to confirm the compositions for 11 strains including 8 strains that showed considerable variation among clones. The reexamination that was carried out 4 months after the initial experiment confirmed that changes in sabinene composition of less than 3% occurred in all of needle samples with seasonal change. Only one clone showed considerable difference in 6 strains (Nos. 20, 25, 26, 28, 60, and 87), whereas the two remaining strains, Nos. 24 and 91, showed considerably differed sabinene proportions in each clone.

Tree-to-tree variation and clone-to-clone variation

The relationship between the proportion of sabinene and the other three principal monoterpene constituents, α-pinene, myrcene, and limonene, were investigated separately in two groups: 22 strains (110 samples) that showed less than 15% clone-to-clone variation (i.e., tree-to-tree variation) in sabinene composition (Fig. 3), and 8 strains (40 samples) that showed over 15% clone-to-clone variation in sabinene composition (Fig. 4). The proportion of sabinene negatively correlated with that of α-pinene ($r = -0.86$, $P < 0.0001$), myrcene ($r = -0.88$, $P < 0.0001$), and limonene ($r = -0.98$, $P < 0.0001$) in the tree-to-tree variation. In the clone-to-clone variation, sabinene also negatively correlated with that of α-pinene ($r = -0.86$, $P < 0.0001$), myrcene ($r = -0.67$, $P < 0.0001$), and limonene ($r = -0.98$, $P < 0.0001$).

Proportions of sabinene in the strains without clonal variations were categorized into three groups (A 28.0%–47.6%, B 50.2%–63.5%, C 67.7%–77.1%). The proportions of limonene acted complementarily in concert with the sabinene and were also able to be categorized (A 10.7%–21.3%, B 7.6%–12.4%, C 2.0%–5.8%). Proportional variations of α-pinene and myrcene in hinoki LBP monoterpenes did not show such a relation with the sabinene content.

Discussion

The wonderful scent of hinoki comes from a special blend of monoterpenes, which are toxic to both beetles and their pathogenic fungal symbionts.[6,10] The bioactive volatile substances are synthesized from geranyl diphosphate (GPP),[11,12] which is derived from the pyruvate/glyceraldehyde-3-phosphate pathway,[13] by monoterpene synthases (cyclases) in the epithelial cells of specialized secretory structures, such as resin blisters in which oleoresin is accumulated.[14,15]

Considerable proportional variation of LBP monoterpenes was observed in hinoki strains. The relative proportion of sabinene in the total LBP monoterpenes, which are the dominant constituents, showed the biggest variation among nine monoterpenes. An individual of hinoki, which contained sabinene as only about 5% of the LBP monoterpenes as measured by headspace technique, was found previously in Shimane Prefecture, Japan.[3] Such an extremely low sabinene profile was not observed in this study. However, despite selection as a cold-wind-resistant tree, these strains showed wide varieties of LBP monoterpene profiles without showing any signs of convergence. It is apparent that the monoterpene composition indicates very specific, undescribed characteristics of hinoki. While the relationship between cold-wind-resistance properties and the monoterpene composition is unknown, further selection of

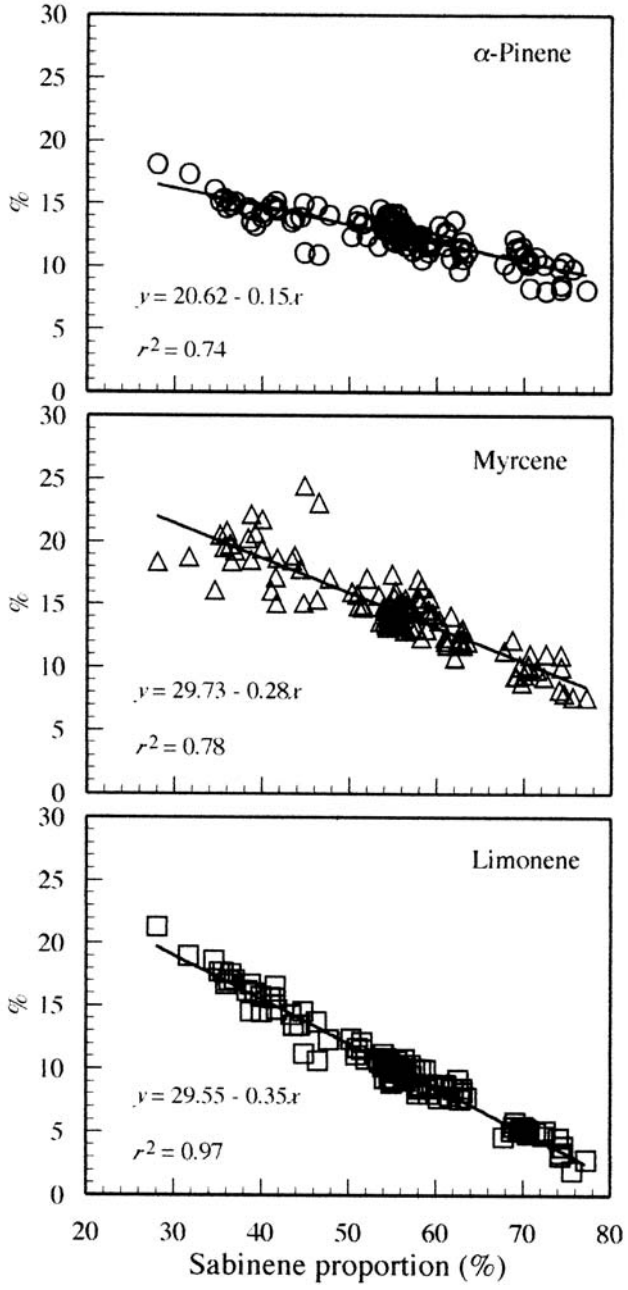

Fig. 3. Relationship between sabinene proportion and α-pinene (*upper*), myrcene (*middle*), and limonene (*lower*) proportions in LBP monoterpenes emitted from needles of hinoki strains that did not show clonal variation in sabinene proportion

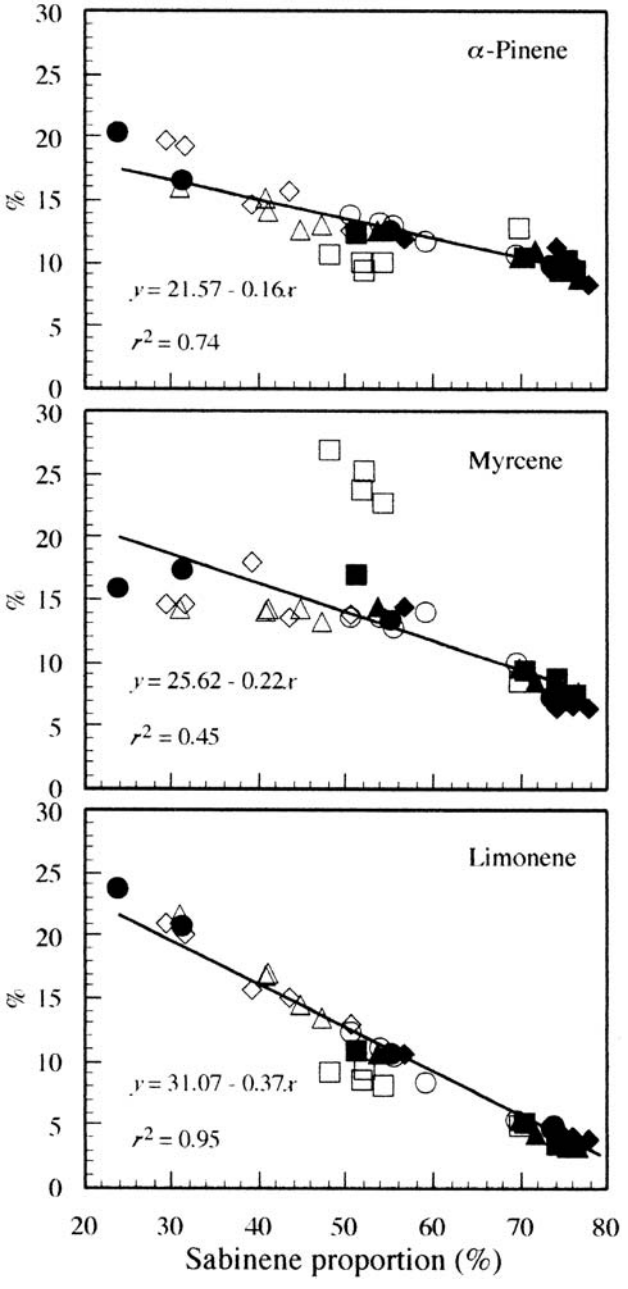

Fig. 4. Relationship between sabinene proportion and α-pinene (*upper*), myrcene (*middle*), and limonene (*lower*) proportions in LBP monoterpenes emitted from needles of hinoki strains that showed clonal variation in sabinene proportion. Data were attained from measurements on eight strains, Nos. 20 (*filled squares*), 24 (*filled circles*), 25 (*filled triangles*), 26 (*filled diamonds*), 28 (*open squares*), 60 (*open circles*), 87 (*open triangles*), and 91 (*open diamonds*)

these strains for cold-wind resistance-properties may be possible by investigating the function of terpenoids.

The clear negative correlation ($r = -0.98$) between the proportions of sabinene and limonene was observed in strains without clonal variation and those with clonal variations in sabinene proportion. Monoterpene synthases show an interesting feature that simultaneously produces multiple products from a single substrate, GPP; for example, pinene synthase from several plant sources produces both α-pinene and β-pinene.[16,17] Moreover, these unusual and intriguing monoterpene synthases dramatically alter the product outcome by a few amino acid substitutions or domain swapping.[18,19] Grand fir (*Abies grandis*) has been developed as a model system for study of the biochemistry and molecular genetics of defensive oleoresin formation in conifers, because this species both accumulates material in resin blisters and markedly increases the rate of oleoresin production in stem tissue upon wounding.[20–22] Limonene/α-pinene synthase obtained from grand fir[23] altered the product distribution and relative velocity by even only one

or a few amino acid substitutions.[19] The variability of sesquiterpenes emitted from two *Zea mays* cultivars is controlled by allelic variation of two terpene synthase genes encoding stereoselective multiple product enzymes.[24] The subtle amino acid differences may also affect the monoterpene distributions of hinoki. The clear negative correlation between the proportions of sabinene and limonene in the strains without clonal variations implies two possibilities. One hypothesis is that combination of multiple alleles, which are mainly codominant and underwent various amino acid substitutions that greatly influence the activity and the product distribution of the encoded protein producing mainly sabinene or limonene, results in the diversity of monoterpene composition. Another is that regulatory mechanisms involved in enzymes producing sabinene or limonene show the clear negative correlation. α-Pinene and myrcene, which were found to be less correlated to sabinene, appear to be produced mainly by monoterpene synthases that are different from the enzymes producing sabinene or limonene.

Different proportions of monoterpenes among five clones were hardly observed in most strains. This result suggests that the monoterpene composition is constitutively steady in most hinoki trees. On the other hand, several strains showed considerable clone-to-clone variation in the monoterpene compositions. Many conifer species respond to wounding, bark beetle attack, and associated fungal infection by secreting de novo-synthesized, wound-induced oleoresin at the wound site.[25,26] The wound-induced oleoresin often has a different composition to that of the constitutive oleoresin. Light and water stresses greatly reduce the constitutive level of monoterpene cyclase activity and abolish the wound-induced response.[27] However, the wound response appears to decline within several weeks and return to the constitutive state, and the response is localized to the wound site.[21] With reexamination being carried out 4 months after the primary experiment and the compositional differences being hardly observed between the two experiments, these clone-to-clone variations are unlikely to be a result of the general wound responses. Airborne chemicals such as methyl jasmonate (MeJA) or ethylene are known to induce a systemic defensive response over foliage.[28–31] A well-known disease in hinoki, resinous stem canker, causes serious resin exudation from the stem over many years.[32] A fungi, *Cistella japonica*, is considered a candidate as the causal agent, resulting in numerous traumatic resin canals in the secondary phloem and lowering the quality of the tree as dimensional lumber.[32] Because the diseased tree does not show resinosis as a visible symptom for nearly 10 years from early infection, the tree is regarded as a healthy tree until the first resinosis appears.[33] The regulatory mechanisms of monoterpene biosynthesis in which the trees showed clone-to-clone variation might be in a long-term defensive state in response to invisible diseases that consecutively induce airborne chemicals such as MeJA or ethylene.

The clear negative correlation ($r = -0.98$) between the proportions of sabinene and limonene in the strains with clonal variation seems to reflect the clear correlation of regulatory mechanisms involving sabinene or limonene synthase genes. This stringent correlation implies that the ratio of sabinene and limonene plays an important role in defense against numerous insects and their pathogenic fungal symbionts in hinoki. Environmental factors affecting growth conditions of trees must also be taken into account for altering monoterpene compositions. To elucidate the molecular genetic and biochemical mechanisms controlling monoterpene diversity and distribution in hinoki, cloning of all of the monoterpene synthase genes from four monoterpene chemotypes is underway.

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