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INDIVIDUAL VARIATION IN CONSTITUTIVE AND INDUCED MONOTERPENE BIOSYNTHESIS IN GRAND FIR*

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Key Word Index—*Abies grandis*; Pinaceae; conifer; grand fir; cortical oleoresin; turpentine; monoterpenes; monoterpene synthases; wound-induction; conifer defense.

Abstract—Variations in the level and distribution of monoterpene synthase activities in stem samples of 46 grand fir saplings maintained under controlled conditions were investigated using sensitive, non-destructive bioanalytical techniques that permitted the assessment of constitutive, wound-induced and ethylene modulated biosynthetic capability of each individual. 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 the definition of seven distinct biosynthetic chemotypes. No correlation was observed between total monoterpene biosynthetic capacity and chemotype, indicating that both broad quantitative and qualitative variation in oleoresin monoterpene production exists in this native population of grand fir. The implications of these findings for oleoresin-based defense against bark beetle predation are described.

INTRODUCTION

Many conifer species respond to bark beetle attack and associated fungal infection by secreting oleoresin (pitch) at the wound site [1–4]. This defensive secretion, composed of roughly equal quantities of turpentine (largely monoterpenes with some sesquiterpene olefins) and rosin (diterpenoid resin acids) [5], is toxic to both beetles and their pathogenic fungal symbionts [3, 4, 6] and, after evaporation of the volatile turpentine, it forms hardened rosin barrier to seal the injury [7].

Constitutive oleoresin is synthesized in the epithelial cells of specialized secretory structures, such as stem resin blisters or ducts in which the oleoresin is accumulated, whereas induced oleoresin appears to originate in nonspecialized cells, adjacent to the site of injury, that are not normally associated with large-scale production of terpenoids [3, 8, 9]. The induced oleoresin often has a different composition than the constitutive oleoresin [3, 4]. Although both constitutive and localized, inducible oleoresin-based defense mechanisms seem to have been selected for in conifers, the major conifer genera [pines (*Pinus*), spruces (*Picea*), larches (*Larix*), and true firs (*Abies*)] do differ considerably in their apparent reliance on constitutive or induced resin defenses [3, 10].

Grand fir (*Abies grandis* Lindl.), a traditional source of paper pulp and dimensional lumber in the Western United States, has been developed as a model system for the study 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 challenge [10, 11]. Additionally, saplings raised in the greenhouse very closely resemble mature trees in the forest setting in their response to injury and in environmental influences on defense chemistry[12–15]. The turpentine of grand fir contains 12 principal monoterpenes (Fig. 1) [5], and at least six distinct monoterpene synthases, responsible for the conversion of geranyl diphosphate to the various olefinic products, have been identified in stem tissue [16].

Since both ethylene biosynthesis and oleoresinosis increase in wounded and fungus-infected conifer stem [17], and ethylene alters the emission rates of monoterpenes in conifers, for example sabinene and terpinolene in *Chamaecyparis obtusa* Endl. (hinoki) saplings [18, 19], preliminary studies were undertaken to examine the possible role of ethylene in mediating monoterpene biosynthesis in grand fir. The sapling population studied was from a seed source widely utilized in grand fir reforestation in the Pacific

* This paper is dedicated to Clarence (Bud) A. Ryan on the occasion of his sixty-fifth birthday.

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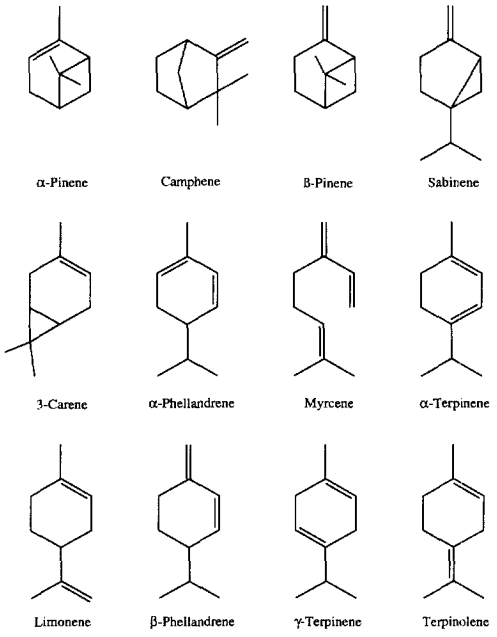

Fig. 1. The major monoterpenes of grand fir turpentine that are generated from geranyl diphosphate.

Northwest region of the United States because of its apparent resistance to fungal root diseases. Considerable individual differences were observed in these experiments in both constitutive and wound-induced monoterpene (turpentine) production. Since the origins of variability are of interest in the context of bark beetle defense at the population level, and an evaluation of such variability is necessary in the design of biochemical and molecular genetic approaches to understanding the regulation of oleroresinosis, a comprehensive assessment of individual variation in monoterpene biosynthetic capability was undertaken. In this paper, we describe the distribution of monoterpene synthase activities in nonwounded (control; constitutive), wounded, and Ethrel-treated stems from 46 individual saplings from two age groups. Quantitative differences were observed and seven 'chemotypes' were defined based on *in vitro* assessment of monoterpene biosynthetic capability.

RESULTS AND DISCUSSION

The biosynthesis of constitutive and wound-induced oleoresin in grand fir has been the object of detailed study [12, 14]. Based on assessment of cell-free extracts, using pooled stem samples from many saplings as the enzyme source, it has been shown that grand fir possesses at least six monoterpene synthases [14]. Some of these enzymes appear to be induced only after stem wounding (e.g., (−)-pinene synthase that produces both α- and β-isomers) (Fig. 1) [20, 21], while others seem to be wound-inducible homologues of constitutive enzymes [16]. Preliminary studies on the influence of ethylene on wound-induced oleoresinosis, using sample sizes of only a few saplings, revealed considerable variation in both the activity levels of the extracts and the distribution of products formed. Since the wound-response is localized [10, 12], and the bioanalytical methods are sufficiently sensitive that only very small tissue samples are required for enzyme assay or determination of oleoresin content [5, 11], it was possible to conduct multiple evaluations on a single stem without permanent damage to the sapling. Therefore, oleoresin turpentine content, and monoterpene synthase activities from both wounded and nonwounded (constitutive, control) stem tissue, were compared for 38 individual three-year-old saplings and eight two-year-old saplings. Constitutive levels of monoterpene synthase activity ranged from 0.03 to 2.59 pkat g fr. wt^{-1} for three-year saplings and 0.12 to 0.74 pkat g fr. $wt.^{-1}$ for two-year saplings (Fig. 2). Based on the product distributions generated in cell-free assays, seven distinct variants in monoterpene synthase content were noted (Table 1). These are presumed to represent genetically-based 'biosynthetic chemotypes', since the plants were grown under ostensibly identical conditions for several months before anlaysis and could have experienced only very minor environmental differences. Total monoterpene synthase activity (in pkat g fr. $wt.^{-1}$) correlated directly with total turpentine content (in mg g fr. $wt.^{-1}$); however, the particular mixture of synthase activities were not correlated with the level of oleoresin or total biosynthetic activity. Thus, both high-level and low-level producers were found for each 'biosynthetic chemotype'. Interestingly, for the saplings surveyed, turpentine composition did not necessarily reflect the observed complement of constitutive monoterpene synthases. This may be explained by the fact that stored oleoresin consists of material accumulated for over two years prior to acquisition of the saplings and thus represents the summary of all epigenetic influences on monoterpene metabolism up to the time that the saplings were placed under controlled growth conditions. It is worth emphasizing here that the classical analytical determination of a chemotype provides only a historical record of secondary metabolism that may or may not reflect the present biosynthetic capability of the plant.

When the 38 three-year-old saplings were subjected to stem wounding and the tissue assayed for monoterpene synthase activity nine days later, at the maximum for induced activity [22, 23], a broad distribution of fold differences was observed, with wounded/non-wounded activity ratios ranging from 0.16 to 19.7. Six of the saplings exhibited high constitutive (non-wounded) levels of monoterpene synthase activity. When the same experiment was carried out with two-year-old saplings, a wide distribution of fold inductions was again observed, with wounded/non-wounded activity ratios ranging from 0.79 to 9.15. The induction of monoterpene synthase activity was also examined in 14 three-year-old saplings and 8 two-year-old saplings that were wounded and simultaneously treated with Ethrel (Fig. 2). Monoterpene

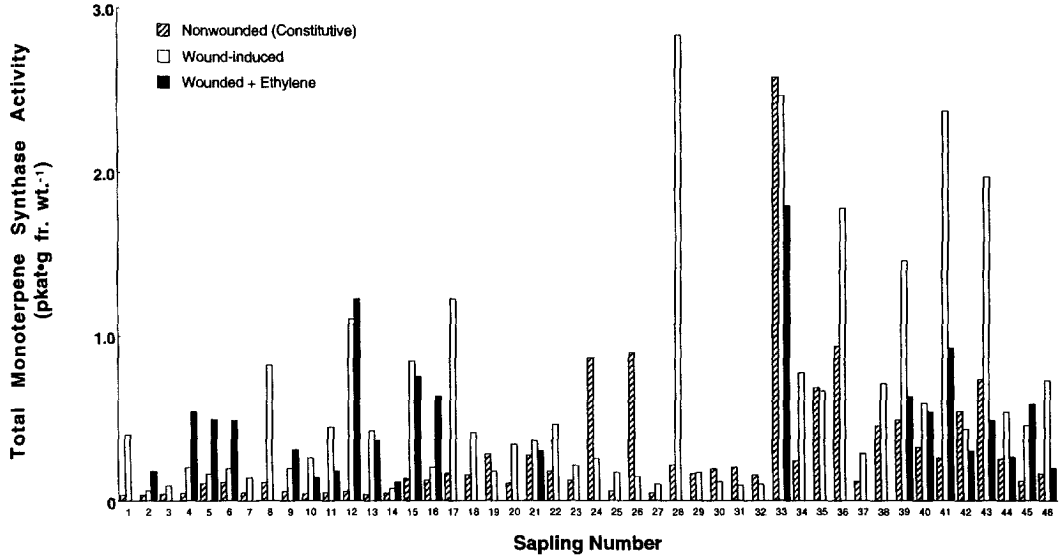

Fig. 2. Total monoterpene synthase activity measured in cell-free extracts of samples from control (nonwounded, constitutive), wounded (induced), and wounded and Ethrel-treated stems from three-year-old grand fir saplings (individuals 1–38) and from two-year-old grand fir saplings (individuals 39–46).

Table 1. Seven monoterpene chemotypes defined by distribution of constitutive and wound-inducible monoterpene synthase activities

	Biosynthetic Chemotype						
	I. α-Pinene	II. β-Pinene	III. 3-Carene	IV. α-Phellandrene (% range)	V. Limonene	VI. α-Pinene/ Limonene	VII. β-Pinene/ Limonene
α-Pinene	25.9–43.7	4.9–32.7	9.7–29.2	2.9–20.4	0.9–20.0	16.4–28.7	3.6–22.4
Camphene	0.5–2.5	0.1–3.3	0.4–6.3	0.1–4.2	0.1–3.0	0.1–4.8	0.2–8.5
β-Pinene	12.1–26.9	37.0–76.4	7.4–25.6	2.1–13.6	6.6–23.0	14.9–21.0	18.4–31.8
Sabinene	1.2–10.8	1.2–8.0	1.0–5.0	0.5–6.0	0.7–10.6	0.9–5.6	0.9–4.3
3-Carene	0.3–22.2	0.3–8.5	9.7–32.5	0.1–8.3	0.5–13.0	0.2–7.9	0.4–7.4
α-Phellandrene	3.0–9.0	1.2–9.6	0.1–11.0	11.2–27.7	1.1–11.7	0.7–9.0	0.7–12.1
Myrcene	0.4–3.2	0.3–11.5	0.1–3.4	0.1–5.9	0.1–3.9	0.1–3.3	0.1–10.7
α-Terpinene	0.7–0.9	0.1–1.5	0.1–2.4	0.1–3.6	0.1–4.5	0.1–3.0	0.1–1.8
Limonene	3.2–19.5	2.9–17.8	6.7–43.6	19.7–44.3	32.3–65.7	20.2–39.6	21.3–36.9
β-Phellandrene	1.9–13.1	1.3–18.8	1.5–14.3	1.8–5.6	1.1–12.8	2.7–9.6	1.4–13.7
γ-Terpinene	0.5–1.5	0.3–3.8	0.5–6.8	0.1–16.5	0.1–7.3	0.1–5.7	0.1–5.3
Terpinolene	1.9–22.2	0.7–9.3	7.0–14.7	10.6–22.7	3.3–18.0	5.8–16.9	6.4–19.0
Constitutive (%)	0	14.8	7.4	11.1	44.4	0	22.2
Induced (%)	4.3	8.7	17.4	15.2	37.0	10.9	6.5

Data given are for the compositional ranges of the saplings described in Fig. 2. The percentage distribution of constitutive and induced chemotypes represents the summation of both two-year-old and three-year-old trees. For monoterpene structures, see Fig. 1.

synthase activity was induced to an even greater degree (than wounding alone) by this additional ethylene treatment in eight of 14 three-year-old saplings and one of 8 two-year-old saplings. This result suggests that the older trees are more sensitive to the influence of exogenous ethylene than are younger saplings. In all cases, the application of Ethrel to unwounded stem sections had no discernible effect on the constitutive monoterpene synthase activities in these control experiments.

Analysis of the products generated in cell-free extracts from the wound-induced stems again showed that the various monoterpene compositions (based on mixtures of the same 12 compounds in Fig. 1) segregated into the same seven groups (Table 1). Indeed, the monoterpene compositions determined by radio-GLC analysis of all 94 samples (all treatments for all saplings) could be readily placed into one of the seven 'chemotype' groups. Although the limonene 'chemotype' was the dominant form overall based on cell-free assay of both constitutive and wound-inducible monoterpene synthases of three-year-old

saplings, most individuals in fact evidenced an alteration in the distribution of monoterpene synthases upon wounding. Thus, half of the individuals exhibiting the limonene 'chemotype' constitutively shifted their pattern of induced monoterpene synthases to that of the α-pinene/limonene, β-pinene/limonene or 3-carene 'chemotype'. Similarly, more than half of the individuals exhibiting β-pinene, β-pinene/limonene and α-phellandrene 'chemotypes' constitutively shifted pattern upon wounding to an alternate 'chemotype', including both the limonene and α-pinene/limonene types. When Ethrel treatment was combined with wounding, a different mix of biosynthetic chemotypes (than either constitutive or wound-induced) was again observed, and in this instance the 'chemotypes' bearing high levels of α- and β-pinene synthase activities dominated.

In case of the two-year-old sapling population, a general alteration in the distribution of monoterpene synthase activities upon wounding was again observed. In this instance, however, more than half of the individuals shifted to 'chemotypes' displaying high levels of α- and/or β-pinene synthase activity. Interestingly, although Ethrel treatment did not markedly increase overall monoterpene synthase activity compared to wounding alone (i.e., only one of eight individuals showed a significant increase), three of the saplings did exhibit a distinct change in the complement of monoterpene synthase activities compared to the display induced by wounding alone (e.g., a shift from α-pinene 'chemotype' to 3-carene 'chemotype', and from 3-carene 'chemotype' to limonene 'chemotype').

Other than the rather general comments indicated above, no other obvious trends could be discerned, and there appeared to be no significant correlation between the levels and distributions of constitutive monoterpene synthase activities and levels and distributions of those induced upon stem wounding, with or without additional Ethrel treatment. While considerable data were gleaned from the nearly 50 individual saplings analysed, it is arguable whether a full assessment of biochemical variation in this grand fir population has been gained from this study, and from earlier, preliminary work in which variation in turpentine content was noted [15]. Nevertheless, it seems highly significant that such great differences in constitutive and induced capacity for monoterpene biosynthesis were observed among this limited number of individuals, implying broad genetic diversity in oleoresin production.

Quantitative and qualitative variation in both constitutive and inducible monoterpene biosynthetic capacity at the population level would appear to have important consequences for defense of the species against predation by bark beetles and their pathogenic fungal associates by the differential display of a range of toxic monoterpene mixtures available immediately (constitutive production) and as a subsequent response to attack (induced production) [24]. Toxicity may not be the singular basis for this apparent evolutionary adaptation, however, since many species of bark beetles are specifically attracted to the monoterpene compounds emitted from their conifer hosts and can utilize components of the host oleoresin in the synthesis of pheromones for promoting aggregation [25, 26]. These same volatile terpenoids can also serve as attractants for beetle predators and parasitoids [27, 28]. Thus, 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. Exploiting such strategies in the transgenic improvement of oleoresin-based conifer defenses requires a biochemical and genetic understanding of the regulation of monoterpene biosynthesis. An appreciation of the extant chemical diversity in the target 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.

EXPERIMENTAL

Plant materials, substrate and standards. Two-year-old grand fir (*Abies grandis* Lindl.) saplings were purchased from the Forestry Research Nursery, University of Idaho, Moscow, ID. The source of seed was the Sears Creed, Clearwater River drainage area, near Harpster, ID, elevation 1.1–1.4 km. Trees from this area are free of root-rotting disease and experience relatively little predation by fir engraver beetles (*Scolytus ventralis*). Saplings were grown in standard potting mix (Sals Inc., Puyallup, WA) with a 16 hr photoperiod (200–300 μE m^{-2} s^{-1}) and 26° day/15° night temp. cycle, and were fertilized (15:30:15 [N:P:K] with trace elements) weekly and watered daily. Upon removal from cold storage and breakage of dormancy, the saplings were grown for at least 6 weeks past bud break to minimize the influence of flushing on induced oleoresinosis [14]. Saplings in active growth (average height of 31 cm for 2-year-old saplings and 34 cm for 3-year-old saplings) were wounded with a 26-gauge hypodermic needle by making a 2 mm deep puncture at midstem. At intervals after wounding (generally 9 days) a sample core was removed from the wound site (to the depth of the woody xylem) using a 5 mm diam. cork borer, and the core was immediately weighed and frozen in liquid N_2. Control samples (non-wounded) for determination of constitutive activity levels and oleoresin content were identically obtained by coring the stem 2 cm above the wound site. For examining the influence of ethylene, a 1 mM aq. soln. of Ethrel (2-chloroethylphosphonic acid, which releases ethylene at pH >5.0) was applied at the wound site with a cotton-tipped applicator (*ca* 50 μl). As a control, Ethrel was applied to a section of intact stem 5 cm above the wound site. All treatments (wounded/

control, ethylene and wounded/ethylene control, and core sample for oleoresin content) could be carried out on the same stem without permanent injury to the sapling. The prepn of [1-^{3}H]geranyl diphosphate (250 Ci mol^{-1}) has been described [29], and the monoterpene standards were from our own collection.

Oleoresin analysis. Tissue samples for oleoresin turpentine analysis were extracted with 1 ml of pentane overnight at 4°. The extracts were analysed by combined capillary GLC-MS, using int. standardization and a protocol described previously [5].

Enzyme extraction. Tissue cores for enzyme extraction were placed in a small manila envelope and, while still frozen, were pulverized with a hammer. The resulting fine powder was thawed by thoroughly mixing into chilled extraction buffer (1.5 ml 10 mg^{-1} fr. wt. of tissue) consisting of 50 mM Hepes (pH 7.5), 200 mM KCl, 20 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM dithiothreitol, 5 mM sodium ascorbate, 5 mM $Na_2S_2O_5$, 1% PVP (M_r = 40000), and 10% glycerol. The suspension was centrifuged at 12000 *g* for 4 min at 0–4° and aliquots of the resulting supernatant were assayed for monoterpene synthase activity [10].

Monoterpene synthase assay. An aliquot of the enzyme extract (generally 100 μl containing 5–10 μg protein) was transferred to a 1.5 ml Eppendorf tube to which 8 μM [1-^{3}H]geranyl diphosphate was added to initiate the reaction. A 1 ml overlay of hexane was then immediately added to the tube to serve as a trap for the volatile monoterpenes, and the mixt. was incubated for 1 hr at 32°. Following incubation, the contents of the tube were vigorously mixed and centrifuged briefly to separate phases. The hexane layer was then transferred to another Eppendorf tube containing about 40 mg of silica gel (Mallinckrodt SilicAR 60 Å) and the contents were thoroughly mixed to allow adsorption to the silica of oxygenated metabolites (i.e., geraniol liberated from the substrate by endogenous phosphatases) and any residual H_2O. Following centrifugation to pellet the matrix, an aliquot of the hexane extract containing the monoterpene olefins (generally 0.6 ml) was transferred to a scintillation vial containing 10 ml of 0.4% Omnifluor (DuPont Biotechnology Systems) in toluene:ethanol (7:3) for determination of ^{3}H content and, thus, monoterpene biosynthetic rate.

The remaining enzyme extract was utilized in prep. incubations (using the same procedure as above) to generate sufficient labelled product for determination of monoterpene composition by radio-GLC [30, 31]. The conditions for analysis on the 3 mm o.d. × 4 m AT1000 column (15% polyethylene glycol ester on Gas Chrom Q) were, 85° isothermal (22 min) then programmed to a final temp. of 200° at 10° min^{-1} with helium (60 ml min^{-1}) as carrier. Since both grand fir oleoresin composition [5] and monoterpene synthase enzymology [16] are well defined, comparison of R_ts of the radio-labelled products to those of authentic standards was sufficient to confirm identifications.

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REFERENCES

1. Johnson, M. A. and Croteau, R., in *Ecology and Metabolism of Plant Lipids*, ed. G. Fuller and W. D. Nes. ACS Symp. Series 325, American Chemical Society, Washington, DC, 1987, p. 76.
2. Nebeker, T. E., Hodges, J. D. and Blance, C. A., in *Beetle–Pathogen Interactions in Conifer Forests*, ed. T. D. Schowalter and G. M. Filip. Academic Press, London, 1993, p. 158.
3. Raffa, K. F., in *Phytochemical Induction by Herbivores*, ed. D. W. Tallamy and M. J. Raupp. Academic Press, New York, 1991, p. 245.
4. Raffa, K. F. and Klepzig, K. D., in *Defense Mechanisms of Woody Plants Against Fungi*, ed. R. A. Blanchette and A. C. Biggs. Springer, Berlin, 1992, p. 354.
5. Lewinsohn, E., Savage, T. J., Gijzen, M. and Croteau, R., *Phytochemical Analysis*, 1993, **4**, 220.
6. Raffa, K. F., Berryman, A. A. Simasko, J., Teal, W. and Wong, B. L., *Environmental Entomology*, 1985, **14**, 552.
7. Croteau, R. and Johnson, M. A., in *Biosynthesis and Biodegradation of Wood Components*, ed. T. Higuchi. Academic Press, Orlando, FL, 1985, p. 379.
8. Cheniclet, C., *Journal of Experimental Botany*, 1987, **38**, 1557.
9. Yamada, T., in *Defense Mechanisms of Woody Plants Against Fungi*, ed. R. A. Blanchette, and A. C. Biggs. Springer, Berlin, 1992, p. 147.
10. Lewinsohn, E. Gijzen, M. and Croteau, R., *Plant Physiology*, 1991, **96**, 44.
11. Lewinsohn, E. Gijzen, M., Savage, T. J. and Croteau, R., *Plant Physiology*, 1991, **96**, 38.
12. Lewinsohn, E. Gijzen, M. and Croteau, R., in *Regulation of Isopentenoid Metabolism*, ed. W. D. Nes, E. J. Parish and J. M. Trzaskos. ACS Symp. Series 497, American Chemical Society, Washington, DC, 1992, p. 8.
13. Lewinsohn, E., Gijzen, M., Muzika, R. M., Barton, K. and Croteau, R., *Plant Physiology* 1993, **101**, 1021.
14. Steele, C. L., Lewinsohn, E. and Croteau, R., *Proceedings of the National Academy of the Sciences of the U.S.A.*, 1995, **92**, 4164.
15. Muzika, R. M., Pregitzer, K. S. and Hanover, J. W., *Oecologia*, 1989, **80**, 485.
16. Gijzen, M., Lewinsohn, E. and Croteau, R., *Archives of Biochemistry and Biophysics*, 1991, **289**, 267.
17. Yamanaka, K., Ph.D. Thesis, Kyoto University, Japan, 1986, p. 48.

18. Katoh, S., Uehara, T., Furuno, T. and Jodai, S., *Journal of the Japanese Wood Research Society*, 1993, **39**, 1084.
19. Katoh, S., Uehara, T., Furuno, T. and Jodai, S., *Journal of the Japanese Wood Research Society*, 1994, **40**, 419.
20. Wagschal, K., Savage, T. J. and Croteau, R., *Tetrahedron*, 1991, **47**, 5933.
21. Lewinsohn, E., Gijzen, M. and Croteau, R., *Archives of Biochemistry and Biophysics*, 1992, **293**, 167.
22. Gijzen, M., Lewinsohn, E. and Croteau, R., *Archives of Biochemistry and Biophysics*, 1992, **294**, 670.
23. Funk, C., Lewinsohn, E., Stofer Vogel, B., Steele, C. L. and Croteau, R., *Plant Physiology*, 1994, **106**, 999.
24. Langenheim, J. H., *Journal of Chemical and Ecology*, 1994, **20**, 1223.
25. Wood, D. L., *Annual Review of Entomology*, 1982, **27**, 411.
26. Gijzen, M., Lewinsohn, E., Savage, T. J. and Croteau, R. B., in *Bioactive Volatile Compounds from Plants*, ed. R. Teranishi, R. G. Buttery and H. Sugisawa. ACS Symp. Series 525, American Chemical Society, Washington, DC, 1993, p. 8.
27. Payne, T. L., in *Potential for Biological Control of Dendroctonus and Ips Bark beetles*, ed. D. L. Kulhavy and M. C. Miller. Stephen F. Austin University Press, Nacogdoches, TX, 1989, p. 55.
28. Gregoire, J.-C., Couillien. D., Krebber, R., König, W. A., Meyer, H. and Francke, W., *Chemoecology*, 1992, **3**, 14.
29. Croteau, R., Alonso, W. R., Koepp, A. E. and Johnson, M. A., *Archives of Biochemistry and Biophysics*, 1994, **309**, 184.
30. Croteau, R., and Cane, D. E., *Methods in Enzymology*, 1985, **110**, 383.
31. Satterwhite, D. M., and Croteau, R., *Journal of Chromatography*, 1988, **452**, 61.