Biochemistry of Oleoresinosis

MONOTERPENE AND DITERPENE BIOSYNTHESIS IN LODGEPOLE PINE SAPLINGS INFECTED WITH CERATOCYSTIS CLAVIGERA OR TREATED WITH CARBOHYDRATE ELICITORS

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ABSTRACT

Elevated levels of monoterpenes and diterpene resin acids are produced in the stems of lodgepole pine (Pinus contorta var latifolia) saplings when wounded and inoculated with the blue-stain fungus Ceratocystis clavigera or when wounded and treated with a pectic fragment from tomato leaves (PIIF) or a fungal cell wall fragment (chitosan). This induced defensive response (hyperoleoresinosis) is the result of a transient rise in the ability to biosynthesize cyclic monoterpenes and diterpene resin acids as measured by the in vivo incorporation of label from [U-14C]Sucrose relative to untreated controls, and is accompanied by a corresponding rise in the levels or activities of the relevant terpene cyclases as determined by in vitro assay using labeled acyclic precursors. The results indicate that juvenile P. contorta responds to infection and biotic elicitors much like the mature tree, and they suggest that the Pinaceae possess a mechanism for elicitor recognition and induced defense similar to that of other higher plants.

The terpenoid fraction of pine resin contains 20 to 50% volatile monoterpenes (C10), minor levels of sesquiterpenoids (C15), and 50 to 80% nonvolatile diterpene acids (C20) (8, 18), and the induced secretion of these materials constitutes an important component of the defense response of pines to attack by bark beetles and their associated pathogenic fungi (2, 13, 26). Resistant lodgepole pine (Pinus contorta Douglas) produces high levels of α-pinene and limonene (Fig. 1) in the secondary resin elicited by attack (relative to the constitutive [preformed] resin contained in the resin ducts) (19); however, the major factor in resistance to the mountain pine beetle (Dendroctonus ponderosae Hopkins) and associated fungi (Ceratocystis clavigera Robinson et Davidson) appears to be a rapid and vigorous secondary resinosis resulting from the increased production of all constitutive terpenoids (21, 23). Mature lodgepole pine inoculated with C. clavigera, a pectic fragment from plant cell walls, or a fungal cell wall fragment (chitosan) respond by producing resin monoterpenes to the same or greater extent as control trees in which the response is elicited by living mountain pine beetle, suggesting that pines, like other higher plants, possess a common recognition-defense mechanism (17).

The chemistry of pine resin and the role of resin components in resistance to infestation and infection have been discussed (20, 25, 26) and the broad outlines of pine defense biology have been delineated (2, 15). The biosynthesis of resin terpenoids, however, is poorly understood, as is the biochemistry of the defense reaction; present concepts are based largely on analogy to terpenoid metabolism and phytoalexin production in herbaceous species (7, 15, 17). The lack of fundamental information about this defense response is, in part, a result of the difficulties encountered in experimenting with mature trees in the forest setting, the system in which the phenomenon was first discovered and with which most early observations were made (17, 19, 21, 23, 24). In this communication we provide an account of induced oleoresinosis in 2-year-old lodgepole pine saplings (maintained in a greenhouse) in response to inoculation with 'blue staining' fungus (C. clavigera) and with carbohydrate elicitors, we report in vivo experiments which demonstrate the elevated formation of monoterpenes and diterpene resin acids, and we describe the isolation and enhanced activity of the terpenoid cyclases which catalyze the committed steps of monoterpenene and diterpene biosynthesis in this species.

MATERIALS AND METHODS

Plant Materials, Substrates, and Reagents. Two-year-old lodgepole pine saplings (Pinus contorta Douglas var latifolia Englemann) of 22 to 28 cm in height were obtained from the U.S. Forest Service Pine Nursery (Bend, OR; courtesy of J. Wojtowych) and the Potlatch Corp. (Lewiston, ID; courtesy of Dr. R. Blair) and, following transfer to 10-in deep pots (620 cc) in Grace Forestry Mix (pH 4.5–5.0) (W. R. Grace & Co., Memphis, TN) and cold conditioning at 0 to 4°C for 1 month, they were maintained in a greenhouse (30°C day/18°C night), fertilized weekly with a complete fertilizer of N:P:K = 15:30:15 (with trace nutrients) for a minimum of 6 weeks to break dormancy before use.

The 'blue stain' fungus Ceratocystis clavigera (Robinson et Davidson) was obtained from ATCC (No. 24286) and was maintained on malt agar at 24°C. Two weeks after transfer the fungal mats were harvested, washed by repeated suspension in 25 mM sodium phosphate (pH 7.0) and centrifugation, and the wet paste was used as the inoculum.

[1-3H]Geranylpyrophosphate (100 Ci/mol) and [1-3H]farnesyl pyrophosphate (84.8 Ci/mol) were prepared as described previously (6). [1-3H]Geranylgeranyl pyrophosphate (10.6 Ci/mol) was a gift from R. M. Coates, University of Illinois and abietic acid (a mixture of abietic and dehydroabietic acids) was from Aldrich Chemical Co. Methylation of the mixture with BF3 in methanol (see below) gave largely methyl dehydroabietate which was converted to the corresponding alcohol by reduction with LiAlH4 in ether, and thence to the abietirole by transformation to and reduction of the primary iodide with NaBH4 (14). [U-14C]Sucrose (514 Ci/mol) was obtained from New England Nuclear. The sources of other reagents and standards have been

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the resin acids of *P. contorta* are largely of the abietic type (1, 23) and are thermally transformed to dehydroabiatic acid (9) upon solvent extraction-steam distillation, no additional precautions (10) were taken and the conversion to methyl esters (primarily methyl dehydroabiatic) was carried out by 1 h reflux in excess 15% BF₃ in methanol. The methyl esters formed were recovered by pouring the reaction mixture into 15 ml of brine followed by ether extraction (2 × 10 ml), and the pooled ether phases were dried over anhydrous Na₂SO₄, concentrated to small volume, and the contents examined by combined capillary GLC-MS. Calibration of the method with α-pinene and abietic acid indicated that volatile monoterpenes were recovered in about 85% yield, whereas resin acids were recovered (as methyl dehydroabiatic) in an overall yield of approximately 80%.

**In vivo Tracer Studies.** The protocol for these experiments was essentially identical to that described above. In this instance, wounded saplings and wounded saplings treated with PIIF or chitosan were employed, along with unwounded controls. Three saplings subjected to each treatment were harvested at 4-d intervals, and each sapling was injected 24 h before harvest with 15 µl of an aqueous solution containing 5 µCi of [U-14C]sucrose (5 µl injections at three sites radial from the wound site at 90° intervals). Isolation of volatile terpenoids and diterpene resin acids (as methyl esters) was as before; however, in this case, products were analyzed by radio-GLC following the addition of appropriate monoterpenes and resin acid carrier standards, which were obtained from the oleoresin.

**Isolation and Assay of Terpene Cyclases.** A similar protocol was employed for these studies carried out with wounded saplings, and wounded saplings treated with PIIF or chitosan, as well as with unwounded controls. Harvest was again at 4-d intervals; however, in this instance any exuded oleoresin was wiped from the stem with a pentane soaked tissue before removal of the 2 cm stem segment. A dozen such segments for each treatment at each time point were obtained and the outer tissue isolated by scraping down to the secondary xylem with a razor blade. The scappings were frozen in liquid N₂ and ground to a fine powder with a mortar and pestle. The powder was then transferred to 15 ml of chilled 100 mM Na-phosphate buffer (pH 6.4) containing 20% glycerol, 5 mM DTE, 10 mM Na₂SO₄, 10 mM Na-ascorbate, 15 mM MgCl₂, and 1 g each of insoluble PVP (GAF Corp.) and powdered polystyrene resin (methanol washed XAD-4, Rohm and Haas, Inc.), and thoroughly homogenized in a loose-fitting Ten-Broeck homogenizer. The homogenate was filtered through several layers of cheesecloth and the resulting filtrate was centrifuged at 105,000g to provide the soluble supernatant used as the enzyme source. This preparation was concentrated to about 3 ml by ultrafiltration (Amicon PM-30) and passed through a 2 × 10 cm column of Sephadex G-25 equilibrated with 25 mM Mes-5 mM Na-phosphate buffer (pH 6.5) containing 10% glycerol, 0.5 mM DTE, 15 mM MgCl₂ and 1.5 mM MnCl₂ to accomplish adjustment to assay conditions (6).

The assay for terpene cyclase activity was initiated by the addition of a saturating concentration (20 µM) of the appropriate 1-³¹H-labeled acyclic precursor for monoterpenes (geranyl pyrophosphate), sesquiterpenes (farnesyl pyrophosphate) and diterpenes (geranylgeranyl pyrophosphate) to 1 ml of the enzyme solution (40–80 µg protein by the Bio-Rad dye-binding assay), and the mixture was incubated for 1 h at 30°C in a Teflon-capped vial. The reaction was terminated by chilling the vial in ice and extraction of the contents with pentane (2 × 1.5 ml). The pooled pentane extract, brought to 1% ether, was passed through a 1 × 5 cm column of silicic acid to isolate the terpene olefins (6). The tritium content of this fraction was determined by scintillation counting of a aliquot and the material analyzed by radio-GLC following the addition of appropriate internal standards. The original reaction mixture was next extracted with
ether (2 x 1.5 ml) and the combined extracts passed through the same silicic acid column to provide the oxygenated terpenoids (6), which were analyzed as before by aliquot counting and radio-GLC following the addition of internal standards.

**Analytical Procedures.** The general procedures for radio-TLC on silica gel G (with or without 12% AgNO₃) and for radio-GLC have been described (6, 11). The developing solvent for analyzing diterpene olefins was hexane:diethyl ether (99:1, v/v). The column used for radio-GLC was stainless steel (10 ft x 1/8 in. o.d.) packed with 10% SE-30 on 80/100 mesh Gas-Chrome Q and operated at a He flow of 45 cm³/min (80°C for monoterpenes olefins, 220°C for diterpene resin acid methyl esters). Analytical capillary GLC analysis (FID) and combined GLC-MS analysis (70 eV) were performed on a 25 m fused silica column coated with SE-30 and programmed from 80 to 240°C at 5°C/min with He as carrier gas. Quantitation was by electronic integration of detector output with respect to the internal standard, and by assuming a response factor of one.

Procedures for liquid scintillation spectrometry have been described (6, 11). Samples were quench-corrected by internal standardization ([¹H] or [¹⁴C]toluene) and counted to <1% probable error (efficiency for ¹H = 30%; for ¹⁴C = 72%).

**RESULTS**

**Time-Course of Induced Oleoresinosis.** Lodgepole pine saplings were examined for alterations in oleoresin content in response to fungal infection and to carbohydrate elicitors of plant cell wall and fungal wall origin. Two-year-old lodgepole pines were wounded and inoculated with *C. clavigera*, a symbiont of the mountain pine beetle, or treated with the biotic elicitors PIIF (3) or chitosan (12), and the total monoterpenes content of the reaction zone was monitored over a 12-d period by combination of steam distillation and gas chromatographic analysis. Fungus-infected stems accumulated 3 times the monoterpenes level of untreated controls after 12 d, whereas the PIIF and chitosan treatments resulted in nearly a 5-fold increase in monoterpenes content with a perceptibly more rapid rate of accumulation (Fig. 2a). Wounding alone led to a lesser increase (1.5-fold) in monoterpenes level, which is a characteristic wound response in conifers (2, 19, 21, 23) while inoculation with spores rather than mycelia led to a relatively slow increase in monoterpenes content to slightly over twice the control level in 12 d (data not shown).

Monoterpenes content of the pine stem oleoresin was examined by GLC-MS at the 10-d period following wounding and fungal infection (Table I). Significant increases in the levels of the monoterpenes α-pinene, β-pinene, 3-carene, and β-phellandrene (for structures see Fig. 1), which are thought to be toxic to this fungus (19, 23), were found in infected stem tissue compared to controls. The levels of α-phellandrene and limonene decreased, probably because of volatility losses with little compensating production of these compounds. Examination of the monoterpenes content of the oleoresin elicited by PIIF and chitosan treatment (at 12 d) revealed a composition similar to that of the infected tissue, but with slightly higher proportions of β-pinene and 3-carene, as well as limonene.

The alteration in diterpene resin acid content of the oleoresin was also measured (by gravimetric determination of free organic acids) in response to the various treatments, and was shown to roughly double upon infection and to increase nearly 3-fold upon treatment with PIIF or chitosan (Fig. 2b). GLC-MS analysis of the methyl esters verified the presence of dehydroabietaoate (major) and chromatographically similar resin acids (minor components) of mol wt = 316 (30), and incidently indicated a slight decrease in the levels of the fatty acids C₁₈:₁ and C₁₈:₂ which comprised about 10% of the oleoresin of untreated controls. Little can be said about the composition of the native diterpene resin acid fraction since the oleoresin isolation procedure (steam distillation

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**Table 1. Effect of Wounding and Infection on the Monoterpene Composition of Lodgepole Pine Stems**

Two-year-old lodgepole pine saplings were infected with *Ceratocystis clavigera* or wounded aseptically and the monoterpenes content of the reaction zone was determined 10 d after treatment. The controls were intact saplings. The monoterpenes were isolated by solvent extraction-steam distillation, identified by GLC-MS, and quantitated by integration of chromatographic peak areas with reference to the internal standard as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Monoterpene</th>
<th>Control</th>
<th>Aseptic Wound</th>
<th>Infected Wound</th>
<th>mg g⁻¹ tissue dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene</td>
<td>0.44</td>
<td>0.69</td>
<td>1.68</td>
<td></td>
</tr>
<tr>
<td>Camphene</td>
<td>trace</td>
<td>trace</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>β-Pinene</td>
<td>1.55</td>
<td>2.79</td>
<td>5.97</td>
<td></td>
</tr>
<tr>
<td>3-Carene</td>
<td>0.27</td>
<td>0.34</td>
<td>1.33</td>
<td></td>
</tr>
<tr>
<td>α-Phellandrene</td>
<td>0.14</td>
<td>0.14</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Limonene</td>
<td>0.85</td>
<td>0.70</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>β-Phellandrene</td>
<td>1.22</td>
<td>2.14</td>
<td>3.14</td>
<td></td>
</tr>
<tr>
<td>Total monoterpenes</td>
<td>4.47</td>
<td>6.79</td>
<td>12.51</td>
<td></td>
</tr>
</tbody>
</table>
in air, exposure to Lewis acid) resulted in the isomerization/oxidation of the acids present with the resulting production of dehydroabietate (9). The mass spectral identification of the latter, as the methyl ester (30), did however confirm the presence of abietic-type resin acids in *P. contorta* saplings, as anticipated from studies on the resin acid content of mature trees (1, 23).

The nonvolatile neutral components of the oleoresin were also subjected to preliminary (gravimetric) analysis, and were shown to increase roughly 2.5-fold in response to infection. This material comprised about 15% of the oleoresin produced in response to infection (volatile monoterpenes about 40%, and free acids about 40%) and GLC analysis of this fraction revealed a very complex mixture of more than twenty components (at >2%), as expected for this class of wood extractives (22).

**In Vivo Tracer Studies.** In order to determine if the induced accumulation of oleoresin was due to *de novo* synthesis, a preliminary attempt was made to assess oleoresin biosynthesis by application of labeled acetate, mevalonate, or sucrose to the wound site. These efforts were thwarted by the presence of oleoresin which prevented either adequate application of substrate to, or uptake by, the relevant tissue. An alternate approach, whereby tracer was injected at sites distributed radially on the stem from the wound site, was taken; yet, even under these circumstances the incorporation of acetate and mevalonate was quite poor presumably due to compartmentation barriers. [U-14C]Sucrose proved to be the most suitable precursor of oleoresin terpenoids and was used to examine biosynthetic capability over a 12-d time course by radial injection of this substrate 24 h prior to harvest and product analysis.

Incorporation of label from sucrose into monoterpenes of the untreated controls was quite low (<0.1%), whereas wounding produced a notable (5-fold at maximum), but transient, increase in the rate of incorporation into these materials (Fig. 3a). Wounded saplings that had been treated with either PIIF or chitosan exhibited the most rapid rate of [U-14C]sucrose labeling (after 24 h), reaching a maximum 4 or 8 d following elicitor treatment and declining to about 50% of maximum at 12 d (Fig. 3a). The rates of *de novo* resin synthesis observed following the elicitor treatment were some 15 to 20 times higher at maximum than were those of untreated controls, a rather higher ratio than the 3- to 5-fold difference in oleoresin content observed analytically between treated and control saplings in the earlier time course experiments (Fig. 2). It should be noted, however, that the oleoresin content of the untreated controls represents primary (preformed) resin which is stored in the resin ducts and not resin produced in response to external stimulus. Radio-GLC analysis of the labeled monoterpenes generated from [U-14C]sucrose in response to chitosan treatment after 8 d indicated the presence of α-pinene, β-pinene, 3-carene, and β-phellandrene, with lesser quantities of camphene, α-phellandrene, and limonene (Fig. 4a), the *de novo* biosynthesis of which could be anticipated from the earlier analytical studies (Table 1).

Incorporation of label from sucrose into diterpene resin acids (measured as [4C-methyl dehydroabietate] was low in untreated controls (<0.1%), whereas wounding resulted in a transient increase in incorporation to about twice that of control levels, and both PIIF and chitosan treatment resulted in an increase of incorporation rate (at maximum) to over 10 times that of the untreated control (Fig. 3b). For the reasons noted earlier, precise definition of resin acid composition was not possible; however, the presence of methyl dehydroabietate as the major component of the labeled resin acid fraction at each time point was verified by radio-GLC, confirming that a transient increase in the biosynthesis of abietane-type resin acids had occurred.

A roughly 10-fold increase in the incorporation of label into neutral, nonvolatile substances was also observed in response to wounding and treatment with PIIF or chitosan (data not shown) but the absolute incorporation rate was too low (i.e. about 0.2% incorporation at maximum) to permit detailed radiochemical analysis of this fraction.

**Measurement of Terpene Cyclase Activity.** To confirm the transient increase in the biosynthesis of oleoresin terpenoids in *P. contorta* saplings in response to treatment with PIIF and chitosan, an effort was made to measure alteration in the activity of the cyclase enzymes responsible for generating parent C10, C15, and C20 terpenoid compounds from the universal acyclic branch-point metabolites geranyl, farnesyl, and geranylgeranyl pyrophosphate. Cell-free extracts (105,000g supernatants) from control and treated tissue were prepared at 4-d intervals following initiation of the experiment and they were assayed under general conditions developed for comparable enzymes from herbaceous species (6).

Assay of the 105,000g supernatant prepared from treated tissue with [2-3H]geranyl pyrophosphate as substrate gave rise to readily detectable levels of monoterpane olefins, well above the levels observed in controls (Fig. 5a). Maximum activity was observed between 4 and 8 d following treatment, with a diminution of activity to roughly 50% at maximum at 12 d. Radio-GLC analysis of the olefin fraction (obtained by assay at d 4 following chitosan treatment) revealed the presence of labeled α-pinene, β-pinene, 3-carene, and β-phellandrene, with lesser quantities of...
Fig. 4. Radio gas-liquid chromatograms of the monoterpenes isolated from lodgepole pine stems (8 d after wounding and treatment with chitosan) that had been administered 5 μCi of [U-14C]sucrose for 24 h (panel a), and of the monoterpenes isolated from a cell-free extract of lodgepole pine stems (4 d after wounding and treatment with chitosan) that had been incubated with 20 μM [1-3H]geranyl pyrophosphate for 1 h (panel b). The smooth lower tracing (panel c) is the detector response obtained from coinjected authentic standards of α-pinene (1), camphene (2), β-pinene (3), 3-carene (4), α-phellandrene (5), limonene (6), and β-phellandrene (7). For experimental conditions and analytical procedures see "Materials and Methods."

camphene, α-phellandrene, and limonene as expected (Fig. 4b). The oxygenated monoterpane fraction was comprised largely of geraniol, released by phosphatases in the preparation, and the level of this activity was similar to that of the control and changed little over the time-course (i.e. 15 ± 3% conversion of substrate).

Assay with [1-3H]farnesyl pyrophosphate as substrate of the soluble enzyme system prepared from treated tissue gave rise to detectable levels of sesquiterpene olefins which were well above those of controls. The time-course of activity paralleled that of the monoterpane cyclase activity from tissue treated with PIIF or chitosan, but the overall level of activity was about 8-fold less, precluding radio-GLC analysis. Radio-TLC analysis, by scraping and counting regions of the plate, indicated that the labeled compounds in this fraction chromatographed in a broad range of R_f values in the region of the sesquiterpene olefins caryophyllene and humulene. Analysis of the oxygenated sesquiterpene fraction indicated the presence of labeled farnesol (by radio-TLC), and the putative phosphatase activity changed little over the time-course.

Although little is known about the biosynthesis of diterpene resin acids, a sufficient theoretical framework exists to suggest that the initial cyclic products of the pathway are olefins of the pimaradiene and/or abietadiene type (7, 29). Assay with [1-3H]geranylgeranyl pyrophosphate as substrate of the soluble enzyme system prepared from treated tissue gave rise to diterpene olefins at overall rates comparable to those observed for cyclization of geranyl pyrophosphate to cyclic monoterpane olefins, and the time-course was also similar with respect to the controls (Fig. 5b). Radio-GLC evaluation of this material suggested the presence of at least two components; however, the lack of suitable standards and the low specific activity of the precursor (and thus low count levels) prevented adequate analysis of these products. Radio-TLC separation indicated the labeled material to chromatograph with an R_f value above that of abietriene (aromatic C-ring). The oxygenated diterpene fraction was shown to contain primarily geranylgeraniol by radio-TLC analysis.

**DISCUSSION**

The results presented here indicate that lodgepole pine saplings respond to fungal infection and challenge with biotic elicitors by the production of oleoresin, much like their mature counterparts (17, 19, 21, 23, 24). The chemical composition of the "induced" oleoresin differs somewhat between saplings and mature trees, most notably in the minor contribution of limonene to the sapling resin and in the greater proportion of diterpene resin acid (19, 23). Similar differences were observed between the primary (constitutive) resin of saplings and mature trees, and such developmental variation is not unexpected (27).
The observation that lodgepole pine responds to the presence of fungus and to the same carbohydrate fragments of plant and fungal cell walls that elicit defensive reactions in the Solanaceae and Leguminosae (28) suggests that the Pinaceae possess a recognition mechanism for induced defense similar to that of other higher plants (2, 17). Although the role of blue stain fungi in pathogenesis has been questioned (4) and the detailed influence of various oleoresin components is still controversial (5), there can be little doubt that *C. clavigera*, as well as biotic elicitors which might be expected to arise in the process of infection, do elicit the production of a complex oleoresin which in turn is detrimental to the growth and development of both the fungus and the bark beetle which serves as the vector (19-21, 25). By these criteria, induced oleoresinosis can be seen to resemble phytoalexin production (16); however, it need be emphasized that the process represents greatly enhanced, although relatively slow, production of constitutive metabolites, which is quite unlike the typical phytoalexic response.

Finally, evidence based on *in vivo* tracer studies is presented for a transient increase in the *de novo* biosynthesis of relevant monoterpene and diterpene oleoresin components in the tissue of the reaction zone in response to challenge. Furthermore, this transient increase in biosynthetic capability is manifested at the level of the cyclases which catalyze the committed steps of terpenoid biosynthesis from branch-point acyclic precursors. Whether the levels or activities of other enzymes of the terpenoid pathway are similarly enhanced in response to challenge is presently uncertain. This is a question which can be readily addressed with pine saplings; a model system for which the induction and analytical procedures are now being optimized.

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