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COMBINED CHEMICAL DEFENSES AGAINST AN INSECT–FUNGAL COMPLEX

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Abstract—This study considered how host plant allelochemicals may contribute to defense against insects and fungi that jointly colonize the subcortical tissues of trees, the relative roles of constitutive and inducible chemistry in these defenses, and how the actions of two different feeding guilds might be interrelated. Our model consisted of the coniferous tree *Pinus resinosa*, the root- and lower stem-colonizing beetles *Hylastes porculus* and *Dendroctonus valens*, and their associated fungi *Leptographium procerum* and *L. terebrantis*, and the stem-colonizing bark beetle *Ips pini* and its associated fungus *Ophiostoma ips*. In a novel bioassay, extracts from reaction tissue elicted by wound inoculation with *L. terebrantis* were more repellent to beetles than were similar extracts from constitutive or mechanically wounded tissue. The effect on beetle behavior was more pronounced in nonpolar extracts, which contain mostly monoterpenes, than in polar extracts, which contain mostly phenolics. Synthetic monoterpenes at concentrations present in the various tissues exerted similar effects and were likewise repellent in dose–response experiments. Growth of *L. procerum* and *L. terebrantis* was inhibited by polar extracts from constitutive and reaction tissue. Inhibition was higher in wounded than control tissue, but the inhibition response did not vary with the type of wounding. Synthetic monoterpenes strongly inhibited spore germination and mycelial growth of both fungi. Colonization of red pine roots by *Leptographium* spp. altered the subsequent effects of extracts of stem phloem tissue on *I. pini*. These effects varied with host condition. Beetles preferred extracts from constitutive stem phloem tissue of healthy trees to that of root-diseased trees. However, extracts from reaction tissues of healthy trees were more repellent

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to *I. pini* than were the reaction tissues of root-diseased trees. The implications of these results to plant defense against insect-fungal complexes and interactions among different feeding guilds are discussed.

Key Words—Insect-fungal interactions, host selection, plant stress, forest decline, monoterpenes, phenolics, bark beetles, *Ophiostoma ips*, *Ips pini*, *Dendroctonus valens*, *Hylastes porculus*, *Leptographium procerum*, *Leptographium terebrantis*, *Pinus resinosa*.

INTRODUCTION

Plant parasitic insects and plant pathogenic microorganisms are confronted with similar defensive mechanisms when invading potential host plants (Schultz, 1983; Krischik et al., 1991; Hammerschmidt, 1993); yet the chemical ecology of plant defense against these organisms has largely been studied separately. Recent discussions of plant defense theory emphasized the need for integrated studies on plant-insect-microbial interactions (e.g., Barbosa et al., 1991; Schowalter and Filip, 1993). Bark beetle (Coleoptera: Scolytidae)-ophiostomatoid fungi complexes that colonize living conifers provide a valuable system for comparing host plant defenses against herbivory and disease. Most bark beetle species vector only a few, or at most several, ophiostomatoid wood-colonizing fungi. These insect-fungal symbioses may initially be mutually advantageous in overcoming host tree resistance (Berryman, 1972; Hemingway et al., 1977; Raffa and Berryman, 1983a; Berryman et al., 1985; Whitney, 1982), but fungi may reduce beetle success during subsequent development (Barras, 1970; Bridges and Perry, 1985; Fox et al., 1983).

The physiology and chemistry of conifer constitutive and induced defenses against attack by phloem-invading complexes have received significant attention. Constitutive defenses include tough outer bark, several classes of allelochemicals, and elaborate networks of resin ducts or glands. Attacks by invading bark beetles elicit localized host responses including rapid formation of resinous lesions around the attack sites (Berryman, 1972; Raffa and Smalley, 1995). In instances of successful tree defense, the beetle and its associated microbes are contained within these lesions. When the tree is unsuccessful, the beetle and microbes colonize and kill host tissue. In these cases, host defensive reactions are only barely apparent and appear to have been exhausted by the rapid arrival of numerous beetle-fungal units mediated by aggregation pheromones (Raffa and Berryman, 1983a,b).

Two classes of allelochemicals, monoterpenes and phenolics, are particularly abundant in conifer subcortical tissue and increase in concentration in response to invasion of living phloem (Jorgensen, 1961; Shain, 1967; Russell and Berryman, 1976; Raffa and Berryman, 1982, 1983b; Hain et al., 1983;

Cook and Hain, 1986; Faeth, 1986; Lorio, 1986; Miller et al., 1986; Paine et al., 1987; Lewinsohn et al., 1991; Herms and Mattson, 1992; Klepzig et al., 1995b). Hence, these compounds have been proposed as important components of chemical defense. Evidence in support of this hypothesis includes observations that monoterpenes can inhibit mycelial growth of beetle-vectored fungi and repel or kill bark beetles under some conditions (Smith, 1963; Cobb et al., 1968; Shrimpton and Whitney, 1968; DeGroot, 1972; Coyne and Lott, 1976; Bordasch and Berryman, 1977; Raffa et al., 1985; Bridges, 1987; Delorme and Lieutier, 1990; Paine and Hanlon, 1994). However, we know little about how host defenses may affect beetle and fungal establishment during the early and most critical phases of this interaction. In particular, integrated studies on how host chemistry affects fungal propagule germination and beetle host-selection behavior are lacking.

We have utilized an insect–fungal complex associated with a forest decline disease to investigate chemical defenses in a coniferous host plant (Klepzig et al., 1991, 1995a,b). This complex includes root-infesting (*Hylastes porculus* Erichson) and stem-infesting (*Dendroctonus valens* LeConte) bark beetles (Scolytidae) that vector two ophiostomatoid fungi, *Leptographium terebrantis* Barras and Perry and *L. procerum* (Kendrick) Wingfield, to red pine (Klepzig et al., 1995a). Colonization by this complex is not lethal to mature red pine. The pine engraver, *Ips pini* (Say) (Scolytidae), colonizes the stem above ground, vectors the fungus *Ophiostoma ips* (Rumb.) Nannf., and ultimately kills declining trees (Klepzig et al., 1991).

In this study we sought to determine the role of monoterpenes and phenolics in the constitutive and inducible defenses of red pine against two insect–fungal complexes from different feeding guilds and examine the effects of previous activity by one feeding guild on subsequent plant-defense reactions against another.

METHODS AND MATERIALS

Two sets of experiments were conducted. The first series evaluated the effects of constitutive and induced allelochemicals on subcortical beetles and their associated fungi. Healthy, mature red pines were wound-inoculated with *L. terebrantis* to simulate natural attacks. Phloem tissue was collected from these inoculations at one and three weeks after inoculation to determine if the host reaction changed with time since inoculation. Extracts from constitutive (unwounded) and reaction (wound-inoculated) phloem tissue were tested in insect behavioral, fungal germination, and fungal growth assays. The monoterpene and phenolic compositions of these extracts were previously reported in Klepzig et al. (1995b). The bioassays were also conducted using defined concentrations

of synthetic monoterpenes that simulated the composition of host constitutive and reaction tissue. The tests with synthetic monoterpenes included both individual compounds and combinations, as recent work demonstrates the importance of synergistic interactions in some systems (e.g., Berenbaum et al., 1989).

A second set of experiments considered how colonization by a member of a root-colonizing guild (*L. terebrantis*, the fungal associate of *H. porculus*) affected host plant interactions with a member of a stem-colonizing guild (*I. pini*). Our previous research demonstrated an association between colonization by *I. pini* and prior root infection with *Leptographium* spp. (Raffa and Klepzig, 1996). In the current set of experiments, the effects of extracts from healthy and root-diseased trees on behavior of stem-colonizing beetles were compared.

Insect and Fungal Culture

Adult *H. porculus* and *D. valens* were collected from red pine plantations and held at 7°C until use. Adult *I. pini* were obtained from a laboratory colony reared in red pine logs using previously described methods (Raffa and Smalley, 1995). Refrigerated slant cultures of *L. terebrantis* (isolate K9013, from an adult *D. valens*, and isolate LT23, from a *Pinus resinosa* Ait. root) and *L. procerum* [isolate LP5, obtained from an adult *Hylobius radicis* Buchanan (Curculionidae)] were transferred to plates of potato dextrose agar (PDA) and allowed to grow for 10–14 days at 24°C.

Host Treatment and Extraction

Healthy, mature red pines were wound-inoculated (Wright, 1933) to the phloem–xylem interface with a 12-mm disk of PDA colonized by *L. terebrantis*, mechanically wounded with a sterile 12-mm cork borer, or left unwounded (constitutive). Reaction lesions were sampled at one or three weeks after inoculation. In the wounding and inoculation treatments, the xylem and phloem tissues containing the resinous reaction lesion were removed with a chisel and transported to the laboratory on Dry Ice. Samples of constitutive tissues of similar size were removed from a randomly chosen site at the same height on the stem, but were otherwise treated the same as wounded and inoculated tissues. The phloem was separated from the xylem and weighed. The phloem was chopped into small ($< 1 \times 1$ mm) pieces and extracted for 24 hr at 25°C, in either polar or nonpolar solvents. Polar solvents were used to extract phenolics. In 1986, tissue samples were extracted in 10 ml of 50% methanol for 24 hr at 25°C. In 1993, tissue samples were extracted in 10 ml of 70% acetone for 24 hr at 25°C. Extracts were filtered through medium-porosity filter paper and stored at −30°C in sealed vials. Constitutive and reaction tissues were extracted in 10 ml of pentane for 24 hr at 25°C to extract monoterpenes. Extracts were

filtered through medium-porosity filter paper and stored at −30°C in sealed vials.

Effects of Induced and Constitutive Allelochemicals on Beetle Host Selection Behavior

Polar extracts (primarily phenolics), nonpolar extracts (primarily monoterpenes), or synthetic pure monoterpenes (Klepzig et al., 1995b) were incorporated into artificial media.

Polar Extracts. A host selection medium was developed to elicit tunneling and feeding behaviors. Melted 1.5% PDA (500 ml) was combined with 80 g of ground, freeze-dried, autoclaved, constitutive red pine phloem from healthy unwounded mature trees. A strip of plastic transparency film was sealed with melted paraffin wax across the middle of a half-section of a two-compartment I-plate Petri dish (Fisher Scientific, Pittsburgh, Pennsylvania). Test extracts were added directly to the two quarter-sections of the Petri plate. A 0.5-ml aliquot of one extract was added to one section of the assay medium, and 0.5 ml of a different extract was added to the other section, such that the resulting concentrations within the medium were similar to those found in an equivalent weight of red pine phloem. Molten host selection medium (15 ml) was mixed thoroughly with the extracts on each side and allowed to solidify, after which the plastic divider was removed. This assay was used to compare the biological acitivity of polar extracts from unwounded phloem tissue and reaction phloem tissue that had been wound-inoculated with *L. terebrantis* and sampled after three weeks on the tunneling behavior of *D. valens*, *H. porculus*, and *I. pini*. Polar extracts from mechanically wounded tissue were not available for use in these assays.

Nonpolar Extracts. A modified medium was prepared from freeze-dried, ground red pine phloem, water, and Bacto-agar (Difco, Detroit, Michigan) 2:0.002:1 (phloem–water–agar). Approximately 1.5 g of medium was pressed into a 5-mm layer within one of the half-sections of a two-compartment I-plate Petri dish, allowed to dry, and divided into two quarter-sections with a razor blade. Nonpolar extracts from constitutive, mechanically wounded, or inoculated trees were concentrated from 2.0 to 0.5 ml under nitrogen. Subsamples (0.25 ml) of these extracts were added to one or the other quarter-section of medium. The pentane was evaporated under nitrogen, and the razor blade divider was removed.

This assay was used to compare the biological activity of nonpolar extracts from unwounded phloem tissue, phloem tissue that had been mechanically wounded and sampled after three weeks, and phloem tissue that had been wound-inoculated with *L. terebrantis* and sampled after one or three weeks. Preliminary assays of the effects of these extracts on *H. porculus* and *I. pini* tunneling

behavior (Klepzig, 1994) revealed no significant differences between extracts of 1-week-old and 3-week-old inoculations. In addition, these two types of extracts were not found to differ significantly in monoterpene composition (Klepzig et al., 1995b). Due to this, and to the availability of extracts, extracts from 3-week-old inoculations were compared to extracts from constitutive phloem, and extracts from 1-week-old inoculations were compared to extracts from 3-week-old mechanical wounds. *D. valens* was only sporadically available during this time period and was excluded from further assays. However, because *H. porculus* was readily available from the field, and a laboratory colony of *I. pini* was readily available during this time period, these two species were used in this assay.

In all of the above assays, a semicircular piece of plastic transparency film was placed on top of the medium and sealed in place with molten paraffin wax. A hole (2–3 mm in diameter) was drilled through the midpoint of the half-section divider, and 4 mm into the medium. The plate was tilted and either one female *D. valens* or *H. porculus*, or one male *I. pini* (the host-selecting sex, respectively) was introduced into the hole. The hole was sealed behind the insect with cotton and transparent tape. The beetle could then enter either of the two quadrants and move freely between them. The lid of each dish was replaced and sealed with Parafilm. All assay chambers were incubated in a dark growth chamber at 24°C and examined daily for three to five days. Each beetle's path was traced, and the distance tunneled was recorded daily. The location of the beetle was also recorded for each assay dish. For purposes of this assay, the beetle was considered to have made no choice between the two extract types if any part of its body lay along the midline separating the two sections of medium. If, at the time of measurement, the beetle was entirely within one of the two sections, it was recorded as being located on that side for that day. For this reason, the percentages of time on either side of the assay chamber often did not add up to 100%. The total amount of tunneling and the percentage of time located in each side were analyzed by ANOVA (Abacus Concepts, 1989). Where significant treatment effects were observed, the means were compared using the protected least squares means procedure.

Synthetic Monoterpenes. Two-way choice assays were used to compare the effects of concentrations of α-pinene, camphene, β-pinene, 3-carene, myrcene, and limonene equivalent to those found in unwounded, mechanically wounded, and wound-inoculated mature red pine phloem (Klepzig et al., 1995b) on tunneling by *I. pini*. *I. pini* was used because of availability from a laboratory colony and also because of its pivotal role as a final mortality agent in red pine decline disease (Klepzig et al., 1991).

The monoterpenes tested comprise approximately 99% of the monoterpene fraction of red pine phloem (Raffa and Smalley, 1995). We calculated the amount

of each monoterpene necessary (milligrams per gram of medium) to simulate the concentrations (milligrams per gram phloem) found in constitutive, mechanically wounded, and wound-inoculated phloem tissues (Klepzig et al., 1995b). Each section of medium (3.65 g) meant to simulate constitutive phloem received only 0.004 ml of α-pinene. All the other major monoterpenes occurred at levels too low to incorporate effectively into the medium. Each section of host selection medium meant to simulate mechanically wounded phloem sampled after three weeks received 0.066 ml α-pinene, 0.022 ml β-pinene, 0.002 ml 3-carene, and no camphene, myrcene, or limonene. Each section of host selection medium meant to simulate *L. terebrantis* wound-inoculated phloem sampled after three weeks received 0.168 ml α-pinene, 0.001 ml camphene, 0.068 ml β-pinene, 0.005 ml 3-carene, 0.001 ml myrcene, and 0.002 ml limonene. The effects of α-pinene and β-pinene on tunneling by *I. pini* were also compared to each other in two-way choice assays using the concentrations of these compounds described above.

The effects of increasing concentrations of α-pinene and β-pinene on *I. pini* feeding were examined in no-choice assays. In one series of assays, beetles were introduced into host selection medium containing 3.4, 6.8, 17.1, 34.3, 171.6, or 343.2 mg α-pinene/g of medium. A separate series of assays was conducted using the same range of concentrations of β-pinene. Mean amounts of tunneling were measured and analyzed as described above.

Effects of Induced and Constitutive Allelochemistry on Fungal Germination and Growth

The effects of host allelochemicals on fungal growth and germination were determined by exposing spores and/or mycelia to polar host extracts and/or synthetic monoterpenes.

Fungal Propagule Germination. The effects of monoterpenes on fungal germination were determined using the synthetic monoterpenes described above. Molten PDA (0.05 ml) was dispensed into the wells of sterile Falcon 96-well tissue culture plates (Becton Dickinson Labware, Lincoln Park, New Jersey), and allowed to solidify. Spore suspensions (200,000 spores/ml) were prepared from actively growing colonies of *L. terebrantis* or *L. procerum* and dispensed in 0.005-ml aliquots into each well. Sterile glass fiber filter paper disks (0.7 cm diameter) were placed so that they fit snugly in the top of each well but did not touch the agar surface 0.5 cm below. Each of the common red pine monoterpenes was assayed at saturated concentrations (200 μl applied to each filter paper disk) against both fungi. Varying concentrations of α-pinene and β-pinene were also tested. A total of 12 assay wells were tested per fungus–monoterpene combination. Plates were incubated, with lids in place, at 20°C in darkness. Mean percentages of germination were calculated for each fungus–monoterpene com-

bination, and compared using the protected least squares means procedure in ANOVA (Abacus Concepts, 1989).

Fungal Growth. Polar extracts from phloem of mature red pine trees (N = 25) that had been left unwounded, aseptically mechanically wounded, or wound-inoculated with actively growing *L. terebrantis* were incorporated into PDA. The crude extracts were first partitioned in chloroform, and the residue was dissolved in 40 μl of a 1 : 1 acetone–water mixture. Molten PDA (1 ml) was dispensed into the vial, the vial was capped and agitated, set horizontally until the agar solidified, and uncapped to allow the acetone to evaporate. A 2-mm-diameter disk of actively growing *L. terebrantis* (isolated LT23) mycelia on PDA was placed face down on the surface of the medium. The vials were capped and incubated at 24°C in the dark. Mean linear growth (millimeters) at three days was calculated. Means were compared using Duncan's multiple range test in the general linear models procedure in SAS (SAS Institute, 1982).

Nonpolar extracts could not be incorporated effectively into molten PDA. Therefore, mycelia of *L. terebrantis* and *L. procerum* were expoed to atmospheres saturated with pure synthetic monoterpenes found to be most prevalent in red pine—α-pinene, camphene, β-pinene, 3-carene, myrcene, limonene, and γ-terpinene (Klepzig et al., 1995b; Raffa and Smalley, 1995). Each assay chamber consisted of a sterile 90- × 15-mm plastic Petri plate (Fisher Scientific, Pittsburgh, Pennsylvania) containing PDA. Each monoterpene (200 μl) was dispensed directly onto a filter paper disk (0.45 cm diameter) fastened to the plate lid with paraffin wax. A disk (0.5 cm diameter) of either *L. terebrantis* (isolate K9013) or *L. procerum* (isolate LP5) mycelia on PDA was placed face down in the center of the assay plate. The lid was sealed into place with Parafilm, and assay chambers were incubated inverted at 20°C in darkness. Chambers with sterile filter paper fastened to their lids served as controls. This experiment was replicated 10 times for each fungus–monoterpene combination (control and myrcene treatments were replicated 20 times). Mean colony diameters at five days were calculated and compared using the protected least squares means procedure in ANOVA (Abacus Concepts, 1989).

Effect of Root Disease on Bark Beetle Host Selection Behavior

We extracted phloem tissue from mature, field-grown red pine to examine the effects of root infection with *Leptographium* on defense against insects and fungi. Test trees were in a 25-year-old red pine plantation in Sauk County, Wisconsin. We had previously determined the levels of infection with *Leptographium* spp. in these trees, relative to their distance from the decline epicenter, by examining and culturing from excavated roots (Klepzig et al., 1991). Inoculations, sampling, and chemical extractions were conducted as described above for trees estimated to have high and low levels of root infection, based on prior

excavations. Monoterpene extracts from constitutive and reaction tissue from these trees, as well as synthetic monoterpenes at concentrations found within these tissues, were incorporated into medium and applied in two-way choice host selection assays with *H. porculus* and *I. pini* as described above.

RESULTS

Effects of Constitutive and Induced Allelochemistry on Host Selection Behavior of Subcortical Insects

Polar Extracts. The relative effects of polar extracts of constitutive versus induced-reaction tissue on beetle tunneling varied between species (Table 1A).

TABLE 1. EFFECT OF CONSTITUTIVE AND INDUCED RED PINE PHLOEM EXTRACTIVES ON HOST SELECTION BEHAVIORS BY *H. porculus* AND *I. pini*[a]

		Distance		Time	
Insect	Comparison	cm	*P*	%	*P*
A. Polar extracts					
D. valens	Constitutive	4.7 (0.8)	0.06	62.5 (9.5)	0.05
	Inoculated	2.3 (0.9)		35.0 (10.3)	
H. porculus	Constitutive	2.2 (0.6)	ns	34.0 (6.6)	0.07
	Inoculated	1.3 (0.3)		19.0 (4.9)	
I. pini	Constitutive	0.7 (0.2)	ns	20.0 (4.2)	ns
	Inoculated	0.6 (0.1)		17.5 (4.0)	
B. Nonpolar extracts					
H. porculus	Constitutive	6.9 (1.0)	0.0001	74.2 (7.1)	0.0001
	Inoculated	1.1 (0.4)		15.5 (6.0)	
	Constitutive	4.5 (0.8)	ns	49.0 (8.3)	ns
	Mechanical Wound	2.9 (0.7)		34.0 (7.9)	
	Mechanical Wound	5.1 (0.9)	ns	50.0 (8.2)	ns
	Inoculated*	4.0 (1.2)		31.0 (8.2)	
I. pini	Constitutive	7.0 (1.1)	ns	63.0 (8.1)	0.0002
	Inoculated	4.4 (1.4)		20.0 (6.4)	
	Constitutive	3.1 (0.9)	ns	29.0 (5.5)	ns
	Mechanical Wound	3.7 (0.7)		37.0 (7.0)	
	Mechanical Wound	8.5 (1.7)	.01	71.5 (5.7)	0.0001
	Inoculated*	3.3 (1.1)		14.7 (5.0)	

[a]Media amended with extracts from mature red pine trees that were left unwounded (constitutive), mechanically wounded, or wound-inoculated with *L. terebrantis* and sampled three weeks after inoculation, except*, sampled one week after inoculation. Mean (standard error) tunneling and percent of assay period spent on each side in two-way choice assays. Duration of assays = 5 days. $N = 20$ for all comparisons.

D. valens tunneled further and remained for longer periods in extracts of constitutive than reaction tissue. The distances tunneled by *H. porculus* in medium containing extracts of constitutive and reaction tissue did not significantly differ. However, they remained on the side of the assay chamber containing constitutive extracts for a longer period. *Ips pini* showed no significant preferences between polar extracts of constitutive and reaction tissue.

Nonpolar Extracts. Host condition significantly affected tunneling by *H. porculus* and *I. pini* in nonpolar extracts from red pine. In two-way choice assays, *H. porculus* tunneled over six times as far, and spent nearly five times as much time, in extracts from constitutive than reaction tissue (Table 1B). These beetles exhibited no significant preference between extracts from constitutive and mechanically wounded tissue.

In choice assays involving nonpolar extracts of constitutive and reaction tissue, *I. pini* exhibited preferences similar to those of *H. porculus* (Table 1B). *Ips pini* spent over three times as much time within the side of the assay chamber containing extracts from constitutive tissue than in extracts from reaction tissue. However, the distances tunneled by beetles in these extracts did not significantly differ. *Ips pini* did not exhibit clear preferences between extracts from constitutive versus mechanically wounded tissue.

Synthetic Monoterpenes. Synthetic monoterpenes significantly affected *I. pini* behavior. *Ips pini* tunneled nearly three times more, and spent over four times more time, within the side of the assay chamber containing medium amended with the concentration of α-pinene found in constitutive tissue than in reaction tissue (Table 2). *Ips pini* also tunneled over five times farther, and spent over twice as much time within medium amended with the concentration of α-pinene found in mechanically wounded tissue than in the concentration found in reaction tissue. However, *I. pini* did not exhibit significant preferences between α-pinene concentrations found in constitutive versus mechanically wounded tissue.

In two-way choice assays, *I. pini* exhibited no significant preference between medium amended with the concentrations of β-pinene found in constitutive versus reaction tissue or between concentrations found in mechanically wounded versus reaction tissue (Table 2). *Ips pini* did tunnel over twice as much within medium containing the concentrations of β-pinene found in mechanically wounded as in concentrations found in constitutive tissue.

In no-choice assays, monoterpene concentration significantly affected beetle behavior. *Ips pini* tunneled over five times more in medium containing the lowest concentration of α-pinene tested (3.4 mg/g, only slightly lower than the concentration found in constitutive red pine phloem) than in medium containing the highest concentration of α-pinene tested (343.2 mg/g, only slightly higher than the concentration found in wound inoculated red pine phloem) (Figure 1A). A linear equation described the effects of α-pinene on *I. pini* feeding: $y = -0.004x$

TABLE 2. EFFECTS OF SYNTHETIC MONOTERPENES ON HOST SELECTION BEHAVIOR BY *I. pini*[a]

Extracts simulated using synthetic monoterpenes	Comparison	*N*	Distance		Time	
			cm	*P*	%	*P*
α-Pinene	Constitutive	10	1.6 (0.4)	0.05	46.0 (9.9)	0.003
	Inoculated		0.6 (0.2)		10.0 (4.4)	
	Constitutive	10	4.3 (0.9)	ns	30.0 (6.8)	ns
	Mechanical wound		3.3 (1.2)		22.0 (5.5)	
	Mechanical wound	19	5.9 (1.6)	0.009	57.8 (9.5)	0.008
	Inoculated		1.1 (0.4)		22.3 (8.5)	
β-Pinene	Constitutive	10	2.3 (0.8)	ns	12.0 (5.3)	ns
	Inoculated		0.7 (0.5)		4.0 (4.0)	
	Constitutive	10	3.8 (1.4)	0.0008	22.0 (5.5)	ns
	Mechanical wound		10.2 (1.5)		34.0 (5.2)	
	Mechanical wound	10	1.9 (0.5)	ns	10.0 (3.3)	ns
	Inoculated		4.8 (2.2)		26.0 (9.9)	
All	Constitutive	20	8.2 (1.8)	ns	40.0 (5.6)	ns
	Inoculated		5.2 (1.6)		35.0 (10.3)	

[a] Mean (standard error) tunneling and percent of assay period spent on each side in two-way choice assays. Media amended with pure monoterpenes at concentrations similar to those found in nonpolar extracts of mature red pine trees which were left unwounded (constitutive), mechanically wounded, or, wound-inoculated with *L. terebrantis* and sampled three weeks after inoculation. Duration of assay = 4 days. All = α-pinene, camphene, β-pinene, 3-carene, myrcene, and limonene combined.

+ 1.717; $r^2 = 0.974$; where y is the distance tunneled by the beetle, and x is the α-pinene concentration in the media. Likewise, *I. pini* tunneled over eight times more in medium containing the lowest concentration of β-pinene tested (3.4 mg/g, over 30 times higher than the concentration found in constitutive red pine phloem) than in medium containing the highest concentration of β-pinene tested (343.2 mg/g, nearly three times higher than the concentration found in wound inoculated red pine phloem) (Figure 1B). A linear equation described the effects of β-pinene on *I. pini* feeding: $y = -0.004z + 1.323$; $r^2 = 0.903$; where y is the distance tunneled by the beetle, and z is the β-pinene concentration in the media.

Effects of Induced and Constitutive Allelochemistry on Fungal Germination and Growth

Germination of both *L. terebrantis* and *L. procerum* was reduced by increasing concentrations of both α-pinene and β-pinene (Figure 2). At most doses tested, β-pinene was more inhibitory to both fungi than α-pinene. The

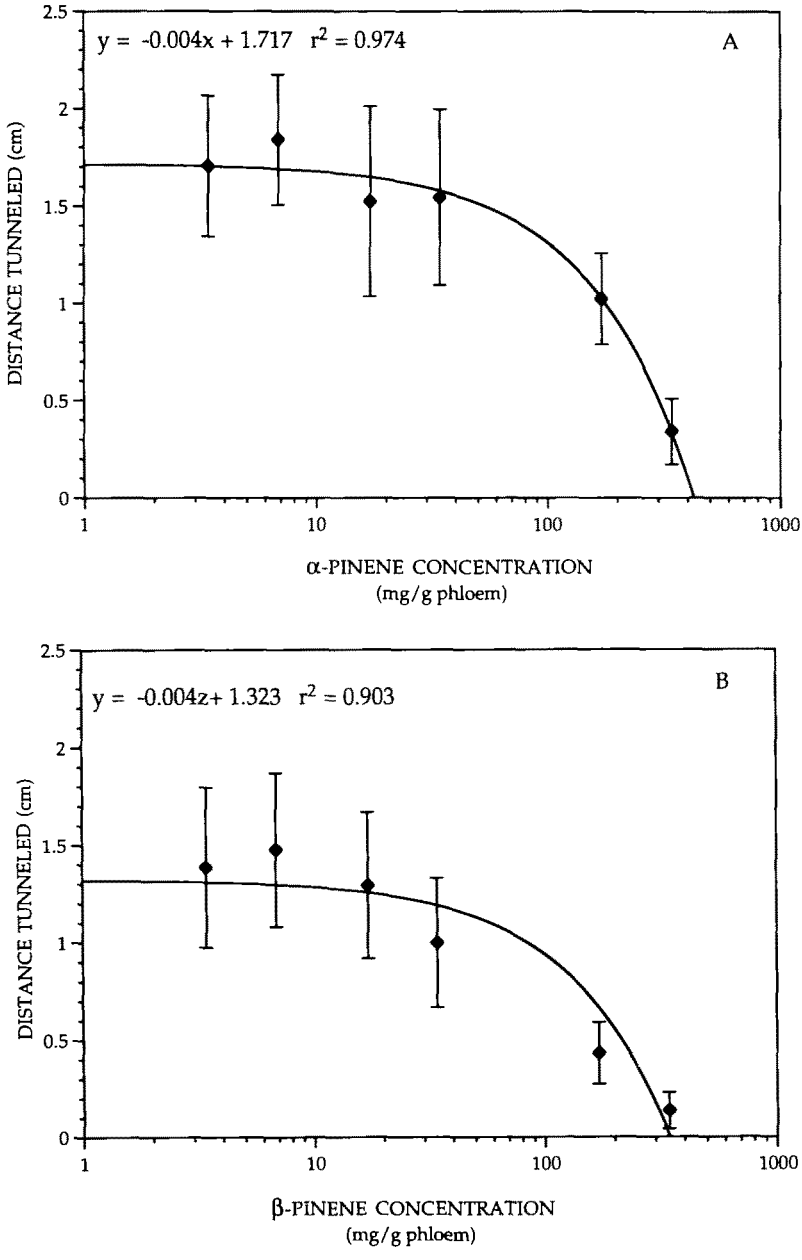

FIG. 1. Effects of synthetic monoterpenes on *I. pini* tunneling (mean ± SE). No-choice assay for two days in phloem-based medium amended with (A) α-pinene, $df = 20$, F 2.93, $P < 0.0001$, or (B) β-pinene, $df = 10$, $F = 2.15$, $P < 0.02$.

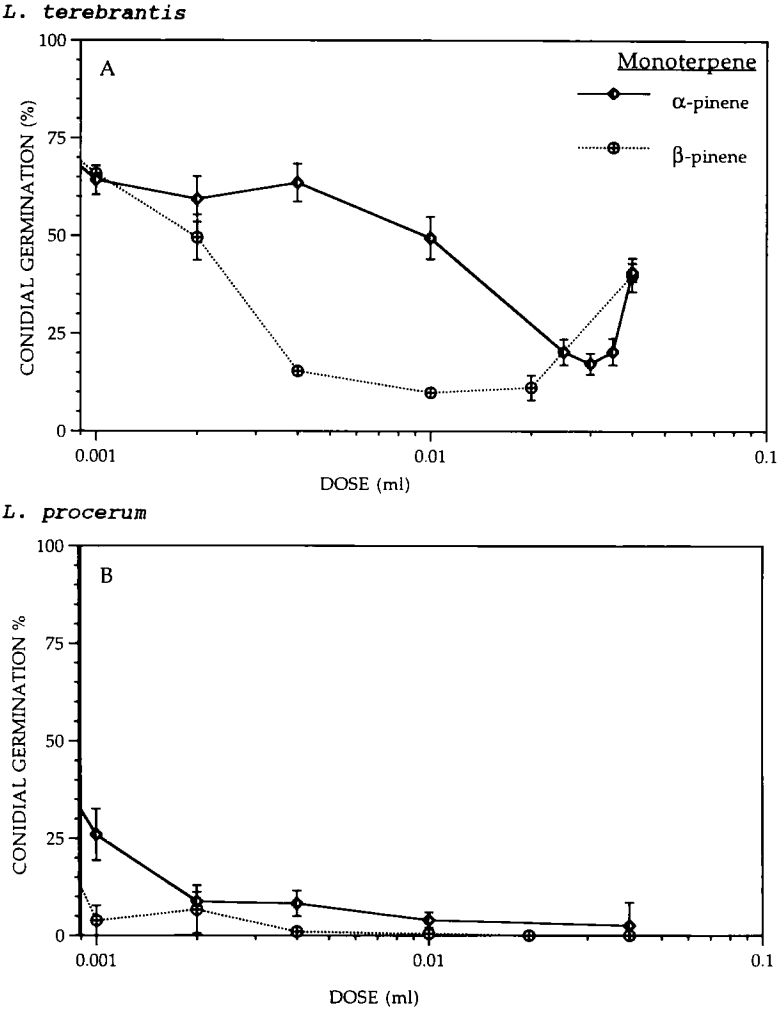

FIG. 2. Effects of α-pinene and β-pinene on germination (mean ± SE) of *L. terebrantis* and *L. procerum* conidia. Fungus by monoterpene interaction: $df = 24$, $F = 93.9$, $P < 0.0001$. Mean percent germination (and standard error) of fungi not exposed to monoterpenes: *L. terebrantis* = 98.0 (0.6) and *L. procerum* = 89.9 (2.3).

highest concentrations of both α-pinene and β-pinene, however, resulted in less inhibition of *L. terebrantis* germination than did intermediate concentrations. No such reduction in inhibitory activity at high concentrations was observed with *L. procerum*.

Polar Extracts. Polar extracts from both constitutive and reaction tissue reduced fungal growth (Figure 3). Extracts from constitutive tissue inhibited mycelial growth of *L. terebrantis* by approximately 15% relative to the control. Extracts of reaction tissue that had been mechanically wounded, inoculated with killed *L. terebrantis*, or inoculated with living *L. terebrantis* inhibited growth equally. Growth was approximately 44% lower on media containing these extracts than on media containing no additives. Medium amended with acetone alone did not differ in inhibitory activity from unamended medium.

Synthetic Monoterpenes. Mycelial growth of *L. terebrantis* was significantly inhibited by vapors of pure α-pinene, camphene, β-pinene, limonene,

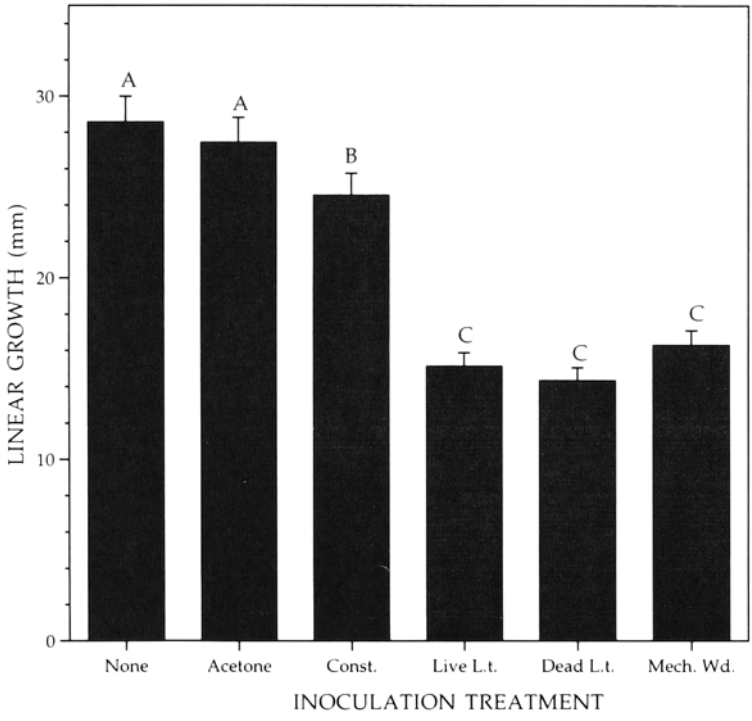

FIG. 3. Effect of acetone–methanol extracts on linear growth (mean ± SE) of *L. terebrantis*. Means followed by different letters are significantly different at $P < 0.05$ by Duncan's multiple range test. Controls: potato dextrose agar (PDA) only (None); PDA + acetone (Acetone). Sources of extracts: constitutive stem tissue (Const.); stem tissue one week after inoculation with viable *L. terebrantis* (Live L.t.); stem tissue 1 week after inoculation with nonviable *L. terebrantis* (Dead L.t.) stem tissue one week after mechanical wounding (Mech. Wd.). $df = 5$, $F = 63.79$, $P < 0.0001$.

TABLE 3. EFFECTS OF PURE MONOTERPENES ON GROWTH AND GERMINATION OF FUNGI[a]

	Mean linear growth (cm)		Mean conidial germination (%)	
Monoterpene	*L. terebrantis*	*L. procerum*	*L. terebrantis*	*L. procerum*
α-Pinene	2.71 (0.04) d	1.25 (0.02) b	40.7 (2.3) e	27.9 (3.0) e
Camphene	2.20 (0.00) e	0.02 (0.01) c	45.9 (2.6) ce	46.6 (3.5) d
β-Pinene	2.61 (0.05) d	1.19 (0.01) b	58.8 (2.5) bd	25.3 (5.2) e
3-Carene	3.34 (0.02) ab	1.35 (0.02) ab	52.7 (2.3) bc	49.7 (4.4) d
Myrcene	3.36 (0.07) ab	1.29 (0.04) ab	60.7 (3.0) ad	68.2 (2.7) c
Limonene	3.26 (0.10) c	1.24 (0.06) ab	76.2 (2.2) a	74.9 (2.5) b
γ-Terpinene	1.60 (0.03) f	0.96 (0.99) c	68.4 (3.2) a	23.2 (2.1) e
Control	3.52 (0.16) a	1.33 (0.02) a	98.0 (0.6) a	89.8 (2.3) a

[a]Mean (standard error) linear growth (cm) of mycelia and mean (standard error) germination (%) of conidia exposed to saturated atmospheres of pure monoterpenes found in red pine reaction tissue and to no monoterpenes (control). Linear growth measured at five day's exposure. Percent germination measured at 1 day of exposure. Means within a column followed by different letters are significantly different at $P < 0.05$ as determined by protected least squares means procedure.

and γ-terpinene (Table 3). Growth of *L. procerum* was significantly inhibited by α-pinene, camphene, β-pinene, and γ-terpinene. The greatest inhibition against both fungi was exerted by camphene and γ-terpinene, which are only trace components in red pine. Among the major components of host monoterpenes, α-pinene and β-pinene were the most inhibitory.

Saturated atmospheres of the predominant monoterpenes in red pine also reduced spore germination of both *L. terebrantis* and *L. procerum* (Table 3). All monoterpenes significantly inhibited germination of *L. procerum* relative to the control. Germination by *L. terebrantis* was significantly inhibited by α-pinene, camphene, β-pinene, and 3-carene. α-Pinene and camphene were the most potent inhibitors of *L. terebrantis*, inhibiting germination by 59% and 53%, respectively. γ-Terpinene, α-pinene, and β-pinene were the most potent inhibitors of *L. procerum*, inhibiting germination by 74%, 69%, and 72%, respectively.

Effect of Root Disease on Bark Beetle Host Selection Behavior

Nonpolar extracts from stem phloem of trees with high and low levels of root infection differentially affected the behavior of *I. pini* and *H. porculus*. In two-way choice assays, *H. porculus* showed no significant preferences between nonpolar extracts from healthy versus root diseased trees (Table 4A). This was true regardless of whether the extracts came from constitutive or reaction tissue.

Ips pini tunneled over four times more, and spent nearly three times more

TABLE 4. EFFECT OF NONPOLAR PHLOEM EXTRACTS AND SYNTHETIC MONOTERPENES FROM HEALTHY AND ROOT-DISEASED RED PINES ON HOST SELECTION BEHAVIORS BY *H. porculus* and *I. pini*[a]

	Comparison		Distance		Time	
Insect	Tree condition	Tree tissue	cm	*P*	%	*P*
A. Nonpolar extracts						
H. porculus	Healthy	Constitutive	6.3 (2.1)	ns	53.3 (14.9)	ns
	Diseased	Constitutive	4.7 (1.7)		46.6 (14.9)	
	Healthy	Inoculated	4.2 (1.9)	ns	50.0 (13.4)	ns
	Diseased	Inoculated	2.4 (0.8)		46.0 (14.6)	
I. pini	Healthy	Constitutive	4.8 (1.2)	0.01	52.0 (13.0)	0.05
	Diseased	Constitutive	1.1 (0.6)		18.0 (9.6)	
	Healthy	Inoculated	2.4 (1.0)	0.06	16.0 (8.8)	0.01
	Diseased	Inoculated	6.0 (1.5)		56.0 (11.0)	
B. Synthetic monoterpenes						
I. pini	Healthy	Inoculated	1.0 (0.6)	ns	7.5 (3.6)	ns (0.13)
	Diseased	Inoculated	2.5 (1.3)		22.5 (8.8)	

[a]Mean (standard error) tunneling and percent of assay period spent on each side in two-way choice assay. A: Nonpolar extracts of healthy vs. root-diseased mature red pine trees that were unwounded (constitutive) or wound-inoculated with *L. terebrantis* and sampled three weeks after inoculation; B: Media amended with concentrations of pure monoterpenes similar to concentrations found in extracts of healthy and root-diseased mature red pine trees that were wound inoculated with *L. terebrantis* and sampled three weeks after inoculation. Duration of assay = 3 days. $N = 20$ for all comparisons.

time, within extracts of constitutive tissue from healthy trees than in extracts from trees with high levels of root infection. However, this pattern was reversed when tree defensive capacity was challenged by wound inoculation with *L. terebrantis*. *Ips pini* tunneled over twice as much, and spent over three times more time, within extracts of reaction tissue from trees with high than low levels of root infection (Table 4A).

Synthetic pure monterpenes incorporated into media at concentrations equivalent to those found in reaction tissue from trees with high and low levels of root infection did not significantly affect *I. pini* behavior (Table 4B).

DISCUSSION

These experiments demonstrate the ability of allelochemically based defenses in red pine to affect both insect and fungal parasites. In this system, the early stages of combined parasitic invasion can apparently be interrupted by host

chemical defenses. Beetles and fungi invading red pine are only slightly (or not at all) inhibited by the levels of polar compounds (phenolics) and nonpolar compounds (monoterpenes) present in the constitutive tissue that they first encounter. However, as the beetles tunnel into subcortical tissue, they elicit an induced response from the host (Cheniclet et al., 1988; Miller et al., 1986; Berryman, 1988; Raffa and Smalley, 1995). The resulting elevated concentrations of phenolics and monoterpenes can significantly inhibit the germination of fungal spores or inhibit subsequent hyphal development. Continued insect tunneling and fungal development elicit further host reactions, which are usually sufficient to repel the attack in vigorous, healthy trees.

The effects of particular components of the induced host response vary with the type and species of invading parasite. In this system, phenolics appear to affect fungi to a greater degree than insects but are only moderately inhibitory to both. Conversely, monoterpenes in red pine are highly active in inhibition of both insects and fungi. Based on the results with synthetic monoterpenes, these compounds can partially, but not fully, explain beetle preferences for various host extracts.

H. porculus and *I. pini* both tended to prefer constitutive to induced reaction tissue, at least as indicated by beetle location. However, only *I. pini* discriminated between reaction tissues elicited in response to mechanical wounds versus fungal inoculations. Likewise, only *I. pini* discriminated between extracts from healthy versus diseased trees. These results suggest that *I. pini* is more discriminating than *H. porculus* with regard to monoterpene concentrations and that responses to induced reactions are an essential component of *I. pini*'s overall host selection process. These preferences may relate to host selection behavior by *I. pini* in the field. That is, *I. pini* males must discriminate between vigorous hosts capable of mounting an effective defense and stressed hosts that might be more suitable for colonization and subsequent brood production. *I. pini* preferred extracts from constitutive tissue from healthy trees to extracts from similar tissue from diseased trees. However, monoterpene concentrations were very low in constitutive tissues (Klepzig et al., 1995b), and this may merely represent a stimulatory effect of low concentrations of monoterpenes on *I. pini* tunneling. In reaction tissue resulting from wound inoculation with *L. terebrantis*, this preference was reversed. Soon after *I. pini* enters red pine, it inoculates its associated fungus, *O. ips*, into the phloem and initiates a rapid induced response (Raffa and Smalley, 1988). This is the type of tissue with which *I. pini* is most likely confronted as it begins the process of gallery construction that may lead to pheromone production and subsequent mass attack by other *I. pini*. Thus, the ability of a tree to mount an effective induced response may be as important, or more important, than its constitutive allelochemical composition. We currently know very little about the biology and behavior of *H. porculus*, so results with this root-feeding species are more difficult to interpret (Klepzig, 1994).

The closely related fungi studied here also differed in their sensitivities to host extracts. The germination and growth of *L. procerum* were strongly inhibited by saturated atmospheres of monoterpenes. The germination and growth of *L. terebrantis* were also inhibited by monoterpenes, but to a lesser extent. The biological significance, if any, of the decreased levels of inhibition of germination of *L. terebrantis* conidia by the highest concentrations of α- and β-pinene is unclear. These higher doses may be artificially high and/or the decreased level of inhibition at these doses may be an artifact of the assay chamber design. The differences between the two fungi in allelochemical tolerance, however, may relate to life history traits of these two fungi. *L. procerum* is only weakly virulent in red pine and most other conifers. *L. terebrantis*, the more allelochemically tolerant species, may be among the most virulent of the bark beetle-associated fungi (Harrington, 1993). In two growth-chamber studies *L. terebrantis*, but not *L. procerum*, was able to kill red pine seedlings (Klepzig, 1994). Although this pattern does not demonstrate a definite link between allelochemical tolerance and virulence in these plant parasites, it is consistent with this hypothesis and argues for further work on this aspect of conifer–scolytid–fungal interactions.

Biotic and abiotic stresses can impair the defense mechanisms described above. Trees whose roots have been extensively colonized by parasitic organisms are less able to produce reaction tissue that is inhibitory to *I. pini*. These behavioral reactions correspond to reduced levels of induced allelochemical accumulation, following *I. pini–O. ips* attack in root-infected trees (Klepzig et al., 1995b). Thus, successful colonization by the root-colonizing beetle *H. porculus* and its associated fungus *L. terebrantis* appears to predispose red pine to subsequent attack and colonization by the main stem-colonizing beetle *I. pini* and its associated fungus *O. ips*. Tree mortality data are consistent with this proposed mechanism (Klepzig et al., 1991; Raffa and Klepzig, 1996). Abiotically stressed trees can also be more susceptible to infection by these facultatively pathogenic fungi (Klepzig, 1994). As with most infection, the stem subcortical tissue of light-limited trees showed altered patterns of allelochemical induction in response to simulated attack (Klepzig et al., 1995b). Both of these examples of increased susceptibility in relation to biotic and abiotic stress can be at least partially explained by changes in the carbon-based defensive chemistry. That is, the observed chemical changes are consistent with physiological changes associated with impaired host abilities to compete for resources. For example, diseased roots and shaded needles are less able to capture water and nutrients, and sunlight, respectively.

The relationships among trees physiology and plant parasites described here have implications for the ecology and management of forest ecosystems. In particular, they suggest a probable sequence for the multiple species interactions involved in a decline disease affecting red pine (Klepzig et al., 1991). The most

frequent and most severe examples of this decline typically occur in trees growing off site, often on poor soils, and under potentially resource-limiting conditions. The resulting chronic stresses may represent predisposing factors as defined by Manion (1991). Likewise, some short-term stresses that can serve as inciting factors in this decline have been identified. These include reduced light availability, root disease, lightning strike, and/or root damage due to temperature or moisture extremes. However, the exact identity, relative incidence, and overall importance of various inciting factors in red pine decline disease remain unclear. Contributing factors appear to include a complex of subcortical root- and stem-infesting insects and their associated fungi partially described here (Klepzig et al., 1991). Induced defensive reactions to attack by this multispecies complex appear to be compromised by both the predisposing and contributing factors. This may allow increasingly successful attacks by root insects and fatal attacks by *I. pini–O. ips* complexes (Klepzig et al., 1991). This hypothetical framework has also led to suggested strategies for managing red pine decline disease, including severing root grafts in advance of the spread of the root-infecting fungi and planting trees in mixed-species blocks.

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