Effects of Terpenoid Compounds on Growth of Symbiotic Fungi Associated with the Southern Pine Beetle

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ABSTRACT

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The blue-stain fungus *Ceratocystis minor* and the two mycangial fungi of the southern pine beetle (*Dendroctonus frontalis*) were grown in saturated atmospheres of volatile compounds from loblolly pine. The monoterpenes α- and β-pinene significantly stimulated the linear growth of one of the mycangial fungi, a *Sporothrix* species. Growth of the other, a basidiomycete, was significantly inhibited by α- and β-pinene and by most of the other compounds tested. Linear growth of *C. minor* was not significantly affected by α-pinene but was inhibited by β-pinene. A phenylpropanoid, 4-allylanisole, was highly inhibitory to all three fungi. Results suggest that the production of this compound may be an important loblolly pine defense mechanism against attack by the southern pine beetle and fungi associated with it.

Additional key words: resin system, tree resistance.

The southern pine beetle (*Dendroctonus frontalis* Zimmermann) is the most destructive pest of southern pine forests. During outbreaks, this aggressive insect attacks and kills healthy trees and is a very important economic problem. The evolutionary success and destructive power of the southern pine beetle and other bark beetles can be attributed, in part, to their symbiotic association with certain fungi (5). The southern pine beetle carries two species of fungi in a specialized structure called a mycangium (1,8). One of these is the *Sporothrix* anamorph of *Ceratocystis minor* (Hedg.) Hunt var. *barrasii* Taylor (2). The other is an unidentified basidiomycete. Although the role of these fungi in the beetle's biology is not well understood, *Sporothrix* is thought to be a tree pathogen and the basidiomycete is thought to contribute to beetle nutrition (9).

Like most bark beetles, the southern pine beetle usually carries a blue-stain fungus, *C. minor* (28), which is pathogenic to pines (3,19) and has been thought necessary for optimal beetle development (7,21,22). However, because southern pine beetle infestations have been observed without this fungus, its role in bark beetle development has been questioned (10).

The resin system of pines is an important resistance mechanism against stem invasion by bark beetles and fungi associated with them (6). Trees respond to attack by secreting resins containing high concentrations of terpenoid compounds. Although the physical properties of the resin are closely related to host resistance to bark beetle attack (17,18), its chemical composition is also thought to be important in resistance (13,16,18). Because terpenoid compounds are deemed important in tree resistance to invasion by microorganisms (13), knowledge of their effects on symbiotic fungi could be important in understanding the role of these fungi in tree pathology.

The goal of our research was to acquire a better understanding of the relationship of symbiotic fungi to bark beetle biology and host tree resistance. The objective of this study was to test the effects of volatile compounds on the linear growth of selected fungi in culture. This paper reports the results of growing the two mycangial fungi of the southern pine beetle and a blue-stain fungus in saturated atmospheres of volatile compounds (terpenoids and a phenylpropanoid) found in loblolly pine (*Pinus taeda* L.), a preferred host of the beetle.

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MATERIALS AND METHODS

The fungi were isolated just before the initiation of the study: *C. minor* from the inner bark of a beetle-infested loblolly pine and *Sporothrix* and the basidiomycete from the mycangium of the southern pine beetle as described by Barras and Perry (1). The basidiomycete was cultured on a medium containing malt extract (1.5%), casamino acids (1%), and agar (1.5%). The other two fungi were cultured on malt extract (2.5%) and agar (1.5%).

The effects of the volatiles on fungal growth were determined by measuring the growth of the fungi in saturated atmospheres (11,12). The compounds studied were terpenoids (14,24) and a phenylpropanoid (14) identified from loblolly pine; they were obtained commercially (Table 1). Three grams of each compound were placed in a petri dish in the bottom of a 3.5-L widemouthed glass jar. The jar was closed and the volatiles were allowed to vaporize for 24 hr before the fungi were added. A jar without chemical was used as the control. Culture media in 9-cm glass petri dishes were inoculated with 3-mm plugs of inoculum cut from the advancing edge of an actively growing colony of each fungus. Two dishes of each fungus were placed in each jar and the jars were kept in the laboratory at ambient light and temperature. The experiment was repeated at four different times.

Diameters of the fungal colonies were measured in two directions at 90° angles. *C. minor* was measured after 5 days. Growth of the other two fungi was measured weekly for 3 wk. Growth times were selected on the basis of growth rates of the fungi. *C. minor* normally covers a plate within about 7 days, whereas the other two fungi require more than 3 wk. For the mycangial fungi, data for the third week are reported in this paper.

Data were analyzed using a two-way analysis of variance. One factor was treatment chemical, the other was replicate. The mean square for the interaction was used as the error term for testing for significant treatment effects. Dunnett's procedure (two-tailed test) was used to compare each treatment with the control (27).

RESULTS AND DISCUSSION

The effects of volatiles on the growth of the three fungi are shown in Table 1. The fungi varied considerably in their responses; the basidiomycete was the most sensitive to the compounds. Its

growth was inhibited by 18 of the 21 compounds tested. Linear growth of *C. minor* was inhibited by about half of the compounds. This fungus was not significantly affected by α-pinene, the major monoterpene component of southern pine oleoresin. However, growth of *C. minor* was inhibited by β-pinene, the second most abundant monoterpene in loblolly pine oleoresin (20). Growth of *Sporothrix* was significantly increased by both α- and β-pinene. The linear growth of *Sporothrix* was increased by 59% by *d*-1-α-pinene. β-Pinene increased *Sporothrix* growth by 44%. The other compounds either had no significant effect on *Sporothrix* or inhibited its growth (Table 1).

Three of the compounds, *t*-caryophyllene, α-humulene, and *l*-borneol, had no significant effect on the growth of any of the three fungi. α-Terpinene, terpinolene, *p*-cymene, *t*-anethole, and 4-allylanisole significantly inhibited the growth of all of the fungi (Table 1).

Both enantiomers of α-pinene and limonene were tested. There were no significant differences in the effects of the two enantiomers of limonene on any of the fungi. The growth of the basidiomycete or *C. minor* did not differ between the enantiomers of α-pinene. However, racemic α-pinene inhibited *C. minor* compared with either enantiomer alone. The reasons for this are not known.

Each of the enantiomers of α-pinene produced different effects on the growth of *Sporothrix*. Growth was stimulated by *d*-α-pinene, whereas the levorotatory antipode had no significant effect. In loblolly pine, *d*-α-pinene is the naturally occurring antipode (20), and *Sporothrix* has apparently evolved a tolerance to it. Because the mode of action of these compounds on fungi is not known, it is impossible to explain the variation in response of fungi to terpenoids.

Because the linear growth of fungi is generally inhibited by terpenoid compounds (11,12,25,26), stimulation of *Sporothrix* by α- and β-pinene is somewhat unusual. The observed differences in the effects of volatiles on the fungi may have ecological significance related to bark beetle biology. Although the mycangial fungi are thought to be obligate symbionts for the southern pine beetle, the impact of each fungus is not fully understood. There is evidence that the fungi influence beetle biology differently because they affect the beetle in different ways (9). In natural populations, beetles usually carry only one of the two mycangial fungi. Only about 20% of beetles carry both (10). Beetles that carry the basidiomycete alone have been shown to be significantly heavier than those that carry only *Sporothrix* (8,9). Beetle survival and progeny production have been shown to be positively correlated with the incidence of the basidiomycete in beetle populations. Beetle survival and progeny production have been shown to be negatively correlated with the occurrence of *Sporothrix* (8,9). On the basis of these findings, Bridges (9) proposed that the basidiomycete may contribute to beetle nutrition.

It has been suggested that *Sporothrix* could be a tree pathogen facilitating the process of tree death during beetle colonization, although there is no direct evidence to support this theory (10). Growth stimulation by α- and β-pinene, the major monoterpene components of southern pine oleoresin, indicates that this fungus has evolved the ability to overcome one of the resistance mechanisms of the tree. Stimulation of its growth by the pinenes suggests that it could be a successful early colonizer of beetle-attacked trees, as would be expected for a pathogen. On the other hand, the basidiomycete may be inhibited initially by some volatiles in living trees and subsequently able to grow only after terpene concentrations have decreased following tree death. For this fungus to be used as a source of nutrition, growth would need to occur only after the tree has been killed, beetle galleries have become established, and larvae have begun their development.

The significance of *C. minor* in southern pine beetle biology is unclear. It is pathogenic to pines (3,19) and has been thought to be the major pathogen of southern pine beetle-killed trees (21). However, recent descriptions of southern pine beetle infestations without *C. minor* indicate that this fungus is not the primary pathogen (10). Paine et al (23) have suggested that the mycangial fungi may be more highly adapted as pathogens to loblolly pine than the blue-stain fungus. Their conclusion was based on the finding that these fungi do not stimulate lesion formation by the tree in response to fungal inoculation. The results of the experiment described here are consistent with a similar conclusion for *Sporothrix*. Because the linear growth of *Sporothrix* is stimulated by α- and β-pinene and the growth of *C. minor* is either inhibited or not affected by the compounds, *Sporothrix* may be more highly adapted for pathogenicity.

TABLE 1. Linear growth of southern pine beetle mycangial fungi after 3 wk and growth of *Ceratocystis minor* after 5 days in saturated atmospheres of volatiles from loblolly pine

Chemical[a]	Mean colony diameter (mm)		
	Sporothrix	Basidiomycete	*C. minor*
Control	48.2	73.4	46.8
d-*l*-α-Pinene	76.6*[b]	47.2*	38.4
d-α-Pinene	71.8*	48.8*	51.2
l-α-Pinene	62.8	46.0*	50.2
β-Pinene	69.4*	34.8*	32.7*
δ-3-Carene	61.1	37.9*	31.4*
Myrcene	55.3	31.8*	16.9*
α-Phellandrene	55.4	50.4*	35.8
d-Limonene	54.6	26.1*	25.0*
l-Limonene	52.8	19.7*	19.4*
t-Caryophyllene	56.8	71.2	40.9
α-Humulene	52.6	77.1	41.5
l-Borneol	52.1	68.4	44.1
Camphene	51.2	44.2*	37.8
γ-Terpinene	30.9	21.8*	18.2*
α-Fenchol	25.6*	14.6*	49.8
α-Terpinene	24.4*	18.4*	31.6*
Terpinene-4-ol	22.8*	12.3*	50.2
Terpinolene	19.8*	38.5*	6.3*
p-Cymene	13.1*	6.2*	6.3*
t-Anethole	10.7*	4.4*	21.9*
4-Allylanisole	4.4*	4.4*	10.6*

[a] The following commercial sources were used: δ-3-carene (Pfaltz and Bauer, Inc.); myrcene (Sigma Chemical Co.); α-phellandrene, *t*-caryophyllene, and α-humulene (Fluka Chemical Corp.); *d*-limonene (Eastman Kodak Co.); terpinolene (SCM Corp.); and all others (Aldrich Chemical Co.).

[b] Values followed by an asterisk are significantly different from the control at $P = 0.05$ based on Dunnett's test.

These results may have further ecological significance in terms of pine resistance to attack by bark beetles and their associated fungi. Two defensive systems are present in loblolly pine (15,23). The primary one is the preformed oleoresin system. Physical properties of the oleoresin are thought to be important in resistance, and the flow of the resin acts to flush the wound of invading organisms (18). The other system is the wound response initiated following invasion of pathogens (4,15,23). Lesion tissue becomes soaked with resin, and analysis of lesion tissue in loblolly pine has shown that the volatile compounds in such resin were similar to those of preformed oleoresin (14). However, 4-allylanisole was also found in significant quantities in lesion tissue but not in preformed resin (14). This compound was the most inhibitory one to the mycangial fungi of all of the compounds tested (Table 1). It almost completely stopped the growth of the basidiomycete and *Sporothrix* while inhibiting the growth of *C. minor* by 77% (Table 1). Thus, the production of this compound during lesion formation may be involved in loblolly pine resistance to the southern pine beetle and its symbiotic fungi.

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