Effects of Oleoresin and Terpenoids on Fungal Growth associated with Pine Decline in the Southern United States

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Abstract

As a means of exploring pine resistance to root disease and declines, the effects of host plant secondary metabolites on the growth of root colonizing fungi associated with three diseases ⁄ declines of southern pines – loblolly pine decline, littleleaf disease and annosum root rot were tested. The associated fungi – *Leptographium huntii, L. serpens, L. terebrantis, L. procerum, Heterobasidion annosum* and *Phytophthora cinnamomi* – were grown in saturated atmospheres or in direct contact with, pure monoterpenes and crude oleoresin collected from the four southern pines (*Pinus taeda, P. eschinata, P. palustris* and *P. elliotti)* for 7 day. Fungal growth was measured at 3, 5 and 7 day. Root-infecting fungi differed significantly in sensitivity to crude oleoresin and pure monoterpenes. All fungi tested were inhibited, to some extent, by the resins tested. *H. annosum* and *P. cinnamomi* were strongly inhibited by all the monoterpenes tested. The ophiostomatoid fungi were significantly less affected by the compounds tested. *L. huntii* and *L. serpens* were less inhibited by monoterpenes than either *L. terebrantis* or *L. procerum*. These fungal growth studies show that the kind and amount of secondary metabolite produced by the host plant have a profound effect on tree pathogens. Alterations of tree physiology may have implications for defenses against tree pathogens as well as to the ecology and management of forest ecosystems. Difference in incidence of root disease observed in the field may be explained by the ability of the fungus to tolerate these host defense mechanisms.

Introduction

The defense system of conifers to biotic agents such as pathogens and herbivores consists of: (i) a constitutive; (ii) a preformed oleoresin response; and (iii) an induced oleoresin response that develop simultaneously and complement each other. The constitutive and preformed mechanisms exist in the absence of a pathogen and include tough outer bark, several classes of secondary metabolites and an elaborate network of resin ducts. However, the induced response and mechanisms are activated only when a pathogen or herbivore attacks the tree and consists of a several classes of secondary metabolites and uses the network of pre-formed resin ducts.

Many secondary metabolites of wood are toxic or inhibitory to pathogenic fungi. The two most abundant monoterpenes in southern pines are (-)-a-pinene and (-)-b-pinene, while others such as camphene, myrcene, limonene and b-phellandrene are common in the four southern pines. *Pinus elliottii*. and *P. palustris* oleoresin contains significantly less total monoterpenes than *P. taeda* and *P. echinata*, as a result of the lower content of b-pinene. *P. elliottii* oleoresin contains more b-phellandrene, *P. palustris* oleoresin contains more a-pinene and *P. taeda* oleoresin has more myrcene and limonene, than oleoresin from the other tree species (1).

Chemical analysis of *P. taeda* resin soaked tissues has shown that volatile monoterpenes are similar to those of preformed oleoresin (2) formed in response to infection ⁄ inoculation of bluestain fungi. Some differences may occur, however. For example, the phenylpropanoid 4-allylanisole (4-AA) may be found in significantly higher quantities in lesion tissue than in preformed resin (2). This compound has shown some activity as a repellent of bark beetles and an inhibitor of their associated fungi (3, 4).

Two classes of secondary metabolites, monoterpenes and phenolics, are particularly abundant in conifer subcortical tissue and will increase in response to invasion of living phloem by fungi and insects. Therefore, monoterpenes and phenolics have been proposed as important chemical defense components in trees (5, 6, 7, 8). Evidence for defense against infection comes from trials that report monoterpenes inhibiting mycelial growth (9, 10, 11, 12). Schuck (13) suggests that monoterpenes may be toxic to fungi and Hinejima et al. (14) reported that resin from *P. ponderosa* has antimicrobial activity against fungi and some gram-positive bacteria.

Methods

Effects of oleoresin and synthetic resin constituents on fungal growth:

For the fungal growth experiments, 20 ml of 3% PDA (potato-dextrose agar) was poured into Petri dishes. Each fungal species (Table 1) was grown on PDA for 10–14 days at 25C. A disk (4 mm dia) was cut using a cork borer from the actively growing margin of the source of fungus and transferred to the center of each study plate.

The direct contact growth study plates were prepared by pipetting 1 ml of test chemical or oleoresin onto the center of each plate and gently swirling over the agar surface before inoculation. The two most abundant [(-⁄+)-a-pinene and (-)-b-pinene] and four other common (camphene, myrcene, limonene, b-phellandrene) monoterpenes in this system, as well as, a phenylpropanoid (4-AA) which are common among the four southern pines were chosen for testing. Oleoresin from *P. taeda, P. echinata, P. palustris* and *P. elliottii* was collected from living trees with and without decline symptoms to determine if fungi had an advantage in symptomatic trees. Resin was collected by drilling into the xylem and inserting an 8 dm amber vial into the hole. The vial and the accumulated resin were removed after 4 h, placed on dry ice, returned to the laboratory, and stored at -70C until needed.

Agar in plates was inoculated with one of the fungi within 1 h after the resin had been applied to the agar surface. Colony diameters were traced at 3, 5 and 7 days after inoculation. Areas were calculated using a digital planimeter. At the end of seven days, fungal plugs that showed no growth were removed from chemical plates and placed on new PDA plates to determine if the various monoterpenes had fungistatic or fungicidal activity.

Culture plates for the saturated atmosphere study were prepared similarly without the chemical treatment and inoculated with fungi. Each test chamber consisted of a 3.79-liter paint can with a wire rack bottom to support a stack of twelve plates (two plates of each fungus species). Two milliliters of one of the test chemicals was placed in an open glass dish beneath the wire rack. Then the inoculated culture plates were stacked in a random sequence on top of the rack and the lid tightly sealed on the test chamber. Three cans were prepared for each of the 21 treatments including a dH2O and a blank control. Colony diameters were traced at 7 days after inoculation and area calculated. At the end of seven days, fungal plugs that showed no growth were removed from chemical plates and placed on new PDA plates to determine if the various monoterpenes had fungistatic or fungicidal activity.

Methods

Effects of oleoresin and synthetic resin constituents on fungal germination:

The effects of fungal germination were determined using the chemicals listed above. Molten PDA (0.05 ml) was dispensed into the wells of sterile Falcon 96-well tissue culture plates and allowed to solidify. Spore suspensions (200,000 spores ⁄ ml) were prepared from actively growing colonies of *L. terebrantis, L. procerum, L. serpens* and *L. huntii* and dispensed in 0.005-ml aliquots into each well. Sterile glass fiber filter paper disks (0.7 cm dia) 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 test chemical was assayed at saturation concentrations (.002 ml applied to each filter paper disk) against all four fungi. A total of 12 assay wells were tested per fungus-chemical combination. Plates were incubated for 72 h, with lids in place, at 25C in the dark. Fungal germination was determined using a microscope and mean percentages of germination were calculated for each fungus-chemical combination and compared using the protected least square means procedure in ANOVA.

Results

1. Fungi differed in their sensitivity to crude oleoresin and terpenes. (Fig. 1 and 2)
2. *H. annosum* and *P. cinnamomi* were the most inhibited by both the oleoresin and terpenes. (Fig. 1 – 4)
3. Ophiostomatoid fungi were less inhibited, but *L. procerum* was more inhibited than *L. terebrantis* which was more inhibited than *L. serpens* and *L. huntii.* (Fig. 1-4)
4. Most of the time *L. procerum* was not different from *H. annosum* or *P. cinnamomi.* (Fig. 1-4)
5. Crude oleoresin from *P. palustris* and *P. elliottii* were the most inhibitory.
6. In germination experiments, *L. huntii* and *L. serpens* were less inhibited than *L. terebrantis* and *L. procerum.* (Fig. 5-6)

Saturated Atmosphere Results:

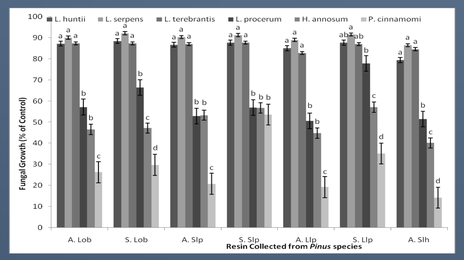

Fig. 1. Effects of crude oleoresin on fungal growth.

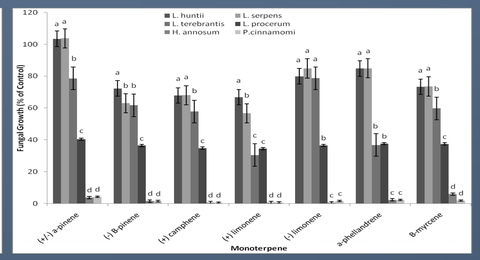

Fig. 2. Effects of monoterpenes on fungal growth.

Tactile Results:

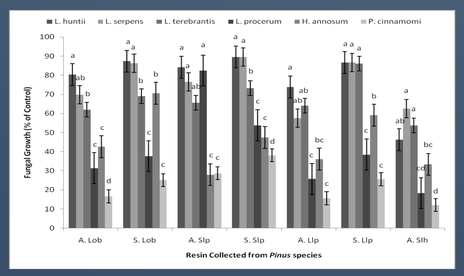

Fig. 3. Effects of crude oleoresin on fungal growth.

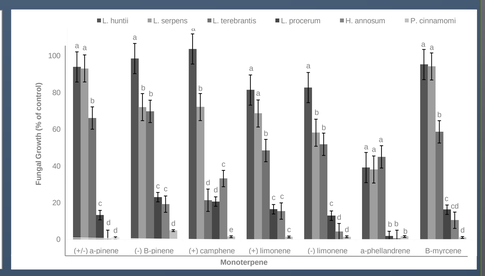

Fig. 4. Effects of monoterpenes on fungal growth.

Germination Results:

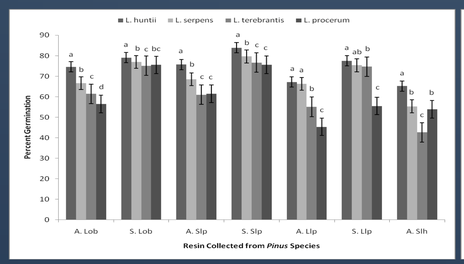

Fig. 5. Effects of crude oleoresin on fungal germination.

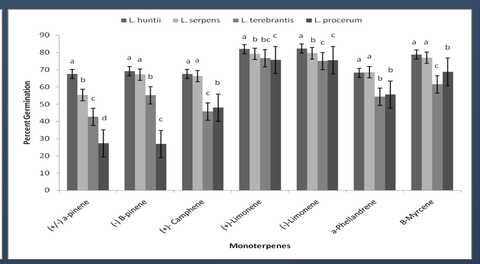

Fig. 6. Effects of monoterpenes on fungal germination.

Table 1. Source of fungal isolates used for growth (volatile and tactile) and sporulation studies

Isolate	Isolate # / ATCC Accension #	Collection Site	Host Source
Leptographium huntii	LLP-R-02-100 / MYA-3311	Military Reservation, GA	Longleaf pine root
L. serpens	LOB-R-00-309 / MYA-3315	Westervelt (previously Gulf State Paper)	Loblolly pine root
L. terebrantis	LOB-R-00-805 / MYA-3316	TNF, Oakmulgee Ranger District	Loblolly pine root
L. procerum	LOB-R-00-456 / MYA-3313	TNF, Shoal Creek Ranger District	Loblolly pine root
Phytophthora cinnamomi	LOB-S-00-825 / MYA-3317	TNF, Oakmulgee Ranger District	Soil from loblolly root zone
Heterobasidion annosum	LLP-R-01-223 / MYA-3318	International Paper Land AL	Longleaf pine root

Summary

The differences between the growth of the fungi exposed to the various chemicals may explain the disease expression caused by these fungi. *Leptographium procerum* is known as a weak pathogen in *P. taeda* and other conifers, while the more allelochemical tolerant *L. terebrantis* is known as a moderate pathogen (15, 16). In greenhouse studies *L. terebrantis,* but not *L. procerum,* was able to kill *P. taeda* seedlings. *Leptographium huntii* and *L. serpens*, which were the most allelochemical tolerant are less studied. Current pathogenicity studies demonstrate that these two fungi are more virulent than either *L. terebrantis* or *L. procerum* (16, 17). Although these inhibition patterns do not demonstrate a definite link between allelochemicals tolerance and virulence in this system, they are consistent with findings by Zamponi et al. (18), Klepzig et al. (12) and Paine and Hanlon (11).

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