For. Path. doi: 10.1111/j.1439-0329.2008.00570.x

Effects of oleoresins and monoterpenes on *in vitro* growth of fungi associated with pine decline in the Southern United States

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Summary

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.

1 Introduction

Reports of forest decline and tree mortality have increased in recent years and are presently considered to be a major threat in temperate ecosystems (Manion and Lachance 1992; Hess et al. 1999; Eckhardt et al. 2007). Forest decline diseases are characterized by the interactions of predisposing abiotic factors and biotic agents that come together in an orderly fashion resulting in tree death (Manion 1991). The biotic agents involved in most declines are generally opportunistic, able to grow either saprophytically or parasitically and function as part of the decline complex (Manion 1991). An example of one such tree decline complex may be found in Loblolly Pine Decline. This decline, occurring in pines in the southeastern region of the United States, is associated with at least four ophiostomatoid fungi: *Leptographium procerum* (Kendrick) Wingfield, *L. terebrantis* Barras & Perry, *L. serpens* (Goid.) Wingfield, and *L. huntii* MJ Wingfield and their insect vectors, *Hylastes* spp. (Eckhardt et al. 2007). Within a forest stand the decline is characterized by thin crowns, chlorotic foliage, reduced radial growth and premature tree mortality.

Received: 19.07.2007; accepted: 18.06.2008; editor: S. Woodward

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 (Wagener and Davidson 1954; Cartwright and Findlay 1958). The two most abundant monoterpenes in southern pines are (−)-α-pinene and (−)-β-pinene, while others such as camphene, myrcene, limonene and β-phellandrene are common in the four southern pines (Hodges et al. 1979; Strom et al. 2002). *Pinus elliottii* Englem. and *P. palustris* P. Mill. oleoresin contains significantly less total monoterpenes than *P. taeda* L. and *P. echinata* Mill., as a result of the lower content of β-pinene. *P. elliottii* oleoresin contains more β-phellandrene, *P. palustris* oleoresin contains more α-pinene and *P. taeda* oleoresin has more myrcene and limonene, than oleoresin from the other tree species (Hodges et al. 1979).

Chemical analysis of *P. taeda* resin soaked tissues has shown that volatile monoterpenes are similar to those of preformed oleoresin (Gambliel et al. 1985) 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 (Gambliel et al. 1985). This compound has shown some activity as a repellent of bark beetles and an inhibitor of their associated fungi (Hayes et al. 1994; Joseph et al. 2001).

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 (Jorgensen 1961; Shain 1967; Russell and Berryman 1976; Hain et al. 1983; Cook and Hain 1986; Miller et al. 1986; Paine et al. 1987; Lewinsohn et al. 1991; Klepzig et al. 1995). Therefore, monoterpenes and phenolics have been proposed as important chemical defense components in trees (Kopper et al. 2005; Bonello et al. 2006; Keeling and Bohlmann 2006; Ockels et al. 2007). Evidence for defense against infection comes from trials that report monoterpenes inhibiting mycelial growth (Cobb et al. 1968; Bridges 1987; Paine and Hanlon 1994; Klepzig et al. 1996). Schuck (1982) suggests that monoterpenes may be toxic to fungi and Hinejima et al. (1992) reported that resin from *P. ponderosa* P. & C. Lawson has antimicrobial activity against fungi and some gram-positive bacteria.

While these studies emphasize how different resin components may serve different physical and biochemical roles in tree defenses, relatively little is known about the roles, that pine terpenes play in root diseases and declines in the southern pine ecosystem. The objective of this study was to determine if volatile monoterpenes affect the growth of root-infecting fungi *in vitro*. This paper reports the results of growing four blue-stain fungi (*L. huntii*, *L. serpens*, *L. terebrantis*, *L. procerum*), *Heterobasidion annosum* and *P. cinnamomi* in crude oleoresin and individual resin constituents (saturated atmospheres and tactile) found in southern pines.

2 Methods and materials

The effects of host allelochemicals on fungal growth and spore germination were determined by exposing spores and/or mycelia to oleoresin and/or synthetic individual resin constituents. Strains used in this study are shown in Table 1 and representative isolates were deposited with the American Type Culture Collection (ATCC).

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

Isolate	Isolate no./ ATCC accession no.	Collection site	Host source
Leptographium huntii	LLP-R-02-100/ MYA-3311	Fort Benning Military Reservation, GA	Longleaf pine root
L. serpens	LOB-R-00-309/ MYA-3315	Westervelt (previously Gulf State Paper) Company Land, AL	Loblolly pine root
L. terebrantis	LOB-R-00-805/ MYA-3316	Talladega National Forest, Oakmulgee Ranger District AL	Loblolly pine root
L. procerum	LOB-R-00-456/ MYA-3313	Talladega National Forest, Shoal Creek Ranger District AL	Loblolly pine root
Phytophthora cinnamomi	LOB-S-00-825/ MYA-3317	Talladega National Forest, Oakmulgee Ranger District AL	Soil from loblolly root zone
Heterobasidion annosum	LLP-R-01-223/ MYA-3318	International Paper Land AL	Longleaf pine root

2.1 Effects of oleoresin and synthetic resin constituents on fungal growth

For the fungal growth experiments, 20 ml of 3% PDA (potato-dextrose agar – Difco, Voigt Global Distribution, Lawrence, Kansas, USA) was poured into Petri dishes (100 × 15 mm, plastic plates were used for direct contact growth study and glass for saturated atmosphere study – Thermo Fisher Scientific, Atlanta, GA, USA). Each fungal species (Table 1) was grown on PDA for 10–14 days at 25°C. A disk (4 mm diameter) was cut using a cork borer from the actively growing margin of the source of fungus and transferred to the centre of each study plate.

The direct contact growth study plates were prepared by pipetting 1 ml of test chemical onto the centre of each plate and gently swirling over the agar surface before inoculation. The two most abundant [(−/+)-α-pinene and (−)-β-pinene] and four other common (camphene, myrcene, limonene, β-phellandrene) monoterpenes in this system, as well as, a phenylpropanoid (4-AA) which are common among the four southern pines (Hodges et al. 1979; Strom et al. 2002) were chosen for testing. Test chemicals were obtained from commercial sources [4-AA, (+)-Camphene, (−)-Limonene, (+)-Limonene, β-Myrcene, (±)-α-Pinene, (−)-β-Pinene (Sigma-Aldrich, St Louis, MO, USA) and α-Phellandrene (TCI America, Portland, OR, USA)]. Both enantiomers of α-pinene and limonene were tested as well as the racemeric mixture of α-pinene.

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. Decline/disease symptomology was determined by crown condition using Forest Health Monitoring techniques (Dunn 1999) and published crown symptomology (Campbell and Copeland 1954; Hodges 1969; Eckhardt et al. 2007). Resin was collected by drilling into the xylem and inserting an 8 dm amber vial into the hole (two trees per category and four vials per tree). The vial and the accumulated resin were removed after 4 h, placed on dry ice, returned to the laboratory, and stored at −70°C until needed. Agar in plates was inoculated with one of the fungi (as described above) within 1 h after the resin had been applied to the agar surface. Inoculated plates were sealed with Parafilm® (Fisher Scientific, Atlanta, GA, USA) to retard desiccation and evaporation. Each treatment was replicated six times. Colony diameters were traced at 3, 5 and 7 days after inoculation. Areas were calculated using a digital planimeter (Lasico 1281-12; Lasico, Los Angeles, CA, USA). 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.

The 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 dH_2O and a blank control. Each treatment was replicated six times. Colony diameters were traced at 7 days after inoculation and area calculated as described above. 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. Mean area and percent growth of control for contact and saturated atmosphere tests were calculated and were analysed using repeated measures (contact only), protected least square means procedure and contrasts in ANOVA version 8.02 (SAS Institute Inc., Cary, NC, USA).

2.2 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 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 test chemical was assayed at saturation concentrations (200 μl 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 25°C 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 version 8.02 (SAS Institute Inc., Cary, NC, USA).

3 Results

3.1 Effects of volatiles of oleoresin and individual resin constituents on fungal growth

All fungi tested were inhibited by the resins tested. Resin from asymptomatic *P. elliottii* and *P. palustris* caused the greatest growth reduction (60–85% less growth than controls) for *L. procerum*, *H. annosum* and *P. cinnamomi. L. huntii*, *L. serpens* and *L. terebrantis* were less affected (17–20% less growth than controls). Resin from both asymptomatic and symptomatic *P. echinata* had the least affect on any fungi with only an 8–10% reduction in growth of *L. huntii, L. serpens* and *L. terebrantis* and a 42, 23 and 68% reduction in growth of *L. procerum*, *H. annosum*, and *P. cinnamomi*, respectively. When comparing fungal growth as a percent area relative to that in controls, *L. procerum*, *H. annosum* and *P. cinnamomi* grew significantly less than *L. huntii*, *L. serpens* and *L. terebrantis* (Fig. 1). Growth of *H. annosum* was more inhibited than *L. procerum* for asymptomatic *P. elliotti* and symptomatic *P. taeda* and *P. palustris*. Growth of *P. cinnamomi* was more inhibited than *L. procerum* except for symptomatic *P. echinata*. *P. cinnamomi* was more inhibited than *H. annosum* in all treatments except symptomatic *P. echinata* (Fig. 1).

The effect of various monoterpenes on fungal growth is shown in Fig. 2. Overall, *L. huntii* and *L. serpens* were less inhibited than *L. terebrantis* and *L. procerum* which were less inhibited than *H. annosum* and *P. cinnamomi* (Fig. 2). While the fungi were inhibited at some level by most of the chemicals, *L. huntii* and *L. serpens* were not inhibited at all by

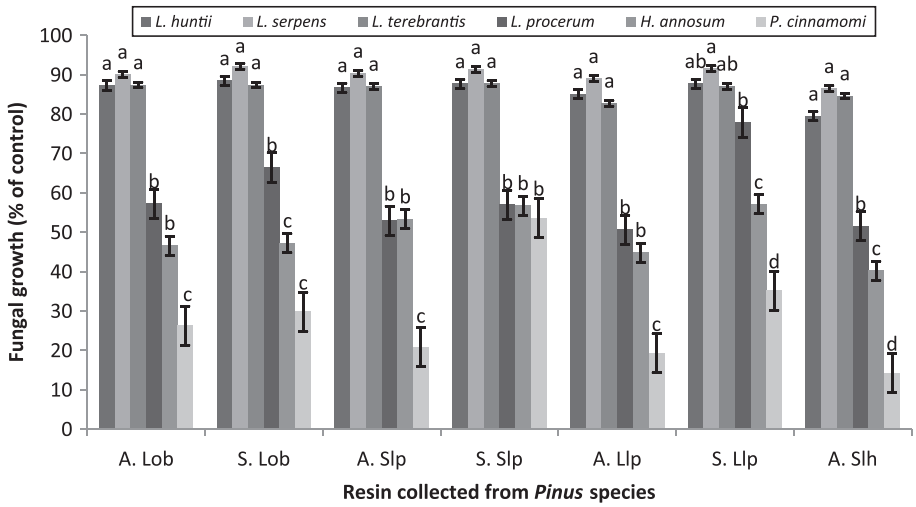

Fig. 1. Effects of crude oleoresin on fungal growth. Mean (standard error) area (cm^2) of colony growth of six fungi associated with root disease grown on 3% Potato-Dextrose Agar for 7 days in a saturated atmosphere of oleoresin from healthy and root-diseased southern. Resin was collected from asymptomatic (a) and symptomatic (b) trees.

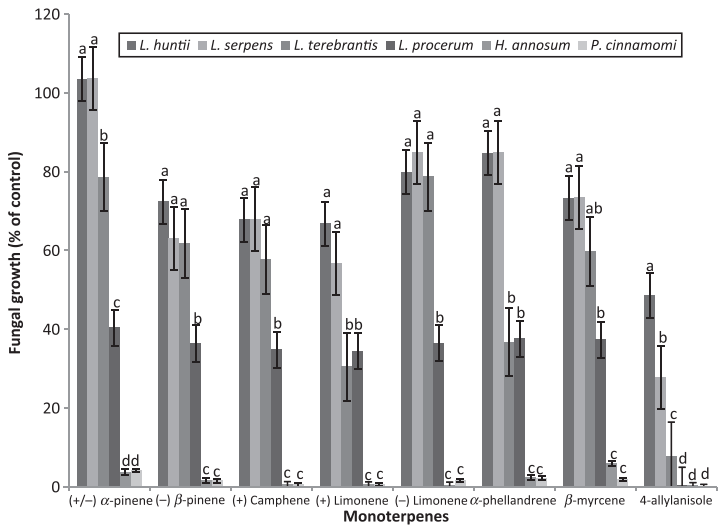

Fig. 2. Effects of pure monoterpenes on fungal growth. Mean area (cm^2) of colony growth of six fungi associated with root disease grown on 3% Potato-Dextrose Agar for 7 days in a saturated atmosphere of resin constituents.

α-pinene (Fig. 2). 4-AA caused the greatest reduction (50–100% less growth than controls) in fungal growth, but *L. huntii* was the least affected. The effects of 4-AA on growth of *L. procerum*, *H. annosum* and *P. cinnamomi* did not significantlly differ (Fig. 2). *H. annosum*

and *P. cinnamomi* growth was negatively affected by all chemicals and there was no difference in the degree of effect on fungal growth among the chemicals tested (Fig. 2). Growth of *L. huntii* was the least inhibited of all the fungi for all chemicals (4-AA 50%, camphene, (+)-limonene, and β-pinene (the second most abundant monoterpene in *P. taeda* pine oleoresin and least abundant in *P. palustris* oleoresin) 26–36%, α-phellandrene and (−)-limonene15–26%, and no inhibition by α-pinene (the main monoterpene component of southern pine oleoresin). *L. serpens* was similar to *L. huntii* in all chemicals tested, except for 4-AA which inhibited *L. serpens* growth by 82%.

Both enantiomers of α-pinene and limonene were tested. Inhibition of fungal growth was greater in response to (+)-limonene than (−)-limonene for *L. huntii, L. serpens* and *L. terebrantis.* Growth of *L. huntii* and *L. serpens* did not differ when the two fungi were exposed to enantiomers of α-pinene, but growth of *L. huntii* was enhanced by the racemic α-pinene relative to growth of controls. Growth of *L. terebrantis, L. procerum, H. annosum* and *P. cinnamomi* were significantly inhibited by both enantiomers and the racemic α-pinene.

When fungi were removed from atmospheres containing various monoterpenes, the fungi responded by growing more rapidly until growth equaled that of controls in 5 day except for those growing initially with 4-AA. The 4-AA appeared to act fungistatically on *L. huntii* and *L. serpens,* as when cultures of these fungi were removed from saturated 4-AA atmospheres they recovered only very slowly taking 2 and 3 weeks to reach growth of controls, respectively. *L. terebrantis* took 4 weeks to reach growth of controls when removed from 4-AA atmospheres. In contrast, 4-AA appeared to act fungicidal on *L. procerum*, *H. annosum* and *P. cinnamomi,* as when these fungi were removed from atmospheres containing 4-AA, there was still no colony growth after 8 weeks.

3.2 Effects of oleoresin and individual resin constituents on fungal growth when applied to substrate surface

With few exceptions, growth of fungi was less when exposed to crude oleoresin than when exposed to saturated atmospheres (Fig. 3). All fungi were inhibited at Day 3 and 5 (data not shown), but by Day 7 growth for *L. huntii* in symptomatic *P. taeda*, *P. palustris* and *P. echinata*, *L. serpens* in symptomatic *P. palustris* and *L. terebrantis* in symptomatic *P. taeda*, *P. palustris* and *P. echinata* were not different from controls. *L. huntii*, *L. serpens* and *L. terebrantis* growth was less inhibited than *L. procerum* for all resins tested (Fig. 3). *L. huntii* and *L. serpens* growth was less inhibited than *L. terebrantis* for all resins except asymptomatic and symptomatic *P. palustris* and asymptomatic *P. elliottii*. While *L. huntii* and *L. serpens* growth was less inhibited than *H. annosum*, *L. terebrantis* and *H. annosum* had similar growth for symptomatic *P. taeda* and asymptomatic *P. palustris* and *L. procerum* and *H. annosum* had similar growth for asymptomatic *P. taeda*, *P. palustris* and *P. elliottii* and symptomatic *P. echinata*.

Growth of fungi was, with few exceptions, less when exposed to crude oleoresin than when exposed to saturated atmospheres (Figs 3 and 4). All fungi were inhibited at Day 3 and 5 (data not shown), but by Day 7, growth reduction for *L. huntii* in camphene, β-myrcene, α-pinene, and β-pinene and *L. serpens* in β-myrcene, (+/−)-α-pinene, (−)-α-pinene was not different from controls. Similar to results seen in the vapors only, inhibition, or tolerance to the individual components differed among the fungi tested. When fungal growth was compared to the percent area of the control, *L. huntii* and *L. serpens* growth was less inhibited than the other fungi in all test chemicals (Fig. 4). The bluestain fungi growth was less inhibited than *H. annosum* and *P. cinnamomi* (Fig. 4). *L. huntii* was the least inhibited by 4-AA and *L. procerum*, *H. annosum* and *P. cinnamomi* the most (Fig. 4). When fungi were removed from plates containing monoterpenes, colony growth equaled that in controls in 7 d except for 4-AA.

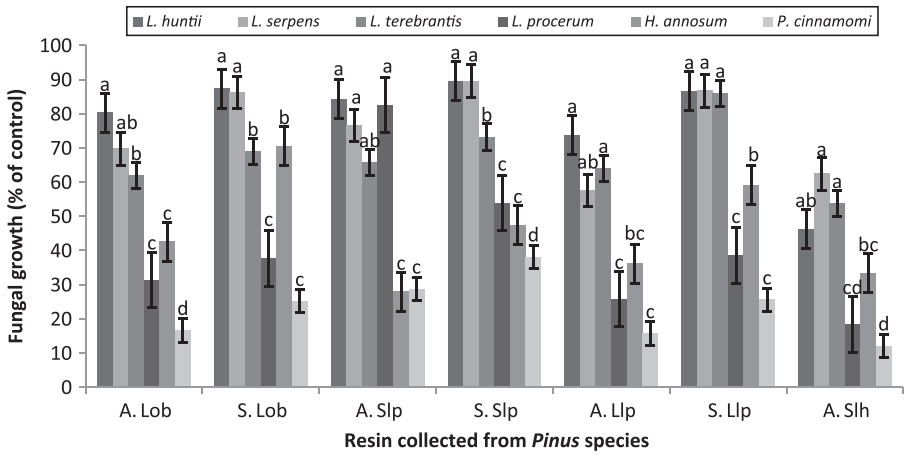

Fig. 3. Effects of crude oleoresin on fungal growth. Mean area (cm^2) of colony growth on 3% Potato-Dextrose Agar with 1 ml of oleoresin from healthy and root-diseased southern pines spread across surface of culture plates at day 7.

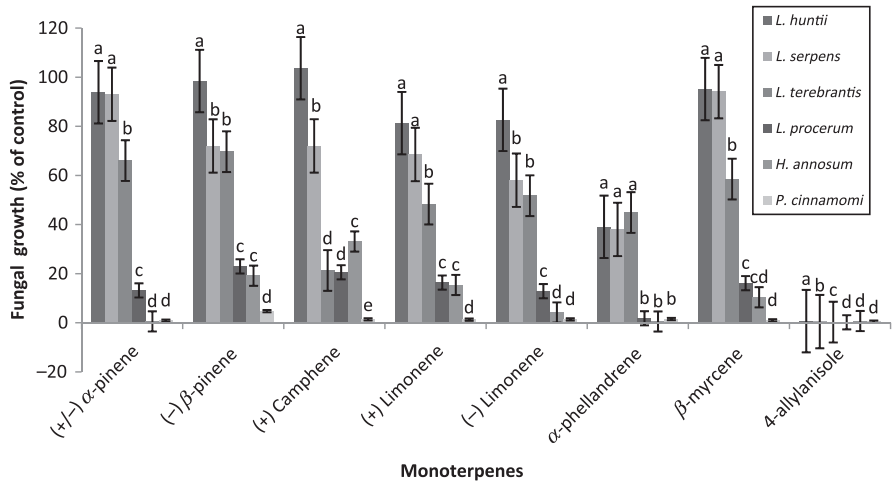

Fig. 4. Effects of pure monoterpenes on fungal growth. Mean area (cm^2) of colony growth on 3% Potato-Dextrose Agar with 1 ml of individual resin constituents spread across surface of culture plates at day 7.

3.3 Effects on spore germination

Saturated atmospheres of crude oleoresin significantly reduced spore germination in all *Leptographium* spp. (Fig. 5). Resin collected from asymptomatic *P. elliottii* and *P. palustris* trees inhibited spore germination in all *Leptographium* spp. tested, while resin collected from symptomatic *P. echinata* trees was the least inhibitory. Sixty-five percent of the *L. huntii* spores were able to germinate in all resins tested. *L. huntii* and *L. serpens* were the least affected followed by *L. terebrantis* and *L. procerum.*

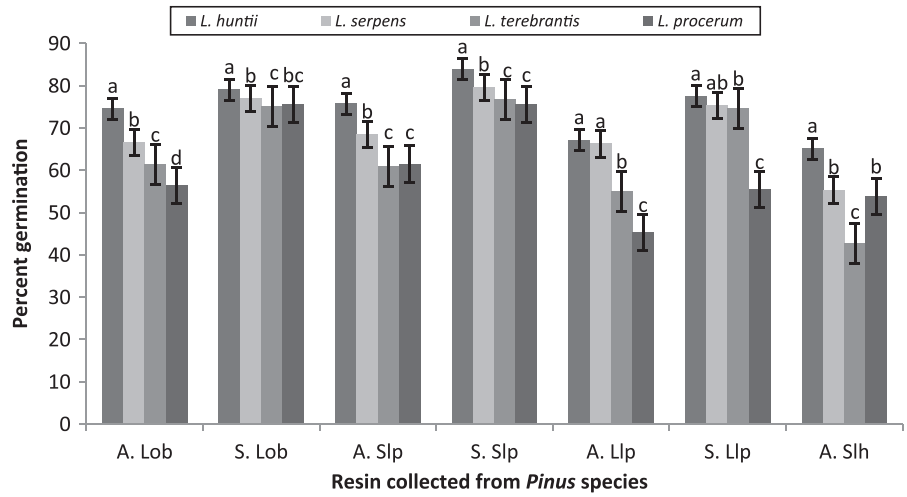

Fig. 5. Effects of crude oleoresin on fungal spore germination. Resin was collected from asymptomatic (A) and symptomatic (S) trees.

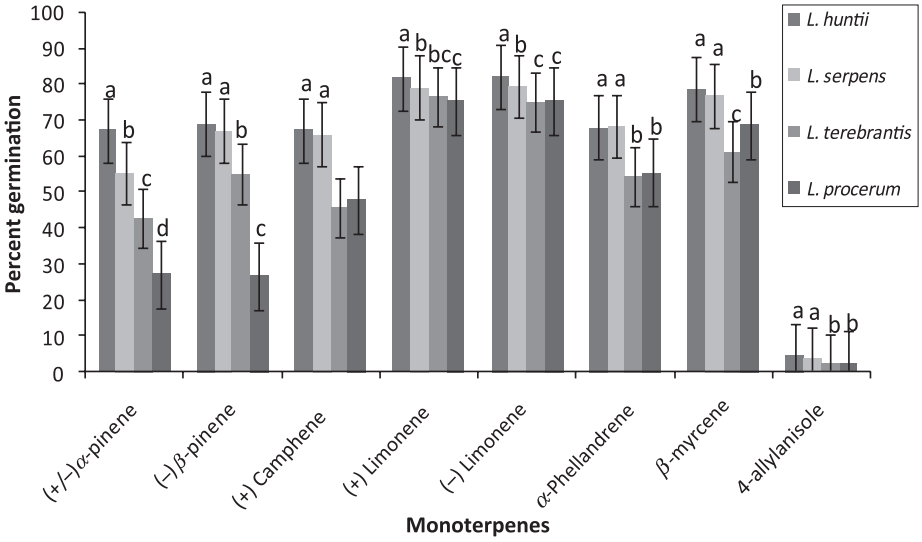

Fig. 6. Effects of pure monoterpenes on fungal spore germination.

Saturated atmospheres of individual resin components significantly reduced spore germination in all species of *Leptographium* (Fig. 6). *L. huntii* and *L. serpens* were the least affected followed by *L. terebrantis* and *L. procerum*, with 4-AA being the most inhibitory (Fig. 6).

4 Discussion

In this system alleolchemical based defenses in southern yellow pines significantly inhibited the growth and sporulation of some root infecting fungi. This inhibitory action of

some resin components may affect the ability of the fungi to infect and proliferate within the host. The early stages of invasion of fungi are known to be affected by host chemical defenses. In some beetle-ophiostomatoid fungus associations, for example, the host tree initiates an induced response in reaction to entry by the beetle fungal complex (Raffa and Smalley 1995). The resulting elevated concentrations of monoterpenes can significantly inhibit the germination of fungal spores or inhibit subsequent hyphal development (Klepzig et al. 1996).

The six root disease fungi examined differed in their sensitivity to crude oleoresin and pure monoterpenes. The fungal growth of *H. annosum* and *P. cinnamomi* were strongly inhibited in pure monoterpenes and oleoresins in contrast to the ophiostomatoid fungi. This result is surprising as both *H. annosum* and *P. cinnamomi* are considered primary root pathogens of pine, while *Leptographium* species are reported to be mild to moderately pathogenic or acting saprophytically. These findings suggest that Ophiostomatoid fungi may have a higher tolerance for host performed or induced oleoresin defense system than either *H. annosum* or *P. cinnamomi*. These results may be of considerable importance in the diagnosis of pine root diseases and the future study of pine decline in the southeastern US.

When looking only at spore germination in the ophiostomatoid fungi, *L. procerum* was inhibited the most of all the fungi by saturated atmospheres of allelochemicals and oleoresins. *L. terebrantis* was moderately inhibited, with *L. serpens* and *L. huntii* inhibited the least. This inhibition trend held true for contact presentation of the allelochemicals and oleoresins as well. However, there may also have been physiological differences in the response of the fungi to the different presentation modes, as seen by the fungal growth in α- and *β*- pinenes. For example, *β*-pinene was more inhibitory when presented in saturated atmosphere than tactile for all fungi and α-pinene acted oppositely. The racemic mixture of α-pinene, however, enhanced growth of *L. huntii* and *L. serpens.* Resin collected from *P. palustris* and *P. elliottii* trees were more inhibitory than the other resins on all fungi. *Pinus palustris* and *P. elliottii* oleoresin contains significantly less total monoterpenes than either *P. taeda* or *P. echinata,* as a result of the lower content of *β*-pinene (Hodges et al. 1979). Although, Hodges et al. (1979) found that in more resistant pines resin was slower to crystalize (*P. elliotii*) or had a higher resin flow (*P. palustris*) compared to more susceptible trees (*P. taeda* and *P. echinata*). 4-AA was the most inhibitory of all chemicals tested. This compound composes 1–11% of resin in pines (Hodges et al. 1979). Bridges (1987) also reported 4-AA as being highly inhibitory, but species specific.

The differences between the growth of the fungi exposed to the various chemicals may explain the disease expression caused by these fungi. *L. procerum* is known as a weak pathogen in *P. taeda* and other conifers, while the more secondary metabolite tolerant *L. terebrantis* is known as a moderate pathogen (Harrington 1993; Eckhardt et al. 2004). In greenhouse studies *L. terebrantis*, but not *L. procerum*, was able to kill *P. taeda* seedlings. *L. huntii* and *L. serpens*, which were the most secondary metabolite tolerant are less studied. Current pathogenicity studies demonstrate that these two fungi are more virulent than either *L. terebrantis* or *L. procerum* (Eckhardt et al. 2004; Matusick et al. 2007). Although these inhibition patterns do not demonstrate a definite link between secondary metabolite tolerance and virulence in this system, they are consistent with findings by Zamponi et al. (2006), Klepzig et al. (1996) and Paine and Hanlon (1994).

Biotic and abiotic stresses can impair the tree defense mechanisms which result in the modification or reduction of various secondary metabolites (Klepzig et al. 1996). In this system, trees with extensively colonized roots were less able to produce inhibitory compounds against invading root colonizing fungi. This is consistent with previous work showing that abiotically stressed trees appeared to be more susceptible to infection by saprophytic fungi than healthy vigorous trees (Klepzig 1994). The relationship among tree physiology and pathogens has implications for the ecology and management of forest ecosystems. These fungal growth studies show that the kind and amount of secondary

metabolite produced may have a profound effect on tree pathogens and saprophytes. Differences in the expected type and occurrence of root diseases observed in the field may be explained by the ability of the fungus to tolerate these host defense mechanisms.

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