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Melanin and the ecology of southern pine beetle associated fungi

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Abstract
I report here a series of initial investigations into effects of melanins on the interactions of the three primary species of fungi associated with the southern pine beetle (SPB), and into possible means for mitigating the damaging activities of the melanistic fungus, *Ophiostoma minus*. Growth of the SPB mutualistic fungus *Entomocorticium* sp. A on high melanin medium was less than half of the growth on control medium. Growth of *O. ranaculosum* was not significantly affected by the addition of melanin to malt extract agar. Four species of biological control (of bluestain) fungi showed the ability to decolorize melanistic *O. minus* cultures to various extents.

Keywords: Mutualism, *Ophiostoma*, *Entomocorticium*, *Dendroctonus*, competition

1. Introduction

Melanins are dark biological macromolecules composed of phenolic or indolic monomers (Butler and Day, 1998). These secondary metabolites can protect fungi against irradiation, enzymatic lysis, temperature extremes, and dessication (Butler and Day, 1998). At the same time, melanins can be toxic (Henson et al., 1999), and can act as virulence factors. They may account for as much as 30% of the dry weight of a cell (Butler and Day, 1998). The role of melanins in the ecology and pathology of some plant pathogenic fungi has been reviewed (Henson et al., 1999), however, virtually nothing is known about the roles of these compounds in the biology of the bark beetle associated stain (ophiostomatoid) fungi. I report here a series of initial investigations into effects of melanins on the interactions of fungi associated with the southern pine beetle, and into possible means for mitigating the damaging activities of the melanistic fungus, *Ophiostoma minus* (Hedgcock) H. and P. Sydow responsible for bluestain.

Bark beetles (Coleoptera: Scolytidae, altern. Curculionidae: Scolytinae), are consistently associated with fungi in a variety of interactions, from casual phoresy to mutualistic symbioses (Paine et al., 1997). The southern pine beetle (SPB) *Dendroctonus frontalis* Zimmermann is among the most damaging of North American forest insects (Price et al., 1992) and carries three primary fungi into the trees it attacks. *Ophiostoma minus* is an ascomycete fungus carried phoretically on the SPB exoskeleton and by phoretic mites (Rumbold, 1931; Bridges and Moser, 1983).

While this fungus, in concert with beetle tunneling, may hasten tree death (Paine et al., 1997), its main economically damaging activity may be the blue staining that its highly melanized hyphae impart to infected wood. Southern pine beetles and their arthropod associates (especially mites) are the only means by which *O. minus* can gain access to new host tissue (Dowding, 1969). Thus, at least at the early stages of SPB attack, a mutualistic relationship exists between SPB and *O. minus*.

However, by the time larvae begin developing within the host tissues, *O. minus* becomes a competitor and antagonist of SPB (Barras, 1970). The mechanism of this antagonism is likely due, in large part, to the interactions of SPB with its two other significant fungal associates. Each female SPB possesses a mycangium (Barras and Perry, 1972; Happ et al., 1971) within which she maintains a pure culture of either *Ophiostoma ranaculosum* (syn. *Ceratocystiopsis ranaculosus*, Jacobs and Kirisits, 2003; Barras and Taylor, 1973) or *Entomocorticium* sp. A (aka, SJB122, Barras and Perry, 1972; Happ et al., 1976), an amber colored basidiomycete (Hsiau, 1996). These fungi are not virulent in their pine hosts, rather they grow within the phloem, sporulating heavily in beetle tunnels within which the SPB larvae graze and likely obtain the majority of their nutrition (Ayres et al., 2000). Both mycangial fungi are nutritional mutualists of SPB, though *Entomocorticium* sp. A appears to be of greater benefit to the beetles than *O. ranaculosum* (Bridges, 1985; Goldhammer et al., 1990; Coppedge et al., 1995; Klepzig and Wilkens, 1997). All three of these fungi compete for uncolonized pine phloem (Klepzig and Wilkens,

1997). The results of these competitions can have significant impacts on their beetle hosts. Areas fully colonized with *O. minus* are correlated with reduced developmental success – inhibited egg production, slower larval growth and development, even larval mortality (Barras, 1970; Franklin, 1970). In addition, overall levels of *O. minus* within SPB infestations are negatively correlated with SPB population increase (Bridges, 1985; Lombardero et al., 2000).

In addition to direct effects on SPB biology, *O. minus* directly impacts the economic value of its host tree through causing blue stain in infected tissues (Seifert et al., 1988; Zink and Fengel, 1988). This staining is mostly attributed to the presence of melanin (Seifert, 1993). Though slight reductions in wood toughness, specific gravity and hardness have been noted in association with infection by stain fungi, these are generally considered insignificant (Seifert, 1993; Schirp et al., 2003).

The detrimental effects melanins have on lumber as well as their role in protecting plant pathogens, have made these compounds the target of biological control efforts. In particular, various fungi and bacteria have been tested for their ability to 'decolorize', or break down melanin (Rättö et al., 2001). Four fungal species have shown particularly high abilities to decolorize melanin in one bluestain fungus species, *Aureobasidium pullulans* (Rättö et al., 2001); *Bjerkandera adusta*, *Galactomyces geotrichum*, *Trametes hirsuta*, and *Trametes versicolor*. The focus of these studies was to gain a better understanding of the role of melanin in interactions among SPB fungi through assays examining the effects of melanin on fungal growth. As a second objective, I sought to quantify the possible utility of four fungi as biological control agents of *O. minus*, the SPB associated stain fungus.

2. Materials and Methods

Effects of melanin on growth of mutualist fungi. Estimates of melanin content in *O. minus* were based on the results of previous workers who had found hyphae of dark colored fungi to contain 100–200 μg melanin/mg dry weight (Frederick et al., 1999). The weight of *O. minus* hyphae in a fully colonized 9 cm petri dish of malt extract agar (MEA) was found to be approximately 1 mg. A low dose (0.004 g) of melanin (from *Sepia officinalis*, Sigma-Aldrich Corp., St. Louis, MO, USA) was suspended in 4 ml of 1% hydrogen peroxide and added to 500 ml molten MEA. The resulting plates each contained 200 μg of melanin ("low melanin"). To challenge the fungi with a higher concentration of melanin, I prepared media containing a 0.04 g of melanin (otherwise, as above). These plates contained 2 mg of melanin each. Equal numbers of plates containing 'Peroxide' media (4 ml 1% H_2O_2) or 'Control' media (4 ml sterile distilled water) were also prepared. Comparisons of pH among the various media revealed no significant differences (all approximately equal, 6.4).

To assess the effects of melanin on the growth of the two SPB mycangial fungi, 10 plates of each medium were inoculated with a 0.5 cm disk of MEA colonized with actively growing *O. ranaculosum*, or *Entomocorticium* sp. A. Each Parafilm sealed plate was incubated inverted, at 20°C in a growth chamber (8:16, L:D photoperiod). Growth of each fungus was traced every two days until day 30. A digital planimeter (Numonics Corp., Lansdale, PA) was used to measure cumulative area grown by each fungus (cm^2) at each sampling date. Effects of melanin on fungal growth were analyzed using a repeated measures ANOVA in Statview (SAS Institute 1992) and means separated using Tukey/Kramer at the $p<0.05$ level.

Decolorization of O. minus *produced melanin*

Four melanin decolorizing fungi (Rättö et al., 2001) were obtained from the VTT culture collection (VTT Biotechnology, Finland): *Bjerkandera adusta* (strain VTT-D-99746), *Galactomyces geotrichum* (strain VTT-D-84228), *Trametes hirsute* (strain VTT-D-95443), and *Trametes versicolor* (strain VTT-D-99747).

Forty plates of MEA were inoculated with an isolate of *O. minus* (from SPB collected on the Homochitto National Forest, MS, USA, isolate #OM8MS03, deposited in the Forest and Agricultural Biotechnology Institute Culture Collection, Pretoria, RSA). Each of the cultures grew for 6 days (mycelial growth within 2 cm of the plate edge). A 0.5 cm diameter disk of MEA colonized with one of the four test fungi was place on the uncolonized agar (8 plates for each fungus). Eight control plates were only 'inoculated' with an uncolonized disk of MEA. The Parafilm sealed plates were incubated inverted, in a 20°C growth chamber (8:16, L:D photoperiod). The area of *O. minus* which had been decolorized after 37 days was traced, on the bottom of each plate. Zones of interaction were identified as: 'Uncleared' (*O. minus* hyphae dark brown to black), 'Partly cleared' (hyphae sparse, tan to light brown), 'Mostly cleared' (hyphae very sparse, hyaline to light tan), or 'Completely cleared' (hyphae absent, agar hyaline to white). Completely cleared zones were so due to the complete absence of growth by *O. minus* in the area. In these cases, only the biocontrol fungi had grown. A digital planimeter was used to measure the cumulative area (cm^2) of each type of zone. The effects of these fungi on *O. minus* were expressed as a percentage of the total area of the original *O. minus* colony at day 37, and analyzed using ANOVA (Statview, SAS Institute 1992) and Tukey/Kramer ($p<0.05$) mean separations.

3. Results and Discussion

Effects of melanin on growth of mutualist fungi. Fungal species significantly affected fungal growth ($F_{1,70}=645.7$, $p<0.0001$), so I analyzed the growth of *O. ranaculosum*,

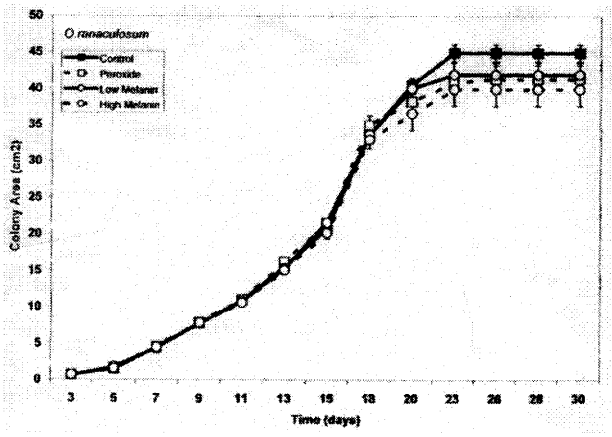

Figure 1. Growth of *Entomorticium* sp. A as affected by melanin at high and low levels, and on control media either with or without hydrogen peroxide. Bars are standard errors about the mean.

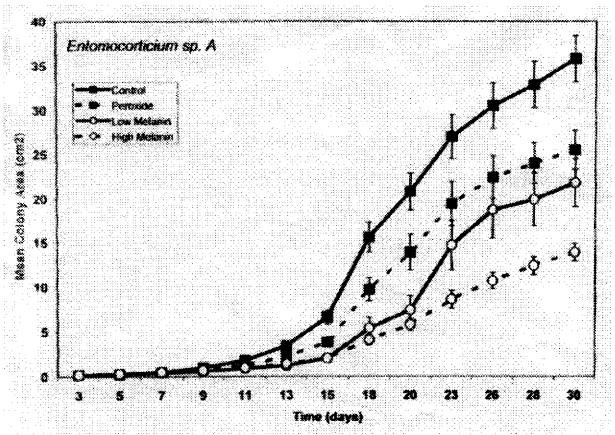

Figure 2. Growth of *Ophiostoma ranaculosum* as affected by melanin at high and low levels, and on control media either with or without hydrogen peroxide. Bars are standard errors about the mean.

and *Entomocorticium* sp. A separately from one another. Time × Treatment interaction significantly affected growth of both *Entomocorticium* sp. A. (F_{36}=13.3, p<0.0001), and *O. ranaculosum* (F_{36}=12.4, p=0.03). Considered alone, growth of *Entomocorticium* sp. A was significantly ($F_{3,35}$=15.6, p<0.0001) affected by the type of medium (Fig. 1). Growth of this fungus was most rapid on the control medium. The addition of hydrogen peroxide to the medium significantly inhibited growth of this fungus, but the addition of melanin at both the low and high levels resulted in the greatest impact. Growth of *Entomocorticium* sp. A on the high melanin medium was less than half what it was on control medium. Towards the end of the assay period, *Entomocorticium* sp. A growth was significantly less on high melanin medium than it was on all other media. Growth of this fungus was significantly inhibited by low levels of melanin at a few measurements dates, though by the end of the assay this difference was no longer significant. Growth of *O. ranaculosum* was not significantly ($F_{1,35}$=1.8, p=0.16) affected by the addition of melanin to MEA (Fig. 2). Only slight inhibition was evident in any of the amended media, and this seems likely to have been due to effects of the added hydrogen peroxide.

Decolorization of O. minus *produced melanin*

Only the control plates (*O. minus* alone) consisted entirely of uncleared zones, being completely colonized by *O. minus*. Conversely, the *O. minus* vs. control plates were the only plates to show no partly cleared zones. While fungal treatment was significant in determining the percentage of the plate which was partly cleared ($F_{3,28}$=4.2, p=0.01), *T. versicolor* plates showed significantly less partly cleared areas (8.9 cm^2) than did *B. adusta* (26.4 cm^2) and *G. geotrichum* plates (23.5 cm^2). Both *B. adusta* vs. *O. minus* and *G. geotrichum* vs. *O. minus* plates, however, showed partly cleared areas. Fungal treatment was again significant in determining the percentage of mostly cleared areas ($F_{3,28}$=4.9, p<0.01), but *T. versicolor* plates showed significantly more mostly cleared areas (84.3 cm^2) than did the other biological control fungi (60.3 to 64.4 cm^2). Few of the fungi tested produced sizable completely cleared zones, and fungal treatment was not significant in determining this parameter ($F_{3,28}$=0.7, p<0.6). All of the biological control fungi exhibited similar growth rates, and all showed the ability to decolorize the melanistic *O. minus* cultures to some extent (data not shown).

The inhibition of *Entomocorticium* sp. A growth in response to naturally occurring levels of melanin suggests that at least part of the competitive edge that *O. minus* enjoys over this important mycangial fungus is due to chemically based, localized antibiosis. In areas where the bluestain fungus is growing abundantly, *Entomocorticium* sp. A growth could be inhibited by 50% or more due to the effects of melanin alone. In contrast, *O. ranaculosum* was not affected by melanin in our assays. This fungus is, however, easily overcome by *O. minus* in head-to-head competition (Klepzig and Wilkens, 1997). Interactions between *O. minus* and *O. ranaculosum* may involve strategies other than the type of antibiosis seen with *Entomocorticium* sp. A. I have observed signs that *O. minus* may even be lysing the cell walls of *O. ranaculosum* as it grows through cultures of this fungus.

The ability of *B. adusta*, *G. geotrichum*, *T. hirsuta*, and *T. versicolor* to decolorize melanin within cultures of *O. minus* indicates promise for their use as biological control agents to counter the staining effects arising from infection with this SPB associated fungus. Of the four fungi I tested, *T. versicolor* was slightly more effective at altering the appearance of *O. minus* cultures than the other three.

However, the difference between the four fungi were usually slight, and often insignificant. The degree to which these fungi might be efficacious in natural substrates (harvested logs, standing/ beetle-attacked trees) remains to be seen and should be tested. In addition, the mechanism behind the inhibition we report here, as well as the role of melanin in the biology of other ophiostomatoid fungi, is worthy of further consideration.

The interactions discussed here may have larger implications to the biology of the beetle by which many of these fungi are vectored. For example, *O. ranaculosum* appears to increase within infestations relative to *Entomocorticium* sp. A, when *O. minus* abundance is high (Hofstetter, 2004). The melanin based inhibition we see here may be one mechanism by which the prevalence of *Entomocorticium* sp. A is reduced while the association between beetles and *O. ranaculosum* is favored. Such changes in the relative abundance of the two mycangial fungi may have consequences to beetle population growth.

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