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Effects of available water on growth and competition of southern pine beetle associated fungi

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Competitive interactions among bark beetle associated fungi are potentially influenced by abiotic factors. Water potential, in particular, undergoes marked changes over the course of beetle colonization of tree hosts. To investigate the impact of water potential on competition among three southern pine beetle associated fungi, *Ophiostoma minus*, *Entomocorticium* sp. A and *Ceratocystiopsis ranaculosus*, we utilized artificial media with water potentials of 0, −5, −10, and −20 MPa. Growth of all three fungi, when grown alone, decreased on media with lower water potentials. Growth rates of all three fungi were likewise reduced in competition experiments. At −5 to −10 MPa, *C. ranaculosus* (a fungus with beneficial effects toward southern pine beetle) was nearly equal in competitive ability to *O. minus* (a fungus with antagonistic effects towards southern pine beetle). This was not true on control media, nor at other water potentials tested. The range of water potentials used in our assays was similar to the range of water potentials we measured in loblolly pines within a southern pine beetle infestation. This study indicates that water potential may alter the outcome of competitive interactions among bark beetle-associated fungi in ways that favour bark beetle success.

INTRODUCTION

Bark beetles (*Coleoptera: Scolytidae*, altern. *Curculionidae: Scolytinae*), interact with their symbiotic fungi in interesting and complex ways (Paine, Raffa & Harrington 1997). The southern pine beetle, *Dendroctonus frontalis* (SPB) is among the most damaging of North American forest insects (Thatcher *et al.* 1980, Drooz 1985, Price *et al.* 1992). All trees within which SPB develop die, and tree death must occur for the beetle to reproduce successfully. The SPB carries three major fungi as it attacks host trees.

Ophiostoma minus, the causal agent of the 'blue stain', often found in the xylem and phloem of SPB infested wood is an ascomycetous fungus carried phoretically on the SPB exoskeleton and by phoretic mites (Rumbold 1931, Bridges & Moser 1983). Although artificial inoculations of southern pines with *O. minus* do cause resinosis and tissue damage, they do not result in mortality of mature trees (e.g. Nelson 1934, Cook & Hain 1986) and the fungus is apparently not necessary for tree death to occur (Bridges 1985). Southern pine beetles and their arthropod associates serve as the only effective means by which this fungus can gain access to new host tissue (Dowding 1969). However, as SPB eggs hatch within the pine phloem, the fungi inoculated by the attacking adult female, begin growing and colonizing the phloem. Higher levels of phloem colonization 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* with SPB infestations are negatively correlated with SPB population increase (Bridges 1985, Lombardero *et al.* 2000). The mechanism of this antagonism remained unclear until recently, but may be partially explained by examining interactions of SPB with its two other significant fungal associates.

Each female SPB possesses a prothoracic mycangium specialized for transporting fungi (Happ, Happ & Barras 1971, Barras & Perry 1972). Within each side of the mycangium, the female SPB is able to maintain a pure culture of either *C. ranaculosus* (a hyaline ascomycete) (Barras & Taylor 1973) or *Entomocorticium* sp. A (aka, SJB122; Barras & Perry 1972, Happ, Happ & Barras 1976, Hsiau 1996).

The SPB mycangial fungi are not highly virulent in their pine hosts. It seems more likely that the proper time to evaluate the role of the mycangial fungi in the SPB life cycle is post-mass attack. Once the tree's

resistance has been overcome, and the eggs hatch, early instar larvae begin feeding, constructing fine, sinuous galleries in the phloem as they go (Payne 1983). Eventually, the larvae enlarge their feeding area into obovate feeding chambers that become lined with luxuriant growth of either of the two mycangial fungi. It appears extremely likely that larval SPB get the majority of their nutrition from the fungal growth within their feeding chambers rather than directly from the phloem itself. The mycangial fungi may, in fact, provide their most substantial benefits to SPB by concentrating dietary N for larvae (Ayres *et al*. 2000). Beetles carrying *Entomocorticium* sp. A within their mycangia are more fecund, heavier, and have higher lipid contents, than those containing *C. ranaculosus*. In turn, beetles containing *C. ranaculosus* tend to be more fit than those whose mycangia contain no fungi (Bridges 1985, Goldhammer, Stephen & Paine 1990, Coppedge, Stephen & Felton 1995).

Ophiostoma minus, *Entomocorticium* sp. A, and *C. ranaculosus*, compete for the rare and ephemeral resource of uncolonized pine phloem (Klepzig & Wilkens 1997). These fungi first engage in primary resource capture, followed by direct interaction, which can lead to defence, and/or secondary resource capture. Competitive 'wins' by mycangial fungi will result in successful development and emergence of fit adults, 'wins' by *O. minus* will likely result in poorly fed, weakened larvae and little to no emergence of adults.

Biotic and abiotic factors may influence, and even alter the nature of the interactions among closely associated organisms (Callaway & Walker 1997), yet the response of host trees to these fungi have typically involved unattacked, relatively moisture rich, trees (e.g. Cook & Hain 1985, Paine & Stephen 1987). Our own investigations into competitive interactions among SPB associated fungi have utilized relatively dry pine bolts or moisture rich artificial media (Klepzig & Wilkens 1997, Klepzig 1998). None of these approaches approximate actual conditions of tissues within which beetles and fungi develop and interact (Coulson 1980). In particular, soon after SPB attack, the phloem tissue the beetles inhabit rapidly dehydrates (Webb & Franklin 1978, Wagner *et al*. 1979). Subsequent changes in water relations occur which seem very likely to affect the ability of SPB associated fungi to grow and compete with one another. No studies have been conducted on the growth of SPB associated fungi in relation to water potential.

Previous work has demonstrated the importance of water potential in determining the colonization success of tree pathogens (Hong & Michailides 1999, Whiting & Rizzo 1999). Our objectives for this study were to quantify the effects of a range of decreasing water potentials on the ability of SPB associated fungi to grow and compete on artificial media. To determine a biologically meaningful range of water potentials to consider, we also measured water potentials of pines at different stages of SPB colonization.

MATERIALS AND METHODS

Range of water potentials in attacked trees

We conducted phloem sampling and water potential measurements in an active SPB infestation on the Homochitto National Forest, Mississippi. We selected 11 trees for sampling which were either unattacked by SPB, recently attacked by SPB, or attacked and colonized by SPB. We collected two phloem discs (1 cm diam) from each tree. We used a HR-33T Dew Point Microvoltmeter with a C-32 sample chamber (Westcor, Logan, UT) to measure the water potential of each disc either immediately upon collection or upon return to the laboratory.

Growth and competition assays: fungi

Based on previous work in which we noted low variability in growth rate and colony morphology among isolates of SPB associated fungi (Klepzig & Wilkens 1997), we selected representative isolates of the three major fungal associates of SPB – *Entomocorticium* sp. A, *Ceratocystiopsis ranaculosus* and *Ophiostoma minus* from beetles emerging from SPB infested loblolly pines on the Bankhead National Forest in Alabama. We isolated the mycangial fungi by surface sterilizing and dissecting mycangial (Barras 1972), and placing them on the surface of plates of benomyl amended malt extract agar (modified from Ross, Fenn & Stephen 1992). We isolated *O. minus*, by crushing SPB adults and streaking them across plates of cycloheximide-streptomycin amended malt extract agar (Ross *et al*. 1992). Subcultures of the fungi we used in this study are deposited in the Forest and Agricultural Biotechnology Institute Culture Collection (University of Pretoria, South Africa).

Growth and competition assays: media

Using previously published methods (Whiting & Rizzo 1999), we prepared fungal growth media of various water potentials. By varying the ratio of KCl and sucrose in malt extract agar (MEA) (15 g l^{-1} agar, 10 g l^{-1} malt extract; Fisher Scientific), we prepared media with osmotic potentials of 0 (unamended MEA), −5 MPa (MEA amended with 8.2 g l^{-1} KCl and 68.1 g l^{-1} sucrose), −10 MPa (MEA amended with 16.6 g l^{-1} KCl and 134.2 g l^{-1} sucrose), and, −20 MPa (MEA amended with 33.5 g l^{-1} KCl and 261 g l^{-1} sucrose). We dispensed 25 ml of each medium into 100 × 15 mm plastic petri dishes and allowed the plates to equilibrate for 24 hours under sterile conditions.

Growth assays

To assess the growth of each of the three SPB associated fungi experiments, we inoculated five plates of each of the water potential media with a 0.5 cm disc

of MEA colonized with actively growing fungi. We sealed each plate with Parafilm and incubated each at 20 °C in a growth chamber (8:16, L:D photoperiod). Beginning on day 3, we traced the growth of each fungus on the bottom of each plate every two days until day 18. At the termination of the experiment, we measured the area covered by growth by each fungus (cm² per 2 d) using a digital planimeter (Numonics, Lansdale, PA). Growth varied substantially across (though not within) fungal species across all media, so we analysed growth of the three species separately using Repeat Measures ANOVA in Statview (SAS Institute 1998).

Competition assays

We conducted a second set of experiments testing the ability of these fungi to compete with one another on those media which had supported fungal growth in the first experiment (Control, −5, −10 and −20). We inoculated 10 plates of each medium with two 0.5 cm discs of MEA colonized with different species of actively growing fungi. The discs were placed opposite each other near the edge of the plate (approx. 7 cm apart). We sealed all plates with Parafilm and incubated them at 20 ° in a growth chamber (8:16, L:D photoperiod). We measured the growth as described above from day 3 until day 18. At the termination of the experiment, we measured the area occupied by each fungus (cm² per 2 d) using a digital planimeter. Growth varied substantially across (though not within) fungal species across all media, so we analysed growth of three species separately, on each type of medium, using Repeat Measures ANOVA in Statview (SAS Institute 1998). In some cases fungi were measured for different amounts of time (e.g. some reached the plate edge sooner than others) resulting, in some cases, in differing numbers of measurements.

RESULTS

Attacked trees

Water potentials of trees in various stages of SPB colonization varied widely, ranging from −5.6 to −12.5 in SPB infested trees, and from −7.3 to −13.2 in healthy/unattacked trees. Although, we found no apparent correlation between attack status and water potential, all the phloem samples we measured fell well within the range of water potentials found in the media we formulated.

Growth assays

The growth of *Ophiostoma minus*, *Ceratocystiopsis ranaculosus* and *Entomocorticium* sp. A were all significantly affected by water potential (in interaction with day) ($P<0.0001$). The growth of *O. minus* was significantly affected by water potential × day interaction ($F_{15,75}=4.37$, $P<0.0001$). Growth of *O. minus* was essentially the same (and sometimes even somewhat more rapid) on −5 medium as it was on the control medium (Fig. 1). Media with water potentials of −10 and −20 reduced growth of *O. minus* substantially (by approx. 32%).

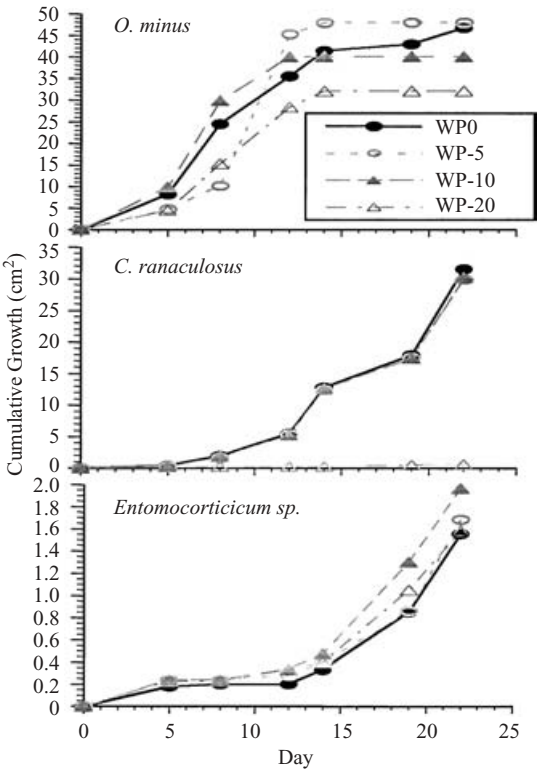

Fig. 1. Individual growth (cm²) of fungal associates (*Ophiostoma minus*, *Ceratocystiopsis ranaculosus*, *Entomocorticium* sp. A) of the southern pine beetle on media of varying water potentials, as measured with a digital planimeter.

The growth of *C. ranaculosus* was significantly affected by water potential × day interaction ($F_{21,105}=426.97$, $P<0.0001$). Growth of *C. ranaculosus* did not differ noticeably or significantly, between control, −5 or −10 media (Fig. 1). However, *C. ranaculosus* growth was almost completely inhibited (approx. 94% reduction) by the −20 medium by experiment's end.

The growth of *Entomocorticium* sp. A was significantly affected by water potential × day interaction ($F_{24,112}=3.54$, $P<0.0001$). Growth of *Entomocorticium* sp. A was essentially the same on control, −5, −10, and −20 media throughout the experiment (Fig. 1). However when cultures were allowed to continue growing up to 37 days post-inoculation, the −20 medium substantially reduced *Entomocorticium* sp. A growth (by approx. 54%, as compared to the control).

Competition assays

The three fungal species grew at varying rates on all media tested, and media type significantly affected competitive interactions. Growth of *Ophiostoma minus* and *Ceratocystiopsis ranaculosus* was significantly

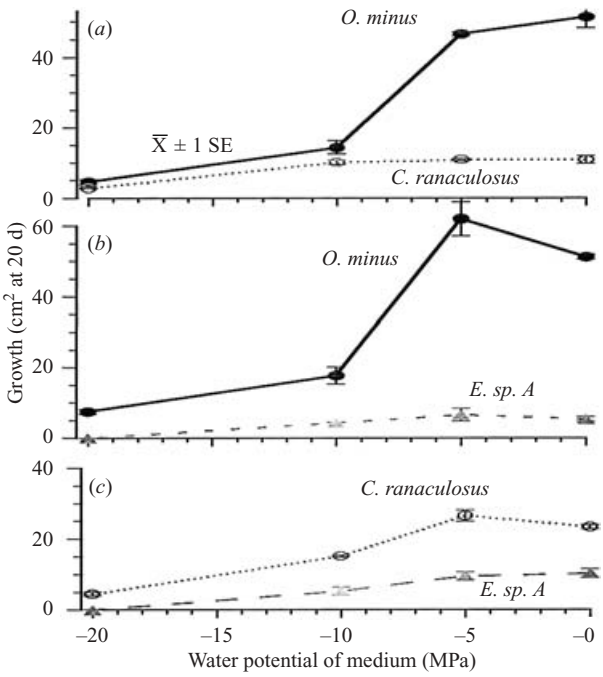

Fig. 2. Competition of fungal associates of the southern pine beetle (*Ophiostoma minus*, *Ceratocystiopsis ranaculosus*, *Entomocorticium* sp. A) on media of varying water potential. Area colonized (cm^2, as measured with a digital planimeter) by each fungus in one on one competitive assays: (*a*) *O. minus* vs. *C. ranaculosus*; (*b*) *O. minus* vs. *Entomocorticium* sp. A; and (*c*) *C. ranaculosus* vs. *Entomocorticium* sp. A.

affected by fungus × day interaction on 0 medium ($F_{4,40}=61.07$, $P<0.0001$), −5 medium ($F_{4,40}=41.08$, $P<0.0001$), −10 medium ($F_{5,90}=5.16$, $P<0.0003$) and −20 medium ($F_{5,90}=11.94$, $P<0.0001$). Growth of *O. minus* and *Entomocorticium* sp. A was significantly affected by fungus × day interaction on 0 medium ($F_{5,25}=296.24$, $P<0.0001$), −5 medium ($F_{5,85}=126.19$, $P<0.0001$), −10 medium ($F_{5,90}=26.29$, $P<0.0001$) and −20 medium ($F_{5,90}=96.02$, $P<0.0001$). Growth of *C. ranaculosus* and *Entomocorticium* sp. A was significantly affected by fungus × day interaction on 0 medium ($F_{5,90}=33.17$, $P<0.0001$), −5 medium ($F_{5,90}=102.76$, $P<0.0001$), −10 medium ($F_{5,100}=91.22$, $P<0.0001$) and −20 medium ($F_{5,90}=10.25$, $P<0.0001$). In most cases, the fungi had grown far enough to come into contact by the end of the assay. Even in the cases where fungi did not actually come into contact, they were competing *via* primary substrate capture (Rayner & Webber 1984) and/or possible short range antibiosis, and the outcome of such struggles for available substrate would have serious implications for SPB larvae.

Ophiostoma minus *vs.* Entomocorticium *sp. A*

As we observed in the growth experiment, *Ophiostoma minus* grew more extensively on the −5 medium than on the control medium, allowing it to capture more substrate in competition with *Entomocorticium* sp. A (Fig. 2). Although growth of *O. minus* was greatly reduced in −10 and −20 media, it was still readily able to outcompete *Entomocorticium* sp. A in these media as well. By 22 d of growth, *Entomocorticium* sp. A had captured only 10% of the amount of substrate captured by *O. minus*.

Ophiostoma minus *vs.* Ceratocystiopsis ranaculosus

Ophiostoma minus easily outcompeted *Ceratocystiopsis ranaculosus* for substrate on control and −5 media (Fig. 2). However, at lower water potentials (−10 and −20 media) the two fungi captured approximately the same amount of substrate.

Ceratocystiopsis ranaculosus *vs.* Entomocorticium *sp. A*

Ceratocystiopsis ranaculosus easily outcompeted *Entomocorticium* sp. A on all media, though progressively less so as water potentials decreased from control (0) to −10 (Fig. 2). On the lowest water potential media (−20), however, *Entomocorticium* sp. A growth was so reduced that, as was the case in its competition with *Ophiostoma minus*, it only captured 10% of the amount of substrate captured by *O. minus*.

DISCUSSION

Bark beetle-associated fungi are faced with highly variable environments within which they must obtain nutrients, grow, reproduce and become available for dispersal. Among the factors which may affect the relative success of these fungi are host nutrients, defensive chemicals, temperature and moisture (Klepzig & Wilkens 1997). Our experiments determined that the growth of the three major fungal associates of SPB is strongly affected by decreasing water potential. Although pines under attack by SPB have drier phloem than unattacked trees (Webb & Franklin 1978), our sample sizes may not have been large enough to detect significant differences in water potentials based on degree of SPB attack. However, Phloem moisture decreases rapidly and severely soon after successful SPB attack and reaches its lowest levels during larval development (Wagner *et al.* 1979, Coulson 1980). Within the range of water potentials found within the trees we sampled, competitive interactions remained largely similar to those found previously (Klepzig & Wilkens 1997, Klepzig 1998). At the lower water potentials, fungal growth was reduced to the extent that one of the SPB mycangial fungi (*Ceratocystiopsis ranaculosus*) could equally compete with *Ophiostoma minus*. It should be noted, however, that *O. minus* grew faster than *C. ranaculosus* when the two were grown separately on the −10 medium. When these fungi were grown on the same plate, there was more of a reduction of growth in *Ophiostoma* than in *C. ranaculosus*, but *Ophiostoma* grew significantly faster than either mycangial fungus when they were grown together at

−10 MPa, and faster than *Entomocorticium* sp. A at −20 MPa. While the media contained increasing amounts of sucrose with increasingly negative water potentials, any potentially stimulatory effects of this carbon source were apparently outweighed by the effects of the additional KCl and altered water relations. These media have previously been used successfully to test the effects of water potential on fungal growth (Whiting & Rizzo 1999).

These demonstrated impacts of one abiotic factor on fungal interactions may help explain the success of mycangial fungi in SPB infested hosts, despite the nearly overwhelming competitive ability exhibited by *O. minus* in most cases (Klepzig & Wilkens 1997). Our future research will seek to determine the impact of other host and microclimate related variables on the growth and competitive interactions of these important associates of the most destructive forest insect in the southern USA.

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