Nitrogen Concentration in Mountain Pine Beetle Larvae Reflects Nitrogen Status of the Tree Host and Two Fungal Associates

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ABSTRACT Individual lodgepole pines (Pinus contorta) were fertilized with urea at nitrogen (N) inputs equivalent to 0, 315, or 630 kg/ha. Four months after application of the fertilizer, inner bark tissue N concentrations were significantly higher in the trees that had received the low dose (315 kg/ha) fertilization treatment than in the control trees; trees that had received the high-dose treatment (630 kg/ha) were intermediate and not significantly different from either of the other treatments. There was a significant positive correlation between N concentration in inner bark tissue and larval mountain pine beetle, Dendroctonus ponderosae (Coleoptera: Curculionidae, Scolytinae). In vitro studies on synthetic growth media examined effects of temperature and N concentration on N concentration of two common fungal associates of the mountain pine beetle (Ophiostoma clavigerum and Ophiostoma montium). Increasing N concentration in growth media significantly increased fungal N concentrations in both O. clavigerum and O. montium. Furthermore, N concentration was consistently higher in O. clavigerum than in O. montium. Neither species had sufficient growth at 30°C, nor did O. clavigerum at 15°C, to test N concentration. However, for O. montium, increasing temperatures decreased fungal N concentrations. There was no correlation between N concentration of O. clavigerum and growth temperature. Potential impacts of ingestion of the fungal species by developing mountain pine beetle larvae-infesting trees under various environmental conditions such as increasing temperatures are discussed.

KEY WORDS Dendroctonus, Ophiostoma, nitrogen fertilization, temperature, symbiotic fungi

Mountain pine beetle, Dendroctonus ponderosae Hopkins (Coleoptera: Curculionidae, Scolytinae), is one of the most serious insect pests of pines in western North America. The beetle is frequently associated with lodgepole pine (Pinus contorta Douglas), but is also capable of attacking and killing many other native pine species within its range, including ponderosa pine (Pinus ponderosa Lawson) and western white pine (Pinus monticola Douglas) (Furniss and Carolin 1977, Furniss and Johnson 2002). Outbreaks of the beetle can persist for years, during which time the beetle can cause extensive tree mortality (Furniss and Carolin 1977, Amman et al. 1990, Raffa et al. 2008).

Because of its wide distribution (from northern Mexico, throughout the western United States, and into southwestern Canada), local populations of mountain pine beetle may encounter very different environmental conditions and host populations. In addition, factors that affect levels of tree stress and/or development of bark beetles could also affect beetle outbreaks. Three such factors are temperature, fungal associates, and nitrogen (N) status.

Fertilization with N is a silvicultural activity that can be used to attempt to improve stand/tree vigor of coniferous as well as deciduous trees. Fertilization is typically used early in stand establishment, a time during which conifers would not typically be attractive to most tree-killing bark beetles. However, more mature trees/stands can also be fertilized. Fertilization can change the nutritional value of foliage and has been demonstrated to impact the development of some forest defoliators (e.g., western spruce budworm, Choristoneura occidentalis Freeman, on grand fir, Abies grandis Douglas [Mason et al. 1992] and large aspen tortrix, Choristoneura conflictana [Walker] [Leidoptera: Tortricidae] on quaking aspen, Populus tremuloides Michx. [Bryant et al. 1987]).

N fertilization has been demonstrated to increase growth rates in mature stands of lodgepole pine, but did not prevent attacks by mountain pine beetle. Attacks occurred until a threshold of tree growth efficiency was exceeded (Waring and Pitman 1985). In another bark beetle system (southern pine beetle, Dendroctonus frontalis Zimmermann attacking loblolly pine, Pinus taeda L.), higher N content within the host resulted in larger adult beetles (Ayres et al. 2000). A model developed by these authors suggests that mycangial fungi that colonize from the sites of beetle attack benefit the beetles by concentrating...
plant N into fungal biomass that may serve as a source of dietary N for the developing larvae.

Two fungi commonly associated with the mountain pine beetle are *Ophiostoma clavigerum* (Robinson-Jeffrey & Davidson) Harrington and *Ophiostoma montium* (Rumbold) von Arx (Whitney and Farris 1970, Six 2003). Earlier work on bark beetle-fungal-tree interactions examined the direct effects of inoculating trees with bark beetle-associated fungi (i.e., Raffa and Berryman 1983, Cook and Hain 1986; Lietzelt et al. 1991). Both *O. clavigerum* and *O. montium* are capable of staining the sapwood of infected trees, and *O. clavigerum* was demonstrated to be capable of causing mortality of mature trees (Yamaoka et al. 1995). However, another role that these fungal associates may play is to provide a nutritional benefit to the developing bark beetle larvae (Ayres et al. 2000, Bentz and Six 2006). Of the two fungi, *O. clavigerum* appears to provide greater benefit to *D. ponderosae*, as progeny production was higher and adult emergence greater for beetles reared in bolts of lodgepole pine inoculated with *O. clavigerum* versus bolts inoculated with *O. montium* (Six and Paine 1998).

Previous studies examining the growth of various species of *Ophiostoma* under different temperature conditions reported that several species can be distinguished from one another based upon growth and survival at differing temperatures (Brasier and Stephens 1993, Solheim and Krokene 1998, Kim et al. 2003). In addition, the relative prevalence of the two fungi within mountain pine beetle populations is temperature dependent, with *O. montium* increasing in prevalence above 25°C (Six and Bentz 2007). However, *O. clavigerum* was demonstrated to grow for a longer period of time under limited oxygen conditions than did *O. montium* (Solheim and Krokene 1998).

Three hypotheses relating to N and temperature environment of the mountain pine beetle-fungus-host interaction were tested in the current study, as follows: 1) that N fertilization would increase the N concentration in the inner bark tissue of lodgepole pine, which would consequently elevate the N concentration of mountain pine beetle larvae that developed within that tissue; 2) that increasing the N content of media on which *O. clavigerum* and/or *O. montium* were grown would increase the N content within the fungi; and 3) that changing the temperature at which the fungi were grown would alter the N concentration within the fungi as a result of potential changes in fungal growth related to temperature.

**Materials and Methods**

**Field Locations, Enhancement of Tree N, and Tissue Sampling.** An ongoing, active mountain pine beetle infestation was identified in Nez Perce County, Idaho, ≈24 km southeast of Lewiston during early 2004. In April, 30 apparently vigorous lodgepole pine trees along the periphery of the outbreak that had no signs of bark beetle attack were randomly assigned to receive one of three N treatments. Ten trees received no additional N (control trees), 10 were individually treated with urea to receive the equivalent of 315 kg/ha N, and 10 received the equivalent of 630 kg/ha. Amounts of N to be applied were calculated based upon the green crown area of each tree. Urea was applied on 21 May 2004 to an area of ground corresponding to twice the diameter of the crown. A 5 × 5-cm sample of inner bark tissue was removed from each tree on 23 May 2004 and placed on dry ice. A second inner bark sample was removed from each tree on 2 July 2004 and placed on dry ice. Both samples were subsequently analyzed for N content.

A mountain pine beetle lure (PheroTech International, Delta, British Columbia, Canada) was placed on thebole of the tree at ≈1.5 m on 5 July 2004. Attacks were noted for each tree, and larvae were collected in late June of the following year, 2005. Individual larvae were dried at 70°C, ground, and analyzed for N concentration.

Fungi for the study were obtained from a second infestation of mountain pine beetles attacking lodgepole pines at Lookout Pass in Shoshone County, Idaho, ≈55 km east of Coeur d’Alene, Idaho. Fungi were isolated from the mycangia of mountain pine beetle adults excised from infested trees on 22 June 2006. Beetles were decapitated, and the heads were incubated at 20°C on *Ophiostoma*-selective media containing 1.5% agar, 1.5% malt extract, 100 ppm each of cycloheximide and streptomycin, and 250 ppm of penicillin G (Harrington 1981). Single hyphal tips of developing fungi were plated on nonselective media (as above, but without the stock solutions) and grown at 20°C to obtain genetically uniform isolates. Isolates tentatively identified to species based upon morphology were confirmed as *O. clavigerum* and *O. montium* using DNA sequence analysis of the rDNA insulin-transferrin-sodium selenite region (Kim et al. 2003) before use in N concentration experiments.

**Laboratory Tests With Ophiostoma.** For the first laboratory experiment, growth of the two species of *Ophiostoma* was compared on agar media containing different concentrations of organic N (2.05, 2.19, 4.01, and 4.60% wet weight) at a constant temperature of 20°C. Concentrations of N in the media were achieved by adjusting the N source (Difco Bacto peptone) in a basal medium. The basal medium contained 1.4% Difco peptone agar, 3.5% dextrose and the vitamin thiamine HCl (0.1 ppm), pyridoxine HCl (0.075 ppm), and biotin (5.0 ppb). Vitamins were added to the media after autoclaving as a filter-sterilized 100× stock solution in 50% EtOH (Zambino and Harrington 1990). After ≈1-wk acclimation on the media, four 2 × 2-mm pieces of fungal cultures from the edge of the upper surface of the colony were transferred to 10-mm-pore nylon membranes on plates of the same nutritional media. The membranes allowed the fungi to absorb nutrients, but were not penetrated by the fungi. New fungal tissue from beyond the inoculum was scraped from the nylon membranes after ≈1 wk, placed in glass vials, and dried for 24 h at 50°C before being analyzed for N concentration. Fungus-free media was also dried and analyzed for N concentration. Only the media with 2.19% N was used for the second laboratory experiment in which the two species of *Ophiostoma* were grown at four temperatures (15°C, 20°C, 25°C, and 30°C) before being scraped, dried, and
analyzed for N concentration along with the appropriate noncolonized media.

**N Analysis.** The inner bark samples were split along the cambial layer and placed flat in a drying oven for 24 h at a temperature of 70°C. The first 3 mm of inner bark tissue from along each side of the cambium was scraped and ground into a fine powder. Samples were packed, and N concentration was determined using a mass spectrometer (Stable Isotope Core Laboratory, Washington State University, Pullman, WA). Beetle larvae excised from trees were dried, ground, and analyzed for N concentration in the same manner as the fungal samples and control (noninoculated) media. Tissue was converted to N₂ using an elemental analyzer (ECS4010, Costech Analytical, Valencia, CA) in a 3-m gas chromatography column equipped with a continuous-flow mass spectrometer (Brenna et al. 1997, Qi et al. 2003). Samples were normalized using two internal standards (acetanilide and keratin).

**Statistical Analyses.** The size of trees (crown radius) and the pretreatment N concentration of the inner bark tissue before and after the fertilization treatment was calculated. The relationship between fertilization treatment and posttreatment N concentration was examined using a protected least significant difference test with an analysis of variance procedure on the differences between pre- and posttreatment inner bark N. Correlation analysis was used to examine the relationship between tree inner bark N concentration and the N concentration of mountain pine beetle larvae collected from those same trees. Because no upper level of N concentration for these fungal species is established, the relationship between the dry weight N concentration of the growth media and the subsequent concentration of N in the *O. clavigerum* and *O. montium* grown on those media was examined using linear regression analysis. Because fungi did not grow enough to sample N concentration at all of the temperatures tested, the relationship between temperature and N concentration of the *O. clavigerum* and *O. montium* grown on media containing 2.05% N was examined using correlation analysis. The concentration of N contained in *O. clavigerum* and *O. montium* was compared using a paired Student’s *t* test, in which pairing was conducted on treatment similarity. All analyses were conducted using the STATISTIX software package (Analytical Software 1998).

**Results and Discussion**

**Field Results: Tree and Beetle Parameters.** There were no significant differences in the mean crown radius (\(F = 0.89; \text{df} = 2, 57; [P > F] = 0.4149\) or the prefertilization inner bark N concentration (\(F = 1.15;\)
df = 2, 47; \( [P > F] = 0.3253 \) among study trees in the three treatments (Table 1). Fertilization did result in a significant increase in the N concentration of inner bark tissue \( (F = 3.30; \text{df} = 2, 26; [P > F] = 0.0527) \) for trees that had received the low-dose treatment, but apparent increases in the trees that had received the high-dose treatment were not significant (Table 1). The fertilization rates used in this study were selected to result in a change in tree chemistry, not as recommended rates for silvicultural applications. However, other studies that used a fertilization regime similar to our low-rate treatment have demonstrated an increase in phloem N after fertilization (Warren et al. 1999).

Although the sample size was limited to eight pairs of tree and beetle data, there was a significant and positive correlation \( (r = 0.6727; [P > r] = 0.0675) \) between the N concentration in tree inner bark tissue and the subsequent N concentration of mountain pine beetle larvae that developed within that tree tissue (Fig. 1). Similarly, Ayres et al. (2000) reported a positive relationship between tree and beetle N concentrations in the loblolly pine-southern pine beetle system. Those authors suggested that fungal symbionts may concentrate N within the phloem tissue. We designed our subsequent laboratory experiments to examine that possibility.

Fig. 2. Relationship between the concentration of N (%) in growth media and the subsequent concentration of N within \textit{O. clavigerum} and \textit{O. montium}.
Laboratory Results: Media and Temperature Change *Ophiostoma*. The first laboratory experiment examined the impact of changing N concentration on the concentration of N in *O. clavigerum* and *O. montium*. There was a significant and positive relationship between the concentration of N in the growth media and the subsequent concentration of N in both *O. clavigerum* (R² = 0.8116; F = 73.36; df = 1, 38; [P > \(F\)] = 0.0001) and *O. montium* (R² = 0.1801; F ≈ 8.35; df = 1, 38; [P > \(F\)] = 0.0063) (Fig. 2). This positive relationship between growth media and fungal N concentration was stronger for *O. clavigerum* and, while significant, it was a weaker relationship for *O. montium*. However, it should be noted that we only tested a single isolate for each of the fungi and that the dry weight N concentrations in our growth media were higher than the dry weight N concentrations in our experimental trees.

If the relationship of increasing N concentration in the bark beetle-associated *Ophiostoma* in response to increased N concentrations in tree tissue holds across several geographic areas, it could have implications for the maintenance of these symbiotic fungi by local populations of mountain pine beetle. Both *O. clavigerum* and *O. montium* can be carried either as mycangial or phoretic fungi on individual mountain pine beetles (Six 2003), and temperature can determine the prevalence of the fungal species carried by mountain pine beetle populations (Six and Bentz 2007).

Our second laboratory experiment examined the impact of changing temperature on the concentration of N in *O. clavigerum* and *O. montium*. We attempted to grow the fungi on one growth medium containing 2.05% N at four different temperatures (15°C, 20°C, 25°C, and 39°C). We were not successful growing *O. clavigerum* at the lowest (15°C) and highest (39°C) temperatures, and there was no statistically significant difference (\(r = -0.0299, [P > r] = 0.8922\)) in the concentration of N in the *O. clavigerum* grown at 20°C or 25°C (Table 2). We were also not able to grow enough *O. montium* for N analysis at 30°C, but there was a significant negative correlation between temperature and N concentration in the *O. montium* (\(r = -0.5467; [P > r] = 0.0069\)) (Table 2). A difference in prevalence of the fungi as determined by temperature has previously been reported (Six and Bentz 2007), and these authors speculated that the difference in optimal growth temperatures could facilitate the stable coexistence of the two fungal symbionts. However, the fungus that these authors reported to be most prevalent at the higher temperatures (*O. montium*) had concentrated less N within its hypae when grown at the higher temperatures during the current study. The fact that the fungus is able to survive and continue to concentrate N for potential nutritional purposes even if it is at reduced concentrations compared with lower temperatures should benefit the developing beetle larvae. The relationship also suggests that under changing climate conditions, the prevalence of *O. montium* may be favored in some geographic locations.

The N concentration in *O. clavigerum* was consistently higher (\(t = 4.35; df = 5; [P > t] = 0.0074\)) than the N concentration in *O. montium* when comparing the two species in the laboratory experiments (Table 3). If mountain pine beetle larvae do receive nutritional benefits in the form of N from feeding on the fungi during development, we would expect selection pressure to maintain the fungus that provides a higher level of the nutritional compound, and indeed the species that is more adapted to for mycangial dissemination is *O. clavigerum* versus *O. montium*, which is isolated more frequently from the exoskeleton of the beetle (Six 2003).

**Implications for Bark Beetle-Fungal Associations.** Increasing the N available to pines increases the inner bark concentration of N within those trees. Trees that are successfully attacked by mountain pine beetle have the fungal symbionts of the beetle introduced into this inner bark tissue. These fungi may act to concentrate available N (Ayres et al. 2000) in such a manner as to provide an increased nutritional benefit to the developing larvae. This increased N availability is expressed in the beetles and results in increased progeny production and adult emergence (Six and Paine 1998). Of the two fungi tested, *O. clavigerum* consistently had a higher concentration of N than did *O. montium*. The higher N concentration could provide the explanation for why *O. clavigerum*-inoculated bolts produced more progeny than did bolts inoculated with *O. montium* (Six and Paine 1998). *O. clavigerum* has also been demonstrated to be pathogenic to mature lodgepole pines (Yamaoka et al. 1995). When coupled with the pathogenicity of the fungus, *O. clavigerum*’s tendency to have a higher N concentration would suggest that there should be selection pressure for mountain pine beetle to maintain this species as a symbiont. However, *O. montium* has been reported to grow better at higher temperatures than

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**Table 2.** N concentration (%) of two mountain pine beetle-associated fungi (*O. clavigerum* and *O. montium*) grown on media containing 2.05% N at different constant temperatures

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>N content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>O. clavigerum</em></td>
</tr>
<tr>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>

* Number of samples of a total of 15 plates inoculated for each temperature and fungus combination.

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**Table 3.** Paired comparison of the N concentration (%) within *O. clavigerum* and *O. montium* grown under the same conditions during the laboratory experiments

<table>
<thead>
<tr>
<th>Media (%) N</th>
<th>Temperature (°C)</th>
<th><em>O. clavigerum</em></th>
<th><em>O. montium</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>2.05</td>
<td>5.50 ± 0.06</td>
<td>5.46 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>2.05</td>
<td>5.78 ± 0.06</td>
<td>4.55 ± 0.20</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>2.05</td>
<td>4.41 ± 0.12</td>
<td>3.75 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>2.19</td>
<td>5.78 ± 0.07</td>
<td>5.35 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>4.01</td>
<td>6.84 ± 0.15</td>
<td>5.92 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>4.60</td>
<td>6.88 ± 0.09</td>
<td>5.27 ± 0.13</td>
</tr>
</tbody>
</table>
O. clavigerum (Six and Bentz 2007), and in our current work, O. montium also grew better than O. clavigerum at the lowest temperature tested. Under a scenario of increasing temperatures, there may be a change in prevalence of the fungal symbionts that favors O. montium in some geographic locations. Mountain pine beetle has a large geographic range, and additional geographic isolates of both fungi should be examined to test the robustness of our results.

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