

USING NON-WOVEN BANDS IMPREGNATED WITH THE
ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE*
(METCHNIKOFF) FOR BIOLOGICAL CONTROL OF THE ASIAN
LONGHORNED BEETLE, *ANOPLOPHORA GLABRIPENNIS* (MOTSCHULSKY)

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ABSTRACT

The Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky), is a tree-boring cerambycid that has invaded northeastern North America, where it poses a serious threat to urban and forest trees. The use of non-woven fiber bands impregnated with *Metarhizium anisopliae* F 52 (fungal bands) for control of adult *A. glabripennis* is in development. Studies were conducted to evaluate whether conidia from fungal bands disperse in the environment and whether adult *A. glabripennis* can acquire infection through exposure to environments contaminated with conidia. In the field, fungal bands containing *M. anisopliae* were hung at a height of 3 m on 15 trees. Bark samples were taken 10, 20 and 30 cm above the bands and 10, 30, and 60 cm below the bands 2, 5, and 9 days after band placement to quantify conidial densities. There were significantly more conidia in samples taken below bands (average = 4.7 ± 1.1 conidia/sample) compared with samples taken above bands (average = 1.0 ± 0.3 conidia/sample). A significant positive correlation was found between rainfall and the occurrence of conidia on any of the bark samples. A laboratory study was conducted to determine whether conidia from *Metarhizium anisopliae* F 52 fungal bands could be spread to other parts of the environment, and whether *A. glabripennis* could become infected by conidia dispersed from fungal bands. One or five adult *A. glabripennis* were used to contaminate artificial environments with conidia after being exposed to fungal bands. All adults subsequently exposed to contaminated environments were killed by fungal infection. Furthermore, beetles exposed to environments that had been contaminated by five beetles died in significantly fewer days (16.2 ± 1.1 d) compared with environments contaminated by one beetle (27.9 ± 2.5 d). Beetles in both density treatments died in significantly fewer days than beetles exposed to environments without *M. anisopliae* conidia (114.9 ± 16.2 d). These results indicate that

environmental contamination with conidia from fungal bands can occur, and that adult *A. glabripennis* can acquire infection from a contaminated environment. Lastly, in a laboratory bioassay, beetles were immobilized on *M. anisopliae* F 52 fungal bands for 30 seconds to quantify the median lethal dose, measured in number of viable conidia per square centimeter of a fungal band, for *M. anisopliae* F 52 fungal bands against adult *A. glabripennis*. Correlations were found between dose and mortality and between dose and beetle longevity for beetles presumed to have died as a result of fungal infection. A median lethal dose of 6.80×10^6 conidia/cm² was calculated. These results suggest that *M. anisopliae* F 52 fungal bands, which have been shown to retain densities greater than 1×10^7 viable conidia/cm² for at least 112 days (in New York City), would only need to be hung on trees once per season to enable exposure to the majority of adult *A. glabripennis* in US infestations each year.

BIOGRAPHICAL SKETCH

The author was born and raised in Rochester, New York. He attended the State University of New York, College of Environmental Science and Forestry (SUNY ESF) in Syracuse where he studied environmental biology. Upon completion of his Bachelor's Degree in 2004, the author began a Masters of Science degree program in the Entomology Department at Cornell University. In the summer of 2005 he began working with Dr. Ann Hajek, professor of insect pathology.

to my parents

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CHAPTER 1

ENVIRONMENTAL CONTAMINATION WITH *METARHIZIUM* *ANISOPLIAE* FROM FUNGAL BANDS FOR CONTROL OF THE ASIAN LONGHORNED BEETLE, *ANOPLOPHORA GLABRIPENNIS* (COLEOPTERA: CERAMBYCIDAE)

INTRODUCTION

The Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky), was recently introduced to the United States (Haack 2006), where it poses a serious economic and ecological threat to urban and forest trees (Nowak et al. 2001). Eradication efforts in the U.S. involve cutting and chipping infested trees that are detected, but the biology of *A. glabripennis* makes detection and eradication of this pest difficult. Mature larvae tunnel into the sapwood and the long-lived adults tend to stay high in the tree canopy (Haack et al. 1997). While susceptible trees are often treated with trunk and soil injections of imidacloprid (USDA-APHIS 2007), this systemic insecticide exhibits strong antifeedant effects and is not distributed evenly within trees (Poland et al. 2001, Poland et al. 2006). Therefore, it is questionable whether beetles receive lethal doses of imidacloprid.

Following emergence, many tree-boring cerambycids walk on trees and are reluctant to fly during what is referred to as a prematuration or preoviposition period (Iba 1993). For *A. glabripennis*, this period can last more than one week (Keena 2002) or possibly for as long as 11-16 days (Smith et al. 2002). Based on this cerambycid trait, Higuchi et al. (1997) developed a 50 x 500 mm non-woven fiber band product (Biolisa Kamikiri; Nitto Denko, Japan) containing the entomopathogenic fungus

Beauveria brongniartii (Sacc.) Petch that can be wrapped around the trunk or main branches of trees in orchards for control of tree-boring Cerambycidae in Japan. In addition to orchard pests, fungal bands have been investigated for the control of several other species of wood-boring cerambycids (e.g. Shibata et al. 1991, Higuchi et al. 1997, Tsutsumi 1998, Shimazu 2004). These non-woven fiber bands are impregnated with a fungal pathogen, that grows throughout the material and sporulates on the surface, so that adult beetles walking across the bands can acquire infection. Under favorable conditions, fungal bands retain infectivity in the field for over 40 days (Higuchi et al. 1997, Matsuura et al. 1997). In contrast, conidial viability is significantly reduced 10 days after spraying fungal spores onto tree trunks (Dubois et al. 2004a). Bands can be stored at low temperatures for long periods of time (>400 days at 5°C), and conidial viability of dried band cultures can be restored by adding water (Higuchi et al. 1997).

The efficacy of fungal bands against *A. glabripennis* has been investigated for use as an alternative control strategy in the US. Optimally, beetles would cross fungal bands shortly after emergence, minimizing oviposition and feeding. It is unknown how often *A. glabripennis* would contact bands in the field. In the lab, Dubois (2003) tested strains of *B. brongniartii*, *B. bassiana* (Balsamo) Vuillemin, and *Metarhizium anisopliae* (Metchnikoff) Sorokin, several of which exhibited strong virulence against *A. glabripennis*. In addition, field studies have been conducted in China to compare the effects of fungal bands against *A. glabripennis* using several fungal species and isolates. Significant reductions in adult *A. glabripennis* longevity, in fungal-treated compared with control plots, were observed for most strains tested (Dubois et al. 2004a, Dubois et al. 2004b, Hajek et al. 2006). A sublethal effect was also observed in the field, as fungal band treatments resulted in reduced oviposition (Dubois et al. 2004a, Dubois et al. 2004b, Hajek et al. 2006). Oviposition reduction as a sublethal

effect of fungal bands has been shown in the past for other tree-boring cerambycids (Ninomiya and Higuchi 1998, Tsutsumi 1998), and contributes to the efficacy of this method of control.

It has been shown that beetles exposed to fungal bands can pass conidia to healthy beetles during mating (Tsutsumi and Yamanaka 1995, Hajek, unpubl. data). The objective of the current study was to determine whether *A. glabripennis* adults can acquire fungal infection without directly contacting fungal bands or other beetles. Of the fungal isolates that have demonstrated virulence against *A. glabripennis* adults in lab and field settings (Hajek et al. 2007), *M. anisopliae* F52 (ARSEF 7711) was chosen for the current study because this strain is produced commercially (Novozymes Biologicals Inc., Salem, VA; formerly Earth Biosciences Inc., New Haven, CT) and is registered with the US Environmental Protection Agency (EPA). A field study was conducted to investigate the degree to which conidia can move from a fungal band to adjacent tree bark, and survive on the bark, which would increase the likelihood of infection for beetles not walking on bands. In addition, a laboratory study was conducted to determine whether beetles could acquire fungal infection from an environment contaminated by other *A. glabripennis* adults.

METHODS

Fungal band production

Metarhizium anisopliae F 52 (ARSEF 7711) was originally isolated in 1971 from a mycosed cadaver of *Cydia pomonella* L. in Austria, and is maintained at the US Department of Agriculture (USDA) Agricultural Research Service Entomopathogenic Fungal (ARSEF) culture collection (Ithaca, NY) . Fungal bands were made with 238.6

g/m² Soft & Bright® polyester quilt batting (The Warm Company, Lynnwood, WA), using sheets measuring approximately 30.5 x 45.7 cm. Slits were partially cut in each sheet at 5 cm intervals to yield 6 bands measuring 5 x 45.7 cm that could be separated. The sheets were attached with pins to racks measuring 30.48 x 45.72 cm made of hardware cloth. These racks, with the sheets attached, were autoclaved prior to fungal inoculation. *M. anisopliae* F 52 was cultured on Sabouraud dextrose agar with yeast (SDAY) in 100 ml Petri dishes for 10 to 14 days at 28°C. Scraping with a sterile swab, conidia from fungal cultures were showered onto the surface of 2 l of Sabouraud dextrose with yeast (SDY) in a 2.8 l Fernbach flask, and incubated for 3 days on a rotary shaker at 175 rpm and 28°C.

Seven liters of SDAY were autoclaved and poured into a pan measuring approximately 50.8 x 45.7 x 15.2 cm (length x width x depth) under a fume hood. Once the temperature of the SDAY had decreased to approximately 45-50°C, 2 l of the liquid culture was added to the 7 l of SDAY and mixed with a sterile spatula. Individual racks with fabric sheets attached were dipped into the SDAY cultures. Once the quilt batting was saturated, excess inoculum was wiped from the surface of the batting. The racks were then transferred immediately to a metal rack (175.9 x 51.4 x 66 cm, 7.6 cm shelf spacing; Big Tray, Inc., San Francisco, CA), that was covered with a large plastic bag. The top of the rack was covered, shelves with inoculated sheets attached were added starting at the top (2 sheets per shelf), and the rack was sequentially covered by the plastic bag. Each batch of inoculum made approximately 12 sheets of fungal bands, and the entire rack had 19 shelves for a total of 38 sheets (= 228 bands).

Once a rack was full, the plastic bags were taped shut and racks were incubated in the dark at high relative humidity (presumed to be at or near 100% RH due to the saturation of the bands) and 27°C for 10-14 days. Following the incubation period, the

plastic bags were cut from the racks and the relative humidity was decreased to 80% for a drying period of approximately 2 days. The final water activity of the fungal bands was measured with a water activity meter (Decagon Devices, Pullman, WA).

Conidial movement from fungal bands to bark

This field experiment was carried out at Arnot Teaching and Research Forest (Newfield, NY; N 42° 16.764' W 76° 39.587') between the dates July 27 - August 25, 2006. Fungal bands were attached to 15 red maples (*Acer rubrum* L.), with an average diameter of 27.0 ± 1.4 cm. The trees selected had no branches or bark lesions within the area of the trunk used in this study. All trees were within the closed canopy of the forest, so that fungal bands were shaded from sunlight. Rainfall was collected in a rain gauge and recorded at approximately the same time each day during the time of the study. One band was hung around the main trunk of each tree at a height of 3 m and attached with 2-3 nails. Fungal bands had an average (\pm SE) of $4.87 \times 10^7 \pm 2.12 \times 10^7$ viable conidia per square centimeter of material ($n = 8$). All bands measured approximately 50 x 457 mm, and multiple bands were spliced to fully encircle trees with circumferences greater than 50 cm.

Sample sets consisted of 6 bark disks, taken at 10, 20, and 30 cm above, and 10, 30, and 60 cm below the band (i.e. measured from the center of the band). All bark samples were extracted using a cordless drill with a hole saw attachment. The hole saw had an inner diameter of 5.08 cm, and a center arbor measuring 0.64 cm in diameter, thus each donut-shaped disk of bark had a surface area of 19.96 cm².

Immediately prior to hanging fungal bands, sample sets were taken from each tree to determine baseline densities of *M. anisopliae* on the bark. These samples served as the controls. Bark disks were extracted with a chisel and placed into a

labeled 532 ml Whirl-Pak bag (Nasco, Fort Atkinson, WI), being careful not to touch the outer surface of the bark. Samples were returned to the laboratory and stored at 4°C prior to processing.

Subsequent sample sets were taken 2, 5, and 9 days after band placement. For each tree, one sample set was taken from each of the four cardinal compass directions, in a random sequence. Three repetitions of five trees each were banded and sampled in this fashion, during distinct 10-day periods and at separate locations within the forest (minimum 50 m between each set of trees). The first, second and third repetitions began on 27 July, 10 August, and 17 August, respectively.

In the laboratory, 50 ml of 0.2% Tween 20 detergent solution was measured into each Whirl-Pak bag, and samples were sonicated for five minutes in an ultrasonic cleaner to suspend conidia. Dilutions were prepared (1:10, 1:100) and 100 µl of each dilution was spread on plates of semi-selective media containing dodine, prepared according to Sneh (1991) (benlate omitted). One plate per dilution was prepared for each sample. All plates were incubated in the dark at 20°C and observed for fungal growth for at least three weeks. *M. anisopliae* colonies were identified by morphology and color, and counted (when colony diameter was approximately 1 cm, and 20-200 colonies per plate) to determine the number of conidia per square centimeter of bark surface. There were no significant differences in tree diameter at breast height ($F_{2,12} = 1.8502$, $P = 0.1994$) or average daily rainfall ($F_{2,24} = 2.2182$, $P = 0.131$) among the three replicates of this study.

Laboratory study of environmental contamination by adult beetles

Laboratory bioassays were conducted using beetles reared on artificial diet from a colony in the US Department of Agriculture, Agricultural Research Service (USDA, ARS) quarantine on Cornell University campus (Dubois et al. 2002). All living beetles and cadavers were kept at 24°C with a 16h:8h (L:D) photoperiod. Squares of fungal bands and fungus-free band material were cut to 25 cm² and stored in individual 100 mm plastic petri dishes at 4°C. Bolts of sapling *A. pensylvanicum* were cut at Svend O. Heiberg Memorial Forest (Tully, NY) and sealed at each end with paraffin wax to prevent desiccation (length = 20.3 ± 0.03 cm; average diameter = 5.1 ± 0.1 cm). Bolts were cut less than a week before use in the study. The beetles used were virgins between 10-30 days old (i.e. days since fully melanized), and were randomly assigned to treatments, with equal numbers of males and females in each treatment (average \pm SE age = 15.9 ± 0.6 d, average \pm SE male weight = 0.75 ± 0.02 g, average \pm SE female weight = 0.99 ± 0.03 g).

For the “one-beetle” treatment, a beetle was held onto a 25 cm² square of fungal band material (average = $4.43 \times 10^7 \pm 1.35 \times 10^7$ viable conidia/cm²; n = 8) for 30 seconds. This “contamination beetle” was then placed into a 3.79 liter glass jar containing a bolt of *A. pensylvanicum*. After 20 minutes, the beetle was transferred from the glass jar to an opaque plastic 473.2 ml cup with a transparent lid, containing 4 short fresh *A. pensylvanicum* twigs for food, each about 10 cm in length. A second beetle (= the “exposure beetle”) was then placed into the same glass jar with three 20 cm long fresh *A. pensylvanicum* twigs, and the original bolt of wood. After one week, this “exposure beetle” was transferred from the glass jar to a plastic 473.2 ml cup (opaque white with a clear plastic lid) with 4 fresh 10 cm long twigs. This process was

replicated 24 times, and each contamination beetle was used to contaminate jars for 6 exposure beetles.

In the “five-beetle” treatment, five contamination beetles were used per repetition, each exposed to its own fungal band square. All five beetles were placed into the jar with a log at the same time. As in the one-beetle treatment, the contamination beetles were removed after 20 minutes, and a single exposure beetle was placed in the jar with twigs. This five-beetle treatment was replicated 24 times, and each group of five contamination beetles was used to contaminate the jars for 6 exposure beetles.

Ten control beetles were treated before any fungal bands were used, and were identical to the one-beetle treatment except that the two contamination beetles used were exposed to a fungus-free square of band material. Each contamination beetle was used for 5 exposure beetles in the control treatment. All contamination and exposure beetles were checked daily for mortality until all beetles had died, and twigs were replaced every 7 days. Cadavers were placed in clear plastic 59.1 ml cups with moist cotton balls, sealed with parafilm, and observed for fungal outgrowth for at least two weeks.

There were no significant differences between treatments for beetle gender (equal numbers of each sex were used), average age ($F_{2,55} = 0.4021$, $P = 0.6709$), average weight ($F_{2,55} = 0.8467$, $P = 0.4343$), or average diameter of the *A. pensylvanicum* bolts ($F_{2,55} = 2.0684$, $P = 0.1361$). These factors, which might have influenced conidial movement, infectivity, and beetle lifespan, were controlled in the experimental setup.

Conidial dose quantification

For each of the three treatments (1-beetle, 5-beetle, and control), the density of conidia deposited onto the bark surface by the contamination beetles was estimated. As above, beetles were individually exposed ventrally to a square of band material for 30 seconds, placed into a 3.79 liter glass jar with a bolt of *A. pensylvanicum*, and removed after 20 minutes. Using a cork borer of 13 mm inner diameter, one bark disc sample was removed from each bolt. Bark disk samples were taken from the middle, the top third, or the bottom third of the bolt, and placement was randomized for each bolt. Each bark disc was placed individually into a 50 ml centrifuge tube with 15 ml of 0.1% Tween 20, being careful not to touch the outer surface of the bark disk. This process was repeated 10 times for each of the two fungal treatments, and 5 times for the control.

Centrifuge tubes were sonicated for five minutes to remove conidia from the bark and break up aggregations of spores. Dilutions of were prepared (1:10, 1:100, 1:1000) and vortexed for 10 seconds, and 200 μ l of spore suspension was spread on each of three plates per dilution on semi-selective media containing dodine, prepared according to Sneh (1991) (benlate omitted). All plates were incubated in the dark at 20°C and observed for fungal growth for at least three weeks. *M. anisopliae* colonies were identified by color and morphology, and were counted when colony diameter was approximately 1 cm, and 20-200 colonies were growing per plate, to determine the number of conidia per square centimeter of bark surface.

Data analysis

In the field study, plates streaked with the 1:1 dilution were used for all sample data. Conidial counts made prior to hanging bands (= day 0) were considered controls, and were excluded from analysis. Due to the prevalence of samples with no conidia, the count data was transformed into a binary variable, which was used for all analyses of conidial presence/absence. A binary parameter was made for position (above vs. below the band) to simplify the model. A binary rainfall parameter was made for presence/absence of rainfall since the last sampling day (Tables 1.1 and 1.2). Nominal logistic regression analysis was used to fit the data to a chi-square distribution using JMP 7.0 (Schlotzhauer 2006). The binary conidial count data were analyzed for all trees except tree 8, from which none of the samples contained conidia. Trees were treated as a repeated measure.

For the laboratory exposure study, beetle longevity was analyzed by treatment using ANOVA, with Tukey-Kramer HSD tests for post hoc comparisons. Differences in longevity across the three treatments were analyzed using one-way ANOVA for both contamination and exposure beetles.

RESULTS

Field study

The highest conidial density found on any one bark sample was 5.01×10^2 conidia/cm² (in the first repetition, tree five, day 9, at 60 cm below the band). A single spore was found in the controls (in the second repetition, tree 6, day 2, at 20 cm above the band),

but no molecular testing was conducted to determine whether the conidium was naturally occurring or due to contamination of a sample during this study. Rainfall varied throughout the study, with greatest amount of rain occurring during the third repetition, and the least amount of rain occurring during the second repetition (Tables 1.1 and 1.2). There were no significant differences (at $\alpha = 0.05$) in the probability of finding conidia in samples among trees, sampling days, replications, or the cardinal directions. Therefore, these parameters were omitted from the model.

Table 1.1. Total rainfall (cm) over sampling intervals within each repetition in the field study. In parentheses: number of days with rainfall during the interval.

	Total rainfall (cm)		
	Repetition 1	Repetition 2	Repetition 3
	26 Jul - 4 Aug	9 Aug - 18 Aug	16 Aug - 25 Aug
Days 1-2	0.97 (1)	0	0
Days 3-5	1.90 (2)	0	5.10 (2)
Days 6-9	1.02 (1)	0.51 (1)	4.22 (2)

Table 1.2. Amount of rainfall (cm) on sampling days within each repetition in the field study.

	Rainfall (cm)		
	Repetition 1	Repetition 2	Repetition 3
	26 Jul - 4 Aug	9 Aug - 18 Aug	16 Aug - 25 Aug
Day 2	0.97	0	0
Day 5	0	0	0
Day 9	1.02	0	3.84

Conidia were more likely to be found in samples taken below the fungal bands versus above ($\chi^2 = 8.38$, $P = 0.0038$), with the greatest number of conidia occurring in samples taken 10 centimeters below the center of the fungal band (Figure 1.1). In addition, conidia were significantly more likely to be found after rainfall ($\chi^2 = 3.99$, $P = 0.0457$) (Figure 1.2). No significant interactions were found between the rainfall and position variables. Sample position and quantitative daily rainfall had a marginally insignificant relationship for samples taken below the fungal band ($F_{1,7} = 5.5133$, $P = 0.0512$) but no relationship for samples taken above the fungal band ($F_{1,7} = 0.3923$, $P = 0.5510$) (Figure 1.3).

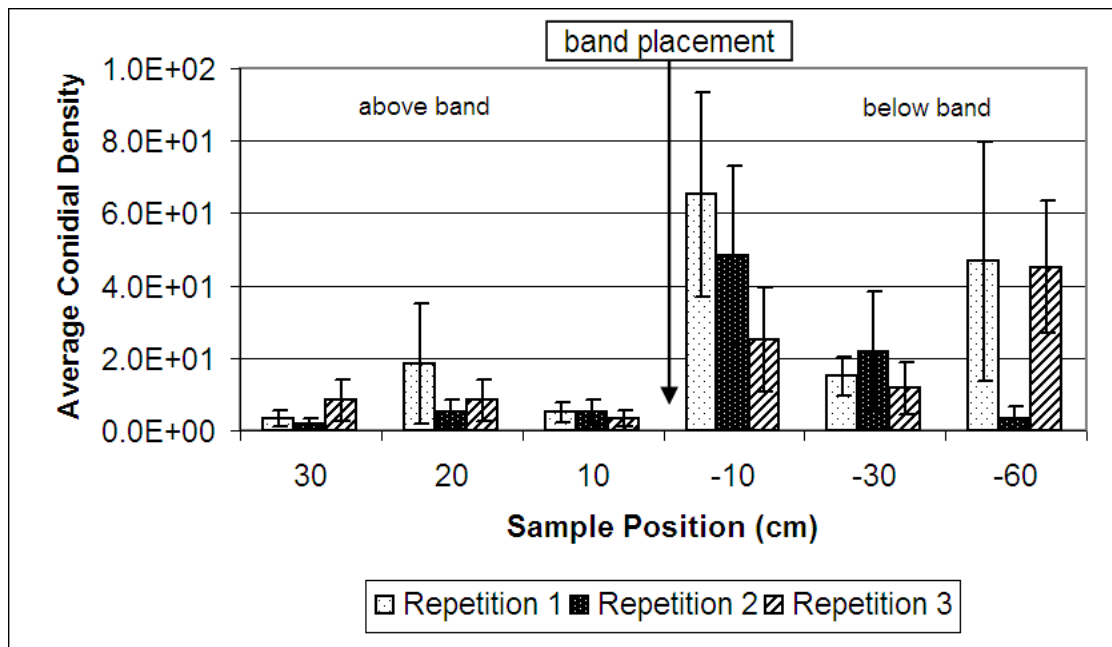


Figure 1.1. Average viable conidia/cm² \pm SE by position (centimeters above or below the center of the fungal band) for each of the three repetitions.

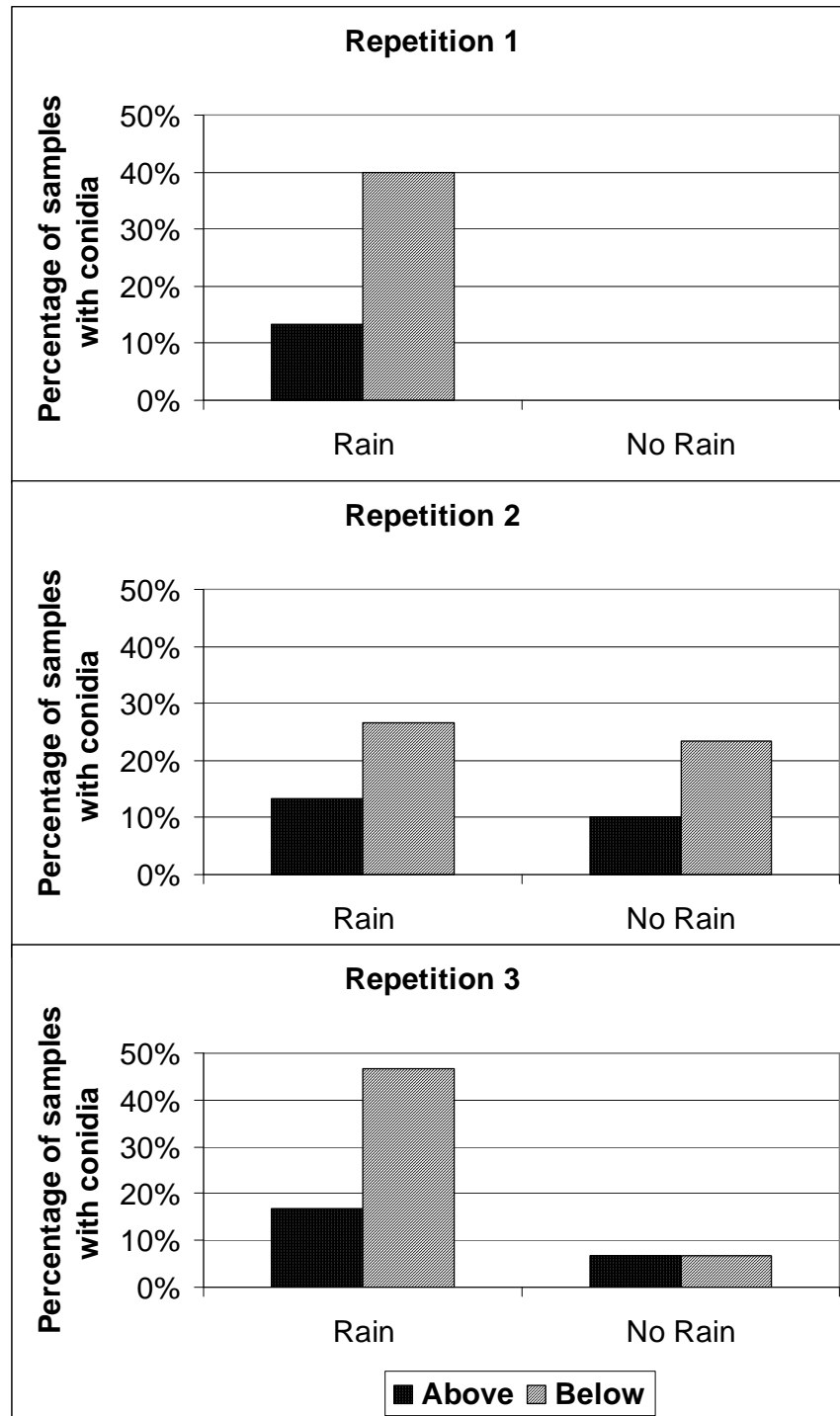


Figure 1.2. Percentage of samples with conidia, for samples taken above and below fungal bands, shown for sampling days following periods with rainfall events since the previous sampling day, versus sampling days following periods without rainfall.

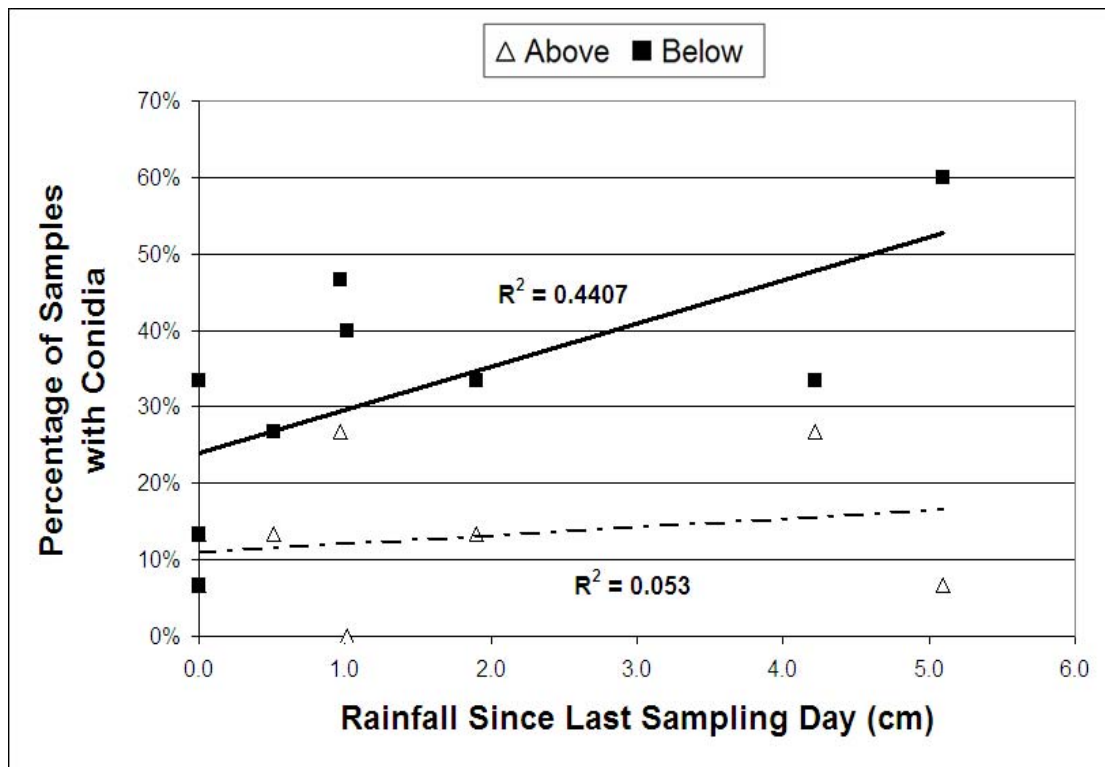


Figure 1.3. Percent of samples with conidia in samples taken above ($F_{1,7} = 0.3923$, $P = 0.5510$) versus below the fungal band ($F_{1,7} = 5.5133$, $P = 0.0512$) as a function of the amount of rainfall received prior to the current sampling day. Data shown for all samples in the three repetitions.

Laboratory exposure study

The average densities of conidia/cm² bark surface deposited by contamination beetles in the one-beetle and five-beetle treatments were $1.52 \times 10^4 \pm 3.46 \times 10^4$ and $2.78 \times 10^4 \pm 8.44 \times 10^3$, respectively. Plates for control samples showed no fungal growth. All cadavers of exposure beetles from the 1-beetle and 5-beetle treatments exhibited fungal outgrowth, while none of the control cadavers showed signs of infection.

The 1-beetle and 5-beetle treatments each differed significantly from the control treatment in terms of contamination beetle longevity ($F_{1,4} = 11.7722$, $P = 0.0265$ and $F_{1,20} = 78.5717$, $P < 0.0001$, respectively) (Table 1.3). However, contamination beetle longevity was not significantly different between the 1-beetle and 5-beetle treatments ($F_{1,22} = 0.0217$, $P = 0.8842$). All pairwise comparisons of the three treatments showed significant differences in longevity of exposure beetles (Tukey-Kramer HSD, $P < 0.05$) (Figure 1.4). Contamination beetles died in significantly less time than exposure beetles for the 1-beetle and 5-beetle treatments ($F_{1,26} = 10.1303$, $P = 0.0038$ and $F_{1,42} = 40.3579$, $P < 0.0001$, respectively). There was no significant difference in longevity between contamination and exposure beetles in the control ($F_{1,9} = 1.6836$, $P = 0.2267$).

Table 1.3. Average days to death (\pm SE) for *A. glabripennis* in the laboratory contamination study. Values followed by different letters within a column are significantly different ($P < 0.05$).

Average Days to Death				
Treatment	n	Contamination Beetles	n	Exposure Beetles
Control	2	79.6 \pm 29.5 a	10	114.9 \pm 16.2 a
1-Beetle	4	8.5 \pm 0.3 b	24	27.9 \pm 2.5 b
5-Beetle	20	8.6 \pm 0.3 b	24	16.2 \pm 1.1 c

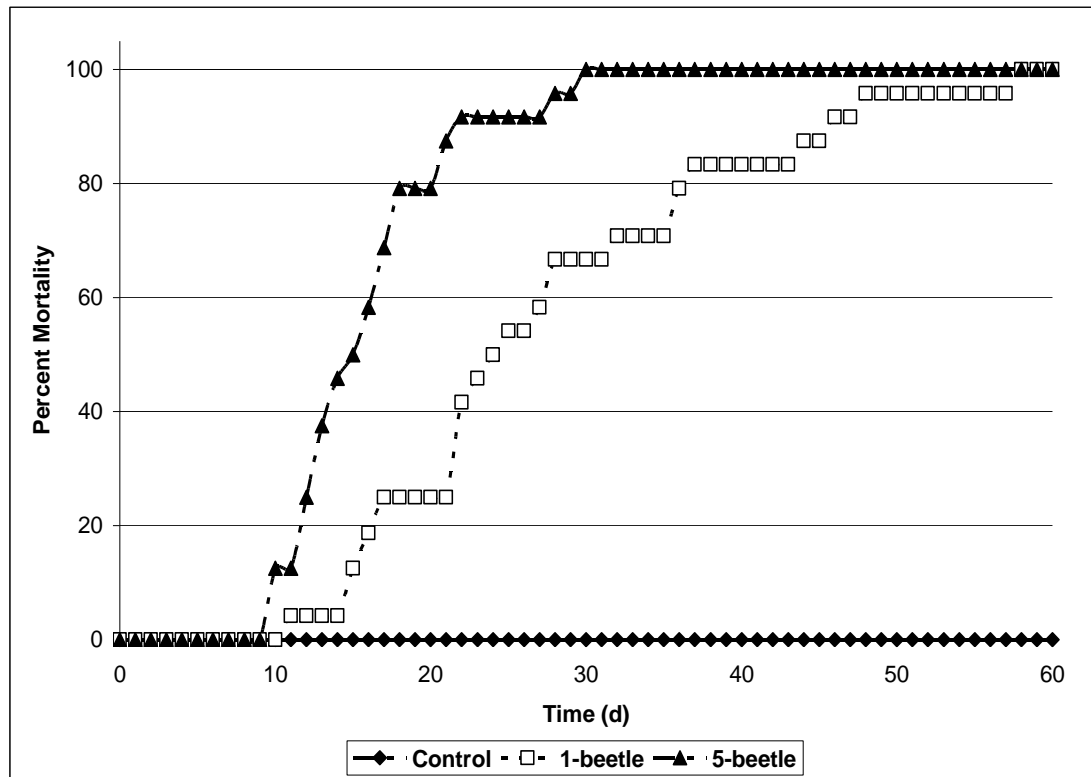


Figure 1.4. Percent mortality over time for *A. glabripennis* adults exposed to environments that were previously contaminated with *M. anisopliae* conidia by one or five adults exposed to fungal bands and then caged in the environment for 20 minutes. One contamination beetle was used in the control and one-beetle treatments, five contamination beetles were used in the five-beetle treatment.

DISCUSSION

In the field, conidia can be spread from non-woven fiber bands impregnated with entomopathogenic fungi to other areas of tree bark. Possible mechanisms of transportation could include passive animal vectors or abiotic factors (e.g. rain, wind, gravity). Adult *A. glabripennis* that contact fungal bands can also transfer conidia within their environment. The movement of viable conidia from fungal bands to other areas of tree bark increases the chances of *A. glabripennis* infection and of subsequent autodissemination of the entomopathogenic fungi. Conversely, the increased risk of infection in non-target arthropods may also be a concern.

Although the actual means of conidial movement were not identified in this field study, several of the results indicate that rain might have been an important mechanism for conidial movement. The probability of finding conidia in any given sample was significantly increased by the occurrence of rainfall since the previous sampling day or on the current sampling day. On the last sampling day of the third repetition, following a significant rainfall event (3.84 cm rain), the majority of conidia were found in samples 60 cm below the center of the fungal band (data not shown). This might indicate that fungal conidia can be moved more than 60 cm down the tree trunk by substantial rainfall, however this was not investigated here. Overall, there were significantly more positive samples found below bands than above, with the greatest number of conidia occurring at the position 10 cm below the center of the fungal bands. In addition, the number of conidia that moved from the fungal bands to the tree bark and failed to germinate was not investigated. Therefore, the number of *M. anisopliae* conidia that moved to the tree bark but died is unknown.

My findings support those of Gyoutoku et al. (1992), who observed movement of lethal doses of conidia from fungal bands, especially downward. Bands with *B.*

brongniartii were hung at specific positions within the branches of citrus trees. Adult *Anoplophora chinensis* (Förster) (= *A. malasiaca* (Thomson)) in 20 x 10 x 10 cm cages were also positioned within the tree branches, above, below, and horizontal (i.e. laterally, at the same height) to the fungal bands. Within 2 days, all beetles caged 50 and 100 cm below fungal bands had obtained a lethal infection. After 9 days, 100 % mortality was found in beetles caged at all distances below the fungal bands, in beetles caged 10 cm above the fungal bands, and in beetles caged 50 cm laterally from the bands at the same height. Most of the beetles (71.3%) caged 100 cm laterally from the bands at the same height obtained a lethal dose. No infections were found among the beetles caged 50 and 100 cm above the fungal bands, however (Gyoutoku et al. 1992).

Adult *A. glabripennis* were used in the laboratory at two densities to contaminate artificial environments with conidia from fungal bands. All beetles exposed to these environments were killed by fungal infections. Moreover, *A. glabripennis* adults exposed to environments that had been contaminated by five beetles (= higher density) died in significantly less time compared to beetles exposed to contaminated environments in the lower density treatment. All exposure beetles in these two treatments exhibited fungal outgrowth, while none of the controls had outgrowth. In the high density fungal treatment, the cadaver of one of the contamination beetles, which had been directly exposed to fungal bands, showed no signs of *M. anisopliae* growth, although this beetle died after only 9 days. Likewise, Dubois et al. (2004b) found that *B. brongniartii* did not grow from all cadavers of *A. glabripennis* that were assumed to have died from fungal infections. Poor mycelial growth has also been reported from cadavers of *Monochamus alternatus* Hope killed by *B. bassiana* (Shimazu and Takatsuka 2006).

Different tree species were unavoidably used for the laboratory and field studies. Diameters of bolts/trees were fairly consistent within each study. While the *A.*

rubrum trees used for the field study had relatively rough/flakey bark, the bark of the *A. pensylvanicum* bolts was smooth and waxy. It is not known however, how differences in bark texture might affect conidial persistence, movement or survival, or beetle infection rates. Duetting et al. (2003) found higher adhesion and germination of the entomopathogenic fungus *Pandora neoaphidis* (Remaudière and Hennebert) Humber, and higher infection of pea aphids, *Acyrtosiphon pisum* Harris, on leaves of pea plants with reduced surface wax (versus normal wax pea plants). Looking at myxomycete diversity and distribution on trees in North American temperate forests, Stephenson (1989) saw some indication that more spores were present on trees that had rougher bark. He concluded however, that because bark texture is not quantifiable, these observations were not testable.

The efficacy of fungal bands as a method for controlling *A. glabripennis*, through significant reductions in beetle longevity, has been demonstrated in both the lab and field (e.g. Dubois et al. 2004b, Hajek et al. 2006). In addition, fungal bands have also been shown to have sublethal effects on *A. glabripennis* by reducing oviposition (e.g. Dubois 2003, Dubois et al. 2004a, Hajek et al. 2006). Fungal infection can also be spread indirectly from infected females to their mates (AE Hajek, pers. comm.). It was shown in the current lab study that fungal infection can be acquired by *A. glabripennis* adults when exposed to an environment that was previously contaminated with conidia from other beetles exposed to fungal bands.

A. glabripennis adults are apt to walk on tree trunks during their prematuration feeding period of 11-16 days (Smith et al. 2002). In the current field study, bark samples with viable spores were few, and maximum densities of viable conidia were relatively low. However, the results presented here suggest that beetles could become infected by walking in close proximity to fungal bands. In dipping assays with *M. alternatus* and suspensions of *B. bassiana*, Shimazu (1994) found limited mortality at

comparably low conidial doses (12.5% mortality at $<10^3$ conidia/beetle), suggesting that some mortality can occur with exposure to low doses. However, beetles exposed to low doses lived significantly longer than beetles exposed to higher doses (Shimazu 1994).

The low densities of viable conidia found in the current study suggest that viable *M. anisopliae* conidia are mostly contained on fungal bands and nearby tree bark, which would minimize impacts on non-target arthropods. Therefore, beetles still need to contact fungal bands to obtain high doses of *M. anisopliae*. Many bark samples in the field study contained no conidia, and the greatest conidial density found on any of the bark samples (i.e. 5.01×10^2 conidia/cm²) was several times smaller than the median lethal dose for *M. anisopliae* against *A. glabripennis* (Chapter 2). Development of long-range attractants for *A. glabripennis* will improve the efficacy of fungal bands by increasing the likelihood of infection through beetles directly contacting bands.

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CHAPTER 2

EVALUATING THE VIRULENCE OF NON-WOVEN FIBER BANDS IMPREGNATED WITH *METARHIZIUM ANISOPLIAE* AGAINST THE ASIAN LONGHORNED BEETLE, *ANOPLOPHORA GLABRIPENNIS* (COLEOPTERA: CERAMBYCIDAE)

INTRODUCTION

Non-woven fiber bands impregnated with fungal pathogens were first developed as a biological control method against wood-boring orchard pests in Japan. The efficacy of such fungal bands has been evaluated for use against several tree-boring cerambycids, with much success (e.g. Shibata et al. 1991, Higuchi et al. 1997, Shimazu 2004, Hajek et al. 2006). A commercial fungal band product named Biolisa Kamikiri (Nitto Denko, Japan), which contains *Beuveria brongniartii* (Sacc.) Petch, has been developed for control of several cerambycid pests in Japanese orchards (Higuchi et al 1997). While tree-boring pests are often difficult to target with chemical insecticides, fungal bands take advantage of the tendency of many tree-boring cerambycids to walk on trees during what is known as the prematuration or preoviposition feeding period (Iba 1993).

The Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky), has been discovered to North America, first in New York in 1996 followed by subsequent discoveries (between 1998 and 2003) in Chicago, Illinois, New Jersey and Toronto, Ontario. Moreover, infestations of this beetle have been found since 2001 in Austria, France, and Germany (Haack 2006). *A. glabripennis* has a wide host range, and attacks both stressed and seemingly healthy trees. This pest poses a serious economic

and ecological threat to urban and forest trees. Therefore, *A. glabripennis* eradication efforts have been ardent (Nowak et al. 2001). Infested trees are removed, and uninfested host trees are often treated with imidacloprid through trunk and soil injections (USDA-APHIS 2005). This systemic insecticide, however, is not distributed evenly within trees and has exhibited strong antifeedant effects for *A. glabripennis* (Poland et al. 2001, Poland et al. 2006). Thus, it is questionable whether beetles are likely to receive a lethal dose of imidacloprid and whether feeding on inoculated trees will ultimately enhance dispersal.

As with other cerambycids, reductions in both adult longevity and female oviposition have been found when *A. glabripennis* adults are exposed to fungal bands (Dubois 2003, Dubois et al. 2004a and 2004b, Hajek et al. 2006). Fungal infection can also be horizontally transmitted during mating for many cerambycids (Shibata and Higuchi 1988, Tsutsumi and Yamanaka 1995, Tsutsumi 1998), including *A. glabripennis* (AEH and RPS, unpubl. data). In addition, Shimazu et al. (1995) reported 80% average larval infection in the Japanese pine sawyer, *Monochamus alternatus* Hope, as a result of field applications of fungal bands containing *Beauveria bassiana* (Balsamo) Vuillemin. Determining the longevity of fungal bands under field conditions is contingent on establishing the critical density of conidia (per square centimeter of band material) required to kill the target pest. Critical doses have been quantified for *B. brongniartii* against the yellowspotted longicorn beetle, *Psacotha hilaris* Pascoe (Higuchi et al. 1997, Tsutsumi 1998), and for *B. bassiana* against *M. alternatus* (Shimazu 2004). Studies investigating critical doses against *A. glabripennis* adults have involved dipping assays with liquid suspensions of *B. bassiana*, *B. brongniartii*, and *Paecilomyces* spp. (Zhang and Liu 1996, Shimazu et al. 2002).

The objective of the current study was to quantify the median lethal dose (viable conidia/cm²) for *A. glabripennis* adults exposed to fungal bands impregnated

with *Metarhizium anisopliae* (Metchnikoff) Sorokin strain F52, which is currently registered with the US Environmental Protection Agency (EPA) and produced commercially (Novozymes Biologicals Inc., Salem, VA; formerly Earth Biosciences Inc., New Haven, CT). To obtain prescribed doses for lethality testing, squares of *M. anisopliae* fungal bands were exposed to high temperature (42°C) for various lengths of time in order to kill some percentage of the conidia. Beetles were exposed to these bands and observed for longevity and mycelial outgrowth. Comparisons will be made with other entomopathogen-cerambycid systems, and implications for the use of fungal bands as part of an integrated *A. glabripennis* control program will be discussed.

METHODS

Fungal band exposures

Fungal bands were produced by dipping polyester bands into liquid cultures of *Metarhizium anisopliae* F 52 (ARSEF 7711) and storing them on racks at high humidity to allow growth and sporulation, as described in Chapter 1. Fungal bands were stored at 4°C until they were used (i.e. up to 10 months). To prepare individual doses for the bioassay, band squares from a single sheet were placed in a drying oven at 42°C for different lengths of time. The density of viable conidia per square centimeter was estimated both before and after exposing bands to heat.

To quantify the total number of conidia per square centimeter of a piece of band material, a slightly modified technique based on that used by Higuchi et al. (1997) was employed. From a fungal band sheet, a 25 cm² square was cut and homogenized in 300 ml of 0.2% Tween 20 detergent using a 2 L blender (Hamilton

Beach). This mixture was filtered through a 150 μm mesh filter into a 500 ml plastic bottle. An additional 200 ml of deionized water was used to wash any contents remaining in the blender into the plastic bottle. This bottle was sonicated for five minutes in an ultrasonic cleaner to suspend conidia. The suspension was agitated and loaded onto both sides of a Thomas hemocytometer to determine the conidial density. After allowing the conidia to settle for five minutes, counts were made in five 0.2 mm² areas on the hemocytometer grid (four corners plus the center) and summed together. The process was repeated for the other side of the hemocytometer, and the average of the two counts was taken. This average was multiplied by 50,000 to find the number of conidia per ml. By multiplying this product by 500 (volume of suspension), and dividing by 25 (area of fungal band square), the number of conidia per square centimeter of fungal band material was obtained.

From the conidial suspension prepared above, 0.75 ml was spread onto each of 3 plates of 2% water agar (in 100 mm plastic petri dishes). Plates were incubated at 20°C for 48 hours, and then transferred to 4°C until counts could be made (within 48 hours). Germination percentage was recorded for 200 conidia on each plate using a compound microscope, counting 20 conidia at each of 10 separate locations on the plate. Conidia were considered germinating if the germ tube was longer than half the major axis of the conidium. From the percent germination counts for the three plates, the average was taken and multiplied by the conidial density obtained above to determine the number of viable conidia per square centimeter of fungal band material.

Adult Beetle Exposures

Ten fungal doses were tested ($n = 70$ beetles), as well as a control treatment involving squares of fungus-free band material placed into the oven at 42°C for 6.3 h (i.e. the average time of heat exposure over all doses) (Table 2.1). Most beetles and beetle cadavers were kept at 24°C and 16h:8h (L:D) photoperiod in the US Department of Agriculture, Agricultural Research Service (USDA, ARS) quarantine on Cornell University campus. The control treatment and two of the fungal doses (2.25×10^6 and 3.21×10^6 conidia/cm²) were conducted in the US Department of Agriculture (USDA) Forest Service quarantine in Ansonia, CT, using methods and environmental conditions identical to those used for the other doses.

The beetles used were between 10-30 days old, and were randomly assigned to treatments, with 4 males and 3 females in each treatment. Each beetle was exposed ventrally by holding it on an individual square of fungal band material for 30 seconds. Before and after being exposed to a fungal band square, each beetle was kept in a plastic 473.2 ml cup and received four fresh 10 cm long *Acer pensylvanicum* twigs every 7 days. Beetles were checked daily for death, and *A. glabripennis* surviving ≥ 40 days were considered uninfected. When possible, survivors continued to be fed and observed for longevity after the 40 day period had ended. Cadavers were placed in plastic 59.1 ml cups with moist cotton balls. The cups were sealed with parafilm, and the cadavers were observed for fungal outgrowth for at least two weeks.

Table 2.1. Fungal band information for each of the 10 fungal doses.

Month of batch production	Sheet	Length of 42C exposure (hours)	Total conidia/cm ²	Percent conidia germinating	Viable conidia/cm ² (dose)
May 2006	1	0	2.28 x 10 ⁷	88.28 %	2.01 x 10 ⁷
May 2006	2	0	4.46 x 10 ⁷	88.50 %	3.95 x 10 ⁷
May 2006	1	1	2.28 x 10 ⁷	2.67 %	6.08 x 10 ⁵
May 2006	2	1	4.46 x 10 ⁷	6.17 %	2.75 x 10 ⁶
August 2006	3	16	5.29 x 10 ⁷	0.42 %	2.20 x 10 ⁵
August 2006	3	20	5.29 x 10 ⁷	0.25 %	1.32 x 10 ⁵
August 2006	4	0	2.50 x 10 ⁸	3.44 %	9.68 x 10 ⁶
August 2006	4	0.25	2.09 x 10 ⁸	1.17 %	2.25 x 10 ⁶
August 2006	4	0.5	2.40 x 10 ⁸	1.33 %	3.21 x 10 ⁶
August 2006	5	0	2.52 x 10 ⁸	1.28 %	3.26 x 10 ⁶

Data analysis

The bioassay data were analyzed using logistic regression in JMP 6.0.2, using the arcsine square root (ASR) transformation of mortality versus dose (Schlotzhauer 2006). This transformation, which is similar to the probit analysis, was used to maximize the fit of the model. All beetles dying in less than 40 days were included in the logistic regression analysis. In addition, the relationship between dose and longevity was analyzed using standard least squares in JMP 6.0.2 for beetles that died within 40 days and/or beetles that exhibited fungal outgrowth after death (Schlotzhauer 2006). The control treatment was excluded from all analyses. There were no significant differences in the probability of mortality among beetles with regard to sex ($\chi^2 = 1.6488$, $P = 0.1991$), weight ($\chi^2 = 2.7208$, $P = 0.0990$), or age ($\chi^2 = 3.5026$, $P = 0.0613$).

RESULTS

A strong significant correlation was found between dose, measured as the density of viable conidia per square centimeter of band material, and the ASR-transformed proportion of beetles dying within 40 days ($F_{1,9} = 29.2628$, $P = 0.0004$, $n = 32$) (Figure 2.1). The median lethal dose for *M. anisopliae* F 52 fungal bands against adult *A. glabripennis* was determined to be 6.80×10^6 viable conidia per square centimeter of band material ($\alpha = 0.05$; 95% confidence interval = 1.31×10^6 , 1.20×10^7).

For beetles exhibiting post-mortem fungal outgrowth, the average number of days to death (\pm SE) was 21.8 ± 2.0 days ($n = 34$), while the average longevity (\pm SE) for beetles not showing outward signs of infection was 85.0 ± 8.6 ($n = 14$; many replicates were censored). For all beetles that were presumed to have been killed by

fungal infection (i.e. if they had died within 40 days and/or exhibited fungal outgrowth after death; $n = 34$), a significant negative relationship between beetle longevity and dose was found ($F_{1,32} = 4.3945$, $P = 0.0440$). Only one beetle died within the 40 day study period without exhibiting fungal outgrowth, dying 20 days after being exposed to a fungal band with 6.08×10^5 conidia/cm². Conversely, two beetles outlived the 40 day study period yet exhibited post-mortem fungal outgrowth, one of which died 56 days after being exposed to a fungal band with 6.08×10^5 conidia/cm², the other dying 55 days after being exposed to a fungal band with 2.75×10^6 conidia/cm². All of the beetles in the control treatment survived for over 40 days.

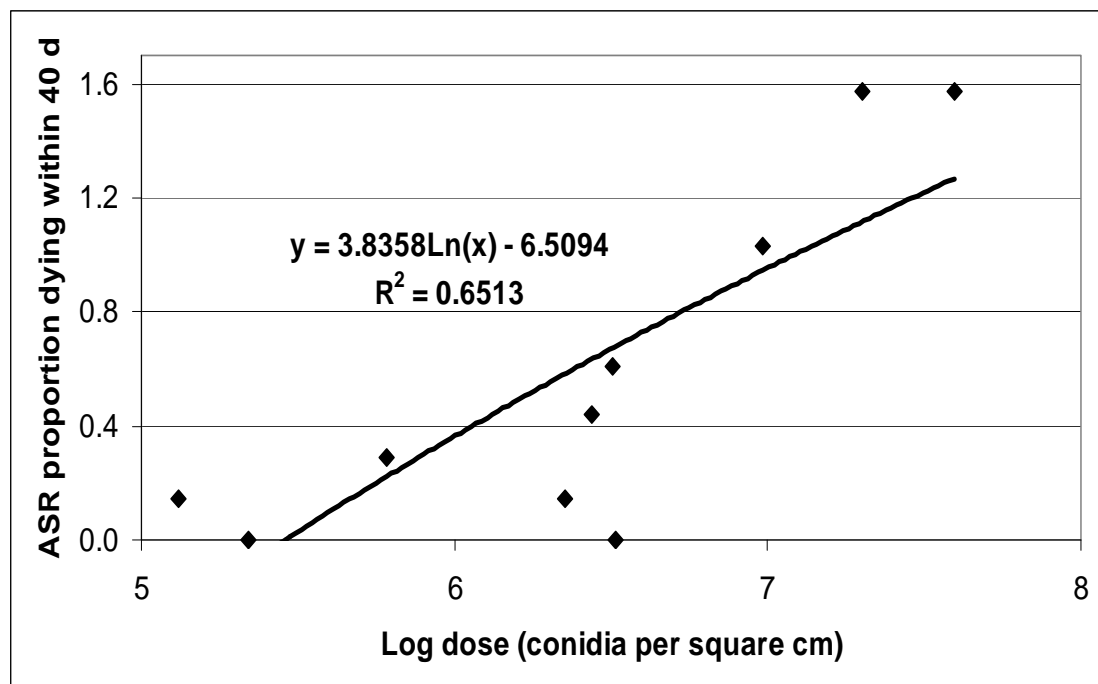


Figure 2.1. Arcsine square root (ASR)-transformed mortality (proportion dying within 40 days) for each fungal band dose (density of viable conidia per cm²) ($F_{1,9} = 29.2628$, $P = 0.0004$). Points represent the proportion dying out of 7 beetles.

DISCUSSION

Non-woven bands impregnated with entomopathogenic fungi provide a potential alternative to systemic insecticides for the control of *A. glabripennis*. The strategy of using fungal bands is based on the premise that during its prematuration feeding period, when *A. glabripennis* is predisposed to walk on tree trunks and branches, the beetle might come in contact with a fungal band and become infected. Although the pathogenicity of fungal bands has been well documented for *A. glabripennis* (Dubois 2003, Dubois et al. 2004a and 2004b, Hajek et al. 2006), questions remain regarding the required number of applications of fungal bands per year in infested areas. To be able to answer these questions, the activity time of fungal bands impregnated with *M. anisopliae* F 52 must first be determined. In turn, to calculate the longevity of fungal bands, the critical dose required to reliably kill adult *A. glabripennis* must be established.

My findings confirmed that beetle mortality due to infection acquired from fungal bands is strongly dose-dependent, and that beetle longevity decreases as dose increases. Most beetles exhibiting fungal outgrowth died within 40 days (32 out of 34, 94.1%), while most beetles not showing outward signs of infection survived the 40 day study period (45 out of 46, 97.8%). This suggests that 40 days was a reasonable length for the bioassay. Furthermore, the large disparity in average days to death between beetles with fungal outgrowth versus those without was not surprising. The beetle that died 20 days after being exposed to a fungal band with 6.08×10^5 conidia/cm² may have been killed by *M. anisopliae*, despite the lack of outward evidence. Cadavers of beetles that were assumed to have died from fungal infections but that failed to exhibit fungal outgrowth have been previously reported for *A. glabripennis* exposed to *M. anisopliae* F 52 (see Chapter 1) and *B. brongniartii*

(Dubois et al. 2004b), as well as for *Monochamus alternatus* Hope exposed to *B. bassiana* (Shimazu and Takatsuka 2006).

A median lethal dose of 6.80×10^6 viable conidia per cm^2 for *M. anisopliae* F 52 fungal bands was estimated here. Fungal band critical doses have been estimated for several other tree-boring cerambycids including *A. glabripennis*. Zhang and Liu (1996) dipped adult *A. glabripennis* into suspensions of *B. bassiana* and *B. brongniartii* and found LD_{50} s of 2.2×10^6 and 2.2×10^9 conidia/ml, respectively. Likewise, Shimazu et al. (2002) dipped adult *A. glabripennis* into fungal suspensions for 30 seconds, and after 19 days found LD_{50} s between 10^6 - 10^7 conidia/ml for *B. bassiana*, and around 10^4 conidia/ml for *B. brongniartii*.

Higuchi et al. (1997) used a walking bioassay to determine the critical dose required for *B. brongniartii* fungal bands against the yellowspotted longicorn beetle, *P. hilaris*. These tree-boring cerambycids acquired a dose of fungi by walking on bands for “more than 5 s,” which resulted in an LD_{50} of 1×10^7 conidia per square centimeter of fungal band material. This value was hypothesized as a threshold of fungal band activity by Hajek et al. (2007) in their investigation of fungal band longevity. Tsutsumi (1998) also examined the lethality of *B. brongniartii* fungal bands against *P. hilaris*, allowing adult beetles to walk on bands for 5-60 seconds. On fungal bands containing 5×10^7 conidia/ cm^2 , 100% mortality was found, while 85% mortality occurred on bands containing 2.5×10^6 conidia/ cm^2 . Shimazu (2004) used a paintbrush to apply dried conidia from *B. bassiana* fungal bands to the tarsi of Japanese pine sawyer, *M. alternatus*, another tree-boring cerambycid. In 4-day old beetles, median lethal doses of 1.9×10^6 and 2.4×10^4 conidia/individual were found after 14 and 30 days, respectively. In “aged adults” (i.e. >10 days since emergence) median lethal doses of 5.5×10^6 and 2.8×10^5 conidia/individual were found after 14 and 30 days, respectively (Shimazu 2004).

There are several factors (e.g. age, sex and weight of the insect being tested) that could potentially influence infection and lethality of an entomopathogenic fungus and must be taken into account when designing fungal band bioassays. Moreover, differences in the application method, dose units, and the number of days at which lethality estimates are calculated can make comparing studies difficult. Compared with dipping, walking is a more realistic application method for fungal band bioassays. Notably, Tsutsumi (1998) found no significant difference in *P. hilaris* longevity regardless of whether the beetles walked on the fungal bands for 5 or 60 seconds. Likewise, Dubois (2004b) found no significant difference in *A. glabripennis* longevity when walking on *M. anisopliae* fungal bands for 5 versus 25 seconds (although a significant difference in longevity was found for one of the two strains of *B. brongniartii* tested). Tsutsumi and Yamanaka (1996) found that adult *P. hilaris* walking on *B. brongniartii* fungal bands containing 10^8 , 10^6 , and 10^5 conidia/cm² acquired around 10^7 , 10^5 , and 10^4 conidia per beetle, respectively. After allowing adult *M. alternatus* to walk on *B. brongniartii* fungal bands containing *B. bassiana* “for more than 5 cm,” Shimazu (2004) found 8.5×10^5 conidia/individual.

The pathogenicity of *M. anisopliae* F 52 against adult *A. glabripennis* has been verified through both dipping and walking bioassays (Hajek et al. 2007). The current study utilized a contact bioassay, designed to emulate the acquisition of infection from walking on fungal bands, in order to estimate the median lethal dose for *M. anisopliae* F 52 fungal bands against *A. glabripennis*, exposing each adult to a 25 cm² square of fungal band material. The calculated median lethal dose of 6.80×10^6 viable conidia per cm² for *M. anisopliae* F 52 fungal bands in the current study is fairly consistent with calculations for other systems involving fungal pathogens against tree-boring cerambycids.

Difficulties arise concerning the preparation of prescribed fungal band doses in a replicable method. Moreover, the resulting doses must have uniform densities of viable conidia throughout the fungal band material (see Appendix A). In “separate experiments,” Higuchi et al. (1997) observed *B. brongniartii* only sporulating on the surface of fungal bands. Any treatment of fungal bands for the production of bioassay doses must therefore either be applied to both sides of the band material equally, or be applied uniformly throughout the material, but without damaging the surviving conidia. In the current study, heat was used to inactivate conidia by exposing fungal band squares to 42°C for different lengths of time. In their evaluation of *B. brongniartii* fungal band activity against *P. hilaris*, Higuchi et al. (1997) obtained doses by incubating bands for 1-4 days at temperatures ranging from 22-30°C. Likewise Shimazu (2004) used high temperatures to prepare doses of dry conidia, mixing live spores at specific ratios with dead spores which had been killed through exposure to extreme heat (100°C for 1 h).

To determine the length of heat exposure needed to produce a certain dose, several exposure times were used, with multiple band squares (all coming from a single sheet) used per length of time. This calibration process established a linear trend between heat exposure time and conidial viability, and improved prediction power. However, difficulties arose with obtaining prescribed doses on pieces of fungal band material due to variability in: 1) conidial density and viability between batches of fungal bands, 2) conidial density and viability between sheets of fungal bands (within batches), 3) the effect of exposure to heat due to differences in fungal band production. This often meant that if exposing bands to heat for a certain length of time didn’t produce the desired dose, an additional set or sets of band squares had to be cut and exposed to heat before the desired dose was acquired. This trial-and-error process was repeated 2-3 times per dose on average. In addition, due to variability among

sheets and batches of fungal bands, new calibrations had to be made every time a new sheet or batch was used.

Drying fungal bands to lower water activity ($= 0.3 a_w$) helps to retain viability during storage and thus increase shelf life (J. Leland, pers. comm.). The optimal drying period is approximately 7-10 days, and it has been found that a shorter drying period ($= 2$ d) can result in decreased viability (J. Leland, unpubl. data). Before application of fungal bands, rehydration at $>95\%$ RH for 1-2 hours can provide significant improvement in viability, compared with unhydrated bands. The duration and environmental conditions during drying and rehydration can differ for fungal bands versus spore powders, as well as for different species and strains of entomopathogenic fungi (J. Leland, pers. comm.).

Studies of fungal band longevity have produced a range of estimates for activity retention time. In particular, for *B. brongniartii* fungal bands against various tree-boring cerambycids, bands have been found to remain active in the field for at least 20 days (Tsutsumi 1998), about 30 days (Dubois et al. 2004b, Matsuura et al. 1997), or for up to 40 days (Higuchi et al. 1997). Recently, Hajek et al. (2007) reported that *M. anisopliae* F 52 fungal bands attached to trees in New York City maintained densities greater than 1×10^7 conidia per square centimeter for at least 112 days. In the US, *A. glabripennis* emergence generally occurs in late June or early July, with the majority of beetles being present in July and August (Nowak et al. 2001). Therefore, the protracted longevity of fungal bands in the field suggests that hanging bands once per season would be sufficient for enabling exposure for the majority of adult beetles.

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