Attraction of Walnut Twig Beetle *Pityophthorus juglandis* (Coleoptera: Curculionidae) to the Fungus *Geosmithia morbida*

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ABSTRACT

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Geosmithia morbida causes thousand cankers disease of *Juglans nigra* and it is transmitted by the walnut twig beetle (WTB, *Pityophthorus juglandis*). Along with WTB, an ambrosia beetle, *Xyleborinus saxeseni*, is commonly associated with thousand cankers disease in the later stages of *J. nigra* decline, although *X. saxeseni* is not a known vector of *G. morbida*. We initiated studies to determine whether WTBs or *X. saxeseni* were attracted to volatiles produced by *G. morbida* and other bark fungi in a laboratory choice-test setting. There was no difference between the numbers of emerged WTB adults that were collected in tubes containing

potato dextrose agar colonized by *G. morbida* and those in tubes containing agar only. More adult *X. saxeseni* were collected in tubes containing agar only compared with *G. morbida*. Walnut twig beetle larvae migrated more frequently toward an agar plug colonized by *G. morbida* and *Fusarium solani* compared with an un-colonized agar plug. No larval preference was observed when agar plugs colonized by *F. solani* and *G. morbida*, or *G. morbida* and *Penicillium solitum*, were placed in the same petri dish. These results suggest that WTB larvae are attracted to bark fungi in general, but not specifically to *G. morbida*.

INTRODUCTION

Thousand cankers disease of *Juglans* (walnut) is the result of feeding by the walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae, Scolytinae), and canker formation caused by its fungal associate, *Geosmithia morbida* M. Kolařík, E. Freeland, C. Utley, & N. Tisserat (Ascomycota: Hypocreales) (28). This disease is responsible for killing thousands of *J. nigra* L. (black walnut) in several western United States (Arizona, California, Colorado, Idaho, Nevada, New Mexico, Oregon, Utah, and Washington; 7,27,28), where the tree is planted for shade, lumber, and/or nuts. In the past four years, the disease has been found established in native stands of *J. nigra* in Tennessee, Virginia, Pennsylvania, North Carolina, and Ohio (9,22,23,24,29), where it has been associated with crown dieback and some tree mortality. Along with WTB, the ambrosia beetle, *Xyleborinus saxeseni* (Ratzeburg), is commonly found during the later stages of thousand cankers disease-related decline, although it is not a known vector of *G. morbida* (27).

The WTB was originally collected in 1896 from *J. major* (Torr.) A. Heller (Arizona walnut), its presumed native host, in New Mexico (4). It has subsequently been found to occur over broad areas of North America (7) and was recently found in Italy (21). In this expanded range, it has been found colonizing all six species of *Juglans* native to North America (*J. californica* S. Watson, *J. cinerea* L., *J. hindsii* (Jeps.) Jeps. ex R.E. Sm., *J. microcarpa* Berl., *J. major*, and *J. nigra*) as well as *Juglans regia* L. *Geosmithia morbida* has been consistently associated with all of these hosts in all locations, being isolated from the WTB and cankers surrounding beetle galleries, but has not been

recovered from other beetles associated with *Juglans* (16,17,18). This type of vector and substrate specificity is similar to that reported for other *Geosmithia* species (18), and supports the hypothesis of a long and stable association between *G. morbida* and the WTB.

Relationships between insects and fungi can range from mutualistic (14,26) to antagonistic (2,13,25). The association between *Geosmithia morbida* and the WTB is not well understood. Although *G. morbida* produces conidia that could be dispersed in air, it appears to be dependent on WTB for its dispersal and ingress into *Juglans* bark. *Geosmithia morbida* grows in WTB galleries and often coats the pupae and pupal chambers with a thick mycelial and conidiophore lining (Fig. 1 A and B). However, whether the fungus provides some type of nutritional source or perhaps serves as a microbial protection is unknown. *Geosmithia morbida* may also benefit WTB by weakening host defenses or otherwise increasing the probability of successful insect reproduction, as has been shown with other bark beetle/fungal associations (1,5). In the case of ambrosia beetles, fungi are introduced into host plants and cultured as the primary diet for developing larvae (3).

Attraction of bark beetles to fungal volatiles is not well documented. However, one attempt by Huler et al. (15) found the redbay ambrosia beetle, *Xyleborus glabratus* Eichhoff, was attracted to fungal volatiles of its associated fungus, *Raffaelea lauricola* T. C. Harr., Fraedrich & Aghayeva, but was not attracted to the volatiles of a non-associated fungus (15). Recently, the major constituents of *R. lauricola* volatiles were identified and a synthetic odor blend was created. When this blend was combined with host plant volatiles, it greatly increased attractiveness to the beetles in a synergistic manner (19). Fungal volatiles as oviposition attractants were shown by Honda et al. (14), who found the yellow peach moth, *Conogethes punctiferalis* (Guenée), was more attracted to fruit inoculated with fungi rather than

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FIGURE 1

(A) Mycelium and conidia of *Geosmithia morbida* often envelope the pupae and pupal chambers of the walnut twig beetle (WTB). (B) A newly emerged WTB in pupal chamber encapsulated with *G. morbida*.

FIGURE 2

Adult choice test chamber. A walnut twig beetle infested log was placed inside of the bucket and the migration of the emerging insects into the two tubes was recorded. Tubes were filled with either ¼-strength potato dextrose agar modified with 100 mg/liter streptomycin sulfate and 100 mg/liter chloramphenicol (¼ PDA ++) or agar colonized by *Geosmithia morbida*.

healthy fruit (14). Fäldt et al. (10) found that volatiles collected in situ from bracket fungi attracted several species of wood-inhabiting beetle generalists (10). The oak wilt pathogen, *Ceratocystis fagacearum* (T. W. Bretz) J. Hunt, produces sticky spores in fungal mats that are disseminated by nitidulids and drosophilids (6,8). Volatiles from *C. fagacearum* may mimic fruit odors that are attractive to these insects (6,11,20). This study was conducted to determine whether *G. morbida* may produce volatiles or other diffusible compounds that are attractive to WTB adults or larvae. A secondary objective was to determine if attraction to *G. morbida* was also observed by *X. saxeseni*.

ADULT CHOICE TEST

The initial study was conducted to determine the attractiveness of *G. morbida* to adult walnut twig beetles and adult *X. saxeseni*. Black walnut trees showing symptoms of thousand cankers disease were felled in Boulder, CO in December 2010. The logs were left uncovered outdoors until April 2011 when they were cut

FIGURE 3

Individual thousand cankers disease infested logs were placed into adult choice test chambers (described in Fig. 2) and insect emergence was quantified after 20 days. Results were analyzed using a chi-square goodness of fit test. Dashed lines represent expected values for the chi square goodness of fit analysis. Bars with different letters are statistically significant at $P < 0.01$. (A) Number of emerged walnut twig beetle (WTB) adults (±SD) per treatment. Columns are divided into proportions of male and female WTB adults; light grey portions are female and dark grey are male. (B) Number of emerged *Xyleborinus saxeseni* (±SD) per treatment.

into five 30-cm-diameter and 20-cm-long sections. Each section was placed inside a 19-liter plastic bucket modified to include two 50 ml translucent plastic tubes on opposite sides such that the bottoms of the tubes were at the same height as the base of the bucket (Fig. 2). Tubes were attached to the bucket using 45° angled polyvinyl chloride elbows. Elbows were sanded along their interior in order to provide a non-slippery surface for beetles to walk. Due to positive phototaxis, beetles moved to the light and fell into the tubes. Preliminary observations indicated that adult beetles easily succumbed to static electricity on the smooth surface of the plastic bucket so 100-grit sandpaper was placed in the bottom to provide traction for walking. The bucket was sealed with a plastic lid containing a 10-cm-diameter hole covered with a fine mesh fabric to provide air circulation. A 6-cm × 4-cm area of bark from each log was removed and dissected to confirm the presence of living WTBs. Here, the presence of *X. saxeseni* was also detected.

The 50-ml collection tubes contained either 15 ml of quarter-strength potato dextrose agar modified with 100 mg/liter streptomycin sulfate and 100 mg/liter chloramphenicol (¼ PDA++) or ¼ PDA++ colonized by *G. morbida* isolate 1217. The buckets were exposed to 14 h light and 10 h dark at a constant 25°C. Collection tubes were left in place for 10 days then switched to account for possible environmental differences between the different sides of the bucket. After 10 additional days adult beetles were removed, identified, counted, and in the case of WTB, sexed. The experiment was repeated four times using the same logs. A chi-square goodness of fit test was performed on untransformed data to compare the beetle counts related to the two treatments (CHITEST, Excel 2007). No post hoc comparisons were made.

There was no difference ($P > 0.10$) between the number of emerged WTB adults that were collected in tubes containing ¼ PDA++ colonized by *G. morbida* to those in tubes containing

¼ PDA++ only (Fig. 3A). The number of male and female WTBs collected in tubes with *G. morbida* was similar; however, more males than females ($\sigma = 287$, $\varphi = 278$; $P < 0.05$) were collected in tubes containing agar only (Fig. 3A). More *X. saxeseni* were collected in tubes containing agar only compared to *G. morbida* ($P < 0.001$) (Fig. 3B).

LARVAL CHOICE TEST

Single-spore isolates of *G. morbida* (1217), *Fusarium solani* (Mart.) Sacc. (1179), and *Penicillium solitum* Westling (1281) were grown for two weeks in 100-mm-diameter × 15-mm-deep petri dishes containing 20 ml of half-strength potato dextrose agar (½ PDA). *Fusarium solani* is commonly isolated from necrotic bark of *J. nigra* during the later stages of thousand cankers disease and is also pathogenic to walnut (27) whereas *P. solitum* was also consistently isolated from decaying walnut bark but is not considered plant pathogenic to *Juglans* spp.

A 5-mm-diameter plug was removed from each side of a petri plate containing water agar. The holes were filled with plugs of ½ PDA or ½ PDA colonized by one of the test fungi in various combinations. Second and third instar WTB larvae were extracted from infested *J. nigra* bark, placed individually into 2 ml tubes, and stored for 48 h in the dark at 4°C until the experiment began. They were then placed in the center of the petri dish immediately after insertion of the fungal plugs (Fig. 4A). The dishes were incubated at 25°C in the dark for 48 hours at which time larvae were removed. The movement of the larva was visualized 24 to 48 h later by observing the small amount of fungal/bacterial growth associated with the larval movement (Fig. 4B). Trails were traced using a permanent marker (Fig. 4C). The movement of the larvae relative to the position of agar plugs containing *G. morbida* was tested against larval movement in relation to plugs of ½ PDA ($n = 55$), agar colonized by *F. solani* ($n = 50$), or agar colonized by *P. solitum* ($n = 50$). Larval movement relative to the position of

FIGURE 4

Larval choice test bioassay. (A) A walnut twig beetle larva (indicated by red arrow) was placed on a petri plate filled with water agar. At each side of the dish, plugs of water agar were removed. The holes were filled with either half strength potato dextrose agar or agar colonized by one of the test fungi (*Geosmithia morbida*, *Penicillium solitum*, or *Fusarium solani*). The larva was removed after 48 hours. (B) Bacterial/fungal growth under magnification outlining the trail of walnut twig beetle larvae. (C) Example of larval trail that was traced with permanent marker.

½ PDA or plugs colonized by *F. solani* was also tested (n = 35). Larval movement was scored as either movement towards one agar plug only, movement towards both agar plugs, or movement toward neither plug. Occasionally, a larva would pupate or die and was omitted from the study.

Comparisons of counts of larval preferences using a chi-square goodness of fit test were made to determine whether there was a preference between the four categories (i.e., one of the two active treatments, both treatments, or neither treatment). Distribution of WTB larvae and adults among the treatments was tested against the distribution of the expected homogeneous values (CHITEST, Excel 2007). Post hoc comparisons looking at the two active treatments were then made using a Z-test of single proportions.

Walnut twig beetle larvae migrated more frequently towards an agar plug colonized by *G. morbida* ($P < 0.001$, $\chi^2 = 20.20$, $df = 3$) or *F. solani* ($P < 0.001$, $\chi^2 = 18.78$, $df = 3$) compared to an uncolonized agar plug (Fig. 5A and B). No larval preference was observed when plugs colonized by *F. solani* and *G. morbida* ($P = 0.32$, $\chi^2 = 0.31$, $df = 3$) were placed in the same plate (Fig. 5C). Similarly no differences in larval preference were observed when plugs colonized by *G. morbida* and *P. solitum*

($P = 0.75$, $\chi^2 = 2.84$, $df = 3$) were placed in the same plate (Fig. 5D).

In experiments comparing larval movement towards a fungus (*G. morbida*, *F. solani*, or *P. solitum*), the larvae usually traveled in a more or less direct route from the center of the petri dish to the colonized plug (Fig. 4C). In many of the bioassays, larvae would stay on the fungal plug or burrow inside of it and remain there for the duration of the experiment (Fig. 6A and B). In experiments where the larvae were given the choice between two different fungi, the larval trails were more or less sporadic and seemed to cover more area of the water agar petri plate.

SUMMARY

There was no preferential movement of emerging adult WTBs to tubes containing *G. morbida*, suggesting that volatile chemicals produced by the fungus were not attractive to WTB adults. However, some aspects of the experimental design may have precluded finding the influence of any fungal volatiles. For example, the length of time the tubes were left on the boxes may have concentrated the volatiles throughout the bucket and prevented a concentration gradient within the still air of the

FIGURE 5

Individual walnut twig beetle (WTB) larva were subjected to a choice test bioassay (described in Fig. 4) and movement of the larva was observed and recorded. Larvae were scored as moving towards one agar plug, towards both plugs, or to neither plug. Results were analyzed using a chi-square goodness of fit test. Dashed lines represent expected values for the chi square goodness of fit analysis. Post-hoc comparisons were made using a Z-test of single proportions. Bars with different letters are statistically significant at $P < 0.01$. (A) Number of WTB larvae that migrated towards a 5-mm plug of half strength potato dextrose agar (½ PDA) or ½ PDA colonized by *Geosmithia morbida*. (B) Number of WTB larvae that migrated toward *Fusarium solani* or ½ PDA. (C) Number of WTB larvae that migrated toward *G. morbida* vs. *F. solani*. (D) Number of WTB larvae that migrated toward *G. morbida* vs. *Penicillium solitum*.

FIGURE 6

(A) Walnut twig beetle (WTB) larva residing on an agar plug colonized by *Geosmithia morbida*. (B) Plug of agar colonized by *G. morbida* that has been burrowed through by WTB. The larva is indicated by the red arrow.

emergence chambers. In the future, a bioassay using a constant flow of air should be tested. Alternatively, many of the beetles were teneral and may have been more physiologically insensitive to differences in volatiles than mature adults. Nevertheless, we think it is unlikely that the volatile attractants produced by *G. morbida* are strongly attractive to the WTB.

Adult *X. saxeseni* preferentially chose containers filled with ¼ PDA++ over containers colonized by *G. morbida*. This avoidant behavior exhibited by *X. saxeseni* toward containers filled with *G. morbida* may be due to the strong association of ambrosia beetles to specific fungi with which they have mutualistic relationships; as a result they may avoid other fungi that may be competitive or otherwise detrimental to these associated fungi (3,12).

Larvae of walnut twig beetle were attracted to several fungi associated with the bark of *J. nigra*. The reason for this attraction is unknown but there are many potential benefits fungi may produce for WTB. For example, WTB larvae may benefit from the fungus incapacitating plant defenses in the area where it develops. Alternatively, the fungus may provide some nutritional benefit either directly (consumption of the fungus) or indirectly (degrading plant material).

The chemical cue for the attraction between larvae and bark fungi could be water soluble and may only be perceivable by larvae growing under the bark. If the chemical cue is volatile, adults may not perceive the cue because it may be too diffusely spread outside of the bark. Additionally, volatiles may be produced by other fungi or the same fungi (*Fusarium solani*) in a non-*Juglans* host, which would not be suitable for larval development. Further studies to determine whether bark fungi are important for the survival and reproduction of the WTB are needed.

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