

Short Communication

Effect of Bark Application With *Beauveria bassiana* and Permethrin Insecticide on the Walnut Twig Beetle (Coleoptera: Curculionidae) in Black Walnut Bolts

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Abstract

Formulations of entomopathogenic (insect-killing) fungi represent alternatives to synthetic insecticides in the management of forest and shade tree insects. We evaluated bark spray applications of the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Cordycipitaceae) strain GHA (BotaniGardES), permethrin insecticide (Astro), and water (control) on colonization of black walnut (*Juglans nigra* L.) (Fagales: Juglandaceae) bolts by the walnut twig beetle (*Pityophthorus juglandis* Blackman) (Coleoptera: Curculionidae), vector of the fungus that causes thousand cankers disease. Treated bolts were baited with a *P. juglandis* aggregation pheromone lure and deployed in infested walnut trees. Bark application of permethrin prevented *P. juglandis* colonization of the phloem. Although treatment of bolts with the *B. bassiana* suspension did not reduce *P. juglandis* colonization or short-term emergence relative to the control treatment, it increased the *B. bassiana* infection rate from 25 to 62% of emerged adults. Results suggest that commercial applications of *B. bassiana* strain GHA may help augment natural levels of infection by this entomopathogen in the eastern United States, and support continued exploration of entomopathogens for biological control of the walnut twig beetle.

Key words: entomopathogen, thousand cankers disease, biological control, *Juglans nigra*, *Pityophthorus juglandis*

Practical and environmental limitations on the use of synthetic insecticides in natural ecosystems have generated interest in using entomopathogenic (insect-killing) fungi for control of forest and shade tree insects (Hajek and Bauer 2007). Entomopathogenic fungi such as *Beauveria bassiana* (Balsamo) (Hypocreales: Cordycipitaceae) Vuillemin and *Metarhizium brunneum* Petch (formerly *M. anisopliae*) (Hypocreales: Clavicipitaceae) occur in nature worldwide, and various strains of these fungi are used as active ingredients in formulations of registered biological insecticides (Shah and Pell 2003). Formulations of *B. bassiana* have exhibited pathogenicity against several important species of bark and ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) (Kreutz et al. 2004, Castrillo et al. 2013, Carrillo et al. 2015, Srei et al. 2017), suggesting that entomopathogenic fungi are worthy of consideration as biological control agents for other scolytine beetles.

The walnut twig beetle, *Pityophthorus juglandis* Blackman (Curculionidae: Scolytinae) transmits a fungal associate (*Geosmithia*

morbida Kolařík, Freeland, Utley and Tisserat) (Hypocreales: Bionectriaceae) that causes cankers on walnut (*Juglans* spp.) and wingnut (*Pterocarya* spp.) (Tisserat et al. 2009, Hishinuma et al. 2016). Synergistic activity of both biotic agents causes dieback and mortality in various species of both tree genera (Kolařík et al. 2011, Serdani et al. 2013, Hishinuma et al. 2016, Seybold et al. 2019). This syndrome, known as thousand cankers disease, develops as a consequence of numerous *P. juglandis* brood galleries and subsequent cankers in the phloem caused by *G. morbida* and potentially *Fusarium solani* (Tisserat et al. 2009, Kolařík et al. 2011, Montecchio et al. 2015). Thousand cankers disease and *P. juglandis* have been detected in the eastern United States on black walnut (*Juglans nigra* L.) (Fagales: Juglandaceae) (Seybold et al. 2019), a host with high economic and ecological value (Newton et al. 2009). Because *J. nigra* produces nuts that may be consumed by both humans and wildlife, options to protect live trees from *P. juglandis* attack using systemic or traditional, topically applied synthetic insecticides are limited

(Daniels et al. 2016). Biologically based management options that reduce the population density of *P. juglandis* on individual trees would be useful for minimizing the impacts of thousand cankers disease.

In a preliminary survey in 2011, natural occurrence of *B. bassiana* was detected on 15% of *P. juglandis* adults ($n = 41$) collected from infested *J. nigra* trees in Knox Co., Tennessee that had no history of commercial *B. bassiana* treatments (J. Juzwik, unpublished data). A pilot study was therefore initiated to determine whether application of a commercial formulation of *B. bassiana* could augment the natural rate of fungal infection in *P. juglandis*. The pilot study was followed by a more extensive, 2-yr study which demonstrated that applications of two entomopathogenic fungi (*B. bassiana* strain GHA and *M. brunneum* strain F52) significantly reduced the number of attacks, brood production, and emergence of *P. juglandis* from treated *J. nigra* bolts (Castrillo et al. 2017). Here, we report the results of the pilot study that evaluated *P. juglandis* colonization, emergence, and *B. bassiana* infection rates in bolts sprayed with 1) a commercial formulation of *B. bassiana* strain GHA, 2) a synthetic insecticide registered for bark beetle stem treatments (permethrin), and 3) water only (control).

Materials and Methods

In June 2013, 54 bolts (60 cm long, mean [SE] diameter 10.9 [0.2] cm) were cut from healthy, asymptomatic *J. nigra* trees in a plantation in McDowell County, NC, 113 km east of any known *P. juglandis* infestations or trees symptomatic of thousand cankers disease. Thousand cankers disease was first detected in North Carolina in 2012, but has not been detected outside of the northwestern portion of Haywood County (North Carolina Forest Service 2017). Bolts were arranged into three groups of 18 bolts of equal group mean diameter ($F = 0.10$; $df = 2,53$; $P = 0.906$), and each group was randomly assigned to one of three spray treatments: 1) BotaniGardES (a.i. *B. bassiana* strain GHA 11.3%; Laverlam International Corp., Butte, MT), 2) Astro Insecticide (a.i. permethrin 36.8%, FMC Corp., Philadelphia, PA), and 3) control (water only). Prior to treatment, the viable *B. bassiana* conidial content of BotaniGardES was 8.6×10^9 conidia per ml, estimated by plating 100 μ l aliquots of a 10^{-2} dilution of the stock suspension on 0.25 strength Sabouraud dextrose agar with yeast extract. For bolt treatment, BotaniGardES was diluted in tap water (946 ml product in 7.6 liters total volume) producing a suspension containing 1.08×10^9 *B. bassiana* conidia per ml. Astro was diluted in tap water (9.46 ml product, 7.6 liters total volume) producing a solution containing 4.8 mg permethrin per ml. Bolts were sprayed by using a pressurized, hand operated tank sprayer delivering approximately 10 ml per second to the point of runoff, and delivering approximately 1.85×10^8 *B. bassiana* conidia and 0.83 mg permethrin per cm^2 of bark surface, respectively. Control bolts were sprayed with tap water only. Spray times per bolt (range 23–45 s) were calibrated by 1) spraying three practice bolts to the point of runoff and calculating the average surface area (cm^2) covered by 1 ml of spray (bolt surface area/tank fluid volume loss), 2) dividing the surface area of each treatment bolt by this value to determine the target spray volume, and 3) dividing the target spray volume by the sprayer delivery rate.

After treatment, one *P. juglandis* pheromone lure (product #300000736, Contech Enterprises Inc., Delta, BC) was stapled to the midpoint of each bolt to attract flying beetles, and a white adhesive card (7.6 \times 17.5 cm, AlphaScents Inc., West Linn, OR) was attached to the opposite side to assess *P. juglandis* landing rates (estimate of beetle pressure). One cut end of each bolt was fitted with a steel screw eye, which was attached to a rope and raised into the crown

of a live, infested *J. nigra* tree. Three bolts per tree (one of each treatment) were raised into three infested *J. nigra* trees at each of six sites in Knox County, TN (18 tree replicates, 54 bolts total). Within each tree, bolts of different treatments were randomly assigned to spots within the crown, separated by ≥ 3 m and hung 5–8 m above ground. On days 10 and 19 post-deployment, bolts were lowered, adhesive cards were replaced, and the number of *P. juglandis* per card were counted. Coincident with adhesive card replacements, bolts in the *B. bassiana* treatment only were re-sprayed with the BotaniGardES suspension, based on label recommendation to re-treat on 5–10 d intervals (permethrin and control bolts were not re-treated).

After 28 d, each 60 cm bolt was returned to the laboratory at the University of Tennessee, Knoxville, and cut into two 30 cm pieces. One piece was randomly assigned to a ventilated rearing container (described in Mayfield et al. 2014) and the dry collection cups were checked daily for adult *P. juglandis* emergence for 10 wk. Emerged adults were placed individually into 1.5-ml microcentrifuge tubes, stored at 10°C, and shipped every 5 d to the USDA Forest Service Northern Research Station laboratory in St. Paul, MN. Each adult was plated on dodine wheat germ agar (DWGA) in 100 mm dia Petri plates and incubated at 24°C. Ingredients of DWGA were 1,000 ml aqueous extract of 30 g wheat germ, 0.25 g chloramphenicol, 0.3 g dodine (65% *n*-dodecyl-guanidine acetate), 10 mg crystal violet, and 15 g granulated agar per 1 liter agar. Presence of *B. bassiana* sporulation on each beetle cadaver was determined after 5, 10, and 15 d. The fungus was identified based on colony morphology and microscopic features (conidia and conidiophores; Dugan 2006) and compared with those of reference cultures. The other 30-cm piece was peeled of its bark and assessed for *P. juglandis* nuptial chambers, egg galleries, adults, and larvae. Numbers of nuptial chambers and egg galleries observed were recorded. Length of larval tunnels emanating from egg galleries were measured by using a Scalex MapWheel (Scalex Corp., Carlsbad, CA) and summarized by bolt. *P. juglandis* larvae and adults (not exceeding 24 specimens of each type per bolt) were plated on DWGA and assessed for *B. bassiana* sporulation as described above.

Mixed-model analysis of variance (ANOVA) was conducted by using the Fit Model procedure in JMP 14.0.0 (SAS Institute Inc. 2018) to test whether means of the following *P. juglandis* variables differed by treatment: adults trapped per week on adhesive cards, number of nuptial chambers and egg galleries per peeled bolt, total length of larval tunnels per bolt, and number of adults emerged per containerized bolt. Treatment and tree (i.e., the infested *J. nigra* in which bolts were hung) were modeled as fixed and random effects, respectively. Models were fit by using a Standard Least Squares approach and the Restricted Maximum Likelihood (REML) method. A contingency table analysis was conducted by using the Fit Y by X procedure in JMP 14.0.0 to test whether the control and *Beauveria* spray treatments differed in the proportion of *P. juglandis* beetles infected with *B. bassiana* post-treatment. Separate analyses were performed for adults emerged from containerized bolts, and adults and larvae extracted from peeled bolts. Bolts treated with permethrin were not colonized and so were excluded from contingency analyses.

Results and Discussion

The number of *P. juglandis* adults trapped on the adhesive sticky cards did not differ by spray treatment (Table 1), indicating that the treatments did not affect the attractiveness of the bolts in the field. No *P. juglandis* were found within the phloem peeled from bolts treated with permethrin (Astro), and with the exception of one adult recovered from one collection cup, no emergence was detected from

Table 1. Mean (SE) and ANOVA statistics comparing *Pityophthorus juglandis* landing rate, colonization, and emergence variables for *Juglans nigra* bolts ($n = 18$ per treatment) subjected to different bark spray treatments in Knox County, TN, 2013

<i>P. juglandis</i> response variable	Spray treatment				<i>F</i>	df	<i>P</i> -value
	Permethrin	<i>Beauveria</i>	Control				
Adults trapped per week on adhesive cards	23.6 (6.6)	19.1 (4.8)	22.1 (4.4)		0.50	2,34	0.611
Nuptial chambers per peeled bolt	0.0 (0.0) ^a	23.2 (3.8)	22.2 (2.9)		0.06	1,17	0.805
Egg galleries per peeled bolt	0.0 (0.0) ^a	72.4 (12.9)	57.0 (8.1)		1.39	1,17	0.255
Larval tunnel length (cm) per peeled bolt	0.0 (0.0) ^a	98.5 (22.3)	85.7 (22.9)		0.38	1,17	0.547
Adults emerged per containerized bolt	0.05 (0.05) ^a	73.3 (22.3)	65.3 (22.9)		0.07	1,17	0.798

^aThe permethrin treatment (Astro insecticide) was excluded from ANOVAs for beetle colonization and emergence variables because means and variances in this treatment were nearly or equal to zero. For these variables, summary statistics reflect the comparison between the *Beauveria* (BotaniGardES, *B. bassiana* strain GHA) and control (water) treatments only.

Table 2. *Beauveria bassiana* (*Bb*) infection rates in *P. juglandis* beetles emerged from containerized *J. nigra* bolts ($n = 18$ per treatment), and extracted from peeled bolts, for *Beauveria* and control spray treatments applied in Knox County, TN, 2013

Response variable	Total no. <i>P. juglandis</i> beetles assessed		No. beetles <i>Bb</i> positive		Infection rate (percent <i>Bb</i> positive)		χ^2	Prob > χ^2
	<i>Beauveria</i>	Control	<i>Beauveria</i>	Control	<i>Beauveria</i>	Control		
Adults emerged (containerized bolts)	764	691	476	171	62.3%	24.7%	207.3	<0.001
Adults extracted (peeled bolts)	345	338	149	9	43.2%	2.7%	157.7	<0.001
Larvae extracted (peeled bolts)	172	378	18	1	10.5%	0.3%	36.9	<0.001

The Pearson chi-square (χ^2) statistic was used to test the null hypothesis of no difference between the *Beauveria* (BotaniGardES, *Bb* strain GHA suspension) and control (water) spray treatments in the proportion of beetles infected with *B. bassiana*.

permethrin-treated bolts (Table 1). Because collection cups were positioned at the bottom of the emergence containers, it is likely that this beetle was killed after contact with permethrin and dislodged from the bark after placement in the container. Although effective for preventing *P. juglandis* colonization of *J. nigra* in this study, Astro insecticide (permethrin) is not labeled for use on commercial fruit and nut trees (FMC Corp. 2016) and is a broad-spectrum insecticide that poses risks to aquatic ecosystems and pollinators if improperly applied (United States Environmental Protection Agency (US-EPA) 2009). Therefore, permethrin formulations may not be viable options for protecting *J. nigra* trees in certain circumstances.

In contrast, *P. juglandis* readily colonized and initiated reproduction in both the *Beauveria*-treated (BotaniGardES) and control bolts. The mean length of larval tunnels and the mean numbers of nuptial chambers, egg galleries, and adult *P. juglandis* emerged per bolt did not differ between the *Beauveria* treatment and the control (Table 1). However, *B. bassiana* infection rates were greater in *P. juglandis* larva and adults recovered from *Beauveria*-treated bolts than from control bolts (Table 2). Beetles infected with *B. bassiana* in the control treatment likely represented naturally infected beetles, but it is also possible that some flying *P. juglandis* adults visited *Beauveria*-treated bolts in the field before visiting control bolts. We maximized the distance between bolts within tree crowns to minimize this possibility.

The voltinism of *P. juglandis* in eastern TN is uncertain but is presumed to include at least two overlapping generations per year; adults and larvae are known to overwinter and adult flight occurs primarily from late spring through the fall (Nix 2013, Daniels et al. 2016). Because *P. juglandis* can complete a generation within 7 wk (Tisserat et al. 2009), emerging adults in this study may have included both founder adults that attacked the bolts in the field and some adults of the next generation that completed development in

the bolt. Although the *Beauveria* treatment in this experiment had no significant effect on total *P. juglandis* emergence, a subsequent study showed that application of BotaniGardES drastically reduced the number of adults emerging from treated bolts over a 5-mo period (Castrillo et al. 2017). Our emergence results may have differed from those of Castrillo et al. (2017) either because emergence monitoring ended too early to reveal treatment differences, or due to a longer period of treated bolt exposure to sunlight in the field. Viability of *B. bassiana* conidia is reduced by exposure to ultraviolet light (Ingliš et al. 1995) and despite our retreatment of bolts in the field every 9–10 d, the bolts may have been subject to sufficient sunlight during the 28-d field exposure to reduce conidial viability. Nonetheless, our results support those of (Castrillo et al. 2017) in demonstrating that commercial applications of *B. bassiana* strain GHA can augment natural entomopathogen infections in the field, and deserve continued exploration as biologically based, environmentally acceptable alternatives to synthetic insecticides in the management of *P. juglandis* and thousand cankers disease.

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References Cited

- Carrillo, D., C. A. Dunlap, P. B. Avery, J. Navarrete, R. E. Duncan, M. A. Jackson, R. W. Behle, R. D. Cave, J. Crane, A. P. Rooney, *et al.* 2015. Entomopathogenic fungi as biological control agents for the vector of the laurel wilt disease, the redbay ambrosia beetle, *Xyleborus glabratus* (Coleoptera: Curculionidae). *Biol. Control* 81: 44–50.
- Castrillo, L. A., M. H. Griggs, and J. D. Vandenberg. 2013. Granulate ambrosia beetle, *Xylosandrus crassiusculus* (Coleoptera: Curculionidae), survival and brood production following exposure to entomopathogenic and mycoparasitic fungi. *Biol. Control* 67: 220–226.
- Castrillo, L. A., A. E. Mayfield III, M. H. Griggs, R. Camp, B. Mudder, A. Taylor, and J. D. Vandenberg. 2017. Mortality and reduced brood production in walnut twig beetles, *Pityophthorus juglandis* (Coleoptera: Curculionidae), following exposure to commercial strains of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium brunneum*. *Biol. Control* 114: 79–86.
- Daniels, D. A., K. A. Nix, P. A. Wadl, L. M. Vito, G. J. Wiggins, M. T. Windham, B. H. Ownley, P. L. Lambdin, J. F. Grant, P. Merten, *et al.* 2016. Thousand cankers disease complex: a forest health issue that threatens *Juglans* species across the US. *Forests* 7: 260.
- Dugan, F. M. 2006. The identification of fungi – an illustrated introduction with keys, glossary and guide to the literature. The American Phytopathological Society, APS Press, St. Paul, MN, p. 176.
- FMC Corp. 2016. Astro® insecticide label. FMC Corp., Philadelphia, PA.
- Hajek, A. E., and L. S. Bauer. 2007. Microbial control of wood-boring insects attacking forest and shade trees, pp. 505–525. In L. A. Lacey, H. K. Kaya (eds.), *Field manual of techniques in invertebrate pathology*. Springer, Dordrecht, Netherlands.
- Hishinuma, S. M., P. L. Dallara, M. A. Yagmour, M. M. Zerillo, C. M. Parker, T. V. Roubtsova, T. L. Nguyen, N. A. Tisserat, R. M. Bostock, M. L. Flint, *et al.* 2016. Wingnut (Juglandaceae) as a new generic host for *Pityophthorus juglandis* (Coleoptera: Curculionidae) and the thousand cankers disease pathogen, *Geosmithia morbida* (Ascomycota: Hypocreales). *Can. Entomol.* 148: 83–91.
- Inglis, G. D., M. S. Goettel, and D. L. Johnson. 1995. Influence of ultraviolet light protectants on persistence of the entomopathogenic fungus, *Beauveria bassiana*. *Biol. Control* 5: 581–590.
- Kolařík, M., E. Freeland, C. Utley, and N. Tisserat. 2011. *Geosmithia morbida* sp. nov., a new phytopathogenic species living in symbiosis with the walnut twig beetle (*Pityophthorus juglandis*) on *Juglans* in USA. *Mycologia*. 103: 325–332.
- Kreutz, J., O. Vaupel, and G. Zimmermann. 2004. Efficacy of *Beauveria bassiana* (Bals.) Vuill. against the spruce bark beetle, *Ips typographus* L., in the laboratory under various conditions. *J. Appl. Entomol.* 128: 384–389.
- Mayfield, A. E., S. W. Fraedrich, A. Taylor, P. Merten, and S. W. Myers. 2014. Efficacy of heat treatment for the thousand cankers disease vector and pathogen in small black walnut logs. *J. Econ. Entomol.* 107: 174–184.
- Montecchio, L. M. Faccoli, D. Short, G. Fanchin, D. Geiser, and M. T. Kasson. 2015. First report of *Fusarium solani* phylogenetic species 25 associated with early stages of thousand cankers disease on *Juglans nigra* and *Juglans regia* in Italy. *Plant Dis.* 99: 118.
- Newton, L. P., G. Fowler, A. D. Neeley, R. A. Schall, and Y. Takeuchi. 2009. Pathway assessment: *Geosmithia* sp. and *Pityophthorus juglandis* Blackman movement from the western into the eastern United States. United States Department of Agriculture Animal and Plant Health Inspection Service Rev., Washington, DC. 1:10.19.2009, p. 50. https://mda.mo.gov/plants/pdf/tc_pathwayanalysis.pdf.
- Nix, K. A. 2013. The life history and control of *Pityophthorus juglandis* Blackman on *Juglans nigra* L. in eastern Tennessee. M.S. thesis. University of Tennessee, Knoxville, TN.
- North Carolina Forest Service. 2017. Thousand Cankers Disease of Walnut Frequently Asked Questions and Thousand Cankers Disease Tracking Map. https://www.ncforestservice.gov/forest_health/forest_health_thousandcankers.htm.
- SAS Institute Inc. 2018. User guide for JMP® 14.0.0 (64 bit). SAS Institute Inc., Cary, NC.
- Serdani, M., J. J. Vlach, K. L. Wallis, M. Zerillo, T. McCleary, J. Romero-Severson, and N. A. Tisserat. 2013. First report of *Geosmithia morbida* and *Pityophthorus juglandis* causing thousand cankers disease in butternut. *Plant Health Prog.* doi:10.1094/PHP-2013-1018-01-BR.
- Seybold, S. J., W. E. Klingeman III, S. M. Hishinuma, T. W. Coleman, and A. D. Graves. 2019. Status and impact of walnut twig beetle in urban forest, orchard, and native forest ecosystems. *J. For.* 117: 152–163. doi:10.1093/jofore/fvy081.
- Shah, P. A., and J. K. Pell. 2003. Entomopathogenic fungi as biological control agents. *Appl. Microbiol. Biotechnol.* 61: 413–423.
- Srei, N., R. Lavallée, and C. Guertin. 2017. Susceptibility of *Dendroctonus simplex* to Hypocreales fungi: towards the development of a biological control strategy. *J. Appl. Entomol.* 141: 487–495.
- Tisserat, N., W. Cranshaw, D. Leatherman, C. Utley, and K. Alexander. 2009. Black walnut mortality in Colorado caused by the walnut twig beetle and the thousand cankers disease. *Plant Health Prog.* doi:10.1094/PHP-2009-0811-01-RS.
- United States Environmental Protection Agency (US-EPA). 2009. Permethrin facts. EPA 738-F-09-001, August 2009. https://www3.epa.gov/pesticides/chem_search/reg_actions/reregistration/fs_PC-109701_1-Aug-09.pdf.