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Walnut Twig Beetle (Coleoptera: Curculionidae: Scolytinae) Colonization of Eastern Black Walnut Nursery Trees

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Abstract

Thousand cankers disease, caused by the invasive bark beetle *Pityophthorus juglandis* Blackman and an associated fungal pathogen *Geosmithia morbida* M.Kolařík, E. Freeland, C. Utley, N. Tisserat, currently threatens the health of eastern black walnut (*Juglans nigra* L.) in North America. Both the beetle and pathogen have expanded beyond their native range via transport of infested walnut wood. *Geosmithia morbida* can develop in seedlings following inoculation, but the ability of *P. juglandis* to colonize young, small diameter trees has not been investigated. This study assessed the beetle's colonization behavior on *J. nigra* nursery trees. Beetles were caged directly onto the stems of walnut seedlings from five nursery sources representing a range of basal stem diameter classes. Seedlings were also exposed to *P. juglandis* in a limited choice, field-based experiment comparing pheromone-baited and unbaited stems. When beetles were caged directly onto stems, they probed and attempted to colonize seedlings across the range of diameters and across sources tested, including stems as small as 0.5 cm in diameter. In the field experiment, beetles only attempted to colonize seedlings that were baited with a pheromone lure and appeared to prefer (though not statistically significant) the larger diameter trees. Despite several successful penetrations into the phloem, there was no evidence of successful progeny development within the young trees in either experiment. Further investigation is recommended to better elucidate the risk nursery stock poses as a pathway for thousand cankers disease causal organisms.

Key words: black walnut, colonization behavior, *Pityophthorus juglandis*, thousand cankers disease

Continued range expansion of the walnut twig beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae), and an associated fungal pathogen, *Geosmithia morbida* M.Kolařík, E. Freeland, C. Utley, N. Tisserat, currently threatens the health of a valuable hardwood lumber and veneer species, eastern black walnut, *Juglans nigra* L. This insect–pathogen complex causes an emerging and potentially fatal disease known as thousand cankers disease (Tisserat et al. 2009, Seybold et al. 2016). Thousand cankers disease can occur in all North American, Eurasian, and South American walnut species and in wingnut from the closely related genus *Pterocarya* (Hishinuma et al. 2016). *Juglans nigra* is particularly susceptible (Tisserat et al. 2011) and is of significant management concern given its economic value (Shifley 2004, Newton and Fowler 2009, Moltzan 2011). The disease results from feeding and gallery formation by the phloeophagous bark beetle, *P. juglandis*, and development of cankers caused by *G. morbida* (Tisserat et al. 2009, Kolařík et al. 2011). Adult *P. juglandis* introduce the pathogen when they enter the phloem of a host tree. *Geosmithia morbida* is not a systemic pathogen and numerous attacks are required to cause mortality of the host (Tisserat *et al.* 2009). Currently, *P. juglandis* is the only known vector of the pathogen (Tisserat *et al.* 2009); however, *G. morbida* has also been recovered from a species of weevil, *Stenomimus pallidus* Boheman, in Indiana (Juzwik et al. 2015) and from two species of invasive ambrosia beetle *Xylosandrus crassiusculus* (Motschulsky) and *Xyleborinus saxeseni* (Ratzburg) (Juzwik et al. 2016). Branch mortality generally progresses downward and inward from the most distal portions of the crown, and dieback is the result of cankers and beetle gallery formation coalescing in the phloem, girdling branches and in severe cases, the main stem (Tisserat et al. 2009).

P. juglandis is historically known from the southwestern United States and northern Mexico (Cranshaw 2011; Seybold et al. 2012b, 2016); however, in recent decades, it has been widely recovered beyond its historic range. The current distribution of the beetle and fungal pathogen encompasses most of the western states (Cranshaw 2011; Tisserat et al. 2011; Seybold et al. 2012b, 2016), and several eastern states within the native range of *J. nigra* including parts of Tennessee (Grant et al. 2011), Pennsylvania, Virginia (Seybold et al. 2012b), North Carolina (Hadziabdic et al. 2014), Ohio (Fisher et al. 2013), Maryland (Seybold et al. 2016), and Indiana (Marshall 2015). Both the beetle and pathogen have also been recovered from a walnut grove in Italy (Montecchio et al. 2014). Given the tremendous distance between and disjunct nature of these detections, human transport of infested walnut wood is strongly suspected as a pathway contributing to the range expansion of these organisms (Newton and Fowler 2009, Turcotte et al. 2013).

Another pathway of concern is the movement of walnut nursery stock (Newton and Fowler 2009). Live plant material represents a significant pathway of global translocations of arthropod pests including subcortical beetles (Haack 2006, McCullough et al. 2006, Hulme et al. 2008, Liebhold et al. 2012). Ports of entry and commercial nurseries are often points of introduction and establishment for non-native bark and ambrosia beetles (Haack 2006, McCullough et al. 2006, Gandhi and Herms 2010, Seybold et al. 2016). Such species can cause significant damage to nursery crops, and are often a management concern (Oliver and Mannion 2001, Adkins et al. 2010).

Currently, nursery stock is a regulated article in state-issued thousand cankers disease quarantines (Newton and Fowler 2009, Moltzan 2011) and once a county has been confirmed positive for the disease, transport of walnut nursery stock out of the area is restricted. Introduction of *P. juglandis* and thousand cankers disease into areas with major nursery growing operations is likely to have economic ramifications (Cranshaw and Tisserat 2011). *Geosmithia morbida* has previously been shown to develop in experimentally-inoculated nursery seedlings of several walnut species including *J. nigra* (Utley et al. 2013), but to our knowledge, there have been no reports of *P. juglandis* infestations in nursery seedlings in the field. To date, no studies have addressed the colonization behavior of *P. juglandis* in young walnut trees. Current knowledge of *P. juglandis* biology suggests that the beetle has a preference for branches ≥ 1.5 cm in diameter, but it has been observed on rare occasion in branch segments of smaller diameters (Seybold et al. 2016). This suggests the beetles could potentially colonize juvenile nursery tree stock.

In order to effectively manage the emerging threat of thousand cankers disease, pathways of introduction and spread of *P. juglandis* and *G. morbida* should be evaluated and understood. The objective of this study was to determine if *P. juglandis* will penetrate and colonize young, small diameter, *J. nigra* nursery trees. In the context of this study, colonization is defined as the processes that occur between adult emergence and oviposition with four distinct phases as defined by Wood (1982): dispersal, selection, concentration, and establishment. A better understanding of *P. juglandis* colonization behavior as it relates to the nursery industry is crucial for managing the threat of thousand cankers disease. Three experiments were designed: one no-choice experiment in which beetles were caged onto stems in a greenhouse; and two choice experiments in which the seedlings were exposed to *P. juglandis* colonization pressure in an open field. We hypothesized that beetles would bore into the bark of the young trees (selection and concentration) and reproduce (establishment), with successful reproduction (i.e., developed and emerged F_1 adults), in the host material under both experimental settings. We also hypothesized the beetles would attack larger diameter (≥ 1.5 cm) seedlings in greater frequency and produce more progeny than in seedlings <1.5 cm in diameter.

Materials and Methods

Nursery Trees

A total of 116 young *J. nigra* trees were procured from five nurseries in five states within the native range of *J. nigra* for two experiments (Table 1). Basal diameters were estimated as the average of two, perpendicular caliper measurements made 1 cm above the root collar for each stem.

All nursery trees were grown from nuts collected from open-pollinated *J. nigra* trees. Trees from the Willis Orchards (GA) and North Carolina Division of Forestry (NC) nurseries were grown in the field and were dug from the field shortly before shipment and kept in walk-in coolers with moistened roots wrapped in plastic prior to shipment. These trees were shipped as bare-root transplants, and upon receipt in Tennessee, were transplanted into 23-liter plastic molded containers (C2800, Nursery Supplies Inc., Kissimmee, FL) using a custom blend of 50% aged pine bark, 20% peat, 20% sand, 5% ash, and 5% perlite mix (Salifu et al. 2006). Stems from GA were dug in December 2013 and shipped to the University of Tennessee (UT) in Knoxville, TN, on December, 31, 2013. Trees were transplanted into 23-liter containers on January 28 and 29, 2014, and kept in a white polyethylene-covered bow house until March 6 when the trees were placed in a greenhouse. The NC stock was picked up from Asheville, NC, on May 22, transplanted on May 23, and placed into the greenhouse on May 30, 2014.

Seedlings from the University of Tennessee (TN), Willoway (OH), and Forrest Keeling (MO) nurseries were all grown in

Table 1. Nursery source, state of origin, and number of containerized *J. nigra* seedlings used in each trial of the greenhouse no-choice and field choice experiments investigating the colonization behavior of *P. juglandis*

	Source	No-choice		Choice	
State	Nursery	Trial 1	Trial 2	Trial 1	Total seedlings
GA	Willis Orchards Cartersville, GA	8	*NA*	*NA*	8
OH	Willoway Nursery Inc. Avon, OH	8	12	22	42
TN	University of Tennessee Department of Plant Sciences Knoxville, TN	4[a]	*NA*	*NA*	4
MO	Forrest Keeling Nursery Elsberry, MO	8	12	22	42
NC	North Carolina Division of Forestry Asheville, NC	8	12	*NA*	20

[a] Only 4 replicates were included from the TN source as a result of poor condition of the tree seedlings. *NA* indicates no replicates from a particular source were included during the corresponding trial.

containers. The Forrest Keeling nursery utilizes a proprietary root production method involving multiple containerized steps (Lovelace 2002). All trees from these sources were in 7.3-liter (TN), 11.4-liter (MO), and 19.6-liter (OH) containers at the time of procurement (C1000S, C1200, and C2100 blow-molded pots, Nursery Supplies Inc.). All trees were placed into the greenhouse at UT by the end of May 2014. The greenhouse bay was maintained between 24 and 30°C, 60–80% RH, and under a ambient photoperiod of 12:12 (L:D) h day length. Although no walnut trees were located within at least 500 m of the greenhouses, all nursery trees were kept in the greenhouse until deployment in the respective experiment to prevent exposure to any non-experimental *P. juglandis*. Seedlings were watered twice a week (960–1600 ml per watering event, based on the diameter of the stem, with the largest trees receiving the most water), throughout the duration of the study.

Greenhouse No-Choice Experiment

Two no-choice trials (trial 1 in July to August 2014 and trial 2 in Sept. 2014) were conducted in a greenhouse under natural light conditions. Temperature was maintained between 24 and 30 °C based on the temperatures associated with observed maximum *P. juglandis* flight activity (Seybold et al. 2012a). Ten beetles were caged onto the stem of each replicate in a similar fashion to Mayfield et al. (2008). Source beetles were reared from *J. nigra* bolts that were hung in trees symptomatic of thousand cankers disease in Knoxville and Maryville, TN. Beetles were grouped by gender using a stereoscope (Seybold et al. 2013), placed into a Petri dish assigned to each replicate, and then refrigerated at 4.5 °C for ≤ 3 d prior to use (Browne 1972). For consistency, a sex ratio of 5F:5M was utilized whenever possible; however, the number of available beetles was limited in trial 1, and several cages received slightly female skewed (+1–2 beetles) sex ratios based on beetle availability. More beetles were available for trial 2 which allowed us to replace dead individuals observed 3 d after the initial introduction. Beetles were replaced in order to keep the exposure to colonizing beetles as consistent as possible at 10 total beetles per stem and were replaced in roughly equal sex ratios.

Beetles were caged onto eight replicates from each of the five sources (except for the TN source in which four trees were excluded for poor health) in trial 1 (36 trees total) and onto 12 replicates from three sources (NC, MO, and OH) in trial 2 (36 trees total) (Table 1). Cages were constructed using 15-cm-long Mylar tubes 4 cm in diameter. Two windows were cut into the tubes and covered with a fine mesh cloth to allow for gas exchange into the cage. Each tube was cut down the length of one side and wrapped around the stem and sealed with packaging tape. Finally, both ends were plugged with 4 cm × 6.5 cm diameter memory foam plugs to fill the space around the stems (Fig. 1A). A middle section was taken out of each plug with a cork borer, using three different diameters, 0.3, 0.5, and 1.0 cm, corresponding to small, medium, and large diameter stems.

Beetles were monitored for 30 s after initial release to ensure they were ambulatory and that the artificial caging arena did not inhibit the beetle's behavior or prevent movement toward the stem. The number of trees with ≥ 1 beetle crawling on the stem within the first 30 s was recorded and suggests that beetles adjusted to the artificial exposure scenario (data not shown). Cages were observed daily for 15 d, the total number of dead and live *P. juglandis*, and all attack holes were recorded (Fig. 1B).

After 15 d of exposure, stems were cut ∼3 cm above and below each plug. Attack holes were observed outside of the caged area on a few stems (within 1–2 cm of the end of the foam plugs), thus the total area analyzed for each stem was increased to reflect this observation. Approximately 27 cm of each stem was analyzed. Plugs were carefully removed over a sheet of white paper to prevent the loss of any beetles, and all dead beetles were counted and sexed. The bark surface was examined and all attack holes were marked and recorded. Phloem width was recorded for each as an average of the top and bottom of each segment. Each stem was placed into 18.9-liter emergence chambers as described by Mayfield et al. (2014).

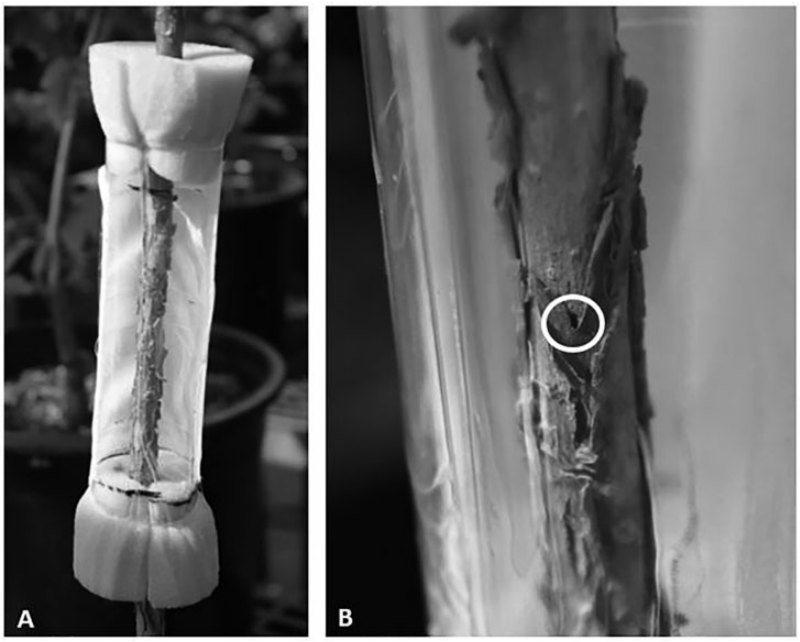

Fig. 1. (A) Example of the cage design implemented on a *J. nigra* seedling in the greenhouse no-choice experiment. (B) Example of an attack hole (inside white circle) as seen through the cage during the no-choice experiment.

Collection cups at the base of the emergence chambers were filled with a small amount of propylene glycol to kill and preserve emerged beetles. Beetles were collected and sexed from the cups every 4 weeks for 20 weeks. Number of progeny (F_1 generation) were determined by subtracting the number of recovered adults from the initial number of parent beetles released. After the emergence period, bark was scraped away around attack holes on each stem with a scalpel blade to reveal beetle galleries. Total gallery lengths were measured using a Scalex MapWheel (Scalex Corp. Carlsbad, CA), and any beetles found were recorded by life stage (larvae, pupae, or adult).

Greenhouse No-Choice Experimental Statistics

The total number of attack holes and presence of attack (presence/absence) were modeled using generalized linear mixed-effect and generalized linear modeling techniques using the lme4 package in R statistical software (R Core Team 2016). Phloem width and basal diameter were used as fixed effects in the models and nursery tree source was modeled as a random factor using the glmer function. In trial 2, the total number of *P. juglandis* per cage was also incorporated as a fixed effect in the models. Attack holes were modeled using a Poisson distribution and the presence of attack was modeled using a binomial distribution. In both trials, the random factor (tree source) had no effect on the models and thus the generalized linear modeling approach was selected. For the number of attack holes, both Poisson and Gaussian distributions were modeled, and in trial 2, one outlier was removed for analysis. Model selection was informed by a combination of the Akaike information criterion (AIC) values and likelihood ratio tests (Zuur et al. 2009).

To compare gallery lengths across trees, the total measured lengths were summed and divided by the total number of attack holes. This value was then modeled using phloem width and basal diameter as fixed effects and the source as a random factor using the lme function and a Gaussian distribution. Again, in trial 2, the total number of beetles caged per tree was also incorporated as a fixed effect. Model selection criteria followed the same procedure as stated previously. The numbers of progeny per stem were not compared as there was no evidence of successful development (see Results). For all relevant tests, P values <0.05 were considered significant.

Field Choice Experiment

A field choice experiment was conducted in 2015 in which containerized trees were exposed to natural populations of *P. juglandis* associated with mature, live *J. nigra* trees instead of infested bolts. Containerized trees of two basal diameter size classes were tested, with the average diameter of the smaller and larger trees blocked to be 1.8 and 2.4 cm, respectively. Four container-grown trees consisting of two trees in each size class were placed beneath each of 11 mature "beetle source" trees distributed across four sites in Knoxville, TN. Each containerized tree was randomly assigned to a cardinal direction and placed 2.5 m from the base of each mature, infested tree beneath the dripline of the crown. For each size class, one containerized tree was randomly assigned a *P. juglandis* pheromone lure that was attached to the main stem, whereas the other tree was left unbaited. The pheromone lure was included as a treatment as a means of assessing whether or not the beetles could be induced to attack young nursery trees. This worst-case scenario was intended to overcome (via pheromone) the case in which the nursery trees might lack a necessary (e.g., visual or olfactory) cue (see Discussion).

The containerized seedlings were deployed in the field from Sept. 11 to Oct. 26, 2015, after which the stems were harvested and placed into emergence chambers as described above. The above-graft stem and enlarged rootstock for each replicate were placed into separate chambers. Emergence was monitored weekly from Oct. 26, 2015 to April 18, 2016. Following emergence monitoring, each stem was removed from the chamber and inspected for holes. Those stems that had holes were dissected and any remaining beetles were recorded, and the total gallery length was measured as described above.

Field Choice Experiment Statistics

A generalized linear mixed-effect model was fit to the number of *P. juglandis* recovered from each stem and the presence of attack (presence/absence) using SAS v. 9.3 statistical software (PROC GLIMMIX) (SAS Institute 2011). Tree basal diameter class, presence of the pheromone, and the interaction were treated as fixed factors in the models. Nursery tree source and location in the field test were treated as random factors in both models. The distribution of the error was either negative binomial or Poisson. Where the interaction was significant, the differences between the two basal diameter sizes were compared separately for each level of the pheromone, presence or absence. Model selection was as described above. The number of holes and gallery lengths were not analyzed (see Discussion). For all relevant tests, P values <0.05 were considered significant.

Results

Greenhouse No-Choice Experiment

In the greenhouse experiment, 92% (33/36) and 61% (22/36) of trees in trials 1 and 2, respectively, had ≥ 1 *P. juglandis* crawling along the stem within 30 s of introduction, indicating the beetles were not inhibited by the conditions inside the cages. In both trials, all but two trees had at least one beetle hole within the first 5 d. In trial 1, new holes were only observed in six trees (17%) beyond day five and in four trees (11%) beyond day 10. In trial 2, new holes from beetles were observed on nine trees (25%) beyond day five and on only two trees (6%) after day 10.

As indicated previously, the random factor of nursery tree "source" had no effect in any of the models for the number of attack holes observed or for the presence of attack in either trial. This factor was not included in any of the subsequent models examined. In trial 1, the model was selected to include the number of attack holes and included basal diameter as the only predictor variable yielding a Gaussian distribution (Table 2). This model indicated that basal diameter was not a variable that yielded significant prediction capability ($t_{35} = 1.493$; $P = 0.145$), of the number of attack holes observed. The final model for predicting the presence of attack on each tree in trial 1 also included basal diameter as the only predictor variable and was fitted with a binomial distribution. In this case, basal diameter was a significant predictor ($Z_{35} = 2.389$, $P = 0.017$), indicating that as basal diameter increases, the probability of attack increases. In other words, the predicted probability of attack given a basal diameter equal to 2 cm is approximately 98%.

In trial 2, one tree from the Ohio nursery source, which had a basal stem diameter of 1.83 cm, yielded a total of 12 entry holes: as this individual tree was an extreme outlier in the models, this individual tree was thus removed from subsequent analyses. Absent this outlier, the only predictor in the final model was the total number of beetles released per cage when modeled with a Poisson distribution

Table 2. Summary statistics for generalized mixed effects models predicting *Pityophthorus juglandis* presence of attack, attack holes, and gallery lengths for the 2014 no-choice experiment. Values in bold indicate statistical significance at $\alpha = 0.05$

Trial	Response variable	Fixed effects variable(s)	df	*Z* value	*t* value	SE	*P* value
Trial 1	Presence of attack	Basal diameter	35	**2.389**		1.379	**0.017**
	Attack holes	Basal diameter	35		1.490	0.954	0.145
	Gallery length/hole	Basal diameter	29		0.487	2.519	0.630
		Phloem width	29		−0.396	11.493	0.695
Trial 2	Presence of attack	Basal diameter	35	**2.389**		1.379	**0.017**
	Attack holes	Total num. beetles	34	**−2.358**		0.073	**0.018**
	Gallery length/hole	Basal diameter	30		0.117	4.156	0.907
		Phloem width	30		0.338	28.741	0.738
		Total num. beetles	30		−1.315	0.537	0.199

(Table 2). The total number of beetles was a significant variable ($Z_{34} = -2.358$, $P = 0.018$) in the model and actually had a negative coefficient of -0.17, which indicates a slight decrease in the number of holes predicted as the number of beetles introduced increases. The model selected for the presence of attack in trial 2 included basal diameter as the only predictor variable when fit with a binomial distribution, and basal diameter was again a significant predictor of attack ($Z_{35} = 2.389$, $P = 0.017$). As with trial 1, basal diameter again yielded a positive correlation with the presence of attack, wherein the probability of attack approached one when basal stem diameters were 2 cm and greater.

The selected model for the total gallery length per attack hole in both trials included the nursery source as a random factor and all fixed effects tested in each trial. In both cases, none of the fixed effects were significant (Table 2). Total gallery length per attack hole appears to be independent of the variables examined in this experiment.

Emergence in trial 1 and trial 2 did not exceed the number of adults introduced (F_0 generation) for any replicate. Had the F_0 generation successfully reproduced within the material, we would expect to see more emerged adults than the number of adults exposed to the stem (after accounting for the dead adults that were recovered). This measure may be confounded in that many beetles were unaccounted for at the conclusion of the experiments. A total of 231 of 360 (64%) beetles were not accounted for in trial 1 and 123 of 539 (23%) beetles were not accounted for in trial 2. Beetles likely escaped by wedging themselves between the foam plug and the bark or between the foam plug and the cage wall. Several individuals were observed escaping via the latter method. Excavation of the bark confirmed the lack of F_1 development. Gallery excavations yielded a total of 15 larvae from only three trees. Twelve larvae (80%) were recovered from a single tree; a tree that also had the most attacks (12 holes) and had the longest gallery length.

Field Choice Experiment

P. juglandis probed or penetrated the bark of six of the 44 (13.6%) nursery trees exposed to colonization pressure in the field experiment, and at least one tree attacked at each of the four sites (Table 3). Five of the six penetrated trees were in the larger stem diameter group, and all six of the attacked trees were baited with a pheromone lure (Fig. 2). The generalized linear mixed-effects modeling revealed that the interaction between the two basal diameter sizes and the presence of the pheromone was highly significant ($F_{1,20} = \text{Infinity}$, $P < 0.001$). For the smaller diameter trees, the presence of the pheromone significantly increased the presence of attack ($F_{1,10} = \text{Infinity}$, $P < 0.001$). Conversely, for the larger diameter

Table 3. Site locations, number of nursery trees attacked, number of *P. juglandis* emerged, and maximum time for emergence for *J. nigra* nursery stock field choice experiment in Knoxville, TN, conducted from Sept. to Oct. 2015

Location	Seedlings[a]	Seedlings with holes	Beetles emerged	Max. time (wk) for beetle emergence
36.095 °N, −83.909 °W	4	1	4	21
36.080 °N, −83.858 °W	16	1	2	10
35.972 °N, −83.992 °W	8	1	1	6
35.822 °N, −84.146 °W	16	3	23	21

[a] Containerized *J. nigra* seedlings deployed such that a group of four trees were spaced evenly around a single mature *J. nigra* known to be infested with *P. juglandis*.

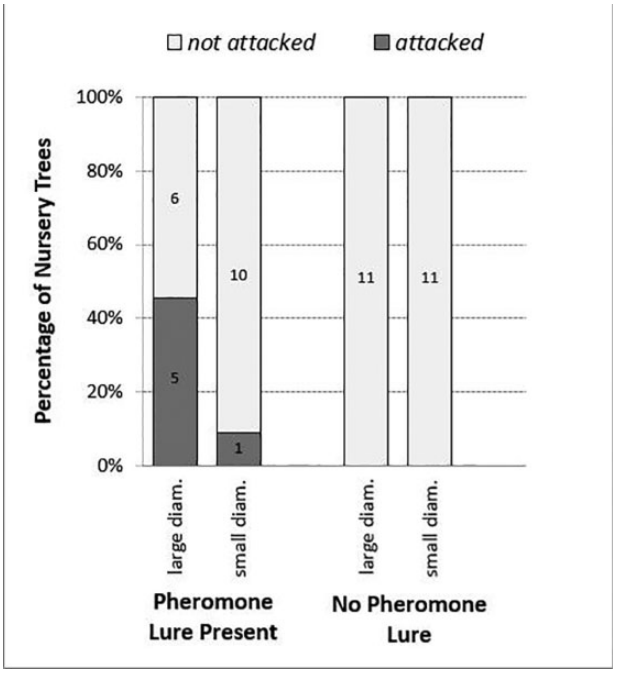

Fig. 2. Percentage (y-axis) and absolute number (within bars) of *J. nigra* seedlings attacked and not attacked by *P. juglandis* when placed beneath infested trees in Knoxville, TN (Sept. to Oct. 2015), by average diameter size class (large = 2.4 cm, small = 1.8 cm) and presence of pheromone lure.

trees, the presence or absence of the pheromone did not significantly affect the presence of attack ($F_{1,10} = 0.00$, $P = 0.995$). For both trees with the pheromone and without the pheromone, the two basal diameter sizes did not significantly affect the presence of attack either ($F_{1,10} = 3.07$, $P = 0.111$ and $F_{1,10} = 0.00$, $P = 0.999$, respectively).

A total of 30 *P. juglandis* were recovered from stems placed into emergence chambers. Adult beetles were recovered from the emergence chambers ranging from 6 to 21 wk post harvest (Table 3). Most of the beetles (90%) were recovered from the larger diameter trees, and 19 (63%) of those beetles came from a single tree. As with the presence of attack, the model for the total number of *P. juglandis* recovered revealed that the interaction between the two basal diameter sizes and the presence of the pheromone was highly significant ($F_{1,20} = \text{Infinity}$, $P < 0.001$). For the smaller diameter trees, the presence or absence of the pheromone did not significantly affect the number of beetles recovered ($F_{1,10} = 0.00$, $P = 0.999$). Similarly, for the larger diameter trees, the presence or absence of the pheromone did not significantly affect the number of beetles recovered either ($F_{1,10} = 0.00$, $P = 0.995$). When the two basal diameter sizes were compared for the trees with the pheromone there was evidence that the larger basal diameter increased the number of *P. juglandis* recovered although the trend was not statistically significant ($F_{1,10} = 4.87$, $P = 0.052$). Neither of the two diameter sizes significantly affected the number of beetles recovered when compared for trees without the pheromone ($F_{1,10} = 0.00$, $P = 0.999$). The bark of the six trees was dissected to measure total gallery lengths; however, all observed entrance holes yielded a depth of ≤ 3.0 mm into the phloem and thus lengths were not measured.

Discussion

In the context of Wood's (1982) definition of bark beetle host colonization, when we limited the beetles by caging them onto the stems of nursery trees, we were able to induce the selection phase on the young small diameter trees. However, under the slightly more realistic condition of field choice experiment 2, *P. juglandis* initiated colonization (dispersal and selection phases) only on trees baited with a pheromone lure, an artificial signal pertinent to the concentration phase. No successful establishment was observed in any of the experiments, despite some evidence of limited—and ultimately unsuccessful—larval development in the largest diameter class of trees tested in the no-choice experiments. Conversely, there was no evidence of reproduction by the colonizing adults even in the largest diameter trees tested in the field-based experiments.

Greenhouse No-Choice Experiment

P. juglandis probed and penetrated young, small diameter *J. nigra* nursery stock when caged directly onto stems in a no-choice exposure experiment. At least one replicate from each of the four diameter classes and from each of the nursery sources tested was attacked in both trials. In part because mean stem diameters differed across the nursery sources, the effects of seedling origin on *P. juglandis* colonization attempts could not be discerned in this experiment. Beetle penetrations were more pronounced on the larger diameter stems, as evident by an increased probability of tunneling as basal diameter increased. This observation is consistent with other reports of a greater rate of *P. juglandis* attack on branch diameters larger than 1.5 cm (Seybold et al. 2016) or 2 cm (Tisserat et al. 2009). Both generalized linear models predicted that the probability of colonization attempts increased to nearly 100% as stem basal diameter approached and exceeded 2 cm.

Interestingly, the phloem width was not a significant of a predictor of attempted colonization by *P. juglandis* as was expected, given that the phloem comprises the reproductive resource required by the beetles. It is possible that outer bark thickness, which was not quantified in this study, may be a more limiting factor in the colonization success than phloem width. Even the smallest trees that had the thinnest phloem (Fig. 3) appear to have had sufficient phloem thickness to allow for some gallery formation; however, the outer bark was split open (Fig. 4), apparently as a result of the tunneling action of the beetles that likely contributed to subsequent desiccation of the phloem and death of the beetles.

Despite replacing dead beetles with new live beetles during trial 2, thus effectively increasing the total number of *P. juglandis* to which each stem was exposed, the smaller diameter stems were less likely to be attacked. This observation provides further evidence that *P. juglandis* colonization behaviors are more likely to occur on larger diameter stems.

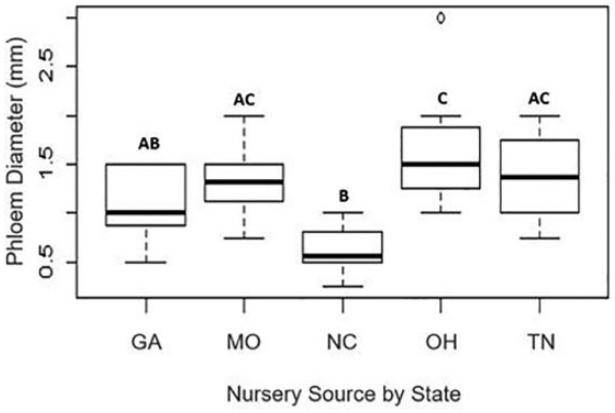

Fig. 3. Comparisons of the mean (±SD) phloem widths (mm) of the *J. nigra* nursery trees from the greenhouse no-choice experiments compared by nursery source. Different letters indicate different means based on Tukey's HSD tests ($\alpha = 0.05$).

Fig. 4. Observed splitting of *J. nigra* outer bark on a seedling with two adult *P. juglandis* found dead in the tunnel. Photograph was taken prior to gallery excavation with a scalpel.

Contrary to our expectations, the mean number of colonization attempts did not differ among the basal diameters tested in either no-choice trial. The large variation in number of observed holes and tunneling on individual stems may help explain the apparent lack in response to basal diameter size. For instance, in all of the replicates with basal diameters > 1.35 cm in trial 1 and > 1.5 cm in trial 2, which were positive for colonization attempts, the number of holes ranged from 1 to 10 and 1 to 12 holes, respectively.

Similarly, no significant differences were found in the mean gallery lengths per hole among the trees tested. We expected to see longer mean lengths in the larger diameter stems, coinciding with the observed increased rate of attack response on the larger stems. Instead, it appeared as though once a given beetle responded to a stimulus triggering colonization behaviors, the total gallery length remained consistent, irrespective of stem diameter. An interesting observation from both trials was that several of the holes on the largest stems yielded short tunnels, apparently abandoned galleries, ranging in length from < 1 to 3 mm. Such instances may constitute evidence of "tasting" or gustation probing (Raffa and Berryman 1982, Wood 1982, Graves 2008) by *P. juglandis* as a means of assessing host suitability, yet could serve as introduction points for *G. morbida* infection. It is unclear why so many of these holes occurred on the largest diameter stems but did not appear on the smaller stems.

There was no evidence of successful reproduction in any of the replicates tested. All larvae found appeared to be dead (Fig. 5), and no pupae were recovered. Several dead adults from the F_0 generation (initial colonizers) were also found dead inside their galleries. During the experiment, the outer bark of several small-diameter, thin-barked nursery trees was observed splitting behind excavating adults (Fig. 4) from several of the smaller diameter seedlings with thin outer bark. The observed bark splitting likely contributed to phloem desiccation, thus may have contributed to prematurely death of adults, developing larvae, and eggs. Wagner et al. (1979) revealed that larvae of *Dendroctonus frontalis* Zimmerman were susceptible to delayed development and mortality when phloem moisture content decreased during larval development. This observation is consistent with a previous study in which *P. juglandis* attacked samples of kiln dried bark but did not produce any offspring in the material (Audley et al. 2016). Harvesting the small stems likely also contributed to the rapid desiccation of phloem as stems and limbs of smaller diameters lose moisture faster than larger diameter material (Hayes et al. 2009, Nicholls and Brackley 2009).

Fig. 5. Desiccated *P. juglandis* larvae (inside white circle) found via gallery excavation of a *Juglans nigra* seedling from the no-choice assay. Bark was carefully peeled back using a scalpel blade.

Although beetles did not successfully reproduce in the nursery stock, it appears the method for monitoring emergence may have influenced that success, at least among the larger diameter seedlings. Beetle reproduction in nursery stock should be further tested with the trees kept intact throughout the duration of the experiment to better control for phloem desiccation. Knowledge of *P. juglandis* fecundity within nursery hosts could better inform management options for thousand cankers disease in a nursery setting. A second issue regarding this experiment was the number beetles that escaped the enclosure, especially in the first trial. In addition to improving conditions during emergence monitoring, future work could improve the method in which beetles are caged onto the stems in order to reduce the incidence of escape.

Field Choice Experiment

Successful colonization efforts by beetles were recorded from seedlings in the field choice experiment, when containerized trees were exposed to infested mature trees. However, only trees baited with the pheromone lure were penetrated. This condition simulates a worst-case scenario, in which *P. juglandis* beetle(s) had already initiated colonization in the area and were subsequently emitting aggregation pheromone to attract additional beetles. Although there appears to be a significant trend of increased colonization on the larger diameter, pheromone lure baited trees (Fig. 2), the power of the statistical models was heavily influenced by the large number of zeros and small number of counts within the data. These results were likely affected by a relatively low number of replicates and perhaps by declining populations of *P. juglandis* at the field sites tested. Recovery of the beetle across eastern Tennessee locations has generally been declined in each season from 2013 through 2016 (J.A., W.E.K., personal observation).

Without the assistance of the lure containing male-produced aggregation pheromone, young, small diameter trees may not have provided a sufficient visual cue (Kogan 1994, Mayfield and Brownie 2013) or the necessary kairomones required by beetles to enable host detection (Wood 1982, Kogan 1994, Bruce et al. 2005). Volatile organic compounds that function in host recognition by some bark beetles (Byers 1996, Bruce et al. 2005) can change in composition and profile depending on tree age (Adams and Hagerman 1976, Nunes and Pio 2001, Pallardy 2010). It is entirely possible that *P. juglandis* may not register *J. nigra* nursery trees of this size range as potential hosts within the landscape, despite our observation of colonization attempts on seedlings in a no-choice setting. Forcing beetles onto the stem may have circumvented some host detection stimuli that was not presented by the young trees.

Our results suggest there is a low likelihood of *P. juglandis* colonizing young, nursery walnut trees in field settings; however, we recommend further investigation before taking steps to remove nursery stock from the list of regulated articles in state quarantines for thousand cankers disease. Given that we were able to induce a small number of colonization attempts on containerized nursery trees when baited with a pheromone lure, other scenarios that could increase the attractiveness of young trees (e.g., successful colonization of water stressed or mechanically wounded trees) should be considered and tested. Although there was no evidence of reproduction, it is worth noting that the colonizing adults remained on or within the bark of the baited nursery trees for 6–21 wk after collection and placement in rearing containers (Table 3). Given the shallow depth of the excavated galleries, it appears the beetles may have

entered winter diapause on the young tree sections, in part explained because the emergence chambers were stored in a self-storage unit with minimal climate control, and thus, the winter temperatures (outside) may have been sufficient enough to influence the climate inside the storage unit, triggering a diapause behavior. Whether or not the beetles remained active or truly in a diapause state, the length of time an individual adult can spend in a young tree is of potential concern to managing the spread of *P. juglandis* and thousand cankers disease. Beetle colonization attempts were best explained by basal diameter, with the larger diameter stems more likely to be probed and penetrated than smaller diameter stems. Even without complete colonization, if young trees can provide a temporary refuge to *P. juglandis* adults, beetles could be unintentionally spread through a nursery stock pathway.

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