

## ABSTRACT

GRESHAM, SEAN DANIEL MOREHU. Association of Xyleborine Ambrosia Beetles and Phytopathogens with Declining Apple Trees in North Carolina. (Under direction of Drs. Sara Villani and James Walgenbach)

Exotic ambrosia beetles from the tribe Xyleborini beetles (Coleoptera: Scolytinae), *Xylosandrus crassiusculus* (Motschulsky) and *X. germanus* (Blandford) are of increasing concern to commercial tree production in eastern North America. Surveys of apple orchards in North Carolina in 2017 experiencing rapid apple decline (RAD) symptoms (high mortality rates of young apple trees without documented known causes) found that ambrosia beetle attacks were associated with dead and declining trees (Ch. 1). Three species were found within declining trees: *Xylosandrus crassiusculus*, *X. germanus*, and *Xyleborinus saxesenii* (Coleoptera: Scolytinae, Xyleborini). These beetles are attracted to physiologically stressed trees that produce ethanol as main attractant, they burrow into the trees where they cultivate their nutritional symbiotic fungus on which they and their offspring feed upon. In addition, fungi were isolated from beetle gallery and non-gallery tissue on declining trees, including opportunistic phytopathogenic organisms such as *Botryosphaeria*, *Fusarium*, and *Diaporthe* spp.

Ethanol-baited traps at apple orchards showed that *Xylosandrus germanus* was dominant in OH, and that *X. germanus* and *X. crassiusculus* were both common in VA and NC. Traps placed at orchard edge bordering woods captured more beetles than traps within the interior. In 2019 and 2020, ethanol-drenched potted apple trees were deployed in two NC apple orchards for 14 day periods. The majority of entries occurred in May and June, and were almost all identified beetles within trees were *X. crassiusculus*, *X. germanus*, or *Cnestus mutilatus*. In 2018 and 2019, the efficacy of insecticides was tested in field trials; neither soil nor foliar-applied neonicotinoid insecticides provided significant control, trunk-applied synthetic pyrethroids provided variable control, and an insecticide-impregnated net provided a high level of control.

The diversity of fungi associated with Xyleborine ambrosia beetles attacking declining apple trees was investigated further. A total of 1229 isolates were collected from beetle galleries in ethanol-drenched potted apple trees deployed in orchards, 335 isolates from 153 Xyleborine ambrosia beetles trapped at orchards, and 470 isolates from non-attacked, non-declining apple trees in commercial orchards. *Fusarium*, *Alternaria*, and *Trichoderma* spp. were commonly isolated across all sample types. *Botryosphaeria dothidea* was common on non-attacked apple trees, less common in galleries and not isolated from beetles. The aggressiveness of selected isolates were tested on detached apple shoots and apple seedlings. The most common isolates found across all samples (*Ambrosiella* spp. and *F. solani*) were not highly aggressive, while the most aggressive isolates were rare or absent on beetles. Finally, *in vitro* assays to assess the relative effect of ethanol on growth of selected isolates of *Ambrosiella*, *Botryosphaeria*, *Fusarium*, and *Trichoderma* spp. showed that *Ambrosiella* spp. were more tolerant to ethanol compared with the other isolates, but in confrontation assays, amending the media with ethanol did not provide a significant advantage to the *Ambrosiella* spp. isolates.

Fire blight, caused by the bacteria, *Erwinia amylovora*, is a devastating disease of apple. Laboratory and field experiments showed that infection by *E. amylovora* elicits an increase in ethanol production within the infected apple tree, which can attract and induce colonization by Xyleborine ambrosia beetles. However, the increased ethanol concentration appeared to be localized to infected tissue and did not induce high frequency of attack by beetles.

Results made it difficult to differentiate the relative impact of the ambrosia beetle attacks versus the underlying physiological stress on RAD. The effect of excluding ambrosia beetles on the health and mortality of apple trees was tested by comparing excluding or allowing beetle colonization on potted trees that were flood stressed or drenched with ethanol to induce ambrosia beetle attack. Excluding ambrosia beetle colonization did not have a marked impact on apple tree health. In addition, apple trees (cv 'MAI-1, "EverCrisp®") grafted to three different dwarfing rootstocks ('M.9', 'G.41', and

'B.9') were subjected to drought stress and flood stress, which showed that flood stress negatively impacted apple tree health across all commercial rootstocks tested, eliciting elevated ethanol and ambrosia beetle attack.

Association of Xyleborine Ambrosia Beetles and Phytopathogens with Declining Apple Trees  
in North Carolina.

by  
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Sean Gresham was born and raised in rural Canterbury, New Zealand. His interest in environmental science and agriculture led him to undertake a double degree in Environmental Management (B.EM.) and Plant Science (B. Sc) at Lincoln University. Sean was awarded a post-graduate fellowship by Pipfruit New Zealand (now 'NZ Apple and Pear') to undertake his Masters of Science (Entomology) at Virginia Tech working on the specialist predator, *Heringia calcarata* (Diptera: Syrphidae) as a biological control agent for woolly apple aphid. Foregoing the opportunity to transition from Masters to PhD, Sean joined Fruitfed Supplies in Hastings, New Zealand as a technical advisor focused on research and development for crop protection and integrated pest management in horticultural crops. During his six years in this role he conducted over 100 field trials and provided crop protection training and advice to customers and staff across the country with a primary focus on apples and winegrapes. In 2019 Sean joined NCSU department of Entomology and Plant Pathology as a PhD student to work on projects exploring the association of ambrosia beetles with rapid apple decline. In his PhD Sean gained skills across multiple disciplines and worked extensively with the fruit pathology lab at the Mountain Horticultural Crops Research and Extension Center in Mills River, NC.

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## Chapter 1 Exotic Ambrosia Beetles and Phytopathogenic Fungi Associated with Rapid Apple Decline in North Carolina.

### Abstract

Rapid apple decline (RAD) is a syndrome primarily affecting young (<6 years old) apple trees planted in high-density orchards throughout eastern USA and Canada. There has been no documented biotic cause of RAD and it continues to concern growers. Surveys of apple orchards in North Carolina experiencing RAD symptoms in 2017 found that ambrosia beetle attacks were associated with dead and declining trees. The exotic ambrosia beetles (Coleoptera: Scolytinae), *Xylosandrus crassiusculus* (Motschulsky) and *X. germanus* (Blandford), have been implicated in damage and decline of ornamental, fruit, and nut trees throughout the USA and are of increasing concern in other regions throughout the world. Trapping surveys across western North Carolina apple orchards over three years revealed that *X. crassiusculus*, *X. germanus*, and *Xyleborinus saxesenii* (Ratzeburg) were the dominant species captured, with annual variation in species composition. The same three species of beetles were found inside the scion, rootstock, and graft union of declining apple trees collected from commercial orchards. A large diversity of fungi were associated with beetle gallery and non-gallery tissue on declining trees, including opportunistic phytopathogenic genera such as *Botryosphaeria*, *Fusarium*, and *Diaporthe*. No phytopathogenic fungi appeared to be exclusively associated with gallery tissue.

### Keywords:

Apple, Ambrosia beetles, Xyleborine, Opportunistic phytopathogens, tree decline

## Introduction

Rapid Apple Decline (RAD) is a syndrome reported in the eastern USA and Canada where apple trees die following a rapid onset of symptoms without defined causal mechanisms (Singh et al. 2019). The condition is typically characterized by stunted tree growth, leaf yellowing and chlorosis, and within a few weeks of symptom onset, defoliation and death of the tree (Singh et al. 2019). To date, there are no common phytopathogens associated with RAD, and roots usually appear unaffected, except for the absence of fine feeder roots (Singh et al. 2019, Peter 2021). Declining trees are often infested with ambrosia beetles, which are attracted to stressed trees (Agnello et al. 2017). RAD-affected trees are usually distributed in clusters throughout the orchard, and decline is most prevalent in young (<6 year old) high-density plantings utilizing dwarfing rootstocks (Peter 2021, Singh et al. 2019). Tree losses in high-density orchard systems imposes a significant financial burden and threaten the sustainability of these farming systems.

While no confirmed causative agents of RAD have been identified to date, a number of biotic factors have been proposed. These include unidentified viruses (Liu et al. 2018, Wright et al. 2020), rootstock incompatibility, stem boring insects, and bacterial and fungal pathogens (Singh et al. 2019). Abiotic stressors such as cold damage, drought (Hossain et al. 2018, Singh et al. 2019, Gattmann et al. 2021), waterlogging (Frank et al. 2017, Marchioretto et al. 2018), and herbicide injury (Rosenberger 2019) have been proposed to weaken trees and increase susceptibility to pathogen and insect attack. Cold damage and drought-stress in particular have been linked with tree decline in eastern USA and Canada, and are known to cause severe damage to trees leading to failure of the vascular system and eventual death of the tree. Abiotic stress can also result in indirect tree mortality as a result of secondary pests and pathogens that are better able to exploit the weakened host. Western North Carolina experienced a drought in 2016. Typically Western NC receives sufficient rainfall throughout the apple growing season so most growers do not have permeant irrigation infrastructure.

Physiologically stressed trees can often be infested by ambrosia beetles, specifically *Xylosandrus germanus* and *X. crassiusculus* (Ranger, Schultz, et al. 2015, Agnello et al. 2017). Ambrosia beetles have been recorded as attacking apple trees in Michigan (Haas et al. 2016) and New York (Agnello et al. 2015, 2017), but there is little knowledge of ambrosia beetles attacking apples in the southeastern US.

Ethanol, emitted from trees in response to stress events serves as an attractant for ambrosia beetles (Ranger et al. 2018). Ethanol is produced and released by plant tissue under anoxic conditions in response to flooding (Ferner et al. 2012, Ranger et al. 2013), heat (Manter and Kelsey 2008), drought (Kelsey et al. 2014, Kelsey and Westlind 2017), freeze damage (Ranger et al. 2019), and oxidative stress (Kelsey et al. 2014). Ambrosia beetle host selection is highly specific to trees releasing ethanol following abiotic stress events, or following exogenously added ethanol (Ranger et al. 2021). Ambrosia beetle attack therefore is only expected to occur following stress events that potentially predispose the tree to decline and necrotrophic phytopathogens. The extent of the contribution of ambrosia beetle attack in the acceleration of apple tree decline and death is unknown (Agnello et al. 2017, Rosenberger 2019).

Ambrosia beetles are fungal gardeners that carry mutualistic fungi in mycangia (Biedermann et al. 2013). After boring into wood and establishing a gallery, the foundress releases spores of the symbiotic fungus, and beetle progeny feed on the resulting fungal growth on the gallery walls. Mycelial growth does not penetrate deep into host tissue (<1 cm) (Biedermann et al. 2009). The symbionts associated with *Xylosandrus germanus* and *X. crassiusculus*, (*Ambrosiella grosmaniae* and *A. roeperi*, respectively) are not known to have direct pathogenic effects on trees (Mayers et al., 2015), unlike *Xylosandrus compactus*, which can carry a phytopathogenic strain of *Fusarium solani* (Skelton et al. 2019), or *Xyleborus glabratus* that vectors the phytopathogenic symbiont *Raffaelea lauricola* (Harrington 2008, Ploetz et al. 2011). However, opportunistic phytopathogenic fungi from the genera *Fusarium*, *Diaporthe*, and *Botryosphaeria* have been isolated from *X. germanus* galleries on apple (Agnello et al. 2017), which may contribute to dieback and accelerated decline of trees.

Here we show that *X. crassiusculus* and *X. germanus* are associated with RAD-affected apple trees in North Carolina, report on certain aspects of their ecology, and examine filamentous fungi associated with declining apple trees and ambrosia beetle galleries.

## **Materials and Methods**

### **Progression of decline across orchards in North Carolina in 2017**

In June 2017, five high-density apple orchards (1-6 years old) with reported tree decline issues, and one young high-density block located at the Mountain Horticultural Crops Research and Extension Center (MHCREC) in Mills River, NC, were surveyed throughout the growing season for progression of RAD symptoms (Table 1). At each orchard, 20-25 trees from each of 10-15 rows per block (200-375 total trees per block) were randomly selected and monitored for decline at 3 to 4 week intervals. On each evaluation date, the health status of each tree was categorized into one of three categories: healthy, in decline, or dead based on visual assessment of trees. Briefly, healthy trees had full green foliage throughout the canopy, had no premature leaf drop and no evidence of necrosis in the graft union or rootstock; in decline had >25% leaf chlorosis, dark red or brown foliage, premature leaf drop, arrested terminal growth, and evidence of necrosis at the graft union or rootstock; and dead trees had no green tissue evident throughout the tree (Fig. SI 1).

### **Beetle entry sites associated with tree decline status**

In July 2017, a survey was conducted in orchards in Henderson and Haywood counties, NC (identified as: AH, JB, TH, and NR) to determine the relationship between frequency of ambrosia beetle entry holes in apple trees and tree health status (Table 1). In each orchard the number of ambrosia beetle entrance holes in the above ground rootstock/graft union region and scion were recorded by

examining a maximum of ten trees from each tree-health category. Briefly, healthy trees had full green foliage throughout the canopy, had no premature leaf drop and no evidence of necrosis in the graft union or rootstock; mild decline had >25% leaf chlorosis, minor premature leaf drop, arrested terminal growth, and evidence of necrosis at the graft union or rootstock; severe decline had primarily dark red or brown foliage, significant premature leaf drop, and evidence of necrosis at the graft union or rootstock; and dead trees had no green tissue evident throughout the tree.

The number of entries on graft union/rootstock and scion were analyzed as a randomized complete block design (RCBD) using an ANOVA with location as a blocking factor using Proc MIXED in SAS (V 9.4, SAS institute, Cary, NC). Means were compared using Tukey's HSD.

#### **Identification of ambrosia beetles and putative fungal phytopathogens in declining trees.**

During the spring and summer of 2017, a total of 169 trees from 27 orchard locations and at varying stages of decline were evaluated for the presence ambrosia beetles, fungal pathogens, the causal bacterial pathogen of fire blight, *Erwinia amylovora*, and the oomycete causal pathogen of collar and crown rot, *Phytophthora* spp. The trees were dissected at beetle entry sites to recover the foundress. Beetles were placed individually into 1.7 mL Eppendorf tubes with 70% ethanol and identified under 40x power dissecting microscope to species level. To isolate fungi, tissue was excised from beetle galleries and from symptomatic areas on the scion and graft union/rootstock where necrotic tissue interfaced with green tissue and was stored at 3-7°C until processing. Samples were individually surface sterilized for 90 s in 70% ethanol then triple rinsed in sterile deionized water. Using sterile tools, 3 x 5-10 mm subsections from each sterilized tissue sample were dissected and placed on potato dextrose agar (PDA; Difco Laboratories) amended with 50 µg mL<sup>-1</sup> streptomycin and 50 µg mL<sup>-1</sup> chloramphenicol (PDA++). For each unique fungal morphotype from each symptomatic region, a 5 mm mycelial plug from the outer edge of each distinct fungus was excised and placed on PDA ++ for 7-10 days under a

16:8 L:D regime. Resulting cultures were identified into morphotypes based on appearance of mycelial growth on the plate. For each morphotype identified throughout the entire survey, a maximum of three isolates were arbitrarily selected for DNA extraction and molecular identification. In addition to PDA++ for fungal isolation, tissue from the graft union was also plated on Crosse Goodman (CG) medium (Crosse J. E., 1973) and PARP-H (Ferguson and Jeffers 1999), to evaluate the presence of *E. amylovora* and *Phytophthora* spp., respectively. Putative fungal or *Phytophthora* cultures were incubated for 5-10 days at 20-25°C under a 16:8 L:D regime. Putative bacterial cultures were incubated in the dark for 2-3 days at 28°C.

For each isolate selected for molecular identification, two 5 mm plugs were transferred to fresh PDA medium and incubated at 25°C, under a 12:12 L:D regime. After 1 week of incubation, approximately 200 mg of mycelium was harvested from each culture and ground in liquid nitrogen. Extraction of genomic DNA was accomplished using the Omega Bio-Tek E. Z. N. A. Plant DNA DS kit (Omega Bio-tek Inc, Norcross, GA) according to manufacturer's instructions. The resulting gDNA was subjected to rDNA- based identification for fungi using the ITS locus (White et al. 1990). Amplification of the ITS region was accomplished using the primers ITS1F and ITS4 (White et al. 1990). PCR reactions were performed in 25 µl volumes and contained 1x EmeraldAmp GT PCR Master Mix (Takara, Mountain View, CA), 0.4 µM of each primer, and 5-10 ng of extracted gDNA. All amplifications were performed in a T100 Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA) with the following cycling parameters: an initial denaturation of 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s; this was followed by a final extension at 72°C for 7 min. PCR products were separated by gel electrophoresis using a 2% agarose gel in 1xTris-borate-EDTA buffer (44.5mM Tris-borate and 1mM EDTA, pH 8.0) at 90 V for 60 min. Single-band PCR products were purified for sequencing using a Zymo DNA Clean & Concentrator kit (Zymo Research, Irvine, CA) according to manufacturer instructions and sequenced at the North Carolina State University Genomic Services

Laboratory (Sanger DNA sequencing) on an Applied Biosystems 3730xl capillary sequencer. Identification of rDNA sequence data analysis was accomplished using the Basic Local Alignment Search Tool (BLAST) on NCBI GenBank databases

In addition to the isolation of microbes, all 169 trees that were evaluated had a minimum of one beetle entry hole on the tree. Trees were dissected to extract ambrosia beetles, which were placed in individual tubes for identification. Beetles were identified under a dissecting microscope to species level with assistance and confirmation of voucher specimens from the NCSU diagnostics laboratory in Raleigh, NC.

#### **Ambrosia beetle phenology using ethanol-baited traps.**

In June 2017, February 2018, and March 2019, ethanol baited traps were deployed at commercial apple orchards in Haywood and Henderson Co., NC (Table 1). All trapping locations contained trees that had been attacked by ambrosia beetles, had symptoms of RAD, and were adjacent to a wooded area. At each location, three traps were placed either within a single orchard row (row perpendicular to the wooded area) or across orchard rows (rows parallel to the wooded area) spaced at 15-20 m intervals. Traps were similar to those used by Agnello et al. (2017), and consisted of an inverted 2 liter juice bottle with “windows” cut in the side, and an ethanol lure pouch (AgBio Incs. Westminster, CO) hung from inside the top of the bottle. Lures released EtOH at a rate of 65 mg/day at 30°C (AgBio Inc., Westminster, CO). Beetles were captured in detergent water, which was contained in the lower portion of the trap. Traps were monitored weekly and samples were sorted in the lab. All Scolytinae beetles were identified to species as described above.

The effect of trap location (edge, 20 m, and 40 m inside orchard) on captures of *X. crassiusculus*, *X. germanus*, and *Xyleborinus saxesenii* were analyzed separately using a one-way ANOVA in SAS V 9.4 (SAS Institute, Cary, NC) based on total annual trap captures across sites and years.

## **Weather data.**

Weather data from 2006 to 2017 were accessed from the Mountain Horticultural Crops Research and Extension Center (MHREC) ECONET weather station using the North Carolina state climate office database (<https://econet.climate.ncsu.edu>). The selected weather station provided the most reliable long-term daily weather data representing Henderson Co., NC apple orchards. Weather data included maximum, minimum, and mean daily temperatures, daily precipitation, and mean daily soil moisture at 1 m depth.

## **Results**

### **Progression of decline across orchards in North Carolina in 2017**

Among the surveyed orchards, RS, located at the Mountain Horticultural Crops Research and Extension Center, had the lowest total percentage of declining trees, while the highest tree loss was observed in orchard AH, where 29% of surveyed trees were dead or in decline by the final evaluation (Fig. 1). The percentage of trees dead or in decline increased from June to August and then stabilized in all orchards except for “RS”, which showed an increase in percentage dead and declining trees through the final evaluation date on 20 September (Fig. 1).

### **Beetle entry sites associated with tree decline status**

A total of 157 apple trees across 4 locations at selected levels of decline were inspected for ambrosia beetle entries in the field. An average of  $5.6 \pm 0.8$  (SEM) entries/tree were observed across all trees. The majority of entries were found on the scion or main trunk (67%) followed by the graft union (22%). This bias was most pronounced on dead trees, whereas attacks were evenly distributed between graft union and the main trunk on healthy and early and late-decline trees (Table 2). There was a significant effect of health status on the number of entries on the graft union ( $F = 5.17$ ; d.f. = 3,150;

p=0.002) and scion (F= 6.12; d.f. = 3,150; p=0.0006). Significantly more entries were observed on trees that were in late decline and dead compared to healthy trees on the graft union, and on the scion and overall (total entries) significantly more entries were observed on trees in early and late decline and on dead trees (Table 2). Although there tended to be a higher number of entries on dead trees compared with early and late-declining trees, there were no significant differences in the number of entries among early and late decline or dead trees.

#### **Identification of ambrosia beetles and putative fungal phytopathogens in declining trees.**

Declining apple trees collected from apple orchards in Haywood and Henderson Co., NC had an average of  $4.4 \pm 0.51$  entries/tree on the scion and  $2.3 \pm 0.11$  entries/tree on the graft union. A total of 313 ambrosia beetles were extracted for identification. Of the 282 identified ambrosia beetles, 81 (25.9%) were *X. crassiusculus*, 108 (34.5%) were *X. germanus*, and 96 (30.7%) were *X. saxesenii*. A small proportion of the beetles (9.8%) were non-ambrosia bark beetles or were unable to be identified due to damage.

Declining apple trees collected from apple orchards in Haywood and Henderson Co. (169) and evaluated for phytopathogens, a total of 62 different fungal species across 31 genera were isolated from the gallery, scion, and graft union tissue. Of the common fungal genera identified, 10 of the 12 genera were isolated at similar frequencies from all tissue types (graft union, scion, and beetle gallery tissue), whereas two genera, *Ambrosiella* spp. and *Chaetomium* spp., were recovered exclusively from gallery tissue (Table 3). The three most common fungal genera isolated from trees (*Fusarium*, *Diaporthe*, and *Botryosphaeria*,) represented 67% of all isolates. Isolates of *Fusarium*, *Penicillium*, *Paraconthyrrium*, and *Flavodon* spp. were recovered more frequently from gallery opposed to non-gallery tissue (Table 3). The opposite trend was observed for isolates of *Epicoccum*, *Clonostachys* and *Botryosphaeria* spp. isolates which were more frequently recovered from gallery tissue samples than non-gallery tissue. Nineteen

fungal genera were infrequently detected (in <2% of samples) and are not presented in results (see full list of species in SI Table 4). Testing indicated that neither *Phytophthora* spp. nor *Erwinia amylovora* were recovered from tissue sampled at or below the graft union.

*Ambrosiella grosmanii*, the nutritional symbiont of *X. germanus* was recovered from nearly a third of all gallery tissue samples, but *A. roeperi* and *Raffaelea sulfurea*, the nutritional symbionts of *X. crassiusculus* and *Xyleborinus saxesenii*, respectively were not detected (Table 4). *Botryosphaeria dothidea* was frequently recovered from scion tissue and moderately common on graft union/rootstock and gallery tissue. A number of *Fusarium* species were frequently recovered from gallery, scion, and graft union/rootstock tissue. The most common *Fusarium* spp. identified was *F. solani*, followed by *F. amermeriacum*, *F. petersiae*, *F. fujikuroi*, and *F. oxysporum* species complex (Table 4).

#### **Ambrosia beetle phenology using ethanol-baited traps.**

Three species of ambrosia beetles, *Xyleborinus saxesenii*, *Xylosandrus germanus*, and *X. crassiusculus*, dominated captures in traps at each site and year across all site, cumulatively representing 99, 84, and 93% of trapped beetles in 2017, 2018, and 2019, respectively. Ten other species of Scolytinae beetles were identified in traps with low frequency (Table 6).

In 2017 trapping did not commence until June, so early season trends were not observed. Captures of all beetle species were minimal after August in 2017 and 2019, but continued through to early October in 2018 (Figs.1-3). The timing of peak captures of *X. saxesenii* were variable across locations. The first *X. saxesenii* were captured in February 2018 and April 2019 (Figs. 2 and 3). *Xylosandrus germanus* tended to be captured earlier than *X. crassiusculus* and had 2-3 peaks with highest captures occurring in the first peak in April-May in 2018 (Fig. 2) and 2019 (Fig. 3) and in 2017 highest captures were in late June across most sites, except site 'TH' which was in late July 2017 (Fig 1). The first captures of *X. crassiusculus* occurred in mid-May across all sites in 2018 (Fig. 2) and late April in

2019 (Fig. 3). Captures of *X. crassiusculus* peaked in June and early July in 2017 (Fig. 1), but the first flight was likely missed.

Across all trapping sites, there was a significant effect of trap placement on captures of *X. crassiusculus* ( $F_{2,36} = 5.95$ ,  $P = 0.0059$ ) and *X. germanus* ( $F_{2,36} = 4.25$ ,  $P = 0.0221$ ), but no significant effect was detected for *Xyleborinus saxesenii* ( $F_{2,36} = 0.64$ ,  $P = 0.533$ ). Traps placed at the orchard-woods edge captured 2 to 4 times more *X. crassiusculus* and *X. germanus* than traps placed 20 and 40 m within the orchard interior (Table 6). There was no significant difference in total captures of *X. crassiusculus* or *X. germanus* between traps placed at 20 or 40 m within the orchard.

#### **Weather data.**

Total precipitation measured at the MHCRC weather station was 995 mm, 19% lower than the 10 year average of 1224 mm. Drought conditions began in spring 2016, with 40% lower total rainfall in March-May, near-normal precipitation June-August and a severe drought during the late summer and into the fall. The total precipitation for September and October was 32.8 mm, only 16% of the 10-year average of 201.0 mm (Fig 5, SI Table 1). Below-average soil moisture at 1 m depth were recorded for May-November with a severe deficit in June and September, October, and November (Fig 5, SI Table 2). Drought conditions continued throughout the winter with average to below average rainfall and soil moisture, but rainfall was above average in spring 2017, 502 mm precipitation was recorded in April-June 2017, 61% higher than the long term average. Soil moisture levels were close to normal throughout the 2017 growing season.

The winter of 2016-2017 was mild compared with the previous 10 year average. In particular, the average daily minimum temperature in January and February 2017 was 4.3 and 2.5 °C higher than the long term average and the absolute minimum of -13.4 and -6.8 °C was well above the 10 year record lows of -18.5 and -16.1 °C (SI Table 3). The average daily high and daily mean temperature in January

and February was warmer than the long term average and record high daily temperatures were recorded, reaching 22.2 and 24.4 °C in January and February respectively. However, a late freeze occurred March 17, 2017 with a record low temperature of -7.4 °C.

## Discussion

Rapid apple decline (RAD) is a major concern for apple growers throughout the eastern USA due to the costs associated with replacing trees and lost revenue from affected trees (Peter 2021). Our surveys found decline and mortality rates of young trees as high as 30% in surveyed blocks in North Carolina in 2017. Decline symptoms were evident throughout affected blocks in June, when surveys began, and decline severity and tree mortality increased throughout the summer. Necrosis of the graft union was commonly observed on declining trees in North Carolina, and has been reported in other regions in relation to RAD (Singh et al. 2019, Xu et al. 2023).

We found three Xyleborine species in declining or dead apple trees in our in NC, *X. crassiusculus*, *X. germanus*, and *Xyleborinus saxesenii*. These species accounted for >84% of all beetles trapped in NC apple orchards over three years. Xyleborine ambrosia beetles have a large host range (>200 species reported), and are problematic pests in a range of ornamental, fruit, and nut production systems (Ranger et al. 2021), and *X. germanus* has been associated with RAD of apples in New York (Agnello et al. 2017, Donahue and Elone 2021). Trapping studies in apple orchards in NY showed a dominance of *X. germanus* and *Xyleborinus saxesenii* (Agnello et al. 2015, 2017), while in GA tree fruit orchards *X. crassiusculus* and *X. germanus* dominated trap captures (Monterrosa et al. 2022). Of the primary ambrosia beetles captured in NC orchards, only *X. crassiusculus* and *X. germanus* are recognized as a pest in fruit trees. First reported in SC on peaches (Kovach and Gorsuch 1985) and is now widespread in the eastern USA, the granulate ambrosia beetle, *Xylosandrus crassiusculus*, has been reported as a pest of woody ornamentals and fruit trees (Gugliuzzo et al. 2021). Black stem borer, *Xylosandrus germanus*, is

another exotic ambrosia beetle that is widespread throughout the USA, first reported in New York in 1932 (Felt 1932). The fruit tree pinhole borer, *Xyleborinus saxesenii*, also is not native to North America, but is thought to have established over 100 years ago (Batra 1985).

Xyleborine ambrosia beetles, especially *Xylosandrus* spp. preferentially attack physiologically stressed deciduous trees with thin bark (Ranger, Schultz, et al. 2015), mediated by ethanol which acts as a short and long range attractant (Ranger, Tobin, et al. 2015). Although declining trees had a higher incidence and severity of ambrosia beetle attacks than healthy trees, we also found that almost a third of apparently healthy apple trees were attacked by ambrosia beetles. Entry and gallery establishment is dependent on elevated ethanol in host tissue which has been shown to occur in response to abiotic stressors such as acute flooding (Ranger et al. 2010, Frank and Ranger 2016), and cold damage (La Spina et al. 2013, Ranger et al. 2019), or other abiotic or biotic stressors (Ranger et al. 2021). Conversely, non-stressed trees with little to no ethanol production are not attractive to beetles even under no-choice conditions (Ranger, Tobin, et al. 2015). Also, trees with elevated ethanol do not always show visual symptoms of decline. (Ranger et al. 2010, 2013, Ranger, Schultz, et al. 2015). Ethanol content was not measured in our survey, but other studies have shown that the relationship between stress, ethanol, and beetle attack on apple trees is comparable to other hardwood species (Ranger et al. 2019, Gugliuzzo et al. 2021, Reding et al. 2021). Hence, for apple trees to be attacked by ambrosia beetles, they must have elevated ethanol resulting from pre-existing abiotic or biotic stress. It is therefore difficult to determine from the association of apple decline and ambrosia beetle attack whether ambrosia beetle attacks were responsive to or causal of RAD. Despite the common association of ambrosia beetles with declining trees, the impact of *X. crassiusculus* and *X. germanus* on tree health and mortality remains unknown (Ranger et al. 2021). Further work is required to quantify the relative impact of ambrosia beetles on tree health.

We observed a broad diversity of filamentous fungi associated with ambrosia beetle galleries and non-gallery tissue on declining apple trees. Two genera were found exclusively from gallery tissue – *Ambrosiella*, the nutritional symbiont for *Xylosandrus* ambrosia beetles (Harrington et al. 2014, Mayers et al. 2015b), and *Chaetomium*, a common epiphyte associated with plants that has been investigated as a potential biological control agent for oomycete and fungal disease control (Mondello et al. 2018). *Chaetomium globosum* has been reported as being associated with ambrosia beetles, particularly *Xyleborinus saxesenii* galleries in-vitro (Diehl et al. 2022). Opportunistic phytopathogenic fungi such as *Fusarium*, *Botryosphaeria*, and *Diaporthe* spp. were frequently isolated from galleries, but no single isolate was consistently recovered across declining trees.

*Botryosphaeria* and *Fusarium* spp. were the most common genera of fungi isolated from ambrosia beetle galleries on declining apple trees in NY (Agnello et al. 2017), and *Botryosphaeria dothidea* was associated with RAD-affected trees in NY (Rosenberger 2019, Donahue and Elone 2021). Physiological stress, especially drought, can increase infection and colonization of host tissue by species from the three most common genera isolated from gallery and graft union tissue in our study: *Botryosphaeria* (Ma et al. 2001, Slippers and Wingfield 2007, Galarneau et al. 2019, Darge and Woldemariam 2021), *Fusarium* (Stack et al. 2020), and *Diaporthe* (Agustí-Brisach et al. 2020). Drought stress and the delayed onset or disruption of dormancy in apple trees, two environmental conditions observed prior to the rapid decline of trees in NC, are considered to be particularly important for infection and disease expression of *B. dothidea* (Pusey 1989, Brown-Rytlewski and McManus 2000, Agustí-Brisach et al. 2020) in apple.

*Fusarium* is a cosmopolitan and diverse genus. Members of the *F. solani* and *F. oxysporum* species complexes are typically saprobic, non-pathogenic to host trees, or of low virulence. However, there are a number of phytopathogens and toxin producing species within the *Fusarium* genus, including *F. solani* and *F. oxysporum* species complexes. *Fusarium solani* was the most common single species

recovered from gallery tissue (40% of galleries) and it has been previously found in association at high frequency in declining apple trees and ambrosia beetles alike (Manici et al. 2003, Bateman et al. 2016, Agnello et al. 2017, Markakis et al. 2017, Cheng et al. 2019, Astapchuk et al. 2020, Gugliuzzo et al. 2020). Surveys of declining trees have also found high prevalence of *F. solani* and *F. oxysporum*, and a lower occurrence of other *Fusarium* species, *F. equiseti*, and *F. accuminatum* in Iran (Esmaili and Sharifnabi 2023), Italy (Manici et al. 2003), Russia (Astapchuk et al. 2020), and Tunisia (Mannai et al. 2018). Typically *F. solani* is not considered pathogenic, or has low aggressiveness, but can be pathogenic on apple (Manici et al. 2003, Yan et al. 2018, Cheng et al. 2019). Infection of M9 rootstocks caused oxidative damage to roots, which resulted in decreased biomass, poor plant health, mortality (Xiang et al. 2021), and severe wilting on apple seedlings (Yan et al. 2018). *Fusarium tricinctum*, which has been associated with declining trees in Korea, contains ice-nucleation metabolites that can decrease cold tolerance of infected apple trees (Avalos-Ruiz et al. 2022). In our survey we did not sample the rhizosphere nor did we test the pathogenicity of isolates detected.

Western North Carolina experienced drought conditions in 2016, with below average rainfall and soil moisture recorded at the MHCREC ECONET weather station. Drought stress can cause irreversible damage to trees and is considered to be a major cause of tree mortality (Choat et al. 2018, Preisler et al. 2021). In high density apple plantings, drought has been implicated in RAD, in as the dwarfing rootstocks utilized in these systems have reduced root biomass and access to soil water compared with standard rootstocks (Singh et al. 2019, Xu et al. 2023). Drought stress has been directly associated with RAD incidence (Singh et al. 2019, Xu et al. 2023) and is known to increase susceptibility to pests and diseases through disruption of vascular system and altered carbohydrate balance (Xu et al. 2023). However, drought stress was not likely to have directly contributed to the observed colonization of declining trees by ambrosia beetles in NC. A recent study by Ranger et al. (2023) showed that drought

stressed apple trees are not attractive to Xyleborine ambrosia beetles and resulted in little ethanol production compared flood stress

The mild winter preceding the spring of 2017 (SI table 3) may have increased the risk of severe cold injury at temperatures that would otherwise be tolerated. A severe freeze occurred in mid-March, reaching a record low temperature for March in Henderson County. Most cultivars were in the early green-tip stage at this time and given the mild winter and above-freezing temperatures in late-February and early-March, trees were likely vulnerable to freeze damage (Ketchie 1985). The tolerance of apple trees to freeze injury is affected by dormancy-related cold hardiness, and trees can deacclimatize within days of warmer temperatures (Ketchie 1985, Moran et al. 2021) and freeze-thaw cycles can be particularly damaging to apple trees rather than extreme cold (Palmer et al. 2003). During endodormancy, cryoprotectants prevent tissue damage through supercooling at temperatures in the range of -20 to -35 °C. However, damage to the vascular system can occur at -8 °C at green-tip, and at -5 °C from flowering onwards (Ketchie 1985). Warm temperatures in late winter have been shown to deacclimate dormant apple tissue by up to 15°C (Moran et al. 2021), and a number of studies have shown increased damage during freeze-thaw cycles compared with consistently freezing temperatures (Palmer et al. 2003). Low temperatures in November, December, and February were found to be a key factor affecting Canadian apple production in study examining various weather data and tree mortality over a 72 year period (Caprio and Quamme 2011). In another study that evaluated the incidence of blackheart damage caused by freezing on apples across the USA, damage was more strongly influenced by temperature fluctuations than by absolute winter temperatures (Domoto et al. 2001). Although winter temperatures in North Carolina are generally higher than in more northern regions, cold injury could still be a major factor in apple tree mortality in NC. Cold injury also likely had a considerable impact on tree decline across southeastern orchards given the large variability in temperature during the winter of 2016-17. Therefore, despite the mild winter of 2016-17, freeze events could have caused

damage due to abnormally warm winter conditions and large temperature fluctuations. In the winter of 2008-2009, large temperature spikes were considered to be responsible for high tree deaths, with higher tolerance to winter injury in trees grafted to 'B.9' and G.16 rootstocks than 'M.9' clones (McArtney and Obermiller 2011) and a late spring freeze in 2007 led to widespread damage and dieback of deciduous trees throughout the eastern USA (Gu et al. 2008). In addition to the mild winter, drought conditions that persisted in 2016 may have also influenced the cold hardiness of apple trees and increased risk of damage. In Canada, winter injury severity was associated with the location of irrigation sprinklers, which the researchers considered to be related to both soil water status and tree water status (Quamme and Brownlee 1989).

In conclusion our survey of RAD-affected apple orchards in NC found that decline and mortality was predominantly observed in early to mid-summer in 2017 and the majority of dead and declining trees were attacked by Xyleborine ambrosia beetles. A multitude of phytopathogenic and non-pathogenic fungi were isolated from galleries and non-gallery tissue on declining apple trees with *Ambrosiella*, *Botryosphaeria* and *Fusarium* being most commonly isolated. Drought stress and freeze damage were likely contributing factors in decline issues observed in 2017 given that the preceding year the affected region experienced a drought which can predispose apple trees to invasion by opportunistic pests and phytopathogens, and the winter had large temperature fluctuations which has been shown to increase the risk of freeze damage on apple. The high occurrence of ambrosia beetles and opportunistic phytopathogenic fungi such as *B. dothidea* is indicative that attacked trees were physiologically stressed. Decline was likely a result of a culmination of abiotic stress, phytopathogens, and damage from ambrosia beetle attack. Further research is required to understand the relative impact of abiotic stressors, ambrosia beetle attack, and phytopathogenic fungi on apple decline.

## References

- Agnello, A., D. Breth, E. Tee, K. Cox, and H. R. Warren. 2015.** Ambrosia Beetle – An Emergent Apple Pest. 2013–2016.
- Agnello, A. M., D. I. Breth, E. M. Tee, K. D. Cox, S. M. Villani, K. M. Ayer, A. E. Wallis, D. J. Donahue, D. B. Combs, A. E. Davis, J. A. Neal, and F. M. English-Loeb. 2017.** *Xylosandrus germanus* (Coleoptera: Curculionidae: Scolytinae) Occurrence, Fungal Associations, and Management Trials in New York Apple Orchards. *J. Econ. Entomol.* 110: 2149–2164.
- Agustí-Brisach, C., D. Moldero, M. D. C. Raya, I. J. Lorite, F. Orgaz, and A. Trapero. 2020.** Water stress enhances the progression of branch dieback and almond decline under field conditions. *Plants.* 9: 1–26.
- Astapchuk, I., G. Yakuba, and A. Nasonov. 2020.** Species diversity of root rot pathogens of apple tree of the genus *Fusarium* Link in Southern Russia . *BIO Web Conf.* 21: 00005.
- Avalos-Ruiz, D., L. N. Ten, C. K. Kim, S. Y. Lee, and H. Y. Jung. 2022.** Isolation and Identification of Ice Nucleation Active *Fusarium* Strains from Rapid Apple Declined Trees in Korea. *Plant Pathol. J.* 38: 403–409.
- Bateman, C., M. Šigut, J. Skelton, K. E. Smith, and J. Hulcr. 2016.** Fungal Associates of the *Xylosandrus compactus* (Coleoptera: Curculionidae, Scolytinae) Are Spatially Segregated on the Insect Body. *Environ. Entomol.* 45: 883–890.
- Batra, L. R. 1985.** Ambrosia beetles and their associated fungi: research trends and techniques. *Proc. Plant Sci.* 94: 137–148.

**Biedermann, P. H. W., K. D. Klepzig, and M. Taborsky. 2009.** Fungus Cultivation by Ambrosia Beetles: Behavior and Laboratory Breeding Success in Three Xyleborine Species. *Environ. Entomol.* 38: 1096–1105.

**Biedermann, P. H. W., K. D. Klepzig, M. Taborsky, and D. L. Six. 2013.** Abundance and dynamics of filamentous fungi in the complex ambrosia gardens of the primitively eusocial beetle *Xyleborinus saxesenii* Ratzeburg (Coleoptera: Curculionidae, Scolytinae). *FEMS Microbiol. Ecol.* 83: 711–723.

**Brown-Rytlewski, D. E., and P. S. McManus. 2000.** Virulence of *Botryosphaeria dothidea* and *Botryosphaeria obtusa* on apple and management of stem cankers with fungicides. *Plant Dis.* 84: 1031–1037.

**Caprio, J. M., and H. A. Quamme. 2011.** Weather conditions associated with apple production in the Okanagan Valley of British Columbia. *J. Plant Sci.* 72: 129–137.

**Cheng, Y., W. Zhao, R. Lin, Y. Yao, S. Yu, Z. Zhou, X. Zhang, Y. Gao, and W. Huai. 2019.** *Fusarium* species in declining wild apple forests on the northern slope of the Tian Shan Mountains in north-western China. *For. Pathol.* 49: 1–20.

**Choat, B., T. J. Brodribb, C. R. Brodersen, R. A. Duursma, R. López, and B. E. Medlyn. 2018.** Triggers of tree mortality under drought. *Nature*, 558(7711), 531-539.

**Crosse J. E., G. R. N. 1973.** A selective medium for and a definitive colony characteristic of *Erwinia amylovora*. *Phytopathology.* 63: 1425–1426.

**Darge, W. A., and S. S. Woldemariam. 2021.** *Botryosphaeria* tree fungal pathogens and their diversity. *Int. J. Phytopathol.* 10: 49–56.

**Diehl, J. M. C., V. Kowallik, A. Keller, and P. H. W. Biedermann. 2022.** First experimental evidence for active farming in ambrosia beetles and strong heredity of garden microbiomes. *Proc. R. Soc. B Biol. Sci.* 289: 20221458.

**Domoto, P. A., W. R. Autio, G. R. Brown, D. C. Ferree, P. M. Hirst, C. A. Mullins, and J. R. Schupp. 2001.** Blackheart injury in “Golden Delicious”, “Jonagold”, “Empire” and “Rome Beauty” apple trees on five rootstocks in the 1990 NC-140 cultivar/rootstock trial. *J. Am. Pomol. Soc.* 55: 146–153.

**Donahue, D. J., and S. E. Elone. 2021.** Case Study of a Declining Apple Orchard Daniel J. Donahue and Sarah E. Elone. *New York Fruit Q. - Summer.* 29: 28–31.

**Esmaili, Z., and B. Sharifnabi. 2023.** Fusarium species associated with apple trees decline in Isfahan , Iran. *Mycol. Iran.* 10: 23–34.

**Felt, E. P. 1932.** A new pest in greenhouse grown grape stems. *J. Econ. Entomol.* 25: 418.

**Ferguson, A. J., and S. N. Jeffers. 1999.** Detecting multiple species of Phytophthora in container mixes from ornamental crop nurseries. *Plant Dis.* 83: 1129–113.

**Ferner, E., H. Rennenberg, and J. Kreuzwieser. 2012.** Effect of flooding on C metabolism of flood-tolerant (*Quercus robur*) and non-tolerant (*Fagus sylvatica*) tree species. *Tree Physiol.* 32: 135–145.

**Frank, S. D., A. L. Anderson, and C. M. Ranger. 2017.** Interaction of Insecticide and Media Moisture on Ambrosia Beetle (Coleoptera: Curculionidae) Attacks on Selected Ornamental Trees. *Environ. Entomol.* 46: 1390–1396.

**Frank, S. D., and C. M. Ranger. 2016.** Developing a Media Moisture Threshold for Nurseries to Reduce Tree Stress and Ambrosia Beetle Attacks. *Environ. Entomol.* 45: 1040–1048.

**Galarneau, E. R. A., D. P. Lawrence, R. Travadon, and K. Baumgartner. 2019.** Drought exacerbates botryosphaeria dieback symptoms in grapevines and confounds host-based molecular markers of infection by neofusicoccum parvum. *Plant Dis.* 103: 1738–1745.

**Gattmann, M., B. Birami, D. Nadal Sala, and N. K. Ruehr. 2021.** Dying by drying: Timing of physiological stress thresholds related to tree death is not significantly altered by highly elevated CO<sub>2</sub>. *Plant Cell Environ.* 44: 356–370.

**Gu, L., P. J. Hanson, W. M. A. C. Post, and P. Dale. 2008.** The 2007 Eastern US Spring Freeze : Increased Cold Damage in a Warming World ? *Bioscience.* 58: 253–262.

**Gugliuzzo, A., P. H. W. Biedermann, D. Carrillo, L. A. Castrillo, J. P. Egonyu, D. Gallego, K. Haddi, J. Hulcr, H. Jactel, H. Kajimura, N. Kamata, N. Meurisse, Y. Li, J. B. Oliver, C. M. Ranger, D. Rassati, L. L. Stelinski, R. Sutherland, G. Tropea Garzia, M. G. Wright, and A. Biondi. 2021.** Recent advances toward the sustainable management of invasive *Xylosandrus ambrosia* beetles. *J. Pest Sci.* (2004). 94: 615–637.

**Gugliuzzo, A., G. Criscione, A. Biondi, D. Aiello, A. Vitale, G. Polizzi, and G. Tropea Garzia. 2020.** Seasonal changes in population structure of the ambrosia beetle *Xylosandrus compactus* and its associated fungi in a southern Mediterranean environment. *PLoS One.* 15: 1–14.

**Haas, M., J. Wilson, and L. Gut. 2016.** Managing Black Stem Borer in Michigan Tree Fruits. 2.

**Harrington, T. 2008.** *Raffaelea lauricola*, a new ambrosia beetle symbiont and pathogen on the Lauraceae. *Mycotaxon.* 2008. № 104. p. 399-404. 399–404.

**Harrington, T. C., D. McNew, C. Mayers, S. W. Fraedrich, and S. E. Reed. 2014.** *Ambrosiella roeperi* sp. nov. is the mycangial symbiont of the granulate ambrosia beetle, *Xylosandrus crassiusculus*. *Mycologia.* 106: 835–845.

**Hossain, M., E. J. Veneklaas, G. E. S. J. Hardy, and P. Poot. 2018.** Tree host-pathogen interactions as influenced by drought timing: Linking physiological performance, biochemical defence and disease severity. *Tree Physiol.* 39: 6–18.

**Kelsey, R. G., D. Gallego, F. J. Sánchez-García, and J. A. Pajares. 2014.** Ethanol accumulation during severe drought may signal tree vulnerability to detection and attack by bark beetles. *Can. J. For. Res.* 44: 554–561.

**Kelsey, R. G., and D. J. Westlind. 2017.** Physiological stress and ethanol accumulation in tree stems and woody tissues at sublethal temperatures from fire. *Bioscience.* 67: 443–451.

**Ketchie, D. O. 1985.** Cold Resistance of Apple Trees Through the Year and Its Relationship To the Physiological Stages. *Acta Hortic.*

**Kovach, J., and C. S. Gorsuch. 1985.** Survey of the ambrosia beetle species infesting South Carolina peach orchards and a taxonomic key for the most common species. *J. Agric. Entomol.* 2: 238–247.

**Liu, H., L. Wu, E. Nikolaeva, K. Peter, Z. Liu, D. Mollov, M. Cao, and R. Li. 2018.** Characterization of a new apple luteovirus identified by high-throughput sequencing. *Virolog. J.* 15: 1–9.

**Ma, Z., D. P. Morgan, and T. J. Michailides. 2001.** Effects of water stress on *Botryosphaeria* blight of pistachio caused by *Botryosphaeria dothidea*. *Plant Dis.* 85: 745–749.

**Manici, L. M., C. Ciavatta, M. Kelderer, and G. Erschbaumer. 2003.** Replant problems in South Tyrol: Role of fungal pathogens and microbial population in conventional and organic apple orchards. *Plant Soil.* 256: 315–324.

**Mannai, S., N. Horrigue-Raouani, and N. M'Hamdi. 2018.** Effect of Six Fungicides against *Fusarium oxysporum* and *F. solani* Associated with Peach Seedlings Decline in Tunisian Nurseries. *Annu. Res. Rev. Biol.* 26: 1–11.

**Manter, D. K., and R. G. Kelsey. 2008.** Ethanol accumulation in drought-stressed conifer seedlings. *Int. J. Plant Sci.* 169: 361–369.

**Marchioretto, L. D. R., A. De Rossi, L. O. do Amaral, and A. M. A. de S. Ribeiro. 2018.** Tolerance of apple rootstocks to short-term waterlogging. *Cienc. Rural.* 48: 1–7.

**Markakis, E. A., N. Kavroulakis, S. Ntougias, G. C. Koubouris, C. K. Sergentani, and E. K. Ligoxigakis. 2017.** Characterization of fungi associated with wood decay of tree species and grapevine in Greece. *Plant Dis.* 101: 1929–1940.

**Mayers, C. G., D. L. McNew, T. C. Harrington, R. A. Roeper, S. W. Fraedrich, P. H. W. Biedermann, L. A. Castrillo, and S. E. Reed. 2015a.** Three genera in the Ceratocystidaceae are the respective symbionts of three independent lineages of ambrosia beetles with large, complex mycangia. *Fungal Biol.* 119: 1075–1092.

**Mayers, C. G., D. L. McNew, T. C. Harrington, R. A. Roeper, S. W. Fraedrich, P. H. W. Biedermann, L. A. Castrillo, and S. E. Reed. 2015b.** Three genera in the Ceratocystidaceae are the respective symbionts of three independent lineages of ambrosia beetles with large, complex mycangia. *Fungal Biol.* 119: 1075–1092.

**McArtney, S., and J. D. Obermiller. 2011.** Effect of dwarfing rootstocks on low temperature tolerance of “Golden Delicious” apple trees during Winter 2008-2009. *J. Am. Pomol. Soc.* 65: 178–184.

**Mondello, V., A. Songy, E. Battiston, C. Pinto, C. Coppin, P. Trotel-Aziz, C. Clément, L. Mugnai, and F. Fontaine. 2018.** Grapevine trunk diseases: A review of fifteen years of trials for their control with chemicals and biocontrol agents. *Plant Dis.* 102: 1189–1217.

**Monterrosa, A., S. V. Joseph, B. Blaauw, W. Hudson, and A. L. Acebes-Doria. 2022.** Ambrosia Beetle Occurrence and Phenology of *Xylosandrus* spp. (Coleoptera: Curculionidae: Scolytinae) in Ornamental Nurseries, Tree Fruit, and Pecan Orchards in Georgia. *Environ. Entomol.* 51: 998–1009.

**Moran, R. E., B. J. Peterson, G. Fazio, and J. A. Cline. 2021.** Low temperature tolerance of apple shoots following exposure to warm temperatures in late winter. *HortScience.* 56: 642–649.

**Palmer, J. W., J. P. Privé, and D. S. Tustin. 2003.** Temperature. *In* Ferree, D.C., Warrington, I.J. (eds.), *Apples Bot. Prod. Uses.*

**Peter, K. A. 2021.** Apple Disease - Rapid Apple Decline. Penn State Ext.

**Ploetz, R. C., J. M. Pérez-Martínez, E. A. Evans, and S. A. Inch. 2011.** Toward fungicidal management of laurel wilt of avocado. *Plant Dis.* 95: 977–982.

**Preisler, Y., F. Tatarinov, J. M. Grünzweig, and D. Yakir. 2021.** Seeking the “point of no return” in the sequence of events leading to mortality of mature trees. *Plant Cell Environ.* 44: 1315–1328.

**Pusey, P. L. 1989.** Influence of Water Stress on Susceptibility of Nonwounded Peach Bark to *Botryosphaeria dothidea*. *Plant Dis.*

**Quamme, H. A., and R. T. Brownlee. 1989.** Observation of winter injury to the roots of apple trees associated with sprinkler irrigation pattern. *Can. J. Plant Sci.* 69: 617–621.

**Ranger, C. M., P. H. W. Biedermann, V. Phuntumart, G. U. Beligala, S. Ghosh, D. E. Palmquist, R. Mueller, J. Barnett, P. B. Schultz, M. E. Reding, and J. P. Benz. 2018.** Symbiont selection via alcohol benefits fungus farming by ambrosia beetles. *Proc. Natl. Acad. Sci. U. S. A.* 115: 4447–4452.

**Ranger, C. M., M. E. Reding, K. Adesso, M. Ginzel, and D. Rassati. 2021.** Semiochemical-mediated host selection by *Xylosandrus* spp. ambrosia beetles ( Coleoptera : Curculionidae ) attacking horticultural tree crops : a review of basic and applied science. 103–120.

**Ranger, C. M., M. E. Reding, A. B. Persad, and D. A. Herms. 2010.** Ability of stress-related volatiles to attract and induce attacks by *Xylosandrus germanus* and other ambrosia beetles. *Agric. For. Entomol.* 12: 177–185.

**Ranger, C. M., M. E. Reding, P. B. Schultz, and J. B. Oliver. 2013.** Influence of flood-stress on ambrosia beetle host-selection and implications for their management in a changing climate. *Agric. For. Entomol.* 15: 56–64.

**Ranger, C. M., P. B. Schultz, S. D. Frank, J. H. Chong, and M. E. Reding. 2015.** Non-native ambrosia beetles as opportunistic exploiters of living but weakened trees. *PLoS One.* 10: 1–22.

**Ranger, C. M., P. B. Schultz, S. D. Frank, and M. E. Reding. 2019.** Freeze stress of deciduous trees induces attacks by opportunistic ambrosia beetles. *Agric. For. Entomol.* 21: 168–179.

**Ranger, C. M., P. C. Tobin, and M. E. Reding. 2015.** Ubiquitous volatile compound facilitates efficient host location by a non-native ambrosia beetle. 675–686.

**Reding, M. E., C. M. Ranger, and P. B. Schultz. 2021.** Colonization of trees by ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) is influenced by duration of flood stress. *J. Econ. Entomol.* 114: 839–847.

**Rosenberger, D. 2019.** Factors Contributing to the Death and Decline of Young Apple Trees. *New York Fruit Q.* 27: 5–8.

**Singh, J., K. J. P. Silva, M. Fuchs, and A. Khan. 2019.** Potential role of weather, soil and plant microbial communities in rapid decline of apple trees. *PLoS One.* 14: 1–19.

**Skelton, J., A. J. Johnson, M. A. Jusino, C. C. Bateman, Y. Li, and J. Hulcr. 2019.** A selective fungal transport organ (mycangium) maintains coarse phylogenetic congruence between fungus-farming ambrosia beetles and their symbionts. *Proc. R. Soc. B Biol. Sci.* 286.

**Slippers, B., and M. J. Wingfield. 2007.** Botryosphaeriaceae as endophytes and latent pathogens of woody plants: diversity, ecology and impact. *Fungal Biol. Rev.* 21: 90–106.

**La Spina, S., C. De Caniere, A. Dekri, and J. Gregoire. 2013.** Frost increases beech susceptibility to scolytine ambrosia beetles. *Agric. For. Entomol.* 15: 157–167.

**Stack, A. J., M. Madra, T. R. Gordon, and R. M. Bostock. 2020.** Seasonal variation in host susceptibility to *Fusarium* canker in young almond trees. *Plant Dis.* 104: 772–779.

**White, T. J., T. Bruns, S. Lee, and J. W. Taylor. 1990.** Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics., pp. 315–322. *In* Innis, M., Gelfand, D., Sninsky, J., T.J., W. (eds.), *PCR Protoc. A Guid. to Methods Appl.* New York Academic Press, New York, NY.

**Wright, A. A., A. R. Cross, and S. J. Harper. 2020.** A bushel of viruses: Identification of seventeen novel putative viruses by RNA-seq in six apple trees. *PLoS One.* 15: 1–20.

**Xiang, L., L. Zhao, M. Wang, J. Huang, X. Chen, C. Yin, and Z. Mao. 2021.** Physiological Responses of Apple Rootstock M.9 to Infection by *Fusarium solani*. *HortScience.* 1–8.

**Xu, H., K. D. Hannam, J. L. MacDonald, and D. Ediger. 2023.** Field Investigation into Tree Fates from Recent Apple Tree Decline: Abrupt Hydraulic Failure Versus Gradual Hydraulic Loss. *Stresses*. 3: 256–269.

**Yan, K., G. Han, C. Ren, S. Zhao, X. Wu, and T. Bian. 2018.** *Fusarium solani* infection depressed photosystem performance by inducing foliage wilting in apple seedlings. *Front. Plant Sci.* 9: 1–10.

## Chapter 1 Tables.

Table 1.1. Site details for tree decline survey conducted across 6 sites in the growing season of 2017 and ethanol-baited trap surveys conducted in 2017, 2018, and 2019.

Orchard ID	County	GPS coordinate s	2017 Surveys				Trap period		
			Cultivar (rootstock)	Tree age (years)	Trees assessed	Survey dates (2017)	2017	2018	2019
AH	Haywood	35.271679 -83.02302	Golden Delicious, Honey crisp (B.9)	4	375	6/7, 6/28, 7/19, 8/9, 9/7	7 Jun – 18 Dec	9 Jan – 18 Oct	15 Feb – 23-Oct
RS	Henderson	35.429260, -82.561736	Gala (M.9-RN29)	5	250	7/24, 8/16, 9/21	7 Jun – 18 Dec	9 Jan – 18 Oct	-
NR	Henderson	35.398746, -82.353587	Red Delicious & Fuji (M9-T337)	5	125 (Red Delicious ), 125 (Fuji)	6/28, 7/19, 8/9, 9/8	7 Jun – 18 Dec	9 Jan – 18 Oct	15 Feb – 23-Oct
JB	Henderson	35.397987, -82.361879	Honey crisp (M9-337)	unknown	245	6/7, 6/28, 7/19, 8/9, 9/7	7 Jun – 18 Dec	9 Jan – 18 Oct	-
TH	Henderson	35.211611 -82.223040	Honey crisp (M.26)	4	250	6/7, 6/28, 7/19, 8/9, 9/8	7 Jun – 18 Dec	9 Jan – 18 Oct	-
NG	Henderson	35.245816 -82.213135	Granny Smith (M.9)	6	250	7/12, 8/9, 9/8	-	-	-

Table 1.2. Mean ( $\pm$  SE) number of beetle entries and mean percentage of trees with at least one entry (% of trees attacked) on apple trees in various states of decline based on a survey of 10 trees/health status per orchard across 4 orchards in Haywood and Henderson Counties, July 2017.

Tree Health Status <sup>1</sup>	n	Graft Union		Scion		Total	
		avg. entry per tree	Incidence <sup>2</sup> (% attack)	avg. entry per tree	Incidence <sup>2</sup> (% attack)	avg. entry per tree	Incidence <sup>2</sup> (% attack)
Healthy	40	0.5 $\pm$ 0.14a	30.0	0.53 $\pm$ 0.19a	32.5	1.03 $\pm$ 0.25a	57.5
Early Decline	40	1.95 $\pm$ 0.42ab	62.5	2.7 $\pm$ 0.76b	60.0	4.65 $\pm$ 0.8b	85.0
Late Decline	38	3.0 $\pm$ 0.75b	60.5	3.34 $\pm$ 0.77b	76.3	6.34 $\pm$ 1.05b	89.5
Dead	39	1.79 $\pm$ 0.39ab	66.7	8.64 $\pm$ 2.66b	87.2	10.44 $\pm$ 2.77b	89.7

<sup>1</sup>Tree health status pre-determined based on visual assessment of canopy health, chlorosis, and presence of cankers, stunting, or die-back. <sup>2</sup>Percentage of trees surveyed with at least one beetle entry. Different letters within a column indicate significant differences in avg. number of entries per tree using Tukey's HSD.

Table 1.3. Frequency occurrence (% of tissue samples with isolate of that genus) and ratio of the occurrence of each genera isolated from gallery vs non-gallery (scion and graft union/rootstock) of fungi isolated from dead and declining trees. Tissue was taken from beetle gallery, graft union area, or scion of declining apple trees..

Fungal Genus	All tissue	Gallery	Graft Union/Rootstock	Scion	Ratio gallery:non- gallery
<i>Fusarium</i>	48.4	67.3	43.5	42	1.57
<i>Diaporthe</i>	41.9	40	42.9	40	0.97
<i>Botryosphaeria</i>	36.8	29.1	46.1	16	0.4
<i>Alternaria</i>	7.8	12.7	3.9	14	1.42
<i>Ambrosiella</i>	6.6	30.9	0	0	-
<i>Penicillium</i>	6.6	16.4	3.9	4	4.15
<i>Paraconiothyrium</i>	5.8	14.5	2.6	6	3.37
<i>Chaetomium</i>	5.4	25.5	0	0	-
<i>Flavodon</i>	5	10.9	1.9	8	2.2
<i>Epicoccum</i>	3.9	1.8	2.6	10	0.29
<i>Cytospora</i>	3.5	5.5	1.9	6	1.39
<i>Clonostachys</i>	3.1	1.8	3.2	4	0.50

Occurrence of genera represented in less than 3% of samples not shown.

Table 1.4. Percentage occurrence (% of tissue samples with isolate of that species) of fungi isolated from dead and declining trees. Tissue was taken from beetle gallery, graft union area, or scion.

Identity	Gallery	Graft Union	Scion
<i>Alternaria alstroemeriae</i>	12.7	3.3	14
<i>Ambrosiella grosmanii</i>	29.1	0	0
<i>Botryosphaeria dothidea</i>	25.5	16.3	66
<i>Botryosphaeria fusispora</i>	0	2.6	28
<i>Chaetomium sp.</i>	27.3	0	0
<i>Clonostachys rosea f. catenulata</i>	1.8	2.6	2
<i>Cytospora mali-spectabilis</i>	5.5	2	6
<i>Diaporthe alnea</i>	9.1	4.6	46
<i>Diaporthe celeris</i>	0	0	10
<i>Diaporthe eucalyptorum</i>	0	0.7	4
<i>Diaporthe mahothocarpus</i>	0	2.6	8
<i>Diaporthe sp.</i>	32.7	12.4	36
<i>Didymella rosea</i>	0	1.3	2
<i>Epicoccum italicum</i>	0	2	8
<i>Flavodon ambrosius</i>	10.9	2	8
<i>Fusarium armeniacum</i>	14.5	7.2	22
<i>Fusarium equiseti</i>	5.5	2.6	6
<i>Fusarium fujikuroi</i>	12.7	8.5	6
<i>Fusarium oxysporum species complex</i>	20	1.3	8
<i>Fusarium petersiae</i>	21.8	2.6	20
<i>Fusarium solani</i>	40	13.7	28
<i>Fusarium solani sp. Complex</i>	0	1.3	2
<i>Fusarium subglutinans</i>	14.5	3.9	8
<i>Neopestalotiopsis rosae</i>	7.3	1.3	0
<i>Paraconiothyrium brasiliense</i>	14.5	1.3	10
<i>Penicillium ludwigii</i>	1.8	1.3	0
<i>Penicillium singorense</i>	14.5	0	0
<i>Peniophora crassitunicata</i>	1.8	0.7	2
<i>Phanerochaete sp.</i>	0	0.7	4
<i>Schizophyllum commune</i>	1.8	0.7	4
<i>Trametes versicolor</i>	1.8	0	8

Table 1.5. Relative abundance of beetle species (total number of beetles) captured in ethanol-baited traps across 5 orchards over 3 years in North Carolina.

	Trapping year across locations			Trapping site across years			
	2017	2018	2019	AH	JB	NR	RS
<i>Xylosandrus crassiusculus</i>	25.4 (892)	41.7 (525)	57.4 (870)	39.5 (467)	54.2 (111)	43.9 (1081)	45.5 (153)
<i>Xyleborinus saxesenii</i>	50.1 (1760)	19.3 (243)	12.3 (187)	28.3 (566)	23.4 (147)	29.9 (1180)	28.5 (142)
<i>Xylosandrus germanus</i>	23.6 (829)	23 (289)	23.1 (350)	27.2 (386)	10.2 (417)	20 (524)	14.4 (61)
<i>Xyleborus ferrugineus</i>	0.7 (24)	2.6 (33)	1.3 (20)	0.5 (7)	1.9 (6)	1.9 (42)	2.1 (7)
<i>Cnestus mutilatus</i>	0 (0)	0.2 (2)	0.7 (10)	0.1 (1)	0 (0)	0.4 (8)	0.4 (2)
<i>Euwallacea validus</i>	0 (0)	0.6 (7)	0.7 (10)	0.6 (4)	0.5 (2)	0.2 (4)	0.5 (0)
<i>Ambrosiodmus rubricollis</i>	0.1 (2)	4.4 (55)	0.6 (9)	0.8 (5)	0.3 (23)	0.5 (8)	3.5 (21)
<i>Hypothenemus sp.</i>	0 (0)	0.6 (7)	1.3 (20)	0.4 (4)	0 (2)	0.5 (10)	1.1 (2)
<i>Ambrosiophilus atratus</i>	0 (0)	0.6 (7)	0.3 (5)	0.2 (1)	0.9 (3)	0.2 (3)	0.3 (0)
<i>Monarthrum spp.</i>	0 (1)	0.1 (1)	0.1 (2)	0 (1)	0.5 (0)	0 (0)	0.2 (0)
<i>Cyclorhipidion pelliculosum</i>	0 (0)	0.1 (1)	0.5 (7)	0 (0)	0.5 (0)	0.1 (2)	0.4 (0)
Unknown	0.2 (6)	6.9 (87)	1.5 (22)	2.2 (17)	7.7 (16)	2.5 (42)	2.8 (13)

Table 1.6. Mean number of beetles ( $\pm$  SE) of *X. crassiusculus*, *X. germanus*, and *Xyleborinus saxesenii* captured in ethanol-baited bottle traps deployed at apple orchards adjoining unmanaged wooded areas. Traps were placed at orchard-woods interface (edge), and 20 and 40 m away from woods edge into orchard.

Year	Site	n	<i>X. crassiusculus</i>			<i>X. germanus</i>			<i>X. saxesenii</i>				
			Edge	Interior 20 m	Interior 40 m	Edge	Interior 20 m	Interior 40 m	Edge	Interior 20 m	Interior 40 m		
2017													
	AR	21	2.8 $\pm$ 1	3 $\pm$ 1.1	2.3 $\pm$ 0.9		4.8 $\pm$ 1.5	2.6 $\pm$ 0.8	2.2 $\pm$ 0.6	10.4 $\pm$ 3.1	7.7 $\pm$ 2.6	5.7 $\pm$ 1.4*	
	JB	22	5.7 $\pm$ 3	2.1 $\pm$ 1.2	1.4 $\pm$ 0.7		0.5 $\pm$ 0.3	0.2 $\pm$ 0.1	0.1 $\pm$ 0.1	3 $\pm$ 2	0.1 $\pm$ 0.1	0.4 $\pm$ 0.2	
	NR	22	10.2 $\pm$ 3.6	4.2 $\pm$ 1.4	2.5 $\pm$ 1	*	5.1 $\pm$ 1.8	3.1 $\pm$ 1.3	2.4 $\pm$ 0.9	19.6 $\pm$ 8.5	7.2 $\pm$ 2.5	18.5 $\pm$ 6.5	
	RS	21	2.2 $\pm$ 0.7	0.3 $\pm$ 0.1	0.4 $\pm$ 0.2	**	0.5 $\pm$ 0.2	0.3 $\pm$ 0.2	0.2 $\pm$ 0.1	1.3 $\pm$ 0.5	0.9 $\pm$ 0.4	0.3 $\pm$ 0.1*	
	TH	22	2.5 $\pm$ 0.9	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	*	8.6 $\pm$ 3	2.2 $\pm$ 0.8	3.9 $\pm$ 1.3	2.7 $\pm$ 0.9	2 $\pm$ 0.6	1 $\pm$ 0.4	
2018													
	AR	38	1.1 $\pm$ 0.3	0.4 $\pm$ 0.1	0.8 $\pm$ 0.3	*	0.7 $\pm$ 0.3	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.5 $\pm$ 0.2	0.4 $\pm$ 0.2	0.3 $\pm$ 0.1	
	JB	37	0.6 $\pm$ 0.3	0.3 $\pm$ 0.1	0.3 $\pm$ 0.2		0.2 $\pm$ 0.1	0.1 $\pm$ 0	0.1 $\pm$ 0.1	0.4 $\pm$ 0.1	0.2 $\pm$ 0.1	0.1 $\pm$ 0*	
	NR	37	3.3 $\pm$ 0.9	2.2 $\pm$ 0.7	1.6 $\pm$ 0.5		1.8 $\pm$ 0.6	0.8 $\pm$ 0.3	0.7 $\pm$ 0.3	1.3 $\pm$ 0.3	0.4 $\pm$ 0.1	0.3 $\pm$ 0.1*	
	RS	37	1 $\pm$ 0.4	0.8 $\pm$ 0.2	0.5 $\pm$ 0.2		0.5 $\pm$ 0.2	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1	0.8 $\pm$ 0.3	1.3 $\pm$ 0.4	0.2 $\pm$ 0.1	
	TH	38	0.8 $\pm$ 0.2	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1	*	0.9 $\pm$ 0.2	0.3 $\pm$ 0.1	0.7 $\pm$ 0.2	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.1 $\pm$ 0.1	
2019													
	AR		3.3 $\pm$ 1	1.8 $\pm$ 0.6	2.7 $\pm$ 0.9		2.3 $\pm$ 0.7	1.5 $\pm$ 0.4	1.5 $\pm$ 0.7	0.4 $\pm$ 0.2	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	
	NR		6.4 $\pm$ 2	2.3 $\pm$ 0.6	4.2 $\pm$ 1.3		3 $\pm$ 0.8	0.6 $\pm$ 0.2	1.2 $\pm$ 0.4	*	1.8 $\pm$ 0.7	0.5 $\pm$ 0.2	0.9 $\pm$ 0.3

## Chapter 1 Figures.

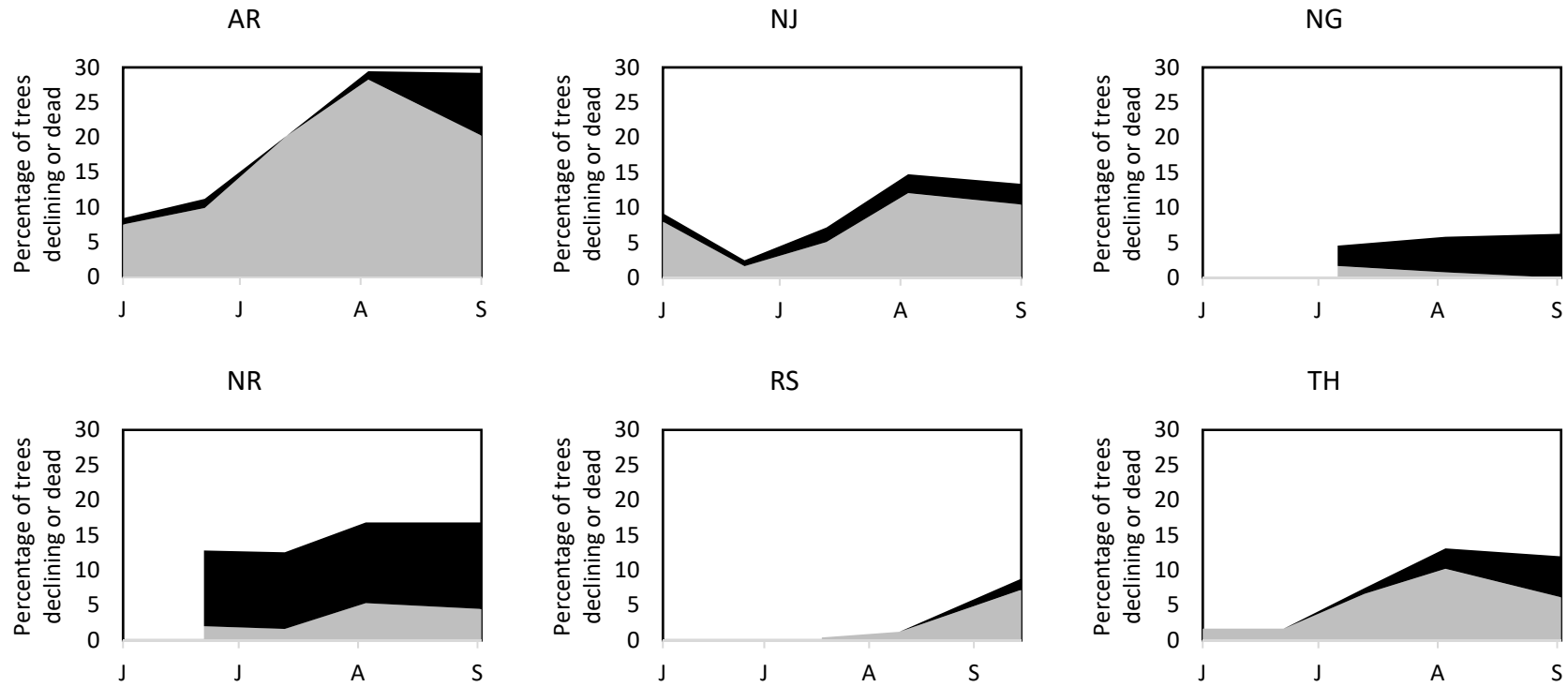


Fig 1-1. Progression of tree decline, percentage of surveyed trees rated as dead (black area) or in decline (grey area) over summer (June-September) 2017 across six orchard sites in Haywood and Henderson counties, NC.

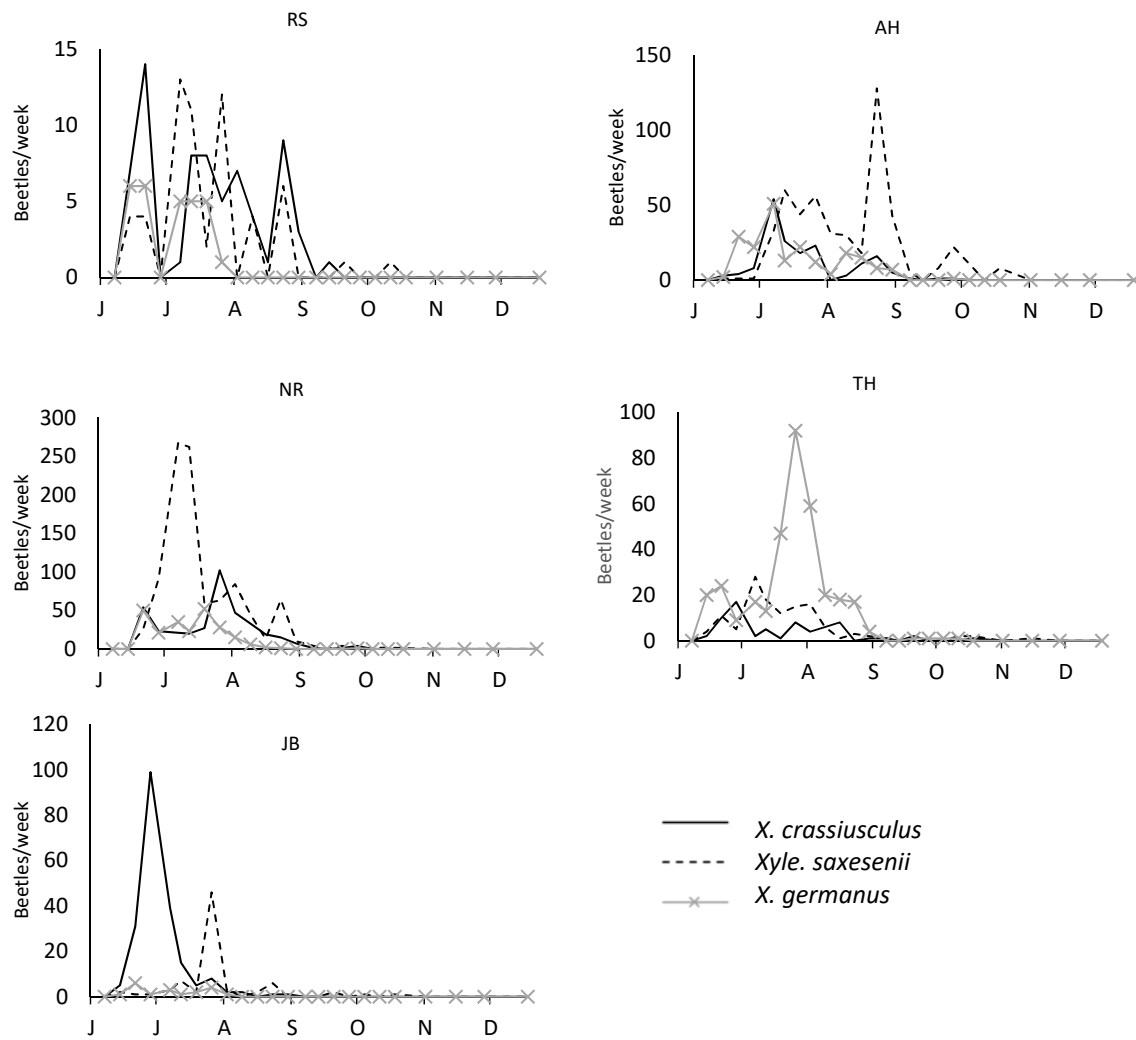


Fig. 1-2. Average weekly trap catches (number beetles per trap week) of *X. crassiusculus* (solid lack line), *Xyleborinus saxesenii* (dashed black line), and *X. germanus* (solid grey line) captured ethanol-baited traps at apple orchards in Haywood and Henderson counties, NC in 2017.

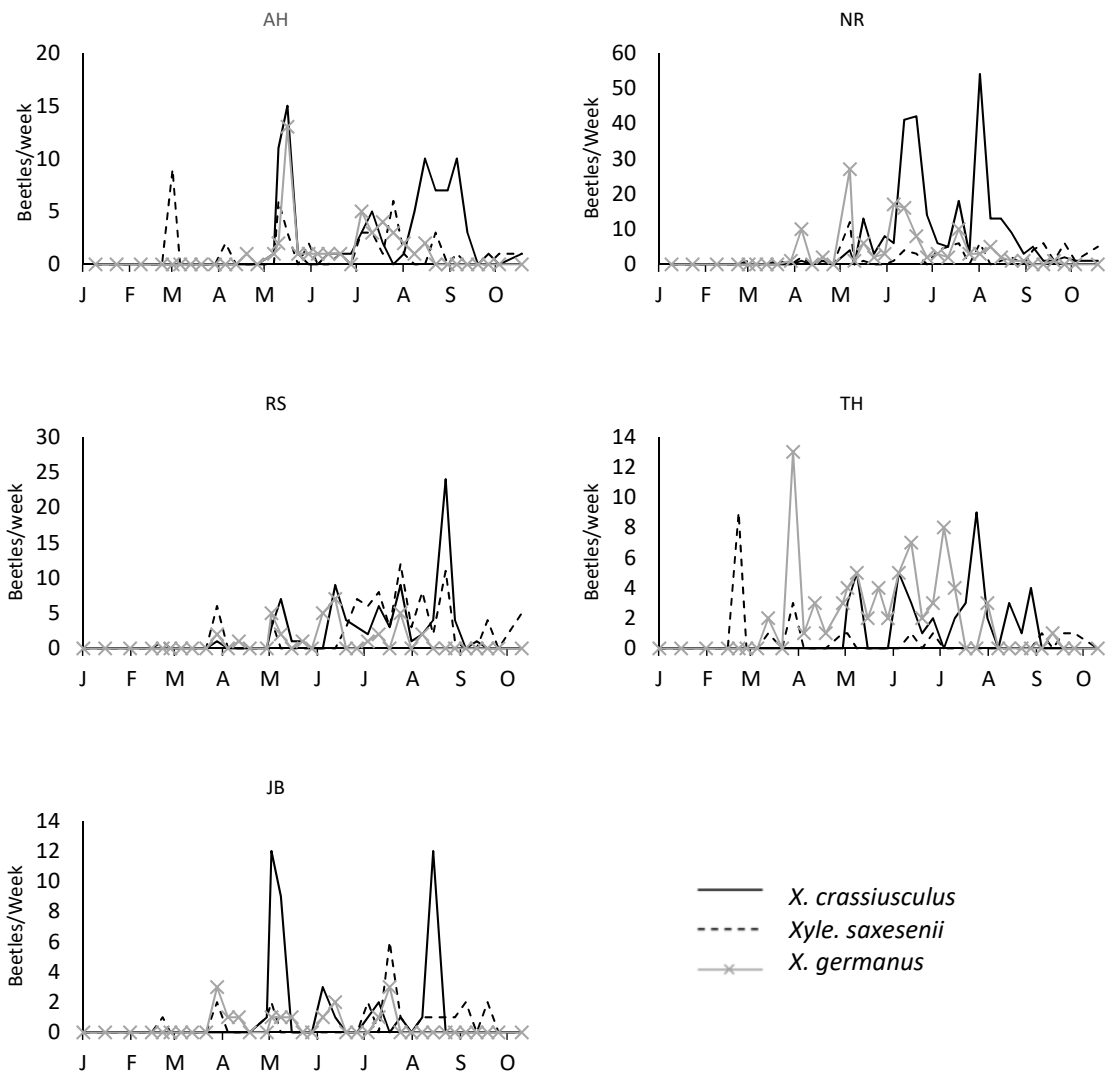


Fig. 1-3. Average weekly trap catches (number beetles per trap week) of *X. crassiusculus* (solid black line), *Xyleborinus saxesenii* (dashed black line), and *X. germanus* (solid grey line) captured ethanol-baited traps at apple orchards in Haywood and Henderson counties, NC in 2018.

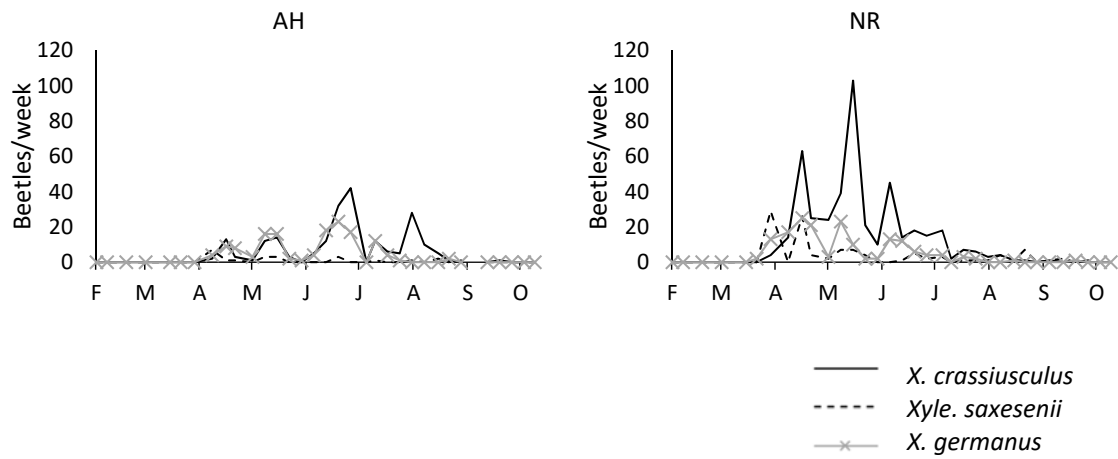


Fig. 1-4. Average weekly trap catches (number beetles per trap week) of *X. crassiusculus* (solid black line), *Xyleborinus saxesenii* (dashed black line), and *X. germanus* (solid grey line) captured ethanol-baited traps at apple orchards in Henderson county, NC in 2019.

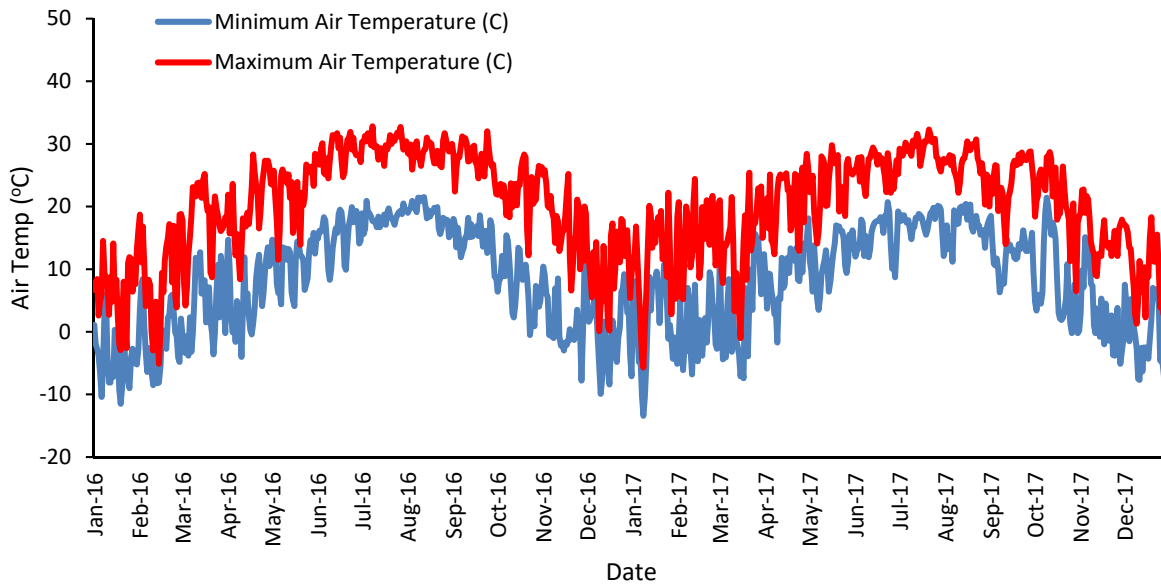
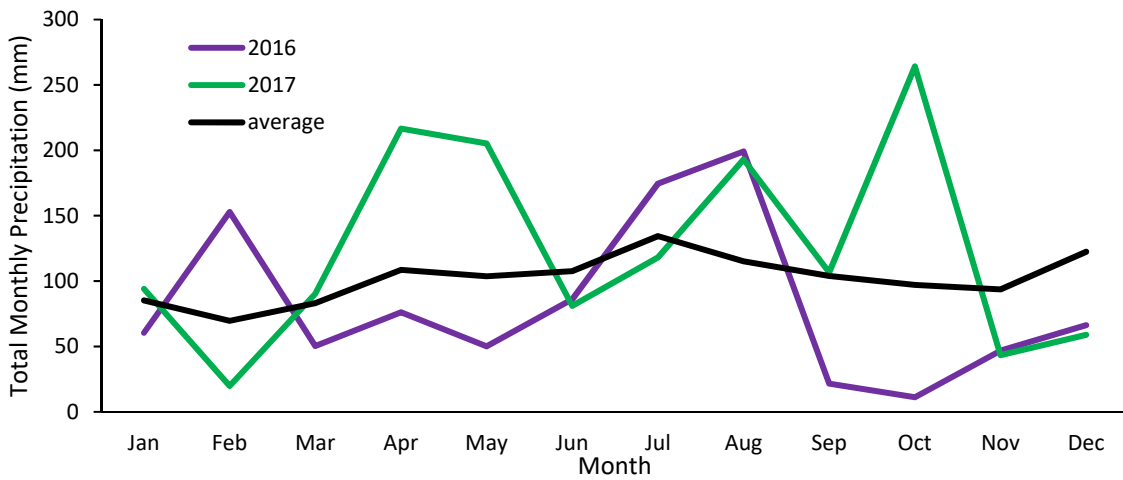
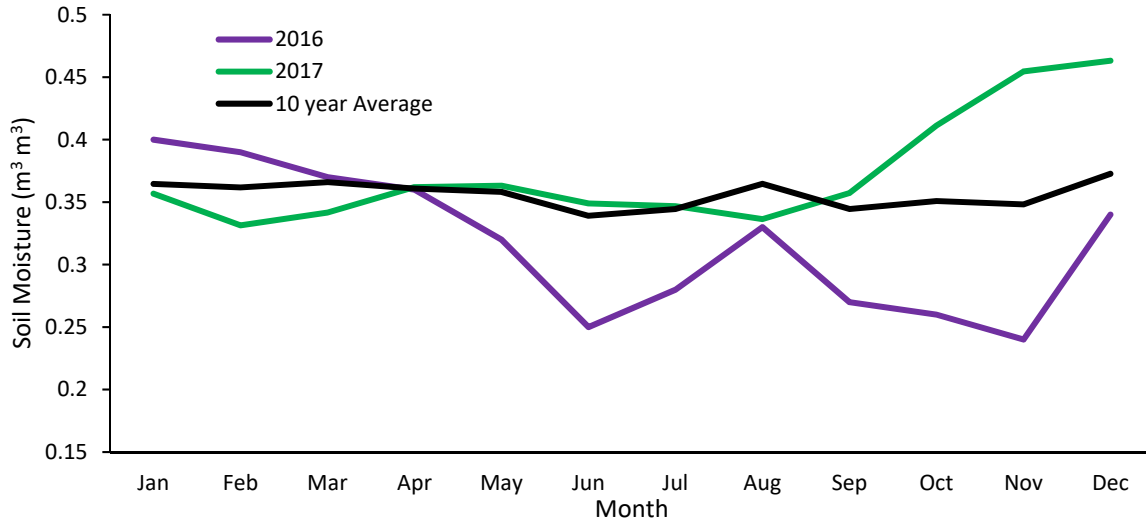


Figure 1-5. Weather Data records for 2016 and 2017 at MHCRC ECONET weather station.

Supplementary Information



SI Fig. 1: Health status of apple trees used for rating status of trees for surveys in 2017, A) Healthy tree, B) Early stages of decline, C) Advanced decline, and D) Defoliated dead tree.

SI Table 1: Total precipitation per month (mm) at MHCREC ECONET weather station from Jan 2006 to Dec 2017

Month	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	average
Jan	92.0	80.0	59.4	57.7	56.6	37.6	101.9	230.9	64.8	86.6	60.5	94.0	85.2
Feb	55.9	37.1	96.5	25.2	79.5	69.3	38.1	99.6	84.1	77.2	152.9	19.8	69.6
Mar	21.8	101.4	110.2	87.1	52.3	172.7	85.6	94.0	71.6	60.5	50.3	90.4	83.2
Apr	97.5	41.4	63.5	81.3	52.6	113.8	117.4	158.5	135.6	149.1	76.2	216.6	108.6
May	39.9	25.7	35.8	200.2	135.1	89.2	85.6	211.6	116.6	49.0	50.0	205.2	103.7
Jun	102.1	65.8	25.9	124.7	46.5	87.6	43.4	294.4	134.6	198.9	86.1	81.0	107.6
Jul	70.4	116.6	63.0	58.7	119.9	105.7	124.5	428.0	135.1	98.3	174.5	118.1	134.4
Aug	174.8	43.4	45.2	98.6	79.8	82.0	62.0	233.2	88.1	82.0	199.1	193.0	115.1
Sep	157.2	89.4	31.5	170.2	101.6	91.7	139.7	85.9	135.4	116.8	21.6	106.7	104.0
Oct	74.7	74.7	41.9	89.9	70.6	63.3	86.9	63.3	111.0	212.9	11.2	264.2	97.0
Nov	108.7	36.8	39.9	137.7	126.0	126.2	21.8	106.2	109.7	221.2	46.7	43.4	93.7
Dec	124.2	104.7	116.1	192.5	9.9	132.3	114.3	220.0	74.4	254.5	66.3	58.9	122.3
Grand Total	1119.1	816.9	729.0	1323.6	930.4	1171.4	1021.1	2225.3	1261.1	1607.1	995.4	1491.4	1224.3

SI Table 2: Average monthly soil moisture measured at 1 m depth (m<sup>3</sup>/m<sup>3</sup>) at MHCREC ECONET weather station from Jan 2006 to Dec 2017.

Month	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	10 year Average
Jan	0.29	0.39	0.35	0.32	0.32	0.40	0.41	0.32	0.40	0.41	0.40	0.36	0.36
Feb	0.28	0.37	0.35	0.28	0.39	0.39	0.41	0.31	0.40	0.41	0.39	0.33	0.36
Mar	0.27	0.38	0.35	0.37	0.39	0.39		0.32	0.40	0.42	0.37	0.34	0.37
Apr	0.28	0.33	0.34	0.39	0.35	0.39	0.40	0.31	0.40	0.42	0.36	0.36	0.36
May	0.32	0.28	0.33	0.40	0.40	0.39	0.39	0.33	0.39	0.39	0.32	0.36	0.36
Jun	0.34	0.27	0.31	0.40	0.33	0.31	0.39	0.34	0.40	0.39	0.25	0.35	0.34
Jul	0.30	0.33	0.31	0.35	0.27	0.33	0.39	0.48	0.42	0.33	0.28	0.35	0.34
Aug	0.33	0.33	0.29	0.37	0.32	0.35	0.40	0.53	0.40	0.36	0.33	0.34	0.36
Sep	0.37	0.34	0.31	0.37	0.34	0.34	0.38	0.33	0.40	0.34	0.27	0.36	0.34
Oct	0.35	0.35	0.30	0.40	0.36	0.35	0.33	0.36	0.41	0.39	0.26	0.41	0.35
Nov	0.38	0.33	0.31	0.40	0.37	0.39	0.23	0.38	0.40	0.40	0.24	0.45	0.35
Dec	0.38	0.36	0.35	0.41	0.39	0.40	0.25	0.40	0.41	0.41	0.34	0.46	0.37

SI Table 3: Average monthly mean temperature (°C) at MHCREC ECONET weather station from Jan 2006 to Dec 2016.

Month	Average monthly mean temperature (°C)												Average
	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	
Jan	5.5	3.7	1.9	1.9	-0.7	0.2	4.4	5.4	-1.3	2.3	0.6	6.2	2.5
Feb	3.1	1.9	5.1	3	-0.1	5.7	6.3	3.8	4	-0.1	3.4	7.8	3.7
Mar	7.8	10.4	7.4	8.7	6.7	8.5	12.6	4.9	6.5	9.7	10.8	7	8.6
Apr	14.4	11.3	12.5	12.4	13.8	14.7	14	12.7	12.9	14.3	13.1	15	13.4
May	15.7	16.9	16.6	17	18.4	17.2	18.6	16.1	17.5	18.5	16.6	17.3	17.2
Jun	19.9	20.7	21.5	21.2	22.4	21.4	20.6	21.2	21.4	21.9	21.9	20.5	21.2
Jul	22.2	20.8	21.8	20.8	23.4	23.6	23.4	22.1	21.3	23.2	23.2	22.7	22.4
Aug	22.4	23.5	21.7	21.3	23.2	22.6	21.1	21.1	21.5	21.6	22.8	21.3	22.0
Sep	17.1	19	18.6	18.4	19	18.3	18.5	18.6	19.7	18.5	20.4	17.7	18.7
Oct	11.5	14.4	11.4	12.2	12.6	11.3	12.1	13.6	13.3	12.6	14.3	13.5	12.7
Nov	7.4	6.8	5.2	8.4	7.4	8.5	6.1	5.8	4.8	10	8.6	8.5	7.3
Dec	4.8	5.8	5.6	1.9	-1	5.9	5.9	5.1	6	9.8	5.1	3.6	4.9

SI Table 4: Absolute minimum and average minimum temperature (°C) at MHCREC ECONET weather station from Jan 2006 to Dec 2016.

Absolute minimum temperature (°C)													
Month	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	Record Minimum
Jan	-7.7	-12.9	-13.7	-15.9	-13.2	-16.6	-11.2	-6.4	-18.8	-15.4	-11.6	-13.5	-18.5
Feb	-8.7	-10.9	-7.7	-13.3	-12.1	-9.6	-9.5	-8.1	-7.7	-16.2	-8.8	-7	-16.1
Mar	-6.4	-5.8	-6.4	-6.1	-7	-4.2	-5.3	-5.7	-6.9	-7.2	-4.2	-7.4	-7.4
Apr	-2	-6.7	-3.8	-3.6	-0.2	-2.2	-0.4	0	-2.7	-3.3	-4.1	-1.7	-4
May	1.7	1.2	2.7	0.9	2.6	0.8	4.7	1.7	1.7	3.5	3.8	3	1.2
Jun	7.5	9.6	9.2	10	11.3	10.3	7.1	10.6	12.5	11.4	8	8.5	7.4
Jul	12.1	11.6	9.2	11.5	12.4	14.6	16.9	13.9	10.3	14.4	15.3	12	8.1
Aug	14	13.5	10.4	13.9	13.1	11.4	11.8	11.3	11.6	12.5	14.8	11.1	10.6
Sep	4.4	3	5.8	8.4	7.6	9.4	5.2	10	7.5	3	8	6.1	3
Oct	-2.4	-3.4	-3.5	-2	-1.4	-3.6	1.4	-5.4	-0.1	-2.7	-0.6	-0.4	-5.3
Nov	-6.7	-7.7	-11.7	-1.5	-6	-5.8	-5.9	-8.4	-8.9	-6.5	-8.1	-5.3	-11.6
Dec	-11.8	-9.7	-12.5	-10.3	-11.6	-7.1	-5	-8.9	-6.4	-5.6	-10.2	-8.3	-12.5

Average Minimum Air Temperature (°C)													
Month	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	10 year Average
Jan	-0.5	-2	-4.1	-3.7	-5.5	-4.9	-1.5	0.6	-7.8	-3.3	-4.6	1.3	-2.7
Feb	-2.8	-4.7	-1.5	-3.7	-3.9	-1.5		-1	-2.1	-5.7	-1.4	0.1	-2.1
Mar	1	2.9		2	1.2	2.7	6.2		-0.5	3.6	3.5	0.4	2.5
Apr	6.1	3.9	5.9	5.3	5.1	7.1	7.4	6.2	5.4	7.9	5.3	9	6.8
May	8.7	8.7	9.7	12.1	12.7	10.7	13	10.2	10.3	11.6	10.7	11.2	11.1
Jun	13.6	14.3	14.2	15.4	16.8	15.5	14.3	16	16.4	16.6	15.4	15.1	15.6
Jul	16.7	15.8	15.1	15.8	18.2	18.6	18.7	18.1	16.3	18.2	18.1	17.5	17.5
Aug	17.2	17.3	15.7	16.8	18.8	17.1	16.4	17.1	16.3	16.4	18.7	16.8	17.4
Sep	12.7	12.6	13.6	14.6	12.7	13.5	13.4	13.9	15.8	13.3	14.4	12.1	13.9
Oct	5.7	8.3	5.1	7	4.9	4.2	6.6	8.1	7.2	7	7.2	7.3	7.0
Nov	1	-0.7	-1.7	2.7	0.6	1.9	-0.7	-0.1	-1.6	4.7	1.3	2.6	1.2
Dec	-2	0.2	-0.1	-2.8	-5.1	0.2	0.7	-0.4	1.7	4.2	-0.2	-1.2	-0.1

## Chapter 2 Understanding and Mitigating the Risk of Ambrosia Beetle (Curculionidae, Scolytinae) Attack on Apple, *Malus domestica* (Borkh).

### Abstract

Exotic ambrosia beetles from the tribe Xyleborini (Coleoptera: Curculionidae) are of increasing concern to tree fruit orchards in eastern North America. These beetles have been associated with dead and dying apple trees in North Carolina and may be a factor in the phenomenon known as Rapid Apple Decline. This study investigated the seasonal activity and relative species abundance of ambrosia beetles in four apple orchards in North Carolina, one in Virginia, and four in Ohio in 2020 and 2021. In 2019 and 2020, ethanol-drenched potted apple trees were deployed in two apple orchards for 14 day exposure periods throughout the season to assess the phenology of tree attack with beetle flight activity. In 2018 and 2019, the efficacy of insecticides in preventing attacks was tested in field trials. Trapping studies showed variability in the seasonal trend in beetle flight activity and relative species abundance across sites and years with *Xylosandrus germanus* dominant in OH, and both *X. germanus* and *X. crassiusculus* common in VA and NC. Traps placed at the interface of apple orchards and unmanaged woods captured significantly more beetles than traps placed within the orchard interior. Almost all successful entries on apple trees were by *X. crassiusculus*, *X. germanus*, or *Cnestus mutilatus*. *Xylosandrus* ambrosia beetles were usually the dominant species in ethanol-baited traps, but a broader a diversity of bark and ambrosia beetles were captured in traps than found colonizing apple trees. The majority of entries on ethanol-drenched trees occurred in May and June in NC, which reflected the main peaks in trap captures. Evaluation of management tactics found soil and foliar-applied neonicotinoid insecticides did not provide significant control, trunk-applied synthetic pyrethroids provided variable control, and an insecticide-impregnated net provide high levels of control.

**Key Words:** Ambrosia beetle, *Xylosandrus*, Xyleborine, Apple, Rapid Apple Decline.

## Introduction

The exotic ambrosia beetles (Coleoptera: Curculionidae, Scolytinae), *Xylosandrus crassiusculus* (Motschulsky) and *X. germanus* (Blandford) have been associated with damage to trees in ornamental nurseries (Ranger et al. 2016) and fruit and nut orchards (Agnello et al. 2015, Monterrosa et al. 2022, Gresham et al. 2023). The highly polyphagous ambrosia beetles in the *Xylosandrus* genus are attracted to physiologically stressed hosts via volatile cues, predominantly ethanol (Ranger et al. 2021). The beetles burrow through the trunk or limb to excavate a tunnel within the sap wood where they propagate their nutritional mutualist fungi on which their offspring feed. (Ranger et al. 2016).

The granulate ambrosia beetle, *X. crassiusculus*, is native to Southern Asia and was first reported in the USA on South Carolina peach trees in 1974 (Anderson 1974). As a sub-tropical species its range appears to be concentrated in the southeastern USA, although detections have been made throughout the eastern USA and it is present in 31 States (Haack and Rabaglia 2013). In recent years *X. crassiusculus* has been reported as an invasive pest in Europe (Contarini et al. 2020), South America (Covre et al. 2021), and Australia (Tran, H. X., Doland Nichols, J., Li, D., Le, N. H., Lawson 2022). The black stem borer, *Xylosandrus germanus*, was first reported on grapevines in New York in 1932 (Felt 1932), and its range extends throughout most of North America; it is present in 34 states and 4 Canadian provinces (Rabaglia et al. 2019). *Xylosandrus germanus* is also found in Europe, first recorded in 1951 in Germany and now occurring in over 21 European countries (Galko et al. 2018, Contarini et al. 2020). The camphor shot borer, *Cnestus mutilatus* (Blandford), is a more recent invader of Asian origin. In the USA, it was first detected in Mississippi in 1999, and has recently been detected in in North Carolina (Gresham et al. 2023) and Pennsylvania in 2013 (Barringer 2016).

Xyleborine ambrosia beetles are able to exploit a large number of hardwood tree species that are physiologically stressed (Ranger et al. 2021). Unmanaged woody habits are considered to be

important sources of beetles for infestations of crops in several production systems (Werle et al. 2015, Ranger et al. 2016, Agnello et al. 2017, Galko et al. 2018). Such habitats commonly border apple orchards in North Carolina (Ogburn et al. 2021), and are suspected of being an important source of ambrosia beetles attacking apple.

Ambrosia beetle flight activity extends throughout the growing season in North Carolina (Gresham et al. 2023) and other eastern states (Ranger, Schultz, et al. 2015, Haas et al. 2016, Agnello et al. 2017, Monterrosa et al. 2022). Traps baited with ethanol lures are used to monitor beetle activity and inform growers of the timing of management tactics (Gugliuzzo et al. 2021, Ranger et al. 2021). Management options for ambrosia beetles are limited to mitigation of predisposing stressors, and application of high rates pyrethroid insecticides to the trunks of trees (Gugliuzzo et al. 2021). However, no insecticides are currently labeled at rates sufficient to control ambrosia beetles on apples and mitigation of predisposing abiotic stress may be outside the control of growers. Alternative management strategies have been investigated, such as repellants (Ranger et al. 2013, Agnello et al. 2021), push-pull semiochemical interruption (Addesso et al. 2019, Werle et al. 2019), systemic acquired resistance inducers (Agnello et al. 2021), microbial pesticides (Castrillo et al. 2011, 2016, Kushiyevev et al. 2021), physical trunk barriers (Ranger et al. 2020), and intercept trapping (Werle, C. T., Sampson B. J. , M. Reding 2017, Addesso et al. 2019). Further development of these strategies is needed to improve efficacy and feasibility before adoption by commercial growers. In this study we sought to understand the relationship between ethanol-baited trap captures and ambrosia beetle attack on apple trees. We also compared ambrosia beetle species diversity in ethanol-baited traps deployed in apple orchards in North Carolina, Virginia, and Ohio, effect of spatial location of traps in apple orchards, and insecticidal control of beetles.

## Methods

### Study Sites

Studies were conducted in apple orchards adjacent to woody habitats. The sites for various aspects of the studies and are shown in Table 1.

### Trapping

Two different trap designs were used across the 2019, 2020, 2021 seasons. In 2019 traps were constructed from a modified 1.75 L inverted juice bottles which had 6x10cm windows cut out of each side (Agnello et al. 2017) baited with ethanol pouches releasing 65 mg/d at 30°C (AgBio Inc., Westminster, CO) and ~ 200 mL of water with a few drops of detergent was used as capture medium. In 2020 and 2021, traps consisted of inverted 2 L soda bottles with three windows cut out of the sides as the top part of the trap and a smaller plastic soda bottle (500-600 mL) filled with water containing a few drops of detergent as the removable trap section as described by (Ranger et al. 2016). The bottles were connected by a female-female threaded Tornado Tube (Steve Spangler Science, Englewood, CO). Traps were suspended at 1.5 m height on the exterior edge of the orchard block adjacent to a wooded area. At each site in NC and OH, a trap was also placed within the orchard interior, 20-30 m from the orchard edge. Orchard interior traps were secured to trellis wires at 1.5 m above the ground. In VA three traps were placed along the orchard-woods interface only. Beetles were collected from traps and trap solution was replaced at approximately weekly intervals.

### **Seasonal variation colonization by Xyleborine ambrosia beetles of ethanol-drenched potted apple trees.**

To evaluate seasonal trends in relative abundance and species composition of ambrosia beetles attacking apple trees, potted apple trees drenched with 3.0% solution of ethanol in water were

deployed and collected every 14 days from May through September at two orchard sites in NC in 2019 and 2020 (Table 1). Apple trees (*Malus domestica* cv 'Granny Smith', 'B.9' rootstock) were planted into 17 L plastic pots with potting media (2:1 bark mulch: vermiculite amended with lime and micronutrients) in 2018. At each site, four trees were placed at the edge of an orchard adjacent to an unmanaged woodlot comprised of mixed hardwoods. The pots were placed inside large contractor bags (156 liter, 3 mil thickness) into which the ethanol solution (3%) was placed to provide a constant source of ethanol to the roots. Pots were topped up with 1-2 L of ethanol solution after 7 days as needed. At each site, an ethanol-baited bottle trap as described above was placed along the woods-orchard interface 20-30 m from the sentinel trees and checked weekly. Scolytinae beetles were identified to species under dissecting microscope.

After 14 days, branches were removed and the trees were cut off at the soil-line and stored at 4°C until processing (maximum of 10 days). The trunks, graft union, and remaining rootstock were inspected for beetle entry holes and galleries were dissected to inspect for the presence of the foundress and species identification. Foundresses were identified to species using a stereo microscope. For each 14-day exposure period, the number of entries per tree and number of recovered *X. crassiusculus*, *X. germanus*, and *C. mutilatus* foundress per tree at each location and deployment date were subjected to ANOVA using Proc GLM in SAS V.9.4 (SAS Institute, Cary NC). The relationship between trap captures and average entries per tree was analyzed using linear regression of two-week trap capture at each site and deployment period and the mean number of entries per tree.

#### **Evaluation of insecticides for reducing ambrosia beetle attacks in apple trees.**

In 2018 and 2019, field trials were conducted to assess the efficacy of various insecticides to prevent beetle attacks and brood establishment on potted apple trees flooded with 2.5% (2018) or 3.0% ethanol (2019) as described above. The insecticide treatments were commercial formulations of

imidicloprid (Admire Pro, 42.8% active ingredient (a.i.), Bayer Cropscience, St Louis, MO); dinotefuran (Venom, 70% a.i., Valent USA, Walnut Creek, CA); chlorpyrifos (Lorsban Advanced, 40.2 % a.i., Corteva, Indianapolis, IL); lambda-cyhalothrin (Warrior II, 22.8 % a.i., Syngenta, Greensboro, NC) and bifenthrin (Brigade 2EC, 25.1 % a.i., FMC Corporation, Philadelphia, PA). Application rates and timings are listed in table 2. Long-lasting insecticide netting (LLIN) impregnated with deltamethrin (Vestergaard-Frandsen, Lausanne, Switzerland) has been previously shown to reduce ambrosia beetle attacks on trunks (Ranger et al. 2020). Soil drench insecticide treatments were applied by irrigating potted trees with 1 liter of insecticide solution as a single application 4 weeks before the exposure period. Foliar spray treatments (Table 2) were applied using a 2.8 liter hand-trigger spray bottle (2018) or a CO<sub>2</sub> backpack sprayer with a twin disc and core nozzle (D3-45) on a hand-held boom (2019) to the point of run-off (363 L Ha<sup>-1</sup> assuming a density of 2500 trees Ha<sup>-1</sup>) from the graft union to about 15 cm above the first scaffold branch when trees were placed in the field and re-applied weekly. Long-lasting insecticide netting (LLIN) wrapped around the trunk and secured with twist ties to cover the trunk from the soil line to a height of ~1 m on trees on the same day insecticide applications began. Both experiments were laid out as randomized complete block designs with five replicates in 2018 and six in 2019. For the 2018 experiment, one-year-old apple trees (cv 'Granny Smith' on Bud.9 rootstock) and in 2019, two-year old potted apple trees (cv 'Golden Delicious' on M.9 Nic-29), were planted in 26.5 liter containers as described above. The container of each tree was placed inside a 78 liter plastic trash bag that was filled with ~10 liters of ethanol solution to induce ambrosia beetle attacks. Weekly counts of beetle entries were conducted beginning 7 days after the first foliar insecticide application, at which time an additional 1 to 2 L of ethanol solution was added as needed. A final destructive assessment was made after 5 weeks all trees were cut at the soil line and stored at 4°C until dissection. Each tree was dissected to inspect beetle entries for species of foundress and determine gallery establishment success based on

presence of progeny and/or symbiotic fungal growth (evidence of dark staining or pale grey mycelium on the inner walls of the gallery).

Field counts of cumulative entries per tree were analyzed as a randomized complete block design ANOVA using SAS (version 9.4) PROC GLM with Tukey HSD used to separate means. Final assessments of beetle entries, successful entries (containing adult beetle, larvae, or symbiont growth) were subjected to ANOVA after testing for normality.

## Results

### Trapping

Ambrosia beetle trap captures in 2020 were dominated at all sites by *X. crassiusculus* and *X. germanus* (Table 3). In North Carolina, 2-3 times more *X. germanus* were captured compared to *X. crassiusculus* in 2020 (Table 3). In 2021, captures were considerably lower for all ambrosia beetles and *X. crassiusculus* was more abundant than *X. germanus*. In Ohio, *X. germanus* was the dominant species across all sites in 2020 and 2021 except the Mooreland site, where *X. crassiusculus* was most abundant. In Virginia, *X. crassiusculus* and *X. germanus* were captured in near equal proportion in 2020, and *X. crassiusculus* was more prevalent 2021.

In North Carolina there was considerable variation in trap captures among sites and years. Captures were generally higher in 2020 than 2021 across all sites. A large number of beetles were captured at first deployment on 16 April in 2020, followed by periods of activity in May, June and July (Fig. 3) and the main summer peak occurred in early July at most sites. Across all sites in OH, peak flight activity of *X. germanus* occurred in late May in 2020 and 2021 (Fig. 1). At two of the sites in 2020, Bauman and Scenic Ridge, there was second period of flight activity in mid-July that was of similar size to the first flight. These July flights were very low or not apparent at other sites. At the Moreland site where *X. germanus* occurred, it also exhibited peak activity in late May, which was most apparent in

2021. At the Virginia site, trends in flight activity in 2020 were difficult to interpret as captures of both species fluctuated from week to week with highest captures occurring from mid-May through late June (Fig 2) and low captures the remaining of the year. In 2021, captures were lower and more condensed than in 2020, with a single peak of *X. germanus* on 5 May. Similarly, *X. crassiusculus* had one distinctive peak on 9 May and low captures the remainder of the trapping period (Fig. 3).

Traps located on the edges of orchards adjacent to woods orchard-woods edge tended to capture more beetles than traps placed 20-30 m into the orchard interior (Table 4). Paired t tests showed significantly higher captures in edge traps in 9 of 17 location-year combinations.

#### **Seasonal variation colonization by Xyleborine ambrosia beetles of ethanol-drenched potted apple trees.**

Three-times more beetle entries were recorded in all trees in 2019 compared to 2020 – 1,093 versus 344. Species composition of extracted beetles differed between years, with *X. crassiusculus* dominant in 2019 (85.3%), and *X. germanus* dominant in 2020 (58.0%) (Table 5). This difference was largely due to considerably lower *X. crassiusculus* numbers in 2020 versus 2019, because the number of *X. germanus* entries were similar in both years (Table 5). In 2019 there was a significant effect of deployment date ( $F_{9,79} = 10.32$ ;  $P < 0.0001$ ) and there was no significant effect of site on average entries per tree ( $F_{1,79} = 0.12$ ;  $P = 0.7255$ ) and no interaction effect of site x deployment date ( $F_{9,79} = 1.48$ ;  $P = 0.1755$ ). The highest number of entries was recorded on trees deployed in mid-May, which had significantly more entries than trees deployed after 10 June. The majority (>60%) of entries and trap captures occurred in May and June in 2019 (Fig 4). In 2020 there was a significant effect of site ( $F_{1,59} = 5.00$ ;  $P = 0.0308$ ) and deployment date ( $F_{8,59} = 6.38$ ;  $P < 0.001$ ) and a significant interaction between site and deployment date ( $F_{8,59} = 3.39$ ;  $P = 0.0044$ ). In 2020, the highest number of entries were recorded on trees deployed mid-June at site 'NR' and late-May at site 'RS' (Fig. 4). There were generally fewer entries

and lower trap catches in 2020 compared with 2019. There were very few entries on trees deployed after mid-July. Overall, there was a significant positive relationship between trap captures and ambrosia beetle entries ( $F_{1,34}=15.49$ ,  $P=0.0004$ ,  $R^2 = 0.31$ ).

#### **Evaluation of insecticides for reducing ambrosia beetle attacks in apple trees.**

In 2018, beetle attacks were relatively low with a cumulative average of only 11.8 attacks per tree in the EtOH control treatment over the 48 day sampling period (Table 6). Significant treatment differences were observed at 7 and 21 days after first foliar application (dafa), with all foliar insecticide treatments significantly reducing attacks compared to the EtOH control (Table 6). Although differences were not significant at 35 and 48 dafa. The chlorpyrifos + lambda-cyhalothrin premix and insecticidal netting treatments consistently had the lowest attacks. Following removal from the field and dissection of galleries, the chlorpyrifos + lambda-cyhalothrin and insecticidal netting treatments were again the only treatments to significantly reduce successful beetle entries (galleries with progeny or fungal establishment) compared to the ethanol control (Table 6). While there was no significant difference between the EtOH and water controls, the EtOH control consistently had higher numbers of beetle attacks. A total of 115 adult beetles were recovered from 304 entries across all trees. Of the 82 beetles that could be identified to species, 40% were *X. germanus* and 57% *X. crassiusculus*. One *Xyleborinus saxesenii* and one *Ambrosidmus rubricollis* were also recovered.

Ambrosia beetle attacks were again relatively low in 2019, but significant treatment effects were observed on all evaluation dates (Table 7). Lambda-cyhalothrin, chlorpyrifos + lambda cyhalothrin, and bifenthrin resulted in significantly lower entries compared with the EtOH control across most evaluation dates, but were not significantly different from the other insecticide treatments (Table 7). The insecticide-impregnated netting completely excluded attacks from the covered trunk area. Of the

272 beetles collected from 441 entries, 89%, 7% and 4% were *X. crassiusculus*, *C. mutilates*, and *X. saxeseni*, respectively.

## Discussion

Trapping studies in North Carolina, Ohio, and Virginia apple orchards showed variation in the abundance and relative species composition of ambrosia beetles captured in ethanol-baited traps among years and locations. In both years at OH sites *X. germanus* was most common with few *X. crassiusculus* captured across most sites. North Carolina and VA showed a similar trend in relative species abundance of *X. crassiusculus* and *X. germanus* with *X. germanus* being more prevalent compared with *X. crassiusculus* in 2020 and the opposite trend in 2021. Our findings concur with other trapping studies conducted in the USA that have found a broad diversity of ambrosia beetles in ethanol-lure bottle traps dominated by the exotic Xyleborine species that showed in northern latitudes in the eastern USA, *X. germanus* is dominant or exclusively captured (Breth, Agnello, and Tee 2016, Haas et al. 2016, Agnello et al. 2017) whereas in southern latitudes it is rare or absent and *X. crassiusculus* and *X. compactus* are more prevalent (Werle et al. 2015, Ranger et al. 2016, Monterrosa et al. 2022). The exception being high-altitude orchard sites in GA where *X. germanus* were captured at similar proportions to *X. crassiusculus* (Monterrosa et al. 2021). Notably, there was a large difference in trap captures between years with higher captures in 2020 compared with 2021 across all sites in NC and VA and most sites in OH. There contributing factors for annual differences in beetle activity should be explored further.

The same exotic Xyleborine ambrosia beetles that dominated ethanol-baited trap captures in NC were found to be responsible for nearly all beetles recovered from ethanol-drenched sentinel apple trees deployed at NC apple orchards. Ethanol-drenched apple trees were almost exclusively attacked by *X. crassiusculus*, *X. germanus*, and *C. mutilatus* in our sentinel tree study over two years. Furthermore,

all identified beetles that attacked trees in the 2019 insecticide experiment and all but two identified beetles in 2018 were these three species. Previous studies using trees that were flood stressed or drenched with ethanol found around 95% of beetle galleries contained exotic Xyleborine ambrosia beetles (Addesso et al. 2019, Werle et al. 2019, Ranger et al. 2023).

Apple trees on the exterior of orchards bordering unmanaged woods are likely to experience higher risk of ambrosia beetle attack than trees within the orchard interior due to a higher beetle abundance at the woods-orchard interface. We captured a significantly higher number of Xyleborine ambrosia beetles in traps placed at the woods-edge than interior rows of apple orchards in NC and OH, supporting previous findings based on trap captures in fruit orchards (Agnello et al. 2017, Monterrosa et al. 2022) ornamental nurseries (Werle et al. 2015, 2019, Werle, C. T., Sampson B. J., M. Reding 2017) in eastern USA, and Black walnut plantations (Williams and Ginzel 2020) in Indiana, USA.

Unmanaged woody habitats commonly found bordering apple orchards in North Carolina are composed of a variety of woody tree species such as *Quercus sp.* (oak), *Juglans nigra* (black walnut), *Prunus sp.* (cherry), *Acer sp.* (maple), and *Cercis canadensis* (redbud) (Bakken et al. 2015, Ogburn et al. 2021) that are hosts for Xyleborine ambrosia beetles (Schedl 1963, Weber and McPherson 1983, Ranger et al. 2016). These unmanaged woodlots are likely to have more physiologically stressed trees compared to managed agricultural systems such as orchards, where dead and dying trees are removed. The risk of tree attack is therefore likely to be concentrated to orchard areas adjacent to unmanaged wooded habitat, presumably due to limited dispersal of the beetles. Further work is needed to understand how surrounding habitats impact ambrosia beetle abundance across landscapes.

The risk of apple trees being attacked ambrosia beetles appears to be highest in spring and early summer. Ambrosia beetle trap captures typically peak in May or early June in NC and the highest number of entries were recorded in May or June in 2019 and 2020 on sentinel apple trees. Although

there were some entries on sentinel trees throughout the summer, they were relatively low. Our results on the phenology of beetle attack of apple trees is similar to attack of other tree species in this region. In Tennessee, 95% of ambrosia attacks of Chestnut occurred in April and May, the majority by *X. crassiusculus* (Oliver and Mannion 2001). The most important factor affecting the risk of beetle attack within an orchard is host ethanol emissions resulting from physiological stress. Ethanol, produced by trees in response to a range of abiotic and biotic stressors acts as an efficient long-range attractant to Xyleborine ambrosia beetles and guides host selection at an individual host level. *Xylosandrus germanus* preferentially landed on ethanol-injected trees but rarely landed on neighboring healthy trees, and only established galleries within hosts with elevated ethanol (Ranger, Tobin, et al. 2015). Furthermore, despite higher populations of beetles along borders with unmanaged habitat, Ranger et al. (2015) found a non-random distribution of attacked trees within an ornamental nursery, whereby beetles infested certain species and individual trees that were impacted by freeze damage, and no edge effect was reported. Therefore, in addition to understanding the risk in relation to temporal and spatial behavior of the beetles, growers should focus management efforts on trees that are vulnerable to attack as a result of damaging stressors such as waterlogging or freeze damage.

In situations where apple trees have experienced severe physiological stress, and are attractive to beetle attack, insecticide applications are the only viable tactic for protection (Breth, Agnello, Cox, et al. 2016, Gugliuzzo et al. 2021). The pyrethroids, bifenthrin and permethrin, have been most effective in protecting trees from ambrosia beetle attack (Mayorquin et al. 2018, Reding and Ranger 2018), and also showed good results in our study. Unfortunately the rate needed for effective control with bifenthrin is not labeled for use on apples. In addition, pyrethroid applications in apples during the early part of the growing season is discouraged to avoid disrupting natural enemy populations (Funayama 2015), and pollinators during bloom. Therefore, preventative control using synthetic pyrethroids targeting the first

beetle flight in April and May in tree fruit production is problematic within an integrated pest management system.

Results of our studies as well as well as others have shown inconsistent or poor results with other types of pesticides (Gugliuzzo et al. 2021). Moderate control was achieved with chlorpyrifos (Agnello et al. 2017), but use of this pesticide has been revoked by the Environmental Protection Agency. Alternative management tactics to insecticides have been explored for managing Xyleborine ambrosia beetles including systemic acquired resistance inducers (Agnello et al. 2021), microbial controls (Kushiyev et al. 2021), fungicides (Kushiyev et al. 2018, Mayorquin et al. 2018), and semiochemical approaches (Hughes et al. 2017, Werle et al. 2019, Agnello et al. 2021, Gugliuzzo et al. 2021). Long-lasting insecticide impregnated netting wrapped around the lower trunk provided the excellent control for preventing ambrosia beetle attack in our studies as well as those by Ranger et al. (2020) in ornamentals. While the deltamethrin-impregnated netting used in our study is not commercially available in the USA, this concept does show promise.

Integrated pest management practices utilize a range of tactics to optimize the benefits of reducing the economic impact of pests with the economic and ecological costs associated with control (Biddinger and Rajotte 2015). The economic impact of ambrosia beetles attacking apple trees is the presumed acceleration of decline leading to tree mortality. Given that Xyleborine ambrosia beetles will preferentially attack trees experiencing stress (Ranger, Tobin, et al. 2015), and to the best of our knowledge, no published studies have quantified the relative impact of beetle attack on tree health, effective controls should focus on the underlying causes of stress that make the apple trees attractive to beetle attack and vulnerable to dieback and decline. Furthermore, a better understanding of the relative impact of ambrosia beetles on tree health is needed so that growers can better prioritize their efforts to limit tree losses in apple orchards.

## References

- Addesso, K. M., J. B. Oliver, N. Youssef, P. A. O'Neal, C. M. Ranger, M. Reding, P. B. Schultz, and C. T. Werle. 2019.** Trap Tree and Interception Trap Techniques for Management of Ambrosia Beetles (Coleoptera: Curculionidae: Scolytinae) in Nursery Production. *J. Econ. Entomol.* 112: 753–762.
- Agnello, A., D. Breth, E. Tee, K. Cox, and H. R. Warren. 2015.** Ambrosia Beetle – An Emergent Apple Pest. 2013–2016.
- Agnello, A. M., D. I. Breth, E. M. Tee, K. D. Cox, S. M. Villani, K. M. Ayer, A. E. Wallis, D. J. Donahue, D. B. Combs, A. E. Davis, J. A. Neal, and F. M. English-Loeb. 2017.** *Xylosandrus germanus* (Coleoptera: Curculionidae: Scolytinae) Occurrence, Fungal Associations, and Management Trials in New York Apple Orchards. *J. Econ. Entomol.* 110: 2149–2164.
- Agnello, A. M., D. B. Combs, C. C. Filgueiras, D. S. Willett, and A. Mafra-Neto. 2021.** Reduced Infestation by *Xylosandrus germanus* (Coleoptera: Curculionidae: Scolytinae) in Apple Trees Treated with Host Plant Defense Compounds. *J. Econ. Entomol.* 114: 2162–2171.
- Bakken, A. J., S. C. Schoof, M. Bickerton, K. L. Kamminga, J. C. Jenrette, S. Malone, M. A. Abney, D. A. Herbert, D. Reisig, T. P. Kuhar, and J. F. Walgenbach. 2015.** Occurrence of brown marmorated stink bug (Hemiptera: Pentatomidae) on wild hosts in Nonmanaged Woodlands and soybean fields in North Carolina and Virginia. *Environ. Entomol.* 44: 1011–1021.
- Barringer, L. E. 2016.** First record of the camphor shot borer, *Cnestus mutilatus* (Blandford), (Curculionidae: Scolytinae: Xyleborini) in Kentucky. *Insecta mundi.* 1–2.
- Biddinger, D. J., and E. G. Rajotte. 2015.** Integrated pest and pollinator management - adding a new dimension to an accepted paradigm. *Curr. Opin. Insect Sci.* 10: 204–209.

**Breth, D., A. Agnello, K. Cox, and E. Tee. 2016.** Black Stem Borer *Xylosandrus germanus*. 81: 6–10.

**Breth, D., A. Agnello, and E. Tee. 2016.** Black stem borer control in apple nurseries and tall spindle plantings Debo. Cornell Coop. Ext. Publ. 2–3.

**Castrillo, L. A., M. H. Griggs, C. M. Ranger, M. E. Reding, and J. D. Vandenberg. 2011.** Virulence of commercial strains of *Beauveria bassiana* and *Metarhizium brunneum* (Ascomycota: Hypocreales) against adult *Xylosandrus germanus* (Coleoptera: Curculionidae) and impact on brood. *Biol. Control*. 58: 121–126.

**Castrillo, L. A., M. H. Griggs, and J. D. Vandenberg. 2016.** Competition between biological control fungi and fungal symbionts of ambrosia beetles *Xylosandrus crassiusculus* and *X. germanus* (Coleoptera: Curculionidae): Mycelial interactions and impact on beetle brood production. *Biol. Control*. 103: 138–146.

**Contarini, M., A. Vannini, F. Giarruzzo, M. Faccoli, C. Morales-Rodriguez, L. Rossini, and S. Speranza. 2020.** First record of *Xylosandrus germanus* (Blandford) (Coleoptera: Curculionidae, Scolytinae) in the Mediterranean scrubland in Southern Italy, and its co-presence with the co-generic species *X. compactus* (Eichhoff) and *X. crassiusculus* (Motschulsky). *EPPA Bull.* 50: 311–315.

**Covre, L. de S., A. A. Melo, and C. A. H. Flechtmann. 2021.** Flight activity and spread of *Xylosandrus crassiusculus* (Motschulsky) (Coleoptera: Curculionidae) in Brazil. *Trees, For. People*. 4: 100076.

**Felt, E. P. 1932.** A new pest in greenhouse grown grape stems. *J. Econ. Entomol.* 25: 418.

**Funayama, K. 2015.** Outbreaks of the two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae) are caused by broad-spectrum insecticide spraying in apple orchards. *Appl. Entomol. Zool.* 50: 169–174.

**Galko, J., M. Dzurenko, C. M. Ranger, J. Kulfan, E. Kula, C. Nikolov, M. Zúbrik, and P. Zach. 2018.** Distribution, habitat preference, and management of the invasive ambrosia beetle *Xylosandrus germanus* (Coleoptera: Curculionidae, Scolytinae) in European forests with an emphasis on the West Carpathians. *Forests.* 10.

**Gugliuzzo, A., P. H. W. Biedermann, D. Carrillo, L. A. Castrillo, J. P. Egonyu, D. Gallego, K. Haddi, J. Hulcr, H. Jactel, H. Kajimura, N. Kamata, N. Meurisse, Y. Li, J. B. Oliver, C. M. Ranger, D. Rassati, L. L. Stelinski, R. Sutherland, G. Tropea Garzia, M. G. Wright, and A. Biondi. 2021.** Recent advances toward the sustainable management of invasive *Xylosandrus* ambrosia beetles. *J. Pest Sci.* (2004). 94: 615–637.

**Haack, R. A., and R. J. Rabaglia. 2013.** Exotic bark and ambrosia beetles in the USA: potential and current invaders, pp. 48–74. *In* Pena, J. (ed.), *Potential Invasive Pests Agric. Crop.* CAB International, Wallingford, United Kingdom.

**Haas, M., J. Wilson, and L. Gut. 2016.** Managing Black Stem Borer in Michigan Tree Fruits. 2.

**Hughes, M. A., X. Martini, E. Kuhns, J. Colee, A. Mafra-Neto, L. L. Stelinski, and J. A. Smith. 2017.** Evaluation of repellents for the redbay ambrosia beetle, *Xyleborus glabratus*, vector of the laurel wilt pathogen. *J. Appl. Entomol.* 141: 653–664.

**Kushiyev, R., C. Tuncer, I. Erper, and G. Özer. 2021.** The utility of *Trichoderma* spp. isolates to control of *Xylosandrus germanus* Blandford (Coleoptera: Curculionidae: Scolytinae). *J. Plant Dis. Prot.* 128: 153–160.

**Kushiyeu, R., M. Türkkan, C. Tuncer, and İ. Erper. 2018.** Evaluation of some fungicides against symbiotic fungus *Ambrosiella hartigii* associated with *Anisandrus dispar* Fabricius and *Xylosandrus germanus* Blandford (Coleoptera: Curculionidae: Scolytinae). *Selcuk J. Agric. Food Sci.* 32: 60–66.

**Mayorquin, J. S., J. D. Carrillo, M. Twizeyimana, B. B. Peacock, K. Y. Sugino, F. Na, D. H. Wang, J. N. Kabashima, and A. Eskalen. 2018.** Chemical management of invasive shot hole borer and fusarium dieback in California sycamore (*Platanus racemosa*) in Southern California. *Plant Dis.* 102: 1307–1315.

**Monterrosa, A., A. L. Acebes, B. Blaauw, and S. V. Joseph. 2021.** Effects of Trap, and Ethanol Lure Type and Age on Attraction of Ambrosia Beetles (Coleoptera: Curculionidae). *J. Econ. Entomol.* 114: 1647–1654.

**Monterrosa, A., S. V. Joseph, B. Blaauw, W. Hudson, and A. L. Acebes-Doria. 2022.** Ambrosia Beetle Occurrence and Phenology of *Xylosandrus* spp. (Coleoptera: Curculionidae: Scolytinae) in Ornamental Nurseries, Tree Fruit, and Pecan Orchards in Georgia. *Environ. Entomol.* 51: 998–1009.

**Ogburn, E. C., A. S. Heintz-Botz, E. J. Talamas, and J. F. Walgenbach. 2021.** Biological control of *Halymorpha halys* (Stål) (Hemiptera: Pentatomidae) in apple orchards versus corn fields and their adjacent woody habitats: High versus low pesticide-input agroecosystems. *Biol. Control.* 152: 104457.

**Oliver, J. B., and C. M. Mannion. 2001.** Ambrosia beetle (Coleoptera: Scolytidae) species attacking chestnut and captured in ethanol-baited traps in middle Tennessee. *Environ. Entomol.* 30: 909–918.

**Rabaglia, R.J., Cognato, A.I., Hoebeke, E.R., Johnson, C.W., LaBonte, J.R., Carter, M.E., and Vlach, J.J. 2019.** Early detection and rapid response: a 10-year summary of the USDA forest service program of surveillance for non-native bark and ambrosia beetles. *American Entomologist*, 65:29–42

**Ranger, C. M., M. E. Reding, K. Addesso, M. Ginzel, and D. Rassati. 2021.** Semiochemical-mediated host selection by *Xylosandrus* spp. ambrosia beetles ( Coleoptera : Curculionidae ) attacking horticultural tree crops : a review of basic and applied science. 103–120.

**Ranger, C. M., M. E. Reding, P. B. Schultz, J. B. Oliver, S. D. Frank, K. M. Addesso, J. H. Chong, B. Sampson, C. Werle, S. Gill, and C. Krause. 2016.** Biology, ecology, and management of nonnative ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) in ornamental plant nurseries. *J. Integr. Pest Manag.* 7.

**Ranger, C. M., P. B. Schultz, S. D. Frank, J. H. Chong, and M. E. Reding. 2015.** Non-native ambrosia beetles as opportunistic exploiters of living but weakened trees. *PLoS One.* 10: 1–22.

**Ranger, C. M., P. C. Tobin, and M. E. Reding. 2015.** Ubiquitous volatile compound facilitates efficient host location by a non-native ambrosia beetle. 675–686.

**Ranger, C. M., P. C. Tobin, M. E. Reding, A. M. Bray, J. B. Oliver, P. B. Schultz, S. D. Frank, and A. B. Persad. 2013.** Interruption of the Semiochemical-Based Attraction of Ambrosia Beetles to Ethanol-Baited Traps and Ethanol-Injected Trap Trees by Verbenone. *Environ. Entomol.* 42: 539–547.

**Ranger, C. M., C. T. Werle, P. B. Schultz, K. M. Addesso, J. B. Oliver, and M. E. Reding. 2020.** Long-lasting insecticide netting for protecting tree stems from attack by ambrosia beetles (Coleoptera: Curculionidae: Scolytinae). *Insects.* 11.

**Reding, M. E., and C. M. Ranger. 2018.** Residue age and attack pressure influence efficacy of insecticide treatments against ambrosia beetles (Coleoptera: Curculionidae). *J. Econ. Entomol.* 111: 269–276.

**Schedl, K. E. 1963.** Scolytidae und platypodidae afrikas. II. *Rev. Entomol. Moçambique.* 5: 1–594.

**Tran, H. X., Doland Nichols, J., Li, D., Le, N. H., Lawson, S. A. 2022.** Seasonal flight and genetic distinction among *Xylosandrus crassiusculus* populations invasive in Australia. *Aust. For.* 85: 224–331.

**Weber, B. C., and J. E. McPherson. 1983.** World list of host plants of *Xylosandrus germanus* (Blandford)(Coleoptera: Scolytidae). *Coleopt. Bull.* 114–134.

**Werle, C. T., Sampson B. J. , M. Reding, E. 2017.** A Role for Intercept Traps in the Ambrosia Beetle (Coleoptera: Curculionidae: Scolytinae) IPM Strategy at Ornamental Nurseries. *Midsouth Entomol.* 2: 14–23.

**Werle, C. T., J. H. Chong, B. J. Sampson, M. E. Reding, and J. J. Adamczyk. 2015.** Seasonal and Spatial Dispersal Patterns of Select Ambrosia Beetles (Coleoptera: Curculionidae) from Forest Habitats into Production Nurseries. *Florida Entomol.* 98: 884–891.

**Werle, C. T., C. M. Ranger, P. B. Schultz, M. E. Reding, K. M. Adesso, J. B. Oliver, and B. J. Sampson. 2019.** Integrating repellent and attractant semiochemicals into a push–pull strategy for ambrosia beetles (Coleoptera: Curculionidae). *J. Appl. Entomol.* 143: 333–343.

**Williams, G. M., and M. D. Ginzel. 2020.** Spatial and Climatic Factors Influence Ambrosia Beetle (Coleoptera: Curculionidae) Abundance in Intensively Managed Plantations of Eastern Black Walnut. *Environ. Entomol.* 49: 49–58.

## Chapter 2 Tables.

Table 2.1. Description of site locations used for ethanol-baited trapping studies, sentinel tree survey, and insecticide experiments.

State	Site Name	GPS coordinates	Dates of experiment/survey				
			Trapping 2020	Trapping 2021	Sentinel tree (2019-2020)	2018 insecticide	2019 insecticide
NC							
	TE	35.371614, -82.337566	Apr-Sep	Apr-Sep			
	KS	35.368067, -82.336477	Apr-Sep	Apr-Sep			
	MK	35.429260, -82.561736	Apr-Sep	Apr-Sep			
	NR	35.398746, -82.353587	Apr-Sep	Apr-Sep	May-Sep	Jul-Aug	
	RS	35.421426, -82.562540			May-Sep		Jul-Aug
OH							
	Bauman	40.5814, -81.4802	May-Oct	May-Oct			
	Hort II	40.4418, -81.5413	May-Oct	May-Oct			
	Moreland	40.4232, -81.5754	May-Oct	May-Oct			
	Scenic Ridge	40.4603, -82.1100	May-Oct	May-Oct			
VA							
	VA-1,2 &3	39.108561, -78.282208	Apr-Sep	Apr-Aug			

Table 2.2. Treatment details for ambrosia beetle insecticide field trials on potted apple trees.

Insecticide a.i. (trade name)	Rate Applied (formulated product)	Application Method	Application Dates
<b>2018</b>			
Imidiclopid (Admire Pro) <sup>1</sup>	0.285 mL/tree	Soil drench <sup>1</sup>	5/17
Dinotefuran (Venom) <sup>1</sup>	0.235 g/tree		
Imidiclopid (Admire pro), <sup>2</sup>	0.55 mL/L	Foliar spray to trunk <sup>2</sup>	6/22, 6/29, 7/6,
Chlorpyrifos (Lorsban Advanced)	7.5 mL/L		7/13, 7/20, 7/27
Lambda-cyhalothrin (Warrior II)	0.2 mL/L		
Chlorpyrifos (Lorsban Advanced)	7.5 mL/L (Chl.)		
+	+		
Lambda-cyhalothrin (Warrior II)	0.2 mL/L (L-Cy.)		
Insecticide impregnated netting <sup>3</sup> (Permanet) <sup>3</sup>	-	Wrapped around tree	6/22
EtOH-Control (2.5% Ethanol)	-	-	
Water-Control (Water)	-	-	
<b>2019</b>			
Imidiclopid (Admire Pro)	0.285 ml/tree	Soil drench <sup>1</sup>	5/17
Imidiclopid (Admire Pro)	2.1 ml/liter	Foliar spray to trunk <sup>2</sup>	6/13, 6/20, 6/27,
Chlorpyrifos (Lorsban Advanced)	3.5 ml/liter		7/5, 7/14
Lambda-Cyhalothrin (Warrior)	1.4 ml/liter		
Chlorpyrifos (Lorsban Advanced) +	2.1 ml/liter +		
Lambda-Cyhalothrin (Warrior)	0.4 ml/liter		
Bifenthrin (Brigade 2EC)	2.4 ml/liter		
Long-lasting insecticide netting <sup>3</sup>	-	Wrapped around tree	6/13
EtOH-Control (3.0% Ethanol)	-	-	

<sup>1</sup> Soil drench applied to potted trees in 1 L of water 2 weeks prior to ethanol drench. <sup>2</sup> Foliar sprays applied with hand-trigger spray bottles to point of runoff. <sup>3</sup> Long-lasting insecticide-impregnated netting was wrapped around trunks of trees 1 day before ethanol drench initiated.

Table 2.3. Total number (and percentage of total) of key ambrosia beetle species captured in ethanol-baited bottle traps deployed in apple orchards in NC, OH, and VA in 2020 and 2021.

Species	2020								
	NC				OH				VA
	RS	KS	NR	TE	Bauman	Hort II	Moreland	Scenic Ridge	AHS-AREC
<i>Xylo. crassiusculus</i>	191 (30.4)	227 (28.3)	183 (19.4)	90 (22.9)	0 (0)	17 (1.3)	47 (8.8)	2 (0.1)	344 (34.1)
<i>Xylo. germanus</i>	332 (52.9)	382 (47.6)	618 (65.7)	234 (59.5)	2577 (96.4)	1212 (91.9)	458 (85.8)	1428 (94.8)	346 (34.3)
<i>Xyle. saxeseni</i>	61 (9.7)	64 (8)	46 (4.9)	43 (10.9)	32 (1.2)	78 (5.9)	20 (3.7)	59 (3.9)	96 (9.5)
<i>A. maiche</i>	-	-	-	-	39 (1.5)	4 (0.3)	0 (0)	6 (0.4)	-
other	44 (7)	129 (16.1)	94 (10)	26 (6.6)	25 (0.9)	8 (0.6)	9 (1.7)	11 (0.7)	222 (22)
Total	628	802	941	393	2673	1319	534	1506	1008
2021									
<i>Xylo. crassiusculus</i>	39 (9.9)	54 (12.4)	71 (2.5)	33 (7.7)	0 (0)	3 (2.3)	159 (52.8)	8 (1.4)	54 (21.3)
<i>Xylo. germanus</i>	24 (6.1)	17 (3.9)	8 (0.3)	9 (2.1)	590 (84)	92 (69.7)	105 (34.9)	492 (86.5)	29 (11.5)
<i>Xyle. saxeseni</i>	70 (17.7)	97 (22.2)	120 (4.2)	116 (27.1)	7 (1)	11 (8.3)	6 (2)	27 (4.7)	33 (13)
<i>A. maiche</i>	-	-	-	-	10 (1.4)	2 (1.5)	1 (0.3)	15 (2.6)	-
other	262 (66.3)	269 (61.6)	2636 (93)	270 (63.1)	95 (13.5)	24 (18.2)	30 (10)	27 (4.7)	93 (36.8)
Total	395	437	2835	428	702	132	301	569	253

Table 2.4. Mean ( $\pm$ SE) number of *Xylosandrus* spp. ambrosia beetles captured in ethanol-baited bottle traps per trap per week located at the orchard-woods interface (“Edge”) or 20-30m from the woods inside the orchard (“Interior”) at apple orchard locations in OH and NC.

State	Site	2020				2021			
		Edge	Interior	t-test	p-value	Edge	Interior	t-test	p-value
OH	Bauman	60.4 $\pm$ 9.8	31.1 $\pm$ 5.8	-5.65	<0.0001	13.4 $\pm$ 2.7	7.2 $\pm$ 2.4	-8.78	<0.0001
	Hort II	15.9 $\pm$ 6.0	28 $\pm$ 11.2	2.13	0.0423	1.3 $\pm$ 0.5	1.4 $\pm$ 0.5	0.55	0.589
	Mooreland	7.6 $\pm$ 2.8	10 $\pm$ 4.0	1.65	0.1104	5.3 $\pm$ 2.3	3.8 $\pm$ 1.7	-1.79	0.084
	Scenic Ridge	24.4 $\pm$ 4.2	26.5 $\pm$ 7.1	0.37	0.718	10.7 $\pm$ 3.0	7 $\pm$ 3.0	-4.18	0.0003
NC	KS	13.3 $\pm$ 3.1	2.7 $\pm$ 0.5	-3.79	0.0005	3.6 $\pm$ 1.1	0.4 $\pm$ 0.2	-3.01	0.0083
	MK	9.6 $\pm$ 1.7	4.2 $\pm$ 0.8	-5.07	0.002	0.6 $\pm$ 0.2	3 $\pm$ 0.8	2.98	0.0089
	NR	15.8 $\pm$ 2.8	4.2 $\pm$ 0.7	-4.47	<0.0001	2.8 $\pm$ 0.9	1.6 $\pm$ 0.6	-1.21	0.243
	TE	5.6 $\pm$ 1.4	2.5 $\pm$ 0.5	-2.53	0.0157	1.1 $\pm$ 0.3	1.5 $\pm$ 0.4	1.38	0.189

Table 2.5. Total number (%) of *X. crassiusculus*, *X. germanus*, and *C. mutilatus* recovered from ethanol-drenched potted apple trees deployed for 14 day intervals at two orchard sites ('NR' or 'RS') in Henderson County, North Carolina in 2019 and 2020.

Species	Site 'NR'		Site 'RS'		Overall
	2019	2020	2019	2020	
<i>X. crassiusculus</i> <sup>1</sup>	320 (82.7)	17 (20)	320 (88.2)	29 (29.9)	686 (73.6)
<i>X. germanus</i> <sup>1</sup>	62 (16)	62 (72.9)	14 (3.9)	43 (44.3)	181 (19.4)
<i>C. mutilatus</i> <sup>1</sup>	5 (1.3)	5 (5.9)	29 (8)	25 (25.8)	64 (6.9)
Total trees sampled	40	30	40	30	140

<sup>1</sup> positively identified beetles recovered from apple trees.

Table 2.6. Effect of soil and foliar-applied insecticides and insecticide-impregnated netting on ambrosia beetle attacks and brood success on ethanol-drenched potted apple trees in 2018.

Treatment	Mean ( $\pm$ SE) cumulative entries per tree				Final destructive assessment		
	7 dafa	21 dafa	35 dafa	48 dafa (Final)	Successful entries <sup>1</sup>	Brood <sup>2</sup> (%)	% Efficacy <sup>3</sup>
Imidiclopid (Soil)	0.2 $\pm$ 0.2 <sup>b</sup>	3.6 $\pm$ 1 <sup>b</sup>	5.2 $\pm$ 1.5	5.4 $\pm$ 1.3	2.8 $\pm$ 0.9 <sup>bc</sup>	30.9 $\pm$ 13.6	55.9
Dinotefuran (Soil)	0.4 $\pm$ 0.2 <sup>b</sup>	6.4 $\pm$ 2.5 <sup>ab</sup>	10.2 $\pm$ 3.8	13.2 $\pm$ 4.8	10.4 $\pm$ 3.9 <sup>a</sup>	38.9 $\pm$ 17	0.0
Imidiclopid	0.0 $\pm$ 0.0 <sup>b</sup>	3.4 $\pm$ 1.1 <sup>b</sup>	5.4 $\pm$ 2	7.4 $\pm$ 2.5	5.2 $\pm$ 1.7 <sup>bc</sup>	29.3 $\pm$ 9.6	37.3
Chlorpyrifos	0.4 $\pm$ 0.4 <sup>b</sup>	3 $\pm$ 0.9 <sup>b</sup>	6.2 $\pm$ 1.5	6.6 $\pm$ 2.1	3.8 $\pm$ 1.7 <sup>bc</sup>	5.9 $\pm$ 3.6	44.1
Lambda-cyhalothrin	0.4 $\pm$ 0.2 <sup>b</sup>	5.4 $\pm$ 2.3 <sup>b</sup>	7 $\pm$ 2.9	6.4 $\pm$ 2.1	3.6 $\pm$ 1.1 <sup>bc</sup>	15.7 $\pm$ 9.6	45.8
Chlorpyrifos + L-cyhalothrin	0.2 $\pm$ 0.2 <sup>b</sup>	2.4 $\pm$ 0.7 <sup>b</sup>	2.6 $\pm$ 0.6	3.2 $\pm$ 1.1	0.6 $\pm$ 0.4 <sup>c</sup>	5 $\pm$ 5	72.9
Permanet netting	0.0 $\pm$ 0.0 <sup>b</sup>	2.6 $\pm$ 1.3 <sup>b</sup>	3 $\pm$ 1.3	2.8 $\pm$ 0.9	1 $\pm$ 0.4 <sup>c</sup>	17.5 $\pm$ 11.8	81.4
EtOH Control	2.2 $\pm$ 0.6 <sup>a</sup>	10.4 $\pm$ 1.9 <sup>a</sup>	12.2 $\pm$ 2.7	11.8 $\pm$ 2.5	7.6 $\pm$ 1.5 <sup>ab</sup>	34.6 $\pm$ 7.3	-
Water Control	0.2 $\pm$ 0.2	5 $\pm$ 1.8 <sup>b</sup>	5 $\pm$ 1.8	4.6 $\pm$ 1.7	3.2 $\pm$ 1.3 <sup>bc</sup>	38.7 $\pm$ 16.5	-
F <sub>8,31</sub>	5.57	2.47	2.04	2.12	3.09	1.45	
p	0.0002	0.0329	0.0725	0.0642	0.011	0.2163	

<sup>1</sup> avg. number of galleries with brood or fungi established. <sup>2</sup> Mean percentage of galleries per tree containing offspring <sup>3</sup> % reduction in total entries at final assessment compared with EtOH control.

Different lower case letters within a column indicate significantly different means ( $\alpha = 0.05$ ) using Tukey's HSD.

2

3 Table 2.7. Effect of soil and foliar-applied insecticides and insecticide-impregnated netting on ambrosia beetle attacks and brood success on  
4 ethanol-drenched potted apple trees 7-47 days after first application (dafa) in 2019.

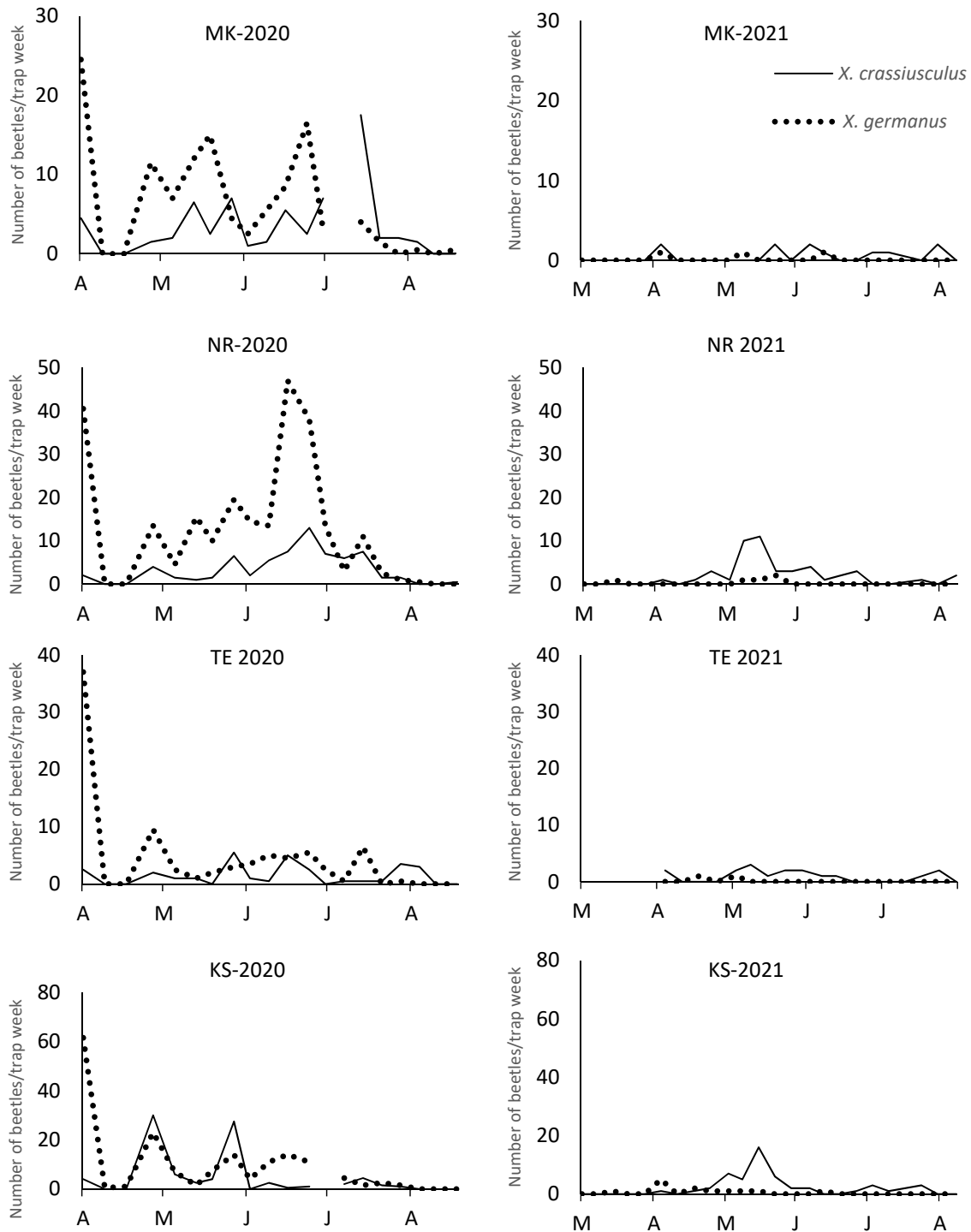
Treatment	Mean ( $\pm$ SE) cumulative entries per tree				Final destructive assessment (47 dafa)		
	7 dafa	22 dafa	40 dafa	47 dafa	Successful Galleries <sup>1</sup>	Brood <sup>2</sup> (%)	Efficacy <sup>3</sup> (%)
Imidicloprid (Soil)	5.3 $\pm$ 1.2 <sup>a</sup>	9.3 $\pm$ 2.5 <sup>ab</sup>	7.5 $\pm$ 1.9 <sup>abc</sup>	11.5 $\pm$ 2 <sup>abc</sup>	10.2 $\pm$ 2.2 <sup>abc</sup>	58.9 $\pm$ 13.9 <sup>abc</sup>	40.2
Imidicloprid	3.5 $\pm$ 1.2 <sup>ab</sup>	10.8 $\pm$ 2.5 <sup>ab</sup>	11.0 $\pm$ 3 <sup>ab</sup>	14.0 $\pm$ 3.9 <sup>ab</sup>	12.2 $\pm$ 3.6 <sup>ab</sup>	64 $\pm$ 10.9 <sup>abc</sup>	29.5
Chlorpyrifos	4.2 $\pm$ 1.5 <sup>ab</sup>	6.7 $\pm$ 3.3 <sup>abc</sup>	6.7 $\pm$ 3.2 <sup>abc</sup>	7.5 $\pm$ 3.1 <sup>ab</sup>	4.3 $\pm$ 3 <sup>ab</sup>	21.8 $\pm$ 11.6 <sup>ab</sup>	59.0
L-cyhalothrin	3.2 $\pm$ 0.9 <sup>ab</sup>	4.5 $\pm$ 0.7 <sup>bc</sup>	5.0 $\pm$ 1.2 <sup>bc</sup>	7.2 $\pm$ 0.9 <sup>bc</sup>	4.5 $\pm$ 0.6 <sup>bc</sup>	45.6 $\pm$ 8.4 <sup>bc</sup>	60.7
Chlorpyrifos + L-cyhalothrin	2.8 $\pm$ 1.1 <sup>ab</sup>	4.7 $\pm$ 1.2 <sup>bc</sup>	5.7 $\pm$ 1.1 <sup>bc</sup>	8.7 $\pm$ 1.3 <sup>bc</sup>	5.0 $\pm$ 1.3 <sup>bc</sup>	26.6 $\pm$ 10.4 <sup>bc</sup>	56.1
Bifenthrin	0.3 $\pm$ 0.2 <sup>b</sup>	0.3 $\pm$ 0.2 <sup>c</sup>	2.2 $\pm$ 0.8 <sup>bc</sup>	4 $\pm$ 1.1 <sup>bc</sup>	1.3 $\pm$ 0.7 <sup>bc</sup>	12.2 $\pm$ 7.8 <sup>bc</sup>	94.3
Permanet netting	0.0 $\pm$ 0 <sup>b</sup>	0.0 $\pm$ 0.0 <sup>c</sup>	0.0 $\pm$ 0.0 <sup>c</sup>	0.0 $\pm$ 0.0 <sup>c</sup>	0.3 $\pm$ 0.2 <sup>c</sup>	-	80.3
ETOH Control	5.3 $\pm$ 1.1 <sup>a</sup>	14.2 $\pm$ 1.8 <sup>a</sup>	15.5 $\pm$ 2.6 <sup>a</sup>	19.3 $\pm$ 3.5 <sup>a</sup>	15.2 $\pm$ 3.4 <sup>a</sup>	69.8 $\pm$ 11.9 <sup>a</sup>	-
F <sub>7,35</sub>	5.1381	6.88	5.71	6.43	5.43	4.11	
P	0.0008	<.0001	0.0002	<.0001	0.0003	0.0022	

5 <sup>1</sup> avg. number of galleries with brood or fungi established. <sup>2</sup> Mean percentage of galleries per tree containing offspring <sup>3</sup> % reduction in total entries at final  
6 assessment compared with EtOH control. Different lower case letters within a column indicate significantly different means ( $\alpha = 0.05$ ) using Tukey's HSD.

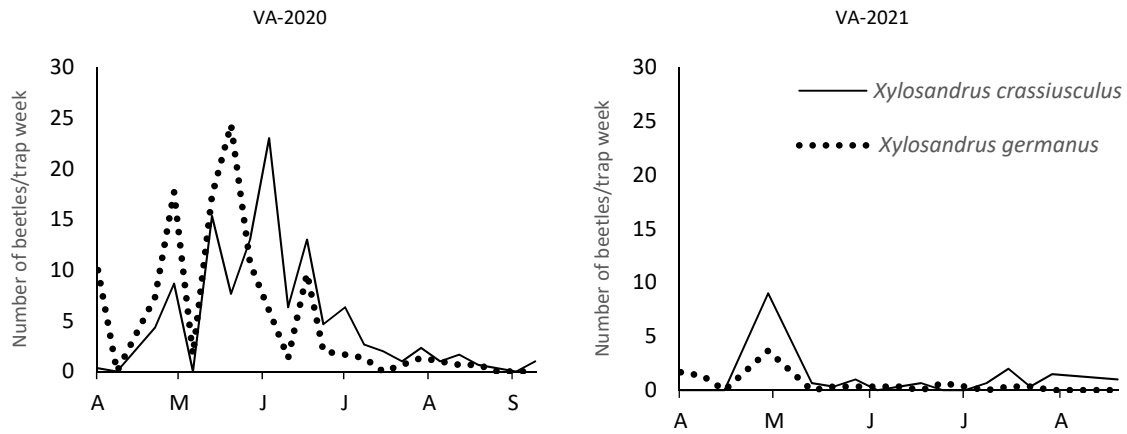
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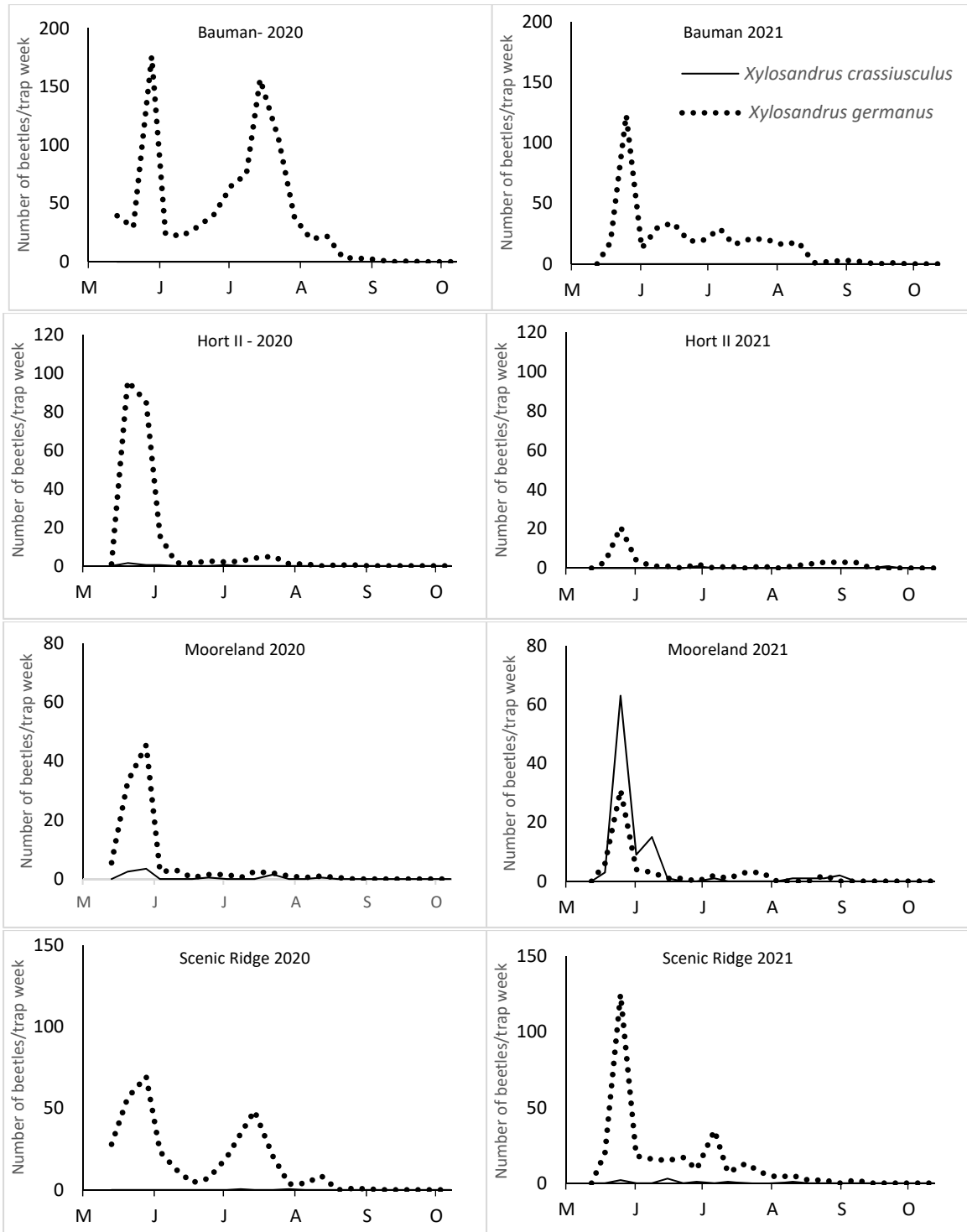
## Chapter 2 Figures:



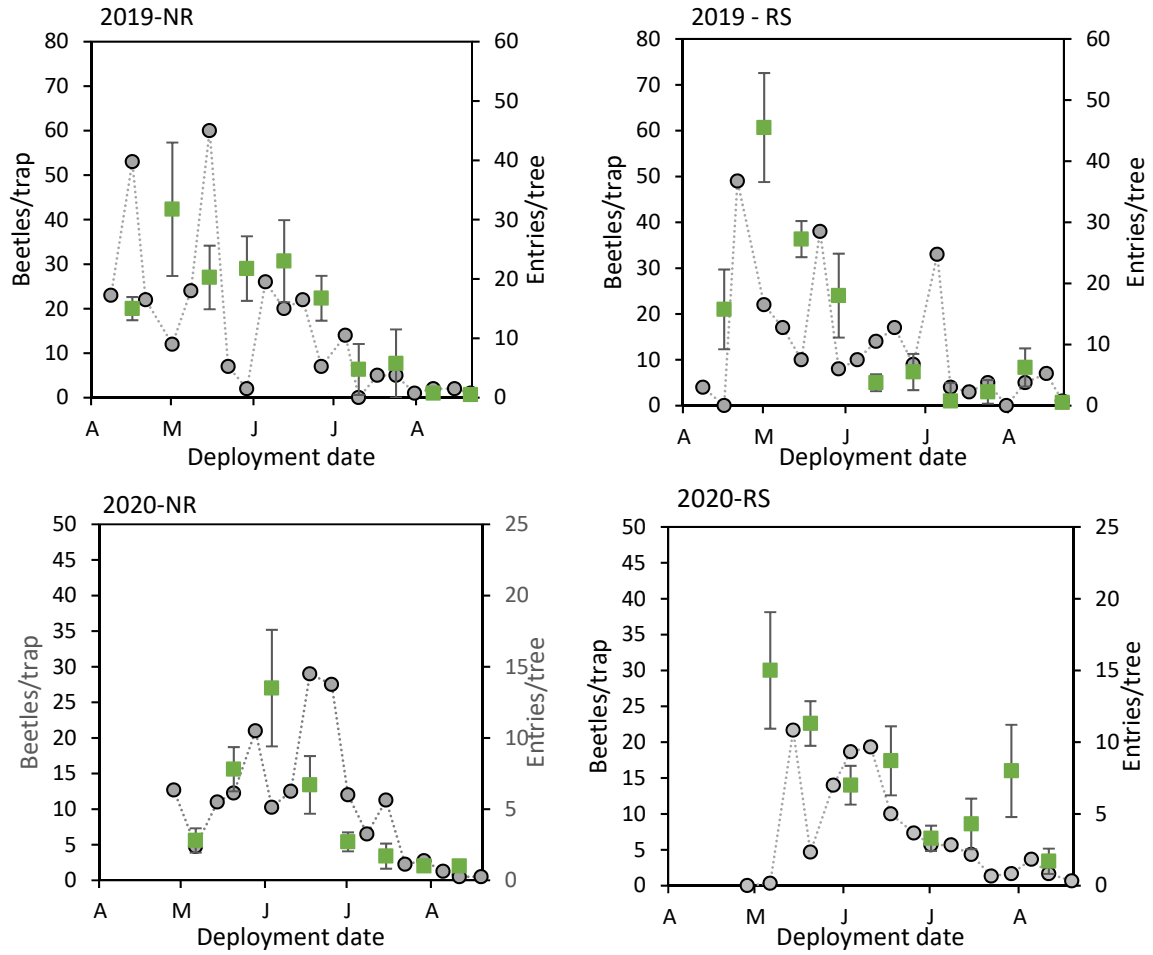
**Fig. 2-1.** Weekly trap captures of *X. crassiusculus* (solid line) and *X. germanus* (dotted line) in ethanol-baited bottle traps deployed along orchard-woods interface at various apple orchards in April-August 2020 & 2021 Henderson County, NC.



**Fig. 2-2.** Weekly trap captures of *X. crassiusculus* (solid line) and *X. germanus* (dotted line) in ethanol-baited bottle traps deployed along orchard-woods interface in April-August 2020 & 2021 at Alson H. Smith Research and Extension Center, Winchester, VA.



**Fig. 2-3.** Weekly trap captures of *X. crassiusculus* (solid line) and *X. germanus* (dotted line) in ethanol-baited bottle traps deployed along orchard-woods interface in March-October in 2020 & 2021 at various apple orchards in Ohio.



**Fig. 2-4.** Number of *Xylosandrus* spp. captured (beetles/week) in ethanol baited traps (solid grey circles with dotted line) and mean number of ambrosia beetle entries ( $\pm$  SE) on ethanol-drenched sentinel apple trees (green square) deployed for 14 day intervals at apple orchards in NC, April to August 2019 and 2020

## Chapter 3 Diversity and Phytopathogenicity of Fungi Associated with Ambrosia Beetles Attacking Apple Trees in North Carolina.

### Abstract

Ambrosia beetles propagate specialized mutualistic fungi on which they and their offspring feed upon within their galleries. Xyleborine ambrosia beetles infest physiologically stressed trees and have been associated with decline and dieback in commercial tree production systems in the USA. In this study, 1229 isolates from beetle galleries in ethanol-drenched potted apple trees deployed in orchards, 335 isolates from 153 Xyleborine ambrosia beetles trapped in orchards, and 470 isolates from non-attacked, non-declining apple trees in commercial orchards were collected and identified using internal transcribed spacer sequences and selected isolates resolved using additional loci. Of the 31 genera isolated from galleries, 48 genera isolated from trapped beetles, and 23 genera isolated from non-attacked apple trees, *Fusarium*, *Alternaria*, and *Trichoderma* spp. were commonly isolated across all sample types. *Botryosphaeria dothidea* which has been previously found associated in high frequency with ambrosia beetle galleries on declining apple trees was common on non-attacked apple trees, less commonly isolated from galleries and not isolated from ambrosia beetles. The aggressiveness of selected isolates of *Botryosphaeria*, *Fusarium*, and *Ambrosiella* were tested on detached apple shoots and potted apple seedlings. The most common isolates found across all samples (*Ambrosiella* spp. and *F. solani*) were not highly aggressive and the most aggressive isolates were rarely found or absent on trapped beetles. Finally, *in vitro* assays were conducted to assess the relative effect of ethanol on growth of selected isolates of *Ambrosiella*, *Botryosphaeria*, *Fusarium*, and *Trichoderma*. Isolates of *Ambrosiella* spp. was more tolerant to ethanol compared with the other isolates, but in confrontation assays, amending the media with ethanol did not provide a significant advantage to the *Ambrosiella* spp. isolates.

## Introduction

Ambrosia beetles are intrinsically linked with fungi that are able to colonize woody tissue. Xyleborine ambrosia beetles are equipped with specialized organs (mycangia) to transport spores of their dedicated nutritional symbiotic fungus. Propagules deposited by the foundress colonize the interior walls of the gallery walls, breaking down lignin and cellulose and providing nutrition to the beetle and its offspring (Batra 1985). The ambrosia symbiosis is believed to have evolved at least 7 times in fungi and 11 times in insects (Massoumi Alamouti et al. 2009, Kasson et al. 2013, Skelton et al. 2019). Although mycangial contents are dominated by their respective nutritional symbiont (Kostovcik et al. 2015, Mayers et al. 2015a), studies have shown a broad and dynamic community of bacteria and fungi are associated with ambrosia beetle mycangia (Carrillo et al. 2014, Kostovcik et al. 2015, Skelton et al. 2018, Saragih et al. 2021), and that horizontal transfer among beetles is possible (Carrillo et al. 2014). While some beetle's nutritional symbionts are able to systemically invade their hosts and cause severe disease, the majority of symbionts are not thought to be phytopathogenic (Hulcr and Dunn 2011, Ploetz 2013). However, phytopathogenic fungi have been associated with mycangia and gallery tissue of *X. crassiusculus* and *X. germanus* (Kessler 1974, Weber and McPherson 1985, Kovach 1986, Dute et al. 2002, Ploetz et al. 2013, Carrillo et al. 2014).

The exotic ambrosia beetles, *Xylosandrus crassiusculus* (Motschulsky) and *X. germanus* (Blandford) in the tribe Xyleborini (Coleoptera: Curculionidae, Scolytinae) are the main species known to attack stressed apple trees in North Carolina (Gresham et al. 2023). These beetles have a wide host range, attacking physiologically stressed, but living trees, and are considered problematic pests in fruit, nut, and ornamental production systems (Ranger et al. 2016). Their association with rapid apple decline (RAD), the sudden and rapid decline and death of young apple trees, has been characterized in New York (Agnello et al. 2017, Donahue and Elone 2021) and North Carolina (Chapter 1). Isolation of fungi from *Xylosandrus* spp. galleries within declining apple trees found a diversity of opportunistic phytopathogens

in the genera *Fusarium*, *Botryosphaeria*, *Diaporthe*, and *Paraconiothyrium* (Breth, Agnello, Cox, et al. 2016, Agnello et al. 2017).

Ambrosia beetles respond to various volatile emissions produced by trees following tissue damage or sub-lethal stress, with ethanol being the most important (Ranger et al. 2015, 2018). Ethanol may also play an important role in successful gallery establishment as it has been shown to increase gallery excavation and improve the growth of *Ambrosiella* spp. fungal symbionts relative to competing fungi such as *Penicillium commune*, *Asoidea* sp., *Aspergillus* sp., and *Chaetomium globosum* (Ranger et al. 2018, Lehenberger et al. 2021). However, limited information is available on the impact of ethanol on the growth of phytopathogens such as *Botryosphaeria* spp. and *Fusarium* spp., or the potential biological control agent, *Trichoderma* spp. (Castrillo et al. 2013, 2016, Kushiyevev et al. 2021, Reverchon et al. 2021). Given that ambrosia beetles preferentially attack physiologically stressed trees, and that physiologically stressed trees are vulnerable to invasion by necrotrophic and saprotrophic fungi, it is expected that interactions between their nutritional symbiont fungi and necrotrophs and saprobes will be commonplace. Therefore, ethanol enriched environments should be expected to benefit the growth of *Ambrosiella* spp. relative to necrotrophic and saprophytic fungicide commonly isolated from the galleries created by ambrosia beetles.

The overall goal of this study was to identify fungi associated with ambrosia beetles and their galleries and to gain insight into the contribution of the most frequently identified fungi involved in apple tree decline. Specific aims were to i) isolate and identify fungi associated with ambrosia beetle galleries created in artificially stressed potted apple trees, ii) isolate and identify fungi associated with ambrosia beetles trapped in and around apple orchards to assess the potential of ambrosia beetles to serve as vectors of fungal phytopathogens, iii) assess the pathogenicity and aggressiveness of fungi frequently isolated from ambrosia beetles and galleries, and iv) evaluate the effect of ethanol on the growth of fungi isolated from ambrosia beetle galleries on apple trees and how ethanol affects the

competition between symbiont isolates and common fungi associated with beetles and their galleries in apple trees. Previous work suggests that elevated ethanol produced within stressed trees should benefit *Ambrosiella* spp. mycelial growth relative to other fungi (Ranger et al. 2018, Lehenberger et al. 2021, Gugliuzzo et al. 2022) and therefore may reduce the inhibitory effects of *Trichoderma* spp.

## Methods

**Isolation of fungi.** For each tissue sample, the outer bark was removed with clean secateurs and woody tissue was sectioned into approximately 1 cm pieces then surface sterilized by immersing in 10% bleach solution (1.2 g/mL NaOCl) for 90 seconds before being triple-rinsed in sterile di-ionized water and air-dried in a laminar-flow hood. Smaller pieces of tissue surrounding the galleries (2-5 mm) were excised using a flame-sterilized scalpel and placed on potato dextrose agar (PDA, Difco Laboratories, Detroit, MI ) medium amended with 50 mg/mL chloramphenicol and 50 mg/mL streptomycin (PDA++) to prevent excessive bacterial growth. Plates were incubated for 5-7 days until unique fungal morphotypes could be identified. Each unique fungus isolated from a tissue sample was further isolated by culturing 2-5 mm sections of mycelium on amended PDA for 7-10 days at 25°C, 16:8 L:D regime, and categorized into morphotype groups based on color, size, texture, and other visual features. Representative isolates from each morphotype were stored long term by placing 6-8 pieces of PDA containing mycelium (3 mm) into Potato Dextrose Broth (Difco Laboratories, Detroit, MI ) with 25% sterile glycerol then stored at -80°C.

**PCR & Sequencing.** Genomic DNA was extracted from approximately 50 mg of fresh mycelium taken from cultures grown on PDA ++ for 7-14 days at room temperature of 2-3 isolates per morphotype group. DNA was extracted using an E.Z.N.A Plant DNA extraction kit (Omega Bio-Tek, Norcross, GA) following the manufacturer's instructions. Partial regions of three loci were amplified using appropriate

primer sets. For all isolates, the internal transcribed spacer (ITS) region was amplified using primers ITS-1F/ITS-4 (White et al. 1990) and sequenced in the forward direction. Additional loci were amplified for a selection of isolates from the *Fusarium*, *Botryosphaeria*, *Trichoderma*, *Diaporthe*, and *Ambrosiella* genera (Table 1). All PCR reactions were conducted using a 25  $\mu$ L reaction volume with 12.5  $\mu$ L Takara Mix (Takara Bio USA, Inc., San Jose, CA, USA), 1  $\mu$ L of each primer (10  $\mu$ M), 1  $\mu$ L DNA template (20-80  $\mu$ M) and 8.5  $\mu$ L nuclease-free water.

Thermocycler conditions for ITS1/ITS4 were initial denaturation at 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 5 min. For *Btub* the conditions were the same except the annealing temperature was 57 °C. For *Tef1/Tef2* the initial denaturation at 94 °C for 4 min, followed by denaturation of 94 °C for 60 s, 30 s annealing with temperature being dropped 1 °C each cycle from 64-52°C, then 15 cycles with annealing temperature of 52 °C 72 °C for 60 s, elongation was 72 °C for 60 s and a final extension at 72 °C for 7 min.

Amplification products were inspected for quality on a 2% agarose gel in 1x TAE (Tris Acetate EDTA) buffer stained with GelRed (Biotium, Haywood, CA) using electrophoresis (80 V for 60 min) and visualized by UV transillumination on Geldoc EZ image (BioRad, Hercules, CA). Single-band PCR products were purified for sequencing using a Zymo DNA Clean & Concentrator kit (Zymo Research, Irvine, CA) according to manufacturer instructions and sequenced at the North Carolina State University Genomic Services Laboratory (Sanger DNA sequencing) on an Applied Biosystems 3730xl capillary sequencer. Identification of rDNA sequence data analysis was accomplished using the Basic Local Alignment Search Tool (BLAST) on NCBI GenBank databases.

**Sentinel tree survey.** Apple trees (*Malus domestica* cv 'Granny Smith', grafted to 'B.9' rootstock) were planted into 27 L plastic pots with potting media (2:1 bark mulch: vermiculite amended with lime and micronutrients) in 2018, and maintained at the Mountain Horticultural Crops Research and

Extension Center (MHCREC) in Mills River, NC with a standard grower pesticide program for pest and disease control and microsprinkler irrigation. Trees were deployed at two different sites in 2019 and 2020: site "RS" was adjacent to a 0.25 research apple orchard at MHCREC (35.421539, -82.562100) and site "NR" was at a commercial apple orchard in Henderson county, NC (35.398746, -82.353587). At each site, four trees were placed at the interface of the orchard and an unmanaged woodlot comprised of mixed hardwoods. At deployment, pots were placed inside large contractor bags (126 L, 3 mil thickness) into which an ethanol solution (3%) was poured and topped up with 1-2 L of ethanol solution after 7 days as needed. Trees were collected and replaced with a new set of four trees every 14 days throughout the growing season (April-September). At collection, the trees were cut off at the soil line and all branches were removed. The remaining trunk section was stored at 4°C until processing for up to 10 days. At first, each tree was inspected for evidence of beetle entry indicating the presence of a gallery. Regions of the tree where beetle attacks were present were dissected to record the presences of a foundress, offspring, and/or fungal gardens within each gallery. Tissue from each gallery was collected into separate paper envelopes and stored at 4°C for up to 2 weeks before being cultured. In 2020, tissue samples from non-gallery areas on the tree were taken from the graft union and the main stem of each collected tree, as well as from approximately 5 cm from the nearest beetle gallery.

**Fungi on trapped beetles.** Beetles were trapped using bottle traps with ethanol lures in accordance with (Ranger et al. 2021) with modifications to capture the beetles without liquid to reduce cross-contamination of beetles. The trap consisted of an inverted 2 L soda bottle with 3 windows (4 x 10 cm) cut into the sides with an ethanol lure with a 65 mg/d release rate at 30°C (AgBio Inc., Westminster, CO) attached to the inside of the bottle. The 2 L bottle was connected to a 0.5 L plastic bottle using a tornado tube connector. In place of trapping liquid, the 0.5 L bottle contained a sterile paper towel that had been moistened with sterile distilled water. Before each trap deployment, the 0.5 L bottle was cleaned and sterilized with 1.2% NaOCl and rinsed with sterile water. The inside of the 2 L bottle was

rinsed with 70% ethanol. Trapping was conducted targeting peak flight activity of ambrosia beetles from April-June in 2020, 2021, and 2022 across several locations in Henderson County, NC (SI Table 1). Traps were collected after 24-48 hours and Xyleborine species were placed individually into sterile 1.5 ml microcentrifuge tubes. Identification of each beetle was confirmed under a dissecting microscope after processing was complete.

To isolate fungi on the outer surfaces of the beetles, 200  $\mu$ l of sterile phosphate buffer was added to each tube containing a beetle and vortexed at 1500 RPM for 15 seconds. Each tube was agitated to re-suspend any particulates and 4 x 25  $\mu$ L aliquots were pipetted onto Potato Dextrose Agar (PDA) amended with 50 mg/mL each of streptomycin and chloramphenicol (PDA++). Plates were incubated initially for 3-7 days at room temperature in the dark. Unique fungi were isolated from the resulting colonies and grouped into morphotypes as described above.

**Orchard fungal survey.** To evaluate the background diversity of opportunistic phytopathogens in apple orchards (i.e. trees not attacked by beetles or undergoing rapid decline), a survey of the culturable mycobiome was conducted in the winter of 2022. Tissue samples were taken from pruning wounds, from the edges of small cankers or blistered areas on the graft union and the main stem (scion), and from tissue adjacent to decaying sections of wood on graft union, or branches. The orchard locations selected had previous observations of ambrosia beetle damage and tree losses but surveyed trees were all living and not under visual decline at the time of sampling. All locations were located in Henderson Co., NC (SI Table 1)

**Pathogenicity & aggressiveness assays.** Isolates of *Ambrosiella*, *Fusarium*, *Botryosphaeria*, and *Paraconiothyrium* were tested for pathogenicity and aggressiveness on detached apple shoots following methods used in previous studies (Marek et al. 2013, Sessa et al. 2018). In 2020, 1 year old shoots were taken from mature apple trees (cv. Fuji) at the Mountain Horticultural Crops Research and Extension

Center (MHCREC). In 2022 1 year old shoots were taken from mature apple trees (cv. Gala) and the experiment was repeated twice, once with dormant shoots collected in January and once using actively growing shoots in May from MHCREC. Isolates used in the pathogenicity and aggressiveness assay were cultured from samples stored at -80°C at MHCREC and identities confirmed by Sanger sequencing using methods described above.

Prior to inoculation, leaves were removed, then shoots were washed twice in tap water, sterilized in 1.2% NaOCl for 90 s, rinsed once with sterile deionized water, and were air dried in a laminar flow hood. Two wounds (5 mm in diameter) were made on an internode area of the shoot, 5-7 cm from each end, to remove the bark and cambium and expose the wood. A 4 mm mycelial plug was taken from the leading edge of a 7 day old culture of each isolate and placed mycelium side on the wound then covered with sterile cotton wool moistened with sterile deionized (DI) water and wrapped in parafilm (2020), or a moistened sterile plastic bandage (2022). Plugs of PDA without mycelium were used as a negative control. For each isolate in the 2020 experiment, a total of 8 shoots per isolate were inoculated and 8 shoots used for the PDA control. For the 2022 experiments, each shoot had one wound inoculated and the second wound mock-inoculated with PDA with 3 replicates repeated on 3 different dates staggered over a 7 day period (9 replicates total). In both years, inoculated shoots were incubated within a surface-sterilized plastic container lined with autoclaved paper towels misted with sterile deionized water. The shoots were stored for 14 days at room temperature with regular misting with deionized water to maintain high humidity.

After 14 days of incubation, the bark was excised to expose the wood around the inoculation site. The length of symptomatic tissue was measured using calipers, differentiating necrotic tissue from discolored tissue. Tissue from the leading edge of the necrotic zone was surface sterilized in 1.2% NaOCl for 90 seconds, rinsed in sterile deionized water, then incubated on PDA for 7 days and compared morphologically against the original isolates to confirm re-isolation.

In 2022, pathogenicity and aggressiveness of selected isolates were assayed on 6 -8 month old apple seedlings. Seeds from 'Gala' apples were surface sterilized, stratified in refrigerator for 40-70 days until germination began, and then planted into seed-raising mix. Once seedlings reached 2-4 true leaf stage they were transferred into 18 cell trays in potting soil and maintained in greenhouse until the experiments commenced. Regular watering, fertilizer and pest control was conducted as required. Two wounds per seedling were made and inoculated as described above for detached shoots, with 6 seedlings per isolate (20 isolates plus PDA control, Table 2). After 22 days each seedling was cut off at the soil line, leaves and lateral branches removed, and lesions measured as described above.

Lesion length data were tested for normality and transformed using ( $\log_{10} + 1$ ) where appropriate to reduce covariate of variation and satisfy the assumptions of ANOVA, individual shoots or seedlings were treated as single replicates. Transformed data were subjected to Analysis of Variance with isolate as main effect using PROC ANOVA in SAS V 9.4 (SAS Institute, Cary, NC). Means were compared using Tukey's HSD test at 95% confidence.

**Effect of ethanol on fungal growth.** To test the hypothesis that the nutritional symbionts of *Xylosandrus ambrosia* beetles show a different growth response to ethanol compared to non-symbiont fungi, an *in vitro* assay was conducted to measure the effect of varying ethanol concentrations on mycelial growth of symbiont and non-symbiont fungal isolates. Malt extract agar (MEA) (Difco Laboratories, Detroit, MI) was amended with 6 different concentrations of ethanol (0.5, 1, 2, 3, 4, and 5% v/v) by adding the appropriate amount of ethanol (200 proof, Sigma Aldrich, St. Louis, MO) to the molten agar (55°C) just prior to pouring into 9 cm petri dishes. Non-amended MEA was used as the control and compared with non-amended PDA to compare the relative effect of media type on fungal growth. Each isolate x media combination was replicated three times. To account for different growth rates of isolates, the average daily mycelial growth was calculated based on colony measurements made at 3, 6, and 10 days after inoculation, ceasing measurements once colony growth reached the edge of

the petri dish. For each isolate, dose response curves were constructed using relative percent inhibition of colony growth at each log-transformed ( $\log_{10}$ ) concentration of ethanol to determine the value of the effective concentration that inhibited isolate growth by 50% ( $EC_{50}$ ). The calculated  $EC_{50}$  values were subjected to Analysis of Variance using PROC ANOVA in SAS version 9.4 (SAS Institute, Cary, NC). Differences among the means were tested using Tukey's HSD at  $\alpha=0.05$ .

**Symbiont-pathogen competition assay.** To test the hypothesis that ethanol-enriched media improves relative growth of *Ambrosiella* spp. (symbiont of *Xylosandrus* spp. ambrosia beetles) in competition with non-symbiont fungi, a dual culture confrontation assay was conducted with several isolates of *Ambrosiella* spp. versus a range of non-symbiont fungi that were associated with ambrosia beetle galleries following methods similar to Gazis et al. (2018). Isolates were grown from stored samples taken from ambrosia beetle galleries or ambrosia beetles in the previous studies outlined above. MEA was used as a control and ethanol-enriched media was made by adding an appropriate amount of 200 proof ethanol (Fisher Scientific, Waltham, MA) to the MEA to achieve 2.5% v/v just prior to pouring into 9 cm Petri dishes.

For the confrontation assay, a dual-inoculation confrontation assay was conducted, a mycelial plug (0.5 cm) of a symbiont and non-symbiont fungal isolate were placed on MEA and MEA + ethanol plates. Mycelial plugs were excised from the actively growing margin of the fungus that was grown on PDA for 7-10 days at 25 °C and a light:dark cycle of 0:24. Each plug was placed mycelial-side down and at opposite sides of the plate, 1 cm from the edge. (For controls, plates were inoculated with a single plug from each isolate placed 1 cm from the edge of the plate). All combinations and controls were replicated in triplicate and the experiment was repeated using the same isolates and methods (6 replicates in total). Plates were incubated at 25°C in the dark. After 3, 5, and 7 days, the radial growth of mycelium of each isolate was measured from the center of the mycelial plug to the colony edge opposite the competing fungus, or towards the opposite side of the plate for singularly-inoculated plates (Fig. 1).

The percentage inhibition of each isolate on each media (MEA or MEA+EtOH) was calculated by comparing the growth of the isolate in competition ( $g_{comp}$ ) with growth of singularly inoculated isolate ( $g_{sing}$ ) [% inhibition =  $(g_{sing} - g_{comp}) \div g_{sing} * 100$ ]. The data were tested for normality and subjected to analysis of variance separately per isolate using PROC ANOVA in SAS V.9 (SAS Institute, Cary NC) to test the effect of ethanol on growth inhibition of each competing isolate pair.

## Results

**Sentinel tree survey.** A total of 627 fungal isolates representing 43 species across 26 genera in 2019 and 607 isolates representing 51 species across 30 genera in 2020 were recovered from sapwood surrounding ambrosia beetle galleries from sentinel potted apple trees deployed in apple orchards for 14 day periods throughout the growing season. In 2020, in addition to gallery tissue, isolations were made from non-gallery tissue in sentinel trees, which recovered 401 isolates representing 56 species across 27 genera (Fig. 1). Across both years the most common fungi isolated from gallery tissue were the nutritional symbionts of the main ambrosia beetles attacking apple, *Ambrosiella grosmanii*, *A. roeperi*, and *A. beaveri* (Table 3) comprising 61% of all isolates, followed by *Fusarium* (24%), *Diaporthe* (17%), *Epicoccum* (9.5%), *Alternaria* (8.9%), *Botryosphaeria* (8.4%), *Paracoiothyrium* (7.0%), and *Didymella* (6.1%).

**Fungi recovered from trapped beetles.** Fungi were recovered from 153 beetles (n = 69 *X. crassiusculus*, 38 *X. germanus*, 34 *Cnestus mutilatus*, 3 unknown spp. and 9 *Xyleborinus saxeseni*), trapped in and around apple orchards in Henderson Co., NC. Across all beetle samples, 71 different species of fungi were identified across 48 genera with 42 species isolated more than once across samples and 14 species recovered 5 or more times (Table 4, Fig. 1). From *X. crassiusculus*, its nutritional symbiont *A. roeperi* was the most common fungal species recovered followed by common non-symbionts associated with the beetle, *Cladosporium crousii*, *C. cladosporioides*, and *Epicoccum nigrum*.

The most common fungal species recovered from *X. germanus* was *C. crousii* followed by *F. solani*, *C. cladosporioides*, and its nutritional symbiont, *A. grosmaniae*. On *Cnestus mutilatus*, *A. beaveri* was most commonly recovered followed by *Aureobasidium pullans*, *Epicoccum nigrum*, *C. cladosporioides*, and an unidentified *Trichoderma* species. Overall, there was a greater diversity of fungi recovered from *X. crassiusculus* compared with the other species, with *X. crassiusculus* being the most abundant beetle sampled. Several other fungi were recovered at low frequencies from beetles including some from phytopathogenic genera such as *Alternaria*, *Fusarium*, *Botryosphaeria*, *Diaporthe*, and *Colletotrichum*.

**Orchard Survey.** A total of 470 isolates consisting of 40 species of fungi (across 23 genera) were recovered from tissue sampled from visually healthy apple trees at commercial orchards that had previously experienced tree decline issues (Table 5, Fig. 1). *Trichoderma atroviridae* was the most common species recovered from decaying wood followed by *Diaporthe sp.*, *Alteraria alternata*, and then *Diaporthe eres*. The most common species recovered from graft union tissue was *Diaporthe sp.*, *Diplodia seriata*, and *Botryosphaeria dothidea*. Woody tissue from pruning wounds and small stem cankers had similar frequencies of fungi recovered with *Paraconthyrium brasiliense*, *Diaporthe sp.*, *Alteraria alternata*, and *T. atroviridae* being frequently recovered in both type of tissue.

**Pathogenicity and aggressiveness assays.** The detached shoot assay in 2020 found a significant effect of isolate on lesion length on detached one-year old apple shoots ( $F_{26,222} = 5.26$ ,  $P < 0.001$ ). There was variation in aggressiveness of *Ambrosiella* species and isolates within a species. With the exception of a single isolate of *A. roeperi*, which caused moderate necrosis (Table 6) and was re-isolated from infected tissue, the other isolates of *Ambrosiella* spp. lesion length was not significantly different from the control. Isolates of *Ambrosiella grosmaniae* caused little necrosis but a large amount of stem discoloration (staining) was observed. All three isolates of *B. dothidea* resulted in significant necrosis compared with the control (Table 6). There was considerable variation among isolates of *Fusarium* spp. with isolates from the *F. sporotrichoides* complex being the most aggressive and *F. solani* and *F.*

*incarnatum-equiseti* species complex being the least aggressive. A single isolate of *Neofusicoccum parvum* was highly aggressive on detached apple shoots.

There was a significant effect of fungal species on lesion length on detached dormant shoots ( $F_{23,192} = 11.63$ ,  $p < 0.0001$ ), detached summer shoots ( $F_{21,65} = 13.59$ ,  $p < 0.0001$ ), and on living apple seedlings in 2022 experiments ( $F_{19,340} = 2.87$ ,  $p < 0.0001$ ). Isolates of *Ambrosiella* spp. showed mixed levels of aggressiveness on detached shoots and seedlings in 2022 inoculation assays but in general resulted in lower necrosis compared with pathogenic isolates of *Diplodia* spp., *Fusarium* spp., *B. dothidea*. One isolate of *A. grosmanniae* and one isolate of *A. roeperi* had moderate aggressiveness on detached shoots with the mean necrotic lesion length comparable to two of the *B. dothidea* isolates and one third of the most aggressive *B. dothidea* isolate (Table 7). Inoculation of seedlings with *A. roeperi*, *A. grosmanniae*, or *A. beaveri* resulted in little necrosis. There were differences in aggressiveness among the *B. dothidea* isolates with 20EB-A.1 having the largest lesion on detached shoots out of all isolates and the other isolates only resulting in moderate lesion length. On seedlings, *B. dothidea* caused small lesions. *Fusarium* spp. isolates caused low to moderate lesion lengths on detached shoots and apple seedlings. Isolate 20L-FO.1, *F. liriodendra*, was moderately aggressive on detached shoots and seedlings, 20ET-FR.2 was moderately aggressive on detached shoots and highly aggressive on seedlings, and other isolates had moderately low aggressiveness on detached shoots and seedlings except for 20ET-CD.1 that was highly aggressive on seedlings but not detached shoots. Two isolates of *P. brassilense* were tested on detached shoots only and caused moderate necrosis.

**Effect of ethanol on fungal growth.** Media ethanol content of 0.5-1% had little inhibitory effect on mycelial growth and in some cases resulted in greater mycelial growth compared with non-amended MEA of non-symbiont fungal isolates (Figs. 2 & 3) and, there was some stimulatory effect of ethanol on growth of *Ambrosiella* spp. isolates, increasing radial mycelial growth rate by 5-18% compared to non-amended MEA (Fig. 3.). Increasing ethanol concentration in MEA progressively inhibited mycelial growth

of all isolates tested from within the 2-5% EtOH. At 4% EtOH mycelial growth of *Ambrosiella* spp. isolates was inhibited by 47-57% whereas *Trichoderma* spp. isolates were reduced by 82-89%, *B. dothidea* by 63-67%, and *F. solani* by 58-59%. *Ambrosiella* spp. isolates had slightly higher EC<sub>50</sub> values compared with *Trichoderma* spp. and *B. dothidea* isolates and were not significantly different to *F. solani* (Table 8).

**Symbiont-pathogen competition assay.** Co-inoculation of *Ambrosiella* spp. isolates with *F. armeniacum*, *B. dothidea*, *T. atroviridae*, and *T. hamatum* suppressed symbiont growth on MEA with and without 2.5% ethanol; inhibition ranged from 9.7-49.3% (Table 9). *Ambrosiella* isolates co-inoculated with *F. solani* showed limited growth inhibition and for two isolates of *A. grosmaniae*, co-inoculation with *F. solani* resulted in a slight positive growth effect compared with singular isolation on MEA+EtOH media.

The addition of ethanol did not significantly improve the competitive ability of *Ambrosiella* isolates with *Trichoderma* isolates *in vitro* (Table 9). Isolates of *Trichoderma* co-inoculated with isolates of *Ambrosiella* spp. had higher relative growth than solo isolates of *Trichoderma* when grown on EtOH-enriched MEA whereas on MEA with no-ethanol, co-inoculation with *Ambrosiella* resulted in 13-30% reduction in mycelial growth of the *Trichoderma* spp. isolates. Three of the four *Ambrosiella* spp. isolates co-inoculated with *B. dothidea* were inhibited to a lesser amount when grown on ethanol-amended MEA compared with MEA alone. However, the relative growth rate of *B. dothidea* in the presence of *Ambrosiella* isolates was higher on MEA-amended media compared to its growth rate on MEA+EtOH in non-competitive assays or in competition assays on non-amended media. Co-inoculation of *F. solani* and *Ambrosiella* spp. resulted in limited inhibition of the symbiont and no antagonism was observed regardless of the ethanol content of the media (Table 9).

## Discussion

In this study, a wide diversity of fungi were found to be associated with ambrosia beetle galleries and on the surfaces of beetles trapped in and around western NC apple orchards. Overall the most common fungi recovered from ambrosia beetle galleries and trapped ambrosia beetles were *Ambrosiella* spp. (Fig. 1) which are nutritional symbionts of the dominant exotic ambrosia beetles found to attack stressed apple trees – *X. crassiusculus* (Harrington et al. 2014), *X. germanus* (Mayers et al. 2015b), and *C. mutilatus* (Mayers et al. 2015b). With the exception of *Ambrosiella* spp., the most frequently recovered fungi were not unique to ambrosia beetles or their galleries as they were also isolated from pruning wounds, graft unions, scions, or cankers on trees that were not attacked by ambrosia beetles. The most common non-symbiont fungi recovered from trapped beetles were predominantly from genera not considered to be pathogenic on apple including *Aureobasidium*, *Cladosporium*, *Epicoccum*, and *Trichoderma*. Putative phytopathogenic species were recovered from trapped beetles at relatively low frequency.

Given the previously reported association of phytopathogenic fungi with apple trees attacked by ambrosia beetles (Breth, Agnello, Cox, et al. 2016, Agnello et al. 2017, Gresham et al. 2023), we expected a higher frequency of phytopathogenic fungi recovered from trapped beetles and galleries on ethanol-drenched apple trees. However, in the previous studies, beetles and galleries were recovered from young trees planted in high density orchards that were stressed and producing ethanol naturally. The greater frequency of phytopathogens recovered from those trees was likely due to their opportunistic nature and their ability to colonize trees faced with a myriad of stressors, including but not limited to: cold injury, drought and flood stress, and herbicide injury (Pusey 1989, Ma et al. 2001, Galarneau et al. 2019, Agustí-Brisach et al. 2020). Conversely, sentinel trees in the current study were apparently healthy and ethanol volatiles were artificially created to attract ambrosia beetles. Such an environment was likely not ideal for the rapid colonization and growth of opportunistic fungal

pathogens. Furthermore, unlike previous studies in which fungi were recovered from gallery tissue or foundresses within galleries, in this survey fungi were isolated from trapped beetles and were likely not contaminated with fungi colonizing woody tissue.

While only a low frequency of putative and known phytopathogens were isolated from the exterior of ambrosia beetles, there remains a potential risk that exotic ambrosia beetles attacking stressed apple trees could increase spread of damaging fungi and could accelerate invasion of problematic phytopathogens throughout a landscape. Indeed, *Xylosandrus* spp. are able to acquire fungi from the invaded environment (Rassati et al. 2019, Morales-Rodríguez et al. 2021), including damaging quarantine phytopathogens such as *Rafaelella lauricola*, the causal organism of laurel wilt disease that is devastating Avocado orchards in the south-eastern USA (Carrillo et al. 2014, Cruz 2021). Therefore, the diversity of fungi recovered from trapped beetles and galleries on apple trees demonstrates the potential for movement of phytopathogens across the landscape. Future studies should confirm whether fungal propagules transported by dispersing beetles are able to colonize host tissue.

*Fusarium* was one of the most common non-symbiont genera associated with trapped beetles, and galleries on sentinel trees in our study. *Fusarium* species are commonly associated with ambrosia beetles, and for some beetle species serve as primary or secondary symbionts and cause disease on host trees (Kasson et al. 2013, Bateman et al. 2016, Carrillo et al. 2020). *Fusarium solani* and other *Fusarium* spp have been found to be the most common fungal species associated with galleries of *Xylosandrus* spp. (Breth, Agnello, and Tee 2016, Agnello et al. 2017, Tuncer et al. 2018).

*Fusarium* spp., especially members of the *F. solani* and *F. oxysporum* species complexes are cosmopolitan soil-inhabiting fungi that are most often saprobic (Crous et al. 2021). There are species and circumstances in which *Fusarium* spp. can be phytopathogens of importance across a wide range of hosts (Bostock and Gordon 2013, Crous et al. 2021, Stack et al. 2021). On apple, *Fusarium* spp. are

associated with postharvest core rots of fruit and can cause root rots and branch and trunk cankers (Ma et al. 2013). Recently, there have been several reports of *Fusarium* spp. causing dieback on apple in China (Cheng et al. 2019), Iran (Esmaili and Sharifnabi 2023), and *Prunus* spp. dieback in California (Marek et al. 2013) and Tunisia (Mannai et al. 2018). *Fusarium solani* has also been implicated in Apple Replant Disease (ARD) and apple decline in Asia (Manici et al. 2003, 2017, Xiang et al. 2021, Duan et al. 2022). However, *F. solani*, the most common *Fusarium* species in our surveys, showed low aggressiveness on detached apple shoots and seedlings. Conversely *F. armeniacum*, *F. fujikuroi*, and *F. sporotrichioides*, which were isolated at low frequency from trapped ambrosia beetles, beetle galleries, and apparently healthy trees were highly aggressive in our assays. As *Fusarium* in apple has been associated with stressed trees, future studies should focus on evaluation of these species and strains under a diversity of abiotic stress conditions. In addition, given the variation in aggressiveness of strains within a species of *Fusarium*, additional studies evaluating a greater number of strains from the most aggressive species is warranted.

Phytopathogenic *Botryosphaeria*, *Diplodia*, and *Diaporthe* species were isolated from ambrosia beetle gallery tissue on sentinel apple trees and at relatively low frequency from trapped ambrosia beetles. These genera contain important wood-invading pathogens associated with dieback and decline on apple (Brown-Rytlewski and McManus 2000, Sessa et al. 2016, 2018, Ilyukhin et al. 2022, Martino et al. 2023, Sha et al. 2023) and Almond (Moral et al. 2019, Holland et al. 2021). Surveys have found *B. dothidea* to be key causal agent of apple dieback in Northern Italy (Martino et al. 2023), common and highly aggressive on apple in Canada (Ilyukhin et al. 2022), and causing dieback on apple in South Africa (Slippers et al. 2007), China (Sha et al. 2023), and Uruguay (Sessa et al. 2016). *Diaporthe* spp. are not considered to cause major economic diseases on apple but have been associated with canker and dieback in apple growing regions throughout the world (Cloete et al. 2011, Sessa et al. 2018, Ali et al. 2020, Mang et al. 2022). A recent survey of apple rootstocks experiencing dieback in Canada recovered

*D. eres* from over 90% of symptomatic trees (Ali et al. 2020). Stress, particularly water stress, is an important determinant of disease severity associated with necrotrophic phytopathogens such as *B. dothidea*, and *D. eres* (Pusey 1989, Ma et al. 2001, Galarneau et al. 2019, Agustí-Brisach et al. 2020).

Our assays found that *Botryosphaeria dothidea* and *Diplodia seratia* (anamorph = *B. obtusa*) were highly aggressive on detached apple shoots, and moderately aggressive on apple seedlings. Previous surveys of declining apple trees attacked by ambrosia beetles found *Diaporthe* spp. were common but not dominant (Agnello et al. 2017, Gresham et al. 2023). *Paraconiothyrium brasiliense* (Pleosporales: Montagnulaceae) was frequently isolated from ambrosia beetle galleries on sentinel apple trees, non-gallery tissue, and was the most common fungus isolated from stem canker tissue in our survey of apparently healthy apple trees. There are few reports of *P. brasiliense* associated with disease of apple and where it has been reported, it has shown low aggressiveness on apple shoots (Damm et al. 2008, Cloete et al. 2009, 2011, Martino et al. 2023). In our assays it showed low aggressiveness on apple shoots, supporting the assumption that it is not likely to be a damaging phytopathogen of fruit trees.

Several species of *Trichoderma* were isolated from ambrosia beetles, their galleries, and non-attacked apple trees in our surveys. The occurrence of *Trichoderma* associated with ambrosia beetle galleries was lower compared with pruning wounds and rootstock/graft unions in our orchard survey and compared with phoretic associations on trapped ambrosia beetles. Several studies have shown that *Trichoderma* sp. are effective biological control agents against ambrosia beetles – inhibiting growth of their nutritional symbiotic fungus and decreasing gallery establishment and production of offspring (Castrillo et al. 2013, 2016, Kushiyevev et al. 2021, Loera 2021, Reverchon et al. 2021, Gugliuzzo et al. 2022). Our dual culture assays showed that *T. atroviridae* and *T. hamatum* quickly overgrew *Ambrosiella* isolates regardless of media ethanol content, suggesting that the ethanol-enriched substrate on which

ambrosia beetles establish their galleries would not prevent *Trichoderma* out-competing their nutritional symbiont.

Numerous biofungicides containing *Trichoderma* are commercially available and should be further explored as a potential control tool for ambrosia beetles under field conditions. Similar to our native *Trichoderma* isolates, dual culture assays in previous studies have shown that *Trichoderma* outcompetes ambrosia beetle symbiont fungi (Castrillo et al. 2016, Gazis et al. 2018, Kushiyeve et al. 2021, Loera 2021, Gugliuzzo et al. 2022). Dipping ethanol-soaked bolts in spore solutions of *T. harzianum*, *T. hamatum*, *T. asperellum*, *T. atroviridae* prior to exposure to ambrosia beetles does not seem to affect adult survival but significantly suppresses production of offspring of *X. germanus* (Castrillo et al. 2016, Kushiyeve et al. 2021), *X. compactus* (Gugliuzzo et al. 2022), *Xyleborinus affinis* (Loera 2021) and *X. crassiusculus* (Castrillo et al. 2013).

Given that ethanol increased radial mycelial growth of *Ambrosiella* relative to non-symbiont fungi on media enriched with <3% ethanol in our assay and mycelial biomass and density in previous studies (Ranger et al. 2018, Lehenberger et al. 2021), we expected that ethanol-enriched media would provide an advantage for *Ambrosiella*, allowing it to better establish within trees that have elevated tissue ethanol content and be able to better out-compete other saprobic and necrotrophic fungi that are also able to exploit physiologically stressed trees. With the exception of *F. solani*, the slowest growing of the isolates, ethanol did not provide a competitive advantage for *Ambrosiella* spp. in regard to mycelial radial growth. Dual culture competition assays showed that the presence of *B. dothidea*, *F. armeniacum*, *T. atroviridae*, and *T. hamatum* inhibited mycelial growth of *Ambrosiella* spp. isolates regardless of the ethanol content of the media. Therefore, although previous studies have shown that ethanol provides a relative benefit to *Ambrosiella* spp. over non-symbiont fungi, fungi commonly isolated from ambrosia beetle galleries on apple trees, *Botryosphaeria* and *Trichoderma* spp. were able to out compete *Ambrosiella* spp. isolates tested. *Trichoderma* spp. has been shown to be antagonistic to *Ambrosiella*

spp. growth and suppress offspring production on ethanol-soaked bolts and other fast-growing fungi may also decrease brood production (Castrillo et al. 2016, Gugliuzzo et al. 2022). Therefore, the success of exotic *Xylosandrus* ambrosia beetles may be in part due to their ability to establish galleries within hosts before necrotrophic and saprotrophic fungi invade the host tissue compared with other ambrosia beetles that attack hosts at later stages of decline.

These studies support previous findings that a broad diversity of fungi, including putative phytopathogens and opportunistic saprobes are associated with ambrosia beetle galleries, and show that there is a risk of transmission of phytopathogenic fungi by Xyleborine ambrosia beetles colonizing apple trees. However, the isolates of *Botrosphaeria* and *Fusarium* spp. that were most aggressive on detached shoots and apple seedlings were commonly isolated from non-attacked apple trees and rare or absent on trapped beetles and galleries from ethanol-drenched potted apple trees. Future surveys with an expanded geographic range and higher sampling intensity of trapped beetles, combined with transmission assays to confirm whether fungal propagules on beetles can infect hosts would be beneficial for understanding the risk of phytopathogen transmission by Xyleborine ambrosia beetles. Furthermore, inhibition of *Ambrosiella* spp. growth in-vitro by fungi commonly isolated from galleries and non-attacked apple trees, suggests that latent infections and saprobes such as *Trichoderma* spp. could limit the establishment and growth of the beetle's nutritional symbiont and thus brood success. Further work is required to better understand the effect of latent infections and *Trichoderma* spp. on ambrosia beetle host selection and gallery success in-planta.

## References

- Agnello, A. M., D. I. Breth, E. M. Tee, K. D. Cox, S. M. Villani, K. M. Ayer, A. E. Wallis, D. J. Donahue, D. B. Combs, A. E. Davis, J. A. Neal, and F. M. English-Loeb. 2017.** *Xylosandrus germanus* (Coleoptera: Curculionidae: Scolytinae) Occurrence, Fungal Associations, and Management Trials in New York Apple Orchards. *J. Econ. Entomol.* 110: 2149–2164.
- Agustí-Brisach, C., D. Moldero, M. D. C. Raya, I. J. Lorite, F. Orgaz, and A. Trapero. 2020.** Water stress enhances the progression of branch dieback and almond decline under field conditions. *Plants.* 9: 1–26.
- Ali, S., W. Renderos, E. Bevis, J. Hebb, and P. A. Abbasi. 2020.** *Diaporthe eres* causes stem cankers and death of young apple rootstocks in Canada. *Can. J. Plant Pathol.* 42: 218–227.
- Bateman, C., M. Šigut, J. Skelton, K. E. Smith, and J. Hulcr. 2016.** Fungal Associates of the *Xylosandrus compactus* (Coleoptera: Curculionidae, Scolytinae) Are Spatially Segregated on the Insect Body. *Environ. Entomol.* 45: 883–890.
- Batra, L. R. 1985.** Ambrosia beetles and their associated fungi: research trends and techniques. *Proc. Plant Sci.* 94: 137–148.
- Bostock, R. M., and T. R. Gordon. 2013.** Etiology , Epidemiology , and Management of *Fusarium* spp . Causing Cryptic Cankers in Cold-Stored , Bare-Root Propagated Almond Trees in California Nurseries By ABIGAIL JUSTINE SEIDLE Submitted in partial satisfaction of the requirements of the degree of .
- Breth, D., A. Agnello, K. Cox, and E. Tee. 2016.** Black Stem Borer *Xylosandrus germanus*. 81: 6–10.

**Breth, D., A. Agnello, and E. Tee. 2016.** Black stem borer control in apple nurseries and tall spindle plantings Debo. Cornell Coop. Ext. Publ. 2–3.

**Brown-Rytlewski, D. E., and P. S. McManus. 2000.** Virulence of *Botryosphaeria dothidea* and *Botryosphaeria obtusa* on apple and management of stem cankers with fungicides. *Plant Dis.* 84: 1031–1037.

**Carrillo, D., R. E. Duncan, J. N. Ploetz, A. F. Campbell, R. C. Ploetz, and J. E. Peña. 2014.** Lateral transfer of a phytopathogenic symbiont among native and exotic ambrosia beetles. *Plant Pathol.* 63: 54–62.

**Carrillo, J. D., C. Dodge, R. Stouthamer, and A. Eskalen. 2020.** Fungal symbionts of the polyphagous and Kuroshio shot hole borers ( Coleoptera : Scolytinae , *Euwallacea* spp .) in California can support both ambrosia beetle systems on artificial media. 155–168.

**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., M. H. Griggs, and J. D. Vandenberg. 2016.** Competition between biological control fungi and fungal symbionts of ambrosia beetles *Xylosandrus crassiusculus* and *X. germanus* (Coleoptera: Curculionidae): Mycelial interactions and impact on beetle brood production. *Biol. Control.* 103: 138–146.

**Cheng, Y., W. Zhao, R. Lin, Y. Yao, S. Yu, Z. Zhou, X. Zhang, Y. Gao, and W. Huai. 2019.** *Fusarium* species in declining wild apple forests on the northern slope of the Tian Shan Mountains in north-western China. *For. Pathol.* 49: 1–20.

**Cloete, M., U. Damm, P. W. Crous, P. H. Fourie, and L. Mostert. 2009.** Pome fruit trees as alternative hosts of grapevine trunk pathogens. *Phytopathol. Mediterr.* 48: 181–182.

**Cloete, M., P. H. Fourie, U. Damm, P. W. Crous, and L. Mostert. 2011.** Fungi associated with die-back symptoms of apple and pear trees, a possible inoculum source of grapevine trunk disease pathogens. *Phytopathol. Mediterr.* 50.

**Crous, P. W., L. Lombard, M. Sandoval-Denis, K. A. Seifert, H. J. Schroers, P. Chaverri, J. Gené, J. Guarro, Y. Hirooka, K. Bensch, G. H. J. Kema, S. C. Lamprecht, L. Cai, A. Y. Rossman, M. Stadler, R. C. Summerbell, J. W. Taylor, S. Ploch, C. M. Visagie, N. Yilmaz, J. C. Frisvad, A. M. Abdel-Azeem, J. Abdollahzadeh, A. Abdolrasouli, A. Akulov, J. F. Alberts, J. P. M. Araújo, H. A. Ariyawansa, M. Bakhshi, M. Bendiksby, A. Ben Hadj Amor, J. D. P. Bezerra, T. Boekhout, M. P. S. Câmara, M. Carbia, G. Cardinali, R. F. Castañeda-Ruiz, A. Celis, V. Chaturvedi, J. Collemare, D. Croll, U. Damm, C. A. Decock, R. P. de Vries, C. N. Ezekiel, X. L. Fan, N. B. Fernández, E. Gaya, C. D. González, D. Gramaje, J. Z. Groenewald, M. Grube, M. Guevara-Suarez, V. K. Gupta, V. Guarnaccia, A. Haddaji, F. Hagen, D. Haelewaters, K. Hansen, A. Hashimoto, M. Hernández-Restrepo, J. Houbraken, V. Hubka, K. D. Hyde, T. Iturriaga, R. Jeewon, P. R. Johnston, Jurjević, Karalti, L. Korsten, E. E. Kuramae, I. Kušan, R. Labuda, D. P. Lawrence, H. B. Lee, C. Lechat, H. Y. Li, Y. A. Litovka, S. S. N. Maharachchikumbura, Y. Marin-Felix, B. Matio Kemkuignou, N. Matočec, A. R. McTaggart, P. Mlčoch, L. Mugnai, C. Nakashima, R. H. Nilsson, S. R. Noumeur, I. N. Pavlov, M. P. Peralta, A. J. L. Phillips, J. I. Pitt, G. Polizzi, W. Quaedvlieg, K. C. Rajeshkumar, S. Restrepo, A. Rhaïem, J. Robert, V. Robert, A. M. Rodrigues, C. Salgado-Salazar, R. A. Samson, A. C. S. Santos, R. G. Shivas, C. M. Souza-Motta, G. Y. Sun, W. J. Swart, S. Szoke, Y. P. Tan, J. E. Taylor, P. W. J. Taylor, P. V. Tiago, K. Z. Váczy, N. van de Wiele, N. A. van der Merwe, G. J. M. Verkley, W. A. S. Vieira, A. Vizzini, B. S. Weir, N. N. Wijayawardene, J. W. Xia, M. J. Yáñez-Morales, A. Yurkov, J. C. Zamora, R. Zare, C. L. Zhang, and M. Thines. 2021.** Fusarium: more than a node or a foot-shaped basal cell. *Stud. Mycol.* 98.

**Cruz, L. F. 2021.** Phoretic and internal transport of *Raffaelea lauricola* by different species of ambrosia beetle associated with avocado trees. 151–161.

**Damm, U., G. J. M. Verkley, P. W. Crous, P. H. Fourie, A. Haegi, and L. Riccioni. 2008.** Novel *Paraconiothyrium* species on stone fruit trees and other woody hosts. *Persoonia Mol. Phylogeny Evol. Fungi.* 20: 9–17.

**Donahue, D. J., and S. E. Elone. 2021.** Case Study of a Declining Apple Orchard Daniel J. Donahue and Sarah E. Elone. *New York Fruit Q. - Summer.* 29: 28–31.

**Duan, Y. N., W. T. Jiang, R. Zhang, R. Chen, X. S. Chen, C. M. Yin, and Z. Q. Mao. 2022.** Discovery of *fusarium proliferatum* f. sp. *malus domestica* causing apple replant disease in China. *Plant Dis.* 106: 2958–2966.

**Dute, R. R., M. E. Miller, M. A. Davis, F. M. Woods, and K. S. McLean. 2002.** Effects of ambrosia beetle attack on *Cercis canadensis*. *IAWA J.* 23: 143–160.

**Esmaili, Z., and B. Sharifnabi. 2023.** *Fusarium* species associated with apple trees decline in Isfahan, Iran. *Mycol. Iran.* 10: 23–34.

**Galarneau, E. R. A., D. P. Lawrence, R. Travadon, and K. Baumgartner. 2019.** Drought exacerbates *botryosphaeria* dieback symptoms in grapevines and confounds host-based molecular markers of infection by *neofusicoccum parvum*. *Plant Dis.* 103: 1738–1745.

**Gazis, R., L. Poplawski, W. Klingeman, S. L. Boggess, R. N. Trigiano, A. D. Graves, S. J. Seybold, and D. Hadziabdic. 2018.** Mycobiota associated with insect galleries in walnut with thousand cankers disease reveals a potential natural enemy against *Geosmithia morbida*. *Fungal Biol.* 122: 241–253.

**Gugliuzzo, A., D. Aiello, A. Biondi, G. Giurdanella, G. Siscaro, L. Zappalà, A. Vitale, G. T. Garzia, and G. Polizzi. 2022.** Microbial mutualism suppression by *Trichoderma* and *Bacillus* species for controlling the invasive ambrosia beetle *Xylosandrus compactus*. *Biol. Control*. 170: 1–11.

**Harrington, T. C., D. McNew, C. Mayers, S. W. Fraedrich, and S. E. Reed. 2014.** *Ambrosiella roeperi* sp. nov. is the mycangial symbiont of the granulate ambrosia beetle, *Xylosandrus crassiusculus*. *Mycologia*. 106: 835–845.

**Holland, L. A., F. P. Trouillas, M. T. Nouri, D. P. Lawrence, M. Crespo, D. A. Doll, R. A. Duncan, B. A. Holtz, C. M. Culumber, M. A. Yagmour, F. J. A. Niederholzer, D. M. Lightle, K. S. Jarvis-Shean, P. E. Gordon, and E. J. Fichtner. 2021.** Fungal pathogens associated with canker diseases of almond in California. *Plant Dis*. 105: 346–360.

**Hulcr, J., and R. R. Dunn. 2011.** The sudden emergence of pathogenicity in insect-fungus symbioses threatens naive forest ecosystems. *Proc. R. Soc. B Biol. Sci.* 278: 2866–2873.

**Ilyukhin, E., K. Schneider, and W. Ellouze. 2022.** First Report of *Botryosphaeria dothidea* Causing Stem Canker and Dieback of Apple Trees in Ontario, Canada. *Plant Dis*. 106: 2994.

**Kasson, M. T., K. O'Donnell, A. P. Rooney, S. Sink, R. C. Ploetz, J. N. Ploetz, J. L. Konkol, D. Carrillo, S. Freeman, Z. Mendel, J. A. Smith, A. W. Black, J. Hulcr, C. Bateman, K. Stefkova, P. R. Campbell, A. D. W. Geering, E. K. Dann, A. Eskalen, K. Mohotti, D. P. G. Short, T. Aoki, K. A. Fenstermacher, D. D. Davis, and D. M. Geiser. 2013.** An inordinate fondness for *Fusarium*: Phylogenetic diversity of fusaria cultivated by ambrosia beetles in the genus *Euwallacea* on avocado and other plant hosts. *Fungal Genet. Biol.* 56: 147–157.

**Kessler, K. J. 1974.** An apparent symbiosis between *Fusarium* fungi and ambrosia beetles causes canker of black walnut stems. *Plant Dis*. 58: 1044–1047.

**Kostovcik, M., C. C. Bateman, M. Kolarik, L. L. Stelinski, B. H. Jordal, and J. Hulcr. 2015.** The ambrosia symbiosis is specific in some species and promiscuous in others: Evidence from community pyrosequencing. *ISME J.* 9: 126–138.

**Kovach, J. 1986.** Life cycle, seasonal distribution and tree responses to scolytid beetles in South Carolina peach orchards.

**Kushiyev, R., C. Tuncer, I. Erper, and G. Özer. 2021.** The utility of *Trichoderma* spp. isolates to control of *Xylosandrus germanus* Blandford (Coleoptera: Curculionidae: Scolytinae). *J. Plant Dis. Prot.* 128: 153–160.

**Lehenberger, M., M. Benkert, and P. H. W. Biedermann. 2021.** Ethanol-Enriched Substrate Facilitates Ambrosia Beetle Fungi, but Inhibits Their Pathogens and Fungal Symbionts of Bark Beetles. *Front. Microbiol.* 11: 1–12.

**Loera, O. 2021.** *Metarhizium robertsii* in combination with *Trichoderma asperellum* reduce the malathion doses used to control ambrosia beetles : the case of *Xyleborus affinis* *Metarhizium robertsii* in combination with *Trichoderma asperellum* reduce the malathion doses used t.

**Ma, L.-J., D. M. Geiser, R. H. Proctor, A. P. Rooney, K. O'Donnell, F. Trail, D. M. Gardiner, J. M. Manners, and K. Kazan. 2013.** *Fusarium* Pathogenomics . *Annu. Rev. Microbiol.* 67: 399–416.

**Ma, Z., D. P. Morgan, and T. J. Michailides. 2001.** Effects of water stress on *Botryosphaeria* blight of pistachio caused by *Botryosphaeria dothidea*. *Plant Dis.* 85: 745–749.

**Mang, S. M., C. Marccone, A. Maxim, and I. Camele. 2022.** Investigations on Fungi Isolated from Apple Trees with Die-Back Symptoms from Basilicata Region (Southern Italy). *Plants.* 11.

**Manici, L. M., F. Caputo, and M. L. Saccà. 2017.** Secondary metabolites released into the rhizosphere by *Fusarium oxysporum* and *Fusarium* spp. as underestimated component of nonspecific replant disease. *Plant Soil*. 415: 85–98.

**Manici, L. M., C. Ciavatta, M. Kelderer, and G. Erschbaumer. 2003.** Replant problems in South Tyrol: Role of fungal pathogens and microbial population in conventional and organic apple orchards. *Plant Soil*. 256: 315–324.

**Mannai, S., N. Horrigue-Raouani, and N. M'Hamdi. 2018.** Effect of Six Fungicides against *Fusarium oxysporum* and *F. solani* Associated with Peach Seedlings Decline in Tunisian Nurseries. *Annu. Res. Rev. Biol.* 26: 1–11.

**Marek, S. M., M. A. Yagmour, and R. M. Bostock. 2013.** *Fusarium* spp., *cylindrocarpon* spp., and environmental stress in the etiology of a canker disease of cold-stored fruit and nut tree seedlings in California. *Plant Dis.* 97: 259–270.

**Martino, I., C. Agustí-Brisach, L. Nari, M. Gullino, and V. Guarnaccia. 2023.** Characterization and pathogenicity of fungal species associated with dieback of apple trees in Northern Italy. *Plant Dis. E-Pub ahea*: 1–76.

**Massoumi Alamouti, S., C. K. M. Tsui, and C. Breuil. 2009.** Multigene phylogeny of filamentous ambrosia fungi associated with ambrosia and bark beetles. *Mycol. Res.* 113: 822–835.

**Mayers, C. G., D. L. McNew, T. C. Harrington, R. A. Roeper, S. W. Fraedrich, P. H. W. Biedermann, L. A. Castrillo, and S. E. Reed. 2015a.** Three genera in the Ceratocystidaceae are the respective symbionts of three independent lineages of ambrosia beetles with large, complex mycangia. *Fungal Biol.* 119: 1075–1092.

**Mayers, C. G., D. L. McNew, T. C. Harrington, R. A. Roeper, S. W. Fraedrich, P. H. W.**

**Biedermann, L. A. Castrillo, and S. E. Reed. 2015b.** Three genera in the Ceratocystidaceae are the respective symbionts of three independent lineages of ambrosia beetles with large, complex mycangia. *Fungal Biol.* 119: 1075–1092.

**Moral, J., D. Morgan, A. Trapero, and T. J. Michailides. 2019.** Ecology and epidemiology of diseases of nut crops and olives caused by botryosphaeriaceae fungi in California and Spain. *Plant Dis.* 103: 1809–1827.

**Morales-Rodríguez, C., I. Sferrazza, M. P. Aleandri, M. Dalla Valle, S. Speranza, M. Contarini, and A. Vannini. 2021.** The fungal community associated with the ambrosia beetle *Xylosandrus compactus* invading the mediterranean maquis in central Italy reveals high biodiversity and suggests environmental acquisitions. *Fungal Biol.* 125: 12–24.

**Ploetz, R. C. 2013.** Diseases that are associated with ambrosia and bark beetles comprise some of the most significant problems that have emerged on trees in the last century . They are caused by fungi in the Ophi- ostomatales , Microascales , and Hypocreales , and have vecto. *Am. Phytopathol. Soc.* 95.

**Ploetz, R. C., J. Hulcr, M. J. Wingfield, and Z. W. De Beer. 2013.** Active tree diseases associated with ambrosia and bark beetles: black swan events in tree pathology? *Plant Dis.* 97: 856–872.

**Pusey, P. L. 1989.** Influence of Water Stress on Susceptibility of Nonwounded Peach Bark to *Botryosphaeria dothidea*. *Plant Dis.*

**Ranger, C. M., P. H. W. Biedermann, V. Phuntumart, G. U. Beligala, S. Ghosh, D. E. Palmquist, R. Mueller, J. Barnett, P. B. Schultz, M. E. Reding, and J. P. Benz. 2018.** Symbiont selection via alcohol benefits fungus farming by ambrosia beetles. *Proc. Natl. Acad. Sci. U. S. A.* 115: 4447–4452.

**Ranger, C. M., M. Dzurenko, J. Barnett, R. Geedi, L. Castrillo, M. Ethington, M. Ginzel, K. Adesso, and M. E. Reding. 2021.** Electrophysiological and Behavioral Responses of an Ambrosia Beetle to Volatiles of its Nutritional Fungal Symbiont. *J. Chem. Ecol.* 47: 463–475.

**Ranger, C. M., M. E. Reding, P. B. Schultz, J. B. Oliver, S. D. Frank, K. M. Adesso, J. H. Chong, B. Sampson, C. Werle, S. Gill, and C. Krause. 2016.** Biology, ecology, and management of nonnative ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) in ornamental plant nurseries. *J. Integr. Pest Manag.* 7.

**Ranger, C. M., P. C. Tobin, and M. E. Reding. 2015.** Ubiquitous volatile compound facilitates efficient host location by a non-native ambrosia beetle. *Biological Invasions.* 675–686.

**Rassati, D., L. Marini, and A. Malacrinò. 2019.** Acquisition of fungi from the environment modifies ambrosia beetle mycobiome during invasion. *PeerJ.* 2019: 1–18.

**Reverchon, F., S. M. Contreras-Ramos, A. Eskalen, J. A. Guerrero-Analco, E. E. Quiñones-Aguilar, C. Rios-Velasco, and J. B. Velázquez-Fernández. 2021.** Microbial Biocontrol Strategies for Ambrosia Beetles and Their Associated Phytopathogenic Fungi. *Front. Sustain. Food Syst.* 5: 1–13.

**Saragih, S. A., S. Takemoto, D. Kusumoto, and N. Kamata. 2021.** Fungal diversity in the mycangium of an ambrosia beetle *Xylosandrus crassiusculus* (Coleoptera: Curculionidae) in Japan during their late dispersal season. *Symbiosis.* 84: 111–118.

**Sessa, L., E. Abreo, L. Bettucci, and S. Lupo. 2016.** Botryosphaeriaceae species associated with wood diseases of stone and pome fruits trees: symptoms and virulence across different hosts in Uruguay. *Eur. J. Plant Pathol.* 146: 519–530.

**Sessa, L., E. Abreo, and S. Lupo. 2018.** Diversity of fungal latent pathogens and true endophytes associated with fruit trees in Uruguay. *J. Phytopathol.* 166: 633–647.

**Sha, S. S., Z. Wang, C. C. Yan, H. T. Hao, L. Wang, and H. Z. Feng. 2023.** Identification of Fungal Species Associated with Apple Canker in Tarim Basin, China. *Plant Dis.* 107: 1284–1298.

**Skelton, J., A. J. Johnson, M. A. Jusino, C. C. Bateman, Y. Li, and J. Hulcr. 2019.** A selective fungal transport organ (mycangium) maintains coarse phylogenetic congruence between fungus-farming ambrosia beetles and their symbionts. *Proc. R. Soc. B Biol. Sci.* 286.

**Skelton, J., M. A. Jusino, Y. Li, C. Bateman, P. H. Thai, C. Wu, D. L. Lindner, and J. Hulcr. 2018.** Detecting Symbioses in Complex Communities: the Fungal Symbionts of Bark and Ambrosia Beetles Within Asian Pines. *Microb. Ecol.* 76: 839–850.

**Slippers, B., W. A. Smit, P. W. Crous, T. A. Coutinho, B. D. Wingfield, and M. J. Wingfield. 2007.** Taxonomy, phylogeny and identification of Botryosphaeriaceae associated with pome and stone fruit trees in South Africa and other regions of the world. *Plant Pathol.* 56: 128–139.

**Stack, A. J., S. M. Marek, T. Gordon, and R. M. Bostock. 2021.** Genetic diversity and potential inoculum sources of *Fusarium* species causing cankers in bareroot-propagated almond trees in California nurseries. *Plant Dis.* 1–40.

**Tuncer, C., R. Kushiyeu, and I. Erper. 2018.** Determination of fungal flora on *Anisandrus dispar* Fabricius and *Xylosandrus germanus* Blandford (Coleoptera: Curculionidae: Scolytinae). *Acta Hortic.* 1226: 391–397.

**Weber, B. C., and J. E. McPherson. 1985.** Relation between attack by *Xylosandrus germanus* (Coleoptera: Scolytidae) and disease symptoms in black walnut. *The Can. Entomol.* 1275–1277.

**White, T. J., T. Bruns, S. Lee, and J. W. Taylor. 1990.** Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics., pp. 315–322. *In* Innis, M., Gelfand, D., Sninsky, J., T.J., W. (eds.), *PCR Protoc. A Guid. to Methods Appl.* New York Academic Press, New York, NY.

**Xiang, L., L. Zhao, M. Wang, J. Huang, X. Chen, C. Yin, and Z. Mao. 2021.** Physiological Responses of Apple Rootstock 'M.9' to Infection by *Fusarium solani*. *HortScience*. 1–8.

## Chapter 3 Tables.

Table 3.1. Primer pairs for identification of fungi

Target Genera	Locus	Forward	Sequence (5'-3')	Reverse	Sequence (5'-3')	Reference
All	ITS	ITS1F	GAA GTA AAA GTC GTA ACA AGG	ITS4	TCC TCC GCT TAT TGA TAT GC	White et al. 1990
<i>Fusarium</i>	Tef	EF1	ATGGGTAAGGARGACAAGAC	EF2	GGA RGT ACC AGT SAT CAT GTT	O'Donnell et al. 1998
<i>Botryosphaeria,</i> <i>Ambrosiella,</i> <i>Diaporthe</i>	Btub	Bt2a	GGT AAC CAA ATC GGT GCT GCT TTC	Bt2b	ACC CTC AGT GTA GTG ACC CTT GGC	O'Donnell et al. 1998
<i>Ambrosiella</i>	Tef	EFCF1.5	GCYGAGCTCGGTAAGGGYTC	EFCF6	CATGTCACGGACGGCGAAAC	Skelton 2019

Table 3.2. Isolates evaluated for pathogenicity on detached apple shoots and seedlings.

Isolate	Species	Isolate Origin
20ET-AU.4	<i>Ambrosiella beaveri</i>	Gallery tissue
20ET-FZ.1	<i>Ambrosiella beaveri</i>	Gallery tissue
LCG-XG1.F1	<i>Ambrosiella hartigii</i>	Trapped beetle
20ET-CQ.2	<i>Ambrosiella grosmaniae</i>	Gallery tissue
20ET-CQ.1	<i>Ambrosiella grosmaniae</i>	Gallery tissue
20ET-CN.2	<i>Ambrosiella roeperi</i>	Gallery tissue
20ET-FB.1	<i>Ambrosiella roeperi</i>	Gallery tissue
20L-EB.2	<i>Ambrosiella roeperi</i>	Beetle from gallery
SYM-X	<i>Ambrosiella roeperi</i>	Trapped beetle
20L-EM.1	<i>Ambrosiella roeperi</i>	Trapped beetle
19SE-B.1	<i>Botryosphaeria dothidea</i>	Gallery tissue
20EB-A.1	<i>Botryosphaeria dothidea</i>	Beetle from gallery
NC17-105	<i>Botryosphaeria dothidea</i>	Rad tree, scion
NC17-122	<i>Botryosphaeria dothidea</i>	Rad tree, graft union
NC17-19	<i>Botryosphaeria dothidea</i>	Rad tree, gallery
20ET-B.1	<i>Diplodia seriata</i>	Gallery tissue
20ET-B.2	<i>Diplodia seriata</i>	Gallery tissue
20L-FO.1	<i>Fusarium liriodendri</i>	Trapped beetle
20L-FQ.1	<i>Fusarium aberrans</i>	Trapped beetle
20ET-FR.2	<i>Fusarium armeniacum</i>	Gallery tissue
20EB-E.1	<i>Fusarium solani</i>	Beetle from gallery
20EB-E.2	<i>Fusarium solani</i>	Beetle from gallery
20EB-GA.1	<i>Fusarium petroliphilum</i>	Beetle from gallery
20ET-CD.1	<i>Fusarium solani</i>	Gallery tissue
20ET-CZ.1	<i>Fusarium solani</i>	Gallery tissue
21-L3.B	<i>Fusarium solani</i>	Trapped beetle
NC17-53	<i>Fusarium solani</i>	Rad tree, graft union
19ET-AH.1	<i>Fusarium trincinctum</i>	Gallery tissue
20ET-AH.1	<i>Fusarium trincinctum</i>	Gallery tissue
20ET-AH.3	<i>Fusarium trincinctum</i>	Gallery tissue
NC17-11	<i>Fusarium avenaceum</i>	Rad tree, gallery
NC17-13	<i>Fusarium oxysporum</i>	Rad tree, gallery
NC17-33	<i>Fusarium equiseti</i>	Rad tree, gallery
NC17-17	<i>Fusarium fujikuroi</i>	Rad tree, gallery
NC17-12	<i>Fusarium armeniacum</i>	Rad tree, gallery
NC17-1	<i>Fusarium oxysporum</i>	Rad tree, gallery
NC17-14	<i>Fusarium sporotrichioides</i>	Rad tree, gallery
NC17-6	<i>Fusarium solani</i>	Rad tree, gallery
NC17-9	<i>Fusarium subglutinans</i>	Rad tree, gallery
NC17-100	<i>Fusarium armeniacum</i>	Rad tree, scion
NC17-43	<i>Neofusicoccom parvum</i>	Rad tree, graft union
20ET-DJ.1	<i>Paraconiothyrium brassilense</i>	Gallery tissue
20ET-FK.1	<i>Paraconiothyrium brassilense</i>	Gallery tissue

Table 3.3. Frequency (% occurrence per tissue type) of fungi isolated from ambrosia beetle gallery tissue recovered from sentinel apple trees deployed for 14 day intervals at apple orchards in North Carolina in 2019 and 2020.

Species	Gallery tissue – Foundress species <sup>4</sup>					All galleries	Non-gallery <sup>3</sup>
	Aborted <sup>1</sup>	Unknown <sup>2</sup>	<i>C. m</i>	<i>X. c</i>	<i>X. g</i>		
<i>Alternaria alstroemeriae</i>	2.8	3.7	9.2	2.7	3.8	3.9	2.9
<i>Alternaria sp.</i>	2.8	-	2.8	-	0.3	1.2	1.7
<i>Ambrosiella beaveri</i>	2.8	8.8	18.3	21.5	0.7	10.4	1.1
<i>Ambrosiella grosmaniae</i>	2.8	11.8	0.9	7.7	27.1	10.1	5.2
<i>Ambrosiella roeperi</i>	5.6	12.7	2.2	1.9	6.3	5.7	2
<i>Boeremia exigua</i>	-	-	-	1.6	1.4	0.6	0.6
<i>Botryosphaeria dothidea</i>	5.6	2.5	0.9	4.2	1.4	2.9	0.6
<i>Cladosporium pini-ponderosae</i>	-	0.5	0.9	1.1	1	0.7	0
<i>Clonostachys rosea</i>	-	0.5	-	1.3	-	0.4	0
<i>Colletotrichum fioriniae</i>	-	0.7	0.9	0.5	0.3	0.5	0.3
<i>Diaporthe alnea</i>	2.8	4.4	-	1.1	0.3	1.7	0
<i>Diaporthe celeris</i>	-	0.2	-	-	0.3	0.1	0.9
<i>Diaporthe eres</i>	5.6	4.6	7.3	3.2	1	4.3	1.1
<i>Didymella prosopidis</i>	-	1.6	6.4	2.7	2.8	2.7	1.4
<i>Didymella rosea</i>	2.8	0.7	2.8	0.3	1.4	1.6	0.9
<i>Didymella sp.</i>	-	0.2	-	0.3	1.4	0.4	1.1
<i>Diplodia seriata</i>	-	0.9	-	1.1	0.7	0.5	2.9
<i>Epicoccum italicum</i>	-	0.2	0.9	0.3	0.3	0.3	0.3
<i>Epicoccum phragmospora</i>	-	4.9	1.8	2.1	0.7	1.9	2
<i>Epicoccum thailandicum</i>	5.6	-	0.9	0.8	-	1.5	0
<i>Fusarium guttiforme</i>	5.6	1.4	-	1.3	1	1.9	3.2
<i>Fusarium latertium</i>	-	0.7	0.9	0.5	0.3	0.5	0.6
<i>Fusarium nurragi</i>	-	1.2	-	0.8	-	0.4	0
<i>Fusarium perseae</i>	-	-	-	-	-	0.0	0.6
<i>Fusarium petroliphilum</i>	-	0.7	-	-	-	0.1	0.3
<i>Fusarium solani</i>	11.1	5.8	0.9	5.3	13.5	7.3	4.9
<i>Fusarium solani complex</i>	2.8	3.5	-	2.4	2.8	2.3	3.2
<i>Fusarium trincinctum</i>	2.8	5.1	2.8	2.7	0.7	2.8	1.1
<i>Geosmithia proliferans</i>	-	0.5	-	0.5	-	0.2	0.9
<i>Geotrichum silvicola</i>	5.6	3.5	-	0.8	3.8	2.7	3.2
<i>Neopestalotiopsis formicarum</i>	-	0.9	0.9	-	0.3	0.4	7.8
<i>Neosetophoma rosigena</i>	-	-	0.9	0.8	0.3	0.4	0.6
<i>Paraconiothyrium brasiliense</i>	2.8	3.2	2.8	4	5.6	3.7	7.2
<i>Penicillium sp.</i>	2.8	3.7	4.6	2.7	5.6	3.9	8.6
<i>Pestalotiopsis grevilleae</i>	2.8	0.2	0.9	-	-	0.8	2.6
<i>Pestalotiopsis scoparia</i>	-	1.6	0.9	1.3	-	0.8	1.1
<i>Pestalotiopsis telopeae</i>	-	0.5	-	-	-	0.1	4.3
<i>Phaeophlebiopsis ignerii</i>	-	1.2	-	0.3	-	0.3	0.3
<i>Phaeosphaeria phoenicicola</i>	-	0.2	-	0.5	0.7	0.3	0.3
<i>Pyrenophora nisikadoi</i>	-	-	-	0.3	-	0.1	1.1
<i>Trichoderma hamatum</i>	-	0.9	0.9	-	1.4	0.6	2
<i>Trichoderma scalesiae</i>	-	-	-	-	-	0.0	1.4
<i>Trichoderma simmonsii</i>	-	-	-	-	-	0.0	1.1
<i>Trichoderma sp.</i>	-	-	-	-	-	0.0	1.7
Unknown	13.9	3.5	7.3	7.4	5.9	7.6	5.7
yeast	5.6	0.7	-	1.9	4.9	2.6	2.9
Samples (n)	19	228	48	190	114	599	146

<sup>1</sup> Short (>5mm) or abandoned gallery with no foundress or signs of fungi. <sup>2</sup> Gallery which foundress was damaged, missing, or unable to be identified. <sup>3</sup> Tissue samples from >5cm from nearest ambrosia beetle gallery. <sup>4</sup> Gallery tissue from different foundress beetles: *C.m* = *Cnestus mutilatus*, *X.c* = *Xylosandrus crassiusculus*, *X.g* = *Xylosandrus germanus*.

Table 3.4. Frequency (% of samples) of fungal species isolated from exterior of ambrosia beetles trapped at apple orchards during peak flight periods (April-July) in Henderson County NC, 2020-2022.

Species <sup>1</sup>	<i>C. mutilatus</i>	<i>X. crassiusculus</i>	<i>X. germanus</i>
<i>Acremonium</i> sp.	-	2.9	2.6
<i>Alternaria</i> sp.	2.9	7.1	15.8
<i>Ambrosiella beaveri</i>	35.3	11.6	-
<i>Ambrosiella grosmanii</i>	-	5.8	15.8
<i>Ambrosiella roeperi</i>	-	43.5	-
<i>Aureobasidium melanogenum</i>	-	1.4	-
<i>Aureobasidium namibiae</i>	-	5.8	5.3
<i>Aureobasidium pullulans</i>	32.4	11.6	5.3
<i>Ceratocystiopsis collifera</i>	-	2.9	-
<i>Chaetomium</i> sp.	2.9	-	-
<i>Cladosporium cladosporioides</i>	14.8	8.7	15.8
<i>Cladosporium crousii</i>	-	11.6	26.3
<i>Cladosporium pini-ponderosae</i>	-	1.4	1.5
<i>Claussenomyces aff. atrovirens</i>	-	1.4	-
<i>Colletotrichum</i> sp.	2.9	2.8	2.6
<i>Diaporthe celeris</i>	-	-	2.6
<i>Diaporthe ellipsoidea</i>	-	1.4	-
<i>Diaporthe eres</i>	-	1.4	-
<i>Didymella pinodes</i>	5.9	-	-
<i>Didymella rosea</i>	-	1.4	-
<i>Diplodia quercivora</i>	2.9	-	-
<i>Diplodia seriata</i>	-	1.4	-
<i>Epicoccum italicum</i>	-	-	5.3
<i>Epicoccum nigrum</i>	26.5	8.7	-
<i>Fusarium armeniacum</i>	-	-	2.6
<i>Fusarium equiseti</i>	-	1.4	5.3
<i>Fusarium incarnatum-equiseti</i> <i>species complex</i>	-	1.4	5.3
<i>Fusarium solani</i>	-	7.2	18.4
<i>Fusarium solani species complex</i>	-	1.4	5.2
<i>Geosmithia omnicola</i>	-	1.4	-
<i>Geosmithia pulvereana</i>	2.9	8.7	-
<i>Geotrichum silvicola</i>	-	1.4	2.6
<i>Hyphopichia pseudoburtonii</i>	-	2.9	-
<i>Lecanicillium saksenae</i>	-	-	2.6
<i>Meira nashicola</i>	-	2.9	5.3
<i>Microcera rubra</i>	-	1.4	2.6
<i>Neocucurbitaria quercina</i>	-	1.4	-
<i>Neopestalotiopsis formicarum</i>	-	-	2.6
<i>Neosetophoma poaceicola</i>	-	1.4	-
<i>Oidiodendron fuscum</i>	-	1.4	5.3
<i>Paraconiothyrium bishopiae</i>	-	-	2.6
<i>Penicillium</i> sp.	-	4.2	5.3
<i>Periconia homothallica</i>	-	-	2.6
<i>Phaeoacremonium scolyti</i>	-	4.3	-
<i>Phaeophlebiopsis ignerii</i>	-	2.9	-
<i>Phialemoniopsis curvata</i>	-	1.4	2.6
<i>Pichia membranifaciens</i>	-	1.4	-
<i>Ramularia alangiicola</i>	-	1.4	-

Table 3.4. (Continued)

<i>Sarocladium strictum</i>	-	2.9	-
<i>Sporothrix rossii</i>	-	2.9	7.9
<i>Thyronectria sp.</i>	-	1.4	2.6
<i>Trichoderma atroviride</i>	5.9	4.3	1.5
<i>Trichoderma sp.</i>	14.8	2.9	5.3
Unknown	8.8	21.7	13.2
<i>Valsa ceratophora</i>	2.9	-	-
<i>Vexillomyces palatinus</i>	-	1.4	-
<i>Xenoacremonium sp.</i>	-	1.4	2.6
Samples (n)	34	69	38

<sup>1</sup> Identification based on ITS sequences of representative isolates of morphotype groups subjected to BLAST search.

Table 3.5. Frequency (% occurrence within sample type) of fungal species recovered from dormant trees at commercial apple orchards in Henderson Co., NC in 2022.

Species	Decayed Wood	Graft Union	Pruning Wound	Stem Canker	All tissue
<i>Alternaria alternata</i>	2.8	-	19.2	2	15.3
<i>Alternaria sp.</i>	25	4.9	19.2	15.6	15.8
<i>Aureobasidium pullulans</i>	-	-	1.4	2.2	1.1
<i>Biscogniauxia atropunctata</i>	-	-	4.1	-	1.6
<i>Botryosphaeria dothidea</i>	-	17.1	5.5	17.8	1.4
<i>Cladosporium sp.</i>	-	-	2.7	-	1.1
<i>Cytospora leucostoma</i>	-	-	-	2.2	0.5
<i>Diaporthe eres</i>	2.8	12.2	2.7	6.7	8.2
<i>Diaporthe sp.</i>	41.7	31.7	42.5	31.1	37.2
<i>Diplodia seriata</i>	12.5	24.4	8.2	8.9	12.6
<i>Epicoccum nigrum</i>	8.3	2.4	17.8	4.4	9.8
<i>Fusarium armeniacum</i>	12.5	12.2	12.3	4.4	1.4
<i>Fusarium avenaceum</i>	8.3	-	1.4	-	1.6
<i>Fusarium citri</i>	-	-	1.4	-	0.5
<i>Fusarium equiseti</i>	-	-	1.4	-	0.5
<i>Fusarium fujikuroi</i>	4.2	2.4	4.1	2.2	3.3
<i>Fusarium graminearum</i>	8.3	7.3	6.8	4.4	6.6
<i>Fusarium incarnatum-equiseti species complex</i>	4.2	2.4	-	-	1.1
<i>Fusarium ipomoeae</i>	-	7.3	-	-	1.6
<i>Fusarium liriodendri</i>	12.5	7.3	2.7	-	4.4
<i>Fusarium solani</i>	4.2	-	-	4.4	1.6
<i>Fusarium solani species complex</i>	-	-	1.4	-	0.5
<i>Gibberella avenacea</i>	16.7	12.2	6.8	6.7	9.3
<i>Leotiomyces</i>	-	-	-	2.2	0.5
<i>Neopestalotiopsis australis</i>	-	-	1.4	-	0.5
<i>Neopestalotiopsis clavispora</i>	8.3	14.6	2.7	15.6	9.3
<i>Neopestalotiopsis sp.</i>	-	7.3	9.6	13.3	8.7
<i>Nigrospora oryzae</i>	-	2.4	4.1	2.2	2.7
<i>Nigrospora sp.</i>	-	2.4	6.8	4.4	4.3
<i>Nigrospora sphaerica</i>	-	-	2.7	-	1.1
<i>Paraconiothyrium brasiliense</i>	33.3	12.2	5.7	64.4	43.2
<i>Peniophora sp.</i>	-	-	2.8	-	1.0
<i>Peniophora pseudoversicolor</i>	-	7.3	8.2	-	4.9
<i>Pithomyces chartarum</i>	-	2.4	-	-	0.5
<i>Rosellinia sp.</i>	-	-	2.8	4.4	2.1
<i>Schizophyllum commune</i>	4.2	2.4	4.1	2.2	3.3
<i>Setomelanomma sp.</i>	-	-	-	2.2	0.5
<i>Stereum complicatum</i>	4.2	-	-	-	0.5
<i>Trichoderma atroviride</i>	58.3	9.8	15.1	15.6	19.7
<i>Trichoderma citrinoviride</i>	-	-	4.1	-	1.6
<i>Trichoderma citrinoviride</i>	-	-	6.8	2.2	3.3
<i>Trichoderma deliquescens</i>	-	-	1.4	-	0.5
<i>Trichoderma harzianum</i>	4.2	9.8	5.5	4.4	6
<i>Trichoderma sp.</i>	-	4.9	-	-	1.1
<i>Trichoderma virens</i>	4.2	12.2	2.7	-	4.4
<i>Xylaria sp.</i>	-	-	2.8	2.2	1.5
<i>Unknown</i>	12.5	4.9	13.7	13.3	11.5

<sup>1</sup>Identification based on ITS sequences of representative isolates of morphotype groups subjected to BLAST search.

Table 3.6. Average length ( $\pm$  SE) of lesion (necrotic tissue) on detached apple shoots inoculated with fungi isolated from ambrosia beetle galleries on apple trees. Summer 2020.

Genus	isolate	Species	lesion length (mm)
<i>Ambrosiella</i>	20ET-CQ.1	<i>A. grosmanii</i>	5.3 $\pm$ 1.6e
	20L-EB.2	<i>A. roeperi</i>	14 $\pm$ 5.2cde
	20L-EM.1	<i>A. roeperi</i>	35.6 $\pm$ 12.7abcd
<i>Botryosphaeria</i>	17RAD-105	<i>B. dothidea</i>	98.1 $\pm$ 13.3a
	17RAD-122	<i>B. dothidea</i>	65 $\pm$ 8.5abc
	17RAD-19	<i>B. dothidea</i>	74 $\pm$ 9.1ab
<i>Fusarium</i>	20L-FQ.1	<i>F. aberrans</i>	15.1 $\pm$ 5bcde
	17RAD-11	<i>F. acuminatum</i>	22.2 $\pm$ 5.7abcd
	17RAD-12	<i>F. armeniacum</i>	53.1 $\pm$ 8.8abcd
	20ET-FR.2	<i>F. armeniacum</i>	58.8 $\pm$ 12.1abc
	17RAD-100	<i>F. armeniacum</i>	66.5 $\pm$ 8.8abc
	17RAD-33	<i>F. equiseti</i>	28.3 $\pm$ 11.2abcd
	17RAD-17	<i>F. fujikuroi</i>	52.1 $\pm$ 20.1abcd
	20L-FO.1	<i>F. liriodendri</i>	32.4 $\pm$ 8.7abcd
	20L-FO.2	<i>F. liriodendri</i>	21.7 $\pm$ 4.1abcd
	17RAD-13	<i>F. oxysporum</i>	51.9 $\pm$ 8abc
	17RAD-1	<i>F. oxysporum</i>	19.7 $\pm$ 7.4cde
	17RAD-53	<i>F. solani</i>	24.3 $\pm$ 7.3abcd
	17RAD-6	<i>F. solani</i>	29.4 $\pm$ 5.4abcd
	20ET-CD.2	<i>F. solani</i>	11.5 $\pm$ 1.3cde
	17RAD-14	<i>F. sporotrichioides</i>	48.9 $\pm$ 6.5abc
17RAD-9	<i>F. subglutinans</i>	18.5 $\pm$ 8.3de	
<i>Neofusicoccom</i>	17RAD-43	<i>N. parvum</i>	60.8 $\pm$ 21.7abcd
CONTROL		PDA-control	9.4 $\pm$ 3.1de

Mean lesion length of each isolate with the same letter within columns not significantly different using Tukey's HSD  $F_{=26,196}$  5.26,  $P < 0.0001$ .

Table 3.7. Mean ( $\pm$  SE) length of lesion (necrotic tissue) on detached apple shoots inoculated with fungi isolated from ambrosia beetle galleries on apple. Winter 2022.

Genus/complex	Isolate	Species	Necrotic length (mm)		
			Winter	Summer	Seedling
<i>Ambrosiella</i>	20ET-AU.4	<i>A. beaveri</i>	15.31 $\pm$ 2.64cd	--	--
	20ET-FZ.1	<i>A. beaveri</i>	16.02 $\pm$ 1.83cd	10.37 $\pm$ 0.98bcd	2.82 $\pm$ 0.2fg
	LCG-XG1.F1	<i>A. grosmaniae</i>	--	9.89 $\pm$ 6.72cd	8.48 $\pm$ 1.88def
	20ET-CQ.2	<i>A. grosmaniae</i>	37.94 $\pm$ 16.68dc	35.9 $\pm$ 14.15abcd	10.37 $\pm$ 1.75de
	20ET-CN.2	<i>A. roeperi</i>	19.23 $\pm$ 4.49dc	14.08 $\pm$ 4.97bcd	7.56 $\pm$ 1.18def
	20ET-FB.1	<i>A. roeperi</i>	12.61 $\pm$ 1.09d	--	--
	20L-EB.2	<i>A. roeperi</i>	21.8 $\pm$ 5.95dc	31.96 $\pm$ 8.12abcd	6.88 $\pm$ 2.47ef
<i>Botryosphaeria</i>	19SE-B.1	<i>B. dothidea</i>	72.33 $\pm$ 14.34ab	44.64 $\pm$ 11.25abcd	16.85 $\pm$ 5.25cde
	20EB-A.1	<i>B. dothidea</i>	95.21 $\pm$ 13.96a	103.56 $\pm$ 69.1a	7.57 $\pm$ 0.99def
<i>Diplodia</i>	20ET-B.1	<i>D. seriata</i>	16.26 $\pm$ 1.65dc	25.36 $\pm$ 6.34abcd	8.42 $\pm$ 0.99e
	20ET-B.2	<i>D. seriata</i>	17.5 $\pm$ 2.93dc	31.41 $\pm$ 9.81abcd	7.35 $\pm$ 0.71def
<i>Fusarium solani</i> complex	20L-FO.1	<i>F. liriodendri</i>	26.68 $\pm$ 4.35bc	57.44 $\pm$ 19.13abc	41.78 $\pm$ 3.47b
	20EB-GA.1	<i>F. petrophilum</i>	12.44 $\pm$ 0.95d	11.52 $\pm$ 1.19bcd	14.41 $\pm$ 2.18de
	20EB-E.1	<i>F. solani</i>	14.97 $\pm$ 0.95dc	16.76 $\pm$ 2.2abcd	18.01 $\pm$ 2.98cd
	20EB-E.2	<i>F. solani</i>	15.02 $\pm$ 0.6dc	7.22 $\pm$ 4.33cd	--
	20ET-CD.1	<i>F. solani</i>	13.79 $\pm$ 1.87dc	7.37 $\pm$ 2.71cd	79.17 $\pm$ 9a
	20ET-CZ.1	<i>F. solani</i>	14.63 $\pm$ 1.03dc	10.58 $\pm$ 1.54bcd	14.24 $\pm$ 1.4cd
	21-L3.B	<i>F. solani</i>	14.67 $\pm$ 2.04dc	16.9 $\pm$ 2.61abcd	32.83 $\pm$ 7.3c
<i>Fusarium incarnatum-equiseti</i> complex	20L-FQ.1	<i>F. aberrans</i>	14.86 $\pm$ 0.75dc	12.7 $\pm$ 3.79bcd	11.6 $\pm$ 1.03cde
<i>Fusarium sambucinum</i> complex	20ET-FR.2	<i>F. armeniacum</i>	38.18 $\pm$ 9.04ab	64.19 $\pm$ 16.02ab	122.99 $\pm$ 16.06a
<i>Fusarium trincinctum</i> complex	19ET-AH.1	<i>F. trincinctum</i>	15.72 $\pm$ 1.52dc	--	--
	20ET-AH.1	<i>F. trincinctum</i>	15.88 $\pm$ 1.18dc	27.02 $\pm$ 6.56abcd	12.87 $\pm$ 1.17cd
	20ET-AH.3	<i>F. trincinctum</i>	13.44 $\pm$ 0.53dc	--	--
<i>Paraconothyrium</i>	20ET-DJ.1	<i>P. brassilense</i>	12.31 $\pm$ 1.54dc	11.32 $\pm$ 0.84bcd	--
	20ET-FK.1	<i>P. brassilense</i>	15.72 $\pm$ 1.37dc	14.64 $\pm$ 1.88bcd	--
CONTROL	Control	PDA control	12.41 $\pm$ 0.29d	11.36 $\pm$ 0.98d	1.31 $\pm$ 0.12g
		<i>F</i> value	11.63	3.45	27.49
		<i>df</i>	23,192	21,65	19,98
		<i>P</i> value	<0.0001	<0.0001	<0.0001

Mean lesion length of each isolate with the same letter within columns not significantly different using Tukey's HSD.

Table 3.8. Mean ( $\pm$  SE) EC<sub>50</sub> (% EtOH for 50% reduction in mycelial growth) and mean ( $\pm$  SE) growth rate on non-amended PDA and MEA of various fungi isolated from ambrosia beetle galleries.

Isolate	Species	EC <sub>50</sub> (EtOH conc. %)	Mean growth rate (mm/day)	
			PDA	MEA
20ET-FZ.1	<i>Ambrosiella beaveri</i>	3.77 $\pm$ 0.13ab	8.93 $\pm$ 0.37	10.24 $\pm$ 0.14
20ET-CQ.2	<i>Ambrosiella grosmanii</i>	3.50 $\pm$ 0.5abc	2.51 $\pm$ 0.05	2.51 $\pm$ 0.01
NC23-F1.2	<i>Ambrosiella beaveri</i>	4.02 $\pm$ 0.27a	9.90 $\pm$ 0.06	10.24 $\pm$ 0.14
NC25.F1	<i>Ambrosiella hartigii</i>	3.4 $\pm$ 0.10abc	5.55 $\pm$ 0.08	4.74 $\pm$ 0.22
20ET-CN.2	<i>Ambrosiella roeperi</i>	3.48 $\pm$ 0.29abc	8.93 $\pm$ 0.29	7.87 $\pm$ 0.72
20L-EB.2	<i>Ambrosiella roeperi</i>	2.94 $\pm$ 0.24bcd	2.38 $\pm$ 0.15	2.13 $\pm$ 0.07
20EB-A.1	<i>Botryosphaeria dothidea</i>	2.93 $\pm$ 0.02bcd	8.38 $\pm$ 1.67	10.11 $\pm$ 0.07
20ET-B.1	<i>Diplodia seriata</i>	2.21 $\pm$ 0.23de	8.48 $\pm$ 0.46	6.61 $\pm$ 0.17
20ET-FR.2	<i>Fusarium armeniacum</i>	2.48 $\pm$ 0.16cde	5.41 $\pm$ 0.13	2.33 $\pm$ 0.03
20EB-E.1	<i>Fusarium solani</i>	3.34 $\pm$ 0.1abc	10.11 $\pm$ 0.11	10.16 $\pm$ 0.08
20ET-CZ.1	<i>Fusarium solani</i>	1.97 $\pm$ 0.28de	8.34 $\pm$ 1.78	10.11 $\pm$ 0.07
20ET-HM.1	<i>Trichoderma atroviride</i>	2.18 $\pm$ 0.16de	5.51 $\pm$ 0.63	4.64 $\pm$ 0.17
20ET-EZ.2	<i>Trichoderma hamatum</i>	2.56 $\pm$ 0.13cd	4.82 $\pm$ 0.80	1.88 $\pm$ 0.17
20ET-HK.1	<i>Trichoderma simmonsii</i>	1.59 $\pm$ 0.10e	6.51 $\pm$ 0.28	6.82 $\pm$ 0.23

Mean EC<sub>50</sub> of each isolate with the same letter within columns not significantly different using Tukey's HSD,  $F_{13,28}=10.66$ ,  $P < 0.001$ .

Table 3.9. Inhibition of symbiont and non-symbiont fungal isolates in dual plate antagonism assays conducted on MEA and MEA + 2.5% ethanol.

Symbiont Isolate	Pathogen Isolate	Inhibition of symbiont (%)		T-test	P value <sup>1</sup>	Inhibition of non-symbiont (%)		T-test	P value <sup>1</sup>
		MEA	MEA + 2.5%			MEA	MEA + 2.5%		
NC23-F1.2	<i>F. armeniacum</i>	18.6 ±4.1	22.1 ±5.8	0.50	0.626	-0.4 ±6.5	2.8 ±5.8	0.37	0.720
<i>A. grosmanniae</i>	<i>F. solani</i>	2.4 ±4	11.7 ±4.1	1.62	0.136	-7.8 ±13.2	4.7 ±8.9	0.78	0.452
	<i>B. dothidea</i>	12.8 ±8.2	4.6 ±7.4	-0.74	0.475	18.5 ±5.8	-0.7 ±12.1	-1.43	0.183
	<i>T. atroviridae</i>	39.9 ±13.4	42.8 ±9.2	0.18	0.862	13.2 ±6.7	-32.3 ±10.4	-3.68	0.0042
	<i>T. hamatum</i>	37.4 ±16.1	30.2 ±6.8	-0.41	0.695	13.3 ±5.7	-22.3 ±6.1	-4.25	0.002
20ET-FZ.1	<i>F. armeniacum</i>	20.5 ±5.4	25.4 ±2.2	0.85	0.416	7 ±3.2	-8.3 ±8	-1.77	0.106
<i>A. beaveri</i>	<i>F. solani</i>	7.2 ±5.1	18.6 ±6.5	1.37	0.202	14.8 ±1.2	5.1 ±8.6	-1.13	0.284
	<i>B. dothidea</i>	18.9 ±5.5	23.4 ±10.8	0.37	0.71	17.9 ±2.1	-4.8 ±3.8	-5.24	0.0004
	<i>T. atroviridae</i>	46 ±11.8	49.2 ±11.1	0.20	0.848	21.3 ±3.3	-15.9 ±12.3	-2.92	0.015
	<i>T. hamatum</i>	45.3 ±12.1	28.4 ±7.8	-1.17	0.2678	21 ±3.3	-10.5 ±7.9	-3.68	0.0042
NC25.F1	<i>F. armeniacum</i>	28.8 ±1.5	23 ±1.5	-2.78	0.19	21.4 ±3.4	9.2 ±2.3	-2.99	0.014
<i>A. grosmanniae</i>	<i>F. solani</i>	7.3 ±7.9	-1.1 ±4.4	-0.93	0.376	19.7 ±4.4	4.6 ±6.1	-2.02	0.071
	<i>B. dothidea</i>	22.2 ±11	9.7 ±8.4	-0.90	0.388	29.8 ±2.8	10 ±9.1	-2.08	0.064
	<i>T. atroviridae</i>	49.3 ±10.5	32.0 ±4.8	-1.50	0.164	30.1 ±2.8	16.3 ±18.2	-0.75	0.472
	<i>T. hamatum</i>	38.7 ±8.7	20.2 ±6.2	-1.73	0.115	28.3 ±2.4	10.1 ±8.6	-2.04	0.069
20ET-CQ.2	<i>F. armeniacum</i>	38.9 ±3.9	23.1 ±5.7	-2.29	0.045	20.5 ±4.3	18.6 ±8.8	-0.20	0.845
<i>A. grosmanniae</i>	<i>F. solani</i>	5.1 ±13.2	-5.1 ±6.6	-0.69	0.506	14.8 ±1.7	9.4 ±7.4	-0.72	0.490
	<i>B. dothidea</i>	18.6 ±12.9	10.8 ±10.5	-0.47	0.647	27.3 ±2.7	-4.4 ±2.8	-8.10	<0.0001
	<i>T. atroviridae</i>	47.2 ±13.1	39.1 ±6.4	-0.56	0.591	24.8 ±2.4	-16.4 ±6.8	-5.70	0.0002
	<i>T. hamatum</i>	38.5 ±13.2	23.6 ±6.4	-1.02	0.332	30.9 ±3.5	-20.5 ±8.6	-5.55	0.0002

<sup>1</sup> Significant effect of ethanol content (0 or 2.5%) in MEA on % inhibition of symbiont or pathogen compared with single inoculation using unpaired t-test (n=6).

### Chapter 3 Figures.

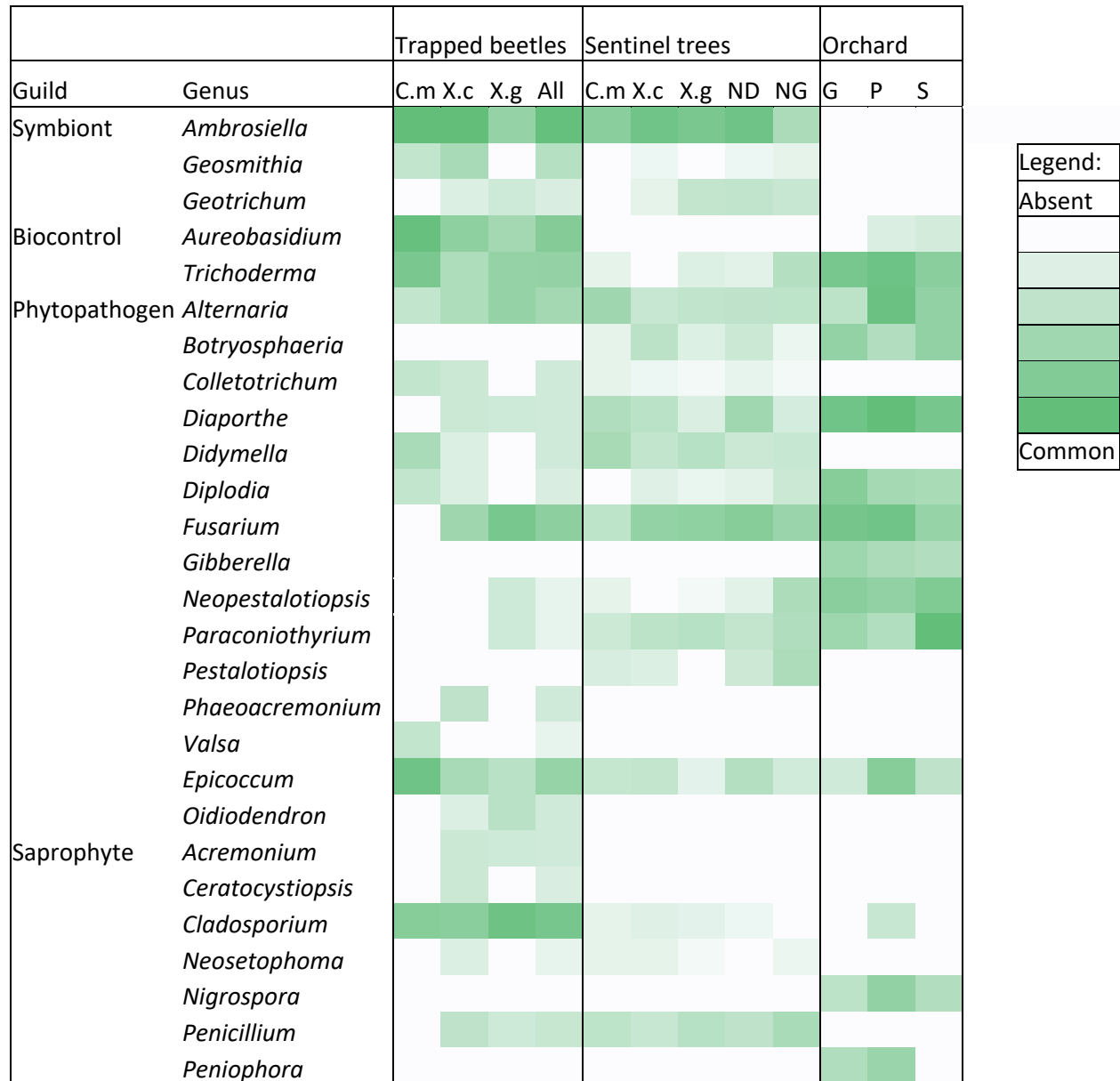


Fig 3-1. Relative frequency of occurrence of fungi by genus isolated from galleries of Xyleborine ambrosia beetles on apple, exterior of Xyleborine ambrosia beetles (C.m = *Cnestus mutilatus*, X.c = *X. crassiusculus*, X.g = *X. germanus*, ND = gallery foundress not identified, NG = non-gallery tissue from sentinel trees), and from non-attacked apple trees at commercial orchards (G = graft union tissue, P = pruning wounds, S = small stem cankers). Darker shades of green indicate higher relative occurrence within each column.

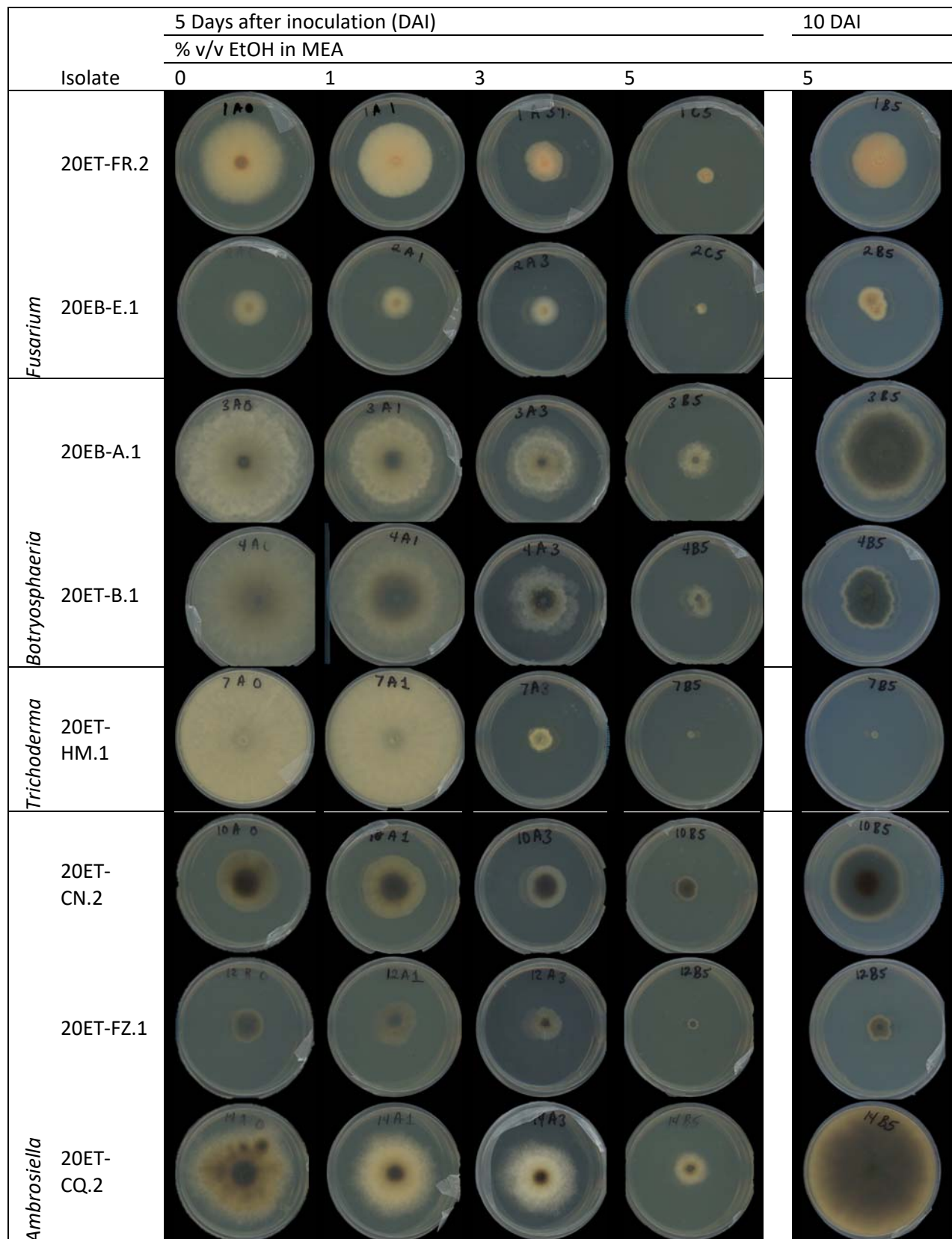


Fig. 3-2. Selected isolates grown on MEA amended with 0-5% ethanol 5 and 10 days after inoculation (DAI)

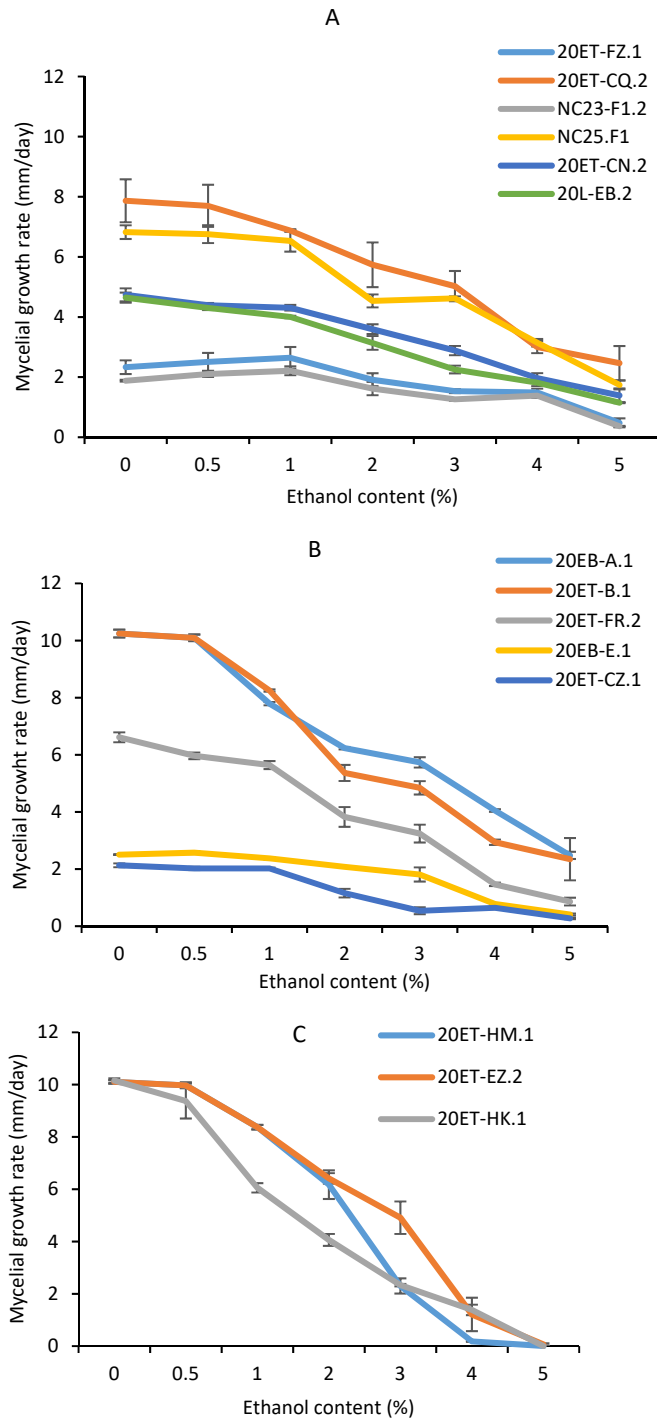


Fig. 3-3. Mean mycelial growth rate (mm/day) of isolates grown on MEA amended with 0-5% Ethanol. (A) *Ambrosiella* spp. (B) *Botryosphaeria* spp. and *Fusarium* spp., and (C) *Trichoderma* spp. Error bars represent  $\pm$  SEM.

## Supplementary information

SI Table 1. Location information for sites sampled for fungi on trapped ambrosia beetles in 2020-2022 and apple orchard fungal survey on non-attacked trees 2022.

<b>Orchard ID</b>	<b>GPS coordinates</b>	<b>Cultivar (rootstock)</b>
RS	35.429260, -82.561736	Gala (M.9-RN29)
NR	35.398746, -82.353587	Red Delicious & Fuji (M9-T337)
TE	35.371614, -82.337566	Honey crisp (M.9)
KB	35.368067, -82.336477	Honey crisp (M.9)
NG	35.407795, -82.358795	Gala (M.9)

## Chapter 4 Fire blight caused by *Erwinia amylovora* Infection Can Attract Ambrosia Beetles to Apple Trees.

### **Abstract**

The exotic ambrosia beetles, *Xylosandrus crassiusculus* and *X. germanus* (Coleoptera: Curculionidae: Scolytinae: Xyleborini) are attracted to physiologically stressed trees, with ethanol being the most important volatile attractant. A range of abiotic stressors and fungal infections have been shown to elicit ethanol emissions from affected trees. Fire blight, caused by the bacteria *Erwinia amylovora*, is a globally important disease of pome fruit. The bacteria can multiply and spread within the tree's vascular system, causing wilting and necrosis. *Xylosandrus* spp. ambrosia beetle attacks have been observed on apple trees expressing fire blight disease symptoms but the association and the mechanism of attraction have not been reported in the literature. These studies infected apple seedlings and trees with *E. amylovora* and measured tissue ethanol emission and ambrosia beetle attacks. Ethanol production increased with fire blight infection, but only in infected and necrotic tissue. Trees were also attractive to and led to entries by *X. crassiusculus* and *X. germanus*. However, ambrosia beetle attacks on *E. amylovora* infected trees were much lower (0.2-1.2 entries/tree) compared to flood-stressed trees (12.4-21.3 entries per tree), which were likely more attractive to ambrosia beetles due to greater ethanol production.

### **Key words:**

Fire blight, *Erwinia*, Ethanol, *Xylosandrus*, exotic ambrosia beetles, apple, decline.

## Introduction

The enterobacterium *Erwinia amylovora* is pathogenic on a number of Rosaceae hosts and is one of the most damaging plant pathogens causing fire blight disease of apple and pear throughout the world (Vrancken et al. 2013, Kharadi et al. 2021). Infections primarily occur via floral infection in the spring. The bacterial population initially grows epiphytically on stigmas and can invade the host through nectary openings. Shoot infections can occur through leaves, with young, new growth being the most susceptible to infection (Suleman and Steiner 1994). Once inside the host, the bacterium produces adhesive pili to adhere to plant surfaces. Simultaneously, it forms a protective biofilm containing exopolysaccharides (EPS) such as amylovoran, which protect the bacteria from host defenses and can be exuded from the host as 'ooze' that contains up to  $1 \times 10^9$  mL<sup>-1</sup> *E. amylovora* (Slack et al. 2017) and plays an important role in disease spread (Kharadi and Sundin 2021, Zeng et al. 2021). Virulence of *E. amylovora* is dependent on the type III secretion system that is used to inject effector proteins into host cells (Vrancken et al. 2013). These effectors manipulate the host's defense responses, suppressing salicylic acid-dependent and jasmonic acid signaling pathways while increasing ethylene production and induces cell death through oxidative bursts (Venisse et al. 2002, Iakimova et al. 2013). The hypersensitive response of susceptible hosts and associated reactive oxygen bursts have been associated with virulence of *E. amylovora* (Oh et al. 2005) as the bacteria are protected within biofilm matrix and may benefit from host cell death (Kharadi and Sundin 2021). The combination of changes to the hormonal balance, ROS damage, and impact of EPS production leads to severe necrosis and the development of characteristic fire blight symptoms, including wilting, blackening, and tissue necrosis (Kharadi et al. 2021). Disruption of the vascular system by EPS and collapse of the parenchyma, in addition to causing wilting and the distinctive 'shepherd's crook' symptom on shoot tips, facilitates systemic movement of the bacteria through the xylem (Kharadi and Sundin 2021).

Anecdotal reports and field observations have noted the association of ambrosia beetle attacks and fire blight infected trees. In New York, ambrosia beetle entries were found on apple trees with fire blight (Agnello et al. 2017) and *E. amylovora* was isolated from *X. germanus* taken from galleries on infected trees (Tancos et al. 2016). Following severe fire blight infestations in Ohio, Hall et al. (1982) found a high prevalence of *Xylosandrus germanus* entries on infected trees and postulated that rootstock and interstem infections could have been vectored by the beetle. Some historical accounts have proposed that fire blight infections on apple (Orton and Adams. 1915) and pear (Jones 1911) may have been transmitted by ambrosia beetles. However, this hypothesis was not tested and to our knowledge, no studies have confirmed that *Xylosandrus* spp. ambrosia beetles are attracted to apple trees with fire blight disease.

Ambrosia beetles from the tribe Xyleborini (Coleoptera: Curculionidae, Scolytinae) attack stressed and dying trees and are considered a problematic pest in eastern USA apple growing areas (Agnello et al. 2021, Gresham, Walgenbach, et al. 2023), nut orchards (Monterrosa et al. 2022) and ornamental nurseries (Ranger, Schultz, et al. 2015, Frank and Ranger 2016, Ranger et al. 2016). Ethanol is a ubiquitous attractant for a number of bark and ambrosia beetles and it has been shown as the key volatile attractant for inducing beetle colonization of stressed but living trees including apple (Ranger et al. 2010, 2018, Ranger, Tobin, et al. 2015). Tissue ethanol content has been shown to be elevated in response to a wide variety of stressors including freeze damage, air pollution, flood, and heat damage (Copolovici and Niinemets 2010, Ferner et al. 2012, Kelsey et al. 2016, Kelsey and Westlind 2017, Ranger et al. 2019). Therefore, host tissue ethanol content is a reliable indicator of vulnerability to attack by Xyleborine ambrosia beetles (Ranger, Tobin, et al. 2015).

Apple trees with fire blight are likely to have localized elevated levels of ethanol as a result of bacterial fermentation, in-planta anoxic conditions induced by vascular occlusion by EPS, and plant tissue necrosis in response to the disease. The occlusion of the vascular system resulting from fire blight

infections reduces oxygen content of the sap (Zamski et al. 2006), reduced internal oxygen supply increases ethanol production by plant cells (Gibbs and Greenway 2003). Furthermore, *Erwinia amylovora* is a facultative anaerobe, with ethanol being a major fermentation byproduct under anaerobic conditions (Sutton and Starr 1959, Haq and Dawes 1971, Cellini et al. 2018). Fungal and Oomycete pathogens have been shown to increase host ethanol concentrations and elicit ambrosia beetle attack (Kelsey et al. 2013, Adesso et al. 2018). The objectives of this study are to investigate the effect of advanced fire blight infection on ethanol content of affected apple seedlings and stems, and to characterize the effect of fire blight infection on attraction and colonization by ambrosia beetles.

## Methods

### General Methods and Plant material.

To evaluate the relative production in relation to progression of fire blight disease on apple, a laboratory-based analysis of ethanol using 4-8 month old apple seedlings was conducted. Seeds from 'Gala' apples were surface sterilized, stratified in at 4°C for 40-70 days until germination, after which they were planted into seed-raising mix (1:1 peat compost:vermiculite plus nutrients). Once seedlings reached the 2-4 true leaf stage they were transferred into 18 cell trays in potting soil and maintained in a greenhouse until the experiments commenced. Just prior to inoculation, seedlings were transferred to 75 mm diameter pots in potting media (peat compost, vermiculite). Regular watering, fertilizer and pest control was conducted as required.

Grafted apple trees purchased from commercial nurseries as bare-rooted one year old trees were used for field studies conducted in 2022 and 2023 to evaluate the effect of *E. amylovora* infection on ethanol production and attraction and colonization by ambrosia beetles. The 2022 field experiment was conducted on 2<sup>nd</sup> leaf 'Honey crisp' and 'Gala' trees, both cultivars were grafted on 'M.9' rootstock and were planted at the Mountain Horticultural Crops Research and Extension Center (MHCRC),

35.430044, -82.562088) in Mills River, NC during the spring of 2021. The experiment was repeated in 2023 using 2<sup>nd</sup> leaf 'Gala' grafted to 'G.41' rootstock grown in 26 L pots with fine ground bark based potting media that were deployed in a wooded area with expected high ambrosia beetle activity at MHCREC. In 2022, a field-cage choice assay experiment was conducted that used 1<sup>st</sup> leaf 'Aztec Fuji' grafted to 'M.9' rootstock planted into 26 L pots with fine ground bark based potting media.

#### **Preparation of *E. amylovora* cultures and inoculation.**

All inoculations of *Erwinia amylovora* were performed with the moderate to high virulent strain Ea110, which has a naturally-occurring mutation that confers resistance to the antibiotic rifampicin (Zhao et al. 2005). This strain allows for rapid selection for the *E. amylovora* strain used to inoculate trees through use of agar amended with rifampicin and Ea110 has moderate to high virulence on apple (Wang et al. 2010).

Inoculum was cultured for 13-16 hours in Luria-Bertani (LB, Difco) broth amended with 100 µg mL<sup>-1</sup> Rifampicin at 28°C on a shaker incubator at 200 RPM in the dark. Final inoculum was adjusted to 1 x 10<sup>6</sup> cfu/mL in 1x phosphate buffered saline (PBS) solution using aLKB Biochrom Ultrospec II spectrophotometer (optical density 600 nm) and the bacterial suspensions were stored on ice for a maximum of 4 hours before inoculations were conducted. Inoculations were performed using 'dip-and-snip' method (Singh et al. 2019). Briefly, sterile scissors were dipped into the inoculum solution then used to bisect the first three unfolded leaves per shoot, ensuring the mid-vein was exposed to inoculum. Inoculations were conducted in the evening and plastic bags were placed over inoculated shoots overnight to maintain high humidity and promote infection.

#### **Ethanol emissions from apple seedlings with fire blight.**

To evaluate the effect of fire blight disease progression on ethanol production in apple, repeated measurement of headspace volatiles were conducted on apple seedlings (6 inoculated

seedlings plus 3 mock-inoculated) 10-15 cm in height that were inoculated with  $1 \times 10^6$  cfu/mL Ea110 using dip and snip methods described above. The experiment was conducted February 2022 and repeated April 2022. Headspace analysis of volatiles was conducted 3, 7, 11, and 17 days after inoculation (DAI) in February and 7, 10, and 14 DAI in April. Just prior to measurement, disease severity was rated by measuring the length of necrotic tissue from the inoculation point and counting the number of symptomatic leaves per plant (Fig 1. b. & c.), then the pots were wrapped in Teflon film barrier to limit media and root-derived volatiles. Seedlings were placed inside a 4 L glass container which had been washed, rinsed, and pre-heated for 30 min at 40°C. A plastic screw-top lid with a rubber septum through which the SPME fiber was inserted was fitted over the glass container (Fig 1.a). The container with the seedling was placed in an oven set at 40°C for 20 min, then the SPME fiber was exposed and held in the oven for another 40 min. Following the absorption period, the SPME fiber was thermally desorbed at 225°C into the injection port of an Agilent 8860 gas chromatograph with a Flame Ionizing Detector (FID), (Agilent Technologies, Palo Alto, CA) with a Merlin Microseal and SPME-liner (0.75 mm × 6.35 mm × 78.5 mm, i.d. × o.d. × length; Restek, Bellefonte, Pennsylvania) under splitless mode. A DB-5ms column (30 m, 0.25 mm i.d., 0.25 µm thickness) with an initial temperature of 40°C was held for 2 min then ramped at 15°C/min to 225°C and held for 7 min with nitrogen as carrier gas set at 1.2 mL/min flow rate. The quantity of ethanol in the tissue was calculated based on an external standard curve ranging from 0 -1000 µg · L<sup>-1</sup> ethanol in water. For the standard curve, 50 µL of each concentration was pipetted onto a 30 mm diameter piece of Whatman filter paper placed inside the 4 L capacity jar subjected to the same conditions as plant samples.

#### **Host selection and colonization of apple trees infected with *E. amylovora* by *X. crassiusculus*.**

*Field cage choice assay.* An experiment in insect-proof field cages (1.8 m x 1.8 m x 1.8 m Bioquip field cages with mesh siding) was conducted to evaluate the effect of fire blight infection in apple on colonization by *X. crassiusculus* ambrosia beetles at MHCREC in July 2022. Inside of each of two cages, 6

potted 'Fuji' apple trees infected with *E. amylovora* Ea110 and 3 non-infected control trees were placed around the perimeter with non-inoculated and inoculated trees alternating. Infected trees were inoculated using the dip-and-snip method described above with  $1 \times 10^6$  cfu mL<sup>-1</sup> *E. amylovora* Ea110. Approximately 100 *X. crassiusculus* were introduced into each cage 5 weeks after inoculation, once disease symptoms were evident on the main trunk of inoculated trees. Beetles were from a laboratory colony.

Five days after beetle introduction, trees were removed from the cages to assess for beetle entries and disease symptoms. Stem sections were analyzed to determine *E. amylovora* population and ethanol content. Stem sections were taken from (1) the necrotic stem section, (2) at the transition zone where internal symptoms stopped, and (3) 15 cm below the symptom transition zone. The location of beetle entries was recorded in relation to disease symptoms and the inoculation point.

Analysis of the *E. amylovora* populations from stem tissue was conducted the same day as sampling. Each sample was cut into small (~0.5 mm) pieces using sterile pruning shears and razor blades then a subsample of approximately 300 µg of tissue was weighed and transferred to a sterile 1.7 mL microcentrifuge tube containing 0.9 mL sterile 1x Phosphate buffer (PBS). The tubes were sonicated for 7 minutes then serial diluted in 1 x PBS at 1:10 each dilution step to achieve a final dilution of 1:10<sup>4</sup>. Serial dilutions were drop-plated (3 x 25 µL) onto LB agar (Difco) plates amended with 50 µg mL<sup>-1</sup> cyclohexamide (Sigma Aldrich) and 100 µg mL<sup>-1</sup> rifampicin (Sigma Aldrich) and incubated at 28°C for 24-48 hours in the dark, after which colonies were counted. The resulting colony counts were used to determine the population of *E. amylovora* Ea110 within the tissue at different sampling locations within the stem tissue.

Tissue for ethanol analysis was taken from stem segments using a 5 mm steel hole punch. Each sample consisted of 4 subsamples that was immediately transferred to -20°C for storage. For analysis,

each sample was weighed then placed in a 2 mL glass vial and capped with a screw cap with a PTFE septum. The sample was incubated for 30 min at 90°C to denature enzymes and pre-heat the headspace (Manter & Kelsey, 2008). The sample was removed from the water bath, and a solid phase micro extraction (SPME) fiber (75 mm fiber (CAR/ PDMS; 75 µm coating; Sigma-Aldrich, St. Louis, Missouri) was immediately inserted through the septum and exposed to the headspace within the vial for 5 min at room temperature. The SPME fiber was removed and manually injected into an Agilent GC-FID using parameters described above.

The *E. amylovora* population, ethanol concentration, and disease data for trees that were attacked by ambrosia beetles was compared to results for non-attacked inoculated trees using ANOVA in SAS (V. 9.4, SAS Institute, Cary, NC).

*Field experiment 2022.* To evaluate the effect of fire blight disease on attraction and colonization of apple by Xyleborine ambrosia beetles under field conditions, a field experiment was conducted at the MHCREC in 2022 using second leaf apple trees (cv. Honeycrisp and Gale Gala on 'M.9' rootstock) spaced 1.2 m apart within rows which were on 4.2 m centers. The trees were planted in alternating groups of 3-trees of each cultivar two rows ~100 m long. For each cultivar, a randomized complete block design was laid out with two treatments, *E. amylovora* inoculated (Fire blight), and mock-inoculated control. Treatments were randomly assigned to a tree within each group of three trees, with 15 single tree replicates per cultivar. Trees in the fire blight treatment, were inoculated on 1 April 2022 with *E. amylovora* by dipping scissors into a solution of 10<sup>6</sup> CFU/mL strain Ea110 suspended in 1x phosphate buffer saline solution (PBS). Three shoots emerging from the scion and within the top 0.5 m of the tree were selected for inoculation. The first three leaves completely unfolded leaves on the distal end of each shoot were bisected with the inoculum-dipped scissors and bagged overnight to maintain high humidity. The untreated control trees were mock-inoculated in the same manner described above, with sterile PBS immediately before inoculations with *E. amylovora* were conducted. Inoculation timing targeted

peak beetle flight activity that usually occurs in May in NC (Gresham, Villani, et al. 2023). Disease symptoms were monitored every 3 to 4 days to evaluate success of inoculation and ideal timing for sampling to commence.

Destructive sampling for analysis of *E. amylovora* and ethanol concentration commenced after disease symptoms had progressed to the proximal end of the shoot on the majority of inoculated trees. On 24 and 31 May, and 6 June, 5 replicates from each cultivar were randomly selected for destructive sampling. Trees were initially inspected for ambrosia beetle entry and disease symptoms progression. The lowest point on the main trunk with visible symptoms of disease was marked and six samples were taken for analysis of *E. amylovora* and ethanol: i) the necrotic lesion area close to inoculation point, ii) at the transition of symptomatic and non-symptomatic tissue (0 cm), and iii) at 5, 10 and 30 cm below the symptom transition zone. Samples for *E. amylovora* quantification were processed immediately as described above, and tissue samples for EtOH quantification were stored at -20°C until analysis.

Population estimates for *E. amylovora* and were log transformed and the non-transformed EtOH concentrations were subjected to a generalized linear model using PROC GLM in SAS (V 9.4, SAS Institute, Cary, NC) to test the effect of sample date, cultivar, and tissue sample location and interactions. There were significant differences and significant interaction effects of sample date but no interaction effects of cultivar so data from each sample date were analyzed separately with cultivar as a blocking factor using PROC MIXED in SAS. To evaluate the effect of *E. amylovora* population on tissue ethanol content, samples with  $< 1 \times 10^3$  CFU g<sup>-1</sup> tissue were excluded and the remaining values across all samples were subjected to a Generalized Linear Model with Gamma distribution and Log link function (lowest AIC = 811, and BIC = 818).

*Field experiment 2023.* Another field experiment was conducted at the MHCREC in 2023 to evaluate the effect of fire blight disease on attack by ambrosia beetles. Potted 'Gala' trees grafted to G.

41 rootstocks were subjected to three treatments, (i) no stress (negative control), (ii) infected with *E. amylovora* Ea110, or (iii) flood-stressed (positive control).. The experiment was conducted twice and each consisted of 10 replicates with treatments arranged in randomized complete blocks with 1 m between trees within blocks and 5-10 m between blocks spread across two locations within wooded areas at the MHCREC in an area with a high occurrence of ambrosia beetles. Trees were deployed on 3 May and a new set of trees on 6 June 2023 for three weeks. Flood stress was initiated in the field when trees were deployed into the woods by placing the potted tree within a 38 L plastic pot lined with plastic contractor bag to retain water. Water was applied to the pot until media was saturated and standing water was at least 1 cm above the top of the media to ensure complete saturation of the roots which causes an anaerobic root zone, and was topped up weekly as needed. The fire blight treatment was inoculated 4 weeks before trees were deployed using the dip-and-snip method described above on three shoots per tree on the upper part of the tree (>1.6 m from ground) on actively growing shoots emerging from the main stem. Before inoculation, flowers from all trees were removed to prevent excessive disease spread.

Trees were collected 21 days after being deployed in the woods to assess for ambrosia beetle entries, and sample tissue for analysis of ethanol and *E. amylovora* population using methods similar to the 2022 experiment. Tissue samples for ethanol and *E. amylovora* population analysis were taken from various stem locations according to treatment: Control and flood-stressed trees, samples taken from 5 cm above the graft union (base), 0.7 m above graft union (middle), and 1.4 m above the graft union (top); for the fire blight treatment, samples were taken from necrotic tissue close to the inoculation point (lesion), 5 cm above the symptom boundary, 5 and 20 cm below symptoms, and at base of trunk (5 cm above graft union). Samples for *E. amylovora* population determination were analyzed the same day using methods described above, and samples for ethanol analysis were stored at -20°C until analysis on Agilent 8860 GC-FID using methods described above.

The number of ambrosia beetle entries at each height position on the tree (below graft union, lower trunk, mid-trunk, and top of tree) and total attacks per tree were subjected to ANOVA using Proc ANOVA in SAS. No entries were recorded on control trees and they were excluded from the analysis. The population of *E. amylovora* and ethanol concentration within tissue sampled at different height positions on the tree were log transformed and analyzed separately for each deployment date as a one-way ANOVA using PROC ANOVA in SAS.

## Results

### **Ethanol emissions from apple seedlings with fire blight.**

Apple seedlings inoculated with *E. amylovora* Ea110 emitted elevated ethanol after fire blight disease symptoms were visible on leaves. The headspace ethanol concentration increased for all infected apple seedlings over the observation period on the first experiment (Fig. 2.a) and the second experiment headspace ethanol concentration peaked at 7 days after inoculation (DAI), and tended to decrease from 7-14 DAI (Fig. 2.b). Low amounts or no ethanol was detected in headspace of mock-inoculated control seedlings over the same observation period.

### **Host selection and colonization of apple trees infected with *E. amylovora* by *X. crassiusculus*.**

*Field-cage choice assay.* Trees infected by *E. amylovora* were attacked by *X. crassiusculus* and no mock-inoculated control trees were attacked. Half of the 12 apple trees inoculated with *E. amylovora* and exposed to *X. crassiusculus* had beetle entries, with 14 entries in total across all trees (1-4 entries per tree) and neither Ea110 nor ethanol were detected on sampled tissue of control trees.

All beetle attacks on infected trees occurred 3.5 - 40.5 cm from the inoculation site on symptomatic tissue (Fig. 3). The average length of necrotic symptoms was significantly greater on trees that were attacked by *X. crassiusculus* compared with trees that were not attacked (Table 1). The mean

population of *E. amylovora* was similar for both attacked and non-attacked trees within the necrotic tissue, but at the symptomatic transition and 15 cm below symptoms, the populations of *E. amylovora* were significantly higher on attacked trees compared with inoculated trees that were not attacked by *X. crassiusculus* (Table 1). There were no significant differences in tissue ethanol concentration between the attacked and non-attacked inoculated trees, although attacked trees tended towards higher ethanol concentration on all tissue types (Table 1).

*Field experiment – 2022.* The majority of inoculated trees developed severe disease symptoms on the upper parts of the tree by 21 DAI. Initial assessments 7-14 DAI found limited evidence of disease spread beyond the inoculated leaves; necrosis on inoculated leaves increased from 7-14 DAI. From 21-66 DAI there was considerable progression of disease symptoms with evidence of bacterial ooze on inoculated shoots and rapid increase in leaf necrosis spreading beyond the inoculated leaves. There were high populations of Ea110 ( $>1 \times 10^6$  CFU/gm) on all necrotic lesion tissue sampled. Disease symptoms on the main stem were difficult to discern without partial dissection of the bark, which revealed discoloration of the cambium layer on infected wood. Tissue sampling revealed that *E. amylovora* spread up to 55 cm below the lowest inoculation point on 'Gala' and 85 cm on 'Honeycrisp' 66 DAI.

Ethanol content in tissue was highest on lesion tissue followed by symptomatic main-stem tissue (Fig. 4). Ethanol was also elevated on stem sections up to 20 cm below visible symptoms (0 cm) but were accompanied with detections of Ea110 at levels  $> 1 \times 10^3$  CFU g<sup>-1</sup> (Figs. 4 & 5). Ethanol concentration of tissue with  $1 \times 10^5$  CFU g<sup>-1</sup> (considered here as active infection) had ethanol concentrations on average over 10x that of tissue with less than  $1 \times 10^5$  CFU g<sup>-1</sup> (Fig. 5). For tissue samples with active fire blight infection, there was a significant positive effect of *E. amylovora* population on tissue ethanol content (95% CI 0.238, 0.447, Wald-Chi = 39.07,  $P < 0.0001$ ). Across all

samples, the mean ethanol content of tissue with no active infection was  $1.95 \pm 0.31$  mg EtOH g<sup>-1</sup> and across all infected tissue the average ethanol content was  $47.90 \pm 5.5$  mg EtOH g<sup>-1</sup>.

*Field Experiment 2023.* Potted apple trees that had been inoculated with *E. amylovora* were deployed in the field alongside potted trees that were subjected to flood stress and non-stressed control trees to evaluate the effect of fire blight disease on attraction of ambrosia beetle attack. In the first trial on 3 May, flood-stressed trees were attacked by ambrosia beetles with an average of 21.3 entries per tree (Table 2). Of the 99 entries containing a foundress, 65 were *X. crassiusculus* and 34 were *X. germanus*. Of the 10 fire blight diseased trees, only one successful ambrosia beetle entry occurred, which contained a *X. germanus* female. With 6 June deployment, all flood stressed trees were attacked by ambrosia beetles, with an average of 12.4 entries per tree (Table 2)/ Of the 47 identified foundresses, 39 were *X. crassiusculus*, 7 *X. germanus*, and one *Cnestus mutilates*. Entries were located predominantly (65%) on the lower portion of the trunk (< 0.7 m from soil line), 28% were located on the mid trunk (0.7-1.4 m) and only 7% of entries were found on the upper portion of the trees (> 1.4 m). Only 4 of the 10 inoculated trees were attacked by ambrosia beetles, with an average of 1.2 entries per tree and all entries were located on the top portion (> 1.4 m) of the tree, closest to the inoculation point (Table 3).

Analysis of the tissue ethanol content across samples showed there was minimal to no ethanol on control trees. Fire blight infected trees had elevated ethanol concentration on tissue close to the inoculation point that had active infection, but was not elevated on non-infected tissue 5-20 cm below symptoms or at the base of the tree (Fig 6). Ethanol concentration on flood stressed trees was elevated with a trend toward higher ethanol content at the base compared with the top of the tree (Fig. 7).

## Discussion

These studies showed that fire blight disease caused by *E. amylovora* can elicit ethanol production in infected apple tissue and can be attractive to ambrosia beetles. Ethanol production on inoculated trees was limited to infected tissue and not occur in non-*E. amylovora* infected tissue. The bacteria and elevated ethanol were measured at up to 30 cm below visible symptoms of disease (browning of inner bark and cambium). Although fire blight foliar disease symptoms are typically conspicuous, the bacteria are able to move systemically within the tree and cryptic infections have been found on apple (Aćimović et al. 2023). There have been some limited reports in the literature and anecdotal observations reported by growers and consultants associating ambrosia beetle attacks on apple trees with fire blight infections (Hall et al. 1982, Breth et al. 2016). Our experiments confirm that ethanol, a key volatile involved in attracting and eliciting burrowing by ambrosia Xyleborine ambrosia beetles, is elevated in tissue infected with *E. amylovora*.

Experiments conducted on apple seedlings and young apple trees inoculated with *E. amylovora* revealed that headspace ethanol content was associated with infection, and that there was a positive correlation between *E. amylovora* population and tissue ethanol content on fire blight infected 2-year old apple trees. Stem sections with high *E. amylovora* populations consistently had the highest ethanol content, and non-infected tissue below visible symptoms of disease had little to no detectable ethanol. *Erwinia amylovora* forms a thick biofilm within the xylem and intercellular spaces of infected plants, which limits sap flow through the vascular system (Kharadi and Sundin 2021). Virulent infection by *E. amylovora* also increases necrosis of infected tissue through upregulation of ethylene biosynthesis and corresponding increase in hypersensitive cell death associated with ROS bursts. Therefore, infected tissue is likely to be anaerobic due to the reduced xylem flow and high rate of bacterial growth (Gansert 2003, Zamski et al. 2006), which could increase ethanol production by *E. amylovora*, (Haq and Dawes 1971) and can lead to elevated ethanol synthesis in trees (Gansert 2003, Gibbs and Greenway 2003).

Analysis of liquid cultures of *E. amylovora in vitro* have been shown to emit ethanol (Sutton and Starr 1959, Haq and Dawes 1971, Cellini et al. 2018), although detection of ethanol have been inconsistent *in planta* (Spinelli et al. 2012, Cellini et al. 2016, 2018). Volatile profiles of apple plants infected with *E. amylovora* found a number of significant volatile compounds, but ethanol was not elevated (Spinelli et al. 2012, Cellini et al. 2016). In another study analyzing the volatiles associated with *E. amylovora* infected apple found high ethanol in liquid cultures of *E. amylovora*, but no increase in ethanol *in planta* as a result of fire blight disease compared with the control. (Cellini et al. 2018). However, the methods for volatile quantification used in these studies either focused on higher molecular weight compounds, which may have missed ethanol, or took measurements early in infection (<72 hours post inoculation) before significant necrosis or occlusion of xylem had occurred. Conversely, elevated ethanol in apple tissue infected with *E. amylovora* was observed in our study, but only after disease symptoms were apparent.

The ethanol content of tissue is a good indicator of risk of ambrosia beetle attack as it is a long and short range attractant for Xyleborine ambrosia beetles (Ranger, Tobin, et al. 2015). Several plant phytopathogens infection have been shown to increase tissue ethanol content and influence attraction of Xyleborine ambrosia beetle on coniferous and angiosperm trees (Kelsey and Joseph 1998, Kelsey et al. 2013, 2016, Rassati et al. 2020). Live oak, *Quercus agrifolia* infected with large cankers caused by *Phytophthora ramorum* were preferentially attacked by ambrosia beetles and had over 4 times higher ethanol concentration than trees with spot cankers; ethanol concentrations were highest within 5 cm of canker edge and lower on non-infected trunk sections (Kelsey et al. 2013). In our study, elevated ethanol appeared to be localized to infected tissue with little to no ethanol on non-infected tissue adjacent to diseased trunk sections. Rassati et al. (2020) found that Chestnut logs infected with *Cryphonectria parasitica* had a significant effect on the number of entries of *Anisandrus dispar*. The evidence suggests that ethanol accumulation is a ubiquitous response to tissue damage from multiple causes, including

phytopathogen infection, and it is likely that necrosis associated with fire blight disease leads to ethanol production, a prerequisite for attack by Xyleborine ambrosia beetles that have been associated with stressed and declining apple trees.

Our field experiments showed that fire blight infected apple trees did attract ambrosia beetles, but at a much lower rate than trees that were flood stressed. Only 16 ambrosia beetle entries were recorded across 20 fire blight infected trees, compared with an average of 16.9 per tree on flood-stressed apple trees exposed to ambrosia beetles over the same period of time. In other fire blight inoculation studies (not ambrosia beetle related), we seldom observe ambrosia beetle entries unless disease progresses to at least half the tree (unpublished). In the current studies, Analysis of ethanol in tissue showed that elevated ethanol was localized to the upper trunk that was infected on inoculated trees, but on flood-stressed trees, ethanol was elevated throughout the tree, tending towards higher ethanol content on lower trunk sections. Although necrotic tissue on fire blight infected trees had higher ethanol than flood stressed tree tissue samples, the total ethanol emission from flood-stressed trees was likely higher given the larger total area of tissue with elevated ethanol. Furthermore, tissue samples were taken 21 DAF and based on previous work it is expected that ethanol production likely peaked at 10-14 DAF on the flood-stressed apple trees (Reding et al. 2021).

In summary, experiments conducted in this study confirmed fire blight infection increases ethanol concentration in apple tissue, which appears to be localized to infected areas and necrotic diseased tissue. Elevated ethanol content in infected tissue is the most likely explanation for previous observed associations between fire blight infections and ambrosia beetle attacks on apple trees. However, the number of entries on infected trees were low in our experiment compared with flood-stressed trees. Therefore, severe flood stress and other physiological stress that elevate ethanol in trees are more likely to be stronger drivers of ambrosia beetle attack risk compared with fire blight infections. Future studies should investigate the attractiveness of apple trees with different levels of fire blight

disease severity and test whether females emerging from fire blight infected wood are able to transmit *E. amylovora* to other trees.

## References

**Aćimović, S. G., R. D. Santander, C. L. Meredith, and Ž. M. Pavlović. 2023.** Fire blight rootstock infections causing apple tree death: A case study in high-density apple orchards with *Erwinia amylovora* strain characterization. *Front. Hortic.*

**Addesso, K., F. Baysal-Gurel, J. Oliver, C. Ranger, and P. O'neal. 2018.** Interaction of a preventative fungicide treatment and root rot pathogen on ambrosia beetle attacks during a simulated flood event. *Insects.* 9.

**Agnello, A. M., D. I. Breth, E. M. Tee, K. D. Cox, S. M. Villani, K. M. Ayer, A. E. Wallis, D. J. Donahue, D. B. Combs, A. E. Davis, J. A. Neal, and F. M. English-Loeb. 2017.** *Xylosandrus germanus* (Coleoptera: Curculionidae: Scolytinae) Occurrence, Fungal Associations, and Management Trials in New York Apple Orchards. *J. Econ. Entomol.* 110: 2149–2164.

**Agnello, A. M., D. B. Combs, C. C. Filgueiras, D. S. Willett, and A. Mafra-Neto. 2021.** Reduced Infestation by *Xylosandrus germanus* (Coleoptera: Curculionidae: Scolytinae) in Apple Trees Treated with Host Plant Defense Compounds. *J. Econ. Entomol.* 114: 2162–2171.

**Breth, D., A. Agnello, and E. Tee. 2016.** Black stem borer control in apple nurseries and tall spindle plantings Debo. *Cornell Coop. Ext. Publ.* 2–3.

**Cellini, A., E. Biondi, S. Biasioli, L. Rocchi, B. Farneti, I. Braschi, S. Savioli, M. T. Rodriguez-Estrada, F. Biasioli, and F. Spinelli. 2016.** Early detection of bacterial diseases in apple plants by analysis of volatile organic compounds profiles and use of electronic nose. *Ann. Appl. Biol.* 168: 409–420.

**Cellini, A., G. Buriani, L. Rocchi, E. Rondelli, S. Savioli, M. T. Rodriguez Estrada, S. M. Cristescu, G. Costa, and F. Spinelli. 2018.** Biological relevance of volatile organic compounds emitted during the pathogenic interactions between apple plants and *erwinia amylovora*. *Mol. Plant Pathol.* 19: 158–168.

**Copolovici, L., and Ü. Niinemets. 2010.** Flooding induced emissions of volatile signalling compounds in three tree species with differing waterlogging tolerance. *Plant, Cell Environ.* 33: 1582–1594.

**Ferner, E., H. Rennenberg, and J. Kreuzwieser. 2012.** Effect of flooding on C metabolism of flood-tolerant (*Quercus robur*) and non-tolerant (*Fagus sylvatica*) tree species. *Tree Physiol.* 32: 135–145.

**Frank, S. D., and C. M. Ranger. 2016.** Developing a Media Moisture Threshold for Nurseries to Reduce Tree Stress and Ambrosia Beetle Attacks. *Environ. Entomol.* 45: 1040–1048.

**Gansert, D. 2003.** Xylem sap flow as a major pathway for oxygen supply to the sapwood of birch (*Betula pubescens* Ehr.). *Plant, Cell, Environ.* 26: 1803–1914.

**Gibbs, J., and H. Greenway. 2003.** Mechanisms of anoxia tolerance in plants. I. Growth, survival and anaerobic catabolism. *Funct. Plant Biol.* 30: 1–47.

**Gresham, S. D. M., S. M. Villani, and J. F. Walgenbach. 2023.** Seasonal and Regional Trends of Exotic Ambrosia Beetles Attacking apple. Submitted.

**Hall, F. R., M. A. Ellis, and D. C. Ferree. 1982.** Influence of fire blight and ambrosia beetle on several apple cultivars on M9 and M9 interstems. *Ohio State Univ. Res. Circ.* 272: 20–24.

**Haq, A., and E. A. Dawes. 1971.** Pyruvic acid metabolism and ethanol formation in *Erwinia amylovora*. *Microbiol. (United Kingdom)*. 68: 295–306.

**Iakimova, E. T., P. Sobiczewski, L. Michalczyk, E. Wegrzynowicz-Lesiak, A. Mikiciński, and E. J. Woltering. 2013.** Morphological and biochemical characterization of *Erwinia amylovora*-induced hypersensitive cell death in apple leaves. *Plant Physiol. Biochem.* 63: 292–305.

**Jones, D. H. 1911.** Scolytus rugulosus as an agent in the spread of bacterial blight in pear trees. *Phytopathology*. 1: 155–158.

**Kelsey, R. G., M. M. Beh, D. C. Shaw, and D. K. Manter. 2013.** Ethanol Attracts Scolytid Beetles to *Phytophthora ramorum* Cankers on Coast Live Oak. *J. Chem. Ecol.* 39: 494–506.

**Kelsey, R. G., and G. Joseph. 1998.** Ethanol in Douglas-fir with black-stain root disease (*Leptographium wageneri*). *Phytopathology*. 1212: 1207–1212.

**Kelsey, R. G., G. Joseph, D. Westlind, and W. G. Thies. 2016.** Ethanol and acetone from Douglas-fir roots stressed by *Phellinus sulphurascens* infection: Implications for detecting diseased trees and for beetle host selection. *For. Ecol. Manage.* 360: 261–272.

**Kelsey, R. G., and D. J. Westlind. 2017.** Physiological stress and ethanol accumulation in tree stems and woody tissues at sublethal temperatures from fire. *Bioscience*. 67: 443–451.

**Kharadi, R. R., J. K. Schachterle, X. Yuan, L. F. Castiblanco, J. Peng, S. M. Slack, Q. Zeng, and G. W. Sundin. 2021.** Genetic Dissection of the *Erwinia amylovora* Disease Cycle. *Annu. Rev. Phytopathol.* 59: 191–212.

**Kharadi, R. R., and G. W. Sundin. 2021.** Dissecting the process of xylem colonization through biofilm formation in *Erwinia amylovora*. *J. Plant Pathol.* 103: 41–49.

**Monterrosa, A., S. V. Joseph, B. Blaauw, W. Hudson, and A. L. Acebes-Doria. 2022.** Ambrosia Beetle Occurrence and Phenology of *Xylosandrus* spp. (Coleoptera: Curculionidae: Scolytinae) in Ornamental Nurseries, Tree Fruit, and Pecan Orchards in Georgia. *Environ. Entomol.* 51: 998–1009.

**Oh, C. S., J. F. Kim, and S. V. Beer. 2005.** The Hrp pathogenicity island of *Erwinia amylovora* and identification of three novel genes required for systemic infection. *Mol. Plant Pathol.* 6: 125–38.

**Orton, C. R., and J. F. Adams. 1915.** Collar-blight and related forms of fireblight. PA Agri. Exp. Sta. Bull. 136: 23.

**Ranger, C. M., P. H. W. Biedermann, V. Phuntumart, G. U. Beligala, S. Ghosh, D. E. Palmquist, R. Mueller, J. Barnett, P. B. Schultz, M. E. Reding, and J. P. Benz. 2018.** Symbiont selection via alcohol benefits fungus farming by ambrosia beetles. Proc. Natl. Acad. Sci. U. S. A. 115: 4447–4452.

**Ranger, C. M., M. E. Reding, A. B. Persad, and D. A. Herms. 2010.** Ability of stress-related volatiles to attract and induce attacks by *Xylosandrus germanus* and other ambrosia beetles. Agric. For. Entomol. 12: 177–185.

**Ranger, C. M., M. E. Reding, P. B. Schultz, J. B. Oliver, S. D. Frank, K. M. Adesso, J. H. Chong, B. Sampson, C. Werle, S. Gill, and C. Krause. 2016.** Biology, ecology, and management of nonnative ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) in ornamental plant nurseries. J. Integr. Pest Manag. 7.

**Ranger, C. M., P. B. Schultz, S. D. Frank, J. H. Chong, and M. E. Reding. 2015.** Non-native ambrosia beetles as opportunistic exploiters of living but weakened trees. PLoS One. 10: 1–22.

**Ranger, C. M., P. B. Schultz, S. D. Frank, and M. E. Reding. 2019.** Freeze stress of deciduous trees induces attacks by opportunistic ambrosia beetles. Agric. For. Entomol. 21: 168–179.

**Ranger, C. M., P. C. Tobin, and M. E. Reding. 2015.** Ubiquitous volatile compound facilitates efficient host location by a non-native ambrosia beetle. 675–686.

**Rassati, D., M. Contarini, C. M. Ranger, G. Cavaletto, L. Rossini, S. Speranza, M. Faccoli, and L. Marini. 2020.** Fungal pathogen and ethanol affect host selection and colonization success in ambrosia beetles. Agric. For. Entomol. 22: 1–9.

**Reding, M. E., C. M. Ranger, and P. B. Schultz. 2021.** Colonization of trees by ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) is influenced by duration of flood stress. *J. Econ. Entomol.* 114: 839–847.

**Singh, J., J. Fabrizio, E. Desnoues, J. P. Silva, W. Busch, and A. Khan. 2019.** Root system traits impact early fire blight susceptibility in apple (*Malus × domestica*). *BMC Plant Biol.* 19: 1–14.

**Slack, S. M., Q. Zeng, C. A. Outwater, and G. W. Sundin. 2017.** Microbiological examination of *Erwinia amylovora* exopolysaccharide ooze. *Phytopathology.* 107: 403–411.

**Spinelli, F., A. Cellini, J. L. Vanneste, M. T. Rodriguez-Estrada, G. Costa, S. Savioli, F. J. M. Harren, and S. M. Cristescu. 2012.** Emission of volatile compounds by *Erwinia amylovora*: Biological activity in vitro and possible exploitation for bacterial identification. *Trees - Struct. Funct.* 26: 141–152.

**Suleman, P., and P. W. Steiner. 1994.** Relationship between sorbitol and solute potential in apple shoots relative to fire blight symptom development after infection by *Erwinia amylovora*. *Phytopathology.*

**Sutton, D. D., and M. P. Starr. 1959.** Anerobic Dissimilation of Glucose by *Erwinia amylovora*. *J. Bacteriology.* 78: 427–431.

**Tancos, K. A., S. Villani, S. Kuehne, E. Borejsza-Wysocka, D. Breth, J. Carol, H. S. Aldwinckle, and K. D. Cox. 2016.** Prevalence of streptomycin-resistant *Erwinia amylovora* in New York apple orchards. *Plant Dis.* 100: 802–809.

**Venisse, J.-S., M. Malnoy, M. Faize, J. Paulin, and M. Brisset. 2002.** Modulation of defense responses of *malus* spp. during compatible and incompatible interactions with *erwinia amylovora*. *Plant-Microbe Interact.* 15: 1204–1212.

**Vrancken, K., M. Holtappels, H. Schoofs, T. Deckers, and R. Valcke. 2013.** Pathogenicity and infection strategies of the fire blight pathogen *Erwinia amylovora* in Rosaceae: State of the art. *Microbiol. (United Kingdom)*. 159: 823–832.

**Wang, D., S. S. Korban, and Y. Zhao. 2010.** Molecular Signature of Differential Virulence in Natural Isolates of *Erwinia amylovora*. *Phytopathology*. 100: 192–198.

**Zamski, E., D. Shtienberg, and D. Blachinsky. 2006.** The role of ooze exudation in the migration of *Erwinia amylovora* cells in pear trees infected by fire blight. *Isr. J. Plant Sci.* 54: 301–307.

**Zeng, Q., J. Puławska, and J. Schachterle. 2021.** Early events in fire blight infection and pathogenesis of *Erwinia amylovora*. *J. Plant Pathol.* 103: 13–24.

**Zhao, Y., S. E. Blumer, and G. W. Sundin. 2005.** Identification of *Erwinia amylovora* Genes Induced during Infection of Immature Pear Tissue. *J. Bacteriol.* 187: 8088–8103.

## Chapter 4 Tables.

Table 4.1. Infection severity (Mean  $\pm$  SE necrotic length) Mean ( $\pm$  SE) *E. amylovora* population and ethanol concentration of tissue samples taken from apple trees with fire blight disease and exposed to *X. crassiusculus* within insect-proof field cages. N = 6 attacked, 6 non-attacked.

Ambrosia beetle entry	Necrotic length (mm)	<i>E. amylovora</i> Ea110 Population (Log 10 CFU g <sup>-1</sup> FW)			Ethanol (mg EtOH g <sup>-1</sup> FW)		
		Necrotic	Symptomatic	15 cm below symptoms	Necrotic	Symptomatic	15 cm below symptoms
Not-attacked	17.6 $\pm$ 1.6	7.4 $\pm$ 0.2	2.9 $\pm$ 1.1	0.8 $\pm$ 0.5	75.5 $\pm$ 19.7	39.3 $\pm$ 22.2	0.5 $\pm$ 0.3
Attacked	32.1 $\pm$ 4.8	6.3 $\pm$ 0.8	6.2 $\pm$ 0.7	3.1 $\pm$ 0.7	91.2 $\pm$ 16.8	51.4 $\pm$ 24.2	2.7 $\pm$ 1.2
F-value d.f	8.26	1.73	6.43	7.73	0.37	0.14	3.18
1,5							
p-value	0.0166	0.2178	0.0296	0.0195	0.5573	0.7194	0.1047

Table 4.2. Effect of infection by *E. amylovora* and flooding on the mean ( $\pm$  SE) number of ambrosia beetle attacks on apple, cv. Gala/G.41 at the MHCRC in 2023

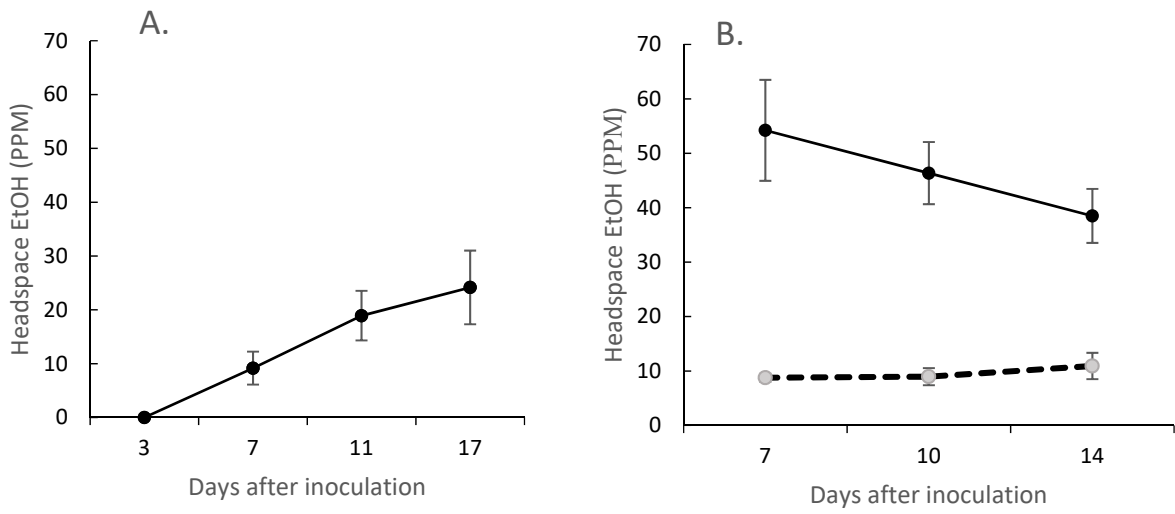
Deployment date	Treatment	Below Graft				
		Union	Lower trunk	Mid-trunk	Upper trunk	Whole-tree
3 May 2023	Fire blight	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
	Flooded	2.5 $\pm$ 0.7	8.9 $\pm$ 2.1	8.9 $\pm$ 2.2	1 $\pm$ 0.3	21.3 $\pm$ 3.2
	Control	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
	F-value (1,20)	12.57	18.31	16.57	6.33	44.41
	P value	0.0020	0.0004	0.0006	0.0205	<0.0001
6 June 2023	Fire blight	0 $\pm$ 0	0 $\pm$ 0	0.3 $\pm$ 0.2	0.9 $\pm$ 0.6	1.2 $\pm$ 0.6
	Flooded	2.4 $\pm$ 0.7	5.6 $\pm$ 1.2	3.5 $\pm$ 0.7	0.9 $\pm$ 0.4	12.4 $\pm$ 2.3
	Control	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0	0 $\pm$ 0
	F-value (1,18)	12.33	20.10	20.66	1.03	21.46
	P value	0.0026	0.0003	0.0003	0.67	0.0002

One-way ANOVA performed on number of attacks at each position separately by date, non-stressed control trees were excluded from analysis as there were no attacks.

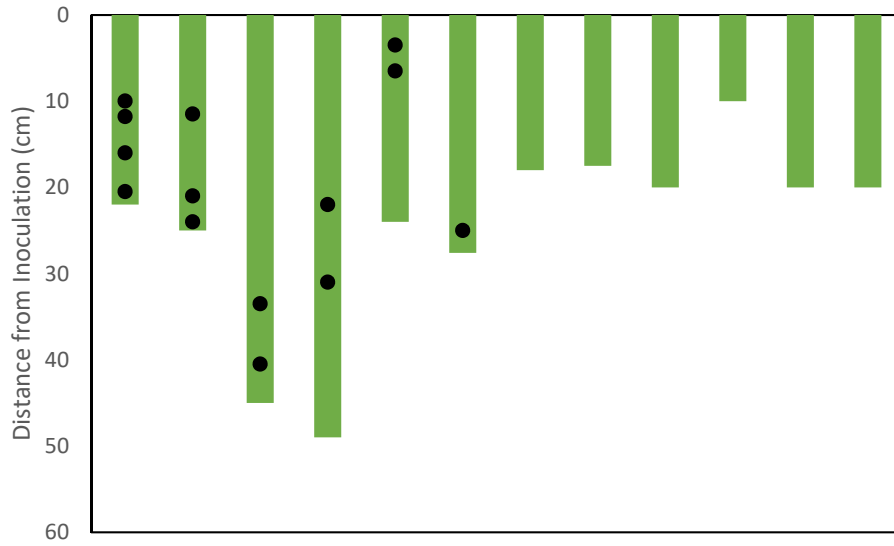
## Chapter 4 Figures



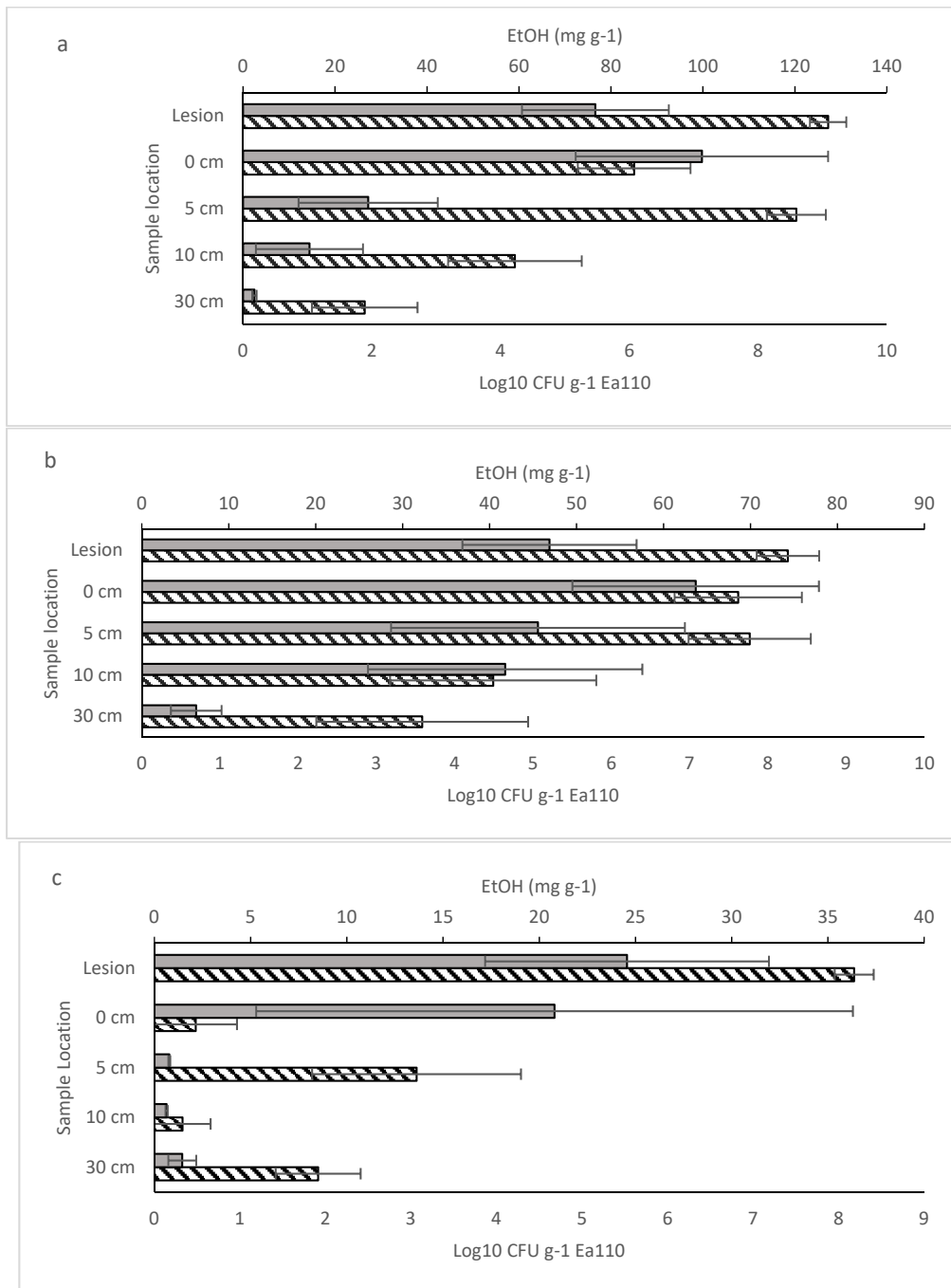
**Fig. 4-1:** Headspace analysis of ethanol using SPME of apple seedlings inoculated with *E. amylovora* Ea110. (a) 4 L glass jar with an apple seedling and SPME fiber inserted through rubber septum fitted in lid, (b) apple seedling 7 DAI with wilting and interveinal necrosis, and (c) apple seedling 14 DAI with leaf necrosis and wilting.



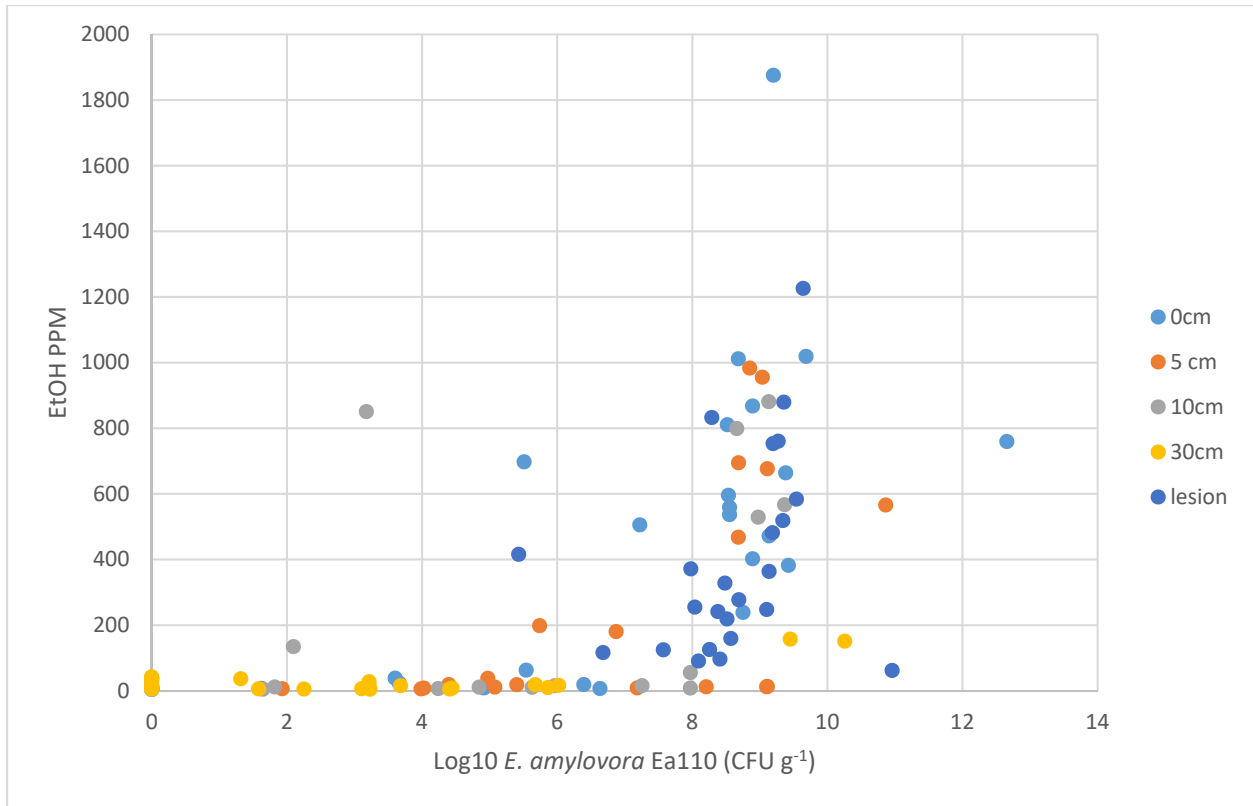
**Fig. 4-2.** Mean ( $\pm$  SE) headspace ethanol concentration (PPM) of apple seedlings inoculated with *E. amylovora* Ea110 (solid line with black marker) and non-inoculated control (dashed line with grey marker) experiment 1 (A.) conducted February 2022, Experiment 2 (B.) conducted April 2022. Error bars represent SEM.



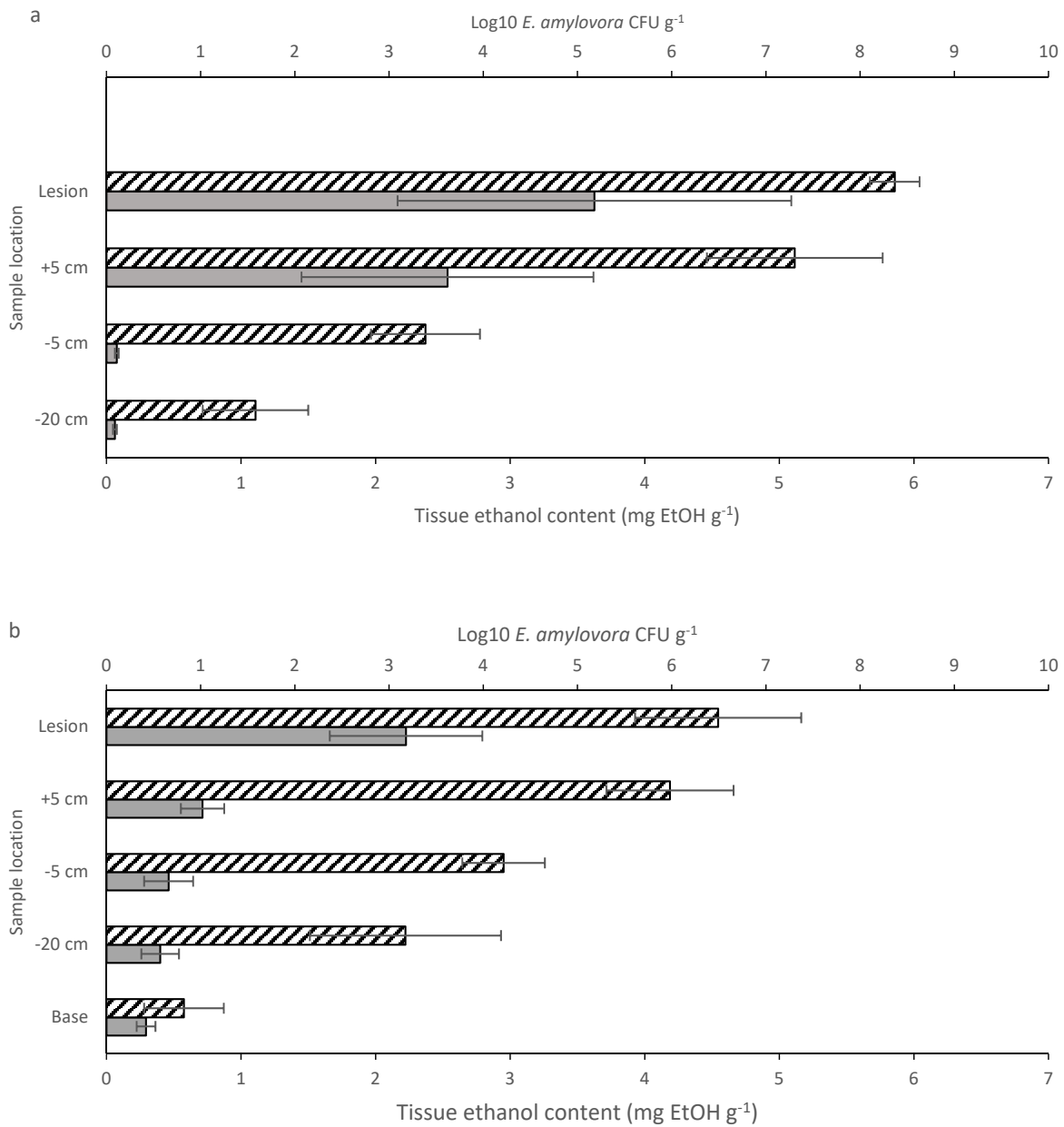
**Fig. 4-3.** Location of *X. crassiusculus* entries (black dots) and fire blight symptoms (green bars) in relation to inoculation point on 'Fuji' apple trees inoculated with *E. amylovora* Ea110 5 weeks before exposure to beetles within insect-proof field cages.



**Fig. 4-4.** Mean (+/- SE) Ethanol concentration (grey bars) and *E. amylovora* population (bars with black diagonal lines) of tissue samples taken from apple trees inoculated with *E. amylovora* Ea110 on 1-Apr-2022. Tissue samples taken from necrotic tissue (lesion) and 0, 5, 10, and 30 cm below symptoms on main trunk. (a) 24 May collection, (b) 30 May collection, (c) 6 June collection. Experiment conducted at MHCREC 2022.



**Fig. 4-5.** Relationship between the population of *E. amylovora* Ea110 (CFU g<sup>-1</sup> tissue) and tissue ethanol content (PPM) from tissue samples taken from the necrotic lesion, and 0, 5, 10, and 30 cm below visible symptoms of disease on apple trees inoculated with *E. amylovora* Ea110 on 30 April 2022. Regression results: 95% CI 0.238, 0.447, Wald-Chi = 39.07, P < 0.0001.



**Fig. 4-6.** Mean (+/- SE) Ethanol concentration (grey bars) and *E. amylovora* population (bars with black diagonal lines) of tissue samples taken from apple trees inoculated with *E. amylovora* Ea110. Tissue samples taken from necrotic tissue (lesion), 5 cm above symptom boundary, and 5 and 20 cm below symptoms and at base of trunk (5 cm above graft union). (a) 3 May deployment n = 11, (b) 6 June deployment n = 10. Experiment conducted at MHCRC 2023.

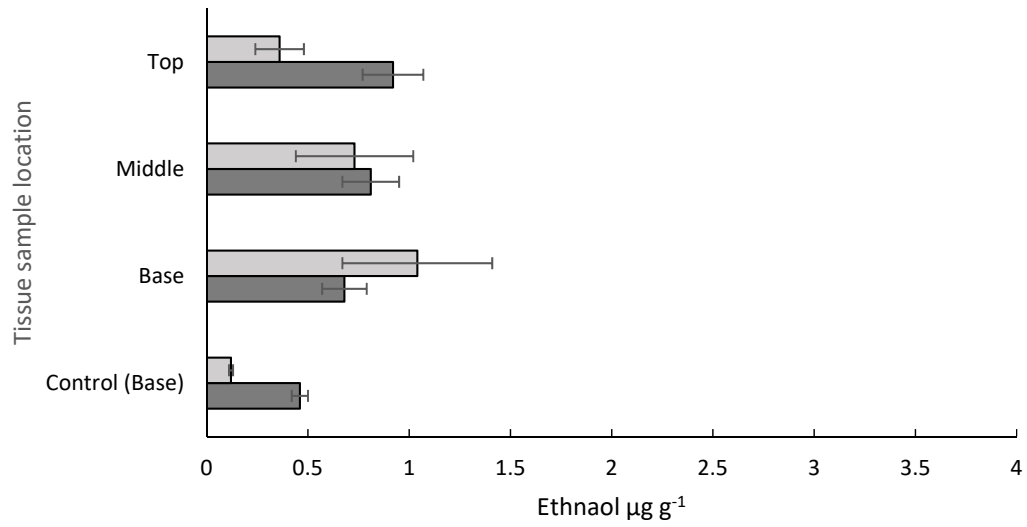
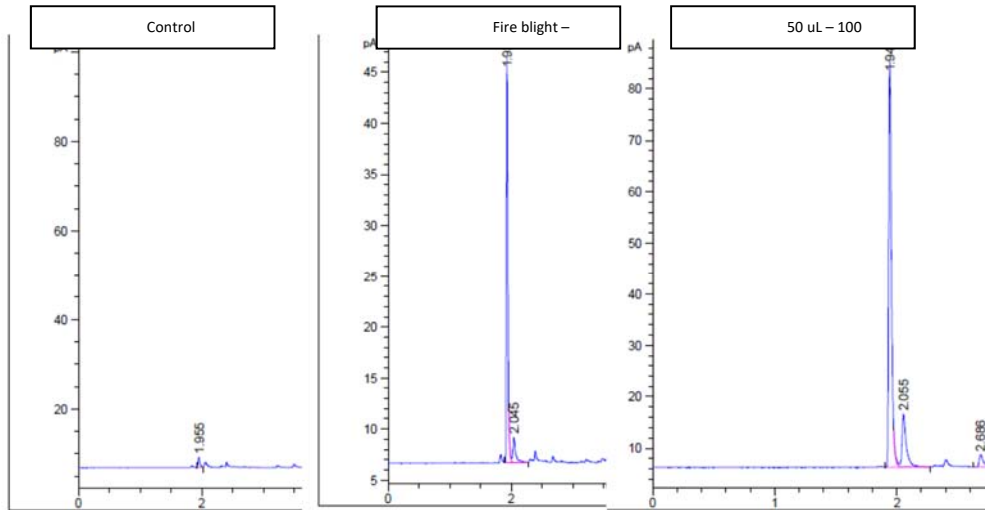


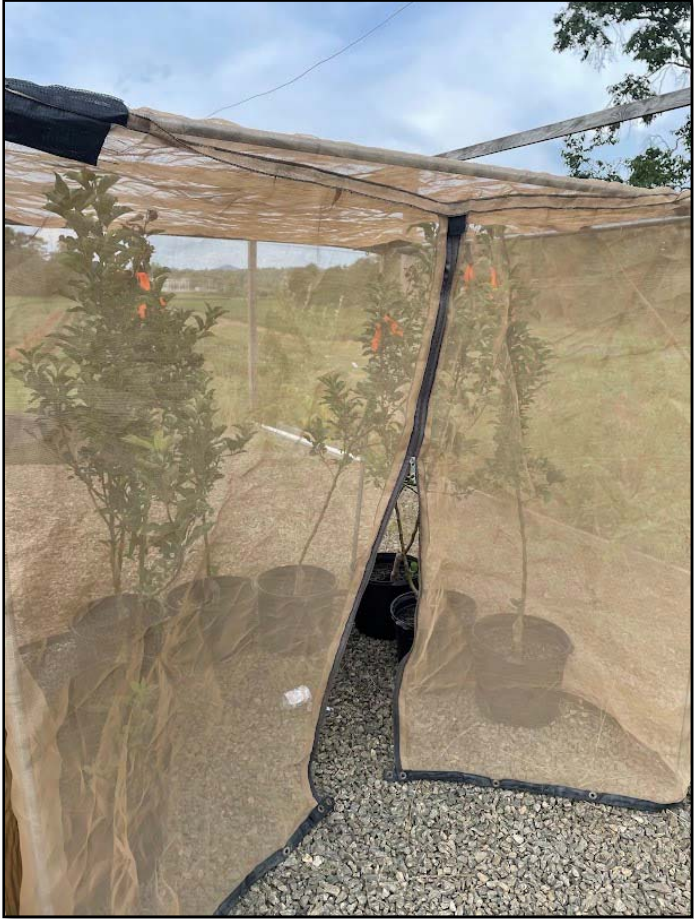
Figure 4-7. Mean (+/- SE) Ethanol concentration of tissue samples taken from apple trees Flood stressed for 21 days. Tissue samples taken from the top canopy (1.4 m above graft union), mid-trunk (0.7 m above graft union), and base (5 cm above graft union). (light grey bars) 3 May deployment n = 11, (dark grey bars) 6 June deployment n = 10. Experiment conducted at MHCREC 2023.

## Supplementary

SI Figure 1 – ethanol emission from diseased seedlings



SI Figure 2: Insect-proof field cage used for field cage no-choice assay



## Chapter 5 Interaction of Abiotic Stress and Ambrosia Beetles on Health and Mortality of Apple, *Malus domestica* (Borkh).

### **Abstract**

Ambrosia beetles in the genus *Xylosandrus* have been associated with decline and death of trees in a number of tree production systems including a role in Rapid Apple Decline (RAD), a syndrome impacting eastern USA in recent years. Physiological stress that causes tissue damage or anaerobic/hypoxic conditions elevate tissue ethanol, which is an important attractant to ambrosia beetles and necessary for successful burrowing and establishment of galleries. Because ambrosia beetle attacks follow stress events, it is difficult to differentiate the relative impact of the ambrosia beetle attacks compared with the underlying physiological stress on tree decline. In this study, interactions of flood stress and ambrosia beetle attack on commercial cultivars of apple with dwarfing rootstocks are explored. The effect of excluding ambrosia beetles on the health and mortality of apple trees was tested on potted trees that were flood stressed or drenched with ethanol to induce ambrosia beetle attack. Ambrosia beetle attacks under natural and no-choice conditions did not have a marked impact on apple tree health. 'Evercrisp' apple trees grafted to three different dwarfing rootstocks were subjected to drought stress and flood stress. We found that flood stress negatively impacts apple tree health across all commercial rootstocks tested (M.9, G.41, and B.9), eliciting elevated ethanol and ambrosia beetle attack.

### **Key Words**

Drought, water stress, ethanol, abiotic stress, flooding, ambrosia beetle, decline, apple

## Introduction

High density apple, *Malus domestica* (Borkh), orchards utilize dwarfing and semi-dwarfing rootstocks for improved precocity of production, yield efficiency, and fruit quality compared with standard low density plantings, but require a higher initial investment in trees and support structures (Robinson et al. 2004). Given the high capital investment required for trees and the trellis infrastructure, decline of young trees in high density systems is a major concern to growers. Over the past 10 years Rapid Apple Decline (RAD) has been an issue concerning apple growers in the eastern USA (Singh et al. 2019) and Canada (Xu et al. 2023). There is some concern that high density systems that utilize dwarfing rootstocks are less resilient to water and nutrient stress due to increased root competition and lower root biomass (Rosenberger 2019, Peter 2021). To date, no phytopathogen has been shown to cause RAD, and roots usually appear unaffected, except for the absence of fine feeder roots (Singh et al. 2019, Peter 2021). Declining trees are often distributed in random clusters throughout the orchard, and decline is most prevalent in young (<6 year old) high-density plantings utilizing dwarfing rootstocks (Peter 2021).

Abiotic stressors such as cold damage (Avalos-Ruiz et al. 2022), drought (Singh et al. 2019, Xu et al. 2023), and herbicide injury (Rosenberger 2019) have been proposed to weaken trees and increase susceptibility to pathogen and insect attack. The association of RAD with several biotic causes have been investigated, including viruses (Liu et al. 2018, Wright et al. 2020), stem boring insects (Agnello et al. 2015, 2017, Donahue and Elone 2021, Gresham et al. 2023), and bacterial and fungal pathogens (Singh et al. 2019, Donahue and Elone 2021, Avalos-Ruiz et al. 2022). Ambrosia beetles, *Xylosandrus crassiusculus* and *X. germanus*, have been associated with declining apple trees in Michigan (Haas et al. 2016), New York (Agnello et al. 2015, 2017, Donahue and Elone 2021) and North Carolina (Chapter 1). Ambrosia beetle species associated with declining apple trees have also been implicated in tree losses in ornamental nurseries, and nut orchards throughout the USA (Ranger, Schultz, et al. 2015, Ranger et al.

2016, Gugliuzzo et al. 2021), and are of concern in natural and managed systems in Europe (Galko et al. 2018, Contarini et al. 2020, Gugliuzzo et al. 2020) and Oceania (Tran, H. X., Doland Nichols, J., Li, D., Le, N. H., Lawson 2022).

Ambrosia beetles in the tribe *Xyleborini* (Coleoptera: Curculonidae) such as *Xylosandrus crassiusculus* and *X. germanus* have successfully invaded managed and natural ecosystems throughout the world from their native habitats in Asia through their ability to successfully establish galleries and fungal gardens in apparently healthy but stressed trees (Ranger et al. 2016, Hulcr and Stelinski 2017). These beetles have been associated with dieback and accelerated tree death in managed tree production systems, but the relative impact of the beetles on tree death has not been experimentally examined to date (Gugliuzzo et al. 2021). Ethanol is considered the most important volatile for long and short range attraction of the beetles to susceptible hosts (Ranger, Tobin, et al. 2015). Induction of host stress events such as flood (Ranger et al. 2010, Frank and Ranger 2016), cold damage (Ranger et al. 2019), heat stress (Kelsey and Westlind 2017), herbicides (Kelsey et al. 2016), mechanical damage, and pollutants (Kimmerer and Kozlowski 1982), have been shown to increase ethanol emissions in trees and attract Xyleborine ambrosia beetles.

Drought stress has not been shown to induce colonization by Xyleborine ambrosia beetles directly (Frank and Ranger 2016, Ranger et al. 2023), however drought conditions in North Carolina during the summer and fall of 2016 have been considered a potential cause of a major outbreak of RAD and ambrosia beetle infestations in North Carolina in the spring of 2017 (Gresham et al. 2023). Therefore the effect of late summer drought on the health and resilience to flood stress the following spring on apple trees was investigated.

The objective of this study was to examine the interaction of abiotic stress and ambrosia beetles on the health and mortality of apple trees grafted to dwarfing rootstocks using flood stress as the

primary stressor based on the well-established link between flooding, ethanol, and ambrosia beetle attack (Ferner et al. 2012, Ranger et al. 2013, Frank and Ranger 2016).

## **Methods**

### **Plant Material.**

Apple trees, used in experiments were purchased from nurseries as 1 year old grafted bare-rooted trees (cultivar and rootstock combination are described for each experiment in sections below). Trees were planted into 38 L plastic pots with pine bark mulch media (1:2 vermiculite: fine ground pine bark mulch with lime and micronutrients) and were maintained outdoors at the Mountain Horticultural Crops Research and Extension Center (MHCREC), Mills River NC (35.417474, -82.558012), subject to a standard pesticide and fertilizer program. Watering to capacity was accomplished using microspinkler irrigation. Herbicide applications were made as needed with glufosinate (Rely 280 SL, BASF, Research Triangle Park, NC) for burn down and a single spring application of flumioxazin (Chateaux, Valent, CA) for residual weed control. All experiments were conducted at the MHCREC or at a commercial orchard in Henderson County, NC (35.417474, -82.558012). Trees were overwintered in a greenhouse at the MCREC with heat provided when temperatures were predicted to be below -4°C.

### **Effect of ambrosia beetle colonization on apple tree health and mortality.**

Field experiments using potted apple trees were conducted to better understand the relative effect of ambrosia beetle attack on health and mortality of apple trees. Two field experiments were conducted in 2020 and 2021 comparing the effect of excluding ambrosia beetles from flooded or ethanol-drenched potted apple trees. Trees were exposed to natural populations of ambrosia beetles and on half the trees beetles were excluded from colonizing. In 2022 the comparative effect of beetle colonization on tree health was evaluated by caging *X. crassiusculus* onto apple trees that were irrigated with ethanol to induce beetle colonization.

*2020 Beetle Exclusion Experiment.* In 2020 total of 60 'Honey crisp' apple trees grafted to 'M.9-Nic 29' rootstock, planted in 38 L containers in 2019. The experiment had a 2 x 3 factorial treatment structure with beetle exposure (exposed to beetles versus beetles excluded) and stress (flood stress, ethanol drench, and regular-watered control) as main factors with 10 replicates per treatment combination. A 3% ethanol solution was used for the ethanol-flooded stress treatment. The trees were placed adjacent to a woods near an apple orchard at the MHCREC in Mills River NC. To exclude ambrosia beetles, non-exposed trees were placed in two insect exclusion field cages measuring 1.8 x 1.8 x 1.8 m (1 mm mesh, BioQuip Products, CA, USA). To account for the shading effect of the insect exclusion cages, shade cloth (50% black polypropylene woven cloth) was placed over the trees exposed to ambrosia beetles and held up by a custom-designed structure using PVC pipe and Y-bar metal stakes (Fig. 1). The shade cloth covered the foliage on the trees, but the lower 1 m of trunks were exposed to ambrosia beetles. Light, temperature, and humidity conditions were not measured within cages or under shade. Flood stress conditions were created using a pot-in-pot system based on (Ranger et al. 2013). Potted trees were placed into another 38 L container lined with a 156 L contractor bag (3 mil thickness) and water was added to completely fill the pot, saturating the substrate until there was standing water on the surface. Excess plastic liner was tied around the base of the tree to encase the pot and cover the surface of the substrate. Additional water was added weekly as needed to maintain complete saturation of the roots. The ethanol drench treatment was applied by placing the potted tree inside a 38 L pot lined with contractor bag, but instead of flooding with water, 4 L of 3% ethanol solution was applied to each tree and 1-2 L was re-applied twice every 3 days then irrigated with water only to maintain adequate soil moisture. The no stress treatment was irrigated with water every 1-3 days as needed.

The experiment was established in the field on 19 June 2020, and on 6 July the outer pots and liners were removed to remove stress when flood-stressed trees were showing wilting and some marginal necrosis on leaves. Both exposed and caged trees were monitored for beetle attacks weekly for

21 days and again on 30 September. On 14 July all trees were moved to a container pad at the MHCREC equipped with a microsprinkler irrigation system that watered trees daily to maintain a non-stressed condition. After moving to the irrigation pad, beetles were excluded from appropriate treatment by wrapping the lower trunk with long-lasting insecticide netting (LLIN) impregnated with deltamethrin (Vestergaard-Frandsen, Lausanne, Switzerland). The exclusion netting remained on beetle-excluded trees through the following spring to assess for long-term impacts of stress and beetle attack. Tree health was assessed on 9 July and 30 September 2020, and on 14 May 2021, based on visual assessment of leaf condition of trees. Each tree was rated on a scale of 1-5 scale by the same assessor for each assessment as follows 1 = most leaves (>60%) wilted and/or brown; 2 = 40-60% of leaves yellow, wilting, or with some necrosis/browning; 3 = 20-40% leaves with wilting and yellowing; 4 = 5-20% leaves with wilting and marginal yellowing or browning; and 5 = healthy, <5% leaves symptomatic.

The number of beetles entries per tree on exposed trees (n=30) were subjected to an ANOVA with means separated using Tukey's HSD ( $P = 0.05$ ) using PROC ANOVA in SAS (V 9.4, SAS Institute, Cary, NC). Exclusion caged treatments were excluded due to all treatments having no attacks. The health score for each observation was analyzed as a two-way factorial using PROC GLM in SAS with stress (ethanol drench, flooding, or no-stress) and beetle exposure (beetle exclusion cage, or exposed) as main effect factors. Differences among treatments for tree mortality were tested using Generalized Linear Model using a binomial distribution using PROC GLM in SAS.

*2021 Beetle Exclusion Field Experiment.* In 2021 a total of 60 'Honey crisp' trees grafted on to M.9-Nic29 rootstock, planted in 38 L containers in 2019 were deployed at a commercial apple orchard in Henderson Co., NC (35.368067, -82.336477). The location was selected based on an expected high population of ambrosia beetles based on previous issues with tree decline and ambrosia beetle attack. The experiment was a randomized complete block design with three treatments and 20 single tree replicates as blocks. Treatments included: i) normal watering (Control), ii) flooding with exposure to

beetle attack, and iii) flooding with beetles excluded from attacking the trunk. Flood stress was imposed in the field on 19 May using the same pot-in-pot method described above. Ambrosia beetles were excluded from colonizing the lower trunks on beetle-excluded trees using LLIN wrapped around the trunk from the soil line to the first scaffold branch. Trees were spaced 1 m within blocks and 5-7 m between blocks along the interface between an unmanaged woodlot and apple orchard. Trees were checked weekly for beetle attacks for 21 days. On 1 June, 13 days after flooding, the outer pot and liner were removed from the flood-stressed trees and all trees were moved to a container irrigation pad at the MHCREC and irrigated daily with micro sprinklers. Tree health assessments were made 8, 13, 29, 42, and 133 days after trial initiation using the same rating scale described above. At the final assessment, 133 days after set up, the percentage of canopy experiencing defoliation and dieback (necrosis, wilting, chlorosis) was estimated. Trees were not assessed for health and mortality the following spring due to winter mortality of a large proportion of the trees caused by rodent damage.

Ambrosia beetle entries as counts per tree, health scores, defoliation, and dieback, were analyzed as a mixed model in SAS (PROC GLIMMIX, V9.4; SAS Institute 2013). Least square means were compared using the lsmeans statement. Mortality was analyzed as a Generalized Linear Model using a binomial distribution using PROC GLM in SAS.

*2022 Beetle Colonization Field Experiment.* To isolate the effect of ambrosia beetle tunneling on tree health, an experiment was conducted in 2022 under controlled infestation levels and in the absence of flood stress. Second leaf EverCrisp® trees grafted on to 'M9.337', 'B.9', or 'G.41', planted in 38 L plastic pots (rootstocks were blocked by replicate with 4 replicates per rootstock-treatment combination). Treatments were: i) water irrigation + no damage, ii) ethanol-irrigated + 20, 2 mm diameter holes drilled into the lower trunk, iii) ethanol irrigated + 20 *X. crassiusculus* caged to the lower trunk, (4) ethanol irrigated + 20, 2 mm diameter holes drilled on the lower trunk and inoculated with mycelium from an isolate of *Botryosphaeria dothidea*, an aggressive wood-invading pathogen of apple.

To elicit beetle entry, trees were irrigated with 4 L of 2.5% ethanol solution followed by application of 2L/day for 3 days then watered normally. Ambrosia beetles (lab-reared *Xylosandrus crassiusculus* that had emerged 5-10 days before attaching to trees) were attached to trees in individual cages using methods adapted from Ranger et al. (2015). The cages consisted of 10 mm diameter plastic tubing bisected longitudinally. 3 cm long, with each end capped with a small amount of hot glue and a 5 mm hole drilled in the middle to insert the beetle. Five sets of four cages were spaced 5-10 cm between sets on the lower 50 cm of the main trunk, each set of cages were arranged around the cardinal directions of the trunk. Beetle cages were inspected one day after attachment, and if beetles did not enter trunks, a new beetle was placed in the cage. To simulate beetle damage 20, 2 mm diameter holes were drilled to a depth of 2-3 cm into the lower trunk with the same orientation and spacing as the beetle cages. The surface of the bark was wiped with 95% ethanol before drilling and the drill bit was cleaned with 95% ethanol and flamed between trees. For the inoculated drilled holes treatment, 20, 2 mm holes were drilled into the lower trunk and inoculated with an isolate of *Botryosphaeria dothidea* by inserting a 2 mm plug of mycelium taken from a 7 day old colony grown on potato dextrose agar (PDA). Parafilm was wrapped around the trunk to cover drilled holes and maintain moisture within inoculated holes. All treatments were applied on 15 July 2022.

Dieback symptoms were estimated on 27 July and 25 October as percentage of branches with dieback and a visual health score rating on a 1-5 scale as described previously. A portable handheld LI-600 porometer system integrated with a fluorometer (LI-COR Biosciences, Lincoln, USA) was used to measure stomatal conductance, transpiration, electron transport rate, leaf temperature, and quantum efficiencies of photosynthetic electron transport through photosystem 2 (PhiPS2) of 6 light-adapted leaves per tree between the hours of 10:00 and 13:00, 1 day before initiation of experiment and 14, 21, and 28 days after initiation of experiment.

A final destructive assessment was made on 22 December to examine the success of beetle colonization within the wood tissue and to observe the internal symptoms associated with beetle entries, simulated entries (drilled holes), and drilled holes inoculation with *B. dothidea*. The trees were cut off at the soil line and stored at 4°C for up to a week. The main trunks were dissected to record average diameter and measured the area of discoloration of the stem cross section at: 2-5 cm below the graft union, at the graft union, and 2, 5, and 10 cm above the graft union. For the beetle treatment, the number of successful galleries were recorded by dissecting the stem section using a chisel. Successful gallery establishment was determined as galleries > 5 mm in length with evidence of establishment of the symbiotic fungus, *Ambrosiella* spp., or the presence of offspring (eggs, larvae or multiple adults).

Health scores and trunk diameter were analyzed as a randomized complete block design (RCBD) using the GLIMMIX procedure in SAS (PROC GLIMMIX, V9.4; SAS Institute 2013). Least square means were compared using the lsmeans statement in SAS.

#### **Effect of rootstock and water stress on attraction of ambrosia beetles to apple trees.**

*Flood stress and beetle colonization.* The effect of flood stress on Evercrisp® apple trees grafted to three different dwarfing rootstocks, 'M9.T337', 'B.9', and 'G.41' on ethanol production and ambrosia beetle colonization was evaluated at MHCREC in 2021. Flood stress was imposed on trees (6 trees per rootstock) using the pot-in-pot method described above on 22 May 2021. Four trees per rootstock were watered normally as controls. Tissue samples for ethanol quantification were taken at 2, 8, and 14 days after flood initiation from each tree by taking 4 subsamples of bark and cambium around the lower main stem (5-10 cm above the graft union) using a handheld 5 mm diameter steel hole punch, then the tissue was stored at -20°C until analysis. The holepunch was cleaned with distilled water and dried with a paper tissue between trees.

To measure ethanol content, tissue samples were placed in a 2 mL glass vial and capped with screw cap fitted with a Polytetrafluoroethylene (PTFE) septum. Each sample was incubated for 30 min at 90°C to denature enzymes and pre-heat headspace (Manter and Kelsey 2008). Following incubation, the sample was removed from the water bath then immediately a solid phase micro extraction (SPME) fiber (75 mm fiber (CAR/ PDMS; 75 µm coating; Sigma-Aldrich, St. Louis, Missouri) was inserted through the septum and exposed to the headspace within the vial for 5 min at room temperature. The SPME fiber was then thermally desorbed at 225°C into the injection port of an gas chromatograph with a Flame Ionizing Detector (FID) (Agilent 8860, Agilent Technologies, Palo Alto, CA) with a SPME-liner (0.75 mm × 6.35 mm × 78.5 mm, i.d. × o.d. × length; Restek, Bellefonte, Pennsylvania) under splitless mode. A DB-5ms column (30 m, 0.25 mm i.d., 0.25 µm thickness) with an initial temperature of 40°C was held for 2 min, then ramped at 15°C/min to 225°C, then held for 7 min with nitrogen as carrier gas set at a 1.2 ml/min flow rate. The quantity of ethanol in the tissue was calculated based on an external standard curve ranging from 0 -1000 ppm ethanol in water. For the standard curve, 10 µL of each concentration was pipetted into a 2 mL glass vial with a 5 mm diameter piece of Whatman filter paper.

To test the effect of flood-stress on beetle colonization, 10 female *Xylosandrus crassiusculus* beetles obtained from a laboratory colony were caged to the tree using the same methods as described above. Caged beetles were arranged on the lower 50 cm of the main trunk in pairs positioned on opposing sides of the trunk with each pair spaced 3-8 cm apart vertically (Fig. 2). Each cage was inspected for beetle entry at 1 and 3 days after introduction and removed after 3 days.

Ethanol was not detected and no beetles entered on control trees so they were excluded from the analysis. Ethanol content (µg EtOH/g tissue) for each rootstock were log transformed and subjected to an ANOVA for each collection date in SAS (SAS V9.4; SAS Institute 2013). The number of beetle attacks per tree by treatment were subjected to ANOVA in SAS.

*Drought and Flood Stress Experiment.* A multi-season experiment was conducted from 2021-2022 to evaluate the effect of chronic drought stress on tree health and resilience to flood stress using 2 year old potted 'Evercrisp' apple trees. The experiment consisted of 6 replicates of 12 treatments in a 2 x 2 x 3 factorial design with the first factor being late summer drought versus non-drought (i.e. well-watered) imposed August-September 2021, the second factor was flood stress (flooded versus normal watering) imposed in spring 2022, and the third was rootstock (G.41, Bud.9, or M9.t337).

To induce drought stress, trees were placed in a plastic covered hoop house to protect from rainfall and given limited water to maintain at or below 50% field capacity based on soil moisture measurements from 24 August to 17 September 2021. Field capacity was established by taking moisture measurements 12 hours after saturating the media of 10 potted trees. Media moisture was measured for the pots twice per week by taking the average of three points around the surface of the pot using Time Domain Reflectometry (TDR) using a Field Scout TDR 350 digital sensor with 70 mm probes (Spectrum Technologies, Aurora, IL, USA) (SI Fig. 1). Trees were individually watered with approximately 1 L of water when they reached 15 % v/v or lower. Flood stress was imposed on 14 June 2022 by saturating the potting media until there was standing water on the surface using the pot-in-pot method described above. Flood stress was removed after 14 days and returned to normal watering regime on microsprinklers.

To assess tree health each tree was assigned a health score rating as described above and estimated dieback symptoms 10 and 5 days before flooding (DBF) and 1, 6, 10 and 17 days after flooding was imposed (DAF). Leaf condition was assessed using a LICOR-600 fluorometer/porometer (Licor, Lincoln, NE) to measure light-adapted photosynthetic capacity, leaf transpiration, and leaf temperature on 6 fully exposed leaves per tree (3<sup>rd</sup> or 4<sup>th</sup> unfolded leaf on extension shoots selected). To determine ethanol content, tissue samples were taken, using methods described above, from 5 cm above the graft

union on 23 June, 9 days after flood initiation and stored at -20°C before being analyzed as described above.

Leaf physiological parameters with ambient temperature as a covariate, tissue ethanol concentration, visual health score, and trunk diameter growth were analyzed as a three-way factorial using PROC GLM in SAS.

## Results

### **Effect of ambrosia beetle colonization on apple tree health and mortality**

*2020 Beetle Exclusion Field Experiment.* Apple trees were attacked by natural populations of ambrosia beetles at a relatively low rate of  $0.8 \pm 0.29$  entries per tree on the flooded treatment and  $2.3 \pm 0.88$  entries per tree on the ethanol-drenched treatments (Fig. 3). Trees excluded from beetle attack and the no stress treatment were not attacked by ambrosia beetles.

On all observations dates, imposed stress had a significant effect on tree health rating (Table 1). Both flooding and drenching with ethanol had a significant negative impact on tree health compared with no-stress control. The majority of these trees showed signs of leaf necrosis and decreased visual health scores in the summer following treatment and the following spring (Table 1). There was no significant treatment effect of beetle exposure or exposure x stress interaction effect on tree health on any of the observation dates. There was a significant effect of stress on tree mortality observed in fall 2020 (Table 1), but no significant effect of beetle exposure or interaction of exposure x stress. Flooded and Ethanol-drenched trees resulted in significantly higher mortality than non-stressed trees but there was no significant difference in mortality between flooded and ethanol-drenched trees. Nearly half of the flood-stressed trees died by the fall of 2020, and 15 of the 20 trees were dead in the late spring of the following year. Trees that were exposed to beetle attack numerically had higher mortality than beetle-excluded trees, but these differences were not significant.

*2021 Beetle Exclusion Field Experiment.* Ambrosia beetle entries on exposed, flooded trees was low with an average of  $2.8 \pm 0.6$  beetle entries per tree one week after flooding and  $4.2 \pm 3.3$  after three weeks. Beetle exclusion by wrapping LLIN around the trunks of flooded trees effectively excluded beetle entry on the covered portion of the trunk but there were a small number of attacks on the upper trunk portions not protected by the netting (avg.  $0.7 \pm 0.1$  entries per tree after 3 weeks). A single entry was recorded on the no-stress treatment. Leaves of flooded trees appeared wilted, chlorotic and necrosis developed over the 15 week observation period. There was a significant treatment effect on the mean health scores, percent defoliation, and percent dieback (Table 2). The mean visual health score was significantly lower, and defoliation and dieback was significantly higher on flooded trees compared with the non-flooded control. No significant differences were observed between the beetle-exposed and beetle-excluded flooded treatments for these parameters. Similarly, flooded, exposed trees had 50-55% mortality compared with 45-50% mortality on flooded trees with exclusion netting and 0% mortality for normal watered control 42-133 daf. The natural levels of ambrosia beetle colonization did not appear to impact the health and mortality of apple trees flooded for 13 days.

*2022 Beetle Colonization Field Experiment.* Ethanol-irrigated 'MAIA-1' trees subjected to damage by i) *X. crassiusculus* tunneling, ii) simulated attack with 20, 2 mm diameter drilled holes, or 20, 2mm diameter drilled holes inoculated with *B. dothidea* mycelium had slightly lower visual health scores compared with the non-damaged untreated control 10, and 102 days after damage treatments were initiated. The stem diameter at 5 cm above the graft union changed very little in the untreated control, the trees with sterile and inoculated drilled holes had significantly more stem growth than the untreated control. None of the damage treatments caused severe decline in the treated trees (Table 3). No significant differences among treatments were observed for stomatal conductance, leaf transpiration, quantum efficiency of PSII, or electron transport rate on any of the dates measured or for any of the treatments in this experiment (Fig. 4). There was a significant effect of damage treatment on internal

browning of sapwood tissue (Table 4, Fig. 5). Trees with holes inoculated with *B. dothidea* or exposed to *X. crassiusculus* tunneling had the highest cross-sectional area with discoloration above and below the graft union and caused significantly more discoloration compared to the untreated control and sterile holes. Longitudinal cross sections of damaged stem and graft union showed the same trend, with a high proportion of internal browning resulting from the *B. dothidea* inoculation or *X. crassiusculus* tunneling (Fig. 5).

#### **Effect of rootstock and water stress on attraction of ambrosia beetles to apple trees.**

*2021 Flood-stress Experiment.* Flooding ‘Evercrisp’ apple trees increased ethanol in tissue on almost all trees on each observation date and for each rootstock. There were no significant differences among the rootstocks (B.9, G.41, and M9 t337) for tissue ethanol measured 2, 8, or 15 days after flooding (daf). The average ethanol content in tissues was higher on 2 and 8 days after flooding compared with 15 days after flooding (Table 5). The number of *X. crassiusculus* caged to trees that burrowed into the flooded trees was highly variable across all rootstocks and no significant effect of rootstock was shown. Ethanol was not detected on non-flooded control trees and no beetles colonized the control trees.

*2022 Drought x Flood-stress Experiment.* Flood-stress and the interaction of flood-stress and fall drought had a significant effect on stomatal conductance 6, 10, and 17 DAF (Table 6). Flood stressed trees had significantly lower stomatal conductance than non-flooded trees and although trees subjected to drought and flood stress had the lowest stomatal conductance, it was not significantly different from flood-stressed trees that were not drought stressed in the fall (Table 7). Similarly, there was a significant effect of flooding and interaction of flood and drought on the estimated transpiration rate 6, 10, and 17 daf. Flood-stressed trees had significantly lower transpiration rate on average with drought-stressed and flooded trees having the lowest mean transpiration rate 6-17 daf. There was also a significant effect of

fall drought on transpiration rate 5 dbf and 1 DAF and a significant interaction effect of rootstocks and flooding 17 daf.

Leaf photosynthetic capacity as measured by quantum efficiency of PSII and electron transport rate was highly variable across sample dates (Fig. 7) and among trees. The variable field conditions likely muted treatment effects due to variation in light and temperature conditions. There was no significant main effects or significant interactions 10 and 5 days before flooding (Table 7). Flooded trees had lower mean quantum efficiency of PSII than non-flooded trees at 10 ( $1.10 \pm 0.17$  vs  $1.15 \pm 0.17$ ) and 17 ( $0.49 \pm 0.03$  vs  $0.65 \pm 0.03$ ) days after flooding and there was a significant rootstock x flood interaction 1 day after flooding. There were no significant or two-way effects on the electron transport rate (ETR) for any of the observation dates. The only significant effect was the three-way interaction of rootstock, flood, and drought at 17 daf.

Flood stressed trees resulted in significantly higher mean tissue ethanol concentration (d.f. = 1, 43,  $F = 23.46$ ,  $p < 0.0001$ ) with flood stressed trees having significantly higher ethanol than non-flooded trees (Fig. 9). There was no significant effect of fall drought stress or interactions with drought stress and other factors on tissue ethanol. There was a significant interaction effect of rootstock x flood-stress (d.f. = 1, 43,  $F = 7.23$ ,  $p = 0.0016$ ) flood-stressed trees grafted to 'B.9' rootstock had the highest ethanol content 8 DAF followed by flood-stressed trees grafted to 'M.9' rootstock which were both significantly higher than flood-stressed trees grafted to G.41. The average ethanol content in trees subjected to flood stress was greater than non-flooded trees on 'B.9' and 'M.9' rootstocks and for 'G.41' tended to be lower but was not significantly different from the other rootstocks.

## Discussion

These studies showed that apple trees grown on dwarfing rootstocks subjected to flood stress had increased tissue ethanol, which induced attacks by ambrosia beetles under natural and no-choice

conditions, consistent with other studies (Schaffer et al. 1992, Agnello et al. 2017, 2021, Reding et al. 2021). We also observed that flood stress had a marked negative impact on the health of apple trees as measured by visual health scores and tree mortality across all root stocks tested. Previous work has shown that apple trees subjected to hypoxic conditions in the field during spring and summer can reduce tree growth and yield (Olien 1987), lower stem water potential and stomatal conductance (Olien 1989), and increased abscisic acid, reduced PSII absorption, stomatal conductance, and photosynthetic acquisition within a relatively short period of hypoxic root conditions (Bhusal et al. 2020, 2023). ‘Evercrisp’ grafted to ‘G.41’ tended to be more tolerant to flood stress in our experiments, exhibiting slightly lower ethanol content and a less pronounced impact on stomatal conductance compared to ‘M.9’ and ‘B.9’ rootstocks. ‘M.9’ is the only rootstock used in our study that others have evaluated for tolerance to flood stress, and it is considered to be sensitive relative to other dwarfing rootstocks (Choi et al. 2020, Marchiorette et al. 2018). The field conditions used in our study lead to highly variable results, future studies conducted in controlled environment could provide improved resolution on differences in to flood stress tolerance among rootstocks.

Drought stress can be a major limiting factor for apple production (Webster 1997) and under severe cases can kill trees. It has been implicated as a factor in RAD (Singh et al. 2019, Xu et al. 2023). Drought and heat stress is considered to be a major driver of tree mortality globally as a result of changing precipitation patterns from climate change (Choat et al. 2018, Preisler et al. 2021). Despite the severe stress inflicted by drought stress, ambrosia beetles have not been observed colonizing drought-stressed trees (Frank and Ranger 2016, Ranger et al. 2023). While *X. germanus* were observed to be attracted to drought-stressed *Cornus florida* trees, very few successfully tunnel into trees. Furthermore, during initial investigations with drought-stressed apple trees, *X. crassiusculus* females failed to enter drought stressed apple trees under no-choice conditions (Unpublished). Fall drought did not appear to impact the leaf physiological health before and after flooding and did not appear to exacerbate the

effect of flood-stress on leaf health or ethanol production on 'MAIA-1' apple trees. The intensity of drought stress may have been insufficient in this case to provoke long-lasting impacts. Future studies using a larger sample size and controlled environment may better elucidate if there is a compounding effect of drought stress and other abiotic stressors across growing seasons.

While flood stress had a negative impact on tree health, increased ethanol production, and elicited ambrosia beetle attack, no significant effect of beetle attacks, primarily by *X. crassiusculus*, was detected. However, there was an apparent trend of higher tree mortality in flood-stressed trees exposed versus not exposed to ambrosia beetles. Internal browning in the heartwood of scions colonized by *X. crassiusculus* caused extensive browning that was the same as that caused by necrotrophic fungi *B. dothidea* inoculated in drilled holes, but the implications of these symptoms are unknown. Apple trees are resilient to internal damage; in one study, apple trees with up to 50% of sapwood with internal injury from freeze stress showed no negative impacts on health and mortality (Warmund et al. 1996), and simulated ambrosia attack with up to 100 drilled holes did not negatively impact peach tree growth (Kovach 1986), but these holes were not inoculated with a pathogen. In addition, tree health of *C. florida* subjected to varying durations of flood stress and exposed to natural populations of *X. germanus* were more strongly affected by flood duration than by the intensity of beetle attack (Reding et al. 2021).

The relationship between ambrosia beetles and tree decline in the absence of a known pathogen is difficult to explain due to the confounding effects of the physiological stressors that induce beetle attacks. The process of decline and death of trees following stress events can be prolonged and multidimensional (Whyte et al. 2016). In severe drought-stressed conifers, for example, ethanol accumulated within tissues (Kelsey et al. 2014) and significant changes in growth and sap flow preceded visual signs of mortality by up to 6 months (Preisler et al. 2021). Visible symptoms of decline were not always evident on flood-stressed hardwood tree species, despite elevated ethanol content and attack by

ambrosia beetles (Ranger et al. 2021, Reding et al. 2021). The mechanical damage inflicted upon the vascular system resulting from gallery excavation and colonization of the symbiotic fungus could block the plants vascular system and could encourage invasion of phytopathogenic fungi (Dute et al. 2002). Clearly further studies are needed to differentiate the impact of the underlying tree stressors and ambrosia beetles indirectly attracted to the stressed trees on the decline of apple trees. Understanding these relationships is important to help guide the need for management of ambrosia beetles in high density apple orchards.

## References

- Agnello, A., D. Breth, E. Tee, K. Cox, and H. R. Warren. 2015.** Ambrosia Beetle – An Emergent Apple Pest. 2013–2016.
- Agnello, A. M., D. I. Breth, E. M. Tee, K. D. Cox, S. M. Villani, K. M. Ayer, A. E. Wallis, D. J. Donahue, D. B. Combs, A. E. Davis, J. A. Neal, and F. M. English-Loeb. 2017.** *Xylosandrus germanus* (Coleoptera: Curculionidae: Scolytinae) Occurrence, Fungal Associations, and Management Trials in New York Apple Orchards. *J. Econ. Entomol.* 110: 2149–2164.
- Agnello, A. M., D. B. Combs, C. C. Filgueiras, D. S. Willett, and A. Mafra-Neto. 2021.** Reduced Infestation by *Xylosandrus germanus* (Coleoptera: Curculionidae: Scolytinae) in Apple Trees Treated with Host Plant Defense Compounds. *J. Econ. Entomol.* 114: 2162–2171.
- Avalos-Ruiz, D., L. N. Ten, C. K. Kim, S. Y. Lee, and H. Y. Jung. 2022.** Isolation and Identification of Ice Nucleation Active *Fusarium* Strains from Rapid Apple Declined Trees in Korea. *Plant Pathol. J.* 38: 403–409.
- Bhusal, N., H. S. Kim, S. G. Han, and T. M. Yoon. 2020.** Photosynthetic traits and plant–water relations of two apple cultivars grown as bi-leader trees under long-term waterlogging conditions. *Environ. Exp. Bot.* 176: 104111.
- Bhusal, N., I. H. Park, S. Jeong, B. H. Choi, S. G. Han, and T. M. Yoon. 2023.** Photosynthetic traits and plant hydraulic dynamics in Gamhong apple cultivar under drought, waterlogging, and stress recovery periods. *Sci. Hortic. (Amsterdam).* 321: 112276.
- Choat, B., T. J. Brodribb, C. R. Brodersen, R. A. Duursma, R. López, and B. E. Medlyn. 2018.** Triggers of tree mortality under drought.

**Contarini, M., A. Vannini, F. Giarruzzo, M. Faccoli, C. Morales-Rodriguez, L. Rossini, and S. Speranza. 2020.** First record of *Xylosandrus germanus* (Blandford) (Coleoptera: Curculionidae, Scolytinae) in the Mediterranean scrubland in Southern Italy, and its co-presence with the co-generic species *X. compactus* (Eichhoff) and *X. crassiusculus* (Motschulsky). *EPP0 Bull.* 50: 311–315.

**Donahue, D. J., and S. E. Elone. 2021.** Case Study of a Declining Apple Orchard Daniel J. Donahue and Sarah E. Elone. *New York Fruit Q. - Summer.* 29: 28–31.

**Dute, R. R., M. E. Miller, M. A. Davis, F. M. Woods, and K. S. McLean. 2002.** Effects of ambrosia beetle attack on *Cercis canadensis*. *IAWA J.* 23: 143–160.

**Ferner, E., H. Rennenberg, and J. Kreuzwieser. 2012.** Effect of flooding on C metabolism of flood-tolerant (*Quercus robur*) and non-tolerant (*Fagus sylvatica*) tree species. *Tree Physiol.* 32: 135–145.

**Frank, S. D., and C. M. Ranger. 2016.** Developing a Media Moisture Threshold for Nurseries to Reduce Tree Stress and Ambrosia Beetle Attacks. *Environ. Entomol.* 45: 1040–1048.

**Galko, J., M. Dzurenko, C. M. Ranger, J. Kulfan, E. Kula, C. Nikolov, M. Zúbrik, and P. Zach. 2018.** Distribution, habitat preference, and management of the invasive ambrosia beetle *xylosandrus germanus* (Coleoptera: Curculionidae, Scolytinae) in European forests with an emphasis on the West Carpathians. *Forests.* 10.

**Gresham, S. D. M., J. Walgenbach, and S. M. Villani. 2023.** Biotic Factors Associated with Rapid Apple Decline in North Carolina. prep.

**Gugliuzzo, A., P. H. W. Biedermann, D. Carrillo, L. A. Castrillo, J. P. Egonyu, D. Gallego, K. Haddi, J. Hulcr, H. Jactel, H. Kajimura, N. Kamata, N. Meurisse, Y. Li, J. B. Oliver, C. M. Ranger, D. Rassati, L. L. Stelinski, R. Sutherland, G. Tropea Garzia, M. G. Wright, and A. Biondi. 2021.** Recent

advances toward the sustainable management of invasive *Xylosandrus ambrosia* beetles. *J. Pest Sci.* (2004). 94: 615–637.

**Gugliuzzo, A., G. Criscione, A. Biondi, D. Aiello, A. Vitale, G. Polizzi, and G. Tropea Garzia. 2020.** Seasonal changes in population structure of the ambrosia beetle *Xylosandrus compactus* and its associated fungi in a southern Mediterranean environment. *PLoS One.* 15: 1–14.

**Haas, M., J. Wilson, and L. Gut. 2016.** Managing Black Stem Borer in Michigan Tree Fruits. 2.

**Hulcr, J., and L. L. Stelinski. 2017.** The Ambrosia Symbiosis: From Evolutionary Ecology to Practical Management. *Annu. Rev. Entomol.* 62: 285–303.

**Kelsey, R. G., D. Gallego, F. J. Sánchez-García, and J. A. Pajares. 2014.** Ethanol accumulation during severe drought may signal tree vulnerability to detection and attack by bark beetles. *Can. J. For. Res.* 44: 554–561.

**Kelsey, R. G., G. Joseph, D. Westlind, and W. G. Thies. 2016.** Ethanol and acetone from Douglas-fir roots stressed by *Phellinus sulphurascens* infection: Implications for detecting diseased trees and for beetle host selection. *For. Ecol. Manage.* 360: 261–272.

**Kelsey, R. G., and D. J. Westlind. 2017.** Ethanol and primary attraction of red turpentine beetle in fire stressed ponderosa pine. *For. Ecol. Manage.* 396: 44–54.

**Kimmerer, T. W., and T. T. Kozlowski. 1982.** Ethylene, Ethane, Acetaldehyde, and Ethanol Production By Plants under Stress. *Plant Physiol.* 69: 840–847.

**Kovach, J. 1986.** Life cycle, seasonal distribution and tree responses to scolytid beetles in South Carolina peach orchards.

**Liu, H., L. Wu, E. Nikolaeva, K. Peter, Z. Liu, D. Mollov, M. Cao, and R. Li. 2018.** Characterization of a new apple luteovirus identified by high-throughput sequencing. *Virology*. 15: 1–9.

**Manter, D. K., and R. G. Kelsey. 2008.** Ethanol accumulation in drought-stressed conifer seedlings. *Int. J. Plant Sci.* 169: 361–369.

**Olien, W. C. 1987.** Effect of Seasonal Soil Waterlogging on Vegetative Growth and Fruiting of Apple Trees. *J. Am. Soc. Hortic. Sci.* 112: 209–214.

**Olien, W. C. 1989.** Seasonal Soil Waterlogging Influences Water Relations and Leaf Nutrient Content of Bearing Apple Trees. *J. Am. Soc. Hortic. Sci.* 114: 537–542.

**Peter, K. A. 2021.** Apple Disease - Rapid Apple Decline. Penn State Ext.

**Preisler, Y., F. Tatarinov, J. M. Grünzweig, and D. Yakir. 2021.** Seeking the “point of no return” in the sequence of events leading to mortality of mature trees. *Plant Cell Environ.* 44: 1315–1328.

**Ranger, C. M., M. Parajuli, S. Gresham, J. Barnett, S. Villani, J. Walgenbach, F. Baysal-Gurel, J. S. Owen, and M. E. Reding. 2023.** Type and duration of water stress influence host selection and colonization by exotic ambrosia beetles (Coleoptera: Curculionidae). *Front. Insect Sci.* 3: 1–11.

**Ranger, C. M., M. E. Reding, K. Adesso, M. Ginzel, and D. Rassati. 2021.** Semiochemical-mediated host selection by *Xylosandrus* spp. ambrosia beetles (Coleoptera: Curculionidae) attacking horticultural tree crops: a review of basic and applied science. 103–120.

**Ranger, C. M., M. E. Reding, A. B. Persad, and D. A. Herms. 2010.** Ability of stress-related volatiles to attract and induce attacks by *Xylosandrus germanus* and other ambrosia beetles. *Agric. For. Entomol.* 12: 177–185.

**Ranger, C. M., M. E. Reding, P. B. Schultz, and J. B. Oliver. 2013.** Influence of flood-stress on ambrosia beetle host-selection and implications for their management in a changing climate. *Agric. For. Entomol.* 15: 56–64.

**Ranger, C. M., M. E. Reding, P. B. Schultz, J. B. Oliver, S. D. Frank, K. M. Adesso, J. H. Chong, B. Sampson, C. Werle, S. Gill, and C. Krause. 2016.** Biology, ecology, and management of nonnative ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) in ornamental plant nurseries. *J. Integr. Pest Manag.* 7.

**Ranger, C. M., P. B. Schultz, S. D. Frank, J. H. Chong, and M. E. Reding. 2015.** Non-native ambrosia beetles as opportunistic exploiters of living but weakened trees. *PLoS One.* 10: 1–22.

**Ranger, C. M., P. B. Schultz, S. D. Frank, and M. E. Reding. 2019.** Freeze stress of deciduous trees induces attacks by opportunistic ambrosia beetles. *Agric. For. Entomol.* 21: 168–179.

**Ranger, C. M., P. C. Tobin, and M. E. Reding. 2015.** Ubiquitous volatile compound facilitates efficient host location by a non-native ambrosia beetle. 675–686.

**Reding, M. E., C. M. Ranger, and P. B. Schultz. 2021.** Colonization of trees by ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) is influenced by duration of flood stress. *J. Econ. Entomol.* 114: 839–847.

**Robinson, T. L., A. M. DeMarree, and S. A. Hoying. 2004.** An economic comparison of five high density apple planting systems., pp. 481–489. *In* VIII Int. Symp. Canopy, Rootstocks Environ. Physiol. Orchard Syst. 732.

**Rosenberger, D. 2019.** Factors Contributing to the Death and Decline of Young Apple Trees. *New York Fruit Q.* 27: 5–8.

**Schaffer, B., P. C. Andersen, and R. C. Ploetz. 1992.** Responses of Fruit Crops to Flooding, pp. 257–313. *In* Janick, J. (ed.), *Hortic. Rev.* Vol. 13.

**Singh, J., K. J. P. Silva, M. Fuchs, and A. Khan. 2019.** Potential role of weather, soil and plant microbial communities in rapid decline of apple trees. *PLoS One.* 14: 1–19.

**Tran, H. X., Doland Nichols, J., Li, D., Le, N. H., Lawson, S. A. 2022.** Seasonal flight and genetic distinction among *Xylosandrus crassiusculus* populations invasive in Australia. *Aust. For.* 85: 224–331.

**Warmund, M. R., W. R. Autio, J. A. Barden, J. N. Cummins, P. A. Domoto, C. G. Embree, R. L. Granger, F. D. Morrison, J. R. Schupp, and E. Young. 1996.** Blackheart injury in ‘Starkspur Supreme Delicious’ on 15 rootstocks in the 1984 NC-140 cooperative planting. *Fruit Var. J.* 55–62.

**Webster, A. D. 1997.** A Review of Fruit Tree Rootstock Research and Development. *Acta Hortic.* 451: 53–74.

**Whyte, G., K. Howard, G. E. S. J. Hardy, and T. I. Burgess. 2016.** The Tree Decline Recovery Seesaw; a conceptual model of the decline and recovery of drought stressed plantation trees. *For. Ecol. Manage.* 370: 102–113.

**Wright, A. A., A. R. Cross, and S. J. Harper. 2020.** A bushel of viruses: Identification of seventeen novel putative viruses by RNA-seq in six apple trees. *PLoS One.* 15: 1–20.

**Xu, H., K. D. Hannam, J. L. MacDonald, and D. Ediger. 2023.** Field Investigation into Tree Fates from Recent Apple Tree Decline: Abrupt Hydraulic Failure Versus Gradual Hydraulic Loss. *Stresses.* 3: 256–269.

## Chapter 5 Tables.

Table 5.1. Mean ( $\pm$  SE) tree health score and percent mortality (% trees dead) on ‘Honey crisp’ apple trees. N = 10 trees/treatment combination subjected to flood stress or ethanol drench and exposed to field populations (“+” beetle exposure) or placed in exclusion cages (“-“ beetle exposure). Experiment commenced 19 June 2020.

Stress	Beetle Exposure	Mean Health score $\pm$ se (1-5 scale)			Mortality (%)		
		20 daf	103 daf	329 daf	103 daf	329 daf	
Ethanol Drench	-	3.2 $\pm$ 0.2	2.7 $\pm$ 0.2	1.8 $\pm$ 0.3	10	50	
	+	3.5 $\pm$ 0.4	2.4 $\pm$ 0.4	2.4 $\pm$ 0.6	30	30	
Flood Stress	-	2.5 $\pm$ 0.4	2 $\pm$ 0.5	1.5 $\pm$ 0.6	40	60	
	+	2.4 $\pm$ 0.4	1.3 $\pm$ 0.6	0.4 $\pm$ 0.6	50	90	
No Stress	-	4.8 $\pm$ 0.3	3.9 $\pm$ 0.5	3 $\pm$ 0.4	0	20	
	+	4.9 $\pm$ 0.1	3.6 $\pm$ 0.2	3.3 $\pm$ 0.6	10	20	
Statistics							
F value	Stress	29.4/<0.000	10.53/0.000	6.71/0.0025	X <sup>2</sup>	9.99	10.56
P value		1	1		P value	0.0068	0.051
F value	Exposure	0.15/0.699	1.34/0.2529	0.02/0.8924	X <sup>2</sup>	1.51	0.24
P value					P value	0.2196	0.621
F value	Stress*exposure	0.20/0.8178	0.13/0.8815	1.14/0.3274	X <sup>2</sup>	1.16	2.90
P value					P value	0.561	0.2341

Health score rated as: 1 = most leaves (>60%) wilted and/or brown; 2 = 40-60% leaves yellow, wilting or browning; 3 = 20-40% leaves with wilting and yellowing; 4 = 5-20% leaves with wilting and marginal yellowing or browning; and 5 = healthy.

Table 5.2. Mean Health Score ( $\pm$  SE), average ( $\pm$  SE) percentage defoliation, percentage ( $\pm$  SE) dieback on apple tree cv. "Honey crisp" (N = 20 trees per treatment) subjected to flooding and either exposed to field populations of ambrosia beetles or with trunks wrapped in LLIN to exclude beetle entry compared with no-stress control. Experiment commenced 19 May 2021.

Treatment	Mean ( $\pm$ SE) Health score <sup>1</sup>					Avg. ( $\pm$ SE) Defoliation (%)	Avg. ( $\pm$ SE) Dieback (%)
	8 daf	13 daf	29 daf	42 daf	133 daf	133 daf	133 daf
Flood + Exclusion	3.9 $\pm$ 0.1a	3.3 $\pm$ 0.2a	2.8 $\pm$ 0.4a	2 $\pm$ 0.5a	1.3 $\pm$ 0.3a	84 $\pm$ 5.4a	57.5 $\pm$ 10.8a
Flood + Exposed	3.7 $\pm$ 0.1a	2.9 $\pm$ 0.2a	1.8 $\pm$ 0.4a	2.2 $\pm$ 0.5a	1.4 $\pm$ 0.3a	85.5 $\pm$ 4.3a	52.8 $\pm$ 10.9a
No-stress (control)	4.7 $\pm$ 0.1b	4.7 $\pm$ 0.1b	4.4 $\pm$ 0.2b	4.8 $\pm$ 0.1b	2.8 $\pm$ 0.1b	67.4 $\pm$ 5.7b	3.5 $\pm$ 1.5b
F value (df 2,38)	18.36	32.10	12.87	17.41	11.27	4.67	13.96
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0153	<0.0001

Different letters within a column indicate significant differences among the mean health score, defoliation or dieback using least square means test,  $\alpha = 0.05$ .

<sup>1</sup> Health score rated as: 1 = most leaves wilted and/or brown; 2 = leaves mostly yellow (>50%), <50% wilting and browning; 3 = 20-40% leaves with wilting and yellowing; 4 = 5-20% leaves with wilting and marginal yellowing or browning; and 5 = healthy

Table 5.3: Mean visual health score ( $\pm$  SE) and mean trunk growth ( $\pm$  SE) on 'Evercrisp' apple trees subjected to damage treatments after irrigating with 2.5% EtOH solution. "Beetle" = 20 *X. crassiusculus* per tree exposed to lower trunk; "Sterile holes" = 20 x 2 mm holes drilled into lower trunk, "Inoculated holes" = 20 x 2mm holes inoculated with *B. dothidea* mycelium.

Treatment	Health score <sup>1</sup> (27 July)	Health score <sup>1</sup> (25 October)	Trunk diameter growth <sup>2</sup> (mm)
Beetle damage	4.0 $\pm$ 0.2b	3.7 $\pm$ 0.4	0.8 $\pm$ 0.2bc
Sterile holes	4.3 $\pm$ 0.2b	3.4 $\pm$ 0.4	1 $\pm$ 0.3b
Inoculated holes	4.0 $\pm$ 0.3b	3.7 $\pm$ 0.2	2 $\pm$ 0.3a
No-damage Control	4.9 $\pm$ 0.1a	4 $\pm$ 0.3	0.2 $\pm$ 0.1c
F value (df 3,38)	4.24	0.59	9.65
P value	0.0112	0.625	<0.0001

Different letters within a column indicate significant differences among the mean health score or trunk diameter growth using least square means test.  $\alpha = 0.05$ .

<sup>1</sup> Health score rated as: 1 = most leaves wilted and/or brown; 2 = leaves mostly yellow (>50%), <50% wilting and browning; 3 = 20-40% leaves with wilting and yellowing; 4 = 5-20% leaves with wilting and marginal yellowing or browning; and 5 = healthy

<sup>2</sup> growth of trunk determined by measuring diameter of trunk at initiation of experiment and again 102 days after initiation.

Table 5.4: Mean ( $\pm$  SE) percentage area of internal discoloration measured on transverse and longitudinal cross sections of apple, cv. 'Evercrisp', subjected to damage treatments after irrigating with 2.5% EtOH solution. "Beetle" = 20 *X. crassiusculus* per tree exposed to lower trunk; "Sterile holes" = 20 x 2 mm holes drilled into lower trunk, "Inoculated holes" = 20 x 2mm holes inoculated with *B. dothidea* mycelium.

Treatment <sup>1</sup>	Transverse cross-sectional discoloration below and above graft union (GU)					Longitudinal discoloration	
	- 2 cm	At GU	+ 2 cm	+ 5 cm	+ 10 cm	Stem	Graft union
Beetle	19.5 $\pm$ 6a	29.6 $\pm$ 5.4	37.5 $\pm$ 8.9ab	53.3 $\pm$ 6.6a	54.4 $\pm$ 3.5a	64.1 $\pm$ 8.7a	18.6 $\pm$ 5.6a
Sterile holes	10.1 $\pm$ 3.2ab	22.2 $\pm$ 3	32.6 $\pm$ 6.5b	25.9 $\pm$ 7b	5.8 $\pm$ 5.8c	20 $\pm$ 3.3b	9.6 $\pm$ 4.1ab
Inoculated holes	14.8 $\pm$ 4.8a	30.9 $\pm$ 9.7	52.1 $\pm$ 5.1a	60.6 $\pm$ 5.5a	37.1 $\pm$ 8.3b	63 $\pm$ 7.6a	14.2 $\pm$ 3.9a
Non-damaged control	0.7 $\pm$ 0.7b	10.8 $\pm$ 3.3	1.1 $\pm$ 1.1c	0 $\pm$ 0c	1.7 $\pm$ 1.7c	0.4 $\pm$ 0.3c	0.8 $\pm$ 0.5b
F value (df 3,36)	3.57	2.20	13.48	23.11	20.97	28.36	4.09
P value	0.023	0.1045	<0.0001	<0.0001	<0.0001	<0.0001	0.0135

Different letters within a column indicate significant differences among the mean area of internal discoloration using least square means test,  $\alpha$  = 0.05.

Table 5.5: Mean ( $\pm$  SE) ethanol concentration and avg. ( $\pm$  SE) number of beetle entries per ten caged to tree on flood-stressed potted apple trees, cv. "MAIA-1", on three different rootstocks. (n=6/treatment)

Rootstock	Mean Ethanol Concentration ( $\mu\text{g EtOH/g tissue} \pm \text{SE}$ )			Beetles Entered <sup>1</sup>
	2 daf <sup>2</sup>	8 daf <sup>2</sup>	15 daf <sup>2</sup>	10 daf <sup>2</sup>
B.9	42.8 $\pm$ 16.9	64.4 $\pm$ 21.8	20.8 $\pm$ 12.1	4.0 $\pm$ 1.2
G.41	82.8 $\pm$ 14	16.3 $\pm$ 8.7	5.0 $\pm$ 3.6	2.3 $\pm$ 1.0
M.9	35.0 $\pm$ 11.3	40.6 $\pm$ 11.4	1.2 $\pm$ 0.3	2.5 $\pm$ 1.3
All RS	54.2 $\pm$ 9.2	40.4 $\pm$ 9.5	9.0 $\pm$ 4.5	2.9 $\pm$ 0.7
F value (df 2,5)	1.56	2.54	2.02	0.58
p value	0.24	0.11	0.17	0.57

<sup>1</sup>ten *X. crassiusculus* per tree were placed in individual cages 7 days after flooding.

<sup>2</sup>daf = days after flooding initiated.

Table 5.6: Effect of drought stress ('drought'), rootstock genotype ("rs") and flood stress ("flood") on stomatal conductance and transpiration rate observed on 'Evercrisp' apple trees (n = 6 trees per treatment combination). Transpiration rate measured on 6 leaves per tree with Licor 600 porometer/flourometer 1-16 days after flooding.

Parameter	10 dbf		5 dbf		1 daf		6 daf		10 daf		17 daf	
	F-value	P	F-value	P	F-value	P	F-value	P	F-value	P	F-value	P
Stomatal conductance												
rs	0.21	0.8103	0.9	0.4143	0.29	0.7512	0.22	0.805	1.05	0.3562	2.71	0.0749
drought	0.01	0.9564	3.78	0.057	3.63	0.0616	0.39	0.5365	0.4	0.527	0.02	0.8988
flood	0.33	0.5695	1.92	0.1713	2.5	0.1193	<b>15.83</b>	<b>0.0002</b>	<b>25.98</b>	<b>&lt;.0001</b>	<b>45.26</b>	<b>&lt;.0001</b>
rs*drought	0.07	0.9352	0.55	0.5824	0.84	0.4354	0.23	0.796	1.14	0.3256	0.58	0.5619
rs*flood	0.23	0.7973	0.25	0.7795	1.9	0.1584	1	0.3747	1.74	0.1847	2.58	0.0842
drought*flood	0.06	0.7999	2.54	0.1162	0.12	0.7323	<b>4.53</b>	<b>0.0378</b>	<b>6.57</b>	<b>0.0129</b>	<b>8.16</b>	<b>0.0059</b>
rs*drought*flood	0.06	0.9464	0.13	0.8747	1.95	0.1515	0.9	0.4129	1.67	0.1978	1.02	0.3671
Transpiration												
rs	0.11	0.9003	0.56	0.5739	0.82	0.4453	0.03	0.9684	1	0.3753	1.89	0.1598
drought	0.06	0.8083	<b>5.96</b>	<b>0.0177</b>	<b>6.73</b>	<b>0.0119</b>	0.11	0.746	0.33	0.5686	0.03	0.8551
flood	1.39	0.2432	0.7	0.4066	0.91	0.344	<b>18.23</b>	<b>&lt;.0001</b>	25.7	<b>&lt;.0001</b>	<b>45.91</b>	<b>&lt;.0001</b>
rs*drought	0.21	0.8114	0.52	0.5995	1.16	0.3219	0.02	0.9848	1.65	0.2004	0.53	0.59
rs*flood	1.45	0.2439	0.11	0.895	1.93	0.154	2.26	0.1135	3.06	<b>0.0545</b>	<b>4.41</b>	<b>0.0165</b>
drought*flood	0.03	0.8666	2.32	0.1331	0.02	0.8908	<b>4.48</b>	<b>0.0387</b>	3.04	<b>0.0863</b>	<b>7.23</b>	<b>0.0093</b>
rs*drought*flood	0.5	0.6063	0.15	0.8617	1.3	0.2795	1.3	0.2798	1.11	0.3363	0.63	0.5347

Table 5.7. Mean ( $\pm$  SE) stomatal conductance and Mean ( $\pm$  SE) leaf transpiration rate of potted apple (cv Evercrisp) subjected to drought stress and flood stress combinations. (n = 6 trees per treatment combination). Transpiration rate measured on 6 leaves per tree with Licor 600 porometer/flourometer.

Drought stress	Flood stress	Stomatal Conductance (Mol m <sup>-2</sup> s <sup>-1</sup> )				Transpiration (mMol m <sup>-2</sup> s <sup>-1</sup> )			
		01 daf	06 daf	10 daf	17 daf	01 daf	06 daf	10 daf	17 daf
+	+	1.19 $\pm$ 0.26	0.26 $\pm$ 0.13a	0.02 $\pm$ 0.09a	0.33 $\pm$ 0.15a	5.43 $\pm$ 1.48	0.89 $\pm$ 0.31a	0.64 $\pm$ 0.18a	0.87 $\pm$ 0.31a
+	-	1.26 $\pm$ 0.27	0.38 $\pm$ 0.13b	0.18 $\pm$ 0.09d	0.6 $\pm$ 0.15d	5.79 $\pm$ 1.52	2.92 $\pm$ 0.32b	1.85 $\pm$ 0.18b	3.7 $\pm$ 0.31b
-	+	1.14 $\pm$ 0.27	0.29 $\pm$ 0.13a	0.06 $\pm$ 0.09ab	0.4 $\pm$ 0.16ab	4.21 $\pm$ 1.53	1.46 $\pm$ 0.32a	0.93 $\pm$ 0.18a	1.66 $\pm$ 0.31a
-	-	1.18 $\pm$ 0.27	0.32 $\pm$ 0.13b	0.11 $\pm$ 0.09c	0.51 $\pm$ 0.16c	4.7 $\pm$ 1.53	2.14 $\pm$ 0.33b	1.54 $\pm$ 0.18b	2.91 $\pm$ 0.31b
Avg. +/-	+	1.17 $\pm$ 0.26	0.27 $\pm$ 0.13	0.04 $\pm$ 0.09	0.37 $\pm$ 0.15	4.82 $\pm$ 1.47	1.17 $\pm$ 0.22	0.78 $\pm$ 0.13	1.27 $\pm$ 0.22
Avg. +/-	-	1.22 $\pm$ 0.27	0.35 $\pm$ 0.13	0.14 $\pm$ 0.09	0.56 $\pm$ 0.16	5.24 $\pm$ 1.49	2.53 $\pm$ 0.23	1.69 $\pm$ 0.13	3.31 $\pm$ 0.22

Means followed by a different letter within each column indicate significant difference using least square means test.

Table 5.8: Effect of drought stress (“drought”), rootstock genotype (rs) and flood stress (“flood”) on quantum efficiency of Photosystem-II measured with Licor 600 on 6 leaves per tree on ‘Evercrisp’ apple trees.

Effect	10 dbf		5 dbf		1 daf		6 daf		10 daf		17 daf	
	F	P	F	P	F	P	F	P	F	P	F	P
	PS-II											
rs	0.02	0.9839	1.03	0.3649	4.84	0.0113	0.15	0.8574	0.38	0.6827	1.69	0.1936
drought	0.54	0.4657	0.62	0.4339	1.56	0.2173	0.32	0.5716	1.25	0.2674	3.32	0.0733
flood	0.26	0.615	0	0.9705	0.49	0.4853	1.52	0.2233	<b>5.04</b>	<b>0.0285</b>	<b>17.08</b>	<b>0.0001</b>
rs*drought	1.83	0.1694	2.48	0.0927	0.94	0.3966	1.05	0.3566	1.03	0.3634	1.83	0.1698
rs*flood	0.26	0.7703	1.92	0.1562	<b>3.62</b>	<b>0.0328</b>	0.59	0.5595	0.99	0.3792	2.45	0.0947
drought*flood	0.01	0.9067	0.13	0.7195	0.65	0.4225	3.18	0.0802	2.96	0.0904	3.18	0.0797
rs*drought*flood	0	0.9998	0.14	0.8704	1.26	0.2899	2.8	0.0694	0.21	0.8127	0.36	0.6969
	ETR											
rs	0.31	0.7355	1.96	0.1501	0.83	0.4394	0.59	0.5554	1.67	0.1975	0.27	0.7626
drought	0.59	0.4465	0.32	0.5751	3.38	0.0709	0.14	0.7084	0	0.9948	1.22	0.2735
flood	0.12	0.7323	6.12	0.0164	0.01	0.9363	2.19	0.1448	2.36	0.1297	3.69	0.0595
rs*drought	0.79	0.4604	0.57	0.5661	0.37	0.6932	2.42	0.0986	0.94	0.3963	0.26	0.7743
rs*flood	0.1	0.9018	0.42	0.6563	0.52	0.5993	1.07	0.3496	1.2	0.3081	2.45	0.0953
drought*flood	0.26	0.6108	0.56	0.4579	0.35	0.5569	0.24	0.6263	0.85	0.3607	2.95	0.091
rs*drought*flood	1.15	0.3221	0.34	0.7129	0.89	0.4173	2.66	0.0791	0.74	0.4803	<b>3.64</b>	<b>0.0322</b>

Significant effects highlighted in bold.

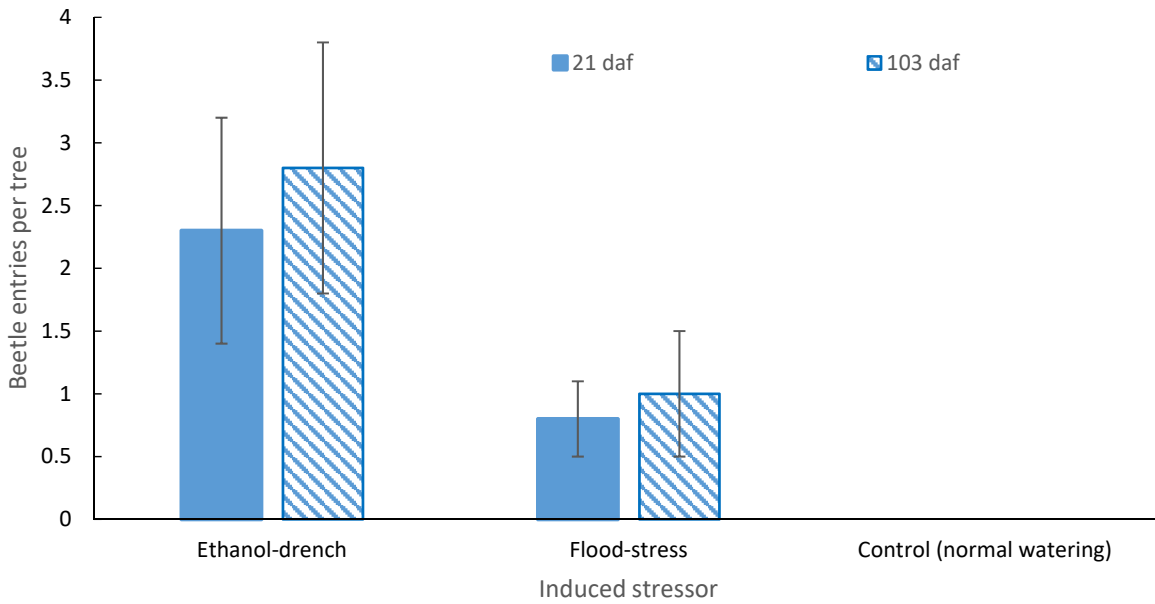
## Chapter 5 Figures.



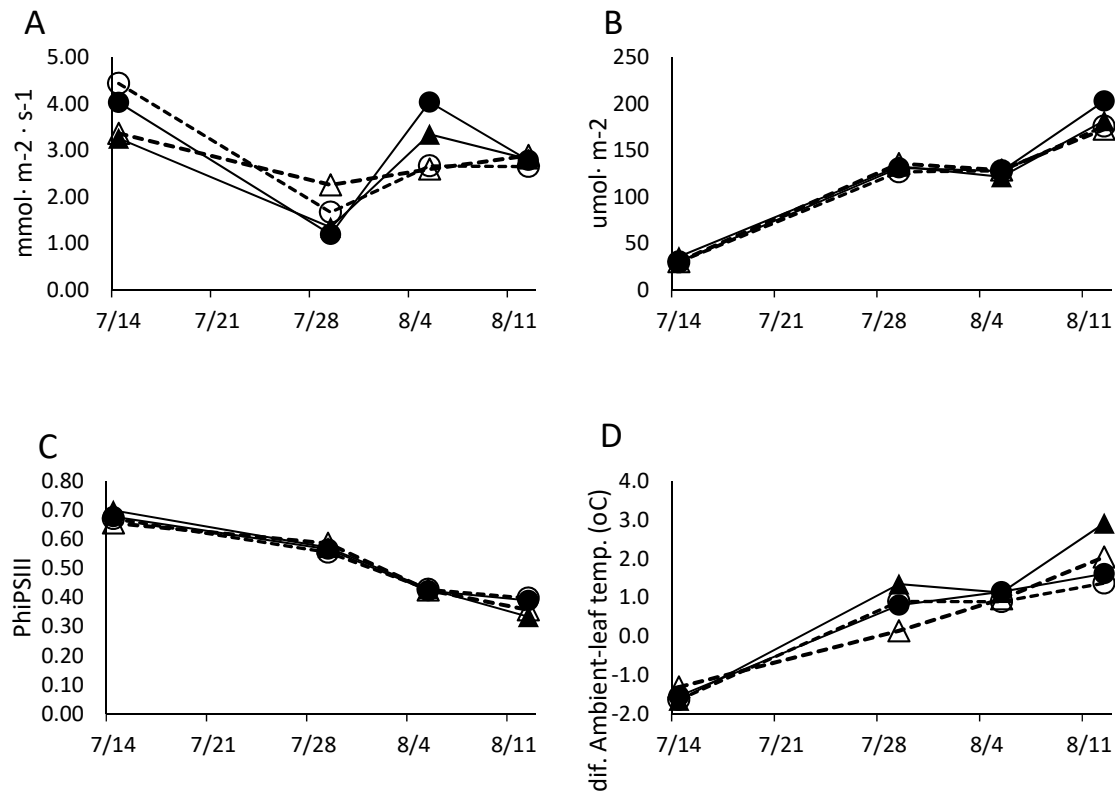
**Fig.5-1.** Beetle exclusion cage (left) with flooded, ethanol-drenched, and non-stressed control trees and open-sided shade structure with same arrangement (right)



**Fig. 5-2.** Cages attached to trunk for no-choice ambrosia beetle assay on flooded apple trees.



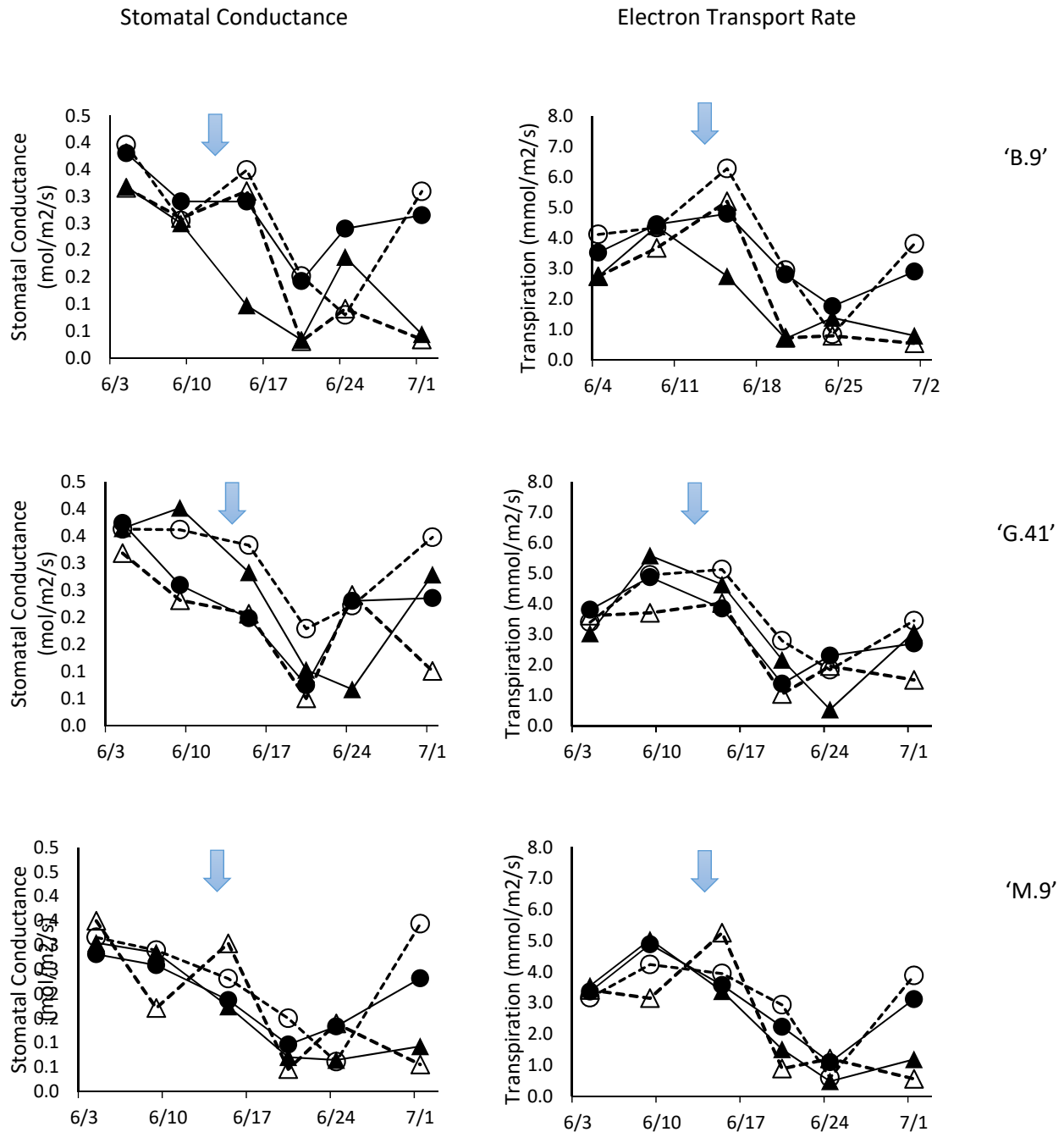
**Fig. 5-3.** Mean ( $\pm$  SE) ambrosia beetle entries per tree on ‘Honey crisp’ apple trees subjected to flood stress, ethanol drench or no stress (regular watering) exposed to natural population of ambrosia beetles at MHCREC on 21 June 2020. n = 10 trees per treatment.



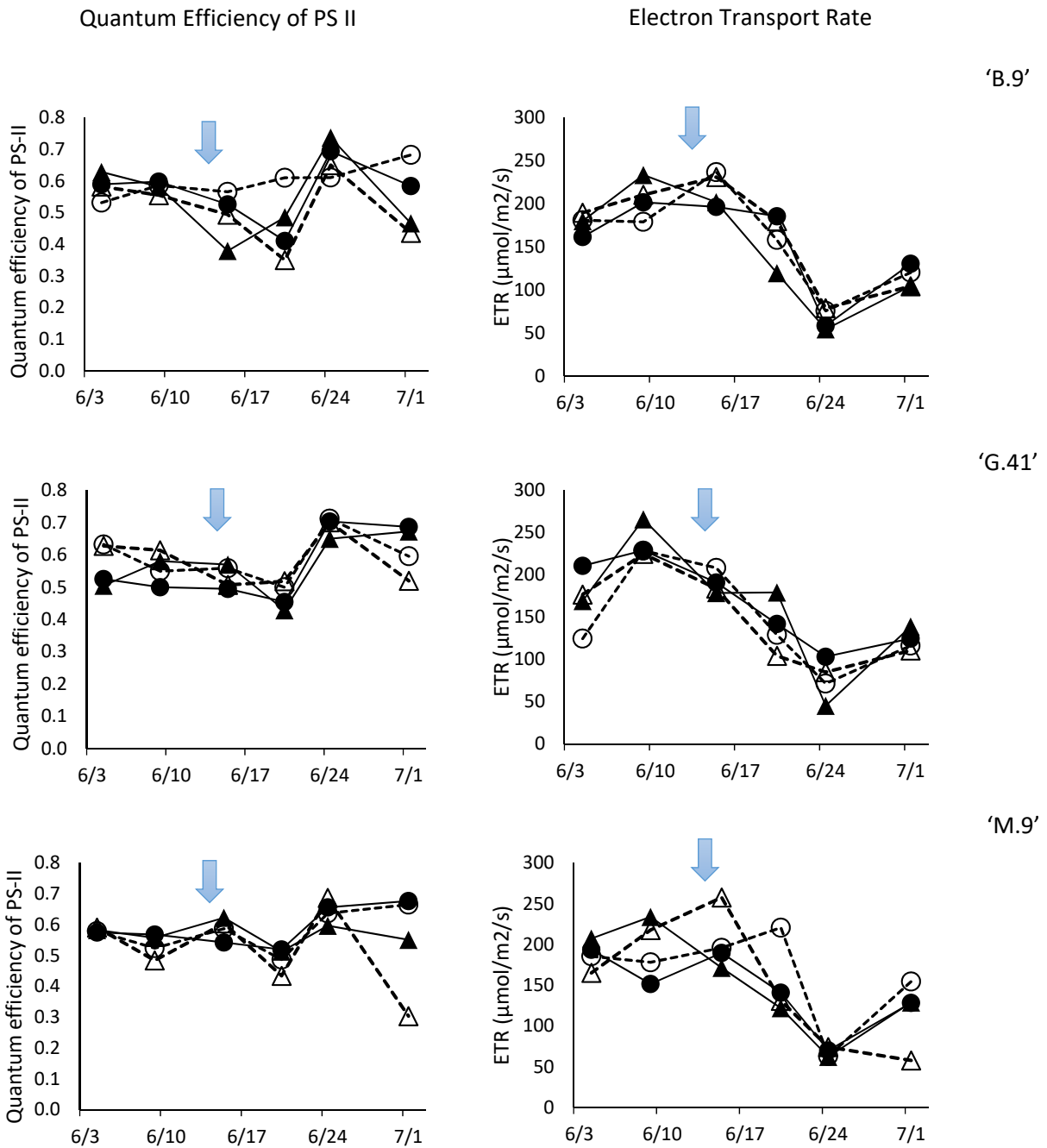
**Fig. 5-4.** Leaf transpiration rate (A), electron transport rate (*ETR*) (B), quantum efficiency of photosystem II (C), and net difference in ambient and leaf temperature (*dT*) (D) on apple trees (cv “Evercrisp”) subjected to different stressors: Ambrosia beetle damage (black circle), 20 x sterile drilled holes simulating beetle damage (open circle with dashed lines), 20 x 2 mm holes inoculated with *B. dothidea* mycelium.



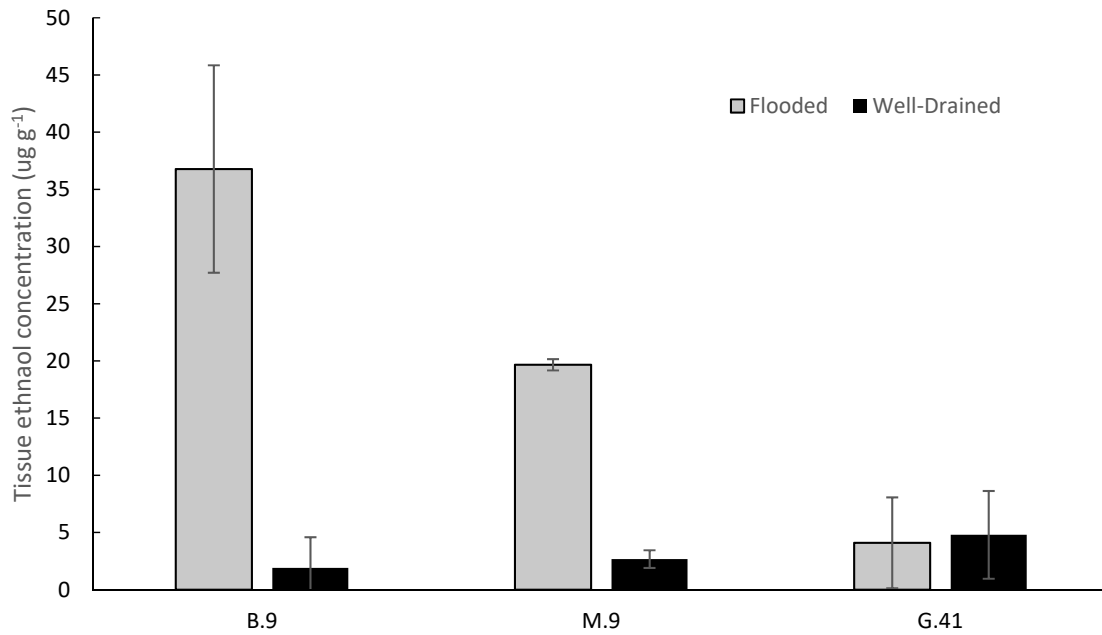
**Fig. 5-5.** Internal discoloration within apple scion resulting from: (A) no damage (control), (B) colonization by *X. crassiusculus*, (C) sterile 2 mm dia. Holes drilled into trunk, or (D) 2 mm dia. holes inoculated with *B. dothidea*. Treatments imposed in 15 July 2022, trees destructively assessed 160 days after treatments imposed.



**Fig. 5-6.** Average stomatal conductance (left column) and leaf transpiration rate (right column) of potted apple (cv Evercrisp) grafted to different rootstocks and subjected to drought (open markers and dashed lines = drought, closed marker and solid line = no drought stress) and flood stress (triangle marker = flooded for 13 day, circle marker non-flooded) (n = 6 trees per treatment combination). Parameters measured on 6 leaves per tree with Licor 600 promter/flourometer. Blue arrow indicates initiation of flooding.

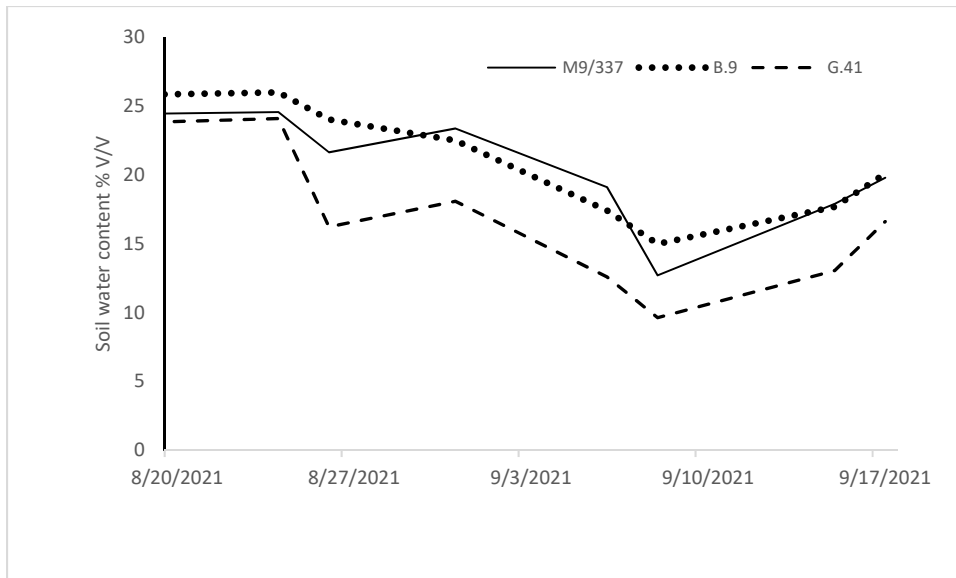


**Fig. 5-7.** Quantum efficiency of Photosystem-II (left column) and Electron Transport Rate (right column) potted apple (cv Evercrisp) grafted to different rootstocks and subjected to drought (open markers and dashed lines = drought, closed marker and solid line = no drought stress) and flood stress (triangle marker = flooded for 13 day, circle marker non-flooded) (n = 6 trees per treatment combination). Parameters measured on 6 leaves per tree with Licor 600 porometer/flourometer. Blue arrow indicates initiation of flooding.



**Fig. 5-8.** Tissue ethanol (ug EtOH per g tissue) taken from 5-10 cm above the graft union on apple trees subjected to drought stress Aug-Sep 2021 and flood stress in June 2022. Samples taken 8 days after flood treatment began. N = 6 replicates of each rootstock-stress treatment combination. Error bars indicate  $\pm$ SEM.

## Supplementary



SI 1. Soil moisture measured at 7 cm depth (average of 3 readings/pot across 20 reps/rootstock) with TDI soil meter with 70 mm probes.

## Chapter 6 Summary and Future Directions

The research work encompassed in this dissertation spans 6 years of field and laboratory experiments, and observational surveys. The underlying motivation for these projects stems from the need to address concerns from apple growers who experienced major losses due to Rapid Apple Decline (RAD) in 2017, a syndrome primarily affecting younger apple trees (<6 years old) planted in high-density orchards in eastern USA and Canada (Singh et al. 2019, Xu et al. 2023). Understanding the causes of RAD is therefore critical to the long-term viability of apple production for many growers as high tree losses within young, high density orchards is not sustainable. Based upon our reported findings in Chapter 1, other published studies, and our own observations, it is apparent that RAD is a syndrome caused by a culmination of abiotic stresses damaging and weakening trees beyond the point of recovery and that a variety of opportunistic biotic agents exploit this vulnerability.

Ambrosia beetles were associated with declining apple trees, significantly more ambrosia beetle entries were observed on dead and declining trees compared with apparently healthy trees in 2017 surveys of commercial orchards in NC (Chapter 1). The observed association combined with reported association of ambrosia beetles with tree apple tree decline in NY and extensive reports of a link between tree death and ambrosia beetles in ornamental nurseries throughout eastern USA motivated further investigation into ambrosia beetles in NC orchards. Four species of ambrosia beetles were found to be associated with declining apple trees. *Xylosandrus crassiusculus* and *X. germanus* are the most dominant species, accounting for 56.4% of identified species colonizing declining apple trees. *Xyleborinus saxesenii* were recovered in high frequency from dead and declining trees in 2017 but rarely colonized trees that were artificially stressed (ethanol-drenched or flooded). *Xyleborinus saxesenii* is an exotic species, but has been established in USA for many decades and appears to colonize trees at later

stages of decline and therefore are unlikely to contribute to decline. *Cnestus mutilatus* was recovered from artificially stressed apple trees in lower frequency compared to *Xylosandrus* spp.

Trapping studies in Chapter 1 and 2 showed that *X. germanus* and *X. crassiusculus* were the most dominant species captured in ethanol baited traps deployed at apple orchards in NC and accounted for almost all beetles recovered from ethanol-drenched potted apple trees deployed in insecticide trials or as sentinel trees (Chapter 2). Traps deployed at apple orchards in Ohio in 2020 and 2021 showed that *X. germanus* was the most dominant species, and that *X. crassiusculus* was rare at most sites, supporting previous reported studies at ornamental nurseries in OH (Ranger et al. 2021, Ranger et al. 2015) and apple orchards in NY (Agnello et al 2017). The species composition in northwest Virginia was similar to NC orchards, with both *X. crassiusculus* and *X. germanus* highly common. The sentinel tree study conducted in 2019 and 2020 showed that the majority of beetle entries occurred in May and June, when catches within the ethanol traps were highest. Trapping studies also showed that traps placed at the outer edge of apple orchards bordering unmanaged woods captured more beetles than traps placed within the orchard interior. This edge effect supporting previous findings based on trap captures in fruit orchards (Agnello et al. 2017, Monterrosa et al. 2022) ornamental nurseries (Werle et al. 2015, 2019, Werle, C. T., Sampson B. J. , M. Reding 2017) in eastern USA, and Black walnut plantations (Williams and Ginzel 2020) in Indiana, USA . Higher captures at the outer edge of orchards suggest that unmanaged woods are a key source for ambrosia beetle populations and studies are currently underway to understand dispersal patterns across a range of landscapes and production systems. Higher edge captures also suggest that placing traps on the outer edges of orchards will be most efficient for monitoring (higher beetle activity), which is also more efficient logistically than placing traps within the orchard interior.

A number of abiotic stressors have been shown to elicit ethanol production in trees and subsequently attract colonization by Xyleborine ambrosia beetles. In addition, there is some evidence

showing that fungal and oomycete phytopathogen infection can effect beetle attraction. There are limited reported observations of an association between that *Xylosandrus* spp. colonization and fire blight caused by the bacteria *Erwinia amylovora*. In Chapter 4 we investigated the effect of fire blight on attraction and colonization by *Xylosandrus* spp. ambrosia beetles. We found elevated ethanol on fire blight diseased trees, but ethanol production was localized to infected tissue and not a systemic response. Although *X. crassiusculus* were found to colonize fire blight infected trees, under field conditions, we observed very low rates of colonization fire blight infected trees, with 10 x fewer attacks compared with flood-stressed trees deployed at the same time and location. Therefore, the necrotrophic bacterial phytopathogen, *E. amylovora* can effect host preference, but does not appear to be a major driver of ambrosia beetle attacks on apple.

Understanding the impact of *Xylosadrus* spp. on RAD is important for targeting appropriate management strategies for RAD. If the beetles are contributing to accelerating decline, it would be wise to optimize control of the beetles in order to limit tree loss. Ambrosia beetles inflict high economic damage in ornamental nurseries as beetle colonization directly reduces the value of the tree, regardless of their impact on tree health. Therefore there is a low economic threshold for ambrosia beetle colonization within ornamental nursery systems, which warrants intensive management intervention. However, in apple production there is no direct impact of ambrosia beetles on the value of trees, rather the economic impact of ambrosia beetle colonization is tree mortality and health. Therefore, we aimed to understand the relative effect of ambrosia beetle colonization on apple tree health. In Chapter 5 we attempted to isolate the effect of *Xylosandrus* spp. ambrosia beetles on the health and mortality of apple trees. Two field experiments were conducted in 2020 and 2021 with flooded trees exposed to natural populations of ambrosia beetles or excluded to prevent colonization. Exposed trees experienced relatively low rates of colonization, but under natural conditions there was no detectable effect of exposure on mortality of flooded apple trees and a weak effect on visual tree health. In 2022, a field

experiment was conducted to evaluate the effect of *X. crassiusculus* colonization on 2 year old apple trees that were irrigated with ethanol to elicit beetle colonization without causing damage from flooding. Despite considerable staining and apparent damage to internal sapwood of colonized trees, no tree mortality occurred and no negative health effects were detected as a result of beetle colonization. Overall we failed to find a strong effect of *Xylosadrus* spp. colonization on apple tree health and mortality, but we did confirm that flood stress can have a significant negative effect on tree health. *Xylosandrus* spp. are attracted to and colonize trees that have experienced physiological stress, and that physiological stress such as freeze damage and flood stress can cause significant mortality in the absence of beetle colonization. Therefore, despite the observed association of *Xylosandrus* spp. with RAD, it is unlikely that they are a major contributing factor to tree decline. Ambrosia beetle attack on apple trees should instead be considered to be an indication of underlying physiological stressors that may result in decline and death of the colonized tree.

Previous studies conducted in Canada, New York, and Pennsylvania have not identified a singular phytopathogen as a causative agent of RAD (Singh et al. 2019, Peter 2021, Xu et al. 2023); in Chapter 1 we showed that there is a wide diversity of wood-invading fungal phytopathogens associated with declining trees, but no single species was isolated consistently across all declining trees. Isolates of *Botryosphaeria*, *Diaporthe*, and *Fusarium* spp. were the most common fungi isolated from declining trees. Fungal phytopathogens from these genera exploit weakened hosts, commonly residing within trees on pruning wounds and small cankers as latent pathogens, and can invade large portions of the tree following stress events such as drought or freeze injury. Our survey of non-attacked apple trees showed there was a similar high occurrence of potential phytopathogens associated with stem cankers, graft union tissue, and pruning wounds on apple trees in commercial orchards. Furthermore, surveys of fungi associated with ambrosia beetle galleries on sentinel apple trees generally found a lower frequency of phytopathogens such as *B. dothidea* and *Diaporthe* spp. compared with galleries in

declining trees and these phytopathogens were very rarely isolated from beetles trapped at orchards in NC (Chapter 3). We showed that there is some potential for movement of fungal microbes by ambrosia beetles, however it is not clear whether the beetles are able to transmit fungi into hosts. A large number of isolates found on the external surfaces of ambrosia beetles were *Aureobasidium* or *Trichoderma* spp. which have been shown to be antagonistic to the beetle's symbiont. Successful gallery establishment and offspring production would therefore require some degree of sanitation. Recent work by Biedermann et al has shown that *X. saxesenii* engage in active sanitation to reduce occurrence of competing fungi within their galleries and it seems likely that *X. germanus* and *X. crassiusculus* would need to engage in similar behavior to maintain their galleries.

Pathogenicity and aggressiveness assays conducted on detached shoots and apple seedlings found some highly aggressive isolates but the most commonly isolated species tended to have lower aggressiveness. Future research should explore the combined effect of abiotic stress and common isolates to gain a better understanding of the aggressiveness of common fungi under conditions that weaken a tree's capacity to defend against infection.

Given the lack of supporting evidence for *Xylosandrus* spp. ambrosia beetles and associated fungal phytopathogens as causative agents of RAD, we are left with a conundrum. RAD continues to be a major concern for apple growers throughout eastern USA and Canada, and without a definitive cause, there appears to be little options for management. If ambrosia beetles were major causative agents of RAD, management efforts targeting the beetles could be further developed to protect growers against tree losses. In the absence of a singular identifiable biotic agent, it appears that RAD is a multifactorial issue driven by climatic conditions (freeze damage, drought, flooding) that are largely outside the control of apple growers. Future research should focus on finding ways in which growers can lessen the impacts of abiotic stress on trees through understanding how plant nutrition, plant growth regulators, weed management, cultivar selection, rootstock selection, and other management techniques affect

tree survival under challenging conditions. This is of particular importance to ensure the long term sustainability of apple growing given climate change is predicted to result in an increase in extreme weather events.

## References

- Agnello, A. M., D. I. Breth, E. M. Tee, K. D. Cox, S. M. Villani, K. M. Ayer, A. E. Wallis, D. J. Donahue, D. B. Combs, A. E. Davis, J. A. Neal, and F. M. English-Loeb. 2017.** *Xylosandrus germanus* (Coleoptera: Curculionidae: Scolytinae) Occurrence, Fungal Associations, and Management Trials in New York Apple Orchards. *J. Econ. Entomol.* 110: 2149–2164.
- Monterrosa, A., S. V. Joseph, B. Blaauw, W. Hudson, and A. L. Acebes-Doria. 2022.** Ambrosia Beetle Occurrence and Phenology of *Xylosandrus* spp. (Coleoptera: Curculionidae: Scolytinae) in Ornamental Nurseries, Tree Fruit, and Pecan Orchards in Georgia. *Environ. Entomol.* 51: 998–1009.
- Peter, K. A. 2021.** Apple Disease - Rapid Apple Decline. Penn State Ext.
- Ranger, C. M., M. E. Reding, K. Adesso, M. Ginzel, and D. Rassati. 2021.** Semiochemical-mediated host selection by *Xylosandrus* spp. ambrosia beetles ( Coleoptera : Curculionidae ) attacking horticultural tree crops : a review of basic and applied science. 103–120.
- Ranger, C. M., P. B. Schultz, S. D. Frank, J. H. Chong, and M. E. Reding. 2015.** Non-native ambrosia beetles as opportunistic exploiters of living but weakened trees. *PLoS One.* 10: 1–22.
- Singh, J., K. J. P. Silva, M. Fuchs, and A. Khan. 2019.** Potential role of weather, soil and plant microbial communities in rapid decline of apple trees. *PLoS One.* 14: 1–19.
- Werle, C. T., Sampson B. J. , M. Reding, E. 2017.** A Role for Intercept Traps in the Ambrosia Beetle (Coleoptera: Curculionidae: Scolytinae) IPM Strategy at Ornamental Nurseries. *Midsouth Entomol.* 2: 14–23.

**Werle, C. T., J. H. Chong, B. J. Sampson, M. E. Reding, and J. J. Adamczyk. 2015.** Seasonal and Spatial Dispersal Patterns of Select Ambrosia Beetles (Coleoptera: Curculionidae) from Forest Habitats into Production Nurseries. *Florida Entomol.* 98: 884–891.

**Werle, C. T., C. M. Ranger, P. B. Schultz, M. E. Reding, K. M. Adesso, J. B. Oliver, and B. J. Sampson. 2019.** Integrating repellent and attractant semiochemicals into a push–pull strategy for ambrosia beetles (Coleoptera: Curculionidae). *J. Appl. Entomol.* 143: 333–343.

**Williams, G. M., and M. D. Ginzel. 2020.** Spatial and Climatic Factors Influence Ambrosia Beetle (Coleoptera: Curculionidae) Abundance in Intensively Managed Plantations of Eastern Black Walnut. *Environ. Entomol.* 49: 49–58.

**Xu, H., K. D. Hannam, J. L. MacDonald, and D. Ediger. 2023.** Field Investigation into Tree Fates from Recent Apple Tree Decline: Abrupt Hydraulic Failure Versus Gradual Hydraulic Loss. *Stresses*. 3: 256–269.