

AN ABSTRACT OF THE THESIS OF

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Title: Ascospore Viability and Dispersal from Pruned Branches Infected with *Anisogramma anomala*

Abstract approved:

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Ascospore viability and dispersal from pruned branches infected with *Anisogramma anomala*

Viability and dispersal of ascospores of *Anisogramma anomala*, the cause of eastern filbert blight (EFB) on European hazelnut, from diseased branches pruned from trees were measured. In each of two years, branches bearing stromata of *A. anomala* were cut in mid-December and compared to branches cut near budbreak in March, when trees became susceptible to infection. The experiment was replicated three times at separated locations. At each location, 125 diseased branches (source) were piled loosely in a 1 x 1 m area. From March to June, spore traps (rain sampling-type) as well as 2-year-old potted hazelnut trees were placed next to each source, 6.4 m upwind and downwind, and 20 m downwind from each source. During seven significant major rain events over the two seasons, hazelnut seedlings (3-month-old) were placed adjacent to the spore traps. Near sources significantly higher ( $P. < 0.01$ ) ascospores counts were obtained for branches cut near budbreak compared to those pruned in December in the first season; no significant difference in counts of ascospores were observed in the

second season between pruning treatments. For both seasons significantly higher ( $P < 0.05$ ) counts of ascospores were observed at 6.4 m downwind compared to 6.4 m upwind or 20 m downwind of a source. Ascospore viability, as assessed by staining with trypan blue, was similar for both pruning times at all distances and averaged 50%. At least one infected seedling was obtained for 5 of 7 major rain events regardless of pruning time at sources and 3 of 7 major rain events 6.4 m downwind of a source. All of the 2-year-old potted trees for both pruning treatments at the source and 6.4 m downwind became diseased and  $> 50\%$  of trees at 20 m downwind became diseased in the 2010 season. Similar to ascospores counts, disease incidence was significantly higher ( $P < 0.01$ ) in 2-year-old potted trees observed 6.4 m downwind compared to 6.4 m upwind or 20 m downwind in the 2010 season. Significantly higher ( $P < 0.01$ ) disease incidence in 2-year-old potted trees was observed 20 m downwind compared to 6.4 m upwind in the 2010 season. Downwind disease gradients for both pruning treatments were shallow with slopes that were not significantly different than zero ( $p > 0.05$ ) for the 2010 season. Based on these results, ascospores from diseased branches pruned from trees in both pruning treatments remained viable, infectious and were dispersed downwind of each treatment.

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Ascospore Viability and Dispersal from Pruned Branches Infected with *Anisogramma  
anomala*

by  
Stephanie Heckert

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Stephanie Heckert, Author

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## **Chapter 1**

### **Introduction**

The fungus, *Anisogramma anomala* (Peck) E. Müller in E. Müller & Arx (Diaporthales), is the causal agent of eastern filbert blight (EFB) on European hazelnut, (*Corylus avellana* L.) in the Pacific Northwest (20). A perennial canker, that has a sunken appearance on the branch due to the death of the cambium, is a symptom of the disease (20). Within a canker are stromata of the pathogen containing perithecia (26), ascospores within the perithecia mature in the fall and are released beginning in late December with rain events (38). Hazelnut trees in contrast are not susceptible to infection by ascospores during winter dormancy (44). Ascospores infect only juvenile hazelnut tissue during budbreak and early shoot expansion (44). Evidence to date indicates that ascospores from diseased trees are actively discharged and dispersed with the prevailing storm track (39). Hazelnut is susceptible to infection by ascospores from budbreak (March) until the onset of the typical Pacific Northwest dry summer season, ascospore depletion and/or cessation of terminal shoot growth (44, 37).

Growers in infested areas with susceptible cultivars of hazelnuts are advised to scout for disease in their orchards (28). Scouting is done principally in winter, when cankers are easy to see without the obstruction of leaves. It is recommended that growers prune 0.6 to 0.9 m below the visible canker and remove infected branches out of the orchard before budbreak in the spring (28). For sanitation reasons it was advised to destroy the infected branches that were pruned from the orchard (28). However, no data were presented on whether infected branches were sources of inoculum and needed to be destroyed. There are strict regulations on agricultural burning in Oregon (12) and more restrictive laws are possible in the future, which may lead to the discontinuing of agricultural burning in certain areas. The level of risk associated with inoculum originating in pruned brush piles is unknown.

In laboratory studies ascospore release rates from cut branches infected with *A. anomala* were greatly reduced in alternating wet/dry cycles compared to continuous wet cycles (38). The results also showed that diseased branches cut in November had lower release rates at colder temperatures, while having higher release rates at warmer temperatures (38). It was concluded that because the study was conducted in November when perithecia were not fully mature, warmer temperatures caused perithecia to mature faster allowing them to release ascospores at a greater rate than under typical winter conditions (38).

Inoculum studies with diseased cut branches < 1 m from hazelnut trees has resulted in consistent and multiple infections (35). However this distance is within the rain splash dispersal range of spores (16) and there has been no investigations on whether *A. anomala* ascospores in cut diseased branches are infectious outside of this range.

This research dealt with pruned hazelnut branches bearing mature stromata of *A. anomala* and addressed whether the timing of pruning diseased branches affected inoculum release and ascospore dispersal. It was hypothesized that diseased branches pruned from hazelnut trees several months before budbreak and put into brush piles would not be a significant source of inoculum compared to diseased branches pruned immediately before budbreak. The objective of this thesis was to look for differences between early and late timings of pruning in number, viability, and dispersal of ascospores from piles of pruned diseased branches. An additional objective was to observe disease incidence and severity in hazelnut trees at specified distances away from these piles.

**Chapter 2**  
**Literature Review**

## Introduction

*Anisogramma anomala* (Peck) E. Müller in E. Müller & Arx is an obligate parasite that is the casual agent of eastern filbert blight (EFB) on several species of hazelnuts. The genus *Anisogramma* is a fungus in the phylum Ascomycota. This fungus has been classified in the Gnomoniaceae family in the order of the Diaporthales (10). The fungus *A. anomala* is a parasite of the American hazelnut, *Corylus americana* Marchsh (44). The American hazelnut is an understory shrub common in the forests of the eastern United States (39) and *A. anomala* is thought to be endemic to this region. EFB has not been shown to cause infection on the Pacific Northwest native hazelnut *Corylus cornuta* Marchsh var. californica (6).

Eastern filbert blight is an important disease on the European hazelnut, *Corylus avellana* L., with disease severity differing among cultivar (39, 35). European hazelnuts could not be established as a commercial crop on the east coast of the United States due to EFB (35). Commercial orchards however were established in Oregon, Washington and British Columbia, where the pathogen was not present initially. Through exclusion of the pathogen (4) the hazelnut industry in the Pacific Northwest thrived and by the early 1980's produced 3% of the world's hazelnuts (41).

EFB was first reported in Washington in 1973 (9). Davison and Davidson (9) reported that the orchard where it was first detected was 50% infected and that the grower first noticed EFB in 1968. Subsequent studies determined that the fungus was probably introduced in the early 1960's. The first detection in Oregon occurred in the Columbia County during an Oregon Department of Agriculture survey in 1974 (6). The Oregon Department of Agriculture set up regulatory disease control areas in hopes of preventing the disease from entering into the Willamette Valley, Oregon's main hazelnut growing area (6,34). In October of 1986, EFB was found in northern Clackamas County with surveys showing that disease

incidence and severity was greater in the NE of the Willamette Valley (34). This new main focus was 25 km SW of where it was first detected in Washington (34). Disease distribution patterns within orchards showed local spread to the N or NE, suggesting spread on the prevailing winds of spring rains (34). Since then EFB has spread southward and has been established throughout the Willamette Valley as far south as the Eugene area. EFB has also been detected in hazelnut orchards in the Fraser Valley of British Columbia, Canada (40).

### **Biology of *Anisogramma anomala***

The disease is characterized by a perennial canker on European hazelnut. The canker has a sunken appearance on the branch due to death of cambium tissue (20). Depending on size of the canker, the number of stromata varies and can be in single or multiple rows (20). The stroma contains the fruiting bodies of the pathogen: a perithecium with asci and ascospores (26). The ascospore is the only known infectious propagule (22). Ascospores have a 2 to 4 month maturation period before they are discharged, however not all ascospores mature at once but instead mature over the late fall to early winter months (20). Gottwald and Cameron (20) estimated that there were approximately 8,400 asci in a mature perithecium, which would produce 67,200 ascospores. After the ascospores have matured in the asci they are released into the environment. By December, 70% of the ascospores in the asci are mature, with 90% by January (38). Ascospores are released with the start of the rainy season in late fall (38), ascospore numbers start to decline in May and nearly all ascospores have been discharged by June (37).

In laboratory studies of ascospore release from infected branches, ascospore release rate was greatly reduced in a discontinuous-wetting treatment, using an intermittent mist system that was on and off over a 5 day cycle, when compared to continuous wetting treatment (38). Infected branches that were cut in November and were on discontinuous-

wetting treatment had greater ascospore release rate at 20° C than at 5° C (38). In field studies, Pinkerton et al. (37) did not find a correlation between temperature and the release of ascospores on diseased trees. They concluded that since the laboratory study, on cut diseased branches, was conducted in November when perithecia were not fully mature; the higher temperature caused perithecia to mature faster allowing them to release ascospores at a greater rate than at lower temperatures (38).

The environmental factor that most greatly affected release of ascospores was precipitation (37). Pinkerton et al. (37) used Burkhard traps and weather-monitoring equipment for 12 weeks in the spring to study ascospore release in an orchard setting. A strong correlation between precipitation and ascospore release was observed, however dew alone was not enough to consistently result in the release of ascospores. Ascospores would start to release after 1 to 2 hours of rainfall and would continue releasing up to 5 hours after the last rain. The concentration of ascospores increased rapidly until the 5<sup>th</sup> hour and then held constant until the 12<sup>th</sup> hour at which time the density started to decrease.

Stone et al. (44) studied the timing of infection in hazelnuts to determine when hazelnuts were most susceptible. Experiments involved exposing 2-year-old European hazelnuts in a diseased orchard at weekly intervals from February to May and inoculation experiments on seedlings in the greenhouse. The orchard study showed 52% of the trees exposed for a 7-day interval between March to May had symptoms after 15 months. During this time, the 2-year-old trees were at the leaf tip emergence to shoot elongation stage. The inoculation experiments also showed the host was susceptible at the same phenological stage and needed a chilling period before symptom development.

Once an *A. anomala* ascospore landed on susceptible host tissue it adhered irreversibly (34). Ascospores were found mostly at the base of trichomes nearest the apical

bud penetrating the epidermal cell wall in cross sectional studies (36). After infection hyphae in the highly susceptible hazelnut cv. Ennis then colonized the cambium, phloem, and secondary tissues (36). The fungus had haustoria-like cells present within phloem and parenchyma cells of the xylem rays in the host (36). Pinkerton et al. (36) did find a hypersensitive-like response of the host with continued colonization of the pathogen in the formation of callose, cell necrosis, and a formation of a cicatricial layer. The host produces the cicatricial layer to help contain the pathogen from further colonization (36). During this time of colonization by *A. anomala* the only external signs of the pathogen was a series of small bumps 0.5 x 2.0 mm in diameter (20).

Twelve to 14 months after infection, stromata start to form and rupture the bark of the host. By Sep the process is complete and stromata are clearly visible on the host, the cambium under the stromata dies and stromata mature into black carbonized bumps (20). The asci and ascospores are undifferentiated at this point. They then mature and are ready for dispersal with the late fall rains (28). The mature *A. anomala* ascospores are 8 to 12  $\mu\text{m}$  long by 4 to 5  $\mu\text{m}$  wide (20). The latent period from the time of infection to the maturation of the first ascospores for dispersal is around 18 to 20 months (22).

Cankers of EFB increase in size perennial and will eventually girdle an infected branch (22). An infection that started on the main scaffolding branch will take several years to girdle, while a smaller branch may be girdled in the first or second season (22). Gottwald and Cameron's (22) experiments showed that the rate of canker expansion increased significantly with canker age as long as there was susceptible tissue available. Cankers would continue to expand onto secondary and tertiary branches. They estimated that a mature hazelnut cv. 'Daviana', would become commercially worthless within 5 to 15 years and a younger tree would become commercially worthless within 4 to 7 years after infection.



### **Epidemiology of *Anisogramma anomala***

Pinkerton et al. (39) studied the disease gradient of EFB in controlled orchard settings. They assessed disease gradients both by counting ascospores from spore traps as well as determining disease incidence on European hazelnuts planted near a highly infested orchard. The results showed that 76% of the planted trees to the north of the infected orchard were infected with *A. anomala*. No significant difference between the number of diseased planted trees near the edge of the orchard or > 5 m out was found indicating a shallow disease gradient. In contrast, only 5% of the planted trees to the south of the orchard were infected, with few trees infected beyond 10 m. Thus, upwind the disease gradient was very steep.

Weather data analyzed from the time EFB was introduced into the Willamette Valley showed that during spring rainstorms the wind direction were from the SW majority of the time (39). Pinkerton et al. (39) monitored disease spread in a mature orchard with 13 diseased trees at the center. Subsequent increase of EFB into the N sector was at 30%, W sector 1%, E sector 7%, and S sector 2% with distribution of the disease being highly contagious. It was concluded that there was active dispersal in *A. anomala*, even though forcible discharge of ascospores from the perithecium had not been observed. Ascospores were believed to be carried on the prevailing wind during rainstorms in the springtime.

### **Dispersal of Fungal Spores**

Early research indicated that *A. anomala* ascospores were rain splash dispersed, where drops of water fall onto the fruiting body of the fungus causing spores to be carried away with the splash (22). Gottwald and Cameron (21) experimented with ascospore release by putting out petroleum jelly coated microscope slides 1 to 2mm over the stromata of cankered EFB hazelnut branches in an orchard setting. Ten slides were exposed in 5 to 20 day intervals and examined microscopically for ascospores. The whole process was repeated 10 times between

November to April (21). The majority of ascospores observed were typical of ascospores dropping down in a rain droplet, though they did observe an octet of ascospores and a few scattered ascospores, which is more typical with active discharge (21). Pinkerton et al. (38) also showed rain splash dispersal of ascospores by using a spore traps to collect ascospores beneath sporulating cankers in hazelnut orchard canopies. A correlation was made between rainfall and ascospore release using this method and they concluded that rain splash dispersal of ascospores may account for disease spread within the canopy (38,39).

The literature has several examples of experiments that were designed on how far rain splashed inoculum would travel. Fitt et al. (19) reviewed data on dispersal of fungal plant pathogens with rain. They observed that in still air only a few droplets go beyond 1m in distance (19). In one experiment spore traps along with young wheat plants were set out to evaluate splash dispersal of *Pseudocercospora herpotrichoides* spores, 52 x 2  $\mu\text{m}$  in diameter and needle-like. The results showed that only 30% of the wheat plants outside of a 2.5 m radius from the inoculum source became infected (17). They concluded that the disease gradient was steep in all directions from the source (17).

Fitt and Lysandrou (18) stated that splash dispersal of pathogens are affected somewhat by the size of the droplet carrying the spore. The study measured where dyed droplets containing *Pseudocercospora herpotrichoides* spores landed as well as dye droplets not containing spores landed. The resulting splash droplets were collected on fixed photographic film. Positions, sizes of splash droplets and the numbers of spores they carried were determined by microscopic examination. The results indicated that the number of droplets, spore-carrying droplets and spores decreased with increasing distance from targets (the point of impact). No splash droplets were collected at > 1 m from target and most of the spore carrying droplets were between 4 to 5 mm in diameter. They did find evidence that 5

mm droplets dispersed more spores than the 4 mm droplets and that most spore carrying droplets were between 200 to 400  $\mu\text{m}$ . Splash droplets were observed up to a height of 70 cm, with most between 15 and 20 cm.

Ntahimpera et al. (32) did an experiment determining if the size of rain droplets and the differing intensities of rain affected the range of splash dispersal. In their experiments they used *Colletotrichum acutatum* (11 x 5  $\mu\text{m}$ ) colony counts on media instead of spore counts to detect the number of spores that were dispersed. They used varying nozzle sizes to break the water into differing artificial raindrop sizes and varying water pressures to show differing rain intensities. The results showed that largest artificial raindrops and lower pressure of water had the higher amount of colonies. The lower amount of colonies came from the small artificial raindrops and higher pressure of water. Most of the colonies were within 30 cm of the source although there were “sufficient” spores at 60 cm that were able to infect strawberries in a fruit disease incidence study. Their results showed that only the treatment with the largest artificial raindrops and low water pressure had colonies at 72 cm and then only at relatively small amounts.

Fitt and Nijman (16) also studied the effect of wind on rain splash dispersal. The experiment used a raintower/wind tunnel complex to simulate wind and rain at the same time with splash dispersal. Spores were deposited up to 1m upwind and 2.5 m downwind from the source of inoculum. They detected water droplets that did not have spores further out suggesting that the spores in the water droplets weighed more than droplets without spores.

Fatemi et al. (15) studied splash dispersal of *Pseudocercospora herpotrichoides* (52 x 2  $\mu\text{m}$ ) and *Pyrenopeziza brassicae* (12 x 3  $\mu\text{m}$ ), conidia that differ both in shape and size. The splash droplets were collected on photographic film. They found that the distance and the

distribution of spore carrying droplets and spores within droplet size categories were similar for both fungi regardless of size and shape of spore.

Yang et al. (47) did an experiment on the effects of surface topography and rain intensity on splash dispersal. The three surfaces were plastic, soil, and straw. They had two different rain intensities: 15 mm/hr and 30 mm/hr and used *Colletotrichum acutatum* colony counts on petri plates as their means of quantifying dispersal. Their study showed no interaction between rain intensity and surface topography. The data also indicated that colony counts were equal or close to zero at 60 cm from the source regardless of surface, occasionally a colony, however, dispersed up to 120 cm away from the source. The change of rain intensity caused more spores to be released therefore causing more colonies, however there was no change in gradient steepness. The results also showed that dispersal of *Colletotrichum acutatum* in the cross row direction was strongly affected by the presence and density of the plant canopy they used.

Later research on ascospore dispersal of *A. anomala* indicated shallow spore and disease gradients suggesting active dispersal of ascospores. Pinkerton et al. (38,39) has on numerous occasions found *A. anomala* ascospores in Burkhard spore traps and in liquid impinger air traps. Both of these spore traps actively sample the air. The large distances between newly infected sites and sites of probable inoculum sources also gave evidence of active dispersal of *A. anomala* ascospores (39). Pinkerton et al. (37) noticed that the ascospores in the Burkhard collection tapes were usually found in clusters suggesting active discharge of the intact ascus.

### **Viability**

There are several stains that have been shown to indicate spore viability; some require only a compound microscope while others require a compound microscope with fluorescence

filters. The fluorescence stains used for viability assays are based on membrane integrity or intercellular enzyme activity of the cell or fungal spore (5). The literature is filled with conflicting reports on which stains are the best for viability. There are three major fluorescence stains: fluorescein diacetate (FDA), propidium iodide (PI), acridine orange (AO), or FDA and PI combined.

Boyd et al. (5) evaluated three different stains; FDA, PI dyes alone, and a combination of FDA/PI for assessing viability of cells in isolated islets of Langerhans (a region on the mammalian pancreas). The results showed that with PI stain alone one could not “unequivocally” determine cell viability. They concluded that fluorescent reagents could be used to determine viability but to get consistent and reliable results depended on the cell and tissue type being examined and the staining procedure being used.

In Yang et al.’s (48) studies on FDA stain showed they were able to discern between viable and dead spores in two out of the four fungi species. They also noted that the stain might be sensitive to the pH of the dissolving solution (48). Sincock and Robinson (43) discounted FDA because the stain did not efficiently penetrate the membrane. They also stated that the stain could leak out of the cell giving false results (43).

Hassan et al. (25) evaluated AO and FDA for viability of *Trichoderma harzianum* hyphae. Their results showed that that FDA quickly faded and lost 50% of visibility within 20 seconds, while AO took 125 minutes for it to lose 50% of its visibility (25). However they were unable to observe a reliable difference between viable and non-viable hyphae in AO (25). The FDA stain was more reliable producing a bright green fluorescent, which indicated viable hyphae, however researchers had to take pictures of viable hyphae to deal with the quick fading of the FDA stain (25). Dittmer and Weltzein’s (11) research was also unable to distinguish between living and dead cells with AO, however FDA gave them better results

throughout (11). Lauer et al. (30) liked AO when compared to the gram stain; yeast cells were a brilliant orange from the stain and counting could be done under a lower magnification. They also stated that they had no false positives with AO (30).

Trypan blue has been used for many years to determine cell viability (14). It is similar to AO in that it uses membrane integrity to determine cell viability (29). Trypan blue is advantageous because it requires only a compound microscope and several samples can be processed in a short amount of time (46). The stain is versatile and has been used for viability of plant, animal, and fungi cells with the majority vitality assays on mammalian cells (33,29,31).

### **Disease Management**

As described in Johnson et al., (28) a management plans have been developed to control EFB in Oregon orchards. The plans include scouting, therapeutic pruning, sanitation, replacement of susceptible pollenizer trees, fungicides, and resistant cultivars. The disease management plan has helped the hazelnut industry in Oregon stay competitive in the global market. Even after decades of EFB in the Willamette Valley, Oregon has consistently contributed 3% of the world market (24).

Regular scouting of orchards is a high priority if growers are in an area where EFB is prevalent. Scouting entails going through the orchard looking for symptoms of EFB cankers and canopy dieback. Growers are encouraged to scout during the winter months when leaves do not block the view of cankers and there is more time to allot for scouting (28). Growers can also scout in late summer when EFB can be detected by looking for dead branches in the canopy. After EFB has been identified in the orchard, growers should therapeutically prune (pruning for the sake of disease) 0.6 to 0.9 m below the visible canker. The extra length of the cut is due to the symptomless presence of the fungus within the infected tissue below the

visible canker. Therapeutic pruning is not particularly successful or cost effective in heavily diseased orchards and is not feasible for growers who have extensive orchards to maintain. One reason is that small cankers in the upper parts of the tree canopy are hard to detect and can go undetected for years, building up inoculum and causing more disease. Also, once cankers have been detected, it is likely the disease has been there for 3 to 5 years and probably has infected neighboring trees. However when done correctly, pruning can be effective and useful when disease incidence is light or moderate and combined with other tactics (28).

Susceptible pollenizer trees should be removed and replaced with more resistant cultivars (28). The most widely planted cultivar in the Willamette Valley is the moderately susceptible Barcelona (7). At the time of EFB detection, the widely used pollenizer tree for Barcelona was the highly susceptible cv. 'Daviana' (39). It was noticed that the first detection of EFB in orchards with this combination was on the cv. 'Daviana' trees and replanting the pollenizer trees might extend the production life of Barcelona orchards (28).

Fungicide applications are recommended for the management of EFB (28). The first application is applied at budbreak, when the period of host susceptibility begins in March and is continued into Apr or May (28). There is an active hazelnut-breeding program at Oregon State University that is breeding for quantitative as well as qualitative resistance against *A. anomala*. There are now several cultivars of both traits available to growers for planting (28). Sanitation is the destruction or removal of infected and infested plants or plant parts (8). Removal of all volunteer hazelnuts or escaped hazelnut seedlings that have sprouted outside of the orchards is recommended (28). These seedlings are susceptible and when infected could be a potential inoculum source for orchards (28). It is also suggested to properly dispose of pruned diseased branches by burning (28).

Pruned hazelnut branches bearing mature stromata of *A. anomala*, removed from trees still have ascospores that can be dispersed (28). There has been no research to indicate whether ascospores from diseased branches can be a source of inoculum outside of the rain splash dispersal range once they have been removed from the tree and this is a concern for growers. There are regulations on agriculture burning in Oregon (12) and there are concerns of more restrictions in the future that may affect how growers are able to destroy their cut diseased branches. These concerns lead to the important question: can diseased branches be left lying on the ground throughout the spring months or are they a significant source of inoculum outside the rain splash zone? The main objective of the current research was to look at brush piles harboring ascospores of *A. anomala* to determine if the timing of pruning diseased branches determined whether they were a significant source of inoculum outside the rain splash zone of ascospores.



### **Chapter 3**

#### **Methods and Materials**

## Field Study

To investigate whether the timing of pruned diseased branches affected ascospore release and infectivity from those branches, two different piles of diseased branches were set out in a paired design. The two source piles were: hazelnut branches with mature stromata of *A. anomala* cut 10 to 12 weeks before budbreak (old pruning treatment) and hazelnut branches with mature stromata of *A. anomala* cut immediately before budbreak (fresh pruning treatment).

For the 2010 old pruning treatment, diseased branches were cut on 16 Dec 09 and stored in a shed until 30 Dec 09 when they were positioned outside. The fresh pruning treatment of diseased branches were cut on 20 and 22 Feb 10 and stored in a shed until 2 Mar 10. The diseased branches of both the old and fresh pruning treatments were cut from the same orchard located in Marion County. This mature hazelnut orchard was planted with the cv. 'Ennis' and was severely diseased with *A. anomala*.

In 2011, the old pruning treatment diseased branches were cut on 16 and 17 Dec 10 and stored in a shed until 30 Dec 10 when they were positioned outside. The fresh pruning treatment, diseased branches were cut on 2 and 4 Mar 11 and stored in a shed until 7 Mar 10. The diseased branches of both old and fresh pruning treatments were cut from the North Willamette Research and Extension Center located in Marion County. These hazelnut trees were 4 to 5-year-olds of the cv. 'Ennis' and were infected with *A. anomala*. Only viable branches that were cankered with mature stromata of *A. anomala* were cut for the experiment.

For both years the experimental units were positioned at six sites at the Botany and Plant Pathology Field Laboratory near Corvallis, OR (A-F in Figure 3.1). The six locations were spaced to keep cross contamination between experimental units to a minimum. The sites varied in terrain from open fields, mature tree orchards, young tree orchards, open fields near

orchards (not on the side of the prevailing storm track) and rows of small shrubs. In each replicate both pruning treatments were paired together and were within 75 to 250 m of each other. The distance between replicates was within 150 to 400 m of each other, oriented in the W to SE direction.

There were two other sources in the area but both were downwind of the prevailing storm track. One source was a fungicide trial with infested branches that was 15 m E of D<sup>2010</sup>, 225 m N of F<sup>2010&11</sup>, 250 m E of C<sup>2010&11</sup> and 225 m N of E<sup>2010&11</sup>. Another source was a naturally infected mature cv. 'Ennis' orchard that was due N of the field laboratory. The orchard was 150 m NE of B<sup>2010</sup> and 275 m NW of D<sup>2010</sup> and 200 m N of C<sup>2011</sup> and 425 m NW of E<sup>2010</sup> (Figure 3.1).

Each treatment was replicated three times for a total of six experimental units. Each treatment had 125 branches with mature stromata of *A. anomala* in a brush pile that was approximately 1 x 1 m area and was considered a source (Figure 3.2). Each source pile had a gravity-type spore trap in the middle of the brush pile. Another spore trap was placed upwind of each source and was 6.4 m away in the SW direction at a 225° angle from a source pile. The upwind trap was a control to monitor ascospores that may come from an unknown outside source. Two additional spore traps were placed downwind of source piles to monitor movement of ascospores. The 6.4 m downwind trap was 6.4 m away in the NE direction at a 45° angle from the source pile. The 20.1 m downwind spore trap was 20.1 m away in the NE direction also at a 45° angle from the source pile (Figure 3.2). The spore traps were orientated this way because of the prevailing storm track that mainly comes out of the SW in spring rainstorms in the Pacific NW (39). The distance of 6.4 m was selected to be outside the range of potential rain splash dispersal of ascospores from the source, and thus would not be a factor in capture of ascospores at traps distal from source piles (16).

The spore traps were gravity-type traps, which collected ascospores that either landed on the surface of the sampler or were scrubbed out during rainy periods. The spore traps were constructed by using a  $\frac{3}{4}$ " PVC pipe leaving 20.3 cm intact on one end and sawing longitudinally for 218.4 cm making a semi-circle shaped trough where the rainwater collected. The surface area of the spore traps was 635.5 cm<sup>2</sup> and the altered PVC pipe was fastened with wire to a 2.5 x 15.2 cm board making a completed spore trap that was mounted onto metal posts. The spore traps were orientated W to E with the W end at a height of 81.3 cm above ground and the E end 48.3 cm from the ground at an angle of 65° to facilitate the run-off of water into a bucket that collected the rainwater. The bucket was covered with a snap lid and the PVC pipe connection to the container was caulked to seal it from the rainwater that landed on the lid.

Rainwater collection and tree exposure ended on 9 Jun 10 and on 7 Jun 11. Weather was monitored with an Adcon A730 weather station (Adcon Telemetry, Klosterneuburg, Austria) for rainfall and wind directions for the two seasons.

### **Collection of Ascospores in Rain Water**

Rainwater samples were collected from spore traps after each major rainstorm of 5 hours or more in duration (37). To collect ascospores each collection bucket was gently shaken to homogenize the contents. Amount of rainwater was recorded for each spore trap and a subsample was put into a screw top plastic container for transport back to the lab. The bucket was then rinsed with water three times and re-positioned for the next rain events. Thirty ml of a 0.5% CuSO<sub>4</sub> solution was added to each collection bucket to inhibit ascospores germination (38).

Samples from major rain events were brought back to the lab and stored at 6° C, until filtered and evaluated using a compound microscope (Zeiss, Standard Series, Oberkochen,

Germany). All source traps were evaluated within 24 hours of collection, and the upwind and downwind traps were generally evaluated within 2 to 4 days after collection.

The method for processing rainwater samples from spore traps was the same procedure used by Pinkerton et al. (38). Rainwater samples from spore traps were filtered through a 20  $\mu\text{m}$  sieve, to remove any excess debris (still allowing the ascospores of *A. anomala*,  $\sim 5 \times 10 \mu\text{m}$ , to go through). Rainwater samples were then diluted depending on the spore counts with each spore trap diluted to at least a 2 x dilution by adding equal parts of the sample and deionized (DI) water. A 30 to 50 ml volume of this subsample was filtered through a gridded, cellulose nitrate filter (Sartorius A.G., Goettingen, Germany), 25mm in diameter with 0.8  $\mu\text{m}$  pore size, held in a syringe holder (PALL Life Sciences, Ann Arbor, MI). The subsample was pushed through the filter with a plastic syringe (B.D., Franklin Lakes, NJ) pre-rinsed with the subsample. The filter was then placed on a glass microscope slide and stained with a 0.05% trypan blue stain (MP Biomedicals LLC, Solon, OH) in lactoglycerol (1:1:1 of water, lactic acid, and glycerol - Appendix A). A drop of the stain ( $\sim 45 \mu\text{L}$ ) was placed on the filter for one minute and then a coverslip was positioned on top of the filter and the slide was put under a compound microscope ( $\times 100$ -400). The number of ascospores in the four corners of the gridded filter and the middle grid were counted. Viability was ascertained at the same time by counting the ascospores that were excluding the trypan blue stain as viable, and the ascospores that took up the stain as dead (Appendix A). A set cut off point for viability counting was determined to be ten minutes (Appendix A) at which time a fresh subsample was filtered through a new cellulose nitrate filter and the procedure was repeated.

Samples were also collected after light rain shower events that were less than five hours in duration from source spore traps only. For the other spore traps, samples were only

collected before major rain events and kept until the end of the season. At the end of the season, these rainwater samples were composited for each month (March, Apr, May, June), and counted for ascospores. If spores were found in one of the composites, each individual light shower event was then filtered for that month and counted for ascospores.

### **Hazelnut Trees and Seedling Samples**

Five healthy, 2-year-old potted hazelnut trees of cv. 'Ennis,' were spaced along the length of each spore trap. Trees were propagated by rooting basal water spouts in sawdust around the base of mature trees. At the end of the season, the rooted sprouts were cut from the mother tree transplanted into rows and allowed to grow an additional year in the field before they were transplanted into 2-gallon pots with Sunshine Professional growing mix (Sun Grow Horticulture, Vancouver, Canada). Experiment trees for the 2011 season were transplanted into 2-gallon-pots straight from the stool beds and grown an additional year in the greenhouse instead of in the field. During propagation, trees were protected from EFB with fungicide (Year 1) or by exclusion in the greenhouse (Year 2) during the 2-year growth phase but were not treated during the experiment. Budbreak of the 2-year-old trees was on 2 Mar 10 and on 21 Mar 11.

After the end of the experiment the 2-year-old potted trees were taken to an outside greenhouse, for dormant season chilling (44), to incubate for symptoms of EFB at the end of the experiment. The 2-year-old potted trees for both years were fertilized on 2 Jun 10 and 20 Jun 11 at a rate of 10 g per 2-gallon container with 14-14-14 of N-P-K (Scotts, Marysville, OH). Herbicide was applied at a rate of 3 g per 2-gallon container (Hi-Yield, Bonham, TX) both years on 3 Mar 11 and on the second year on 14 Jul 11. Disease readings were taken the following summer after the 2-year-old potted trees had been exposed in the springtime (21-23

Jul 11 and summer 2012 pending). Each tree was examined for symptoms of EFB; canker number and length were recorded.

During four major rainstorms in 2010 (30 Mar, 20 Apr, 28 Apr, and 20 May) and three major rainstorms in 2011 (17 Apr, 12 May and 29 May), five seedlings were placed at each of the four spore traps in each experimental unit (29 May 11 only four seedlings per spore trap). These major rain events were referred to as 'seedling rain events'. These seedlings were grown from nuts of the highly susceptible cv. 'Ennis' pollinated with the cv. 'Butler'. The nuts were encouraged to break dormancy by soaking them for 24 hours in a 0.08% solution of Pro Gibbs (Valent BioSciences Corp, Walnut Creek, CA) on 22 Dec 09 and on 4 and 8 Jan 11. They were then put into a mist bed and kept continuously moist until the radical emerged then transplanted into conetainers (Stuewe & Sons, Tangent, OR). They grew for three to four months before placement outside to harden off.

Seedlings exposed in 'seedling rain events' were brought back to the greenhouse and transplanted from conetainers to 1-gallon plastic container with Sunshine Professional growing mix for the first year on 22-24 Jun 10 and on 19 May and 12 Jul 11 for seedlings the second year. The seedlings were fertilized at a rate of 6 g per 1-gallon container with 14-14-14 of N-P-K on 2 Jul 10 (Year 1) and on 20 Jun and 19 Aug 11 (Year 2). Herbicide was applied at a rate of 1.5 g per 1-gallon container and was put on seedlings of the second year on 12 and 22 Jul 11.

Seedlings were destructively sampled within 3 months of exposure (1 Nov 10 to 7 Jan 11 and 29 Aug to 2 Oct 11). Freehand sections of one to two cells thick were made from the subperidermal stem tissue in the seedlings with a razor blade in the region of stem that was susceptible to infection at the time of exposure (44). These sections were transferred to glass slides and then stained with a drop (~60  $\mu$ L) of 0.05% trypan blue (1:1 water to lactic acid). A

coverslip was added and the slides were allowed to sit overnight for the dye to soak into the plant tissue. The slides were then examined microscopically (x100-400) for the presences of *A. anomala* hyphae (44).

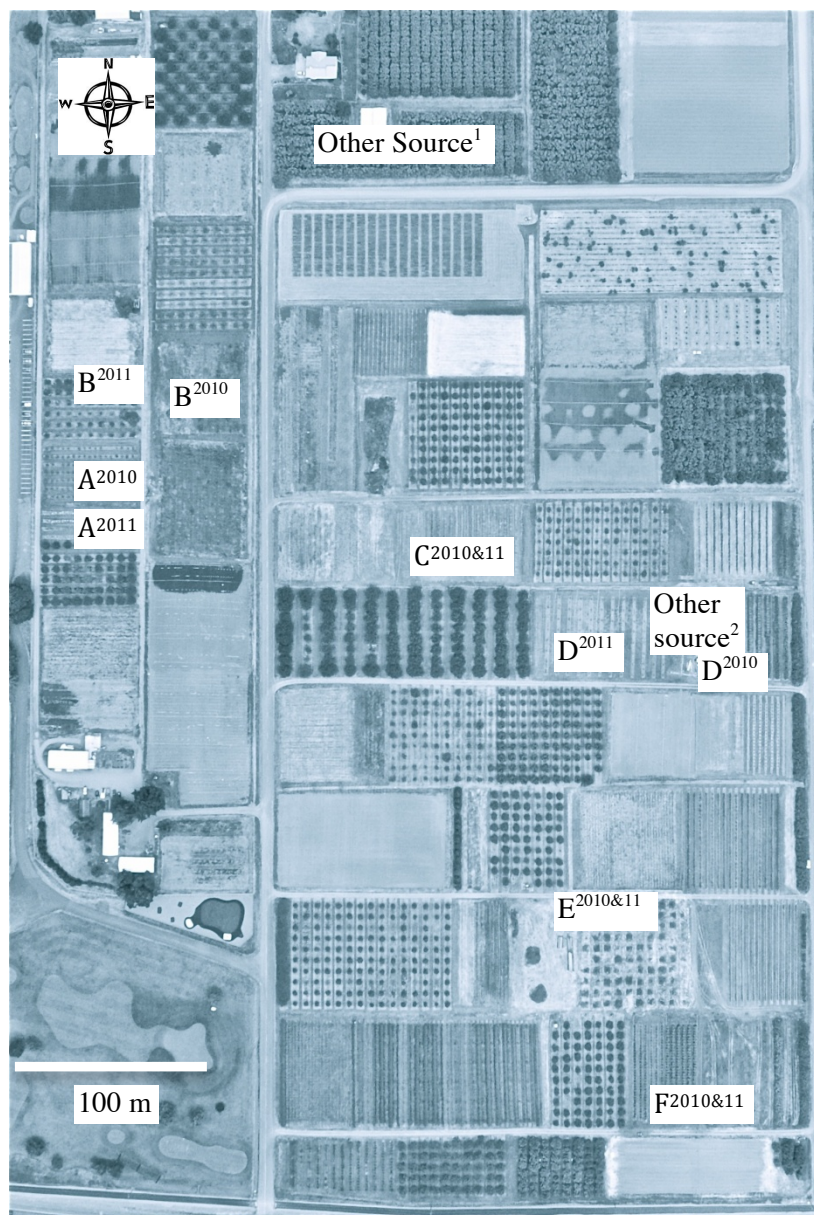
### **Data Analysis**

Data were analyzed for only the major rain events (5 hours or more in duration) by the rate of spores/m<sup>2</sup>/hr. Rain duration for each major rain event was calculated from first rainfall until bark wetness was < 4 U (U = measures electrical resistance on a scale from 0, high resistance (dry) to 10, low resistance (wet)) on the weather station's readout or collection of samples and was calculated for each rain event. Average temperature was determined for each rain event as well as rainfall amounts. For each seedling rain event in 2010 and 2011, the recorded wind direction for each event was tabulated into eight categories by compass direction: S, SW, SE, N, NW, NE, W, and E and wind speed was categorized into: > 8 km/h or < 8 km/h.

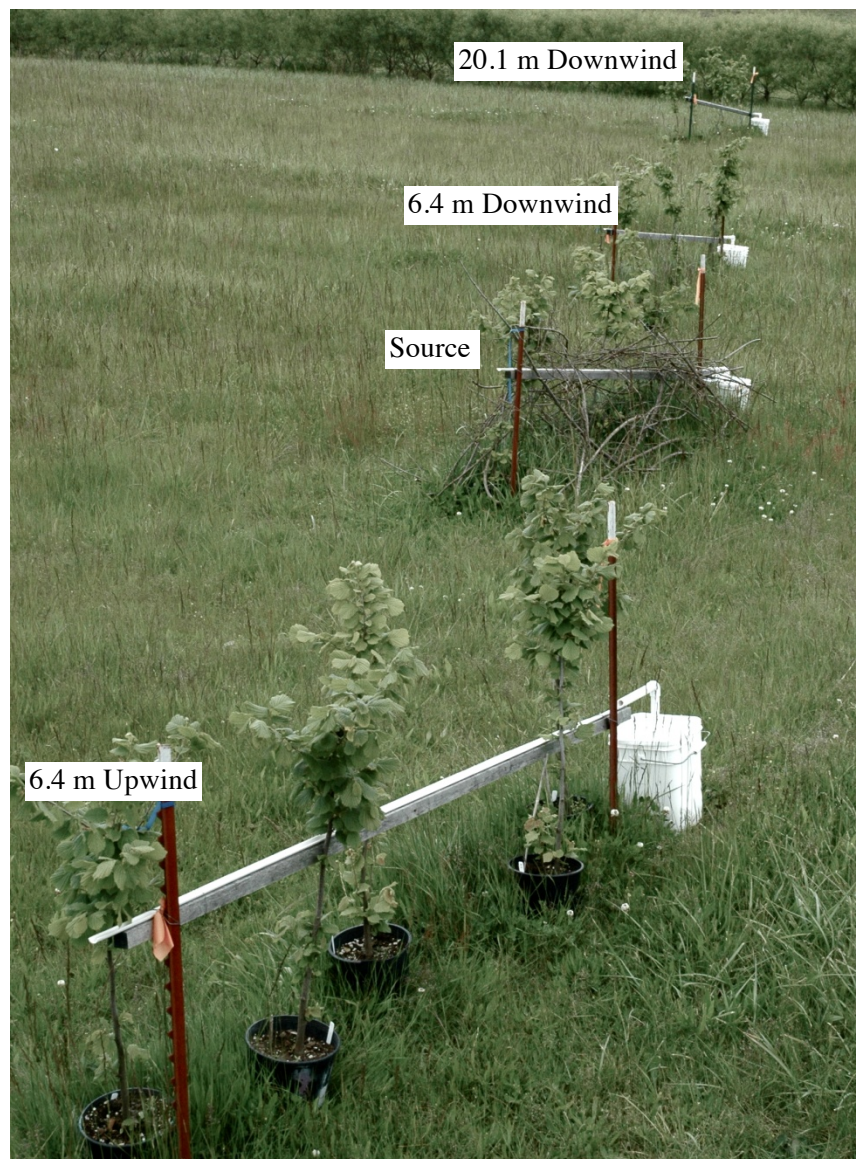
The difference in total ascospore counts for the major rain events (as measured in spores/m<sup>2</sup>/hr) between the two pruning treatments at source traps were analyzed with Satterthwaite's two-sample t-test performed in SAS (SAS, Cary, NC). Total ascospore counts were taken for each replicate over the season because of repeated measurements. T-tests were also used to analyze differences in the two pruning treatments in counts of ascospores for the 'seedling rain events' at the source traps. The difference in the two pruning treatments for the total ascospore count for the major rain events in the upwind and two downwind traps were analyzed by difference of least square means using proc mix in SAS. Total ascospore counts were taken for each replicate over the season because of repeated measurements. Difference of least square means were also used to analyze differences in the two pruning treatments in counts of ascospores for the 'seedling rain events' for spore traps distal to sources.



Differences in ascospore viability (as measured by number of viable ascospores divided by the total ascospores) between the two pruning treatments were analyzed with Satterthwaite's two-sample t-test performed in SAS for the total season viability and the 'seedling rain events'. Differences in disease incidence in seedlings, 2-year-old potted trees and disease severity in 2-year-old potted trees between the two pruning treatments was analyzed by difference of least square means using proc mix in SAS. Disease gradient downwind from the source was determined with polynomial regression model in Excel.



**Figure 3.1.** Aerial photograph of the Oregon State University Botany and Plant Pathology Field Laboratory near Corvallis, OR where the field study was conducted. A,C,E 2010&11 = sites for diseased EFB branches cut 10 to 12 weeks before budbreak (old pruning treatment) for respective year; B,D,F 2010&11 = sites for diseased branches cut immediately before budbreak (fresh pruning treatment) for respective year; Other Source<sup>1</sup> – Diseased EFB cv. ‘Ennis’ orchard; Other Source<sup>2</sup> – Fungicide Trial for EFB.



**Figure 3.2.** Photo of an experimental unit that consisted of a pile of pruned hazelnut branches bearing mature stromata of *A. anomala*, the causal agent of eastern filbert blight and spore traps at locations away from those pruned branches at the Oregon State University Botany and Plant Pathology Field Laboratory near Corvallis, OR. Two-year-old hazelnut trees cv. ‘Ennis’ were placed adjacent to spore traps for the entire spring season (March to June). 6.4 m Upwind = spore trap 6.4 m SW of source; Source = trap near the pile of pruned hazelnut branches bearing mature stromata of *A. anomala*, the causal agent of eastern filbert blight; 6.4m Downwind = spore trap 6.4 m NE of source; 20.1 m Downwind = spore trap 20.1 m NE of source.

## **Chapter 4**

### **Results**

### Major Rain Events

In 2010 there were 12 major rain events with wet durations from 18 to 95 hours and in 2011 there were 13 major rain events with wet durations from 9 to 91 hours. Rainfall for the major rain events in 2010 were from 0.8 to 6.1 cm with a total seasonal accumulation of 28.2 cm. Major rain events in 2011 were from 0.3 to 3.8 cm with a total seasonal accumulation of 22.1 cm. Greater than 80% of wind direction for 3 of the 4 'seedling rain events' in 2010 were from the SW to S directions. While in 2011, < 70% of wind direction in the 'seedling rain events' were from the SW to S directions. Only in the 'seedling rain events' on 30 Mar and 20 May 10 were 50% of the wind speeds > 8 km/h.

### Ascospore Counts

In 2010, spore traps positioned near sources, hazelnut branches bearing mature stromata of *A. anomala*, captured at least two orders of magnitude more ascospores than traps placed at 6.4m upwind or downwind (Figure 4.1). The fresh pruning treatment (hazelnut branches with mature stromata of *A. anomala* cut immediately before budbreak) had significantly higher ( $P. \leq 0.01$ , t-test) counts of ascospores for the entire season of major rain events than the old pruning treatment (hazelnut branches with mature stromata of *A. anomala* cut 10 to 12 weeks before budbreak). In 2011 there was also higher counts of ascospores at source traps by at least two orders of magnitude compared to other traps (Figure 4.2). The counts of ascospores for the two pruning treatments were not significantly different ( $P. > 0.05$ ) for the entire season of major rain events at source traps for the 2011 season. No significant difference ( $P. > 0.05$ ) in counts of ascospores between the two pruning treatments at any of upwind or downwind traps for the 2010 or 2011 seasons was observed (Figures 4.1 and 4.2). Ascospores were recovered from source traps after light shower events at lower counts than in

major rain events for both 2010 and 2011 seasons (data not shown). However, no ascospores were captured in traps located upwind and downwind of sources in these light shower events.

A decrease in counts of ascospores from the beginning of the 2010 season to the end in both the fresh and old pruning treatments were observed (Figure 4.3). Counts of ascospore from the fresh pruning treatment were always greater than counts from the old pruning treatment except for the last rainwater sample collection. In 2011 no apparent decrease of ascospores was observed throughout the season as seen in 2010 (Figure 4.4) and counts of ascospore in both fresh and old pruning treatments were similar.

Counts of ascospores from source traps during ‘seedling rain events’ on 30 Mar 10 and 28 Apr 10 showed that the fresh pruning treatment released a significantly higher ( $P. \leq 0.04$ , t-test) number of ascospore than the old pruning treatment. In contrast, no significant difference ( $P. > 0.05$ , t-test) between the two pruning treatments were detected at any of the ‘seedling rain events’ in the 2011 season (Table 4.1). Counts of ascospores at traps distal to the source were consistently lower than source counts as they were for the entire season of major rain events (Table 4.2). No significant difference ( $P. > 0.05$ , t-test) in counts of ascospores between the two pruning treatments at any of upwind or downwind traps for either the 2010 or 2011 seasons in the ‘seedling rain events’ was observed (Table 4.2).

As there was no difference in pruning treatments in the upwind and downwind traps for counts of ascospores and no significant interactions ( $P. > 0.05$ , difference of least square means) between pruning treatments and locations, pruning treatment data were pooled to look for differences in ascospores counts between locations. For the 2010 major rain events, total counts of ascospores in the 6.4 m downwind traps were significantly higher ( $P. < 0.001$ , difference of least square means) than in the 20.1 m downwind or 6.4 m upwind traps (Figure 4.1). Likewise, for the 2011 major rain events, total counts of ascospores in the 6.4 m

downwind traps were also significantly higher ( $P. \leq 0.04$ , difference of least square means) than in the 20.1 m downwind or 6.4 m upwind traps (Figure 4.2). For ‘seedling rain events’, only on 21 Apr 10 did the 6.4 m downwind traps have significantly higher ( $P. < 0.02$ , difference of least square means) counts of ascospores than the 20.1 m downwind or 6.4 m upwind traps (Table 4.2). In contrast, traps at 6.4 m upwind and 20.1 m downwind showed no significant differences ( $P. > 0.05$ ) in number of ascospores.

### **Ascospore Viability**

The upwind and downwind traps had too few counts of ascospores to give reliable viability results so data were only analyzed for source traps. However, viable ascospores were observed in all six of the 6.4 m downwind traps, in two of the 20.1 m downwind traps and in four of the 6.4 m upwind traps in 2010. For the 2011 season, viable ascospores were observed in three of the 6.4 m downwind traps and in one of the 6.4 m upwind traps. In both the 2010 and 2011 seasons there were no significant differences ( $P. > 0.05$ , t-test) in ascospore viability at the source traps between the two pruning treatments for all spring major rain events combined or at any of the individual ‘seedling rain events’ (Table 4.3). Ascospore viability at the source traps was significantly higher ( $P. < 0.001$ , t-test) in 2010 than in 2011.

### **Seedling Disease**

No significant differences in disease incidence ( $P. > 0.05$ , difference of least square means) between pruning treatments for seedlings near the source during the individual ‘seedling rain events’ in 2010 and 2011 were observed (Table 4.4). In addition, there were no significant differences in disease incidence between pruning treatments for seedlings near traps distal from the source for all events in both years. Since no differences were observed between pruning treatments and no significant interactions ( $P. > 0.05$ , difference of least



square means) between pruning treatments and locations, pruning treatment data were pooled to look for differences in disease incidence in seedlings between locations.

Distance from source had a significant effect ( $P. < 0.001$  difference of least square means) on disease incidence in seedlings (Table 4.4). Seedling disease incidence was greatest at the ascospore source location than on seedlings at other locations on 30 Mar 10, 28 Apr 10 and 17 Apr 11 (Table 4.4). Seedling disease incidence was greater at 6.4 m downwind traps than on seedlings at 20.1 m downwind traps ( $P. < 0.02$ , difference of least square means) or 6.4 m upwind traps ( $P. < 0.01$ , difference of least square means) on 30 Mar 10 and 17 Apr 11. No significant difference ( $P. > 0.05$ , difference of least square means) in disease incidence on seedlings at the 20.1 m downwind or 6.4 m upwind traps was observed. The other 'seedling rain events' (21 Apr 10, 20 May 10, 12 May 11, and 29 May 11) had too few or no diseased seedlings at the source location and no disease on seedlings at the other locations to detect difference among locations (Table 4.4).

### **2-Year-Old Tree Disease**

In 2010, no significant differences ( $P. > 0.05$  difference of least square means) were observed between pruning treatments for disease incidence (Figure 4.5) or canker number (Figure 4.6) on 2-year-old potted hazelnut trees at any of the locations. Also, no significant interactions ( $P. > 0.05$ , difference of least square means) between pruning treatments and locations, so pruning treatment data were pooled to look for differences in disease incidence or canker number on potted trees between locations.

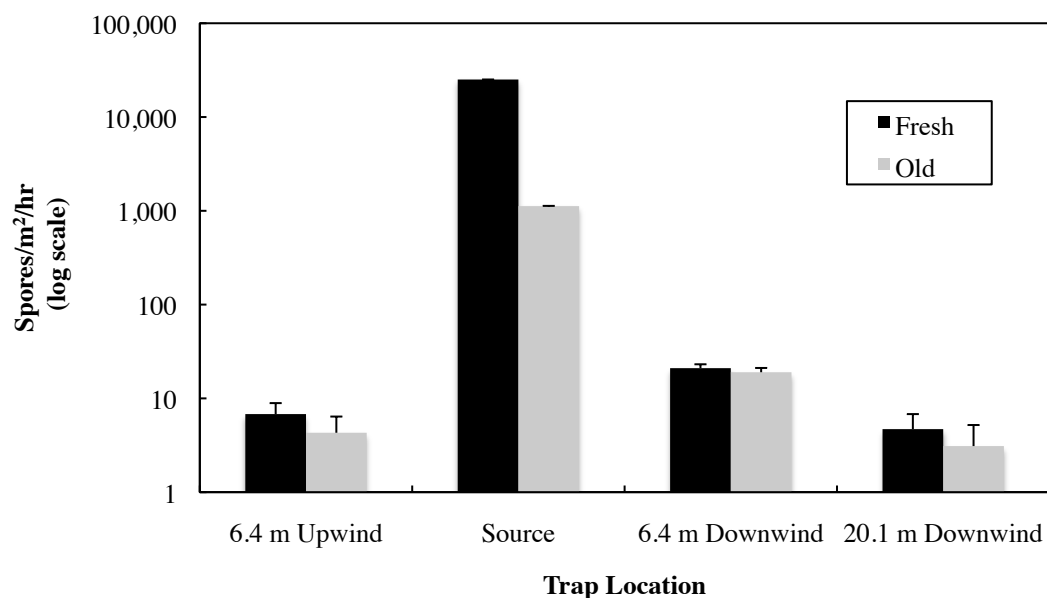
All 2-year-old potted trees adjacent to the source and to the 6.4 m downwind traps were infected (Figure 4.5). Disease incidence on potted trees adjacent to the source and the 6.4 m downwind traps were significantly higher ( $P. < 0.001$ , difference of least square means) than on potted trees adjacent to other traps. Disease incidence on potted trees adjacent to the



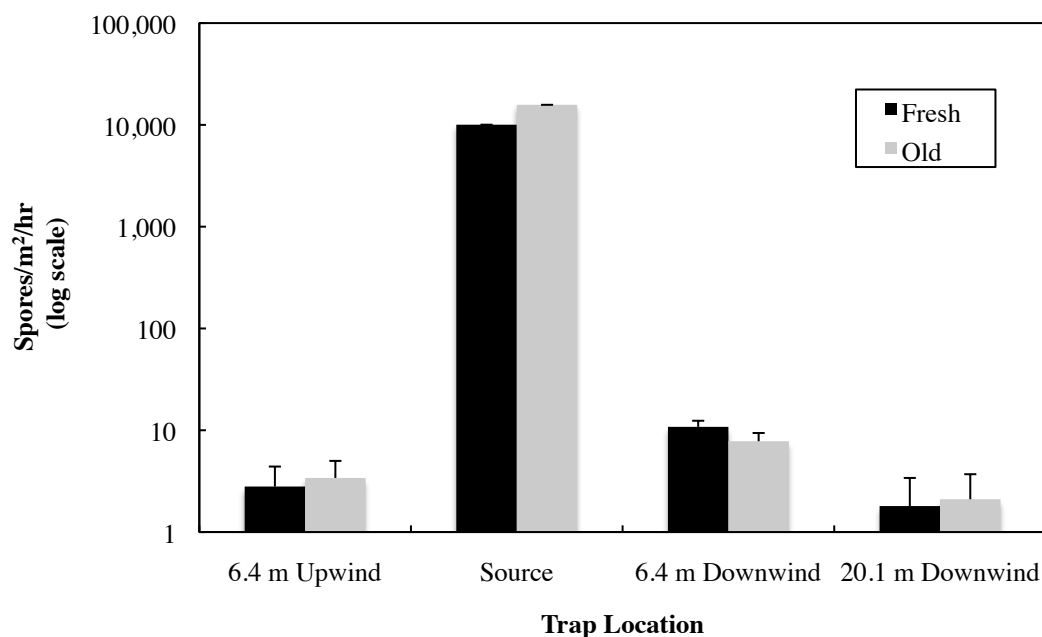
20.1 m downwind traps were significantly higher ( $P. < 0.001$ , difference of least square means) than on potted trees adjacent to the 6.4 m upwind traps.

For the 2010 season, average canker number per 2-year-old potted tree was significantly ( $P. < 0.001$ , difference of least square means) affected by distance from the source of ascospores (Figure 4.6). Disease severity (canker number) was highest on trees located near the source compared to other potted tree locations ( $P. < 0.001$ , difference of least square means). Disease severity on potted trees adjacent to the 6.4 m downwind traps was significantly higher ( $P. < 0.001$ , difference of least square means) than potted trees adjacent to the 20.1 m downwind or 6.4 m upwind traps. Significant higher ( $P. < 0.001$ , difference of least square means) disease severity was detected between potted trees adjacent to the 20.1 m downwind traps than at the 6.4 m upwind traps. The slopes of the disease gradients for both pruning treatments (Figure 4.7) were not significantly different than zero ( $P. > 0.05$ ).

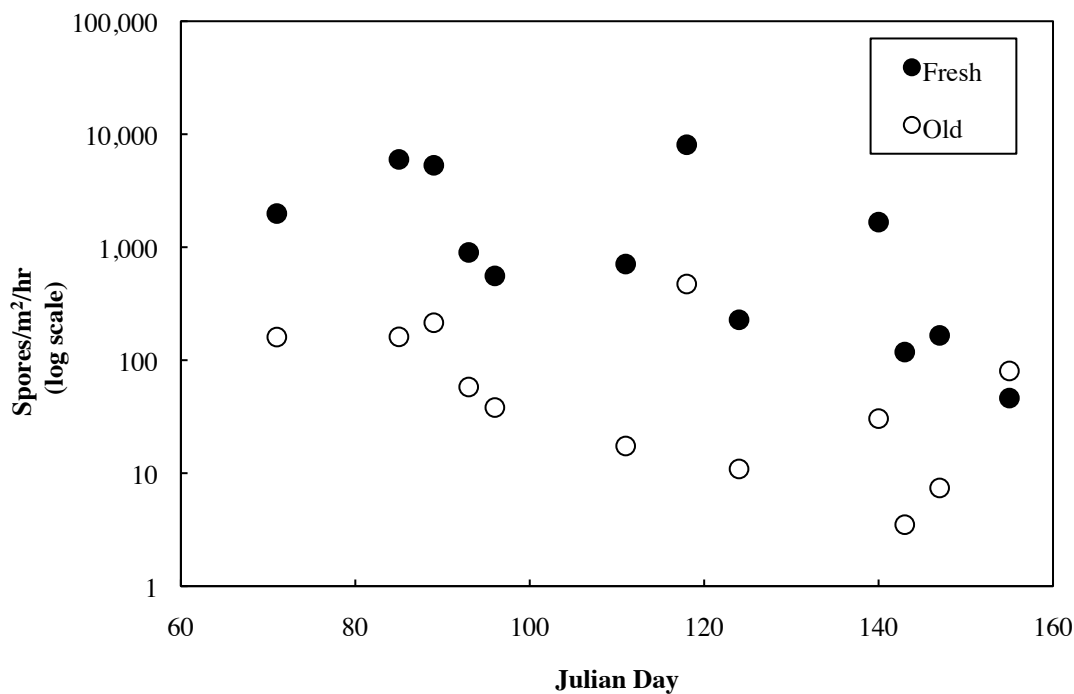
Disease incidence and severity for 2-year-old potted trees for the 2011 season should be determined during the summer of 2012. Results are expected to be similar to the 2010 season. However, I do not expect as many cankers on trees as 2010, especially away from sources. Ascospore counts were lower in downwind traps compared to 2010, which may translate into fewer infections in the downwind trap potted trees.



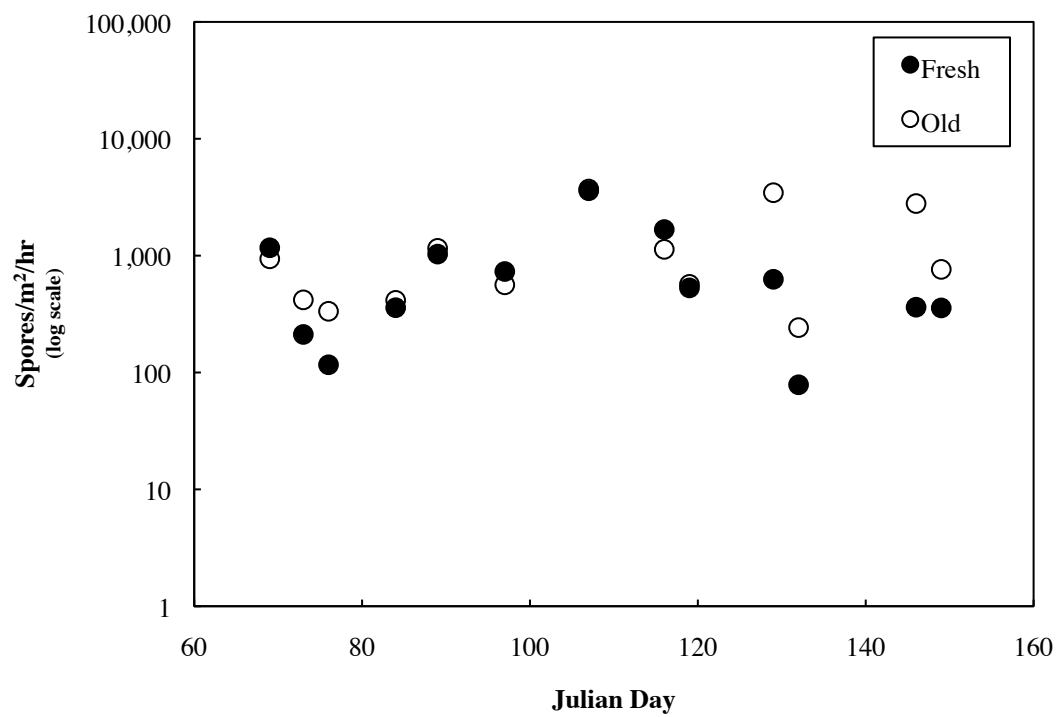
**Figure 4.1.** Total number of *Anisogramma anomala* ascospores captured in 2010 spring major rain events in spore traps located at the Oregon State University Botany and Plant Pathology Field Laboratory near Corvallis, OR. Trap Location: 6.4 m Upwind = spore trap 6.4 m SW of source; Source = trap near the pile of pruned hazelnut branches bearing mature stomata of *A. anomala*, the causal agent of eastern filbert blight; 6.4 m Downwind = spore trap 6.4 m NE of source; 20.1 m Downwind = spore trap 20.1 m NE of source; Fresh = pruning treatment having a source with branches cut immediately before budbreak, when hazelnut becomes susceptible to infection; Old = pruning treatment having a source with branches cut in December, 10 to 12 weeks before budbreak. The vertical line on each bar represents the standard error for each trap.



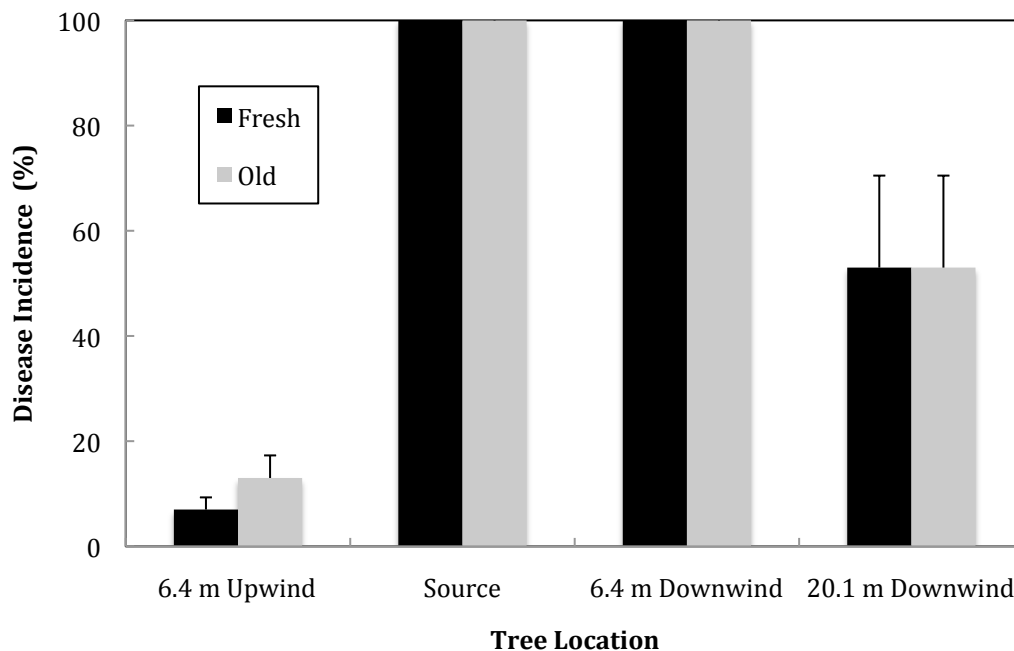
**Figure 4.2.** Total number of *Anisogramma anomala* ascospores captured in 2011 spring major rain events in spore traps located at the Oregon State University Botany and Plant Pathology Field Laboratory near Corvallis, OR. Trap Location: 6.4 m Upwind = spore trap 6.4 m SW of source; Source = traps near the pile of pruned hazelnut branches bearing mature stromata of *A. anomala*, the causal agent of eastern filbert blight; 6.4m Downwind = spore trap 6.4 m NE of source; 20.1 m Downwind = spore trap 20.1 m NE of source; Fresh = pruning treatment having a source with branches cut immediately before budbreak, when hazelnut becomes susceptible to infection; Old = pruning treatment having a source with branches cut in December, 10 to 12 weeks before budbreak. The vertical line on each bar represents the standard error for each trap.



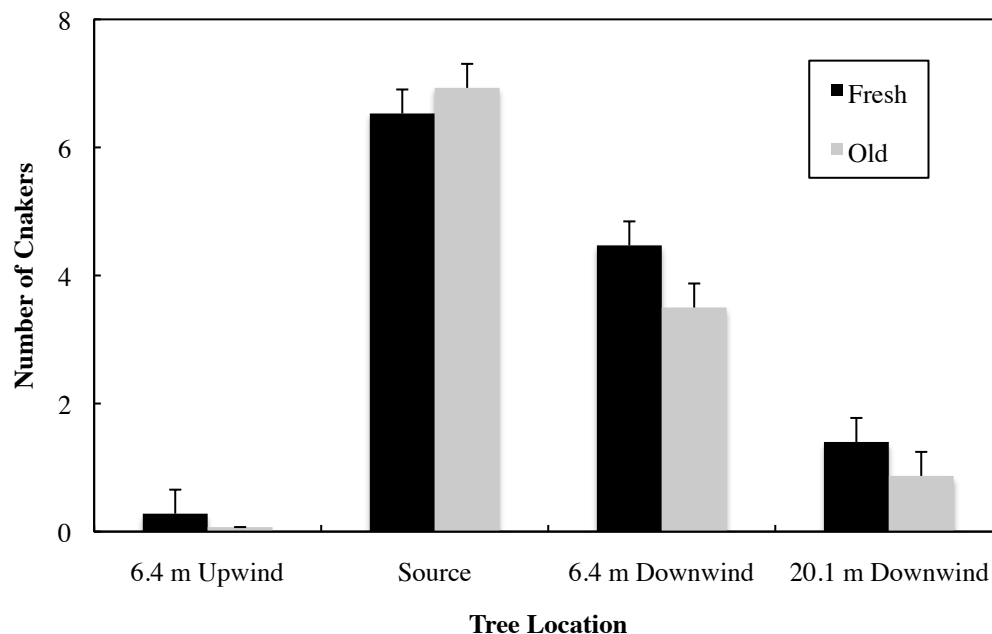
**Figure 4.3.** A scatter plot of the average counts of ascospores of *Anisogramma anomala* for each pruning treatment at the traps near the source piles over the 12 major rain events in 2010. Fresh = pruning treatment having a source pile of pruned hazelnut branches bearing mature stromata of *A. anomala*, the causal agent of eastern filbert blight, cut immediately before budbreak, when hazelnut becomes susceptible to infection; Old = pruning treatment having a source pile of pruned hazelnut branches bearing mature stromata of *A. anomala* with branches cut in December, 10 to 12 weeks before budbreak.



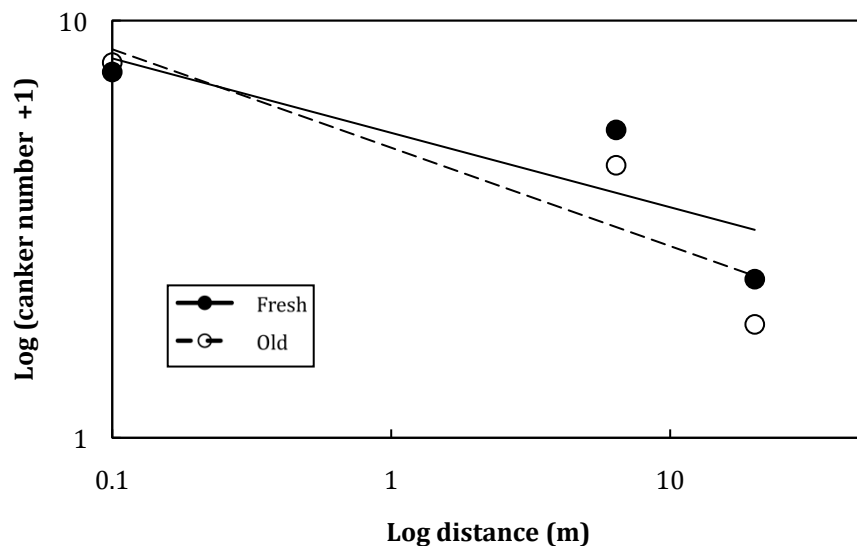
**Figure 4.4.** A scatter plot of the average counts of ascospores of *Anisogramma anomala* for each pruning treatment at the traps near the source piles over the 13 major rain events in 2011. Fresh = treatment having a source pile of pruned hazelnut branches bearing mature stomata of *A. anomala*, the causal agent of eastern filbert blight, cut immediately before budbreak, when hazelnut becomes susceptible to infection; Old = treatment having a source pile of pruned hazelnut branches bearing mature stomata of *A. anomala* with branches cut in December, 10 to 12 weeks before budbreak.



**Figure 4.5.** Incidence of eastern filbert blight in 2-year-old potted European hazelnut trees placed adjacent to the different spore trap locations at the Oregon State University Botany and Plant Pathology Field Laboratory near Corvallis, OR for the entire spring 2010 season from March to June. Tree Location: 6.4 m Upwind = 2-year-old trees placed adjacent to spore trap 6.4 m SW of source; Source = 2-year-old trees placed adjacent to spore traps near the pile of pruned hazelnut branches bearing mature stromata of *A. anomala*, the causal agent of eastern filbert blight; 6.4m Downwind = 2-year-old trees placed adjacent to spore trap 6.4 m NE of source; 20.1 m Downwind = 2-year-old trees placed adjacent to spore trap 20.1 m NE of source; Fresh = pruning treatment having a source with branches cut immediately before budbreak, when hazelnut becomes susceptible to infection; Old = pruning treatment having a source with branches cut in December, 10 to 12 weeks before budbreak. The vertical line on each bar represents the standard error for each trap.



**Figure 4.6.** Severity of eastern filbert blight (average number of cankers per tree) in 2-year-old potted European hazelnut trees placed adjacent to the different spore trap locations at the Oregon State University Botany and Plant Pathology Field Laboratory near Corvallis, OR for the entire spring 2010 season from March to June. Tree Location: 6.4 m Upwind = 2-year-old trees placed adjacent to spore trap 6.4 m SW of source; Source = 2-year-old trees placed adjacent to spore traps near the pile of pruned hazelnut branches bearing mature stomata of *A. anomala*, the causal agent of eastern filbert blight; 6.4 m Downwind = 2-year-old trees placed adjacent to spore trap 6.4 m NE of source; 20.1 m Downwind = 2-year-old trees placed adjacent to spore trap 20.1 m NE of source; Fresh = pruning treatment having a source with branches cut immediately before budbreak, when hazelnut becomes susceptible to infection; Old = pruning treatment having a source with branches cut in December, 10 to 12 weeks before budbreak. The vertical line on each bar represents the standard error for each trap.



**Figure 4.7.** Severity of eastern filbert blight (log average number of cankers +1) in 2-year-old European hazelnut trees positioned downwind from source piles of cut hazelnut branches bearing mature stromata *Anisogramma anomala*. The experiment was conducted at the Oregon State University Botany and Plant Pathology Field Laboratory near Corvallis, OR for the entire spring 2010 season from March to June. Regression lines and points represent the two pruning treatments and their regression lines ( $b = -0.236$ ) were not significantly ( $P > 0.05$ ) different than zero. Fresh = pruning treatment having a source with branches cut immediately before budbreak, when hazelnut becomes susceptible to infection; Old = pruning treatment having a source with branches cut in December, 10 to 12 weeks before budbreak.



**Table 4.1.** Number of ascospores of *Anisogramma anomala* captured at source traps for the ‘seedling rain events’ during the spring of 2010 and 2011.

Source <sup>a</sup> Trap Ascospore Counts (Spores/m <sup>2</sup> /hr)				
2010 Season				
Treatment <sup>b</sup>	30 March	21 Apr	28 Apr	20 May
Fresh	5,128.6	501.2	7,413.1	338.8
Old	125.9	7.1	269.2	24.0
p-value <sup>c</sup>	0.04	0.06	0.03	0.20
2011 Season				
Treatment <sup>b</sup>	17 Apr	12 May	29 May	
Fresh	2,529.3	45.2	312.5	
Old	3,146.3	111.8	301.7	
p-value <sup>c</sup>	0.79	0.63	0.24	

<sup>a</sup> Source = spore trap near a pile of pruned hazelnut branches bearing mature stromata of *A. anomala*, the causal agent of eastern filbert blight.

<sup>b</sup> Treatment: Fresh = pruning treatment having a source with branches cut immediately before budbreak, when hazelnut becomes susceptible to infection; Old – pruning treatment having a source with branches cut in December, 10 to 12 weeks before budbreak, when hazelnut becomes susceptible to infection

<sup>c</sup> p-value of differences between the fresh and old pruning treatment from a t-test (SAS).

**Table 4.2.** Number of ascospores of *Anisogramma anomala* captured at upwind and downwind traps for the ‘seedling rain events’ during the spring of 2010 and 2011.

Upwind & Downwind Trap Ascospore Counts (Spores/m <sup>2</sup> /hr)			
2010 Season			
Treatment <sup>a</sup>	6.4 m Upwind <sup>b</sup>	6.4 m Downwind <sup>c</sup>	20.1 m Downwind <sup>d</sup>
30 March			
Fresh	2.7	2.6	1.2
Old	1.2	5.1	0.0
21 Apr			
Fresh	1.0	6.3	0.0
Old	0.5	3.6	0.0
28 Apr			
Fresh	0.0	0.0	0.0
Old	0.0	2.2	0.0
20 May			
Fresh	0.0	0.0	0.0
Old	0.0	0.5	0.0
2011 Season			
Treatment <sup>a</sup>	6.4 m Upwind <sup>b</sup>	6.4 m Downwind <sup>c</sup>	20.1 m Downwind <sup>d</sup>
17 Apr			
Fresh	0.0	0.9	1.8
Old	0.0	1.0	0.0
12 May			
Fresh	0.0	0.0	0.0
Old	0.0	0.0	0.0
29 May			
Fresh	0.0	0.3	0.0
Old	0.0	0.0	0.0

<sup>a</sup>Treatment: Fresh = pruning treatment having a source with branches cut immediately before budbreak, when hazelnut becomes susceptible to infection; Old = pruning treatment having a source with branches cut in December, 10 to 12 weeks before budbreak, when hazelnut becomes susceptible to infection

<sup>b</sup> 6.4 m Upwind = spore trap 6.4 m SW of source

<sup>c</sup> 6.4m Downwind = spore trap 6.4 m NE of source

<sup>d</sup> 20.1 m Downwind = spore trap 20.1 m NE of source

**Table 4.3.** Viability of ascospore of *Anisogramma anomala* captured at source traps for all spring major rain events combined and individual ‘seedling rain events’ for 2010 and 2011.

Source <sup>a</sup> Trap Spore Viability (% of Viable Spores)					
2010 Season					
Treatment <sup>b</sup>	Seasonal Total	30 March	21 Apr	28 Apr	20 May
Fresh	55.9	68.6	70.0	65.6	55.2
Old	59.0	42.4	46.2	65.8	52.8
p-value <sup>c</sup>	0.28	0.21	0.42	0.98	0.89
2011 Season					
Treatment <sup>b</sup>	Seasonal Total	17 Apr	12 May	29 May	
Fresh	43.5	43.2	53.0	24.7	
Old	39.3	45.4	36.5	27.4	
p-value <sup>c</sup>	0.32	0.83	0.28	0.81	

<sup>a</sup> Source = spore trap near the pile of pruned hazelnut branches bearing mature stromata of *A. anomala*, the causal agent of eastern filbert blight

<sup>b</sup> Treatment: Fresh = pruning treatment having a source with branches cut immediately before budbreak, when hazelnut becomes susceptible to infection; Old = pruning treatment having a source with branches cut in December, 10 to 12 weeks before budbreak, when hazelnut becomes susceptible to infection

<sup>c</sup> p-value of differences between the fresh and old pruning treatment from a t-test (SAS).

**Table 4.4.** Incidence of eastern filbert blight in European hazelnut seedlings placed adjacent to spore trap locations on seven ‘seedling rain events’ during the spring of 2010 and 2011.

Disease Incidence of Seedlings (% diseased seedlings)							
2010 Season							
Date and Treatment <sup>a</sup>	6.4 m Upwind <sup>b</sup>	Source <sup>c</sup>	6.4 m Downwind <sup>d</sup>	20.1 m Downwind <sup>e</sup>	Dur (hr) <sup>f</sup>	Temp (C°) <sup>g</sup>	Rainfall (cm) <sup>h</sup>
30 March							
Fresh	0.0	93.3	13.3	6.7	57	7.8°	6.1
Old	0.0	93.0	46.7	0.0			
21 Apr							
Fresh	0.0	6.7	0.0	0.0	18	9.6°	0.8
Old	0.0	6.7	0.0	0.0			
28 Apr							
Fresh	0.0	86.7	6.7	0.0	40	9.2°	2.8
Old	6.7	73.3	13.3	0.0			
20 May							
Fresh	0.0	0.0	0.0	0.0	25	10.1°	0.8
Old	0.0	6.7	0.0	0.0			
2011 Season							
17 Apr							
Fresh	0.0	66.7	20.0	0.0	91	9.1°	2.8
Old	0.0	73.3	26.7	0.0			
12 May							
Fresh	0.0	0.0	0.0	0.0	9	6.1°	0.3
Old	0.0	0.0	0.0	0.0			
29 May							
Fresh	0.0	0.0	0.0	0.0	46	9.8°	2.1
Old	0.0	0.0	0.0	0.0			

<sup>a</sup> Treatment: Fresh = pruning treatment having a source with branches cut immediately before budbreak; Old = pruning treatment having a source with branches cut in December, 10 to 12 weeks before budbreak

<sup>b</sup> 6.4 m Upwind = seedlings placed adjacent to spore trap 6.4 m SW of source

<sup>a</sup> Source = seedlings placed adjacent to spore trap near the pile of pruned hazelnut branches bearing mature stromata of *A. aomala*, the causal agent of eastern filbert blight

<sup>c</sup> 6.4 m Downwind = spore trap 6.4 m NE of source

<sup>d</sup> 20.1 m Downwind = spore trap 20.1 m NE of source

<sup>g</sup> Dur(hr) = time in hours from beginning of rainfall event until bark wetness was < 4 U (U - measurement of electrical resistance from the weather station’s readout) or collection of samples for each ‘seedling rain event’

<sup>h</sup> Temp(C°) = Average temperature for each ‘seedling rain event’

<sup>i</sup> Rainfall(cm) = Amount of rainfall for each ‘seedling rain event’

**Chapter 5****Discussion**

Pruning European hazelnut branches diseased with EFB at different times during the dormant season did not influence release of *A. anomala* ascospores from these branches. Diseased branches, regardless of time of pruning, were a source of viable ascospores that infected hazelnut seedlings and 2-year-old potted hazelnut trees up to 20 m downwind from their locations. In previous research, (27) diseased branches that were used as sources of inoculum in fungicide trials were placed within the rain splash dispersal range of ascospores and where less than 1 m away from ascospore sources. This thesis research, however, indicated that diseased cut branches were a significant source of inoculum outside the rain splash dispersal range of ascospores, at least 4 m away from ascospore sources. This is consistent with Pinkerton et al. (39) who also observed the spread of disease from orchards to young trees that were planted outside the range of rain splash dispersed ascospores. When removed from diseased trees, cankers remained a source of viable, infective inoculum to susceptible trees at least 6.4 m away from ascospore sources.

In 2010, counts of ascospore from the fresh pruning treatment at source piles were significantly higher than the old pruning treatment. This difference was not observed during the 2011 season. Pinkerton et al. (38) observed year-to-year variability of ascospore release over a six-year period and concluded the variability was mainly due to variation in weather. Weather pattern differences could also explain differences seen between 2010 and 2011. In 2010, it rained 6 cm more than in 2011 and on average individual major rain events had more rainfall in 2010.

Viability of ascospores captured at source locations for either season was not affected by pruning treatment. The overall viability for the 2010 season was 56% and 59% and for the 2011 season was 44% and 39% for fresh and old pruning treatments, respectively (Table 4.3). *Anisogramma anomala* ascospores are encased in asci, which are surrounded by a viscous

ooze in the perithecium and are embedded in a carbonaceous stroma for protection (20). Ascospores are not durable, are susceptible to desiccation and only survive a few days in the environment when they have been released from the perithecium (38). In squash mounts with trypan blue stain, ascospores that were outside the ascus but still within the viscous ooze would stay viable for at least an hour (Heckert *unpublished data*). However, ascospores that were extracted out of the viscous ooze, stained, and mounted on a slide would lose their viability in 10 to 15 minutes (Appendix A). Ascospores from *Calonectria crotalariae* are also surrounded by viscous ooze in a perithecium and in unfavorable environmental conditions survive only a few minutes (42). It could be that the viscous ooze that surrounds the ascospores in *A. anomala* caused them to stay viable until they were released from the perithecium into the environment even when diseased branches had been detached from the tree for several months.

Even though there was a significant difference in counts of ascospores between pruning treatments at source traps during the 2010 season this difference did not result in fewer infections on seedlings or 2-year-old potted trees in the old pruning treatment. In the ‘seedling rain events’ on 30 Mar 10 and 28 Apr 10, > 70% of seedlings in both pruning treatments at source traps became infected (Table 4.4). On these two dates, however, counts of ascospores from the old pruning treatment were only  $10^2$  spores/m<sup>2</sup>/hour compared to the fresh pruning treatment that were  $10^3$  spores/m<sup>2</sup>/hour (Table 4.1). Likewise, all 2-year-old potted trees for both pruning treatments at sources were infected despite a similar order of magnitude difference in total ascospore counts. Johnson et al. (27) observed 52% infection in hazelnut seedlings in their inoculated controls of greenhouse fungicide trials with an ascospore suspension of  $1 \times 10^5$  spores/ml. It is possible that the inoculation rate of ascospores

for consistent infection of hazelnut seedlings or 2-year-old potted trees may be lower in field settings.

Even though there was no difference in disease incidence in seedlings at source traps for the two pruning treatments, there were certain ‘seedling rain events’ where more seedling disease was observed. In three of the 7 ‘seedling rain events’ 60% or more of the seedlings at the source traps became infected (Table 4.4). The weather during these specific ‘seedling rain events’ had wet durations of > 40 hours and at least 2.8 cm of rainfall (Table 4.4). Stone et al. (44) noted that longer wetness periods enhanced infection in hazelnut seedlings. Higher disease incidence in seedlings at sources on 30 Mar 10, 28 Apr 10 and 17 Apr 11 for both pruning treatments suggests counts of ascospores were high enough, ascospores were viable, and environmental conditions were favorable for infection. On the other four ‘seedling rain events’ weather conditions were not as conducive to infection, therefore leading to fewer observed diseased seedlings at source traps (Table 4.4). Therefore the difference in seedling disease in ‘seedling rain events’ was mostly like due to weather conditions at the individual ‘seedling rain events.’

Disease incidence and severity on 2-year-old potted trees located at the ascospore source indicates that both the old and fresh pruning treatment piles in 2010 had enough viable ascospores for consistent, multiple infections (Figures 4.5 and 4.6). These results are similar to what Pinkerton et al. (35) observed in inoculation experiments where cut diseased branches placed on mesh wire < 1 m above cv. ‘Ennis’ hazelnut clone trees that had > 90% infection.

No significant differences were observed between pruning treatments in either the 6.4 m and 20.1 m downwind or 6.4 m upwind traps with respect to counts of ascospores. Even with significant difference in ascospores between the pruning treatments at the ascospore source in 2010 there was no difference in counts of ascospores in either downwind traps.



When data were pooled for pruning treatment, significant differences were observed in counts of ascospores between 6.4 m and 20.1 m downwind traps indicating a spore dispersal gradient away from ascospore source piles for both seasons (Figures 4.1 and 4.2). Most of the ascospores in the downwind traps were captured during major rain events when the source traps also had larger number of captured ascospores, more than 2.5 cm of rainfall, and more than 30 hours of wet duration. All of these conditions would be conducive to active dispersal of ascospores away from the source (37). Most of the ascospores in the downwind traps were captured before May (Table 4.2) suggesting that either the lack of major rain events in May or ascospore depletion in the diseased branches (Table 4.1) was causing a decrease of counts of ascospores observed away from the source (38).

Viable ascospores were found in upwind traps as well as downwind traps in both seasons. However, the majority of viable ascospores were observed in spore traps 6.4 m downwind of sources, suggesting that source piles were the origin of viable ascospores during rain events. The viable ascospores observed upwind from source piles were most likely due to shifting winds during rain events. Analysis of weather data showed that even though the majority of the wind during rain events came from the SW, there were isolated times that wind direction would come from the N to NE. This shift in wind direction was usually brief ( $\geq 15$  minutes) and at the beginning or end of a rain event. It is possible, although unlikely, that the viable ascospores that were collected from spore traps 6.4 m upwind from sources were from unknown natural infestations that were upwind from the field site. Even so, the results of disease incidence on 2-year-old potted trees downwind of source piles indicate a strong local source (ie brush piles) than a potentially unknown source upwind from the field site.

No differences between the two pruning treatments in disease incidence on seedlings or 2-year-old potted trees were observed at the different trap locations away from sources.

However, away from source piles, most seedling infections were at the 6.4 m downwind traps regardless of treatment (Table 4.4). ‘Seedling rain events’ with infected seedlings at the 6.4 m downwind traps corresponded with seedling infections at source piles on those same dates. This indicates that ascospores were viable and environmental conditions conducive for infection away from sources on those dates.

On 21 Apr 10 (Table 4.2) ascospores were found at the 6.4 m downwind traps, however, no seedling infections were observed. Likewise, at sources little infection was observed in seedlings on that date (Table 4.4). The wetness period for that ‘seedling rain event’ was only 18 hours, which might not have been long enough for the pathogen to successfully infect the host (44).

Only on 30 Mar 10 were infected seedlings detected at the 20.1 m downwind traps. Weather conditions were conducive for infection with 6.1 cm of rainfall, wet duration of 57 hours, and a constant SW wind with speeds up to 18.5 km/h. For the remainder of the ‘seedling rain events’, little to no disease occurred suggesting that the rain events had too few of ascospores and/or unfavorable weather conditions for reliable infection of seedlings at locations away from sources.

Infection of 2-year-old potted trees was not limited to NE direction only but was also seen in the N and NW directions as well (Appendix B). In these experiments, there were also a significantly higher disease incidence and severity on downwind trap trees compared to the upwind trap trees. The previous studies of Pinkerton et al. (34, 39) from heavily infested EFB orchards, also observed disease spread in northward directions. Disease gradients downwind for the 2-year old potted trees in 2010 were shallow for both treatments (Figure 4.7) as was observed in previous research with infected EFB orchards (39). The high disease incidence and severity downwind of source piles suggests that ascospores from sources in both pruning

treatments were being dispersed outside of the rain splash dispersal range downwind and were infecting potted trees up to 20 m away.

Source piles were mostly in open areas where wind could blow through them during rainstorms. Forcible discharge of ascospores has not been observed directly in *A. anomala* and the mechanism behind the discharge of ascospores was not explored in this research. *Venturia inaequalis* only needs to forcibly discharge its ascospores 4.1 mm from the leaf surface for it to become dispersed on the wind (2). Fitt and Lysandrou (18) in experiments with *Pseudocercospora herpotrichoides* photographed splash droplets carrying spores consistently 15 to 20 cm above splash surfaces. This height is enough for spores to break the laminar boundary layer and have the potential to be caught and transported by the wind (23). Aylor (1) noted a key to active dispersal was the access of spores to the wind. Whether the mechanism for ascospore release in *A. anomala* is forcible discharge from the perithecium or passive dispersal within splash droplets infected cut branches were a source of disease for hazelnut trees at least 20 m away regardless of time of pruning.

It is recommended that growers continue to destroy all pruned diseased branches before hazelnut budbreak in early March. Growers who are unable to destroy pruned diseased branches are advised to at least put diseased brush piles downwind of their orchards. These diseased brush piles, regardless of date cut, are significant sources of inoculum and can infect susceptible hazelnut tissue at least 6.4 m away. It is suggested that further research be done into chipping diseased branches to determine whether or not they remain significant sources of inoculum.

**Chapter 6****Conclusion**

The timing of pruning branches infected with *Anisogramma anomala*, the causal agent of EFB, in the spring before hazelnut budbreak did not make a difference in whether the cut branches were a source of inoculum. Diseased branches that were cut in December were just as much a source of viable ascospores as diseased branches cut immediately before hazelnut budbreak in March. Inoculum from relatively small brush piles (1 x 1 m, with 125 diseased branches) was able to infect 2-year-old cv. 'Ennis' hazelnut trees with multiple infections 6.4 m away. Shallow disease gradients away from sources for both treatments indicated that ascospores were dispersed outside of the rain splash dispersal range downwind on springtime prevailing storm tracks. These gradients also indicated that with favorable environmental conditions these ascospores infected susceptible hazelnut trees > 20 m away. Further investigation needs to be done on pruned hazelnut branches bearing mature stromata of *A. anomala* to determine whether chipping these diseased branches causes them to remain sources of inoculum.

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## **Appendices**

## Appendix A

### Detecting Ascospore Viability with Trypan Blue

#### Introduction:

Assessing the viability of cells using stains has been practiced since the early 1900's (14). Samples, after proper preparation, are exposed to vital stains for a certain length of time and then examined microscopically for viability. A vital stain is a stain that is able to distinguish viable cells (cells that are capable of performing all cell functions necessary for survival) from dead cells (cells that have lost the ability of performing cell functions necessary for survival) through staining or non-staining of the cells (25).

These vital stains usually require evaluation with a compound microscope with or without fluorescence filters. Fluorescence stains used for viability assays are based on membrane integrity or intercellular enzyme activity to determine cell's vitality (5). Trypan blue also uses membrane integrity to determine viability of a cell. For a viable cell, trypan blue stain is unable to accumulate within the cell so it is then non-stained. However for a dead cell, trypan blue stain does accumulate within the cell and stains it a dark blue (46). Trypan blue is versatile and has been used for assessing viability of plant, animal, and fungal cells with the majority on mammalian cells (33, 29, 31). It is advantageous because it requires only a compound microscope and several samples can be processed in a short amount of time (46). Results and procedural parameters of trypan blue stain vary between the different kingdoms and genera (46), therefore trypan blue stain was evaluated as a vital stain for the ascospores of the fungus *Anisogramma anomala*. Several experiments were conducted to determine its validity.

#### Methods:

##### Collecting Ascospores

Ascospores were collected from hazelnut branches bearing mature stromata of *A. anomala*. Branches had been cut during winter dormancy and stored frozen until needed. Upon removal from the freezer, branches were stored at room temperature for ten minutes. Perithecia were then physically extracted from stromata using a scalpel, hydrated in deionized (D.I.) water and crushed to liberate ascospores. Ascospores were collected with a pipette and diluted to  $1 \times 10^6$  spores/ml (43). Ascospores procured this way were known to result in eastern filbert blight (EFB) when inoculated on hazelnut trees (43).

##### Trypan Blue vs. A.C. Media

Trypan blue staining of ascospores was compared with germination of ascospores on culture media. Trypan blue stain, 0.05% in lactoglycerol (1:1:1 of water, lactic acid, and glycerol), was used in all experiments. Half of an ascospore suspension was placed in boiling water for five minutes and allowed to cool to room temperature to heat kill ascospores (heated solution). The other half of the ascospore suspension remained unaltered (non-heated solution). A 1:10 ratio of trypan blue stain (30  $\mu$ l) to ascospore suspension (290  $\mu$ l) was made for the two solutions and each separate solution was allowed to stand for 1 minute. A drop of each solution was placed on a microscope slide with a cover slip and slides were examined to assess visible differences under a compound microscope (13). Ascospores with and without staining were counted and recorded using a hemocytometer (VWR, Radnor, PA). Each subsample of the two solutions was prepared and examined microscopically 3 times. The entire procedure was repeated a total of 3 times.

Subsamples (30 µl) of non-heated and heated solutions were also plated on a 0.05% activated charcoal (A.C.) agar media (BD, Franklin Lakes, NJ) to check for germination of ascospores (45). The fungus *A. anomala* is an obligate biotroph that does not grow well in standard culture, however, ascospores usually germinate by swelling (2 to 3x their normal size) and in some cases start making hyphal tubes before they lyse (die). This swelling was assessed after 3 to 5 days incubation at 20° C with a compound microscope (45). The number of germinated and non-germinated ascospores were counted and recorded for both solutions. Each subsample of the two solutions was plated out 3 times and later examined microscopically. The entire procedure was repeated a total of 3 times.

In another set of experiments, the non-heated and heated solutions were mixed together in various ratios. Two mixtures were prepared; one with 50% of the non-heated solution and 50% of the heated solution, and another with 25% of the non-heated solution and 75% of the heated solution. The two mixtures were homogenized, diluted and filtered through a gridded, cellulose nitrate filter and then stained with trypan blue and repeated twice for each mixture. A solution of 100% non-heated solution was treated similarly. The first 50 to 100 ascospores were counted for staining. The mixtures were also plated out on culture media (50 µL of each mixture) and then incubated for 3 to 5 days at 20° C and replicated twice for each mixture. The entire process was repeated 3 times.

#### Viability in Copper Sulfate

The effect of copper sulfate on the viability of ascospores was also investigated. Copper sulfate has been used to inhibit ascospores from germinating in rainwater collection containers in the field (38). Half of an ascospore suspension had 1 ml of 0.5% CuSO<sub>4</sub> added to 5 ml of the ascospore suspension while the other half remain unaltered. Trypan blue stain was added to a subsample of each solution as mentioned above and staining was assessed with a hemocytometer twice for both solutions. The two solutions were then kept at room temperature and read 3 and 6 days later.

Ascospore suspensions in 1.0, 0.5, and 0.25% copper sulfate solutions were prepared. Trypan blue stain was added to a subsample of each solution and staining was read with a hemocytometer twice for each solution. The solutions were then kept at room temperature and read 3 days later. This experiment was completed twice.

#### Duration of Viability in Stain

The length of time that ascospores were able to exclude trypan blue stain was determined. An ascospore suspension was prepared, diluted and filtered through a gridded, cellulose nitrate filter. The filter was then placed on a slide and a drop of trypan blue stain (60 µl) was added and allowed to sit for 1 minute and then one grid of the filter was examined under a compound microscope. The ascospores with and without staining were counted and recorded as well as the time it took to read the first grid. A second grid was counted and recorded for stained ascospores as well as the time. The difference in staining between the first and second grid were compared. This whole procedure was repeated 5 different times.

#### Results:

##### Trypan Blue vs. A.C. Media

Most of the ascospores in the non-heated solution examined under the compound microscope excluded the stain and only had a slight shade of blue around their cell walls, these ascospores were considered viable (Figure A.1). The ascospores that were in the heated solution absorbed the stain and turned a dark blue, these were considered dead (Figure A.1).

The average percentage of non-stained ascospores in the non-heated solution was 74.3% (62.5 to 100%). The average percentage of germinated ascospores in the A.C. media was 79.5% (63.2 to 100%). The percentage of stained ascospores in the heated solution for the trypan blue stain and the non-germinated ascospores on A.C. media was 100%.

The average percentage of non-stained ascospores for 100% non-heated solution was 86.3% (73.6 to 94.2%), 50% non-heated solution was 45% (38.7 to 49.2%), and 25% non-heated solution was 25.9% (18.9 to 33.59%). The average percentage of ascospore germination on A.C. media for 100% non-heated solution was 84.14% (82.23 to 85.05%), 50% non-heated solution was 36.28% (32.77 to 42.04%), and 25% non-heated solution was 20.69% (16.67 to 24.57%) (Figure A.2)).

#### Viability in Copper Sulfate

The percentage of non-stained ascospores in the unaltered ascospore solution immediately after sample preparation was 88.9%, and the percentage of non-stained ascospores in the copper sulfate ascospore solution was 84.4%. The percentage of non-stained ascospores in the unaltered ascospore solution dropped to 42.9% after 3 days and the percentage of non-stained ascospores in the copper sulfate ascospore solution was 58.1%. The percentage of non-stained ascospores of unaltered ascospore solution dropped further to 3.1% after 6 days from sample preparation while the percentage of non-stained ascospores of the copper sulfate ascospore solution was 54.17% (Figure A.3).

The average percentage of non-stained ascospores in the 1.0% copper sulfate solution was 87.7% (84.2 to 92.3%), in 0.5% copper sulfate solution was 85.7% (65.8 to 100%), and in 0.25% copper sulfate solution was 80.2% (71.4 to 84.6%). The average percentage of non-stained ascospores 3 days later in 1.0% copper sulfate solution was 69.0% (18.2 to 96%), in 0.5% copper sulfate solution was 63.4% (37.5 to 96.7%), and in 0.25% copper sulfate solution was 58.4% (25.0 to 100%) (Figure A.4)).

#### Duration of Viability

The first grid on average was examined in 4 minutes and a second grid was read before 9 minutes had elapsed. The average similarity in the percentage of non-stained ascospores in first grid compared to second grid was 87.2% (73.4 to 97.3%). Each grid had around 100 total ascospores (Figure A.5).

#### Discussion:

The count of non-stained ascospores from the trypan blue stain solution was comparable to the germination count of the same ascospores solution in culture (Figure A.2). The slightly lower germination rates on the A.C. media may have been due to *A. anomala* ascospores germination inconsistencies. Germination rates were comparable to the results found by Stone et al. (45). Based on this evidence it was determined that trypan blue could be used as vital stain for *A. anomala* ascospores.

Copper sulfate did not have an adverse effect on the viability of the ascospores. After 3 days, ascospores in copper sulfate had greater than 50% viability compared to the unaltered solution that was less than 50% (Figure A.3). The copper sulfate solution was still greater than 50% viability even on the 6<sup>th</sup> day while the unaltered solution ascospore viability was less than 10%.

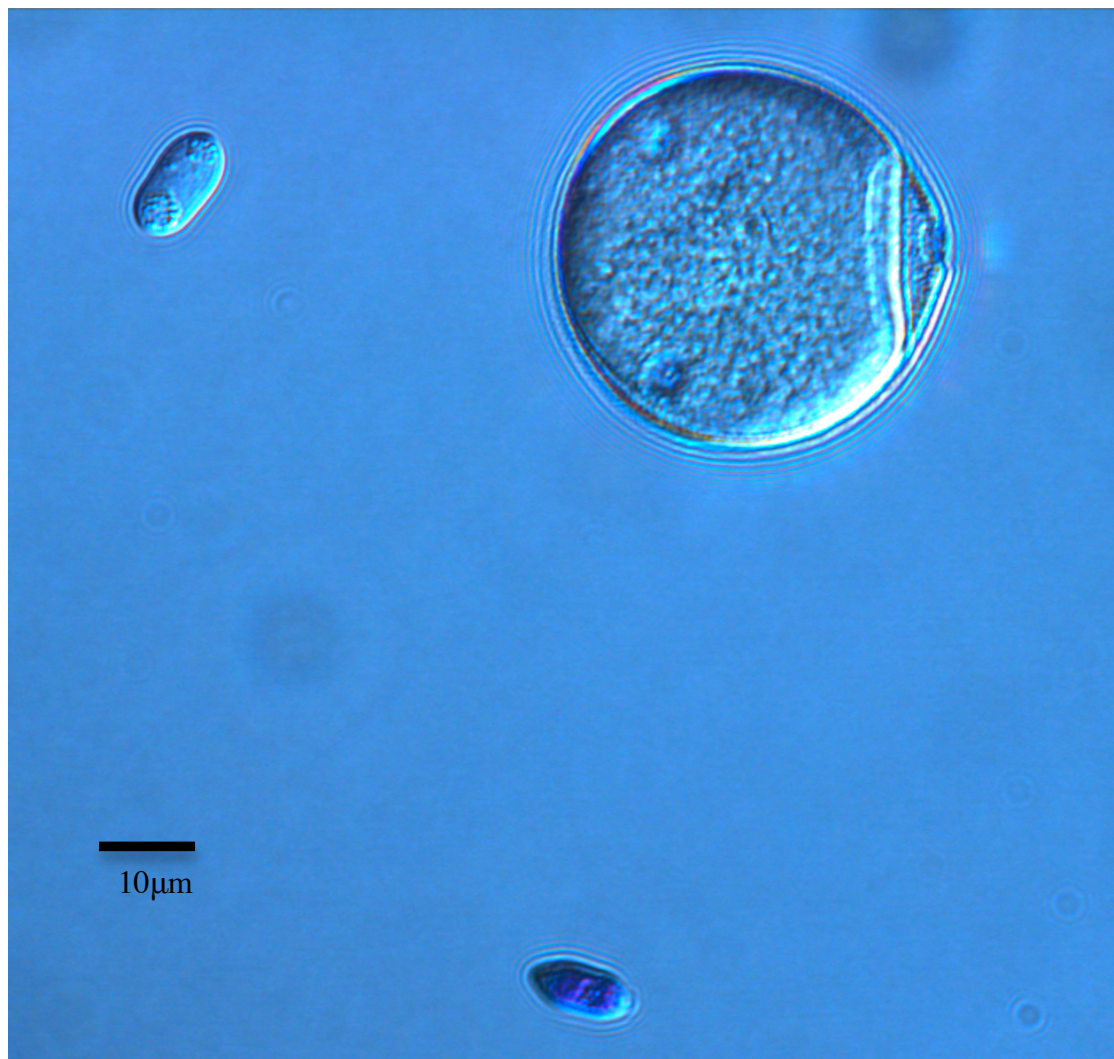
Little variation in the different copper sulfate solutions in regards to the viability of ascospores was observed (Figure A.4). The results in the 0.5% copper sulfate solution had the least variability among the experiments and this was the solution used in spore trapping

experiments of pruned branches bearing stromata of *A. anomala*. The viability recovery 3 days later was consistently lower in the different solutions so it was determined that collecting samples as soon as possible after major rain events had ended would provide the best viability results in the spore trapping experiments.

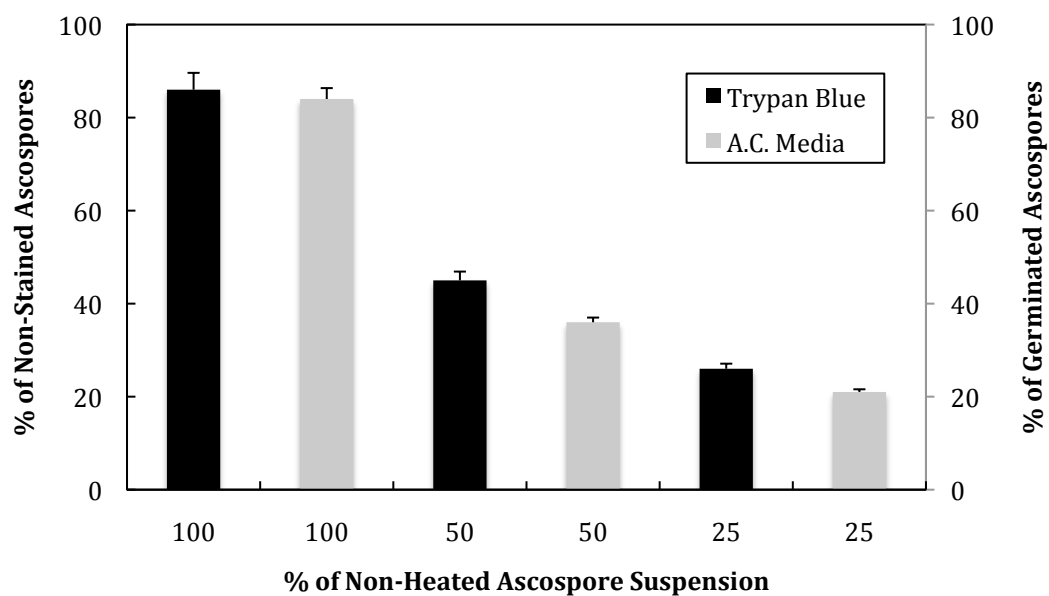
The percentage of non-stained or viable ascospores 0 to 5 minutes after application of the vital stain was similar to the percentage of viable ascospores at 5 to 9 minutes (Figure A.5). Preliminary observation into the length that viable ascospores excluded the stain showed that ascospores initially viable after preparation were mostly dead after 20 minutes. Due to the high recovery percentage at 9 minutes an arbitrary 10-minute cut off point was set to ensure consistent results.

#### Conclusion:

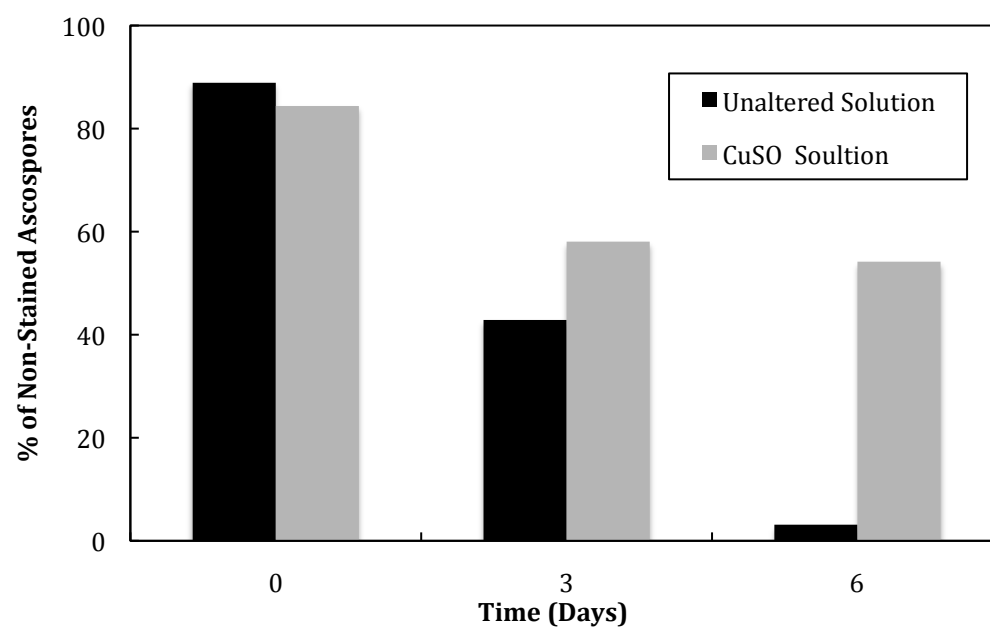
The above studies provide good evidence that trypan blue stain can be used as a vital stain for *A. anomala* ascospores and present guidelines on how to use the stain to obtain accurate results.



**Figure A.1.** A photo of a light blue, viable ascospore (upper left-hand corner) and a dark blue, dead ascospore (middle bottom) of *Anisogramma anomala*, the causal agent of eastern filbert blight on hazelnuts. European hazelnut pollen (upper right-hand corner) added for reference (400x).

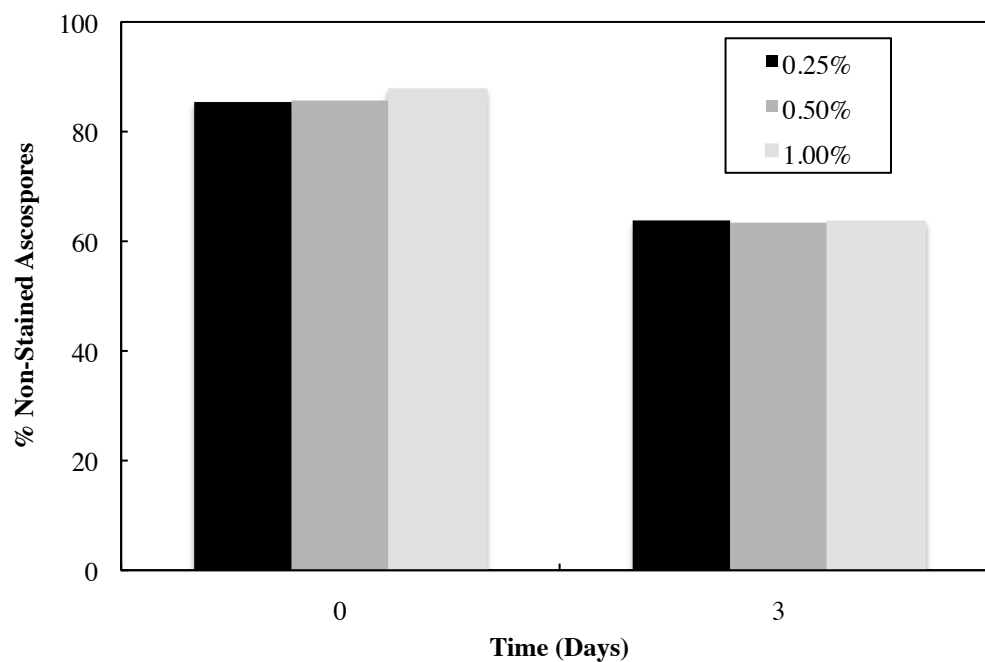


**Figure A.2** - Counts of non-stained ascospores of *Anisogramma anomala* with trypan blue and germination of ascospores counts on A.C. (activated charcoal) culture media in various ascospore solutions. The % of non-heated ascospore solution: 100 = all non-heated ascospore suspension, 50 = 1:1 ratio of non-heated ascospore suspension to heated ascospore suspension, 25 = 1:4 ratio non-heated ascospore suspension to heated ascospore suspension.

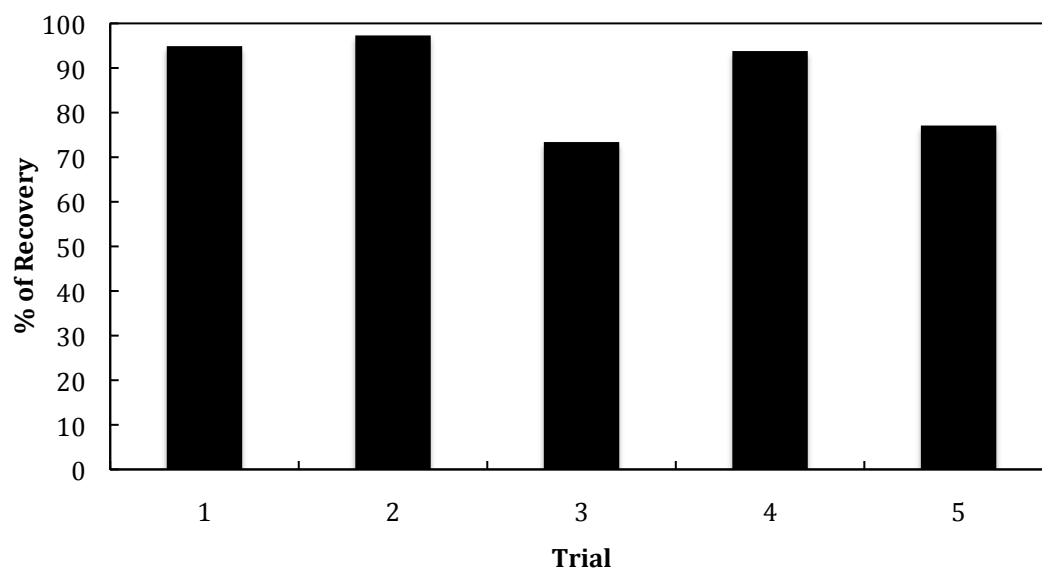


**Figure A.3.** Viability of ascospores of *Anisogramma anomala* in two solutions was evaluated at 0, 3 and 6 days with trypan blue, a vital stain. Unaltered Solution: an ascospore suspension in deionized water; CuSO<sub>4</sub> Solution: an ascospore suspension in a 0.5% copper sulfate solution (CuSO<sub>4</sub>).





**Figure A.4.** Viability of *Anisogramma anomala* ascospores in three different copper sulfate solutions. Viability of ascospores in the three solutions was evaluated at 0 and 3 days after addition of trypan blue, a vital stain. 0.25%: an ascospore suspension in a 0.5% copper sulfate solution ( $\text{CuSO}_4$ ); 0.50%: an ascospore suspension in a 0.5% copper sulfate solution ( $\text{CuSO}_4$ ); 0.25%: an ascospore suspension in a 1.00% copper sulfate solution ( $\text{CuSO}_4$ ).



**Figure A.5.** Duration of the viability of *Anisogramma anomala* ascospores in trypan blue, a vital stain. Above shows the % recovery from the percentage of viable ascospores in 5-10 minute divided by percentage of viable ascospores observed in 0-5 minutes over 5 trials.

## Appendix B

### Additional Spore Traps and Nursery Trees North of a Source

#### Introduction:

Inoculum of *Anisogramma anomala*, the casual agent of eastern filbert blight (EFB) on hazelnut is generally dispersed from the SW to the NE along with springtime storm tracks in the PNW (39). Pinkerton et al. (39) studied disease gradients of EFB in controlled and commercial orchard settings. Their results showed that > 75% of the trees to the north of a heavily infested orchard were infected with *A. anomala* (39). In contrast, only 5% of the trees planted to the south of the infested orchard were infected.

In two source locations additional spore traps were set-up to detect ascospore dispersal and disease incidence on European hazelnuts in directions other than NE during the 2010 season. It was hypothesized that a pile of pruned hazelnut branches bearing mature stromata of *A. anomala* cut immediately before budbreak (fresh pruning treatment) would be a significant source of inoculum and would have more diseased hazelnut trees away from the source than cutting hazelnut branches 10 weeks before budbreak (old pruning treatment).

#### Methods and Materials:

##### Additional Spore Traps and Trees

Two experimental units, one for each pruning treatment (hereafter referred to as section C and D) in 2010 had four additional spore traps (Figure 3.1). Two spore traps were 6.4 m at right angles from each source pile in the N and S directions and two spore traps were at a 135° and 315° angles from source piles in the NW and SE directions at 6.4 m away (Figure B.1). Also, at each spore trap, five healthy 2-year-old potted hazelnut trees of cv. 'Ennis' were spaced out along the distance of the trap. The 2-year-old potted trees were grown from a stool bed and then allowed to grow an additional year in the field before they were transplanted into 2-gallon-pots with Sunshine Professional growing mix. Hazelnut trees were protected from EFB with fungicide during the 2-year growth phase but were not treated during the course of these experiments.

Rainwater from the additional spore traps from sections C (old pruning treatment) and D (fresh pruning treatment) was collected in March and April with major rain events and at the end of May and mid June. Water samples for March and April were composited and processed as mentioned previously. When ascospores were observed, individual major rain events for March and April were processed to ascertain which rain event the ascospores were captured. Viability was not determined for the additional spore traps.

The 2-year-old potted trees in the 2-gallon containers were fertilized on 2 Jun 10 at a rate of 10.0 g per 2-gallon container with 14-14-14 of N-P-K. Herbicide was applied at a rate 3.0 g per 2-gallon container for both years on 3 Mar 11 and on the 2-year-old potted trees on 14 Jul 11. Disease readings were taken the following summer after the 2-year-old potted trees had been exposed in the spring of 2010 (21-23 Jul 11). Each potted tree was examined for symptoms of EFB; canker number and canker length were recorded.

##### Nursery Trees North of a Source

Section C (old pruning treatment) source pile was placed due S of nursery rows of 2-year-old cv. 'Ennis' hazelnut trees (Figure B.2). The trees were 2 to 40 m away from the source pile. These nursery trees were not sprayed with fungicides during the 2010 season.

Disease readings were taken the following summer (20 Jul 11) after the 2-year-old trees had been exposed to the source pile the previous spring (2010).

There were known sources of EFB in the area but both were downwind of the prevailing storm track. One source was a fungicide trial with infested branches that was 15 m E of Section D and 250 m E of Section C. Another source was a natural infestation of a mature cv. 'Ennis' orchard that was due N of the field laboratory. The orchard was 275 m NW of section D and 200 m N of section C and section C was 215 m W of section D (Figure 3.1).

Data were analyzed by Satterthwaite's two-sample t-test and proc mix difference of least square means in SAS for the additional traps in sections C and D. Disease gradient downwind from the source was determined with polynomial regression model preformed in Excel. For each seedling rain event in 2010 the recorded wind direction for each event was tabulated into eight categories by compass direction: S, SW, SE, N, NW, NE, W, & E and wind speed was categorized into: > 8 km/h or < 8 km/h.

#### Results:

##### Additional Spore Traps

Counts of ascospores for the additional spore traps were low and no significant differences ( $P > 0.05$ , t-test) were found between pruning treatments. In section C no ascospores were found in the additional spore traps and in section D a few ascospores were found in March on trap #7 on Julian days 71 and 85 and in May on trap #6 (Table B.1).

There were no significant differences ( $P > 0.05$ , difference of least square means) between old and fresh pruning treatments in disease incidence in 2-year-old potted trees at any of the additional traps. In section C all 5 of the trees located at trap #5 and #6 were infected, trap #7 had 2 infected trees and trap #8 had no infected trees. In section D all 5 trees in trap #5 were infected, trap #6 had 3 infected trees, trap #7 had 2 infected trees and trap #8 had one infected tree. As there was no difference in pruning treatments in the upwind and downwind traps in disease incidence in 2-year-old potted trees and no significant interactions ( $P > 0.05$ , difference of least square means) between pruning treatments and locations, pruning treatment data were pooled to look for differences in disease incidence in 2-year-old potted trees between locations. Significantly ( $P < 0.01$ , t-test) more infection on trees at trap #5 than at trap #7, significantly ( $P < 0.01$ , t-test) more infection on trees at trap #6 than at trap #8, and significantly ( $P < 0.01$ , t-test) more infection on trees at trap #5 than at trap #8 was observed.

##### Nursery Trees N of a Source

Figure B.2 shows a map of the infected (symptoms of EFB) and non-infected hazelnut trees in the nursery rows due north of section C. There were 134 trees in the nursery rows and 99 trees (74%) had EFB cankers by the summer of 2011. Infected trees were found as far as 36.8 m away from the source to the NE. Half of the trees 30 to 40 m from the source were diseased with one canker (Figure B.3). A disease gradient away from the source pile had a slope of  $b = -0.239$  (Power law &  $R^2 = 0.84$  (Figure B.4)).

#### Discussion:

There were no ascospores captured in spore traps in section C and only a few were captured in section D. The few ascospores that were captured compared to the level of infection on potted trees at the various traps suggested that the spore traps used were not adequate for sampling all ascospores. The spore traps used in these experiments passively sampled the air by collecting rainwater. Ascospores either needed to land directly on the

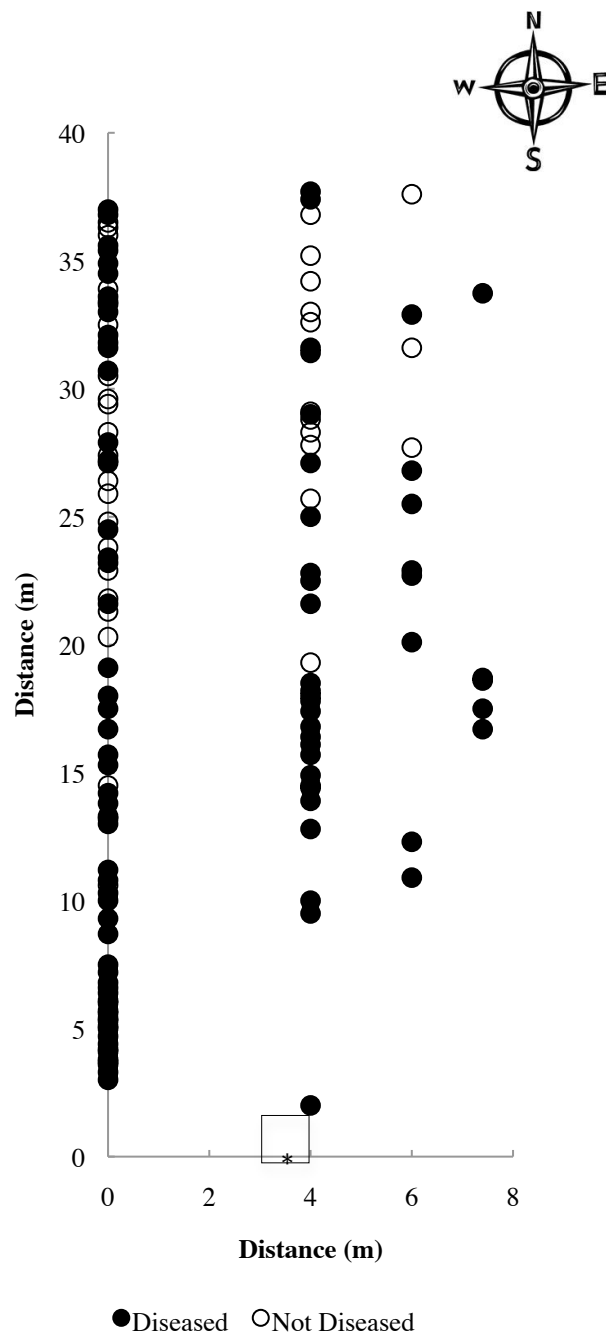
spore traps in rain droplets or rain would “scrub out” (rain drops encounter and absorb ascospores as they fell through the air) the ascospores so that they landed on spore traps. This type of spore trap was more suited for splash dispersed spores (35). Spore traps that actively sample the air (37) would have been more appropriate, however these types of spore traps were expensive and impractical for the amount of traps that were needed for these experiments.

No differences were found in diseased trees for downwind trap #5 (NW) and trap #6 (N) for both pruning treatments indicating that brush from either pruning treatment was capable of infecting trees downwind of sources. There were significant differences in disease incidence between trap #5 (NW) and trap #7 (SE) and between trap #6 (N) and trap #8 (S), when treatments were pooled and analyzed together. This suggests that disease was not due to rain splash dispersal of ascospores or both the upwind and downwind potted trees would have had similar disease incidence (39). Traps #7 and #8 were directly upwind of source piles at least 25% of time throughout the ‘seedling rain events’ and had significantly fewer diseased potted trees than downwind potted trees. This evidence suggests that there might be active dispersal of viable ascospores on the prevailing wind during rain events since trap #5 and trap #6 were downwind from source piles and potted trees were outside rain splash dispersal zone of ascospores. These results are similar to what Pinkerton et al. (39) found in their research on ascospore movement in diseased orchards.

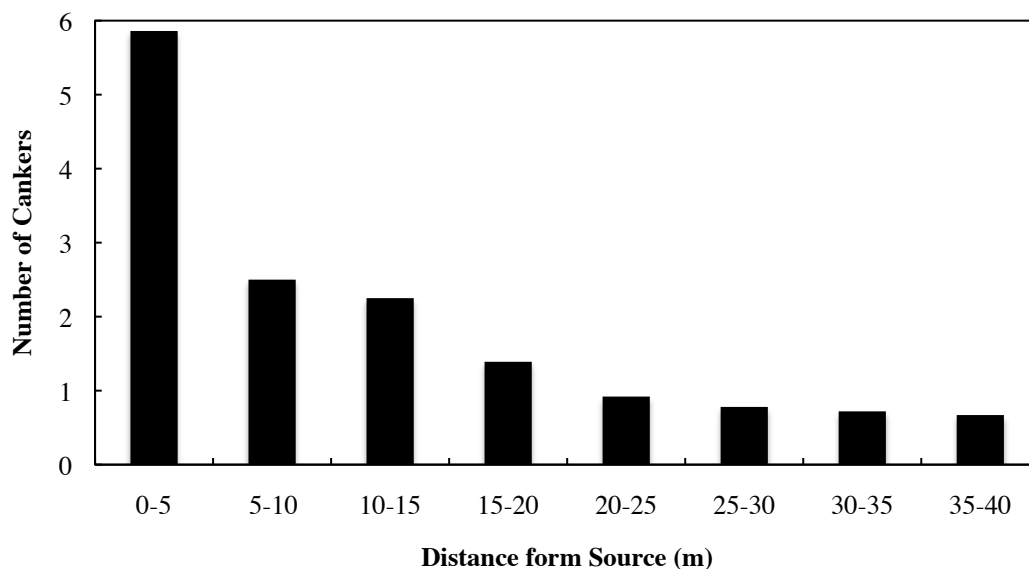
Up to 35 m north of source pile C, 50% of the trees were infected. A shallow disease gradient existed north of this section (Figure B.4) as was observed NE of all sources (see main trial results) and with diseased cv. ‘Ennis’-‘Butler’ mini-orchards (39). These nursery rows were next to a source pile with infected branches from the old pruning treatment. Diseased branches cut any time during the dormant season were a source of viable, infective ascospores.

#### Conclusion:

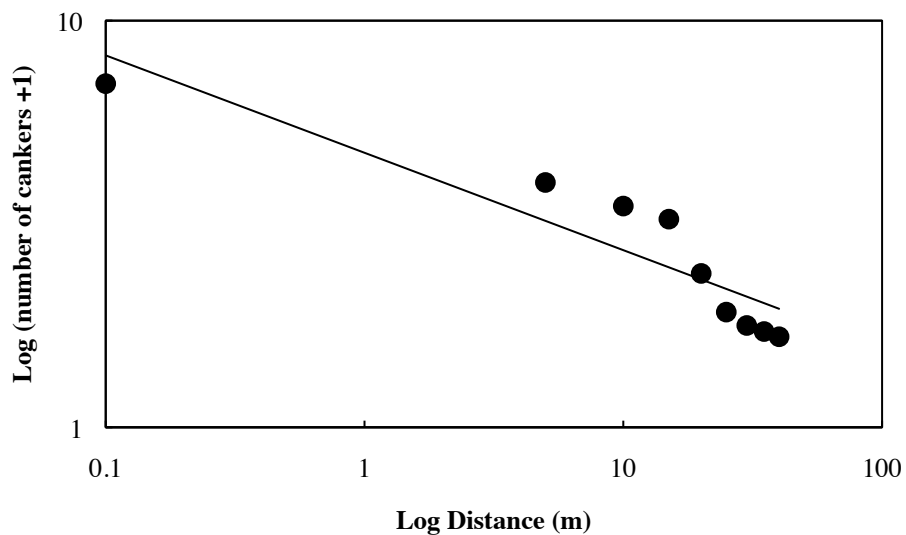
Diseased branches cut 10 weeks before budbreak and diseased branches cut immediately before budbreak are a significant source of *Anisogramma anomala* inoculum. Piles of diseased branches could disperse viable ascospores on the wind of the prevailing storm track and cause consistent infection in 2-year-old hazelnut trees at least 6.4 to 35 m away.



**Figure B.2** Incidence of eastern filbert blight in 2-year-old European hazelnut trees planted in nursery rows north of section C's source pile. Section C's source pile of pruned hazelnut branches bearing mature stromata of *Anisogramma anomala*, the causal agent of eastern filbert blight, was cut in December, 10 weeks before budbreak, when hazelnut becomes susceptible to infection was located at the Oregon State University Botany and Plant Pathology Field Laboratory near Corvallis, OR for the entire spring season from March to June of 2010. \* = source pile location.



**Figure B.3.** Disease severity (average number of cankers per tree) of eastern filbert blight on 2-year-old European hazelnut trees planted in nursery rows north of section C's source pile at 5m increments. Section C's source pile consisting of cut hazelnut branches bearing mature stromata of *Anisogramma anomala* cut in December, 10 weeks before budbreak, when hazelnut becomes susceptible to infection. The nursery rows were at the Oregon State University Botany and Plant Pathology Field Laboratory near Corvallis, OR for the entire spring season from March to June of 2010. (The 5 potted trees near the source pile used in the main trial were included).



**Figure B.4.** Severity of eastern filbert blight in 2-year-old European hazelnut trees planted downwind in nursery rows north of section C's source pile. Section's C's source pile consisted of cut hazelnut branches bearing mature stromata of *Anisogramma anomala* cut in December, 10 weeks before budbreak, when hazelnut becomes susceptible to infection. The experiment was conducted at the Oregon State University Botany and Plant Pathology Field Laboratory near Corvallis, OR for the entire spring season from March to June. Regression line  $b = (-0.239)$ . (Source infection is included)



**Table B.1.** Monthly *Anisogramma anomala* counts of ascospores in additional spore traps in sections C and D for the 2010 season.

Section C & D Ascospore Counts (Spores/ml)				
Month	#5 <sup>a</sup>	#6 <sup>b</sup>	#7 <sup>c</sup>	#8 <sup>d</sup>
March				
C <sup>e</sup>	0.0	0.0	0.0	0.0
D <sup>f</sup>	0.0	0.0	1.2	0.0
Apr				
C	0.0	0.0	0.0	0.0
D	0.0	0.0	0.0	0.0
May				
C	0.0	0.0	0.0	0.0
D	0.0	0.6	0.0	0.0
June				
C	0.0	0.0	0.0	0.0
D	0.0	0.0	0.0	0.0

<sup>a</sup> #5 = spore trap 6.4 m from source in the NW direction

<sup>b</sup> #6 = spore trap 6.4 m from source in the N direction

<sup>c</sup> #7 = spore trap 6.4 m from source in the SE direction

<sup>d</sup> #8 = spore trap 6.4 m from source in the S direction

<sup>e</sup> C = old pruning treatment: having a source with branches cut in December, 10 weeks before budbreak, when hazelnut becomes susceptible to infection

<sup>f</sup> D = fresh pruning treatment: having a source with branches cut immediately before budbreak, when hazelnut becomes susceptible to infection

