Characterization of Injection Wound Damage Associated with Propiconazole Treatments of Northern Pin Oaks

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Alexandra Clay Feltmeyer

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Abstract

Oak wilt, caused by *Bretziella fagacearum*, is one of the most destructive diseases affecting urban and rural oak trees in the Midwest USA. The disease is difficult to control due to the systemic movement of the pathogen through the vascular system of oak trees. Primary approaches used to control oak wilt include disrupting underground spread through root cutting and the prevention of wounding during the high-risk period that the insect vectors of the oak wilt fungus are active. Preventative or therapeutic treatment of oaks using systemic injection techniques are a more recently developed control approach used by arborists and urban foresters. Systemic injection into xylem vascular elements of woody plants, however, involves physical wounding to the lower stem or root crown. The ability of a tree to compartmentalize such damage may affect tree vigor or even a tree's ability to survive after repeated treatments. This thesis summarizes results of research on compartmentalization of damage associated with systemic injection of propiconazole fungicides in northern pin oak in Anoka County, Minnesota.

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Introduction

Oak wilt, caused by the ascomycete fungus *Bretziella fagacearum*, is one of the most destructive forest diseases of the Midwestern United States (Juzwik 2000). Mortality associated with oak wilt in Minnesota is most prevalent in the red oak group, *Quercus* section Lobatae, sometimes occurring within several weeks of first expression of symptomatic leaves (Gibbs and French 1980). Forest disease outbreaks such as oak wilt have negatively impacted timber industry, municipalities and residential property owners, as well as public forest land stakeholders (Lovett et. al 2016).

In Minnesota, management of the oak wilt fungus has focused on disruption of grafted root systems, avoidance of pruning or wounding oaks during time periods of high risk for overland transmission of the pathogen by insects, removal of potential oak wilt spore mat producing trees, and proper disposal of stems and large branches, use of systemic chemical control applied through root flare and stem injections, as well as restricted movement of firewood for prevention of long distance spread (Juzwik et al. 2008, Juzwik et al 2011, Koch et al 2010). Systemic injection of oaks with the ergosterol-inhibiting compound propiconazole (1-[[2-(2,4 Dichlorophenyl)-4-propyl-1-3-dioxolan-2-yl]methyl]-1H-1,2,4 triazole) was found to be effective in preventing oak wilt development Minnesota on *Quercus rubra*, *Q. ellipsoidalis*, *Q. macrocarpa*, and *Q. alba* (Osterbauer and French 1992, Eggers et al 2005). Systemic fungicides are desirable because of their reduced impact on the environment and their direct delivery to the intended target location of the plant. Because of the systemic spread of the oak wilt pathogen within a tree's water-conducting system, systemic fungicide use is limited to intravascular treatment through direct application into the outer sapwood.

Since the first development of a pressurized apparatus for injecting materials into trees in 1971 (Jones and Gregory 1971), there have been many modifications and adaptations to the original injection apparatus and other types developed. Macroinjections, or injections that utilize large volumes of water and rely on low levels of pressure to allow product to be translocated into the vascular system of the tree have been the industry standard when treating systemic vascular wild diseases such as Dutch elm disease and oak wilt. In comparison, microinjections or injections that utilize smaller volumes of water and rely on higher levels of pressure to deliver product more rapidly to the vascular system, have been adapted as the industry standard for injection of emamectin benzoate for the prevention and treatment of emerald ash borer in ash (*Fraxinus spp*.) throughout the Midwest (Doccola and Wild 2012). Microinjections are perceived to take less time to complete due to the reduced time for product suspension to be taken up by the tree because of pressurization. Additionally, they do not require root excavation or large volumes of suspension as the macroinjection method does. Evaluations of tree response to different injections using systemic fungicides and insecticides for control of other pathogens and of insects have been reported (Smith and Lewis 2005, Shigo et al 1977, Doccola and Wild 2012, Tanis and McCullough 2016), but host response to systemic fungicides used in oak wilt management has not been reported in the literature. The purpose of the research presented in this thesis is to document damage associated with the systemic injection of fungicides into northern pin oak, *Quercus ellipsoidalis*, utilizing two different injection methods.

Literature Review

Oak Wilt Biology

Oak wilt is a systemic vascular wilt disease caused by the fungus *Bretziella fagacearum*, formerly known as *Ceratocystis fagacearum* before the recent reclassification (de Beer et. al 2017). *Bretziella fagacearum* causes leaves to become a dullish green often with bronzing, progressing from the margins inward with subsequent wilting and necrosis leading to premature defoliation. Species in the white oak group (*Quercus* section *Quercus*) tends to produce different symptomology than those of the red oak group (*Quercus* sect. *Lobatae*), making diagnosis more difficult (Juzwik, et al. 2011). Parmeter et al. (1956) described the differences in disease development between bur oak (*Quercus macrocarpa*) and northern pin oak (*Quercus ellipsoidalis*). Northern pin oaks were described as wilting rapidly and thoroughly throughout the crown, whereas bur oak expresses symptoms that are more scattered throughout the canopy, sometimes allowing the tree to live for multiple growing seasons. Crown symptoms of the disease progress from the upper canopy downward and range from single branch symptoms in the case of white and bur oak, to full canopy symptoms in northern red and northern pin oak (Tainter and Baker 1996). These symptoms are a result of the pathogen's ability to enter the vascular system and produce hyphae and spores, causing a host response that eventually blocks the vascular system (Schoeneweiss 1959). Beckman et al. (1953) monitored water flow in inoculated trees using radioactive rubidium carbonate and found that water movement in heavily infected trees was reduced by 90 to 99%. The results of that study suggested that vascular plugging was responsible for reduced water flow that subsequently results in foliage wilt.

Oak wilt was first reported in 1943 in Wisconsin and officially described in 1944 (Henry et al. 1943, Henry et al. 1944). There was considerable concern in the U.S. that this pathogen would be a threat to oak populations, especially after the destructive effects of chestnut blight and Dutch elm disease had been documented (Appel 1995, Young 1949). Despite these concerns, chestnut blight and Dutch elm disease caused far greater losses to timber stands in comparison to oak wilt (Juzwik et al. 2008). The disease is most prevalent in states such as Illinois, Iowa, Minnesota, Texas and Wisconsin and is considered to be the most important forest disease in those states (Juzwik 2000, Gibbs and French 1980, Young 1949). All species in the family *Fagaceae* as well as a few related species have been shown to be susceptible to *B. fagacearum* based on observations of naturally infected or artificially-inoculated trees (Bretz 1955). Oak wilt causes rapid wilt to oak hosts in the red oak group, sometimes dying within several weeks after infection (Gibbs and French 1980, Henry et al. 1944). Trees in the white oak group have shown resistance, but they are still susceptible to mortality after multiple infections over time (Gibbs and French 1980).

The oak wilt fungus is transmitted from diseased oaks to healthy ones in two ways: 1) overland spread through insect vectors, and 2) underground spread through functional root grafts (Gibbs and French 1980). Oak wilt's primary source of inoculum is a macroscopically visible, pungent smelling, mycelial mat that resides between the cambium and the bark (Curl et al. 1953). Both asexual (endoconidia) and sexual (ascospores) spores may be produced on these mats. The fungal mats develop in the cambial area with "mirror-image" patterns produced on the outer sapwood and the inner phloem surfaces. Opposing pressure pads (dense, sterile tissue) that form on the mats

exert force as they grow, creating cracks in the bark (Curl et al. 1953). In Curl's description, only red oaks were noted to develop mycelial mats. However, Engelhard (1955) also documented fungal mats on red oaks in Iowa and also infrequently on white oaks species. Curl (1953) reported insect galleries associated with trees that developed the mycelial mat, and their discovery elicited curiosity as to whether there was an insect vector involved.

Hepting et al. (1952) described the sexuality of *B. fagacearum* as having two compatibility types, type A and type B. There was no perithecia formation when type A was paired with another type A isolate; similarly, no formation of perithecia occurred with the pairing of two type B isolates. Only the pairing of the two different types resulted in sexual reproduction. Campbell and French (1955) evaluated the effect of temperature on development of oak wilt mats in Minnesota. They found that mat formation occurred during two periods of the growing season, August through November and May through early June. During the period of August through November, mats formed on trees that had wilted in July, whereas the mats observed in May and June formed on trees that had wilted the previous season. Craighead and Morris (1952) suggested that if the virulent oak wilt fungus had an insect that creates its own wound, such as the European elm bark beetle for Dutch elm disease which transmits the fungus by creating its own wound for transmission of fungal spores, then the disease would have had a much more drastic impact on oak populations. He then described a series of necessary prerequisites for infection to occur, such as a wound to a healthy tree, sporulation occurring on fungal mats, and an insect present to introduce spores to a

healthy tree during an optimal time in the growing season when vessels are forming in the wood.

Wind, rain, and smoke of burning infected wood have not been proven to be modes of pathogen dissemination; however, human-mediated movement of the insect vectors via movement of firewood can lead to initiation of new infection centers (Juzwik et al. 2011). Insect vectors are the means by which new infection centers are started in nature. Craighead and Morris (1952) speculated that there are many insects that could be responsible for the transmission of *B. fagacearum* under normal conditions. Craighead et al. (1953) further hypothesized that the main vectors were nitidulid beetles (Coleptera: Nitidulidae) and two species of *Pseudopityopthorus* (*P. minutissimus and P. pruinosus*) in Pennsylvania. Testing of vector potential of four species of nitidulid beetles was done by Dorsey et al. (1953) in West Virginia by introducing the beetles to pure laboratory cultures of the fungus and then allowing the spore laden beetles to visit naturally and artificially created wounds on oaks in nature. After one month, five of six trees that had been visited by beetles that had been exposed to *B. fagacearum* developed the symptoms of oak wilt. In addition, they collected as many as 50 nitidulid beetles at any time from a single artificially wounded tree. They summarized that nitidulid beetles consistently visited wounded, healthy trees and were able to transmit the pathogen from infected to healthy trees very readily. The authors concluded by suggesting that nitidulid beetles made up one group of the main vectors of the oak wilt fungus. Two other potential insect vectors discovered were the oak bark beetles, *Pseudopityophthorus minutissimus* and *P. pruinosus*. Both were found to feed on twigs of healthy and infected trees in Pennsylvania (Griswold and Neiswander 1953). They were also found to feed in crotches,

leaf axils, bud axils, and immature acorn axils on small twigs of both oak groups (Griswold and Bart 1954). Laboratory experiments confirmed that *P. minutissimus* and *P. pruinosus* could theoretically transmit the fungus after exposure to and acquisition of pathogen propagules from fungal mats, but do not do so frequently enough to consider them primary vectors (Griswold and Bart 1954).

In Minnesota, the primary vectors of *B. fagacearum* are two nitidulid beetle species, *Carpophilus sayi* and *Colopterus truncatus* (Ambourn et al. 2005), while the smaller oak bark beetle, *Pseudopityopthorus minutissimus* is considered a minor vector species (Ambourn et al. 2006). Ambourn et al. (2005) described the abundance of *Ca. sayi* and *Co. truncatus* dispersing over the growing season using insect traps baited with insect pheromones synergized with fermented flour dough. The proportion of the collected beetles carrying viable propagules were determined and used to propose a general risk-based model for nitidulid beetle transmission of the pathogen. Ambourn et al (2006) used non-baited window flight traps placed in crowns of wilted oaks to document the flight periodicitiy of *P. minutissimu*s in Minnesota; however, few of the collected insects were found to be carrying viable propagules of *B. fagacearum*. Many *P. minutissimus* were found to colonize cut and piled branches of red oaks monitored for the insect species' presence in late spring and in August in oak stands.

Spores of *B. fagacearum* are passively carried below-ground in the sap stream of the vascular system from a diseased oak to a healthy one through functional root grafts. This kind of transmission is responsible for most oak mortality attributed to oak wilt (Gibbs and French 1980). Inter-tree root grafting is not uncommon for northern pin oak, making it easy for the pathogen to move from one infected tree to an adjacent healthy one

(Lyford 1980). Inter-tree and self-grafting in northern pin oak was evaluated by Blaedow and Juzwik (2010) in Minnesota by excavating pairs of trees that were grafted to trees known to be wilting from oak wilt. The oak wilt pathogen was isolated from functional, inter-tree root grafts in two of thirteen study trees in northern pin oak in Minnesota (Blaedow et al. 2010). In an early Minnesota study, the north central region was found to have less frequency of root grafting compared to areas of southern Minnesota (Bruhn et al. 1991) and it is this variability that makes frequency of grafting an important factor when considering management (Gibbs and French 1980).

Oak Wilt Management

Root disruption aims to stop below-ground transmission to surrounding healthy trees once primary inoculum has infected a healthy tree nearby (Koch et al. 2010). Species diversity, the ability of the oak species present to graft with one another, and the characteristics of the soil play a large role in the extent of root grafting in a stand (Bruhn et al. 1991). In Michigan, the disease was found to spread over distances of at least 40 ft through root grafting in a single year (Bruhn et al. 1991). Root disruption is commonly executed through use of equipment such as the vibratory plow or a chain trencher. Disruption is often applied along a single primary line or used in conjunction with a secondary line (Koch et al. 2010; Juzwik, et al. 2010). Primary lines are the outermost lines surrounding an oak wilt center, and they are the ones that have the highest probability to protect trees around the infection center (O'Brien et al. 2011). Secondary lines separate trees within the primary line that may look asymptomatic but are close enough to infected trees that they had to be included in the primary line (O'Brien et al.

2011). The secondary line may save some trees in this way, even if they were included in the primary line.

Sanitation, or removal of diseased trees, is another commonly used method for controlling oak wilt. Specifically, recently-killed oak trees that are likely to produce oak wilt mats (= potential spore-producing trees or PSPTs) are marked for removal prior to mat formation. This method is often used after root graft disruption methods are implemented but can also be utilized by itself. Any PSPT left within the primary and secondary root-cutting line, or in a non-treated oak wilt site, represents a future opportunity for insect spread of the pathogen. Removal of such trees reduces the amount of primary inoculum present in the stand for long distance spread (Appel 1995, Juzwik, et al. 2010). Removal of all the trees within the primary line ("cut-to-the-line") versus removal of only the trees that actually wilt ("monitor and remove") is advantageous if the landowner or land manager does not want to annually re-visit the site and likely need to remove trees that wilt in subsequent years. The disadvantage of "cut-to-the-line" is that healthy, non-root grafted trees are sacrificed and a larger number of trees are removed than if the "monitor and remove" is used (Koch et al. 2010, O'Brien et al. 2011 Juzwik, et al. 2010). Koch et al. (2010) noted that simply felling the trees within the line is also not sufficient because they are still able to produce mycelial mats. Appropriate disposal of the main stem and larger branch sections of felled tree is required. Removal of bark, chipping, burning, chemical treatment, girdling or drying of the wood, and stump removal will prevent mycelial mat formation and subsequent long-range primary inoculum dissemination (O'Brien et al. 2011 Tainter and Baker 1996, Koch et al. 2010, Appel 1995, Jones and Bretz 1958).

The use of systemic fungicides was considered as an alternative to root disruption for control of oak wilt by some arborists, but research in the past 15 years with propiconazole has shown this to not be the case (Eggers, et al. 2005; Blaedow, et al. 2010). Demethylation inhibitors (DMI's) are a group of fungicides that contain triazoles, including propiconazole. They have been used since the 1970's and are labeled for field crops and woody plants (Mueller 2006). Members of the triazole family are systemic within the plant, can control ascomycete as well as basidiomycete pathogens, are rapid in action and no resistance has been reported in the field (at least initially) (Schwinn 1983). Chemical control of *B. fagacearum* using triazole compounds has been well documented. Propiconazole based products inhibit a necessary sterol compound for ergosterol biosynthesis, resulting in a toxic effect to the fungal cells (Siegel 1981). Ergosterol is synthesized through the isoprenoid pathway, which synthesizes other important compounds such as hormones and sterols (Fletcher 1985) Thus, propiconazole exhibits both fungicidal activity as well as growth regulator effects.

Appel and Kurdyla (1992) evaluated efficacy of propiconazole intravascular injections in *Quercus virginiana* in Texas in two ways: in inoculated immature trees (30 3-year old live oaks), and in mature live oaks (57 treated trees) that were high-risk for oak wilt fungal infection via common root systems. All the plots were adjacent to expanding oak wilt pockets, being no more than 78 m from a known infected tree. The risk of infection was estimated by proximity to the nearest infected tree. For the immature tree study, fungicide treatment resulted in fewer symptomatic trees and those that were symptomatic had significantly less crown loss (4% after 67 days) when compared to untreated controls (35% after 67 days). In mature live oak plots, a significant reduction in

crown loss and mortality was found after 22 months of observation. Trees that were asymptomatic at time of treatment responded significantly better to the treatment than those that were symptomatic at time of treatment. These findings suggested that systemic treatment of live oaks with propiconazole is best when used preventatively rather than therapeutically.

Osterbauer and French (1992) also evaluated propiconazole as a treatment for oak wilt in Minnesota on *Quercus rubra* and *Q. ellipsoidalis* that were within 15 m of actively wilting trees. The asymptomatic trees used in the study were defined as high risk since they were within root grafting distance. A root flare injection was done using macroinfusion of propiconazole at a constant rate of 0.168 g/cm dbh using a standard root flare injection technique as discussed by Stennes and French (1987). The results indicated that propiconazole was a potentially effective option for control of oak wilt in *Q. rubra and Q. ellipsoidalis*. The number of treated trees that wilted (9 of 88) were significantly less than the number of non-treated treats that wilted (42 of 80) ($P<0.001$). The best control was achieved on sandy sites, rather than sites with other soil types. Vascular tissue samples taken at DBH (1.4m) were analyzed using chromatography to determine the presence of the fungicide at 1, 2, 8, 12, 20 and 23 months after injection. Propiconazole was detected at 1, 2, 8, and 12 months after injection, but was not present at 20 and 23 months after injection. Because of this, the authors suggested that trees be re-treated every 18-20 months. Blaedow et al. (2010) detected propiconazole in 100% of samples (n=68) taken from roots ($\leq$39 inches from injection sites) and in lower stems of treated trees at 2 and 12 months after injection. Detection levels decreased slightly (93%

of 72 samples) based on assay of a similar set of samples at 24 months after treatment. The average concentration of propiconazole decreased by 72% between 2 and 24 months.

An evaluation of operationally treated oaks (i.e. those treated by an arboriculture company for clients) was completed to determine the longevity of propiconazole treatment effect on both the red oak and white oak species (Eggers et al. 2005). The trees in these studies were within 4.6 and 15.2 m (15-50 feet) of an actively wilting tree of the same species. The evaluation study objectives were two-fold: 1) to determine whether propiconazole prevented disease development in visually healthy oaks within root grafting distance of actively wilting oaks over five years, and 2) to determine whether propiconazole treatment of bur oak and of white oaks exhibiting 45 percent or less active wilt in the crown would stop further crown wilt development. The treatment utilized the propiconazole product Alamo® and was applied using standard macro-infusion methods. In the preventative studies, 18 of the 46 total treated red oaks died over a five year period, while only one white oak *(Q. alba)* was found to have active wilt over the same time period for the total of 26 trees preventatively treated white and bur oaks. In the therapeutic study, one of the eight bur oaks that was injected exhibited new oak wilt symptoms during the five seasons of monitoring. Of the 8 white oaks ≤ 56 cm (22 in.) DBH, one showed new oak wilt symptoms in 1999 (one-year post treatment), but no symptoms were exhibited in 2000 (two years post treatment) and 2003 (five years post treatment). Of the five white oaks ≥56 cm DBH, one had new oak wilt in 1999, and two had new oak wilt in 2000.

Compartmentalization of Decay in Trees (CODIT)

The concept of compartmentalization was defined as far back as 1935 and subsequently described in great detail in the early 1970's and 1980's (Hepting 1935, Neely 1979, Shigo and Hillis 1973, Shigo et al. 1977, Shigo and Marx 1984). Hepting (1935) first addressed wounding and decay in trees when he analyzed valuable hardwood species in the Mississippi Delta region following fire scarring. His findings set a framework for which wounding and decay could be analyzed in the future. It was found that there was a definite relationship between age of the tree, diameter of the tree at the time of wounding, and the fungal species found on wounds to the rate of decay of fire scarred trees.

Before discussing the very heavily reviewed and studied topic of compartmentalization of decay in trees, it is important to discuss the progression of discoloration and its importance in trees. Shigo and Larson (1969) detailed the stages of discoloration and decay progression within a tree using data that Shigo had been collecting since 1959. The research was done using destructive dissection sampling on mature trees, a method that had previously not been done due to the invention of the portable gasoline-powered chainsaw not occurring until after World War II.

CODIT, or the Compartmentalization of Decay in Trees, is the concept that describes the ability of the tree to confine infection and decay within three orders of compartments and ending with the new growth being separated from the affected area (Shigo 1985). Trees differ from many other organisms in that they allocate resources to localize or wall off the wounded or desiccated tissue rather than healing or restoring it. Within the tree there are responses to injury that are present in the tree prior to wounding, and some that develop after wounding occurs. CODIT starts with part 1, focused on the

responses within the tree that were present prior to wounding. Three walls are formed and are referred to as reaction zones (Shigo 1985). Wall 1 resists the upward and downward (i.e. vertical) spread of decay, wall 2 resists inward or radial spread, and wall 3 resists lateral (tangential) spread. Part 2 of CODIT concerns responses within the tree that were only formed after wounding (i.e. wall 4) and it involves the localization of wounded tissue from new tissue being formed by the vascular cambium. This last step involves the cambium producing a thin layer of unique cells officially defined as the barrier zone, which is formed after the wounding event occurs.

Systemic Chemical Treatment Methods and Injection site Damage

Access to the vascular system of the tree is necessary for direct delivery of systemic fungicide to the tree when preventatively or therapeutically treating for vascular wilt pathogens. An apparatus was developed by Jones and Gregory (1971) for the injection of various solutions into trees. The purpose of this device was to allow the introduction of solutions directly to the outer xylem. Gregory et al. (1971) used the injection device on oaks (*Quercus rubra* and *Q. velutina*), elms (*Ulmus americana*), and maple (*Acer saccharum*) to test various solutions of benomyl (methyl 1-(butylcabamoyl)-2benzimidazole carbamate) for control of various diseases. The injections were successful in uptake and distribution within all three species tested, but it was concluded that more research was necessary before the technique could be used by practitioners.

Shigo and Campana (1977a) destructively sampled 80 trees that had been injected using various methods of injection and multiple formulations of different chemicals. The purpose of the study was not to determine the efficacy of the different formulations;

rather, it was to emphasize three points that Shigo had made about injection wounds. First, when a tree is wounded it reacts by developing discolored wood. At this point, other pathogens can invade and infect the wounded area, and this colonization can lead to decay. Secondly, coalescence of large columns of discolored and decayed wood may occur with multiple injection treatments. This is important to consider, as injections to therapeutically treated trees occur multiple times throughout the lifespan of the tree. Lastly, the first two events occur at vastly different rates and extents even within trees of the same species. The result of this study was that injections that had been repeated for multiple years yielded severe internal damage and decay.

Andrews et al. (1982) evaluated 16 elm trees to observe varying levels of compartmentalization after injection with Lignasan BLP® and Arbotect 20-S® for the prevention of Dutch elm disease caused by the pathogen *Ophiostoma novo-ulm*i. Lateral extent of internal discoloration in wounds injected with Arbotect 20-S® was significantly (P=0.05) greater than water-injection wounds or non-injected wounds after six months. After six to nine months there was no significant difference in extent of vertical discoloration between treatments. Xylem discoloration was observed immediately above the injection site for all treatments using scanning electron microscopy. Isolation of microorganisms from root flare tissue and stem tissue was done by placing small wood chips of tissue (10x3x3 mm) on four different agar media, and all microorganisms that emerged from the 4,606 wood chips were subcultured for identification. Interestingly, fewer microorganisms were recovered from root flare tissue than from the stem tissue samples. The recovery of basidiomycetes was low, however samples that were treated with the Arbotect 20-S® had an increased incidence in basidiomycetes compared to all

controls. The authors suggested that basidiomycetes could be associated with the Arbotect 20-S® treatment wounds. From these results, the recommendation for systemic injection was to inject healthy and exposed roots. Rainbow Treecare Scientific Advancements utilizes a macroinjection protocol that they developed following this concept, requiring excavation of the roots using a shovel or trowel and thoroughly brushing the soil from the root flares (Prosser et al. N.D.).

Bernick and Smiley (2016 and 2017) provided best management practices for performing tree injections. Macroinjection techniques involve low pressure application of high volumes of diluted chemical products to injection sites on the root flare region. Root flares are preferred for macroinjection because there is significantly more sapwood within that region than sapwood in the stem tissue. This results in much better distribution of the product within the tree, leading to a higher level of protection throughout the canopy. The authors recommend placing injection sites at a minimum of four to eight inches below the top of the root flare. Microinjection techniques do not require root excavation; rather, chemicals are injected under higher pressures (compared to macro-injections) at sites on the main stem. The authors recommended that sharp drill bits be used to create injection wounds, regardless of injection technique. Such bits minimize tearing of tissue (i.e. "cleaner cut") and allow for constant-speed drilling that also minimizes tissue damage.

Tanis and McCullough (2016) conducted multiple year studies evaluating wound responses at the injection sites of ash trees after they were injected with emamectin benzoate for protection against the emerald ash borer. Their objectives were to determine 1) if trunk injections with the product TREE-äge® resulted in external bark cracks and

discoloration in the xylem, and 2) whether injury or the response of trees to wounding varied between ash species, application rates, or frequency of injections. Their study measured wound responses of 46 injection sites two years after injection and an additional 15 injection sites six years after injection. All of the study trees were injected with TREE-äge® (4% ArborJet, Inc., Woburn, Massachusetts, U.S.) utilizing plastic Arborplugs® (#4, 0.95 cm, Arborjet., Inc., Woburn, Massachusetts, U.S.). For their two-year study, 22 of 46 trees were injected utilizing the medium-high rate (0.4 g a.i. per 2.5 cm DBH), applied with the TREE IV Micro-Infusion® system® (ArborJet., Inc., Woburn, Massachusetts, U.S.), while 7 of 15 study trees in the six-year study trees were injected using the same rate and application system. The researchers destructively sampled all the sample trees by felling them and recovering the 50 cm of the bole that contained the injection and associated discoloration. After bringing the samples back to the laboratory, each one was cut into 5 cm wide disks for further examination. The presence of secondary wounds (such as external cracks), xylem necrosis, and any evidence of pathogen infection (discoloration and soft tissue) were documented. Data on callus wound formation was also collected. Height, length, width and area of discoloration around each injection site was also measured. Results from the two-year study were that 77 of the 127 injections that were injected at the medium high rate using the TREE IV system were overlaid with new xylem. The six-year study results also showed that all 121 injections were overlaid with new xylem. Only two trees of the 22 treated with the medium high rate application during the two-year study exhibited external cracks. Results of the six-year study showed only one tree of seven exhibiting external cracks in the bark. For the two-year study trees, area of discolored xylem around

each injection site averaged 9.2±0.86 cm^2 and length of discoloration averaged 32.3±2.4 cm. Results of both studies indicated that injury associated with injection of TREE-äge® using Arborplugs® and the TREE IV Micro-Infusion® system rarely occurred.

Problem Statement

Efficacy of systemic propiconazole based fungicides have proven effective for prevention of oak wilt symptom development for 24 months (Blaedow 2010), providing a control option for tree care professionals who manage oak wilt. However, an evaluation of tree response to the physical wounding associated with injection of systemic fungicides in northern pin oak has not been completed. On sites with high disease pressure, it is necessary to reinject propiconazole products as the chemical degrades in above-ground parts of the plant in 12-18 months and in lower stems and roots in 24 to 36 months after treatment (Osterbauer and French 1992; Blaedow and Juzwik 2010). The host response and any negative side-effects of propiconazole treatment of northern pin oaks has not been previously evaluated. The purpose of this research was to evaluate, and document injection wound site responses in northern pin oak after microinjection and macroinjection with three different formulations of propiconazole for preventative treatment of oak wilt. The specific objectives were to evaluate and document external bark cracks, xylem discoloration, wound closure, and fungi in the wood surrounding the injection sites in northern pin oak.

Materials and Methods

Sampling sites and treatments. Two experiments were established in forest stands located in east central Minnesota. Northern pin oak trees (12 in Lino Lakes; 12 in Blaine) were randomly selected for treatment. Both sites were comprised of Isanti fine sandy loam and Soderville fine sand soil types (USDA NRCS Web Soil Survey). Three treatment methods were tested: (1) propiconazole formulated as the suspension concentrate Alamo® (14.3% active ingredient) (Syngenta Crop Protection Inc., Greensboro, NC) and applied using the standard macroinjection technique (S&S Tree Service, pers. comm.) consisting of 20 ml of the fungicide suspended in 500 ml of water for each 2.5 cm of stem diameter at 1.4 m height (DBH) injected under low pressure (15 psi); (2) propiconazole formulated as the suspension concentrate Propizol® (14.3% active ingredient) (Arborjet, Woburn, MA) using standard microinjection technique consisting of 20 ml of the fungicide in 20 ml of water for each 2.5 cm of stem diameter (DBH) under pressure (40 psi) (Don Grosmann, Arborjet, pers. comm.); and (3) propiconazole formulated as the suspension concentrate Quali-Pro® (14.3% active ingredient) using the standard microinjection technique consisting of 20 ml of fungicide in 20 ml of water for each 2.5 cm of stem diameter at 1.4 m height (DBH) under pressure (40 psi) above. TREE IV Micro-Infusion system was used for the protocol (2) and (3), followed by the insertion of an injection plug (Arborplug®). This plug is a 15mm in length and either 7 or 9 mm in diameter septum that was placed in the drilled hole for injection (Doccola and Wild 2012).

All propiconazole treatments, regardless of injection method, were completed in mid-June 2015. Microinjection treatments were done on the lower stems (0.8 to 1.0 m above soil line) of trees in mid-June 2015 using the Arborjet Tree IV® device. There

were two injection sites per one-inch DBH.. The injection wounds were created by battery-operated drill equipped with a 3/8-inch diameter bit. A plastic plug was inserted to retain any chemical that may not be taken up by the tree. Macroinjections were done in root flares of trees and required removal of 2-4 inches of soil at the base of each tree. A sharp 5/16-inch diameter bit on a power drill was used to create one injection site for each 1.5-inch DBH. In all cases, the drill depth was set at about ¾ inches to allow delivery of the product to active xylem tissue. Four weeks later (mid-July), the trees were challenge-inoculated with *Bretziella fagacearum*. One ml of an aqueous suspension of the pathogen (~ 1 x 10^6 conidia per ml) was placed in a ¾ inch deep x ¼ inch wide drilled hole in each of the excavated roots. The hole was drilled 30 cm distal to the root crown. The hole was covered with moldable epoxy resin and the removed soil placed back over the roots. All trees were monitored for oak wilt symptoms in late August 2015 and in late June and late August of the two subsequent growing seasons. Injection sites on all study trees were examined in mid-summer of 2016 and 2017. External cracks associated with the wounds were first observed on study trees in July 2016.

Destructive sampling. Twenty-four trees (12 per forest site) with a total of 279 injection wounds were selected for evaluation and felling. The selected trees ranged in age from 30 to 110 years. Stem diameters of the trees ranged from 7.2 inches to 17.2 inches DBH. On 29 August 2017, measurements of external cracks were taken on each injection point. The north side of each tree was marked using aerosol paint, and a different mark was made at 3 ft height on the stem of every tree. During tree felling, the apex of the notch of every face and back cut was located at 3 ft, for consistency sake. Once each tree was chainsaw-felled (Husqvarna 550xp, Husqvarna, Sweden, Stihl 180,

Waiblingen, Germany), the main stems were sequentially cut into 4 ft long sections from the lower crown downward until discoloration in the sapwood was observed. Discoloration associated with injections was considered to be any sapwood that differed in color or condition from healthy-appearing sapwood. Once discoloration was observed, the lower stem section from the top of discoloration to 6 inches below the lowest injection point on a tree was cut and removed from the site. Of the 24 trees felled, lower stem sections of two trees were considered too degraded for evaluation; thus, stem sections from these trees were subsequently eliminated from the study.

At the research facility, each tree was further dissected into approximately 3-inch disks and stored in stacks (according to tree number) on mesh benches in the greenhouse (60 to 70 F daytime temp and 55 to 60 F night-time temp) until further processed. Each disk then constituted a "sample". In early September 2017, each disk was further processed using a belt sander (Porter Cable, Syracuse, New York) and reciprocating saw (Bosch, Farmington Hills, Michigan) to make the face of each disk smooth. It was imperative that the faces were as smooth as possible to obtain good quality images. Once smooth, each injection site was numbered clockwise around the disk starting at the northernmost side of the disk. Each disk was stored flat on an indoor storage bench until further processed.

Measurement of external/internal defects and wound closure. Lengths of external cracks and of sapwood discoloration associated with each injection point were measured using all disks taken from the lower stem of a tree. Because a chainsaw was used for processing, a kerf allowance of 0.25 inches was deducted from each sample. The length of internal discoloration for each injection wound was recorded and used later for

volume calculations. Wound closure, expressed as a percentage of the total wound diameter, was recorded for each injection site. More specifically, a ruler was used to measure the initial wound size, and then the percentage of wound closure was obtained based on extent of post-wounding callus tissue formation.

Once the face of a disk was sanded and labeled, it was prepared for imaging. Images were taken in a white imaging tent using an Olympus E-510 digital SLR camera (Olympus America Corporation, Center Valley, PA) as raw images. The disk surface was sprayed with water to amplify the discoloration associated with the injection points. Before the image was taken, a ruler was placed on the face of the disk as a scale to use when calculating area. Images were stored on an external hard drive until further processing could be completed. From each image, area of sapwood discoloration was calculated using a tracing function in the imaging software ImageJ (Schneider et. al 2012) and a modified protocol from Giblin (2013). The area of discoloration and the length (height) of the disk were used to then calculate volume of discolored tissue for that disk. The discoloration was not uniform in that it tapered as it moved upward towards the canopy. To account for taper, each volume of discoloration was taken for each injection in each disk and added to the volume of discoloration in the disk(s) above. All of the calculated volumes were then added together and averaged. This method made for a more accurate assessment compared to using a basic volume calculation using the area of discoloration at the injection point multiplied by the total length of internal discoloration.

Isolation of fungi from injection sites. The disk from each of the 22 trees that contained the injection site(s) was then cut into sections using a reciprocating saw to obtain three randomly selected injection points, one in each wood "slice". The extracted

pieces were sealed with paraffin wax and stored in a 4° C cold room until further processed. The sealed injection sites were cut through their center to expose the axial side of the injection hole. Samples were surface sterilized by spraying the axial surface with 70% EtOH. Once exposed, eight small (0.4 cm^2) wood chips were extracted from the area of discoloration and placed onto plates of acidified potato dextrose agar (Difco bacto potato dextrose agar with 25 drops of 20% lactic acid). Eight additional wood chips were obtained and placed onto plates of BSA (basidiomycete select) medium using standard isolation protocol (Pokorny 1999). After 14-21 days of incubation at room temperature (24ºC), fungal colonies that formed were sub-cultured onto new plates until pure cultures were obtained. Mycelium from pure cultures of each fungal isolate was scraped from the agar surface with a sterile scalpel blade and stored into 200µl of cationic detergent cetyltrimethylammonium bromide (CTAB) lysis buffer.

DNA extraction was done using a Qiagen Stool Kit (Qiagen, Venlo, Netherlands). PCR was completed with the general fungal primer pair ITS1-F (5'-TCCCTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). Each 15 µl reaction contained 0.3 µl of primer ITS1-F (10 µM), 0.3 µl of primer ITS4 (10 µM), 3 µl of genomic DNA, 8.025 µl of molecular-grade water, and 3 µl of GoTaq Green (Promega Corp., Madison, WI). Negative controls lacking DNA were used to test for contamination. DNA amplification was conducted using a Mastercycler (Eppendorf AG, Hamburg, Germany) as follows: 94°C for 2 min; 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 2 minutes; and a final extension of 72°C for 7 minutes. Visualization was done on a 2% agarose gel to check for appropriately sized bands representing amplified fungal DNA from the ITS region.

The PCR product was then cleaned using Qiaquick PCR Purification Kit® (Qiagen, Venlo, Netherlands) before being submitted to the University of Minnesota Genomics Center (St. Paul, MN) for sequencing. Returned sequences were then subjected to BLAST searches of the NCBI database to obtain most likely species identity based on ≥98% match.

Statistical analysis. Data was analyzed using RStudio version 1.1.423 and R version 3.4.4. Assumptions of normality were tested using the Shapiro-Wilk test (Shapiro and Wilk 1965). Percent wound closure data was normalized using log transformations, while all other parameters met assumptions of normality. Sub-sampling was based on a completely randomized design and three-way ANOVA's were used to statistically analyze the differences among the three treatments. When the F statistic resulting from the ANOVA was significant, means comparisons were conducted using Tukey's HSD test (Tukey 1977).

Results

External bark cracks and internal sapwood discoloration. Although the largest number of injection sites were used for the Alamo® – injected trees (13 to 30 per tree) compared to the other two treatments, less than one crack on average was found to be associated with all of injection sites on each tree receiving this treatment. The number of injection holes per tree for microinjected trees were 7 to 11 and 7 to 14 for Propizol® and Quali-Pro® respectively. The average number of external cracks associated with the injection holes on these trees were 6.0 for the Propizol® trees and 7.2 on the Quali-Pro® trees (Table 1).

The average length of external bark cracks for trees treated with Propizol®, Quali-Pro®, and Alamo® was 6.46±0.89 in, 8.52±0.85 in, and 0.22±0.08 respectively (Figure 1). Cracks associated with injection wounds on the trees treated with Alamo® using the macroinjection method were shorter in length than those associated with Propizol® and Quali-Pro® microinjection wounds ($P=0.01$, $P<0.001$ respectively). No differences were found for length of external cracks for trees treated with Propizol® and Quali-Pro® using the micro-injection method ($P=0.51$).

Internal sapwood discoloration was associated with injection sites associated with each chemical x injection method treatment: 143 of 143 sites for Alamo® macroinjections; 60 of 60 sites for Propizol® microinjections; and 49 of 49 for Quali-Pro® microinjections. Average length and standard error of internal discoloration on trees injected with Propizole®, Quali-Pro®, and Alamo® were 28.85±2.4 in, 36.78±1.97, and 19.47±1 cm respectively. Average length of sapwood discoloration associated with injection holes of the three treatments were similar for Alamo® / macro-injection and Propizol® / micro-injection treated trees ($P = 0.17$) (Figure 2). Likewise, average lengths of discoloration were similar on trees treated with Quali-Pro® and Propizol® which were both applied by micro-injection ($P= 0.15$). However, discoloration columns were longer for Quali-Pro® treated trees than Alamo®-treated ones ($P=0.003$).

Wound Closure. Average percent and associated standard area for wound closure estimated for injection holes that were treated with Propizol®, Quali-Pro®, and Alamo® were 60.8±4, 59.0±5, and 98.9±0.06, respectively. Extent of wound closure was greatest for trees receiving Alamo® using macro-injection methods compared to the other two

treatments utilizing micro-injection ($P \leq 0.002$). There was no difference in extent of wound closure for trees treated with Propizol® and Quali-Pro® using micro-injection methods (P=0.91) (Figure 3).

Area and Volume of Internal Discoloration. Area of internal discoloration, which is the area of discoloration looking down at the cross section of the disk, differed by injection types. For trees macro-injected with either Propizol® or Quali-Pro® and for trees micro-injected with Alamo®, the average area and associated standard error of discoloration per injection site was 1.2±0.57 in^2, 1.29±0.59 in^2, and 0.63±0.46 in^2 respectively. Macroinjection utilizing Alamo® yielded a smaller area of internal discoloration compared to discoloration areas found for injection sites on Propizol® and Quali-Pro® treated trees (P=0.005 and P=0.009, respectively). No difference was found for area of discoloration associated with injection sites on trees treated with Propizol® and Quali-Pro® (P=0.99) (Figure 4).

Differences in individual volume of internal discoloration associated with the two injection types were highly significant. Average volume of discoloration associated with the two microinjection techniques utilizing Propizol® and Quali-Pro®, were 11.59 in^3 and 14.45 in^3, respectively, while the macroinjection technique using Alamo® resulted in an average volume of 4.31 in^3. Trees receiving Propizol® and Quali-Pro® had similar volumes of discoloration (P=0.615), but both were significantly larger volumes than those resulting from Alamo® / micro-injection (P=0.025 for Propizol®; P=0.002 with Quali-Pro®).

Total volume of internal discoloration within each tree, i.e. adding all discoloration volumes for a single tree, was less variable among treatment combination (Figures 5 and

6; Table 1). Specifically, the average total volume and associated standard errors for trees injected with Propizol®, Quali-Pro®, and Alamo® were 94.16±8.4 in^3, 123.9±6.7 in^3, and 87.6±2.3 in^3 respectively. There were no significant differences found among these treatments. (Figures 5 and 6, Table 2 and 3).

Fungi Isolated. The success in isolating fungi from injection sites of all three treatments was low and highly variable; thus, only combined results are presented. There were 41 fungal isolates obtained that yielded DNA of sufficient quality to conduct DNA sequencing. All but two of these isolates were ascomycetes. *Diplodia*, *Trichoderma*, and *Penicillium* were the most prominent species. *Diplodia corticola* was isolated 16 times, while *Diplodia mutila* was isolated once (Table 4).

Discussion

The results of this research suggest that there are differences in a tree's ability to respond to injection wounds depending on injection system used. Furthermore, responses measured also suggest that there is an interaction between the tree injection system and the propiconazole product used. This finding is consistent with the results of Andrews et al. (1982) for their study of benzimidazole compounds injected into American elms for the prevention of Dutch elm disease. It is interesting that the results of this study distinctly differ from the evaluation of xylem discoloration associated with microinjection of ash trees for prevention of emerald ash borer (Tanis and McCullough 2016). In the ash study there were no significant bark cracks or visual evidence of fungal colonization when microscopy work was done. Shigo and Shortle (1979) evaluated 40 red oak (*Q. rubra*) that they drilled into eight times at different locations along the stem

of the tree and at different times of the year. The wounding periods were January and August, March and August, March and June, and May and June. They girdled five of each wounding period group, to compare the amount of compartmentalization that would occur between non-girdled and girdled red oak trees. In non-girdled living trees, wounds were strongly compartmentalized by the heartwood, whereas in the girdled trees there was very weak to no compartmentalization responses. This study would suggest that in a living healthy *Quercus rubra*, compartmentalization occurs readily.

In this study, the treatment of macroinjection, utilizing a large volume of water and a significantly higher number of injection holes under lower pressure compared to the microinjection treatment, yielded higher levels of tree response for all measured variables. The microinjection method showed increases in length of external cracking and decreased tree response in the form of lower percent wound closure. External cracking could be associated with the physical wounding caused by the use of a drill bit. The drill bit used for the microinjection protocol is slightly larger (3/8-inch) than the one used for the macroinjection protocol (15/64 inch) which could contribute to significantly greater lengths of external cracks associated with microinjection sites compared to those for macroinjection. Additionally, the microinjection protocol drill-created wounds were made in the root flare tissue for the macroinjection treatments (Bernick and Smiley 2016). This, in conjunction with the use of the rigid plastic Arborplug®, may have inhibited rapid callus formation and, thus, wound closure. If placed too deeply the plastic plug may have contributed to vertical crack formation as newly forming bark callus tissue grows over the plug. If the Arborplug® is not set correctly within the wound, i.e placed too deeply or not deeply enough, the propiconazole product being applied may not reach

the correct conductive tissue. If placed too deeply, the chemical may be released in mostly non-conductive portions of the inner sapwood. In contrast, too shallow placement of the plug could have led to excessive pressure in the cambial region that caused separation of the bark and wood, leading to cambium death. Excessive propiconazole in this same region also could have caused tissue death due to phytotoxicity. This study was not designed to address the potential phytotoxicity interaction. The original treatments were primarily designed to compare efficacy of each propiconazole product x injection method; however, the design did lend itself to evaluation of the unexpected bark crack response to injection site wounding. Since both chemical concentration and volume injected differed between macroinjection and microinjection treatments, the results do lead to a hypothesis that either phytotoxicity or exposure of the cambium to high pressure (100 psi) due to improper plug placement caused the higher frequency of vertical bark cracks when compared to the macroinjection treatment which uses no plug. Rather, the high frequency of complete wound closure in the macroinjection treatments may reflect the fact that no rigid plug was used, and the wound was more conducive to callus overgrowth and wound "healing."

A tree's ability to respond and create a barrier between compromised wood and future growth is what allows it to persist without the introduction of decay. It is clear that there was reduced ability of the trees injected using the microinjection system to set walls 1 and 3 as described in the CODIT model, as lengths of internal discoloration, area and volume were significantly larger than those injected with macroinjection. As previously mentioned, extent of wound closure and callus formation was much less for injection wounds of microinjection treatments than for those macroinjection. Without chemical

and microscopic analysis, a full understanding of the compartmentalization of discoloration and decay in the study trees cannot be obtained. Future research could specifically address the causes or mechanisms responsible for the bark and wood defects results observed in this study in relation to the two types of injection systems.

To continue protecting red oak trees from the oak wilt fungus, propiconazole products are re-injected due to degradation of the chemical over time (Eggers et al. 2005; Blaedow 2010). Injection holes made for propiconazole "re-treatment" are generally placed in between, or otherwise offset, from the wounds made for the first treatment. The results of this study would suggest that re-treatment, regardless of injection system used, would increase incidence of internal and external damage, and potentially introduce plant pathogens within the sapwood of the tree. Thus, overall tree vigor may be an important factor to consider when continued disease pressure on a site justifies re-treatment. Furthermore, re-treatment of a microinjection application with the same system could lead to greater harm to tree health than use of macroinjection. Thus, proper depth placement of plastic plugs is very important to minimize development of unwanted tree defects.

Plant pathogenic fungi were found during the evaluation of fungi colonizing outer sapwood of injection wounds associated with both injection systems. Two other fungal pathogens were isolated, *Diplodia corticola* and *Diplodia mutila,* from the injection wounds and have recently been shown to cause shoot death and stem lesions on bare-root 2-0 and 1-0 red, white and bur oak in greenhouse studies conducted in Wisconsin (Smith and Stanosz 2018). There have been reports of *D. corticola* causing stem and branch cankers (referred to as Bot Canker of Oak) on landscape oaks in West Virginia,

California, Massachusetts, Maine, Florida, and Wisconsin (Smith and Stanosz 2018, Martin et al 2017, Urbes-Torres et al 2010, Munck et al 2017, Dreaden et al 2011, Aćimović et al 2016). It is possible that the presence of *D. corticola* in the wounds of trees in the current study may have interacted with other factors, e.g. mechanical damage and site conditions, to cause the severity of damage observed. Two wood decay-causing basidiomycetes found in this study suggest that organisms may colonize injection wound sites and lead to decay if a tree cannot successfully compartmentalize them. One wood decay fungus detected in this study, *Stereum complicatum*, is known to be a common decay species associated with oaks in the central hardwood region of Ohio, Indiana, Illinois and Missouri (Berry and Beaton 1972). In addition, *S. complicatum* was isolated and it is known to decay wood of oak and other deciduous tree species (Lynch and Eskalen 2014). *Penicillium* was found in abundance and could be functioning as an endophyte in this woody species.

Although the results of the difference in efficacy between the different chemicals or injection types are not part of the investigation covered in this thesis, the findings presented here document a significant difference in tree response to the two injection methods. The differences between the injection types and their impact on how trees respond to wounds could have long term implications regarding the overall health of the trees, and further studies should be conducted to evaluate long term impacts of systemic fungicide tree injections. Such studies should include multiple species of oak, multiple age classes, a combination of site conditions and soils, as well as an evaluation of the potential phytotoxicity that may be contributing to difference in response to damage.

Tables

Table 1. Number of bark cracks associated with injection wounds created for the application of Alamo® (20.0 ml per 2.5 cm DBH), Quali-Pro® (20.0 ml per 2.5 cm DBH) and Propizol® (20.0 ml per 2.5 cm DBH) to northern pin oaks.

Fungicide Product	Injection Method*	No. of Trees	Range in No. Injection Points/Tree	Ave. No. of Cracks/Tree
Propizol	Micro	7	7 - 11	6.02
Quali-Pro	Micro	8	7 - 14	7.15
Alamo	Macro	7	13 - 30	0.54

* Micro = microinjection performed with Tree IV system® applied at high pressure (100 psi); macro = macroinjections performed using macroinfusion system with tubing harness and chemical application at low pressure (15 psi).

Table 2. Volume of internal xylem discoloration associated with injection wounds created for the application of Alamo (20.0 ml per 2.5 cm DBH), Quali-Pro (20.0 ml per 2.5 cm DBH) and Propizol (20.0 ml per 2.5 cm DBH) to northern pin oaks.

Fungicide Product	Injection Method[x]	Volume (inches^3)	Standard Error	N
Propizol	Micro	11.59 a[y]	1.21	49
Quali-Pro	Micro	14.46 a	0.87	60
Alamo	Macro	4.31 b	0.2	142

[x] Micro = microinjection performed with Tree IV system® applied at high pressure (100 psi); macro = macroinjections performed using macroinfusion system with tubing harness and chemical application at low pressure (15 psi).

[y] Significant differences ($P<0.05$) among means are indicated by different letters as determined by Tukey's HSD test.

Table 3. Area of internal discoloration associated with injection wounds created for the application of Alamo (20.0 ml per 2.5 cm DBH), Quali-Pro (20.0 ml per 2.5 cm DBH) and Propizol (20.0 ml per 2.5 cm DBH) to northern pin oaks.

Fungicide Product	Injection Method[x]	Area (inches^2)	Standard Error	N
Propizol	Micro	1.20 a[y]	0.57	65
Quali-Pro	Micro	1.29 a	0.59	60
Alamo	Macro	0.63 b	0.46	142

[x] Micro = microinjection performed with Tree IV system® applied at high pressure (100 psi); macro = macroinjections performed using macroinfusion system with tubing harness and chemical application at low pressure (15 psi).

[y] Significant differences ($P<0.05$) among means are indicated by different letters as determined by Tukey's HSD test.

Table 4. Fungal species isolated from discolored xylem of injection site wounds created on northern pin oaks for systemic fungicide injection of Alamo® (20.0 ml per 2.5 cm DBH), Quali-Pro® (20.0 ml per 2.5 cm DBH) and Propizol® (20.0 ml per 2.5 cm DBH).

Blast Match	Nucleotide Match	Type	GenBank Accession No.
Diplodia agrifolia strain CBS 132778	418/422	Ascomycete	MH866051.1
Diplodia corticola isolate MKJP2_2012 25a_2012	550/552	Ascomycete*	MH384927.1
Diplodia corticola isolate MKJP2_2012 25a_2012	462/465	Ascomycete*	MH384927.1
Diplodia corticola isolate MKJP2_2012 25a_2012	546/548	Ascomycete*	MH384927.1
Diplodia corticola isolate MKJP2_2012 25a_2012	147/153	Ascomycete*	MH384927.1
Diplodia corticola isolate UCR488	348/359	Ascomycete*	JN693501.1
Diplodia corticola isolate UCR488	378/383	Ascomycete*	JN693501.1
Diplodia corticola isolate UCROK1192	387/390	Ascomycete*	JQ512106.1
Diplodia corticola strain BOT22	463/466	Ascomycete*	MF535372.1
Diplodia corticola strain BOT22	320/328	Ascomycete*	MF535372.1
Diplodia corticola strain BOT22	345/346	Ascomycete*	MF535372.1
Diplodia corticola strain BOT23	410/416	Ascomycete*	MG250710.1
Diplodia corticola strain CAA499	556/559	Ascomycete*	MG015741.1
Diplodia corticola strain CAA499	558/560	Ascomycete*	MG015741.1
Diplodia corticola strain CSC63	370/372	Ascomycete*	KX881760.1
Diplodia mutila isolate UCROK1429	541/548	Ascomycete	JQ411412.1
Penicillium coprophilum isolate W50ITS4RC	275/296	Ascomycete	GQ241935.1
Penicillium paneum strain CBS 126218	241/241	Ascomycete	MH863983.1
Penicillium paneum strain CBS 126218	397/398	Ascomycete	MH863983.1
Penicillium paneum strain CBS 126218	402/404	Ascomycete	MH863983.1
Penicillium paneum strain CBS 126218	397/397	Ascomycete	MH863983.1
Penicillium paneum strain CBS 126218	431/434	Ascomycete	MH863983.1
Penicillium paneum strain CBS 126218	231/234	Ascomycete	MH863983.1
Penicillium paneum strain CBS 126218	403/405	Ascomycete	MH863983.1
Penicillium paneum strain K7/1	389/389	Ascomycete	KF267253.1
Penicillium paneum strain Sd44	401/402	Ascomycete	HQ703579.1
Penicillium paneum strain Sd44	399/401	Ascomycete	HQ703579.1
Penicillium paneum strain Sd44	394/397	Ascomycete	HQ703579.1
Penicillium paneum strain Sd44	400/402	Ascomycete	HQ703579.1
Penicillium paneum strain Sd44	395/395	Ascomycete	HQ703579.1
Penicillium paneum strain Sd44	400/404	Ascomycete	HQ703579.1
Pestalotiopsis sp. YM1	335/370	Ascomycete	AB297793.1
Stereum complicatum isolate BHI-F476a	577/596	Basidomycete*	MF161283.1
Stereum complicatum isolate BHI-F476a	564/600	Basidomycete*	MF161283.1
Trichoderma atroviride isolate CTCCSJ-W-HB23823	214/218	Ascomycete	MF408998.1
Trichoderma atroviride strain wxm144	549/557	Ascomycete	HM047764.1
Trichoderma atroviride strain wxm144	567/568	Ascomycete	HM047764.1
Trichoderma atroviride strain ZNCFW5	407/434	Ascomycete	KR868346.1
Trichoderma erinaceum isolate 141010-18_G03_GBT29	816/901	Ascomycete	MF402944.1
Trichoderma sp. isolate L23	569/574	Ascomycete	MG198895.1
Trichoderma sp. isolate yi0549_1	563/569	Ascomycete	MH284961.1

*Indicates species is known to be pathogenic on oak trees.

Figures

Figure 1. Average length (inches) of external bark cracks associated with injection wounds created for the application of Alamo® (20.0 ml per 2.5 cm DBH), Quali-Pro® (20.0 ml per 2.5 cm DBH) and Propizol® (20.0 ml per 2.5 cm DBH). Significant differences among means are indicated by different letters as determined by Tukey's HSD test. Bars indicate +/- standard error; n=143 for Alamo®, n=54 for Propizol®, n=68 for Quali-Pro®.

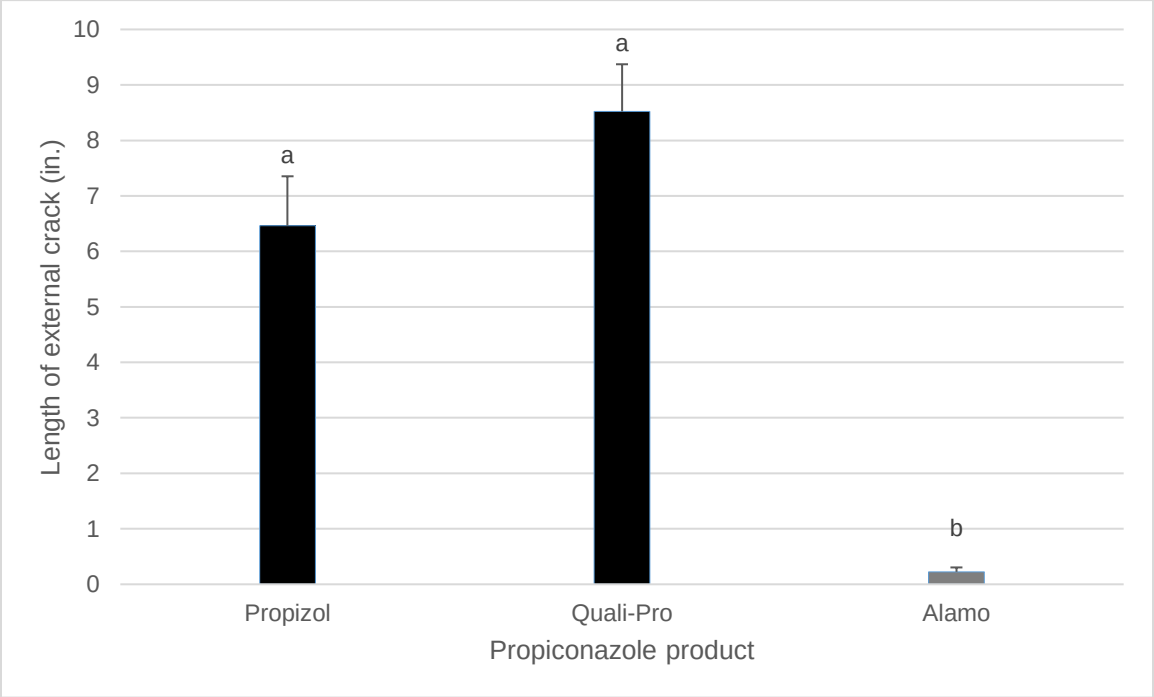


Figure 2. Average length (inches) of internal sapwood discoloration associated with injection wounds created for the application of Alamo® (20.0 ml per 2.5 cm DBH), Quali-Pro® (20.0 ml per 2.5 cm DBH) and Propizol® (20.0 ml per 2.5 cm DBH). Significant differences among means are indicated by different letters as determined by Tukey's HSD test. Error bars indicate +/- standard error: n=143 for Alamo®, n=60 for Quali-Pro®, n=49 for Propizol®.

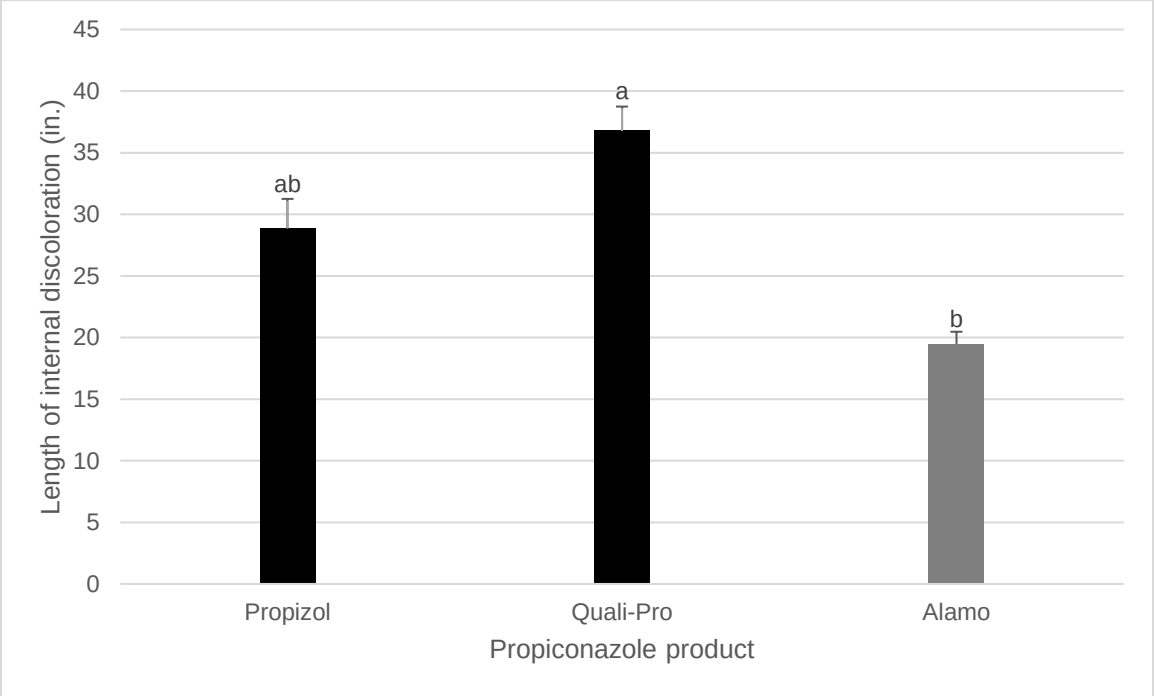


Figure 3. Average percent wound closure due to callus tissue formation after injection of northern pin oaks with Alamo® (20.0 ml per 2.5 cm DBH), Quali-Pro® (20.0 ml per 2.5 cm DBH) and Propizol® (20.0 ml per 2.5 cm DBH). Significant differences among means are indicated by different letters as determined by Tukey's HSD test. Error bars indicate +/- standard error; n=143 for Alamo®, n=60 for Quali-Pro®, n=68 for Propizol®.

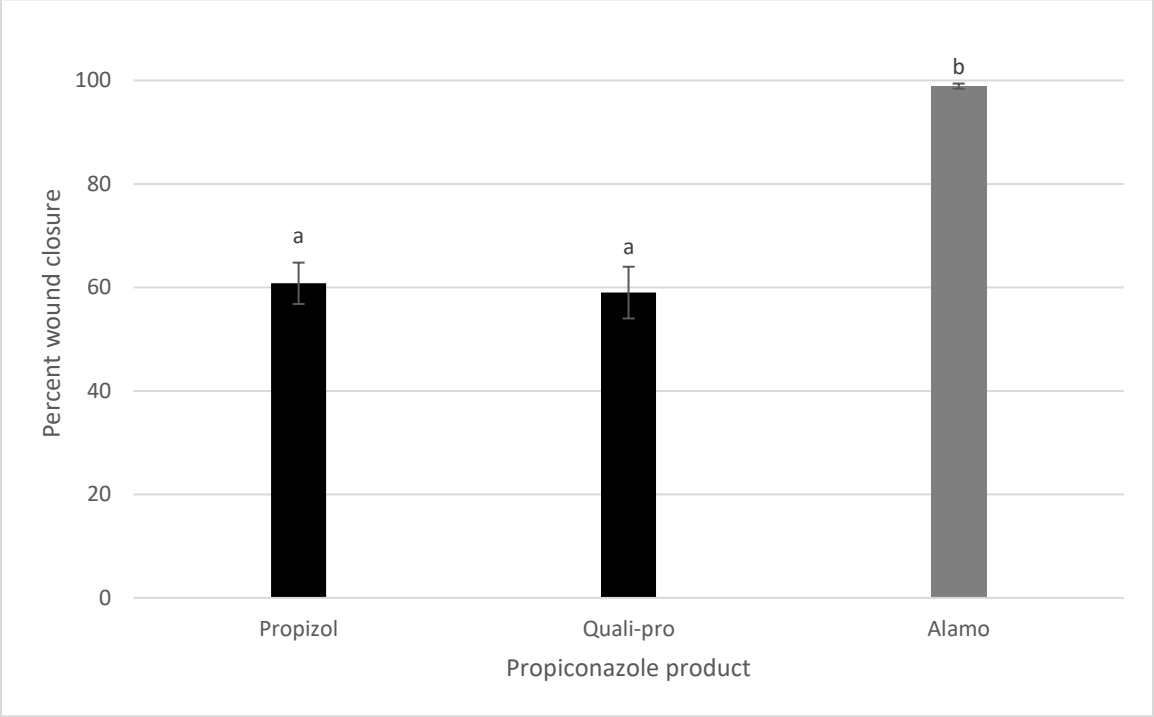


Figure 4. Average area (in.^{2}) of internal discoloration associated with individual injection wounds after systemic injection of Alamo® (20.0 ml per 2.5 cm DBH), Quali-Pro® (20.0 ml per 2.5 cm DBH) and Propizol® (20.0 ml per 2.5 cm DBH). Significant differences among means are indicated by different letters as determined by Tukey's HSD test. Error bars indicate +/- standard error; n=143 for Alamo®, n=60 for Quali-Pro®, n=65 for Propizol®.

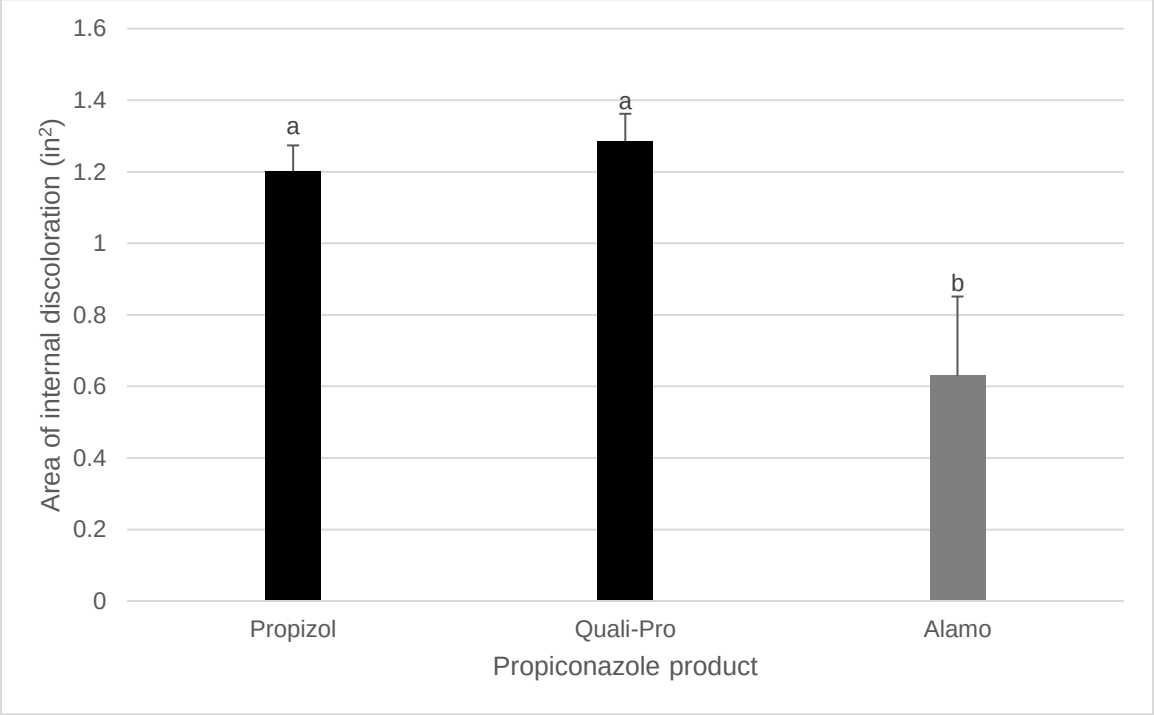


Figure 5. Average volume (in.^{3}) of internal discoloration associated with individual injection wounds after systemic injection of Alamo® (20.0 ml per 2.5 cm DBH), Quali-Pro® (20.0 ml per 2.5 cm DBH) and Propizol® (20.0 ml per 2.5 cm DBH). Significant differences among means are indicated by different letters as determined by Tukey's HSD test. Error bars indicate +/- standard error; n=142 for Alamo®, n=65 for Propizol®, n=60 for Quali-Pro®.

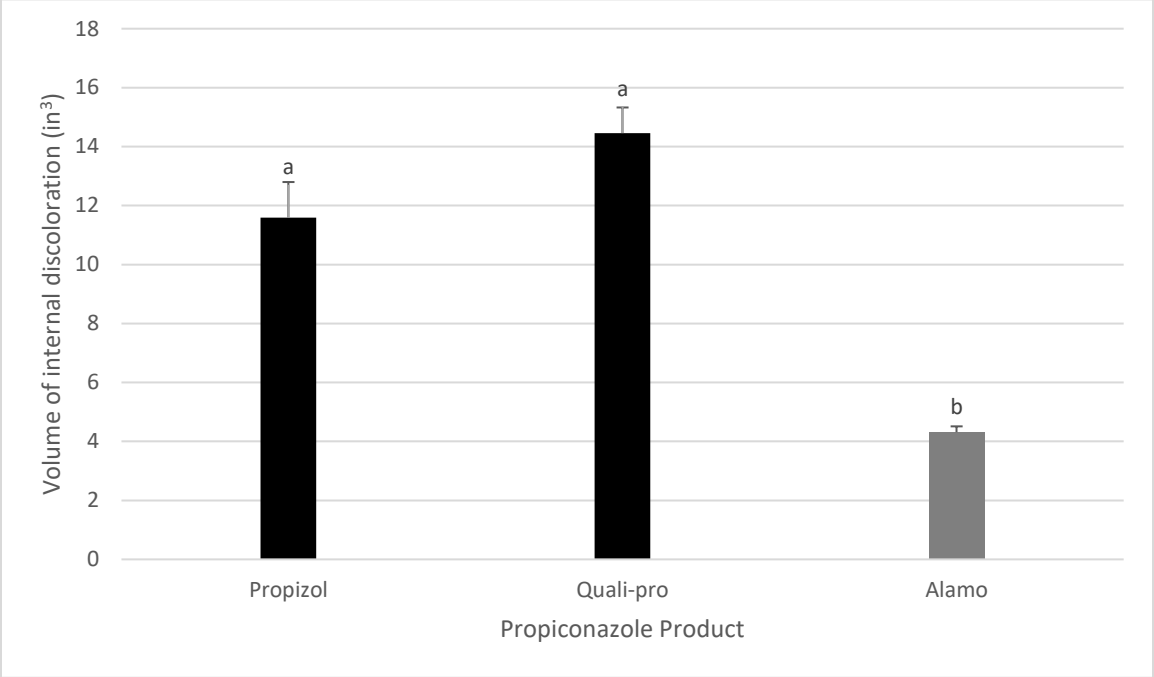
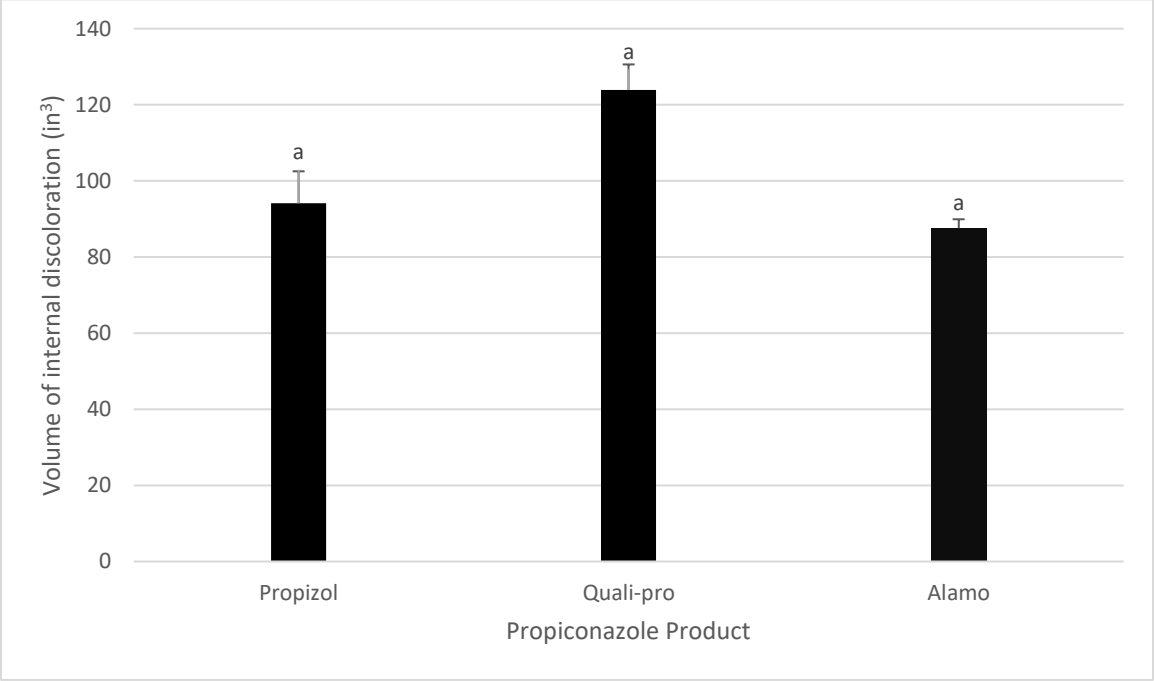


Figure 6. Total average volume (in.^{3}) of internal discoloration associated with all injection wounds within each northern pin oak after systemic injection of Alamo® (20.0 ml per 2.5 cm DBH), Propizol® (20.0 ml per 2.5 cm DBH), or Quali-Pro® (20.0 ml per 2.5 cm DBH). Significant differences among means are indicated by different letters as determined by Tukey's HSD test. Error bars indicate +/- standard error; n=143 for Alamo®, n=60 for Quali-Pro®, n=49 for Propizol®.



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