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EFFECTIVE LONGEVITY OF PROPICONAZOLE FOLLOWING INJECTION INTO QUERCUS RUBRA

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ABSTRACT
Propiconazole was injected into Quercus rubra to determine duration of efficacy against Ceratocystis fagacearum, the fungus that induces oak wilt. Eleven and 13 trees were treated preventatively in 2002 and 2003 respectively; these trees were subsequently inoculated with a conidial suspension of C. fagacearum at 0, 9.5, 14, 21.5, 23, 24, or 34 months following fungicide injection. Five- to six-foot (1.5- to 1.8-m) deep trenches isolated treatment groups. Control trees were located throughout the site and included trees either injected with fungicide, inoculated, or untreated and non-inoculated. Propiconazole-injected trees inoculated in May 2005, as late as 34 months following initial fungicide treatments, did not express wilt symptoms for at least three months; however, all untreated, inoculated control trees developed symptoms within six weeks. As of August 2006, over one year after final inoculations, 14 of the 24 treated and inoculated trees (including five of eleven trees inoculated at 34 months) remained symptomless. Results suggest that inhibition of C. fagacearum may occur even at 34 months post-injection.

Key words: Ceratocystis fagacearum, fungicide, oak wilt

Oak wilt is a lethal disease of oaks caused by the fungus Ceratocystis fagacearum (Bretz) Hunt. The pathogen invades the xylem inducing tylosis and gummosis in the host, which, in addition to fungal material, results in the blockage of water through sap tissues. Overland spread of C. fagacearum occurs via insect vectors, while local spread is primarily through root grafts that form between neighboring trees of the same species. Intravascular injections with a wide variety of antibiotics and fungicides have had limited success in treating or preventing oak wilt (Phelps, Kuntz and Ross 1966, Appel 1995). In 1987, however, Appel determined that live oaks (Quercus fusiformis and Q. virginiana) injected with the triazole fungicide, propiconazole, had significantly lower disease levels compared to untreated trees. Based on this study, propiconazole injection was deemed to be an effective treatment for oak wilt and was registered for use on live oaks in Texas (Appel 1995).

Osterbauer et al. (1992 and 1994) subsequently studied injection treatments in other species of red oak (subgenus Erythrobalanus) and white oak (subgenus Leucobalanus) in Minnesota and found that propiconazole could protect a treated tree for up to two years against root-graft spread. Additional research on propiconazole injection treatments has shown that white oaks typically respond well to fungicide injection and can often be treated therapeutically, whereas red oaks often succumb to wilt despite treatment if they already are infected (Osterbauer and French 1992, Osterbauer, Salisbury and French 1994, Eggers et al. 2005). Therefore, red oak injections are usually limited to high value trees with little or no symptoms of disease. Due to the high cost of treating trees (an average tree may cost a few hundred dollars to treat including chemical and

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labor costs), additional knowledge regarding the activity of propiconazole within a tree in relation to its distribution and to the distribution of the pathogen within the host is necessary for effective management strategies.

As the fungus can remain viable for several years in the roots of wilted trees, and spread through root grafts may take several years to occur, it is difficult to predict where the pathogen is within the root system at any given time (Yount 1955, Rexrode 1978). In addition, disease progression in root-infected trees is often delayed in comparison to trees inoculated above ground (Cobb, Fergus and Stambaugh 1965). Therefore, symptomless trees in naturally-infected stands may or may not already have the pathogen within their roots. Previous research has focused on the efficacy of propiconazole injection against root-graft transmission of *C. fagacearum* in stands where oak wilt was present. We investigated the longevity of propiconazole activity in a wilt-free oak stand in Michigan where root-graft transmission was prevented by trenching prior to experimentation.

**MATERIALS AND METHODS**

All oaks (*Quercus rubra*) used in the study were located on the same site on the Michigan State University campus and ranged from 16 to 51 cm diameter at breast height (dbh), with the average dbh equal to 30 cm (standard error ± 2.4). Trees at the site were arranged in six rows and had been established for several decades. The soil consisted primarily of Colwood-Brookston loam (62%) with Capac loam comprising the rest (38%). These series are characterized by deep, poorly drained, fine loamy soil. Five- to six-foot (1.5 to 1.8-m) deep trench lines were made using a Davis Fleetline 70+4 trencher, which isolated treatment groups by disrupting potential root grafts (Figs. 1 and 2). The experiment was replicated twice, first in July 2002 (Replicate 1) and then in June 2003 (Replicate 2). Forty-two trees were utilized in the study and an additional 21 trees at the site were maintained as negative controls (untreated and non-inoculated). Treatments consisted of trees injected with propiconazole and then inoculated with a wild-type *C. fagacearum* isolate, with the time interval between chemical injection and fungicide inoculation ranging from 0 (inoculated immediately following fungicide injection) to 34 months. Untreated, positive control trees were inoculated with the wild-type strain throughout the course of the study.

Since none of the fungicide-treated and inoculated trees from Replicate 1 developed symptoms by 2005, these 11 trees were inoculated a second time and included into a 34-month inoculation treatment. Therefore, at the conclusion of this study, all non-control trees from Replicate 1 had been inoculated twice: once at 0, 9.5, 14, 21.5, or 24 months after injection, and then again at 34 months post injection. In Replicate 2, trees from treatment groups 0, 9.5, and 14 months also remained symptomless in 2005 and so were incorporated into the 23-month treatment in 2005. Thus, the 23-month treatment group in Replicate 2 consisted of seven previously-inoculated trees and three trees that were only inoculated once, 23 months after fungicide treatment. Trees from Replicate 2, 24-month treatment, were inoculated only once at 24 months following injection.

Fungicide treatments with Alamo® (propiconazole 14.3%) were carried out via pressurized macro-injection into root flares with a 12V Flowjet pump according to the fungicide product label (Novartis Crop Protection, Greensboro, NC) in July 2002 (Replicate 1) and June 2003 (Replicate 2) (Fig. 3). Injection pressure was maintained at 20 pounds per square inch (psi). Trees were treated with 20 ml of fungicide diluted in one liter of water per 2.5 cm (1 inch) of tree dbh (2.8 g active ingredient per 2.5 cm dbh or 0.09 oz per inch dbh), which is the manufacturer’s
recommended dosage for trees under high disease pressure. Injection wounds were painted with wound paint the day after injection before being covered again with soil.

In general, trees absorbed the fungicide solution within a few hours; however, a few trees took much longer and often did not take up the full amount of product even when pressure was increased or the injection apparatus was left connected to the tree overnight. These trees typically received less than two-thirds of the attempted injection amount. Though these trees were retained in the study, their lack of full absorption was noted and any significant variations in results were considered.

Inoculations were performed with one wild-type strain, “Westcott”, recovered from a diseased tree in Ogemaw County, Michigan in 2001. Conidia from Westcott cultures grown on plates containing potato-dextrose agar (PDA) were collected by placing 1-2 ml of distilled water onto the plate and gently rubbing the surface with a glass rod. The resulting suspension was strained through Miracloth™ and the concentration was adjusted to $10^5$ conidia/ml with water and 20% glycerol. This suspension was divided into 1 ml aliquots and maintained at -80º C. The conidial suspension was thawed at room temperature for one hour prior to inoculation studies. Viability of spores was periodically assessed by serial dilution onto Petri dishes containing PDA; spore viability was consistently greater than 90%. For tree inoculations, a 2.5 cm-deep hole was drilled into the north side of the trunk at 1.5 m above ground with a 6 mm (¼ inch) bit. One ml of the $10^5$ conidia/ml suspension was then placed in the hole with a pipette. The suspension was generally absorbed within 5-10 minutes, and holes were subsequently covered with tape to prevent insects from entering the wound.

Trees were visually assessed monthly (May through October) for symptoms of oak wilt to determine the timing between inoculation and initial symptom development. Symptomatic branches from trees expressing disease symptoms were sampled for the presence of C. fagacearum by flame sterilizing samples after dipping in 90% ethanol, removing the outer bark, and then placing pieces of sap wood onto plates containing either PDA or glucose-phenylalanine agar. Final inspection of trees was done in early August 2006, 15 months after final inoculations. For the purposes of this study, trees were rated as either diseased (1) or healthy (0).

The relationship between treatment parameters and disease development was analyzed by exact conditional logistic regression using the LOGISTIC procedure in SAS version 9.1 software (SAS Institute Inc., Cary, NC). Disease was modeled as a function of replicate (1 or 2), month (when inoculation occurred), the number of times a tree was inoculated (once or twice), and whether trees received fungicide prior to injection. Significance of treatment variables to the model was determined according to a Score test and exact parameter estimates were analyzed to determine the type of effect each predictor variable had on disease occurrence. A p value $\leq 0.05$ was used to determine statistical significance.

RESULTS AND DISCUSSION

All 17 positive control trees from both replicates developed wilt symptoms within six weeks following inoculation and were completely wilted within the same year (Table 1). Thirteen of the 21 negative control trees were incorporated into other studies in 2005 leaving eight non-inoculated controls in 2006. None of these trees developed wilt symptoms over the course of the study, indicating that the trench lines initially established in 2002 remained effective and that insects were not moving inoculum.

Possible wilt symptoms on treated trees first appeared in late fall of 2005, but as these symptoms developed just before fall coloration, wilt was not confirmed until the following year.
All symptomatic trees sampled in 2006 were positive for *C. fagacearum*. Of the propiconazole-treated trees from Replicate 1, only six of eleven trees showed disease symptoms in 2006. All six symptomatic trees had been inoculated twice: two at 14 and 34 months after fungicide injection, two at 21.5 and 34 months, and two at 24 and 34 months.

Four of the 13 treated and inoculated trees from Replicate 2 displayed symptoms in 2006: one of the three trees that had been inoculated once only at 23 months and three of the seven trees that were inoculated twice (one at 0 and 23 months and two at 14 and 23 months). All four of the trees from Replicate 2 that expressed wilt symptoms in 2006 failed to absorb the full amount of fungicide when injection was attempted. Only one tree in this study that did not take up the full amount of propiconazole did not develop wilt symptoms. Two of the three trees inoculated once at 23 months and all of the 24 month trees (Replicate 2) remained symptomless over one year after inoculation. Yet, the untreated control trees inoculated at 24 months developed wilt symptoms in 2005, the same year they were inoculated.

All fungicide-treated trees that developed wilt had delayed symptom development both initially and after symptoms appeared. Symptoms were not obvious for at least 3-13 months after inoculation and were confined to scattered branches where the disease progressed slowly. This is contrary to what was observed in the untreated control trees, which expressed symptoms within six weeks following inoculation that progressed rapidly from the top of the crown, downward.

The number of times a tree was inoculated and whether or not a tree received fungicide prior to inoculation significantly contributed to the disease model; however, month (p = 0.43) and replicate (p = 0.28) were not significant explanatory variables and were excluded from the model. Based on parameter estimates, trees that did not receive a fungicide injection and those trees inoculated twice had greater incidence of disease, while fungicide-treated trees (regardless of month) had decreased disease incidence. The null hypothesis that one inoculation had no effect on disease cannot be rejected (p = 1.00).

These results indicate that propiconazole potentially remains effective for at least 24 months and provides some level of protection up to 34 months post-injection. Interestingly, Osterbauer and French (1992) were only able to detect propiconazole using a thin layer chromatography assay up to 12 months following injection. A double (instead of a single) band was observed in sample lanes at 16-18 months post-injection, similar to that found in the fungicide standard lanes when older supplies of propiconazole were analyzed, suggesting degradation of the product. At 20 months post-injection, no propiconazole was detected in any samples. Our results demonstrate that the product may still inhibit fungal growth even after 34 months and that the amount of propiconazole injected may influence the length of efficacy of the product, as Osterbauer and French (1992) used much lower rates in their studies. Additionally, the TLC assay they used may not have been sensitive to low levels of propiconazole that would still affect *C. fagacearum*.

Propiconazole is a triazole-fungicide, one of the classes of sterol-biosynthesis inhibiting fungicides. Wilson and Forse (1997) determined that propiconazole, at sufficiently high levels, was fungicidal to *C. fagacearum*. Therefore, in this study, the pathogen was potentially killed due to high initial levels of the fungicide in trees inoculated soon after injection. However, at lower concentrations of sterol biosynthesis inhibitors, the inhibition of fungal spore germination is incomplete (Kuck and Scheinpflug 1986, Latteur and Jansen 2002, Nogueira et al. 2002). Thus, propiconazole may delay active colonization of the fungus until it degrades to low enough
levels, at which point the pathogen can spread throughout the tree. Incomplete distribution of the fungicide within a tree would also contribute to this effect.

In addition to its fungistatic effects, triazoles, including propiconazole, are known to have plant growth regulating properties (Kuck and Scheinpflug 1986, Wetztein et al. 2002, Hanson et al. 2003). Phelps, Kuntz and Ross (1966) reported that indole 3-acetic acid, a natural growth regulator, delayed symptom development in northern pin oak (Q. ellipsoidalis) up to 12 months or more when injected into the trunk. Although indole 3-acetic acid is an auxin and thus stimulates growth, whereas propiconazole has a growth retardation effect, by changing the balance of growth regulation in the plant (perhaps enhancing the tree’s ability to cope with stress or interfering with the production of tyloses in response to the pathogen), disease development is affected. Thus, it is possible that propiconazole works in two ways to inhibit disease development – first by interfering in ergosterol-biosynthesis and secondly by affecting growth regulation within the host.

Interestingly, the effects of propiconazole on disease development are similar to those found with other compounds tested for the control of wilt. Phelps, Kuntz and Ross’s (1966) research on northern pin oak shows that a few antibiotics and/or chemicals prolonged the incubation period of the disease up to 24 months. The pattern of symptom development on such treated trees differed from untreated trees in that wilt symptoms developed slowly, often branch by branch, and sometimes over one to two years. Similar results of a temporary delay effect were found with trials using thiabendazole for wilt (Appel 1995). We also observed this effect on the treated trees in our plot that eventually developed symptoms: symptoms were initially confined to particular scattered branches and progressed much more slowly than would be expected (in comparison to inoculated controls). Phelps, Kuntz and Ross (1966) also reported that, despite a prolonged incubation period, the fungus was isolated from 75% of symptomless trees 12 months after inoculation. This demonstrates the ability of the fungus to remain within a tree without inciting disease, further supporting the idea that propiconazole may have ultimately only delayed disease development in our plot. However, given that approximately half the trees inoculated for a second time at 34 months post-injection did not develop symptoms over one year later, it may be that by suppressing pathogen growth long enough, a tree could fundamentally be protected.

As the majority of research on oak wilt has focused on the host-pathogen interaction above ground, there remain many unanswered questions regarding the movement and colonization of the pathogen in the root systems. Evidence of pathogen movement through root grafts may take one to three years (Rexrode 1978) and seemingly dormant disease centers may begin wilting again after several years, presumably due to root-graft spread. Additionally, root-inoculated trees often display delayed wilt symptoms up to one year from inoculation (Cobb, Fergus and Stambaugh 1965). Thus, it is difficult to determine when and how the pathogen enters the root system and what happens once it is there. Defensive reactions in response to C. fagacearum are less extensive in the roots than in other parts of a tree (Struckmeyer et al. 1953). This suggests that the pathogen may be able to colonize parts of the root system, which has implications for disease development in fungicide-treated trees. There is evidence that propiconazole is distributed to the root system of injected trees (Tattar and Tattar 1999, Blaedow et al. 2005), but it is unclear to what extent and how this affects pathogen growth and movement within the root system.

Natural infection with C. fagacearum is most likely to occur through branch wounds or root graft movement; thus, the results from this study must be interpreted accordingly. Our experiment tested the effectiveness of propiconazole injection against non-root graft spread of
the pathogen as trees were inoculated in the bole. The observed inhibitory effect may break down with natural overland infections, which probably occur in the crown, as it is believed that the fungicide is not translocated or distributed evenly throughout the upper canopy (Osterbauer and French 1992). While it has been documented that bole inoculations have greater inoculation success than crown-inoculated trees (Jones 1964, Cobb, Fergus and Stambaugh 1965), it is also probable that the greatest amount of fungicide was distributed within the trunk. Therefore, the observed delay in symptom development may or may not be related to the initial distribution of the fungicide in relation to the inoculation site, underscoring the need for further clarification of this relationship.

CONCLUSION

The possible dual inhibitory effects of propiconazole and advances in delivery via macro-injection have made propiconazole a promising fungicide treatment for oak wilt. However, since the early work by Osterbauer and French (1992) on propiconazole injections in red oaks, it has been apparent that red oak treatments are somewhat unpredictable. Our results show that propiconazole injection was an effective preventative treatment for oak wilt in some cases up to 34 months following injection; however, disease pressure apparently affects the duration of efficacy, as intimated by disease occurrence in trees receiving two inoculations. All but one of the treated trees that developed symptoms had been inoculated twice and did not express obvious symptoms for over a year after final inoculations, whereas all positive control trees developed symptoms within six weeks, indicating that treated trees inoculated at the same time were delayed in symptom development. Additionally, all symptomatic trees from Replicate 2 had not taken up the full amount of fungicide administered. Given these results, the effective longevity of propiconazole appears to be dependent on several factors including the amount injected, the level of disease pressure, where the pathogen enters a tree, and the relative distribution of the pathogen and fungicide within a host tree.

ACKNOWLEDGEMENTS

We would like to thank Clifford Zehr and Mario Mandujano for their assistance with the trenching and fungicide injections, respectively.

LITERATURE CITED


Table 1. Proportion of wilting trees in August 2006 per replicate. Treatments consisted of trees injected with propiconazole and then inoculated with *Ceratocystis fagacearum* at A) 0 (inoculated same day as injected), 9.5, 14, 21.5, 23, or 24 months later and B) once at 0, 9.5, 14, 21.5, or 24 months and a second time at 23 or 34 months. Positive, untreated control trees were inoculated at the respective time (in months) after experiments began. Replicate 1 began in July 2002, while replicate 2 was initiated in June 2003. A ‘-’ indicates treatment was not included in that replicate.

**A**

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<th>21.5</th>
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<th>24</th>
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<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>-</td>
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<td>0/3</td>
<td>-</td>
<td>1/3</td>
<td>0/3</td>
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<th>34</th>
<th>Control trees</th>
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<tr>
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**B**

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<tr>
<td>Rep 2</td>
<td>3/7</td>
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Figure 1. Trenches, dug with a Davis Fleetline 70+4 trencher, were used to isolate treatment groups.
Figure 2. Five to six-feet-deep (1.5-1.8 m) trenches break potential root grafts between neighboring trees. Trenches are approximately 4-6 in. (10-15 cm) wide.

Figure 3. Injection apparatus used for propiconazole injection treatments. The fungicide solution was pumped out of the storage tank and into 2.5 cm-deep holes drilled into the xylem of the root flares.