The Proceedings of the 2nd National Oak Wilt Symposium

Edited by:

Ronald F. Billings
David N. Appel

Sponsored by

International Society of Arboriculture – Texas Chapter

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Texas Forest Service
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2009

A Laboratory-Induced Hypovirulent Strain of the Oak Wilt Fungus

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ABSTRACT

A strain of *Ceratocystis fagacearum* with debilitated respiration, as measured by an increase in alternative oxidase activity, was selected in the laboratory after exposure to ethidium bromide and ultra-violet light mutagenesis. The mutant, PM447, showed altered morphology, increased levels of alternative oxidase activity, and reduced virulence when compared to the wild type strain. PM447 protected 28-day-old seedlings from oak wilt disease when seedlings were inoculated two-weeks prior to inoculation by virulent strains. In an effort to apply this technology to a wider application, we expanded the parameters of the original study to include challenges beyond two weeks, varying the strain of virulent wild-type used in the challenge inoculation, varying the number of spores of PM447, and using the mutant to protect saplings and mature trees in field plots. In general, PM447 has been shown to protect challenged seedlings and delay disease onset in mature trees; however, its efficacy as a biocontrol agent in the field is poor and will require a better understanding of the mechanisms of protection.

Key words: Biocontrol agent, *Ceratocystis fagacearum*, hypovirulence

The term 'hypovirulence' was first used to describe strains of the chestnut blight pathogen, *Cryphonectria parasitica* (Murrill) Barr, that were decreased in virulence (Grente 1965). These strains were recovered from non-lethal, healing cankers on chestnut trees and typically displayed abnormal growth and morphology in culture. It was later determined that the hypovirulent phenotype was due to the presence of cytoplasmic double-stranded RNA within the fungal mycelium (Day et al. 1977). Some hypovirulent strains of *C. parasitica* were found that lacked dsRNA hypoviruses (Fulbright 1985, Baidyaroy et al. 2000). Mahanti et al. (1993) established that these isolates had increased levels of alternative oxidase activity, indicative of mitochondrial dysfunction. Additionally, the hypovirulent phenotype was transmissible via hyphal anastomosis and maternally inherited in crosses, suggesting the role of mitochondrial mutations in hypovirulence in these isolates.

Shaw (1999) attempted to duplicate mitochondrial-based hypovirulence in *Ceratocystis fagacearum* (Bretz) Hunt, the fungal pathogen that causes oak wilt. Conidia from a wild-type strain, "Fenn", were exposed to ethidium bromide and UV light and then screened for slow growth, an indicator of possible mitochondrial dysfunction. The mitochondrial origin of the phenotype was determined by testing for alternative respiration (cyanide resistance and salicylhydroxamic acid sensitivity) and maternal inheritance of the trait. One mutant, PM447, appeared to satisfy the above requirements and was subsequently used in seedling assays similar to that developed by Fenn, Durbin and Koontz (1975). Using 28-day-old seedlings maintained in a growth chamber, Shaw found that seedlings first inoculated with the hypovirulent strain PM447 and then challenged with the wild-type Fenn two weeks later, displayed significantly less symptom development when compared to seedlings inoculated with Fenn only or those seedlings

challenged at 0 or 1 weeks. In preliminary efforts to apply this technology to a wider application, we repeated Shaw's earlier work, expanded the parameters to include challenges beyond two weeks, varied the strain of wild-type used in the challenge inoculation, varied the spore load of PM447, and included mature trees from several field plots in these studies.

MATERIALS AND METHODS

Spores from PM447 cultures stored for two years at 4-6ºC were screened for slow growth, and conidia from the slowest growing of these were collected. These conidia were then plated and screened for slow growth following germination. A spore suspension (10^5/ml) was made by combining conidia with water and 20% glycerol. The resulting suspension was stored at -80ºC and used for all subsequent PM447 inoculations. Wild-type isolates used in the studies were originally obtained from diseased trees (Westcott and Beal) or from cultures stored in collections.

Single-spore cultures of all isolates used were plated on potato-dextrose agar (PDA) and allowed to grow for 14 days at room temperature. Conidia were collected by pipetting 2 ml of distilled water onto the plates and rubbing the top of the mycelia with a glass rod. The resulting suspension was strained through Miracloth™ and the conidial density was adjusted to $1x10^5$ conidia/ml with distilled water and glycerol to make a 20% glycerol solution. Suspensions were divided into 1ml aliquots and maintained at -80ºC.

Greenhouse and Growth Chamber Experiments

Experiments performed in the greenhouse and growth chamber utilized 28- to 35-day-old red oak (*Quercus rubra*) seedlings; red oak seedlings were used in the greenhouse study, and pin oak (*Q. palustris*) was used for the growth chamber study (*Q. rubra* seed unavailable). Stratified seed were planted into 16 ounce cups containing Baccto™ planting mix. The growth chamber was maintained at 26º C with a 16-hour daylight period.

For inoculations, a 10 ul drop of conidial suspension was placed at the base of the stem approximately 2 cm above the soil line. A 26-gauge needle was then inserted through the droplet into the stem at a 45-degree angle. Absorption of the droplet was observed, indicating successful uptake of the suspension into the xylem. Inoculated seedlings were monitored weekly for symptom development.

The disease rating of seedlings was based on the degree of symptom expression at six weeks (greenhouse) or eight weeks (growth chamber) post-wild-type inoculation using a 0 to 5 scale (Table 1).

Field Plot Experiments

All trees used in the Beaumont/East Farm study were red oak and trees at the Jackson site appeared to be a mix of red and northern pin oak (*Q. ellipsoidalis*). The Beaumont/East Farm study had three tree category types based on their diameter; type 1 trees were saplings with a diameter at breast height (d.b.h.) of 2-3 cm, type 2 trees had a d.b.h. equal to 5-6 cm, and type 3 trees were mature trees with a d.b.h. greater than 12 cm. All other trees used in the field plots were considered type 3. Inoculation wounds were made at 1.4 meters above ground into the north side of the trunk by drilling a small hole into the xylem. In cases where trees received more than one inoculation, subsequent inoculation sites were located 1/4 of the way around the trunk to the left of the previous inoculation location. Type 1 trees received two doses of 10ul inoculum, five minutes apart, for a total of 20 ul. Type 2 trees received 50 ul inoculum and type

3 trees received 1 ml inoculum. Trees were rated as healthy (0), intermediate (1), or wilted (2). Intermediate ratings were assigned to trees that developed wilt symptoms that did not progress beyond 60% crown wilt over a 1- to 2-year period. A disease rating of 2 was given to trees with advanced stages of wilt that did not recover by the following year.

Statistical analysis of the data was performed using the Genmod procedure with SAS v.9.1 software. In some cases when the model fit using Genmod was in question, the Glimmix procedure was utilized. A p value less than or equal to 0.05 was used to determine statistical significance of the variable/parameter in question.

RESULTS

Greenhouse Inoculation Studies

The greenhouse study was designed to examine how the timing between inoculation with PM447 and wild-type challenge inoculations, as well as varying the wild-type strains (Fenn, Westcott, or Beal), affect symptom development in seedlings. Disease ratings were significantly higher in rep 2, so the data from both experiments were analyzed separately. There was no significant difference between challenges at 0 weeks and seedlings inoculated with the corresponding wild-type strain only (Table 2 and Fig. 1). Disease ratings of seedlings challenged at either 1, 3, or 4 weeks, however, were significantly lower than those of seedlings inoculated with a wild-type strain only or those co-inoculated with PM447 and a wild-type. There was no significant difference between disease ratings for 3 and 4 week (rep 1) or 1 and 3 week (rep 2) challenges for any of the isolates.

Growth Chamber Inoculation Studies

Growth chamber treatments included a range of spore concentrations of PM447 to determine the effect on disease progression in seedlings that were challenged two weeks later with a wild-type strain, Westcott. There were no significant differences in disease ratings among seedlings inoculated with different concentrations of PM447 ($p=0.6$) (Table 3 and Fig. 2). Seedlings that were challenged with Westcott had significantly higher disease ratings than those only inoculated with PM447 ($p=.0004$). However, there was no significant difference in disease rating of challenged seedlings compared to seedlings only inoculated with Westcott ($p=0.1$).

East Farm/Beaumont Field Plot

Treatments within the Beaumont/East Farm study were set up to determine the effect of timing between initial inoculation with PM447 and challenge inoculations with a wild-type strain (Westcott), on disease development on older trees (Tables 4 and 5). All control trees remained symptomless. There was no variation in disease ratings for any type 1 tree regardless of treatment (not including controls); all trees inoculated with either PM447 or Westcott wilted within the first year and did not leaf out the following year. Ratings for tree type 2 trees varied, showing a trend similar to that observed with tree type 3 trees; however, there were no significant differences in treatments. There was a significant effect of the timing of the challenge inoculation on disease rating with type 3 trees ($p=.002$) one year after inoculation. Symptom development on 2- and 3-week-challenged trees progressed much slower than on trees challenged at 1 week or inoculated with the wild-type only. However, all trees eventually wilted completely in subsequent years.

Jackson Field Plot

The Jackson study was designed to assess how varying the spore load of PM447 affects symptom expression when PM447 is inoculated alone. These treatments were done to determine if lower spore loads may reduce symptoms so as to provide protection rather than killing the tree. Conidial concentrations of 10^1, 10^2, 10^3, and 10^1+10^1 did not produce symptoms that were significantly different from each other or the water controls (Fig. 4). Inoculation with 10^5, 10^2+10^2, 10^3+10^3, $10^1+10^1+10^1$, or $10^2+10^2+10^2$ spores produced symptoms that were not significantly different from each other or the Westcott (wild-type) control (Fig. 3). The former treatments (including the water controls) produced significantly lower disease ratings than the latter group of treatments (including the wild-type control) ($p=0.01$) (Table 6 and Fig. 3).

Beaumont Field Plot

The Beaumont study focused on the consequences of varying the spore load of PM447 in combination with two-week-challenge inoculations with the virulent Westcott strain on disease development (Table 7). All water-inoculated (control) trees remained symptomless. There was no significant difference between ratings for those trees inoculated with Westcott only and those trees first inoculated with PM447 and then challenged with Westcott 2 weeks later ($p=0.9$). Ratings for trees inoculated with PM447 only were significantly less than for other treatments ($p=0.02$); however, there was no significant effect in regard to the spore concentrations used in the study.

DISCUSSION

Results from the greenhouse experiments are similar to those reported earlier by Shaw (1999); that is, PM447 was reduced in its ability to cause severe symptoms when inoculated into young seedlings alone, and PM447 provided protection to seedlings subsequently challenged by wild type strains several days later. However, Shaw observed protection only when PM447 was inoculated 2 weeks prior to the wild type challenge inoculation and in this study we observed protection regardless of timing.

Growth chamber results again demonstrated the hypovirulent nature of PM447, but in this study were not as promising as a biocontrol agent. Despite lower disease ratings for challenged seedlings compared to wild type Westcott isolate alone, statistically there was no significant effect of PM447 when inoculated two weeks prior to inoculation with a wild-type strain. Although different oak species were used in these studies, all species of red oaks are thought to have similar susceptibilities. The observed disparity could be due to the smaller sample size used in the growth chamber study coupled with the highly variable seedling response, resulting in a statistically insignificant outcome. It should be noted that Shaw observed significant and reproducible biocontrol outcomes with PM447 in growth chamber studies with red oak (Shaw 1999).

Field plot results varied, and although PM447 appeared to delay symptom development in mature trees, in general it was not an effective biocontrol as trees generally wilted completely within 1-2 years. At the Jackson site, trees inoculated with 10^1, 10^2, 10^3, or 10^1+10^1 (10^1 followed two weeks later by 10^1) spores of PM447 experienced significantly less symptom development than trees inoculated at other (higher) concentrations. These results show that PM447, at relatively low concentrations may not ultimately induce wilt as had previously been

observed using 10^5 spores. This would be promising for its potential role as a biological control agent; however, results at the Beaumont plot indicated that these lower PM447 inoculum loads did not affect the development of wilt upon challenge inoculation with a wild-type strain. While the average disease rating for trees in the "Challenge: 10^1+10^3" treatment in the Beaumont plot appears to be lower than that observed with the other challenge treatments, statistically, there was no significant difference between challenge treatments and inoculation with the wild-type strain, Westcott, only. This lack of significance may be due to the relatively small sample size per treatment and the overlapping range of disease ratings for each treatment.

In general, PM447 appeared to delay symptom development in subsequently challenged seedlings and trees; however, its efficacy as a biocontrol agent is not well understood. The results indicate that red oaks do respond to the presence of PM447 in some capacity that slows the progression of the disease. Perhaps the slow growth of PM447 in culture translates to slower growth and/or reduced fitness within a seedling or tree, thus enabling the host to respond to the presence of this pathogen. It is likely that this response is an induced defensive reaction that restricts the pathogen to some extent, although the pathogen is ultimately able to overcome any defenses produced by the host. Based on these studies, materials capable of inducing an acquired resistance response in oak should be tested for their efficacy to oak wilt.

ACKNOWLEDGEMENTS

We wish to thank Mario Mandujano and Jim Klug for their technical assistance in our work.

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Table 1. Disease ratings (DR) for seedlings based on level of symptom expression. Statistical analysis of greenhouse trial results utilized the DR recorded at 6 weeks post-wild-type inoculation. For the growth chamber study, the DR at 8 weeks was evaluated.

Disease rating	Symptom expression
0	Symptomless
1	Leaves with mild bronzing of tips; less than 25% leaf area affected
2	Mild curling and drying of leaves with bronzing more apparent; up to 50% leaf area affected
3	Leaves curled and dry with bronzing of up to 75% of leaf area
4	Leaves severely curled and dry with bronzing nearly to petiole
5	Leaves entirely brown (though few scattered green flecks may remain); defoliation common; nearly 100% of leaf area affected

Table 2. List of treatments assessed in the greenhouse study to evaluate the effects of wild-type strain used and timing between PM447 and challenge inoculations on symptom development.

Treatment	Description
Control	Seedlings inoculated with water
PM447 only	Seedlings inoculated with the hypovirulent, PM447 strain
Wild-type only	Seedlings inoculated with one wild-type strain (Fenn, Westcott, or Beal)
Challenge: 0 weeks	Seedlings co-inoculated with PM447 and one wild-type strain (Fenn, Westcott, or Beal)
Challenge: 1 week	Seedlings inoculated with PM447, then challenge inoculated 1 week later with a wild-type strain (Fenn, Westcott, or Beal)
Challenge: 3 weeks	Seedlings inoculated with PM447, then challenged 3 weeks later with a wild-type strain (Fenn, Westcott, or Beal)
Challenge: 4 weeks	Seedlings inoculated with PM447, then challenged 4 weeks later with a wild-type strain (Fenn, Westcott, or Beal)

Table 3. List of treatments included in the growth chamber experiment to determine the effect of PM447 spore load on wilt development in seedlings challenged with a wild-type strain two weeks later.

Treatment	Description
Control	Seedlings inoculated with water
PM447: 10^3	Seedlings inoculated with 10ul of 10^3 spores/ml of PM447
PM447: 10^4	Seedlings inoculated with 10ul of 10^4 spores/ml of PM447
PM447: 10^5	Seedlings inoculated with 10ul of 10^5 spores/ml of PM447
PM447: 10^3+10^3	Seedlings inoculated with 10ul of 10^3 spores/ml of PM447, then were inoculated with 10ul of 10^3 spores/ml of PM447 two weeks later
Westcott	Seedlings inoculated with the wild-type strain, Westcott
Challenge: 10^3	Seedlings inoculated with 10ul of 10^3 spores/ml of PM447, then challenge inoculated two weeks later with Westcott
Challenge: 10^4	Seedlings inoculated with 10ul of 10^3 spores/ml of PM447, then challenge inoculated two weeks later with Westcott
Challenge: 10^5	Seedlings inoculated with 10ul of 10^3 spores/ml of PM447, then challenge inoculated two weeks later with Westcott
Challenge: 10^3+10^3	Seedlings inoculated with 10ul of 10^3 spores/ml of PM447, then were inoculated with 10ul of 10^3 spores/ml of PM447 two weeks later, then were challenged with Westcott two weeks after the second inoculation with PM447

Table 4. List of treatments used in the East Farm/Beaumont study to evaluate the effect of the timing between PM447 and challenge inoculations on disease development. Each treatment group included multiple trees of each size type: type 1 trees had a dbh=2-3 cm, type 2 trees had a dbh=5-6 cm, and type 3 trees had diameters greater than 12 cm.

Treatment	Description
Control	Trees were inoculated with water
PM447	Trees were inoculated with the hypovirulent strain, PM447
Westcott	Trees were inoculated with the wild-type strain, Westcott
Challenge: 1 week	Trees were inoculated with PM447, then challenge inoculated with Westcott one week later
Challenge: 2 weeks	Trees were inoculated with PM447, then challenge inoculated with Westcott two weeks later
Challenge: 3 weeks	Trees were inoculated with PM447, then challenge inoculated with Westcott three weeks later

Table 5. Average disease ratings (DR) of type 3 trees (dbh > 12 cm) within the East Farm/Beaumont plot one year (2005) and two years (2006) after inoculation. Trees were assigned a DR of 0 if they expressed no symptoms, a 1 if they had mild to moderate symptoms (less than 60% of crown affected), or a 2 if they developed advanced symptoms or completely wilted.

Treatment	Average DR: 2005	Average DR: 2006
Control	0	0
PM447	1	2
Westcott	2	2
Challenge: 1 week	2	2
Challenge: 2 weeks	1	2
Challenge: 3 weeks	1	2

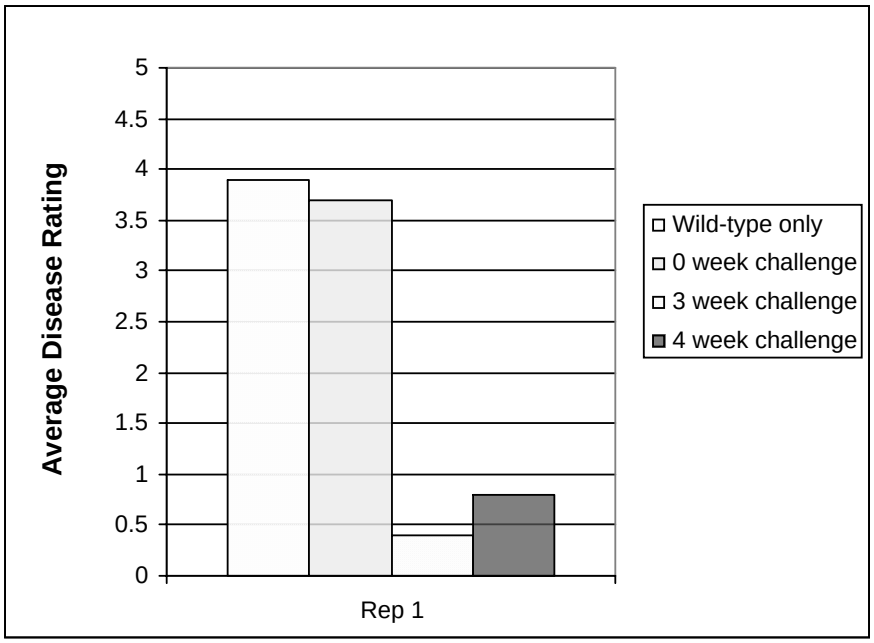
Table 6. List of treatments used at the Jackson site to ascertain the effect of PM447 spore load on symptom development in red oaks. The average disease ratings (DR) for trees within each treatment group are given. Trees were scored based on the level of symptom development one year following inoculation; a DR equal to 0 indicates trees developed no symptoms, a 1 was assigned to trees with mild to moderate symptoms (less than 60% of crown affected), and a 2 represented those trees displaying advanced or complete wilt.

Treatment	Description	Average DR*
Control	Trees were inoculated with water	0 a
Westcott	One tree was inoculated with the wild-type strain, Westcott	2 b
PM447: 10^1	Trees were inoculated with 10^1 spores of PM447	0 a
PM447: 10^2	Trees were inoculated with 10^2 spores of PM447	0 a
PM447: 10^3	Trees were inoculated with 10^3 spores of PM447	0.5 a
PM447: 10^5	Trees were inoculated with 10^5 spores of PM447	2 b
PM447: 10^1+10^1	Trees were inoculated with 10^1 spores of PM447, then were inoculated two weeks later with 10^1 spores of PM447	0.33 a
PM447: 10^2+10^2	Trees were inoculated with 10^2 spores of PM447, then were inoculated two weeks later with 10^2 spores of PM447	1 b
PM447: 10^3+10^3	Trees were inoculated with 10^3 spores of PM447, then were inoculated two weeks later with 10^3 spores of PM447	1 b
PM447: 10^1+10^1+10^1	Trees were inoculated with 10^1 spores of PM447, then were inoculated one week later with 10^1 spores of PM447 and again one week afterward with 10^1 spores of PM447	1.67 b
PM447: 10^2+10^2+10^2	Trees were inoculated with 10^2 spores of PM447, then were inoculated one week later with 10^2 spores of PM447 and again one week afterward with 10^2 spores of PM447	1.33 b

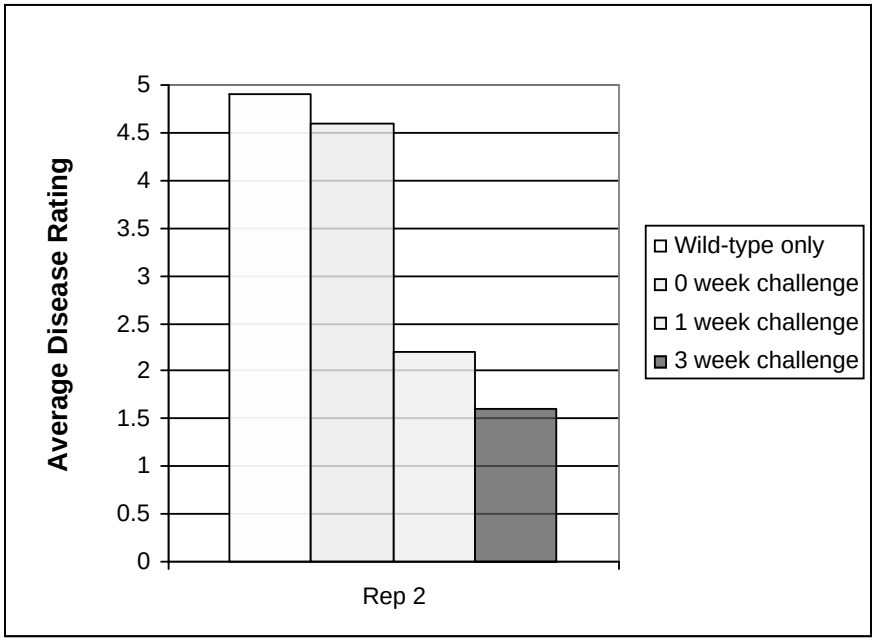
*Disease ratings for each treatment followed by the same letter are not significantly different at p=0.05.

Table 7. List of treatments tested in the Beaumont study to determine the effect of variable spore concentrations of the hypovirulent strain, PM447, on symptom development in trees challenged with a wild-type strain two weeks after inoculation with PM447. The average disease ratings (DR) for trees within each treatment are given. Disease ratings were recorded the year following inoculation and were assigned as follows: 0 = no apparent symptoms, 1 = mild to moderate symptoms (less than 60% of crown affected), and 2 = advanced or complete wilt. In general, trees inoculated with PM447 only had significantly lower disease ratings than trees inoculated or challenged with Westcott when treatments were combined. None of the challenge treatments were significantly different than the Westcott treatment.

Treatment	Description	Average DR
Control	Trees were inoculated with water	0
PM447: 10^1	Trees were inoculated with 10^1 spores of PM447	1
PM447: 10^3	Trees were inoculated with 10^3 spores of PM447	2
PM447: 10^5	Trees were inoculated with 10^5 spores of PM447	2
PM447: 10^1+10^1	Trees were inoculated with 10^1 spores of PM447, then inoculated a second time with 10^1 spores of PM447 two weeks later	0
PM447: 10^1+10^3	Trees were inoculated with 10^1 spores of PM447, then inoculated a second time with 10^3 spores of PM447 two weeks later	0.67
PM447: 10^3+10^3	Trees were inoculated with 10^3 spores of PM447, then inoculated a second time with 10^3 spores of PM447 two weeks later	0.67
Westcott	Trees were inoculated with the wild-type strain, Westcott	2
Challenge: 10^1	Trees were inoculated with 10^1 spores of PM447, then challenged two weeks later with Westcott	2
Challenge: 10^3	Trees were inoculated with 10^3 spores of PM447, then challenged two weeks later with Westcott	2
Challenge: 10^5	Trees were inoculated with 10^5 spores of PM447, then challenged two weeks later with Westcott	2
Challenge: 10^1+10^1	Trees were inoculated PM447: 10^1+10^1 as described above, then challenged with Westcott two weeks later	1.67
Challenge: 10^1+10^3	Trees were inoculated PM447: 10^1+10^3 as described above, then challenged with Westcott two weeks later	0.67
Challenge: 10^1+10^3	Trees were inoculated PM447: 10^3+10^3 as described above, then challenged with Westcott two weeks later	1.67



A



B

Figure 1. Average disease ratings for red oak (*Quercus rubra*) seedlings six weeks after inoculation with a wild-type strain. Data from all wild-type strains utilized was combined as there was no significant strain effect (p=0.9). Challenged seedlings were first inoculated with the hypovirulent strain, PM447, and then challenge inoculated with a wild-type strain at A) 0, 3, or 4 weeks (rep 1) or B) 0, 1, or 3 weeks (rep 2) after PM447 inoculation. Seedlings challenged at 3 and 4 weeks (rep 1) or 1 and 3 weeks (rep 2) had significantly lower disease ratings than seedlings inoculated with a wild-type strain only or challenged at 1 week.

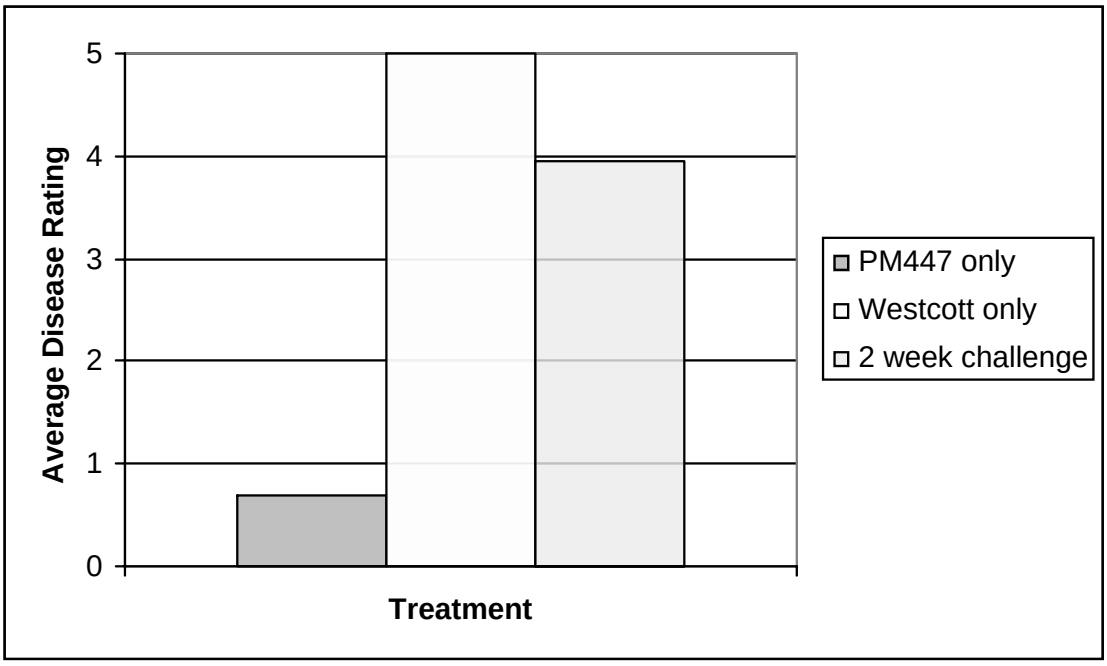


Figure 2. Average disease ratings for pin oak (*Quercus palustris*) seedlings eight weeks after final inoculations. Seedlings were inoculated with either the hypovirulent strain PM447 only, the wild-type strain, Westcott, only, or PM447 followed by a challenge inoculation with Westcott 2 weeks later. All seedlings inoculated with PM447 received one of four different conidial concentrations; however, no spore load effect was observed for either PM447 only or 2 week challenge treatments, so data for all spore concentrations was combined. Seedlings inoculated with PM447 (at any concentration) had significantly lower disease ratings than those inoculated with Westcott only or challenge inoculated. Westcott only and 2 week challenge treatments were not significantly different.

Figure 3. Two treated trees at the Jackson site. The tree on the left was inoculated with 10^2 spores of PM447 and the tree on the left with 10^1 spores. Both trees appear healthy with no symptoms of wilt. Photo was taken June 2006.