ABSTRACT

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| Title of Document: | GENOTYPIC DIVERSITY OF COMMON *PHYTOPHTHORA* SPECIES IN MARYLAND NURSERIES AND CHARACTERIZATION OF FUNGICIDE EFFICACY |
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The genetic diversity of *P. plurivora, P. cinnamomi, P. pini, P. multivora,* and *P. citrophthora,* five of the most common species found in Maryland ornamental nurseries and mid-Atlantic forests, was characterized using amplified fragment length polymorphism (AFLP). Representative isolates of genotypic clusters were then screened against five fungicides commonly used to manage Phytophthora. Three to six populations were identified for each species investigated with *P. plurivora* being the most diverse and *P. cinnamomi* the least. Clonal groups that originated from forest or different nurseries suggest an ongoing pathway of introduction. In addition, significant molecular variation existed for some species among nurseries an indication that unique genotypes being present in different nurseries. Insensitive isolates to fungicides were detected with *P. plurivora* (13), *P. cinnamomi* (3), and *P. multivora* (2). Interestingly, insensitive isolates primarily belonged to the least common genotypic clusters. Because all but two isolates were sensitive to dimethomorph and ametoctradin, the ability of these

chemicals to manage Phytophthora is promising. Nevertheless, the presence of two insensitive isolates could portend general insensitivity to these chemicals as well. Results from this study provide a foundation to future population determination and fungicide sensitivity of the plant pathogenic genus Phytophthora in Maryland's ornamental nursery industry.

# GENOTYPIC DIVERSITY OF COMMON *PHYTOPHTHORA* SPECIES IN MARYLAND NURSERIES AND CHARACTERIZATION OF FUNGICIDE EFFICACY

By

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# Dedication

This thesis is dedicated to my grandfather, Ross Becker: Thank you. I miss you.

# Acknowledgements

First and foremost I would like to thank my advisor, Dr. Yilmaz Balci, for his guidance and constant support throughout this endeavor. Coming back to the University of Maryland to learn about molecular biology was a daunting task and I don't think I would have succeeded without it. I would also like to thank my committee members Dr. Karen Rane and Dr. Kathryne Everts for their suggestions and helpful hints.

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# Chapter 1: Introduction

## *History*

*Phytophthora* species are known as major pathogens of nursery plants and can have devastating impacts in natural ecosystems and on agricultural crops (27). As plant trade continues to increase locally and globally, exotic *Phytophthora* species, are introduced into new ecosystems. The spread of *P. ramorum* exemplifies the scale of this problem: Fourteen years after the first reports of *P. ramorum* in California in 1995, more than 68 countries had either included *P. ramorum* on their lists of regulated pests or mentioned the pathogen in their legislation (55). The movement of *P. ramorum* elevated concerns that other *Phytophthora* species are spreading to locations throughout the US. Consequently, a wide range of surveys have been conducted in nurseries, natural ecosystems, and in agricultural crops in an effort to detect and eliminate these pathogens (3, 7, 24, 54, 61, 78, 80, 83, 102). The survey findings have serious implications, as several reveal the role that nurseries have played in the movement of *Phytophthora*-infested material and, consequently, the introduction of these organisms into forest and agricultural landscapes.

Floriculture and nursery crops account for approximately $17 billion in sales in the US annually (51). Phytophthora root rot, crown rot, and foliage blight are common diseases in the ornamental plant industry, and management of these diseases is challenging (18), especially because environments in nurseries are conducive for their growth. Generally, nursery management of *Phytophthora* consists of schedule-based fungicide application programs. While these practices can help protect plants, it

comes with significant dangers: Overuse of fungicides can lead to pathogen resistance and have major environmental and financial costs. Excess fungicides can contaminate surrounding environments via drift or leaching, contaminating nearby waterways and groundwater resources where they can affect aquatic organisms and be incorporated into the soil (57, 103). Excess use is also a financial problem. Despite spending millions of dollars on fungicide applications annually, fungal pathogens still wreak havoc on nursery plants.

Surveys were conducted from 2010 to 2012 at eight large-scale nursery operations, and 680 *Phytophthora* isolates were collected for this study. Sixteen previously described and two potentially new species were identified (7). Some of these species had not been found in Maryland before. Several species were traced back to shipments from the West coast, again demonstrating the role that nurseries play in the movement of *Phytophthora.* The overarching goal of this study is to characterize both the population structure and fungicide sensitivity of *Phytophthora* in Maryland nurseries. To date, studies such as these have been conducted in numerous other states, but not in Maryland. The hypotheses of this study are: 1) Nursery management practices have selected for a less diverse population of *Phytophthora*, 2) Fungicide applications have selected for a *Phytophthora* population that is less sensitive to fungicides commonly used to manage the pathogens, and 3) A relationship exists between genotype and fungicide sensitivity. The data provided in this study will inform a greater understanding of *Phytophthora* populations in the US and Maryland while enabling nursery managers to create a more targeted,

environmentally and economically sustainable approach to improve yield, reduce pathogen transmission and the use of fungicides.

*Characterization of genetic diversity*

Characterizing the genetic variation of survey isolates is important for several reasons. Previous work has shown that some *Phytophthora* populations, particularly those of *P. infestans*, can be strongly clonal, sometimes with a single clonal lineage in a population (37). Therefore a population should be characterized for both its resistance to fungicides and its genetic variation. The inclusion of forest *Phytophthora* isolates in this study enabled a comparison to be made between population structures in ornamental nurseries and that in a wilder, or less managed, environment. This information would also be useful in the future as comparisons could be made to future survey work to provide an idea of how the population structure of these *Phytophthora* species have or have not shifted. This characterization will also enable the selection of representative isolates to incorporate into a subsequent fungicide sensitivity study.

*Genotyping methods*

The three most common techniques for multilocus genomic fingerprinting are random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphism (AFLP), and inter simple sequence repeats (ISSRs) (72). These techniques use PCR to amplify DNA fragments, allowing for the creation of fingerprints for individuals that have not yet been sequenced (8, 72, 101). Despite their similarities, these techniques vary with respect to data quality, genetic variability, and discriminatory power (72). The RAPD technique is useful, especially

because it can be done without prior sequence knowledge, but it is also very sensitive to reaction conditions, template DNA concentration and purity, and PCR temperature profiles, which can limit its applications (8). In many studies, AFLPs outperform ISSRs and RAPDs in their high reproducibility, robustness, and fewer reported reaction artefacts (72).

These methods are examples of dominant marker systems. In these systems, markers are scored as present or absent. In other words, there is no way to distinguish between homozygous and heterozygous alleles. Methods such as microsatellites (simple sequence repeats (SSRs and single nucleotide polymorphisms (SNPs))) are codominant marker systems, which can distinguish between homo- and heterozygous alleles. Both types are commonly used for linkage analysis, measuring population genetic structure and diversity, and can produce congruent results depending on the number of microsatellites and fragments analyzed (72). Because we do not have sequence information for our isolates, RAPDs and AFLP would be a viable options. This, in combination with the high reproducibility and robustness of the method, AFLP was chosen as the genotyping method for this work.

In general, the AFLP method is a four step process in which unique and reproducible fingerprints are created by 1) Digestion of genomic DNA into restriction fragments, 2) Ligation of adapters to restriction fragments, 3) PCR with primers designed to recognize the adapters, and 4) PCR with "selective" primers designed to recognize adapter ends along with 1-3 additional nucleotides in the template sequences (72). AFLP has been used in numerous studies to characterize various *Phytophthora* populations (5, 16, 31, 45, 47, 59, 99). In fact, many of these studies

also used AFLP to characterize the relationship between the shifts in genetic variation and fungicide resistance (5, 6, 47, 59). These experiments show that using AFLP in conjunction with fungicide resistance studies is a useful way to obtain a clear picture of isolate populations.

*Fungicide use for management of Phytophthora*

The fungicides used for ornamentals are similar to those used in the rest of agricultural production. In fact, 13 of the top 15 agricultural fungicides used throughout the world in 2003 were registered for ornamentals in the US (18). There are many fungicides that can be used to manage *Phytophthora* disease in nurseries. These fungicides are either systemic or protective. Systemic fungicides move through the xylem of the plant and can have some curative activity whereas protectants are applied to the stems and foliage and do not enter the plant. The phenylamides and alkyl phosphonates are systemic fungicides that were shown to be superior to other systemic and protectant fungicides in their ability to inhibit disease development after infection, their lack of vulnerability to weathering, and their longer residual activity (15).

The fungicide chemical metalaxyl and its more recent isomer, metalaxyl-M (mefenoxam), are phenylamide fungicides that have been used widely and intensively to control Oomycete diseases of numerous crops and ornamentals (5, 6, 13, 15, 24, 44, 47, 77, 78, 81, 83). In fact, mefenoxam is one of the major compounds registered as a soil drench to control root infections on ornamental crops (4, 49). In the early 1980s, metalaxyl was shown to successfully control the crown rot phase of *Phytophthora* blight (52, 64, 79, 88) and received federal registration for the control

of *Phytophthora* blight on peppers in the US in the 1990s (87). However, resistance to metalaxyl was reported shortly thereafter in many Oomycetes (74). For this reason, the manufacturer created mefenoxam, which is the more active enantiomer of metalaxyl (77). It is applied similarly to metalaxyl, but at lower rates (87). Mefenoxam acts at the level of DNA translation, selectively inhibiting ribosomal RNA synthesis by affecting the activity of RNA polymerases (19), therefore inhibiting mycelial growth and sporulation (94).

Alkyl phosphonate fungicides are products made up of the salts and esters of phosphorous acid ($HPO(OH)_2$). When mixed with water, phosphorous acid forms phosphonic acid that is too strong to be used on plants and must be combined with other chemicals to raise its pH (60). One method of reducing the acidity of phosphonic acid is to combine it with ethanol to form ethyl-phosphonate. Aluminum ions are added during the manufacturing process to neutralize the ethyl-phosphonate ions and the resulting product is referred to as fosetyl-Al or aluminum tris (O-ethyl phosphonate) (71). The fungicidal properties of phosphonates were discovered by scientists in France during the 1970s (60). Soon after this discovery, fosetyl-Al was formulated under the trade name Aliette and released for commercial use (38).

Unlike metalaxyl and mefenoxam, phosphonate fungicides can move in both the xylem and phloem tissues (15, 60, 98). As a result, fosetyl-Al is the first commercially-produced fungicide that possesses the ability to move in a basipetal direction from shoot to root (14). Noteworthy, however, is the fact that phosphonate fungicides are quite effective when used preventatively, but are not as successful when applied after symptoms are already present (60). The mode of action of these

fungicides is controversial, some scientists suggesting that most of the fungicidal effects of these products are directed towards the fungal pathogen, and others believing that it is both a direct effect on the fungus and a stimulation of natural host defenses that prevent disease (60, 104).

Dimethomorph is another fungicide that can be used to combat Oomycetes. Introduced in 1988, it is a derivative of cinnamic acid and is a member of the morpholine chemical family (BC Ministry of Agriculture Food and Fisheries). Dimethomorph has protective, curative, and antisporulant activities against *Peronosporaceae* and *Phytophthora*, but not *Pythium* (14). The fungicide moves in an upward direction through the plant to the growing leaves. Its mode of action inhibits the synthesis of sterols, thus impairing cell wall production of fungi (BC Ministry of Agriculture Food and Fisheries). Dimethomorph has no cross-resistance to phenylamide fungicides and is highly effective even at relatively low doses (14).

Ametoctradin is a relatively new active ingredient in a fungicide developed by BASF called Initium. Discovered in 2004, it is a mitochondrial respiration inhibitor and belongs to a new class of chemistry, the pyrimidylamines (36). Ametoctradin is a protectant foliar spray and is recommended for use against late blight and downy mildews on agricultural crops. It is not cross-resistant to Oomycete fungicide classes with confirmed field resistance and, at this point, is only marketed in ready-mixtures with other Oomycete active compounds (36).

Fluoxastrobin is a member of the Quinone outside inhibitor (QoI) fungicides, which is an important group of fungicides isolated from decay fungi that interfere with respiration (2, 100). This group can control a wide variety of fungi on many

types of crops including ornamentals, cereals, and vegetables (100). Many of the QoI fungicides, including fluoxastrobin, exhibit translaminar movement. Because not all chemicals in this group are systemic, translaminar movement enables these fungicides to disperse evenly throughout the plant, even when application doesn't provide complete coverage. QoI fungicides are most effective when used as a protective fungicide. While the chemicals effectively kill germinating spores, they are not as effective against mycelium. This, along with the fact that once the chemicals move through the leaf they quickly bind to the cuticle, signifies that this group of fungicides should not be used curatively. Application data for the chemical is not in the 2004 MDA report or in the 2006 and 2009 USDA NASS reports, perhaps because fluoxastrobin did not become available for use in the US until 2005 (1).

*Fungicide use in Maryland*

Fosetyl-Al, etridiazole, mancozeb, and captan are fungicides that can be used to manage oomycetes and are among the top ten fungicides used in Maryland over the last decade (9, 70). Despite its usage decreasing over that same period of time, mefenoxam was still listed in the top 100 pesticides used in Maryland in 2004, and in the top 150 in 2011. Application of most of these fungicides in Maryland decreased drastically between 2004 and 2011. In fact, fungicide use as a percentage of pesticide use in Maryland decreased by 84% between 2004 and 2011 (9, 70). It is worth noting, however, that the decrease occurred during a period of overall increase, where fungicide use increased by 170% between 1997 and 2011. Fungicides commonly used to manage oomycetes and the amounts used in Maryland over the years are listed in Table 1.

| | 2011 | 2004 | 2000 | 1997 | 1994 | 1991 |
|---|---|---|---|---|---|---|
| **Chemical** | | | | | | |
| Azoxystrobin | 5,213 | 1,229 | | | | |
| Captan | 7,127 | 8,816 | | | | |
| **Dimethomorph** | **8** | | | | | |
| Etridiazole | 118,384 | 1,217 | 5,325 | 1,368 | 191 | |
| Fluopicolide | 1 | | | | | |
| **Fluoxastrobin** | **198** | | | | | |
| **Fosetyl-Al** | **1,681** | **90,072** | **19,592** | **12,042** | | **13,355** |
| Mancozeb | 30,280 | 254,254 | 37,405 | 37,343 | 17,572 | 8,710 |
| Maneb | 5,753 | 398 | | | | |
| **Mefenoxam** | **828** | **3,644** | | | | |
| Metalaxyl | | 1,465 | | | | |
| Phosphorous acid | 2,707 | 160 | | | | |
| Propamocarb hydrochloride | 3,415 | 5,563 | | | | |
| Trifloxistrobin | 267 | 365 | | | | |

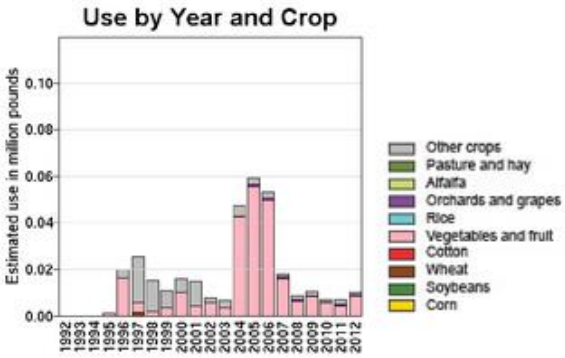
**Table 1.** Estimated use of fungicides that can manage Oomycetes used by Maryland farm operators, certified private pesticide applicators, commercially licensed businesses, and public agencies (total lbs. active ingredient (a.i.))

*Fungicide use in the US*

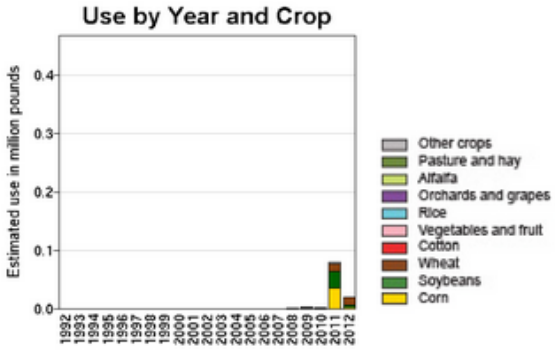
The fungicides commonly used in Maryland to combat Oomycetes along with the fungicides of interest for this study are used throughout the US (Tables 2 and 3; Figures 1 and 2). National usage data for dimethomorph isn't available before 1995. Its use peaked around 2005 and decreased through 2009. Because ametoctradin was developed late in 2012, no usage data is currently available. National data for mefenoxam show that the amount of chemical utilized annually was anywhere from 0.18 to 0.34 million lbs. a.i. in the years between 2005 and 2012 (USGS NAWQA). The data also show relatively consistent usage by crop, despite the large amount of literature indicating that some Oomycetes have developed resistance to the chemical. National usage data for fosetyl-Al show that it has been utilized more than dimethomorph and mefenoxam, but that utilization decreased between the late 1990's and 2009 (USGS NAWQA).

| | 2009 | 2006 |
|---|---|---|
| **Chemical** | | |
| Azoxystrobin | 3,400 | 8,900 |
| Captan | 8,100 | 13,600 |
| ***Dimethomorph*** | ***100*** | ***9,800*** |
| Etridiazole | 2,700 | 1,800 |
| Fluopicolide | (D) | . |
| ***Fosetyl-Al*** | ***34,200*** | ***101,100*** |
| Mancozeb | 90,400 | 145,400 |
| Maneb | 1,700 | 3,600 |
| ***Mefenoxam*** | ***3,200*** | ***76,400*** |
| Metalaxyl | (D) | 800 |
| Phosphorous acid | 1,300 | 10,700 |
| Propamocarb hydrochloride | (D) | (D) |
| Trifloxystrobin | 100 | 2,200 |

**Table 2.** Common fungicides used for combating diseases including Oomycetes in USDA nurseries in program states (lbs.) (D) Withheld to avoid disclosing data for individual operations. (USDA NASS) Note: Fluoxastrobin left out due to lack of data.




(A)

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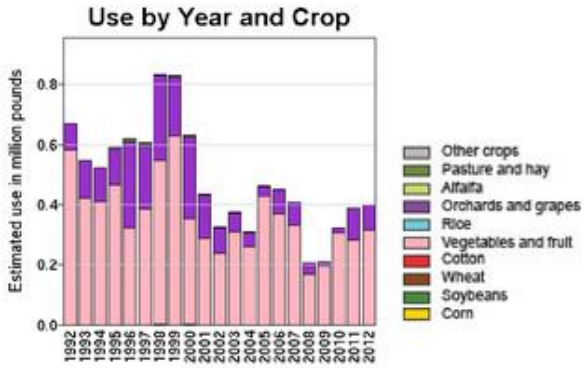


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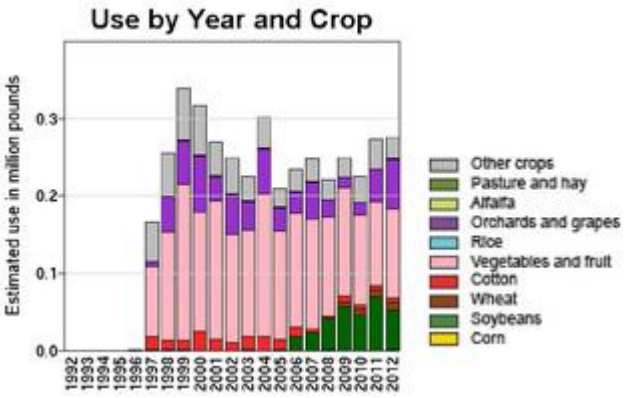
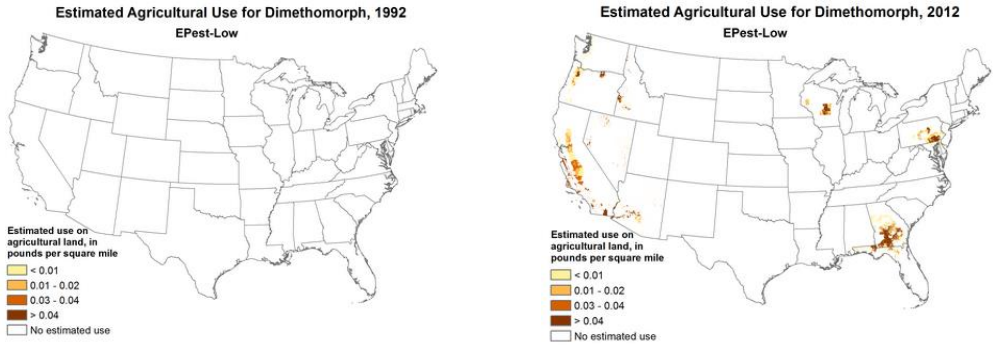


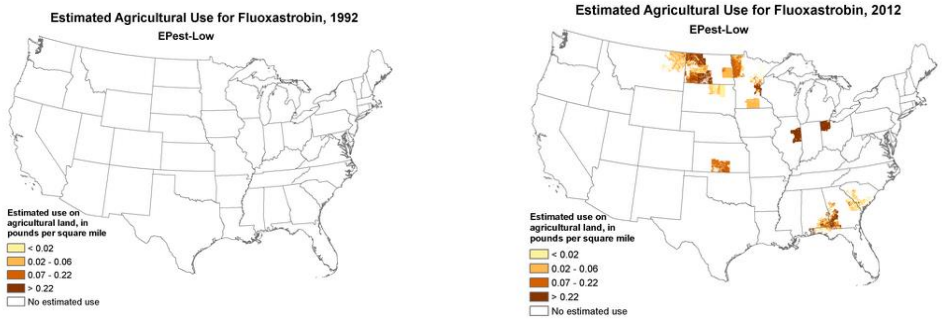

(D)

**Figure 1.** Fungicide use in the US by year and crop for **(A)** Dimethomorph, **(B)** Fluoxastrobin, **(C)** Fosetyl-Al, and **(D)** Mefenoxam (USGS NAWQA).

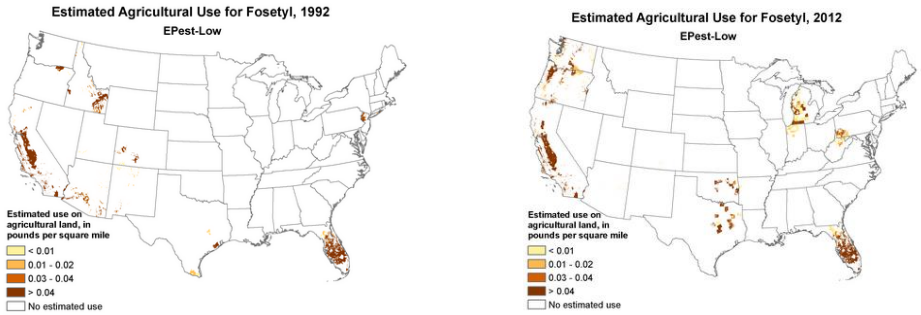
(A)




(B)




(C)




(D)

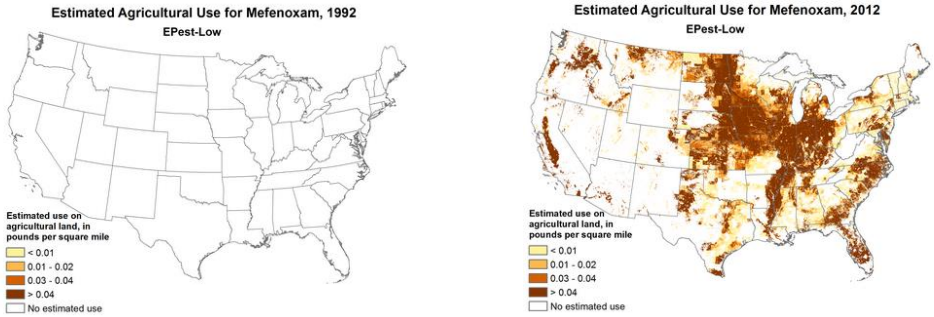



**Figure 2.** Comparison of National Usage Maps from 1992 and 2012 for **(A)** Dimethomorph, **(B)** Fluoxastrobin, **(C)** Fosetyl-Al, and **(D)** Mefenoxam (USGS NAWQA)

*Fungicide resistance*

Fungicide resistance occurs when there is a mutation in the fungus that allows the biochemical process targeted by the fungicide to continue (50). Fungal pathogens are more likely to develop resistance to fungicides with single target sites because only a single mutation is necessary (34). If that same single site fungicide is used repeatedly, resistance will build up in the population, rendering the fungicide ineffective (34). Phenylamides such as mefenoxam are single site fungicides (34, 44), thus it is not surprising that there are reports of mefenoxam resistance in *Phytophthora* species on ornamental plants. Several states that have reported resistance include California (5, 6, 11, 13, 30), North Carolina (47, 78), Virginia (43, 44), and New York (24), suggesting that resistance is becoming more common in horticultural operations. This is not unique to nurseries. Within two years of its introduction to agricultural environments, several incidences of *P. infestans* resistance emerged in potato fields across Europe (35). Since then, metalaxyl/mefenoxam resistance has been frequently detected in other *Phytophthora* species (5, 6, 11-13, 17, 24, 30, 39, 44, 47, 53, 56, 59, 65-67, 73, 78, 97).

Because the mechanism of action of fosetyl-Al is thought to operate by inducing a plant response and not by a site-specific mechanism like mefenoxam, it is reasonable to assume that resistance would not develop as quickly as occurred with the latter. In fact, according to the University of Maryland's Integrated Pest Management (IPM) guidebook (34), its risk of resistance is "medium", while that for metalaxyl and mefenoxam is "high". There have been several studies showing the resistance of *Phytophthora* species to fosetyl-Al (12, 29, 39, 56, 65, 67). Developing

information on resistance of *Phytophthora* species to these fungicides in Maryland nurseries is therefore critical for creating effective practices for managing disease symptoms, presence, and transmission.

In 1982, Fungicide Resistance Action Committees (FRAC) were formed for various chemical groups (15). These committees developed strategies to prevent resistance buildup in pathogen populations. They also grouped fungicides by their mode of action or chemical structure, giving each group a FRAC code (34). Fungicides with the same FRAC code affect pathogens in a similar manner. Just as overuse of a particular fungicide can lead to resistance in the pathogen population, so too can overuse of fungicides in the same FRAC group. With this coding system, fungicide users can easily create a treatment plan using chemicals with different modes of action and thus prevent resistance buildup. See Table 3 for some examples of FRAC codes.

| Chemical | FRAC code |
|---|---|
| Azoxystrobin | 11 |
| Captan | M |
| **Dimethomorph** | 40 |
| Etridiazole | 14 |
| Fluopicolide | 43 |
| **Fluoxastrobin** | 11 |
| **Fosetyl-al** | 33 |
| Mancozeb | M |
| Maneb | M |
| **Mefenoxam** | 4 |
| Metalaxyl | 4 |
| Phosphorous acid | 33 |
| Propamocarb hydrochloride | 28 |
| Trifloxistrobin | 11 |

**Table 3.** FRAC codes for fungicides commonly used to treat Oomycetes
M = Multi-site contact activity

*Integrated use of fungicides*

Although fungicide applications are a valuable component of nursery management of *Phytophthora* species, there is no single fungicide that can effectively manage Phytophthora (44). It is important to use a variety of fungicides with different modes of action to provide broad-spectrum disease control (14, 34). In a study examining mefenoxam resistance of *P. capsici* in a field of bell peppers, the greatest number of resistant isolates was recovered from a field where Ridomil Gold (mefenoxam) was used alone rather than in combination with other fungicides (87). Other examples of this were seen in Europe and Israel. Shortly after metalaxyl was introduced for control of potato late blight and cucumber downy mildew, resistance to the fungicide appeared on a widespread scale in areas where it was used without a protectant (14). For economic reasons, it has been common to combine systemic fungicides like metalaxyl or fosetyl-Al with a protectant (14). Compared to treatment with a protectant alone, combined regimes provide better results and have the advantage of allowing longer intervals between treatments. In addition, combined usage of fungicides with different FRAC codes offers a broader spectrum of protection, as different fungicides can control other non-oomycete diseases (14).

*Methods for testing fungicide resistance*

There are many *in vitro* methodologies for studying fungicide resistance. Mycelial growth rates, spore germination rates, microtiter plates with or without dye, and leaf disc assays are examples of some of these methodologies. Some authors indicate that the leaf disc method is more precise because *in vitro* measurement using metalaxyl-amended agar media provided misleading results (93). Other studies found that *in vitro* methods using agar gave reliable results with fast-growing isolates

compatible with the disc assay as well as with field experiments (37, 68). Other studies suggest a more rapid and reliable assay involving microtiter plates (56, 77, 84, 86, 92).

Mycelial growth is affected by all fungicides used in this work. It can be measured easily and quickly using agar assays. Regardless of whether Petri dishes or microtiter plates are used, these assays are similar. Plates or wells are filled with agar amended with different fungicide concentrations and fresh plugs of mycelial growth are transferred to these wells and plates and incubated in the dark at 18-28°C for 3-10 days. Mycelial growth on agar amended with fungicides is then measured and compared to that on non-amended controls according to established thresholds. Isolates are rated on a scale between sensitive and insensitive (5, 6, 11-13, 24, 29, 30, 39, 44, 45, 47, 53, 56, 59, 65, 67, 78, 95). According to Kuhajek et al (2003), microplate assays are less laborious, faster, and use less space (56). Several trials were conducted with microplates, but it was found that filling the wells with agar and transferring plugs into the smaller wells was actually more laborious than doing so with Petri dishes. Thus for this work 6cm Petri dishes were used to test isolates for fungicide sensitivity.

*Research objectives*

1) **Characterize the genetic diversity of *Phytophthora* species**. We hypothesized that there would be greater genetic diversity in Phytophthora isolates from natural, less managed, forest environments than those from nurseries. AFLP was used to characterize the genetic diversity of isolates representative of the five most common species in Maryland ornamental nurseries and mid-Atlantic forests.
2) **Characterize isolates for fungicide resistance *in vitro***. We hypothesized that fungicide use in Maryland ornamental nurseries over time has selected for Phytophthora isolates that are less sensitive to frequently used fungicides. To test this, a fungicide sensitivity assay was conducted comparing the ability of nursery and forest isolates to grow on agar amended with five fungicides commonly used to manage *Phytophthora* spp.
3) **Evaluate any relationship with isolate genetic variation and fungicide sensitivity**. We hypothesized that a relationship exists between genetic diversity and fungicide sensitivity. To test this, data obtained from objectives 1 and 2 were compared using various statistical methods.

# Chapter 2: Characterization of *Phytophthora* populations using AFLP

This chapter was drafted to be submitted to the peer-reviewed journal PlosOne. The tentative title: Genotypic diversity of commonly occurring *Phytophthora* species in Maryland's nurseries and mid-Atlantic forests.

*Abstract*

Genetic diversity of a collection of *P. cinnamomi* (102 isolates), *P. citrophthora* (24), *P. multivora* (16), *P. pini* (28) and *P. plurivora* (186), common in Maryland nurseries and forests in the Mid-Atlantic United States was characterized using amplified fragment length polymorphism (AFLP). Expected heterozygosity and Shannon's information suggested a high level of diversity among the *P. plurivora* isolates when compared to all other species. Isolates recovered from necrotic tissue and *Pieris* spp. plants had greater genetic diversity with *P. plurivora*. A greater diversity also existed in forest *P. cinnamomi* isolates compared to those that existed in nurseries. Analysis of molecular variance revealed that most of the variations for the five species analyzed were within the groups. However, a significant genetic variation was still detected based on the isolate origin (forest vs. nursery or among nurseries). Clonal groups existed within *P. plurivora* and *P. cinnamomi* and included isolates from both forest and nurseries, suggesting that a pathway from nurseries to forests or visa verse exists. Hierarchical cluster analysis along with UPGMA and STRUCTURE showed between three and four distinct populations. Minimum spanning networks demonstrated that genetic clusters of *P. plurivora* were more separate than those of *P. cinnamomi*. Overall, estimates suggest that isolates representing the five species consist of distinct populations with the greatest diversity observed with *P. plurivora*.

*Introduction*

Numerous studies have explored both the genotypes of *Phytophthora* populations and their resistance to fungicides, particularly metalaxyl and its more active enantiomer, mefenoxam (5, 6, 24, 40, 59, 91, 97). Metalaxyl resistance in Phytophthora is controlled by a single nuclear locus that exhibits incomplete dominance (59, 91). With *P. citricola* a large portion of the population was tolerant to mefenoxam, which may indicate that the species itself might be tolerant (5). *In vitro* selection of resistance to metalaxyl was also demonstrated, which might signify that field resistance to fungicides might evolve as a result of their use (13, 30, 53).

Major differences between the genetic diversity of many *Phytophthora* species have been described. For example, a worldwide population of *P. cactorum* was shown to have a low level of genetic diversity (6, 40), whereas *P. citricola*, *P. capsici*, *P. nicotianae*, and *P. infestans* from agricultural or nursery systems revealed high levels of genetic diversity (5, 24, 59, 91). These differences have examined whether or not correlations between genetic diversity and host species, pathogenicity, geography, and fungicide treatment exist. For example, although California populations of *P. cactorum* had low levels of genetic diversity, they varied greatly in their aggressiveness on almond and strawberry (6). While California populations of *P. citricola* had high levels of genetic diversity, much of the variation was associated with host and geography and not aggressiveness on almond shoots (5). *P. cactorum* isolated from Rhododendron in Germany were found to be genetically similar to both European Union and US isolates from strawberry, but they were non-pathogenic on the fruit and had larger oospores (22). In contrast, *P. capsici* isolates in Michigan

displayed variation in phenotype and mefenoxam resistance, but it was shown that the isolates were similar within specific locations and sample time and were also pathogenic to a variety of bean species and cucumber (33). These studies demonstrate that *Phytophthora* populations can be unique and our understanding of the genetic differences among a population may be related to different factors. Incorporation of population biology data into nursery management plans can improve practices while streamlining fungicide use. This would be particularly useful in Maryland nurseries, where little is known about the Phytophthora population.

A collection of the five most common *Phytophthora* species found in Maryland ornamental nurseries and in mid-Atlantic US oak forests (Supplemental Table 1) were genotyped and compared to explore the hypothesis that nursery management practices have altered the Phytophthora population resulting in limited genetic diversity. We also explored whether or not genotypic differences exist between isolates causing disease and those found associated with asymptomatic plants; different substrates such as soil and irrigation water; among nurseries and various hosts.

## *Materials and methodology*

### Isolate selection

Surveys were conducted between 2010 and 2012 at eight Maryland ornamental nurseries resulting in a collection of 680 *Phytophthora* isolates from various hosts and substrates (7). The five most common species were selected (102 *P. cinnamomi*, 24 *P. citrophthora*, 190 *P. plurivora*, 16 *P. multivora*, and 28 *P. pini*) from those isolates collected in 2010 from five of the seven nurseries (S1).

Mycelia preparation and DNA isolation

Isolates were grown on 10% buffered clarified V8 agar media (10 g $CaCO_3$ per 1 liter of V8 juice spun down at 4,000 rpm for 10 min., 100 ml clarified V8 juice in 900 ml $dH_2O$ with 10 g agar). Two 1 $cm^2$ mycelial plugs were taken from 7-14 day old isolates and placed in a 50 mL Falcon tube filled with 25 mL 10% buffered clarified V8 broth (V8 agar media excluding agar). Tubes were placed on their sides at 23ºC in the dark to produce mycelia. Approximately 200 mg mycelia was produced in each tube (wet), which were harvested using a sterile dissecting needle under a bio hood and rinsed with sterile distilled water once and dried on sterile filter paper. DNA was then isolated using the DNeasy Plant Mini Kit (Qiagen Inc, Valencia, CA). DNA concentrations were adjusted to 14.0 ng/µl with nuclease free water by averaging three measurements using a NanoDrop lite spectrophotometer (Thermo Scientific, Waltham, MA).

AFLP analysis

Isolates were numbered sequentially and, to measure the replicability of peaks, 10 were randomly selected and repeated in each of the five plates. The location of these 10 isolates was determined by generating a set of 10 numbers between 1 and 96 for each plate. Five plates were created. Negative controls were included in Plates 4 and 5. Randomization of plates was performed using www.random.org.

The digestion and ligation steps were performed in the same reaction. Genomic DNA (approximately 0.42 µg) per isolate was incubated for five hours at 37 ºC then for 20 minutes at 80ºC in a C1000 Touch Thermal Cycler (BioRad, Hercules, CA) using an un-heated lid. The solution consisted of 5 U Mse1 restriction enzyme

(New England Biolabs, Ipswitch, MA) and 5 U EcoR1-HF restriction enzyme (NEB Ipswitch, MA) in 20 µL nuclease free water, 10X CutSmart buffer [50mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, and 100 µg/mL Bovine Serum Albumen (BSA)] (NEB Ipswitch, MA), 10 mM ATP (NEB Ipswitch, MA), 1 µL each of Mse1 adapter (Applied Biosystems, Foster City, CA) and EcoR1 adapter (Applied Biosystems, Foster City, CA) and 0.25 µL T4 ligase (400,000 U/mL ) (NEB Ipswitch, MA). After the digestion/ligation, each reaction was diluted 1:10 with nuclease-free water.

A two-step amplification was used (101): an initial pre-amplification followed by a second round of amplification using selective primers. The pre-amplification step used 10 µM *EcoR1* and *Mse1* primers (NEB Ipswitch, MA) in a 10 µL reaction of 10X Taq Buffer [10mM Tris-HCl, 50mM KCl, and 1.5 mM $MgCl_2$] (NEB Ipswitch, MA), 0.125 mM dNTPs (NEB Ipswitch, MA), 0.5 U Taq DNA polymerase (NEB Ipswitch, MA) and 2.0 µL of template DNA (the diluted digestion/ligation product). The pre-amplification cycling protocol was: 95ºC for 30 seconds; then 25 cycles of 95ºC for 30 seconds, 56ºC for 1 minute, and 68ºC for 1 minute; then 68ºC for 5 minutes and 4ºC for 5 minutes (41). Pre-amplified products were diluted 1:40 with nuclease-free water and select amplification was performed using the same 10 µL cocktail. However for the select amplification, two bases (-AC) were added to the *EcoR1* primer (IDT Coralville, IA), which was labeled with fluorescein amidite (FAM) (Integrated DNA Technologies Coralville, IA), and three bases were added to the *Mse1* primer (-CAG) (IDT Coralville, IA). A touchdown-PCR protocol was used (95ºC for 30 seconds then 8 cycles of 95ºC for 30 seconds, 65ºC for 1 minute

(lowering the annealing temperature by 1ºC with each cycle), and 72ºC for 1 minute; then 22 cycles of 95ºC for 30 seconds, 58ºC for 1 minute, and 68ºC for 1 minute; then 68ºC for 5 minutes and 4ºC for 5 minutes) (41). To check quality, we visualized DNA by running final AFLP products on a 1.5% agarose gel (Fisher Scientific, Pittsburgh, PA) in 1X sodium boric acid conductive medium (10) for 12 minutes at 250V and post-stained with a 3X concentration of GelRed (Biotium, Hayward, CA) for 30 minutes.

Fragment analysis

Selectively amplified PCR product was diluted 1:20 to eliminate off-scale peaking and fragment analysis was conducted by McLab (San Francisco, CA), which uses a 500 LIZ size standard (Life Technologies, Carlsbad, CA) and an ABI Genetic Analyzer (3730XL). Electropherograms were scored using GeneMapper Version 4.0 (Applied Biosystems, Foster City, CA). All samples were analyzed together to create the analysis panel. Blue dye color size range was set to 0-999, the marker repeat unit was set to 9, and the stutter ratio was left at 0.0. Peak detection was set to B = 1000 and O = 75. Individual plates were scored separately using the same panel so that any peaks in the negative controls coinciding with peaks in samples on the same plates could be removed. Isolates with bad sizing quality (SQ) or messy peaks were removed from the data set and fragment analysis was repeated. Similarly, individual reactions that resulted in poorly resolved AFLP profiles (e.g., low intensity of signal) were also repeated.

All electropherograms were examined by eye and compared to a matrix of presence/absence alleles between 57 and 489 base pairs (bp). Those that displayed

miscalled or unlabeled peaks were scored manually. Calls for peaks that did not reach or exceed the set threshold of 1000 were removed. Peaks that appeared as "shoulders" of other peaks were only labeled if their height reached or exceeded 10% of the taller peak.

Selective primer optimization

Eleven different selective EcoR1 and Mse1 primer combinations (EcoR1-AA and Mse1-CAC, -CTA; EcoR1-AC and Mse1-CAA, -CAC; EcoR1-AG and Mse1-CTC; EcoR1-AT and Mse1-CAT; EcoR1-TA and Mse1-CTA, -CTC; EcoR1-TC and Mse1-CTC; and EcoR1-TG and Mse1-CAG, -CTC) were tested (Table 4) on five isolates representing each of the *Phytophthora* species with a positive control based on successful combinations in previous studies (5, 6, 59). EcoR1-AC was chosen based on number and resolution of bands produced on gels. A second trial was conducted in which EcoR1-AC was combined with the seven Mse1 selective primers from the first trial (Mse1-CAA, CAC, CAG, CAT, CTA, CTC, CTG) along with Mse1-A and Mse1-C to compare against less specific primers. Based on the number and resolution of bands produced on gels, three Mse1 primers (Mse1-CAG, -CTC, and -C) were chosen to combine with EcoR1-AC for fragment analysis. These primer combinations produced the most, average, and least amount of clear bands on the gel, respectively. This step was conducted for the purposes of identifying whether or not a correlation existed between band quality in a gel and peak quality in electropherograms produced during fragment analysis. Eight dilutions were made of each product (1:1, 2, 5, 10, 20, 50, 100, 200) to determine which dilution would offer the best protection against off-scale peaks while still maintaining good separation

from background noise. Peak number and size in the resulting electropherograms for each of the isolate-primer pair-dilution combinations were compared and the primer combination EcoR1-AC/Mse1-CAG at a 1:20 dilution was selected.

| | | Mse1 Primers | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **-CAA** | **-CAC** | **-CAG** | **-CAT** | **-CTA** | **-CTC** | **-CTG** |
| **EcoR1 Primers** | **-AA** | | X | | | X | | |
| | **-AC** | X | X | | | | | |
| | **-AG** | | | | | | X | |
| | **-AT** | | | | X | | | |
| | **-TA** | | | | | X | X | |
| | **-TC** | | | | | | X | |
| | **-TG** | | | X | | | | X |

**Table 4.** Selective primer combinations tested.

Analysis of AFLP data

A multivariate hierarchical clustering analysis using Ward's method was used to create a distance matrix, which was visualized with heat maps using JMP Genomics 6.0 (SAS Institute, Cary, NC). A dendrogram was built based on distance scales and clusters were then identified using the distance graphs. Genetic similarity among isolates was also calculated using Jaccard coefficient using FAMD (89). The similarity matrix then was subjected to cluster analysis using unweighted pair group method with arithmetic mean (UPGMA) in order to visualize the genetic relationship among clusters. Clustering support was assessed by 1,000 bootstrap replicates. The dendrogram was visualized using MEGA 6 (96). In addition, a minimum spanning network using SplitsTree4 was generated to inference an implicit representation of evolutionary history of the five species analyzed (46).

Evidence of genotypic clustering within the population was assessed through Bayesian model-based clustering in STRUCTURE v.2.3 (28, 85). STRUCTURE determines the most likely number of differentiated clusters (K) represented by the sample and assigns the sampled genotypes to the inferred clusters. Using a random subset of 1,000 markers, we estimated the log likelihood of the data, given different numbers of genetic clusters K. An admixture model with correlated allele frequencies was utilized without sampling locations as priors. Other parameters were left at their default settings. For each k value of 1 through 7, we ran three replicates (100,000 burn-in cycles, 100,000 MCMC iterations), from which we calculated ΔK. The STRUCTURE Harvester software was used to identify the number of populations K with the best support (25).

Parameters of genetic variability and population structure were estimated using GenAlEx 6.5 (82). Analysis of molecular variance (AMOVA) was used to estimate the proportion of genetic variation within and among the samples when defined as; (1) source of isolation (i.e. environment vs. necrotic tissue), (2) origin of isolate (i.e. forest vs. nursery or different nurseries), (3) host symptom (i.e. symptomatic vs. asymptomatic host), and (4) host genus association. The parameter *φpt* (analogous to *Fst* for binary data) was used to determine the differentiation between to subpopulations. If the observed *φpt* value differed significantly from zero the null hypothesis of no genetic differentiation could be rejected. Significance of fixation indices was tested using 1,000 nonparametric permutations.

*Results*

AFLP analysis

The DNA polymorphisms detected by the AFLP experiment were consistent for control samples tested and no plate effect was found. The primer combination EcoR1-AC/Mse1-CAG resulted in a total of 168 clearly resolved fragments in the size range of 57 to 489 bp. The number of polymorphic fragments varied from 45 to 142, and *P. plurivora* had the greatest percentage of polymorphism (85%) followed by *P. cinnamomi* (50%) (Table 5).

Genotypic diversity

The genetic diversity of each population was calculated based on the genotypes present. The expected heterozygosity ($H_E$) was lowest with *P. pini* (0.043) and highest with *P. plurivora* (0.056) (Table 5). Division of populations into origin or host variables had interesting results: Several of the population diversity measures along with $H_E$ and Shannon's Index (*I*) indicated that large differences exist among nursery populations of *P. plurivora.* In particular, isolates collected from Nursery 6 were characterized by the highest heterozygosity (Table 5). When isolates were grouped based on other criteria, those that were directly recovered from lesions were more diverse than those isolated from the soil or environment. Similarly, heterozygosity was higher among isolates that were recovered from symptomatic hosts. Isolate origin (forest or nursery, and among nurseries) was also a significant factor of genetic diversity for *P. cinnamomi* and *P. citrophthora* (Tables 6 and 7).

| Phytophthora spp. | Populations | N | B | P(#) | P (%) | $N_A$ | $N_E$ | *I* | $H_E$ | $uH_E$ |
|---|---|---|---|---|---|---|---|---|---|---|
| *P. plurivora* | Overall | 186 | 10 ± 4.4 | 142 | 84.52% | 1.690 | 1.073 | 0.108 | 0.056 | 0.057 |
| | Origin | | | | | | | | | |
| | Nursery 2 | 99 | | | 49.40% | 0.988 | 1.076 | 0.088 | 0.050 | 0.050 |
| | Nursery 4 | 14 | | | 22.62% | 0.458 | 1.073 | 0.072 | 0.044 | 0.046 |
| | Nursery 6 | 56 | | | 67.86% | 1.357 | 1.076 | 0.118 | 0.061 | 0.062 |
| | Nursery 7 | 12 | | | 17.86% | 0.357 | 1.064 | 0.062 | 0.039 | 0.040 |
| | Origin | | | | | | | | | |
| | Soil | 99 | | | 49.40% | 0.988 | 1.075 | 0.087 | 0.050 | 0.050 |
| | Necrotic tissue | 87 | | | 75.00% | 1.500 | 1.076 | 0.118 | 0.060 | 0.061 |
| | Host | | | | | | | | | |
| | Symptomatic host | 120 | | | 78.57% | 1.571 | 1.074 | 0.116 | 0.059 | 0.060 |
| | Asymptomatic host | 66 | | | 35.71% | 0.714 | 1.076 | 0.080 | 0.048 | 0.049 |
| | Host | | | | | | | | | |
| | Acer | 10 | | | 15.48% | 0.310 | 1.075 | 0.075 | 0.048 | 0.051 |
| | Ilex | 49 | | | 25.00% | 0.500 | 1.078 | 0.075 | 0.047 | 0.048 |
| | Pieris | 55 | | | 60.12% | 1.202 | 1.077 | 0.117 | 0.062 | 0.062 |
| | Rhododendron | 47 | | | 62.50% | 1.250 | 1.076 | 0.109 | 0.057 | 0.058 |
| | | | | | | | | | | |
| *P. cinnamomi* | Overall | 102 | 11 ± 2.7 | 84 | 50.00% | 1.000 | 1.077 | 0.081 | 0.048 | 0.048 |
| | Origin | | | | | | | | | |
| | Forest | 76 | | | 91.95% | 1.839 | 1.148 | 0.160 | 0.093 | 0.094 |
| | Nursery 6 | 26 | | | 44.83% | 0.897 | 1.144 | 0.133 | 0.084 | 0.086 |
| | Host | | | | | | | | | |
| | Symptomatic host | 51 | | | 62.07% | 1.241 | 1.151 | 0.148 | 0.091 | 0.092 |
| | Asymptomatic host | 51 | | | 77.01% | 1.540 | 1.146 | 0.157 | 0.092 | 0.093 |
| | Host | | | | | | | | | |
| | Pieris | 20 | | | 26.79% | 0.536 | 1.079 | 0.080 | 0.049 | 0.050 |
| | Quercus | 70 | | | 42.26% | 0.845 | 1.074 | 0.077 | 0.046 | 0.046 |
| | | | | | | | | | | |
| *P. citrophthora* | Overall | 24 | 12 ± 2.9 | 45 | 26.79% | 0.536 | 1.081 | 0.082 | 0.050 | 0.051 |
| | Origin | | | | | | | | | |
| | Nursery 2 | 11 | | | 14.88% | 0.298 | 1.063 | 0.061 | 0.039 | 0.041 |
| | Nursery 6 | 10 | | | 20.83% | 0.417 | 1.095 | 0.088 | 0.056 | 0.059 |
| | Host | | | | | | | | | |
| | Symptomatic host | 11 | | | 17.86% | 0.357 | 1.082 | 0.077 | 0.049 | 0.052 |
| | Asymptomatic host | 13 | | | 20.83% | 0.417 | 1.077 | 0.073 | 0.046 | 0.048 |
| | | | | | | | | | | |
| *P. pini* | Overall | 25 | 9.6 ± 1.7 | 52 | 27.38% | 0.548 | 1.064 | 0.071 | 0.042 | 0.043 |
| | Host | | | | | | | | | |
| | Symptomatic host | 13 | | | 14.88% | 0.310 | 1.053 | 0.054 | 0.034 | 0.035 |
| | Asymptomatic host | 12 | | | 22.02% | 0.440 | 1.068 | 0.073 | 0.044 | 0.046 |
| | | | | | | | | | | |
| *P. multivora* | Overall | 18 | 9.8 ± 2.2 | 51 | 31.55% | 0.631 | 1.070 | 0.081 | 0.046 | 0.048 |
| | Host | | | | | | | | | |
| | Symptomatic host | 11 | | | 23.21% | 0.464 | 1.067 | 0.074 | 0.044 | 0.046 |
| | Asymptomatic host | 7 | | | 22.02% | 0.440 | 1.072 | 0.080 | 0.048 | 0.051 |

**Table 5**. Population diversity measures for five *Phytophthora* species found in Maryland's nurseries and Mid-Atlantic forests. Only populations >7 isolates were included.

N= Number of isolates tested

B= Mean Band frequency per isolate ± Standard deviation

P(%)= percentage allele that are Polymorphic

P(#)= number of Polymorphic fragments

$H_E$= Expected Heterozygosis

$uH_E$= Unbiased Expected Heterozygosis

$N_E$= No of Effective alleles

$N_A$= No of different Alleles

*I*= Shannon's Information Index

$A_E$ = Effective number of Alleles

## Population structure

Hierarchical clustering revealed distinctly different clades for each of the *Phytophthora* species (Fig. 3). *P. cinnamomi* isolates separated into four clusters, and two of the largest clusters were closely related (cluster 3 and 4), containing 80% of the isolates. Population structure of *P. citrophthora, P. pini* and *P. multivora* was similar to *P. cinnamomi* where two closely related clusters dominating the population. In contrast, *P. plurivora* isolates separated into six clusters, one of which included approximately 40% of the isolates (Fig. 3).

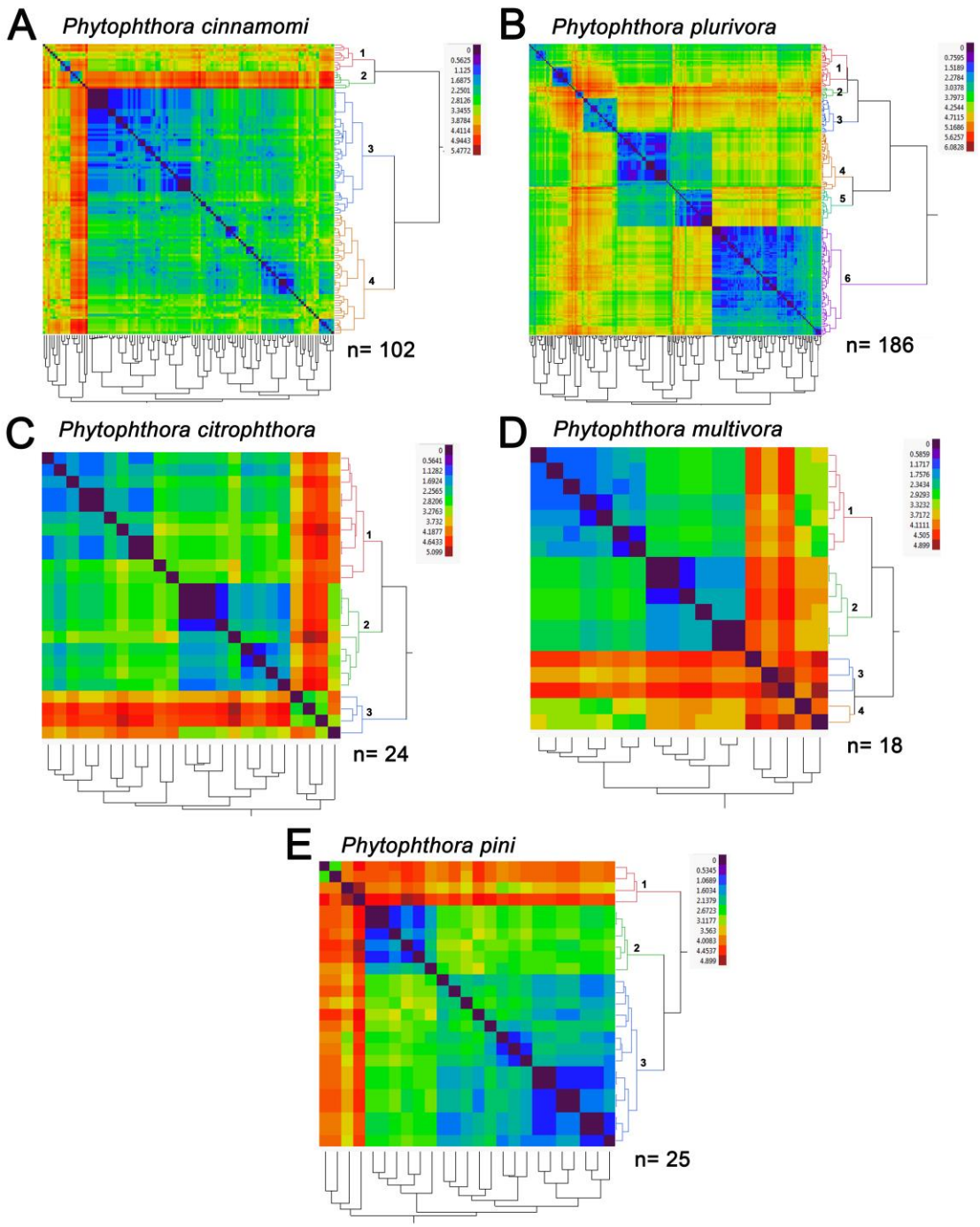



**Figure 3**. Heat maps and dendrograms from hierarchical clustering generated in JMP Genomics using the AFLP data of five *Phytophthora* species.

| Source of Variation | | Variance | Total (%) | [a] $\Phi_{PT}$-[b] Statistic | Variance | Total (%) | $\Phi_{PT}$-Statistic | Variance | Total (%) | $\Phi_{PT}$-Statistic | Variance | Total (%) | $\Phi_{PT}$-Statistic | Variance | Total (%) | $\Phi_{PT}$-Statistic |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Origin (Forest vs. Nursery) | | | | | | | | | | | | | | | | |
| | Among Population | 0.125 | 3% | 0.027* | | | | | | | | | | | | |
| | Within Population | 4.579 | 97% | | | | | | | | | | | | | |
| Origin (different nurseries) | | | | | | | | | | | | | | | | |
| | Among Population | | | | 1.162 | 21% | 0.208* | | | | | | | 2.083 | 24% | 0.240*** |
| | Within Population | | | | 4.434 | 79% | | | | | | | | 6.604 | 76% | |
| Origin (Environment vs. necrotic tissue) | | | | | | | | | | | | | | | | |
| | Among Population | | | | | | | | | | | | | 1.088 | 13% | 0.1304*** |
| | Within Population | | | | | | | | | | | | | 7.259 | 87% | |
| Host symptom (Symptomatic vs. Asymptomatic) | | | | | | | | | | | | | | | | |
| | Among Population | 0.037 | 1% | 0.008 | 0.975 | 18% | 0.182* | 3.610 | 0.000 | -0.042 | 0.155 | 3% | 0.034 | 0.613 | 8% | 0.075*** |
| | Within Population | 4.609 | 99% | | 4.392 | 82% | | 5.524 | 5.524 | | 4.360 | 97% | | 7.521 | 92% | |
| Host association (Different genera) | | | | | | | | | | | | | | | | |
| | Among Population | 0.000 | 0% | -0.005 | | | | | | | | | | 0.838 | 10% | 0.103*** |
| | Within Population | 4.506 | 100% | | | | | | | | | | | 7.261 | 90% | |

**Table 6.** Summary of analysis of molecular variance (AMOVA) of Phytophthora populations based on AFLP.

The UPGMA analysis resolved different species of *Phytophthora* into clusters that diverged from one another at Jaccard's similarity coefficients of <0.20-0.40 (Fig. 4). The UPGMA phylogram showed similar placements of isolates as the hierarchical clustering, but discrepancies were observed for some isolates and some clusters did not have enough bootstrap support (Fig. 4). Isolates that were formerly placed within the *P. citricola* complex (*P. plurivora*, *P. multivora* and *P. pini*) showed high levels of genetic diversity both among and within isolate clusters (Figs. 3B, D and E). *P. cinnamomi* isolates, however, were genetically similar and two distinct clusters were formed with one containing most of the isolates (Fig. 3A).

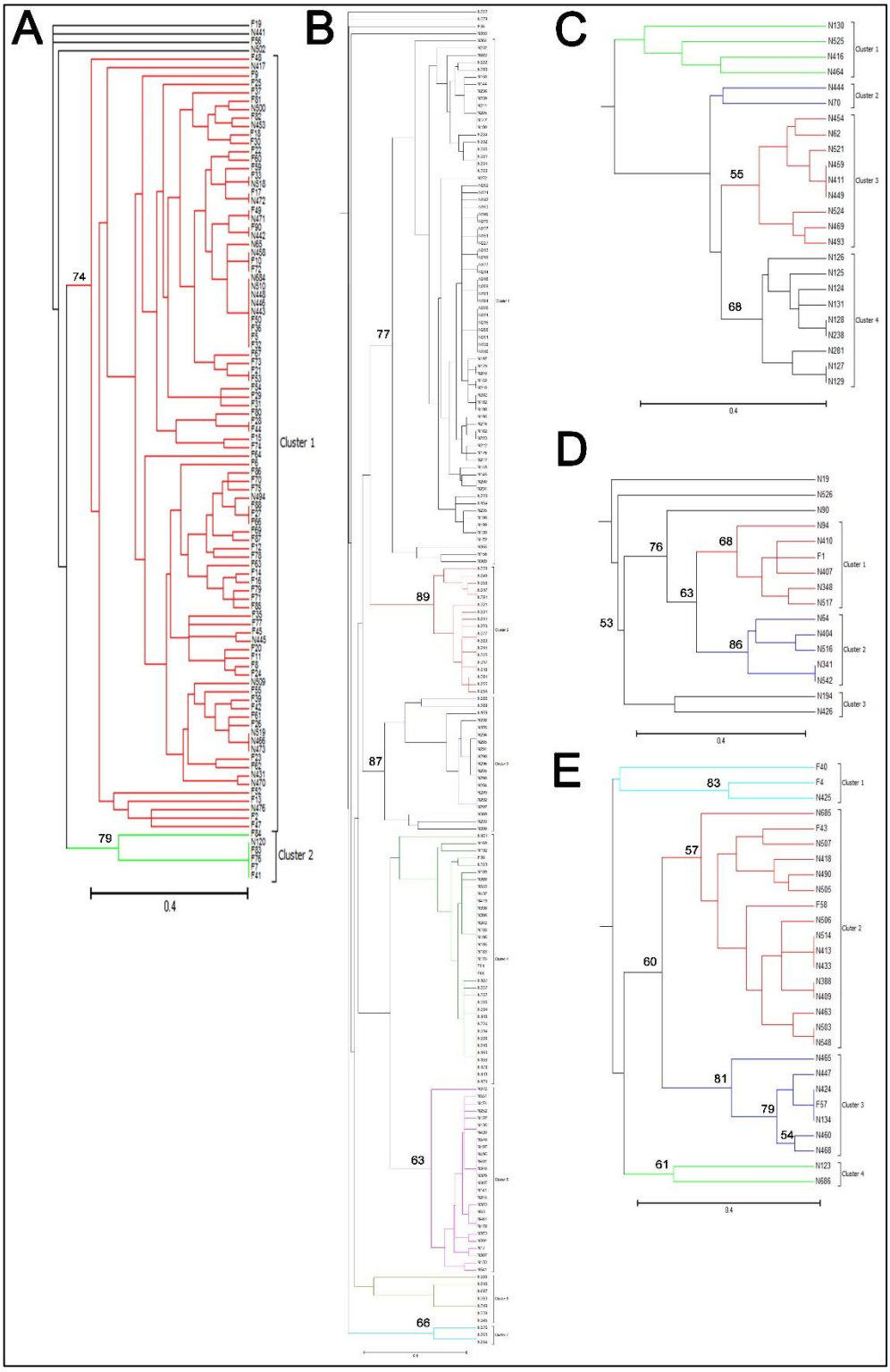



**Figure 4**. UPGMA trees of five *Phytophthora* species with Jaccard coefficient based on AFLP data. A total of 1,000 bootstrap replicates were made, and bootstrap values >%50 are labeled. Scale bar indicates Jaccard's genetic similarity. A *P. cinnamomi*, B, *P. plurivora*. C, *P. citrophthora*, D, *P. multivora* and E, *P. pini*.

The minimum spanning networks were in agreement with genetic distances observed both in hierarchical clustering and in UPGMA. In spite of the wide range of *P. cinnamomi* isolate origins (several nurseries and forests), they were closely related (Fig. 5). In contrast, *P. plurivora* isolates were clearly more diverse with large distances between emerging linages. While the sample sizes were small, populations of *P. citrophthora*, *P. pini* and *P. multivora*, demonstrated similar population structuring as *P. plurivora*.

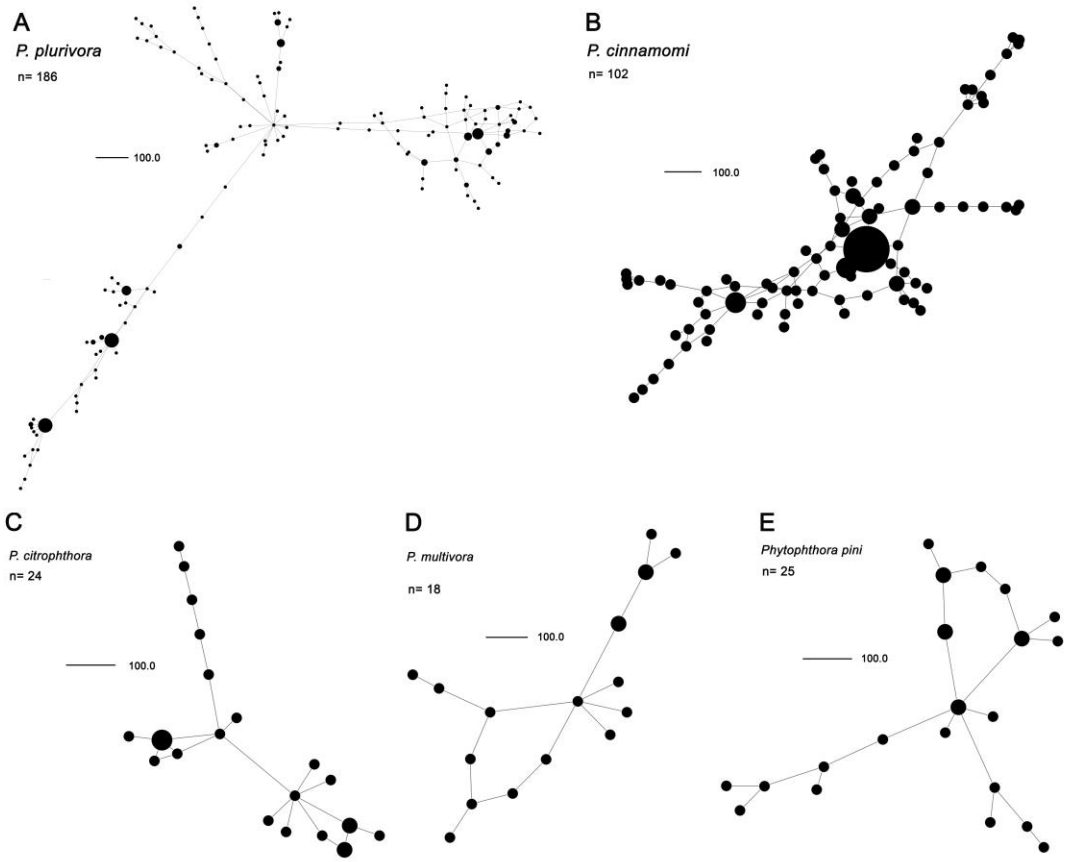



**Figure 5.** Minimum spanning networks of five *Phytophthora* species. Each circle represents a unique AFLP genotype where the size of the circle is relative to the number of individuals represented in the data.

Clonal groups were detected in all species analyzed (Fig 3-5). Interestingly, some clonal groups within both *P. cinnamomi* and *P. plurivora* included a

combination of isolates that were recovered from forests and nurseries. While all clonal groups within *P. cinnamomi* were located in clusters 3 and 4, those within *P. plurivora* were located in clusters 4, 5 and 6 (Fig 3). *P. plurivora* isolates with clonality were found more frequently associated with symptomatic host (Likelihood Ratio: $X^2$= 44.4; *P*= 0.025).

Because prior information about the populations and admixed model was lacking, STRUCTURE was used to estimate that the likelihood of the data was greatest when K= 4 for *P. cinnamomi*, *P. plurivora*, *P. pini* and *P. citrophthora* and K=3 for *P. multivora* (Fig. 6). These genetically distinct clusters primarily corresponded to hierarchical clustering and UPGMA clusters.

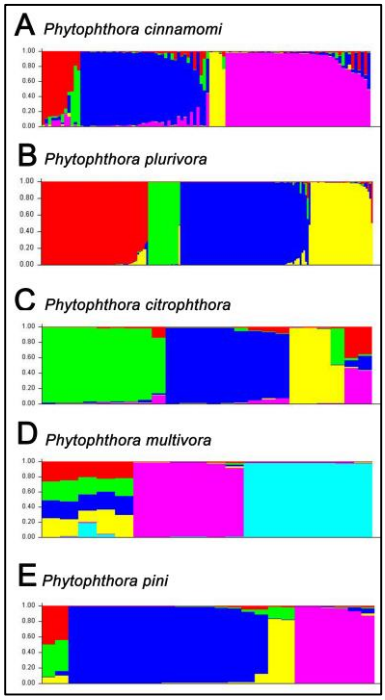



**Figure 6.** Bayesian clustering analysis of all individuals with the program STRUCTURE. Individual assignment to the most probable number of clusters K (3) for *P. multivora* and K (4) for *P. cinnamomi*, *P. plurivora*, *P. citrophthora*, and *P. pini* as inferred from the ΔK statistic

Analysis of molecular variance (AMOVA) indicated that most of the genetic variation resided within the populations (Table 6). However, isolate origin (forest versus nursery or different nurseries) appears to have an effect and significant variations were detected among populations with *P. cinnamomi* (3% of the variance), *P. citrophthora* (21%) and *P. plurivora* (24%). Some contrast was observed between *P. cinnamomi* and *P. plurivora* isolates when grouped based on host association. With *P. cinnamomi* 100% of the variance was attributed to within the population while 90% of the variance in *P. plurivora* was attributed to within the population (Table 6, Fig. 7).

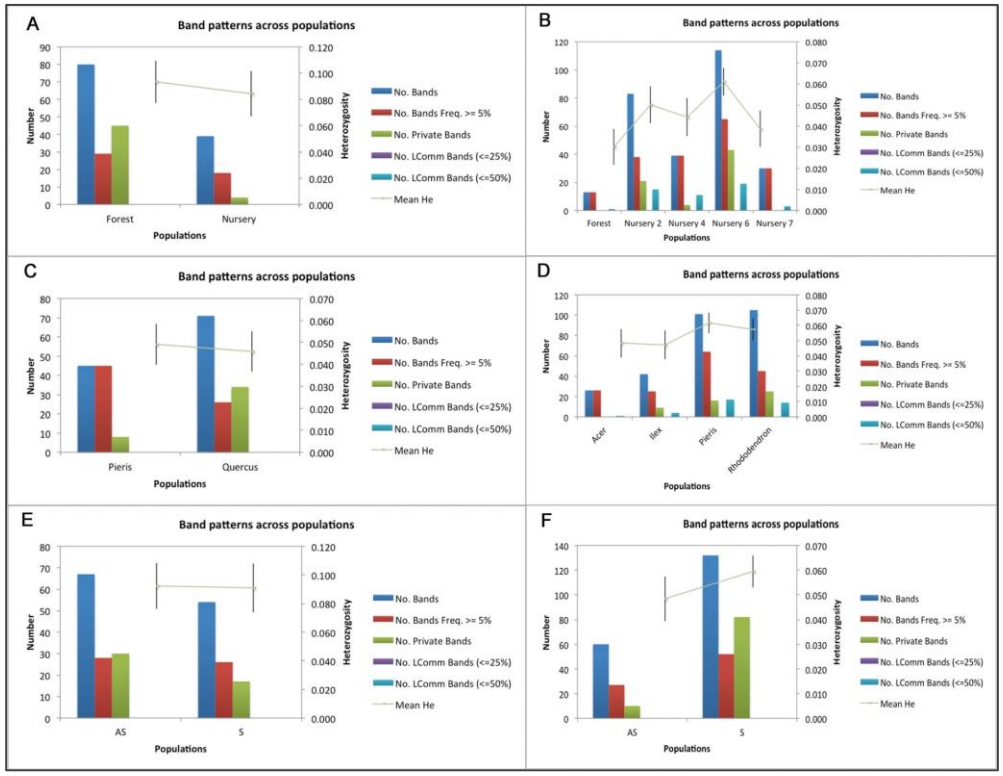



**Figure 7.** Genetic variability of *P. cinnamomi* (A, C, E) and *P. plurivora* (B, D, E)

*Discussion*

This study is the first to examine the genotypic diversity of *Phytophthora* species in Maryland nurseries. AFLP genotyping revealed clear differences between population structures of the five most commonly observed species, in particular between *P. cinnamomi* and *P. plurivora.* Of the five species, *P. cinnamomi* had the least genotypic diversity. *P. cinnamomi* is a cosmopolitan species, demonstrated by the fact that it has become well-established worldwide in nursery, agricultural, and forest systems (3, 7, 23, 26, 65, 69, 75, 76). Our findings support studies conducted in such operations revealing limited genetic diversity and predominance of the A2 mating type (21, 23, 26, 62, 63, 75, 76).

The low genetic diversity seen in *P. cinnamomi* can be explained by a general lack of sexual reproduction observed in the species. There are several explanations for why this might be occurring. Firstly, sexual reproduction has costs and could be unfavorable if oospores fail to germinate or the progeny fail to survive (21). Secondly, in environments where the pathogen is well-established or where there are susceptible hosts, little to no selection pressure would exist, and sexual reproduction would thus be unnecessary. The frequency with which this species is observed on a range of plant genera in nursery and forest systems demonstrates that *P. cinnamomi* is indeed well-established, and can survive and infect a wide range of hosts. Thirdly, sexual reproduction can lead to the breakup of coadapted genes in clonal lineages, which would be unfavorable in low selection pressure environments (21). For these reasons, sexual reproduction is not always advantageous, which could explain why, even when both mating types of *P. cinnamomi* are present, no sexual reproduction occurs (21).

Our study suggests that, although the difference is small, the forest population of *P. cinnamomi* is more diverse than the Maryland nursery population. A lower level of genetic diversity in the nursery population could have arisen from selection pressure created by nursery management practices, in particular fungicide applications. However, when we screened most of the *P. cinnamomi* isolates for their sensitivity to five common fungicides, insensitive isolates were present in both forest and nursery populations (Chapter 3). Alternatively, plant trade could be responsible for the difference between these two systems. Because Phytophthora movement into Maryland nurseries via plant trade occurs regularly (7), it is possible that particular genotypes are being repeatedly introduced and dominating the population. It is also possible that particular genotypes were introduced into these nurseries early on as "founder populations", and have remained dominant over time.

Although *P. cinnamomi* appears to be well-established in both forest and nursery systems, it is plausible that reproduction strategies differ in these systems as a result of nursery plant trade and pest management strategies. Although these populations are not reproducing sexually, they could be reproducing via outcrossing or selfing (42, 58). If both mating type hormones, which are consistent in all heterothallic *Phytophthora* species, are present there is no barrier to alternative reproduction behaviors in heterothallic oomycetes (58). As the A1 mating type of *P. cambivora* occurs both in forests and Maryland nurseries (7, 69), it is possible that outcrossing is responsible for the variations detected among the differing populations.

Environmental factors such as aging, physical damage, fungicides, host-plant exudates, and volatile compounds formed by *Trichoderma* species can stimulate

selfing within heterothallic oomycetes (42, 58). Differences between forest and nursery systems in terms of these environmental factors could be causing different rates of selfing, which could in turn be affecting genetic differences between the two systems. Selfing in heterothallic oomycetes, known as "secondary homothallism", may confer an evolutionary advantage by enabling heterothallic species to form oospores in adverse environmental conditions (58). Another factor to consider is that the formation of oospores by many water molds is favored by salinity levels consistent with those of their natural habitats (58). Perhaps differences in salinity levels between rain water in forests and irrigation systems in nurseries result in different levels of oosporogenesis in the two systems.

In contrast to *P. cinnamomi*, the *P. plurivora* population was very diverse. Unlike *P. cinnamomi, P. plurivora* does not require another mating type to reproduce sexually (inbreeding), which perhaps can explain the differences in diversity observed between the two species. *P. plurivora* is one of seven species once considered part of the *P. citricola* complex (now delineated into *P. citricola*, *P. elongata*, *P. pachypleura*, *P. pini, P. plurivora, P. multivora*, *P. acerina*). Other than Schoebel et al (2014), no other studies investigated the population structure of these species after their delineation as separate species. AFLP genotyping of *P. citricola* in California (5) and Tennessee (22, 45) revealed high genetic diversity. *P. plurivora* is commonly found in both natural and managed environments (90), thus it is possible that this species made up a significant proportion of the *P. citricola* populations genotyped in these studies. Schoebel et al (2014) found a moderate amount of genetic diversity in *P. plurivora*, with more diversity seen in European isolates than in US isolates, which

could signify that the species might have originated from Europe and was introduced into the US (90).

Clonal isolates were commonly observed within the species investigated. Moreover, clonal groups including isolates originating from both forests and nurseries were observed, suggesting that a pathway exists from forests to nurseries or vice versa. The population structure of *P. ramorum* in the US exemplifies the ability of Phytophthora to spread clonally. AFLP profiles showed that 75% of US isolates were comprised of a single genotype (48). In Tennessee, the existence of clonal groups in both managed and natural populations has been also observed with *P. citricola* (45). Nevertheless, it remains unclear why some clonal groups are more common than others. Further study is needed to demonstrate if fitness factors play a role in the success of these clonal populations in nurseries.

The significant molecular variation found for *P. plurivora* on different host genera suggests that host selection may be occurring. Such differences were also reported in California isolates of *P. citricola* where 86 isolates were found on ten host genera (5). Epidemiologically, these results suggest that *P. plurivora* is capable of adapting quickly, which, if true, could explain the frequency with which it was found on a wide range of hosts in Maryland nurseries. In contrast, we did not find any evidence that host selection is occurring in *P. cinnamomi*, which highlights its ability to infect over 5,000 plant species including hundreds of plant genera (27).

This study is the first to characterize the population structure of *P. plurivora*, *P. pini* and *P. multivora* using AFLP. Although the sampling sizes were small, our results suggest that *P. multivora* and *P. pini* consist of a lower genetic diversity than

*P. plurivora*. While these species may be of recent introduction to nursery systems, the small diversity seen could also be the result of less adaptable population behavior, which could, in turn, explain why they were so infrequently isolated as compared to *P. plurivora*. Further study is needed to explore if these theories are correct and why *P. plurivora* is more widespread than the sister taxa that reside in the same environments.

To date, no studies have examined the population structure of *P. citrophthora*. Because the species is heterothallic, we suspected that its genetic diversity would appear similar to that of *P. cinnamomi*. This was not the case, as significant differences were detected in genetic diversity of *P. citrophthora* among different nurseries and from plant tissue. All but one of the 24 *P. citrophthora* isolates in this study were of the A2 mating type, suggesting that a mixed reproductive system (asexual, selfing, outcrossing, parasexual or sexual) might be occurring in nursery populations of *P. citrophthora*.

Differences in genetic diversity between *P. cinnamomi* and *P. plurivora* underscore the concept of diversity versus pathogenicity. Although worldwide populations of *P. cinnamomi* have been shown to be less diverse, the pathogen causes severe damage to thousands of hosts, particularly in Western Australian jarrah forests (27). The host range of *P. plurivora* is still being developed, but disease caused by this species has not been observed on the scale of the dieback caused by *P. cinnamomi*. This raises questions about whether or not genetic diversity is required for a pathogen to cause major losses.

Our work will serve as a baseline to understanding the epidemiology of these pathogens in Maryland ornamental nurseries. Because of the current flaws in regulating pathogen movement in nurseries and natural environments, we can expect to see continued introduction of exotic genotypes into new environments. The consequences of these introductions, however, remain unclear. Can the introduction of new genotypes significantly influence population structures of particular species and, if so, what is the timeline for such alterations? What are the driving forces that leads one species to be more diverse than the others? Answers to such questions will help us to better understand the dynamics of shifting population structures in such intensively managed systems.

# Chapter 3: Fungicide sensitivity of *Phytophthora* in Maryland nurseries

This chapter was drafted to be submitted to the peer-reviewed journal Plant Health Progress. The tentative title: Sensitivity of *Phytophthora spp.* in Maryland Ornamental Nurseries to Oomycete-Targeted Compounds


*Abstract*

*Phytophthora* is a plant pathogen commonly found in Maryland ornamental nurseries. In this study, a collection of isolates from five Maryland nurseries and mid-Atlantic forests was screened against several fungicides commonly used to manage the pathogen. In total, 243 isolates (77 *P. cinnamomi*, 23 *P. citrophthora*, 21 *P. pini*, 15 *P. multivora*, and 107 *P. plurivora*) sampled from a range of hosts and substrates were screened for sensitivity to mefenoxam and fosetyl-Al. Mefenoxam-insensitive isolates [≥50% growth rate relative to controls (RG)] were further tested using higher concentrations of these fungicides along with dimethomorph, dimethomorph + ametoctradin and fluoxastrobin. Mefenoxam and fosetyl-Al-insensitive isolates were detected only for *P. cinnamomi*, *P. multivora* and *P. plurivora* and included 4%, 13% and 12% of screened isolates, respectively. These isolates remained insensitive at the higher concentrations of these fungicides along with fluoxastrobin. Dimethomorph and dimethomorph + ametoctradin, however, were highly effective. When grouped based on a previous genotyping study using amplified fragment length polymorphism (AFLP), most common genotypes found in Maryland nurseries were sensitive or intermediately sensitive (<50% RG) and the least common genotypes included the insensitive isolates. Our studies suggest differences in fungicide sensitivity among these five common *Phytophthora* species, signifying that a genus-level fungicide

application plan is insufficient for the management of all species in ornamental nursery production. Insensitive isolates are present within the population and thus the inclusion of chemicals such as dimethomorph and ametoctradin into management plans is recommended.

*Introduction*

Phytophthora root rot, crown rot, and foliage blight are common diseases in the ornamental plant industry, and management of these diseases is challenging (18), especially because environments in nurseries are conducive for their growth. Generally, nursery management of *Phytophthora* consists of schedule-based fungicide application programs. While these practices can help protect plants, they come with significant dangers: Overuse of fungicides can select for insensitive populations over time. For this reason, a periodic evaluation of fungicide sensitivity of Phytophthora pathogens is necessary.

There are a variety of fungicides that can be used to manage *Phytophthora* disease in nurseries. Among them, the acylanilides and alkyl phosphonates are systemic fungicides that have been shown to be superior to other systemic and protectant fungicides in their ability to inhibit disease development after infection, lack of vulnerability to weathering, and longer residual activity (15). The chemical metalaxyl is an acylanilide fungicide that was created in 1977 and used widely and intensively to control Oomycete diseases of numerous crops and ornamentals (5, 13, 15, 24, 44, 47, 77, 78, 81, 83). However, resistance to the fungicide was reported shortly thereafter in the 1980s (74). For this reason, in 1996 the manufacturer created

metalaxyl-M (mefenoxam), a more active isomer of the fungicide (77), which is applied similarly to metalaxyl, but at lower rates (87). Unlike metalaxyl and mefenoxam, which are xylem-mobile fungicides, phosphonate fungicides can move in both the xylem and phloem tissues (15, 60, 98). The most effective and perhaps well-known phosphonate fungicide is fosetyl-Al which, like metalaxyl, was developed in 1977. Since then there have been few reports of resistance (38, 60). Fosetyl-Al is one of the top ten fungicides used against Oomycetes in Maryland over the last decade (9, 70). Despite its usage decreasing over that same period of time, mefenoxam was still listed in the top 100 pesticides used in Maryland in 2004, and in the top 150 in 2011.

Exploring fungicide resistance in comparison with isolate genotypes has been the focus of numerous studies, particularly metalaxyl and mefenoxam (5, 6, 23, 24, 40, 54, 91, 97). Metalaxyl resistance is controlled by a single nuclear locus that exhibits incomplete dominance and that, as a result, resistance can develop easily (59, 91). Most of a *P. citricola* population was tolerant to mefenoxam, leading to speculation that the species itself might be tolerant (5). *In vitro* selection of resistance to metalaxyl was also demonstrated, which might signify that field resistance to fungicides might evolve as a result of their use (13, 30, 53). To date, there have been no studies examining fungicide resistance in Maryland ornamental nurseries.

A fungicide sensitivity experiment was conducted to explore whether resistance in Phytophthora to commonly used fungicides is occurring and if the frequently used fungicides are effective against five of the most commonly encountered *Phytophthora* species in Maryland nurseries. We also explored whether

there is a relationship between isolate fungicide sensitivity and variables such as isolate genotype, origin (nursery *vs*. forest), host symptom (symptomatic *vs*. asymptomatic), source (soil *vs*. infected tissue) and host genus. The overarching goal of this study was to provide Maryland nursery managers with information about which fungicides are still effective in managing diseases caused by *Phytophthora.*

*Materials and methodology*

Isolates included in this study were sampled from six Maryland ornamental nurseries in 2010 (7). In addition, because Phytophthora is not managed with fungicides in forest settings, isolates from mid-Atlantic US oak forests collected in 2004 and 2012 were included in the study to establish baseline sensitivity (S1) (3, 69). Five species of Phytophthora were tested: *P. cinnamomi, P. citrophthora, P. pini, P. plurivora,* and *P. multivora*. These species represent the most frequently observed isolates in Maryland's nurseries and mid-Atlantic oak forest sites (3, 7, 69). These isolates were previously genotyped using Amplified Fragment Length Polymorphism (AFLP) and clusters were generated based on their genetic distance (Chapter 2). In this study, a subset of 247 isolates (76 *P. cinnamomi,* 24 *P. citrophthora,* 22 *P. pini,* 108 *P. plurivora,* and 16 *P. multivora*) was selected to represent the resulting clusters of each of the five species.

All isolates were maintained on 6 cm Petri dishes with 8 mL of 10% buffered clarified V8 agar (10 g $CaCO_3$ per 1 liter of V8 juice spun down at 4,000 rpm for 10 min., 100 ml clarified V8 juice in 900 ml $dH_2O$ with 10 g agar). Clarified V8 agar was used as base growth media. Once the media was cooled to 50°C, fungicide was

added. While on a magnetic stirrer, a peristaltic pump was used to pour 8 mL of media into 6 cm Petri dishes. Petri dishes with un-amended agar served as the control. A 5 mm diameter sterile cork borer was used to transfer one plug from the growing edge of a 5-10 day old isolate onto the center (mycelia facing down) of each Petri dish. Plates then were incubated in the dark at 25°C, and at 72 hours the diameter of the mycelial growth (mm) was measured along two perpendicular lines, subtracting the diameter of the plug. For each test an equal number of control inoculations were similarly inoculated and measured. Relative growth (RG) was calculated by comparing the averaged diameter measurements on amended plates to those on controls. Using the scale cited by Hwang and Benson (47), isolates were considered sensitive (S) if relative growth was 0%, intermediately sensitive (IS) if relative growth was 1-50%, and insensitive (I) if relative growth was greater than 50%. Three plates were used for each isolate and the experiments were repeated twice. If statistically significant differences were detected among the two trials for an isolate the experiment was repeated twice more for that isolate.

The phenylamide chemical, mefenoxam (Subdue Maxx, 22% a.i., Syngenta) and the phosphonate fosetyl-Al (Aliette WDG, 80% a.i., Bayer) were used to screen for fungicide sensitivity and identify fungicide resistance isolates for the second trial (Table 6). One concentration (100 μg/mL a.i.) of both fungicides was tested based on previous studies (5, 6, 24, 39, 44, 45, 47, 67, 78, 95) and label rates. The subset of isolates labeled “insensitive” were chosen for a second experiment in which their RG was measured on agar amended with additional fungicides or concentrations: 1) Mefenoxam (200 μg/mL), 2) Fosetyl-al (500 μg/mL), 3) Dimethomorph (200 μg/mL)

(Stature SC, 43.5% a.i., BASF), 4) Dimethomorph and Ametoctradin (400 µg/mL) (Orvego, 47.1% a.i., BASF), and 5) Fluoxastrobin (100 µg/mL) (Disarm 480 SC, 40.3% a.i., Arysta LifeScience) (Table 7).

| Chemical | Trade Name | [1]FRAC Code | Label Rate |
|---|---|---|---|
| Mefenoxam | Subdue Maxx (Syngenta) | 4 | 1-2 oz/100 ga |
| Fosetyl-Al | Aliette WDG (Bayer) | 33 | [2]5 oz/10 ga |
| Dimethomorph | Stature SC (BASF) | 40 | 6.12-12.25 oz/50-100 ga |
| Dimethomorph + Ametoctradin | Orvego (BASF) | 40 + 45 | 11-14 oz/50-100 ga |
| Fluoxastrobin | Disarm 480 SC (Arysta LifeScience) | 11 | .15-.6 oz/100 ga |

**Table 7**. Chemicals used in fungicide sensitivity experiments
[1]Fungicide Resistance Action Committee
[2]Label rate based on soil drench recommendation to manage *Phytophthora* root rot in non-bearing avocado

*Results*

Of the five species tested (in total 243 isolates), the majority were intermediately sensitive to mefenoxam at a concentration of 100 µg/mL (Table 8, Fig. 8A). All *P. pini* isolates tested were intermediately sensitive to the fungicide. While *P. citrophthora* and *P. pini* had no isolates that were insensitive, *P. plurivora* had the greatest number of insensitive isolates (12% of the *P. plurivora* isolates screened) followed by *P. multivora* (13%) and *P. cinnamomi* (4%) (Table 8).

A large collection of isolates (in total 202 isolates) was also tested on fosetyl-Al (100 µg/mL) (Table 8, Fig. 8B). In contrast to mefenoxam, none of the isolates were sensitive and a majority of them were completely insensitive to fosetyl-Al. Some isolates grew faster on media amended with fosetyl-Al than on non-amended controls (Fig. 8B).

No significant differences were found in RG rates among the isolates when grouped based on isolation origin (forest or nursery), or isolation environment (soil, water, necrotic root or stem tissue) (Table 8). Similarly, host was not a significant

factor for any of the species except for *P. plurivora*: Isolates recovered from Sophora had significantly greater growth rates than those recovered from other hosts. Interestingly, with almost all species, isolates had significantly greater RG when they were recovered from an asymptomatic than from a symptomatic host. The exceptions were with *P. multivora* and *P. pini* (Table 8).

| ***Phytophthora* spp.** | **[1]Source of isolates** | | **n** | **RG ± StdDv** | ***F*** | **Prob>*F*** |
|---|---|---|---|---|---|---|
| *P. cinnamomi* | Origin | Forest | 66 | 8.1±12 | 0.2211 | 0.6396 |
| | | Nursery | 11 | 10.4 ± 2 | | |
| | Host symptom | Asymptomatic host | 41 | 11.6 ± 19 | 4.2352 | 0.043* |
| | | Symptomatic host | 36 | 4.8 ± 5 | | |
| | [2]Host genus | Six genera | 76 | | 1.0211 | 0.4118 |
| *P. citrophthora* | Host symptom | Asymptomatic host | 12 | 8.9 ± 7 | 8.5303 | 0.008* |
| | | Symptomatic host | 11 | 1.9 ± 4 | | |
| | Host genus | Five genera | 21 | | 0.776 | 0.5551 |
| | Environment | Soil | 19 | 6 ± 2 | 0.4134 | 0.5272 |
| | | Stem tissue | 4 | 3.6 ± 3 | | |
| *P. multivora* | Origin | Forest | 1 | 0 | 0.402 | 0.537 |
| | | Nursery | 14 | 19 ± 29 | | |
| | Host symptom | Asymptomatic host | 4 | 35 ± 43 | 2.0762 | 0.1733 |
| | | Symptomatic host | 11 | 11.6 ± 20 | | |
| | Host genus | Three genera | | | 1.5123 | 0.2629 |
| | Environment | Soil | 11 | 11.2 ± 20 | 9.7126 | 0.003* |
| | | Stem tissue | 3 | 14.9 ± 13 | | |
| | | Water | 1 | 98.2 | | |
| *P. pini* | Origin | Forest | 5 | 12.1 ± 13 | 1.486 | 0.2378 |
| | | Nursery | 16 | 19.3 ± 3 | | |
| | Host symptom | Asymptomatic host | 12 | 16.2 ± 11 | 0.3544 | 0.5587 |
| | | Symptomatic host | 9 | 19.4 ± 13 | | |
| | Host genus | Four genera | 20 | | 0.6505 | 0.5934 |
| | Environment | Soil | 18 | 17.6 ± 11.8 | 0.0005 | 0.003* |
| | | Stem tissue | 3 | 17.7 ± 14 | | |
| *P. plurivora* | Origin | Forest | 4 | 7.7 ± 11 | 1.3839 | 0.2421 |
| | | Nursery | 103 | 21.2 ± 23 | | |
| | Host symptom | Asymptomatic host | 50 | 25.9 ± 25 | 5.3305 | 0.0229* |
| | | Symptomatic host | 57 | 16.1 ± 19 | | |
| | Host genus | *Acer* | 7 | 38.2 ± 27 | AB | 0.001* |

| | | | | | |
|---|---|---|---|---|---|
| | *Buxus* | 2 | 27.9 ± 11 | AB | |
| | *Ilex* | 19 | 16 ± 22 | B | |
| | *Pieris* | 40 | 20.5 ± 22 | B | |
| | *Quercus* | 4 | 7.7 ± 11 | B | |
| | *Rhododendron* | 25 | 16 ± 18 | B | |
| | *Sophora* | 2 | 79 ± 18 | A | |
| | *Tilia* | 1 | 58 | AB | |
| Environment | Soil | 67 | 20.6 ± 3 | 1.7253 | 0.1664 |
| | Stem tissue | 32 | 17 ± 4 | | |
| | Root tissue | 7 | 38.2 ± 27 | | |
| | Water | 1 | 20 | | |

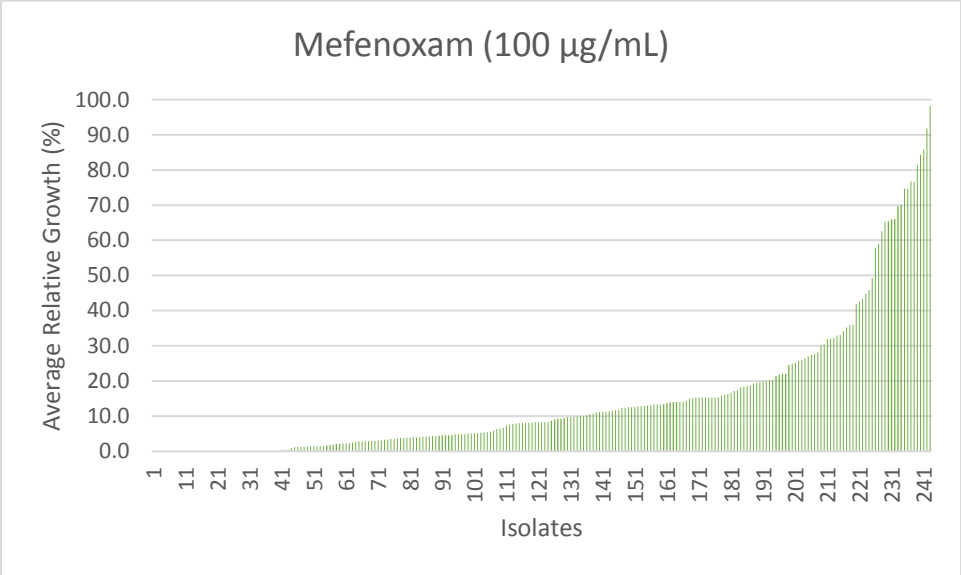
**Table 8.** Analysis of variance of isolate relative growth values for different variables. Figures represent mean relative growth (RG) ± StdDev in media amended with mefenoxam (100µg/mL).
[1] Variables for more than one sample are provided.
[2] When there were no significant differences among hosts, only F values and significance levels are provided.

A total of 18 isolates were identified as insensitive to mefenoxam (100 µg/mL) (Table 9). When these isolates were screened on higher concentrations of mefenoxam (200 µg/mL) and fosetyl-Al (500 µg/mL) they remained insensitive (Table 10). These isolates were also insensitive to fluoxastrobin (200 µg/mL). In contrast, they were sensitive to dimethomorph and dimethomorph + ametoctradin and, with the exception of two *P. plurivora* isolates, all isolates were sensitive.

| *Phytophthora* spp. | n | Sensitivity | Mefenoxam (100 µg/mL) | n | Fosetyl-AL (100 µg/mL) |
|---|---|---|---|---|---|
| *P. cinnamomi* | 77 | Sensitive | 17 | 74 | None |
| | | Intermediate | 57 | | 6 |
| | | Insensitive | 3 | | 68 |
| *P. citrophthora* | 23 | Sensitive | 10 | 17 | None |
| | | Intermediate | 13 | | 6 |
| | | Insensitive | none | | 11 |
| *P. multivora* | 15 | Sensitive | 4 | 7 | None |
| | | Intermediate | 9 | | 2 |
| | | Insensitive | 2 | | 5 |
| *P. pini* | 21 | Sensitive | none | 13 | None |
| | | Intermediate | 21 | | 6 |
| | | Insensitive | none | | 7 |
| *P. plurivora* | 107 | Sensitive | 10 | 91 | None |
| | | Intermediate | 84 | | 19 |
| | | Insensitive | 13 | | 72 |

**Table 9**. Isolate distribution based on relative growth (RG) rates on clarified V8 juice agar amended with mefenoxam and fosetyl-Al

(A)




(B)

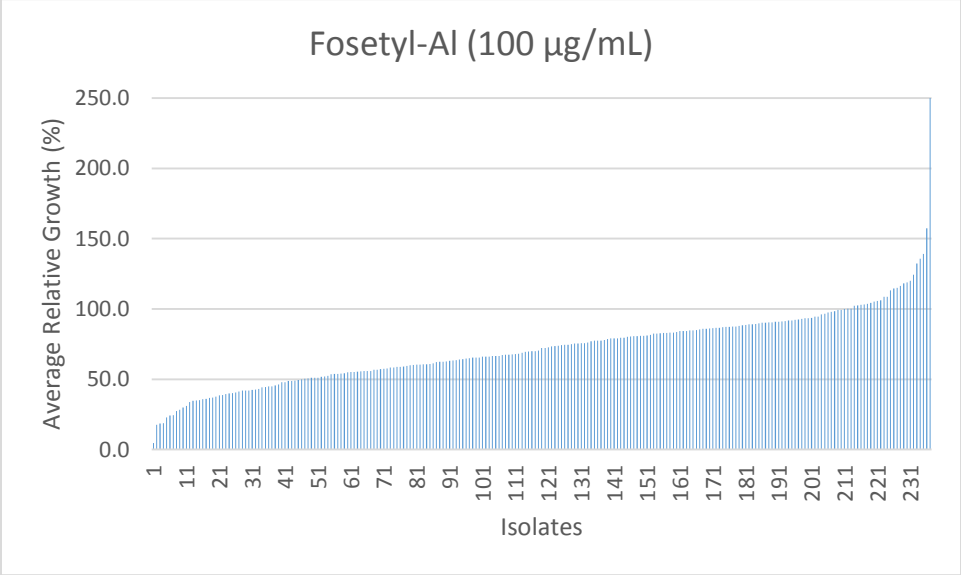



**Figure 8**. Average Relative Growth (% growth compared to controls) of *Phytophthora* spp. isolates grown on **(A)** mefenoxam (100 µg/mL) and **(B)** fosetyl-Al (100 µg/mL).

| *Phytophthora* spp. | n | Sensitivity | Mef (200 µg/mL) | Fos-Al (500 µg/mL) | Flu (200 µg/mL) | Dim (400 µg/mL) | Dim + Ame (100 µg/mL) |
|---|---|---|---|---|---|---|---|
| *P. cinnamomi* | 3 | Sensitive | | | | 3 | 3 |
| | | Intermediate | | | | | |
| | | Insensitive | 3 | 3 | 3 | | |
| *P. multivora* | 2 | Sensitive | | | | 2 | 2 |
| | | Intermediate | | | | | |
| | | Insensitive | 2 | 2 | 2 | | |
| *P. plurivora* | 13 | Sensitive | | | | 11 | 11 |
| | | Intermediate | | | | | |
| | | Insensitive | 13 | 13 | 13 | 2 | 2 |

**Table 10**. Number and mean relative growth (RG) rates of isolates deemed insensitive (see Table 1). These isolates were screened against higher concentrations of mefenoxam (Mef) and fosetyl-Al (Fos-Al) along with three additional fungicides. Flu= Fluoxastrobin; Dim= Dimethomorph, Ame= Ametoctradin

The third objective of this research was to compare the genotyping results with the fungicide study results. To accomplish this, isolates screened in the fungicide study were grouped based on genetic clusters created using AFLP genotyping (Chapter 2) (Table 11). The three insensitive *P. cinnamomi* isolates grouped within the two smallest clusters (genotypic characterization was based on 102 isolates), in particular genotypic cluster two (Table 11). The isolates in cluster two also had a significantly greater average growth rate compared to all other isolates grouped within the three other clusters. Similarly, most of the insensitive *P. plurivora* isolates (8 out of 13) grouped within the least common genotypic cluster (genotypic characterization was based on 186 isolates) (Table 11). These isolates also had a significantly greater growth rate compared to all isolates grouped within the five other clusters.

| *Phytophthora* spp. | [1]Genetic cluster | n | # of insensitive isolates | Relative growth (%) | Tukey HSD |
|---|---|---|---|---|---|
| *P. cinnamomi* | 1 | 16 | 1 | 7.2 ± 16 | B |
| | 2 | 10 | 2 | 23.5 ± 30 | A |
| | 3 | 21 | 0 | 4.5 ± 4 | B |
| | 4 | 30 | 0 | 6.9 ± 7 | B |
| *P. citrophthora* | 1 | 11 | 0 | 10.2 ± 6 | A |
| | 2 | 9 | 0 | 0.5 ± 1 | B |
| | 3 | 3 | 0 | 4.2 ± 7 | AB |
| *P. multivora* | 1 | 9 | 1 | 14.2 ± 21 | A |
| | 2 | 5 | 0 | 9.6 ± 12 | A |
| | 3 | 2 | 0 | 14.4 ± 17 | A |
| | 4 | 2 | 1 | 49.9 ± 68 | A |
| *P. pini* | 1 | 2 | 0 | 10.1 ± 2 | A |
| | 2 | 5 | 0 | 21.6 ± 7 | A |
| | 3 | 10 | 0 | 19.8 ± 14 | A |
| | 4 | 1 | 0 | 3.9 | A |
| *P. plurivora* | 1 | 19 | 3 | 30.7 ± 23 | B |
| | 2 | 9 | 5 | 55.4 ± 26 | A |
| | 3 | 11 | 0 | 3.4 ± 2 | C |
| | 4 | 25 | 4 | 20 ± 26 | BC |
| | 5 | 17 | 1 | 20 ± 13 | BC |
| | 6 | 26 | 0 | 9.9 ± 6 | C |

[1] Chapter 2

**Table 11.** Mean relative growth and Std Dev of isolates when grown in Mefenoxam (100 μg/mL) amended clarified V8 juice based growth media broken down by genetic clusters.

## *Discussion*

This is the first attempt to characterize the fungicide sensitivity of *Phytophthora* species in Maryland ornamental nurseries. The fungicide sensitivity screening of the five most common *Phytophthora* species revealed a wide variety of

sensitivity both within and between the species. A large proportion of isolates were intermediately insensitive and, in at least three species, mefenoxam-insensitive isolates were identified. Isolates that were insensitive to the lower concentration of mefenoxam (100 μg/mL) remained insensitive to the higher mefenoxam concentration, although this was tenfold greater than the recommended label rate concentration.

Sensitivity to mefenoxam is variable within *Phytophthora* species. In Tennessee, *P. hydropathica* isolates were able to grow up to as much as 20% of that on controls on 100 μg/mL while all *P. citrophthora* and *P. citricola* isolates were insensitive (45). Similarly, in Virginia, 26% of *P. nicotianae* isolates were highly resistant to mefenoxam concentrations of 100 μg/mL (44). Variation in sensitivity was also found in California *P. citricola* and *P. cactorum* populations: All 132 *P. cactorum* isolates were sensitive to mefenoxam at 1ppm, whereas all but one of 86 *P. citricola* isolates were tolerant at the same concentration. A subset of these isolates was tolerant even up to 100 ppm (5, 6). Similarly, a range of mefenoxam sensitivity was detected in agricultural ecosystems in New York *P. capsici* populations (24).

Several of the species used in this study were previously classified as *P. citricola.* This is the first study to differentiate their sensitivity to fungicides and suggest that there is considerable variation within these species, which is supported by previous findings from studies conducted with *P. citricola* isolates in California horticultural operations (5) and Tennessee nurseries (22, 45). In California, one of 86 isolates was sensitive to mefenoxam at 1 ppm and only a subset of the isolates was sensitive at 100 ppm. In Tennessee, 10% of a collection of 20 *P. citricola* isolates

from symptomatic leaves on woody ornamentals were resistant to mefenoxam at a concentration of 100 ppm (22). Several isolates collected from symptomatic leaves in nurseries and watersheds in Tennessee were insensitive to the same concentration of mefenoxam while others were not (45).

We observed less variability in sensitivity to fosetyl-Al. Although the concentrations used in our experiments are lower than the label rate, sensitivity to lower concentrations has been reported in previous studies (12, 39, 53). While the majority of isolates we tested were insensitive to the lower concentration, the 18 isolates tested at the higher concentration were still insensitive. Some isolates had even greater RG rates compared to that on non-amended controls. While it has been shown that, at low concentrations, fosetyl-Al activity can be reduced by factors such as pH, temperature, and fungal growth stimulated by nutrient-rich media (39), it remains unclear why growth would be higher on fungicide amended plates than on controls. Further research with higher concentrations of fosetyl-Al are needed to determine what concentration can effectively manage the Phytophthora population in Maryland ornamental nurseries.

The concentration of fluoxastrobin used in this study (200 μg/mL) is 40 times the label rate and all 18 isolates that were insensitive to the low concentration of mefenoxam were insensitive to this concentration of fluoxastrobin. Unlike mefenoxam and fosetyl-Al, which were both introduced in 1977, fluoxastrobin has only been available for use in the US since 2005 (32). However, insensitive isolates tested for fluoxastrobin sensitivity acted similarly to the two former fungicides.

Evaluations should be conducted to reveal whether or not this chemical could be used at higher concentrations to effectively manage these pathogens.

Dimethomorph and ametoctradin were more effective in stemming the growth of insensitive isolates. All but two of the 18 isolates screened were completely sensitive. Dimethomorph has been in use since 1988 (British Columbia Ministry of Agriculture Food and Fisheries), but has been applied in US nurseries by an order of magnitude less than mefenoxam and fosetyl-al (USDA NASS; (9, 70)). Because ametoctradin was discovered in 2004 (36), usage data is not yet available. This is the first report of insensitivity to dimethomorph in the Maryland ornamental nursery population. This finding underscores the need of IPM strategies to enable the long-term use of newly developed fungicides.

There was no significant difference in sensitivity to mefenoxam between nursery and forest isolates. We previously hypothesized that forest isolates, having little to no exposure to fungicides, would be more sensitive to fungicides than isolates collected from nurseries. A similar observation was made in Australia with *P. cinnamomi* in native ecosystems versus managed environments (20). Isolates collected from sites previously treated with phosphite (the active ingredient in fosetyl-Al) were more effective colonizers than isolates collected from sites with no history of phosphite treatment. Our findings suggest that the natural population in mid-Atlantic States already includes insensitive isolates and that the evolution of fungicide sensitivity is not necessarily an outcome of exposure to fungicides over time. Gisi and Cohen (1996) suggested that resistance to phenylamide fungicides originated from naturally occurring insensitive isolates existing at a low proportion in

the population before being exposed to the fungicides (35). This view is further supported by earlier studies where tolerance to *P. citricola* and *P. cinnamomi* was recorded at concentrations as low as 0.25 μg/mL from isolates collected over many years all over the world as early as 1927 (13).

Insensitive isolates accounted for 4 to 13% of the Maryland ornamental nursery Phytophthora population, depending on the species. While this number may appear small, these populations can increase rapidly due to the biology of the organism. For example, Hu et al. (44) found that not only were mefenoxam-resistant isolates able to outcompete sensitive isolates within three to six sporulation cycles, but also they exhibited a greater infection rate and higher sporulation ability. Another study showed that, even after two years without the application of mefenoxam, isolates remained insensitive to the chemical, indicating that insensitivity does not have significant fitness costs in the absence of the chemical (59, 97). Based on these studies, it should not be taken lightly that only a fraction (7%) of isolates were insensitive to mefenoxam, fosetyl-Al, and fluoxastrobin, as it is plausible that this number could increase within a short period of time.

Positive associations between mefenoxam insensitivity and asymptomatic hosts were found in three of the species (*P cinnamomi, P. citrophthora,* and *P. plurivora*). This is surprising because insensitive isolates would be expected to survive mefenoxam applications and be more frequently associated with symptomatic, diseased plants. The association highlights a challenge for nursery managers as it signifies that the mefenoxam-insensitive isolates present in Maryland ornamental nurseries could be spreading throughout nurseries undetected on

asymptomatic hosts, particularly if they are indeed capable of outcompeting sensitive isolates.

Our results demonstrate that discrepancies in fungicide sensitivity exist among and between species, suggesting that a genus-level fungicide application plan is insufficient for the management of Phytophthora in ornamental nursery production. Mefenoxam- and fluoxastrobin-insensitive isolates are present within the population, thus the inclusion of chemicals such as dimethomorph and ametoctradin into management plans is recommended. Although the inclusion of these chemicals into a diverse application program could help prevent fungicide resistance, use of them alone could also lead to resistance, particularly because two *P. plurivora* isolates were insensitive to these chemicals. As movement of these pathogens is continuous (7), regular characterization of Phytophthora populations and fungicide sensitivity is necessary for nursery managers to create effective, sustainable management plans.

# Chapter 4: General discussion, limitations, and future directions

*General Discussion*

Diversity amongst *P. citricola* species has been well-documented, as has the lack of diversity in *P. cinnamomi.* The existence of clonal populations within *P. cinnamomi* and *P. plurivora* from both nurseries and forests suggests that there is a pathway of introduction between the two populations. Our finding that only a small significant difference in genetic diversity exists between forest and nursery populations of *P. cinnamomi* could indicate one or a combination of three scenarios: 1) Serve as an example of the general low genetic diversity within the species, 2) Illustrate the ability of the species to adapt survival strategies in spite of the fitness costs, or 3) Indicate the impracticality of sexual reproduction in established pathogen populations in forests. The second scenario could also explain why we did not observe significant differences in sensitivity to mefenoxam between isolates collected from nurseries and from forests.

One of the most interesting findings of this study was the higher RG rates in isolates collected from asymptomatic hosts. This information is useful from a management standpoint, as it could have serious implications for fungicide applications. While some nursery managers apply fungicides only when they notice disease symptoms, others apply it routinely to prevent disease development. Oftentimes these pathogens can survive on hosts without causing symptoms. The presence of isolates insensitive to fungicides within potting media (e.g. isolates recovered from asymptomatic hosts) warrants a well-planned pest management approach that would stem or even eliminate resistance buildup in these isolates.

Insensitive isolates were associated with the least common genotypes. Future studies will determine if these genotypes are indeed able to outcompete sensitive isolates and become more common. Such findings could provide data necessary for fine-tuning fungicide management strategies. While such data is more available in agricultural systems, it is lacking considerably for ornamental nursery operations. Presently, the most common genotypes appear to be intermediately sensitive to ten times the label rate of mefenoxam. Therefore, mefenoxam applications should be alternated with dimethomorph and ametoctradin to delay further resistance buildup.

Many fungicides suppress disease symptoms, but do not actually cure a plant of disease i.e., kill the pathogen. This "fungicide masking" could account for the association we observed between asymptomatic hosts and insensitive Phytophthora isolates and is important to bear in mind when considering how best to prevent the spread of Phytophthora into other ecosystems. It is probable that the spread of Phytophthora through plant trade occurs in part due to fungicide masking. Seemingly non-infected plants are sold to other nurseries or to the public, only to serve as an avenue for the movement of isolates.

*Limitations and future directions*

These experiments underscore the need for large sample sizes across variables for making correct population descriptions. In spite of conducting extensive surveys of Maryland ornamental nurseries, sample sizes of three of the species examined (*P. multivora, P. pini,* and *P. citrophthora*) were not always represented across all of the isolate origin variables, limiting some of our analyses.

While AFLP is a useful tool for genotyping large populations, newer methods such as Single Nucleotide Polymorphism (SNP) analysis and nextgen sequencing are perhaps preferable. Using AFLP, one can see a clear picture of a population by using several combinations of selective primers in the selective amplification step. In our study, the use of only one primer combination could have limited the completeness of the picture we were able to draw. Further, we found the process to be labor intensive and, due to the sensitivity of the reagents, quite expensive. While AFLP is a useful tool, these newer methods are both faster and less expensive. They are also increasingly available for researchers and could provide an equally clear, if not clearer, picture of what these populations look like.

There is a need for more in-depth and broader fungicide studies in order to determine which chemicals are still effective in managing Phytophthora in ornamental nurseries. Overcoming two particular limitations of our studies, a higher concentration of fosetyl-Al and a broader range of fungicide concentrations should be tested in order to determine effective concentrations (EC50 or EC90 values). Although comparing the sensitivity of our isolates to that in other studies was not a primary objective of the current work, the ability to compare EC values between studies in the future would be useful for putting our results in a broader context and could reveal how fungicide sensitivity may be shifting over time. The sensitivity of *P. plurivora* and *P. cinnamomi* isolates, in particular, should be closely monitored as because these species are most likely still dominating the nursery environment. These longitudinal examinations can help researchers and nursery managers not only stay on top of shifting trends, but also examine patterns and make proactive decisions about

changes in their management practices. It is also important to note that *in vitro* fungicide sensitivity trials do not always translate to what we would see in field trials. Conducting these trials in a greenhouse and (or) nursery would provide more accurate and ecologically valid sensitivity data.

Beyond modifications in research designs for future studies, there is work outside the laboratory that can also greatly aid in the control of these pathogens in ornamental nurseries. Particularly, nursery managers must consider several disease management strategies as fungicides alone cannot effectively manage Phytophthora. Some cultural practices that are effective include good nursery hygiene, proper spacing, temperature control, and soil water management. One specific consideration is controlling and monitoring soil volume water content to determine whether or not fungicide use could be reduced with more efficient water utilization. By decreasing the amount of soil moisture we can likely suppress disease development and reduce the need for fungicide use, which could, in turn, slow resistance buildup.

The ultimate goal of this work was to provide nursery managers with information to create a fungicide application plan tailored to what is actually happening in Maryland nurseries. Prior to this work, characterization of Phytophthora in ornamental nursery populations had not been conducted in Maryland. Our studies provide a foundation for future genotyping work that would reflect shifts in mefenoxam sensitivity, which is particularly important as this chemical is still being utilized in these nurseries. Because movement of these pathogens is continuous, regular characterization of genetic diversity and fungicide sensitivity will continue to

be important for monitoring these shifts and for the creation of effective management strategies.

| Isolate | *Phytophthora spp.* | Origin | Host Species | Environment | Symptoms | State |
|---|---|---|---|---|---|---|
| **F1** | *P. multivora* | Forest | *Quercus alba* | Soil | S | Illinois |
| **F2** | *P. cinnamomi* | Forest | *Q. velutina* | Soil | AS | Indiana |
| **F4** | *P. pini* | Forest | *Q. coccinea* | Soil | AS | Indiana |
| **F5** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Indiana |
| **F6** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Indiana |
| **F7** | *P. cinnamomi* | Forest | *Q. velutina* | Soil | S | Indiana |
| **F8** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Indiana |
| **F9** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Kentucky |
| **F10** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Kentucky |
| **F11** | *P. cinnamomi* | Forest | *Q. rubra* | Soil | AS | Maryland |
| **F12** | *P. cinnamomi* | Forest | *Q. rubra* | Soil | S | Maryland |
| **F13** | *P. cinnamomi* | Forest | *Q. velutina* | Soil | AS | Maryland |
| **F14** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Maryland |
| **F15** | *P. cinnamomi* | Forest | *Q. rubra* | Soil | AS | Maryland |
| **F16** | *P. cinnamomi* | Forest | *Q. montana* | Soil | S | Maryland |
| **F17** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Maryland |
| **F18** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Maryland |
| **F19** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Maryland |
| **F20** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Maryland |
| **F21** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Maryland |
| **F22** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Maryland |
| **F23** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Maryland |
| **F24** | *P. cinnamomi* | Forest | *Fagus americana* | Soil | AS | Maryland |
| **F25** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Maryland |
| **F26** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Maryland |
| **F27** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Maryland |

| | | | | | | |
|---|---|---|---|---|---|---|
| **F28** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Maryland |
| **F29** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Maryland |
| **F30** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Maryland |
| **F31** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Maryland |
| **F32** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Maryland |
| **F33** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Maryland |
| **F34** | *P. plurivora* | Forest | *Q. alba* | Soil | AS | New York |
| **F35** | *P. cinnamomi* | Forest | *Q. rubra* | Soil | S | Ohio |
| **F36** | *P. cinnamomi* | Forest | *Q. montana* | Soil | S | Ohio |
| **F37** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Ohio |
| **F38** | *P. plurivora* | Forest | *Q. rubra* | Soil | S | Ohio |
| **F39** | *P. cinnamomi* | Forest | *Q. rubra* | Soil | S | Ohio |
| **F40** | *P. pini* | Forest | *Q. rubra* | Soil | AS | Ohio |
| **F41** | *P. cinnamomi* | Forest | *Q. palustris* | Soil | AS | Ohio |
| **F42** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Ohio |
| **F43** | *P. pini* | Forest | *Q. alba* | Soil | AS | Ohio |
| **F44** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Ohio |
| **F45** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Ohio |
| **F47** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Ohio |
| **F48** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Ohio |
| **F49** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Ohio |
| **F50** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Ohio |
| **F51** | *P. plurivora* | Forest | *Q. alba* | Soil | AS | Ohio |
| **F52** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Ohio |
| **F53** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Ohio |
| **F54** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Ohio |
| **F55** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | Ohio |

| | | | | | | |
|---|---|---|---|---|---|---|
| **F56** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | Pennsylvania |
| **F57** | *P. pini* | Forest | *Q. alba* | Soil | AS | Pennsylvania |
| **F58** | *P. pini* | Forest | *Q. alba* | Soil | AS | Pennsylvania |
| **F59** | *P. cinnamomi* | Forest | *Q. montana* | Soil | AS | West Virginia |
| **F60** | *P. cinnamomi* | Forest | *Q. rubra* | Soil | S | West Virginia |
| **F61** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | West Virginia |
| **F62** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | West Virginia |
| **F63** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | West Virginia |
| **F64** | *P. cinnamomi* | Forest | *Q. rubra* | Soil | S | West Virginia |
| **F65** | *P. plurivora* | Forest | *Q. coccinea* | Soil | S | West Virginia |
| **F66** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | West Virginia |
| **F67** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | West Virginia |
| **F69** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | West Virginia |
| **F70** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | West Virginia |
| **F71** | *P. cinnamomi* | Forest | *Q. rubra* | Soil | AS | West Virginia |
| **F72** | *P. cinnamomi* | Forest | *Acer saccarum* | Soil | AS | West Virginia |
| **F73** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | West Virginia |
| **F74** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | West Virginia |
| **F75** | *P. cinnamomi* | Forest | *F. americana* | Soil | AS | West Virginia |
| **F76** | *P. cinnamomi* | Forest | *A. saccarum* | Soil | AS | West Virginia |
| **F77** | *P. cinnamomi* | Forest | *Q. alba* | Soil | S | West Virginia |
| **F78** | *P. cinnamomi* | Forest | *A. rubrum* | Soil | AS | West Virginia |
| **F79** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | West Virginia |
| **F80** | *P. cinnamomi* | Forest | *Q. montana* | Soil | S | West Virginia |
| **F81** | *P. cinnamomi* | Forest | *Q. rubra* | Soil | AS | West Virginia |
| **F82** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | West Virginia |
| **F83** | *P. cinnamomi* | Forest | *Rhododendron* | Soil | S | West Virginia |

| | | | | | | |
|---|---|---|---|---|---|---|
| **F84** | *P. cinnamomi* | Forest | *Q. montana* | Soil | AS | West Virginia |
| **F85** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | West Virginia |
| **F86** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | West Virginia |
| **F87** | *P. cinnamomi* | Forest | *Q. alba* | Soil | AS | West Virginia |
| **F88** | *P. cinnamomi* | Forest | *Q. montana* | Soil | S | West Virginia |
| **F90** | *P. cinnamomi* | Forest | *Q. montana* | Soil | AS | West Virginia |
| **N17** | *P. plurivora* | Nursery 2 | *Water* | Water | AS | Maryland |
| **N19** | *P. multivora* | Nursery 2 | *Water* | Water | AS | Maryland |
| **N62** | *P. citrophthora* | Nursery 1 | *Rhododendron 'Percy Wiseman'* | Soil | S | Maryland |
| **N63** | *P. plurivora* | Nursery 1 | *Rhododendron 'Sweet 16'* | Soil | S | Maryland |
| **N64** | *P. multivora* | Nursery 1 | *Rhododendron 'Sweet 16'* | Soil | S | Maryland |
| **N65** | *P. cinnamomi* | Nursery 1 | *Rhododendron 'Herbert'* | Soil | S | Maryland |
| **N70** | *P. citrophthora* | Nursery 1 | *Buxus sempervirens 'Duf. English'* | Stem | S | Maryland |
| **N90** | *P. multivora* | Nursery 1 | *Rhododendron 'Sweet 16'* | Soil | S | Maryland |
| **N94** | *P. multivora* | Nursery 1 | *Rhododendron 'Sweet 16'* | Soil | S | Maryland |
| **N113** | *P. plurivora* | Nursery 2 | *Pieris japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N120** | *P. cinnamomi* | Nursery 2 | *P. japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N123** | *P. pini* | Nursery 2 | *P. japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N124** | *P. citrophthora* | Nursery 2 | *P. japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N125** | *P. citrophthora* | Nursery 2 | *P. japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N126** | *P. citrophthora* | Nursery 2 | *P. japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N127** | *P. citrophthora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N128** | *P. citrophthora* | Nursery 2 | *P. japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N129** | *P. citrophthora* | Nursery 2 | *P. japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N130** | *P. citrophthora* | Nursery 2 | *P. japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N131** | *P. citrophthora* | Nursery 2 | *P. japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N133** | *P. plurivora* | Nursery 2 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |

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| **N134** | *P. pini* | Nursery 2 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N135** | *P. plurivora* | Nursery 2 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N137** | *P. plurivora* | Nursery 2 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N138** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N139** | *P. plurivora* | Nursery 2 | *Ilex crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N140** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N141** | *P. plurivora* | Nursery 2 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N142** | *P. plurivora* | Nursery 2 | *P. japonica 'Browers Beauty'* | Stem | S | Maryland |
| **N144** | *P. plurivora* | Nursery 2 | *P. japonica 'Browers Beauty'* | Stem | S | Maryland |
| **N145** | *P. plurivora* | Nursery 2 | *P. japonica 'Browers Beauty'* | Stem | S | Maryland |
| **N146** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N147** | *P. plurivora* | Nursery 2 | *P. japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N148** | *P. plurivora* | Nursery 2 | *P. japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N149** | *P. plurivora* | Nursery 2 | *P. japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N150** | *P. plurivora* | Nursery 2 | *P. japonica 'Scarlett O'Hara'* | Soil | AS | Maryland |
| **N151** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N152** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N153** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N154** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N156** | *P. plurivora* | Nursery 2 | *Potting Soil* | Soil | AS | Maryland |
| **N158** | *P. plurivora* | Nursery 2 | *Potting Soil* | Soil | AS | Maryland |
| **N162** | *P. plurivora* | Nursery 2 | *Potting Soil* | Soil | AS | Maryland |
| **N169** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | AS | Maryland |
| **N170** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | S | Maryland |
| **N172** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | AS | Maryland |
| **N174** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N175** | *P. plurivora* | Nursery 2 | *Rhododendron catawbiense 'Chionoides'* | Stem | S | Maryland |

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| **N176** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N177** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | AS | Maryland |
| **N178** | *P. plurivora* | Nursery 2 | *Rhododendron catawbiense 'Chionoides'* | Stem | S | Maryland |
| **N179** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N180** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N181** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N182** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N183** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | AS | Maryland |
| **N184** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | S | Maryland |
| **N185** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | S | Maryland |
| **N186** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | AS | Maryland |
| **N187** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | AS | Maryland |
| **N188** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | AS | Maryland |
| **N189** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | AS | Maryland |
| **N189** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | AS | Maryland |
| **N189** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | AS | Maryland |
| **N190** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | AS | Maryland |
| **N192** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | AS | Maryland |
| **N193** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | AS | Maryland |
| **N194** | *P. multivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N195** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N196** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N197** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N198** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N199** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N200** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N201** | *P. plurivora* | Nursery 2 | *Rhododendron catawbiense 'Chionoides'* | Stem | S | Maryland |

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| **N202** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N205** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N208** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N209** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N210** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N211** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N212** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N213** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N214** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N215** | *P. plurivora* | Nursery 2 | *Rhododendron catawbiense 'Chionoides'* | Stem | S | Maryland |
| **N216** | *P. plurivora* | Nursery 2 | *Rhododendron catawbiense 'Chionoides'* | Stem | S | Maryland |
| **N217** | *P. plurivora* | Nursery 2 | *Rhododendron catawbiense 'Chionoides'* | Stem | S | Maryland |
| **N219** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N220** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N221** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N222** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N223** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N224** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N226** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N227** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N232** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N234** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Stem | S | Maryland |
| **N235** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N236** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N237** | *P. plurivora* | Nursery 2 | *Rhododendron catawbiense 'Chionoides'* | Stem | S | Maryland |
| **N238** | *P. citrophthora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |
| **N239** | *P. plurivora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Stem | S | Maryland |

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| **N240** | *P. plurivora* | Nursery 2 | *Rhododendron catawbiense 'Chionoides'* | Stem | S | Maryland |
| **N241** | *P. plurivora* | Nursery 2 | *Rhododendron catawbiense 'Chionoides'* | Stem | S | Maryland |
| **N242** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Soil | AS | Maryland |
| **N244** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Soil | S | Maryland |
| **N245** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Soil | AS | Maryland |
| **N246** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Soil | AS | Maryland |
| **N247** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Soil | S | Maryland |
| **N248** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Soil | AS | Maryland |
| **N249** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Soil | S | Maryland |
| **N250** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Soil | S | Maryland |
| **N252** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Soil | AS | Maryland |
| **N255** | *P. plurivora* | Nursery 2 | *Rhododendron 'Yaku Princess'* | Soil | S | Maryland |
| **N256** | *P. plurivora* | Nursery 2 | *Potting Soil* | Soil | AS | Maryland |
| **N263** | *P. plurivora* | Nursery 2 | *Potting Soil* | Soil | AS | Maryland |
| **N264** | *P. plurivora* | Nursery 2 | *Potting Soil* | Soil | AS | Maryland |
| **N272** | *P. plurivora* | Nursery 2 | *Potting Soil* | Soil | AS | Maryland |
| **N275** | *P. plurivora* | Nursery 2 | *Potting Soil* | Soil | AS | Maryland |
| **N276** | *P. plurivora* | Nursery 2 | *Potting Soil* | Soil | AS | Maryland |
| **N281** | *P. citrophthora* | Nursery 2 | *Potting Soil* | Soil | AS | Maryland |
| **N282** | *P. plurivora* | Nursery 2 | *Potting Soil* | Soil | AS | Maryland |
| **N284** | *P. plurivora* | Nursery 4 | *A. rubrum* | Root | AS | Maryland |
| **N285** | *P. plurivora* | Nursery 4 | *Sophora japonica* | Stem | S | Maryland |
| **N286** | *P. plurivora* | Nursery 4 | *A. rubrum* | Root | AS | Maryland |
| **N288** | *P. plurivora* | Nursery 4 | *A. rubrum* | Root | AS | Maryland |
| **N289** | *P. plurivora* | Nursery 4 | *S. japonica* | Stem | S | Maryland |
| **N290** | *P. plurivora* | Nursery 4 | *A. rubrum* | Root | AS | Maryland |
| **N291** | *P. plurivora* | Nursery 4 | *A. rubrum* | Root | AS | Maryland |

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| **N292** | *P. plurivora* | Nursery 4 | *Oxydendron arboreum* | Stem | S | Maryland |
| **N293** | *P. plurivora* | Nursery 4 | *A. rubrum* | Root | AS | Maryland |
| **N294** | *P. plurivora* | Nursery 4 | *A. rubrum* | Root | AS | Maryland |
| **N295** | *P. plurivora* | Nursery 4 | *A. rubrum* | Root | AS | Maryland |
| **N296** | *P. plurivora* | Nursery 4 | *A. rubrum* | Root | AS | Maryland |
| **N297** | *P. plurivora* | Nursery 4 | *A. rubrum* | Root | AS | Maryland |
| **N298** | *P. plurivora* | Nursery 4 | *Tilia cordata* | Stem | S | Maryland |
| **N306** | *P. plurivora* | Nursery 7 | *I. meserveae 'Blue Maid'* | Soil | AS | Maryland |
| **N307** | *P. plurivora* | Nursery 7 | *I. meserveae 'Blue Maid'* | Soil | S | Maryland |
| **N314** | *P. plurivora* | Nursery 7 | *I. meserveae 'Blue Maid'* | Soil | AS | Maryland |
| **N317** | *P. plurivora* | Nursery 7 | *I. meserveae 'Blue Maid'* | Soil | S | Maryland |
| **N318** | *P. plurivora* | Nursery 7 | *I. meserveae 'Blue Maid'* | Soil | S | Maryland |
| **N321** | *P. plurivora* | Nursery 7 | *I. meserveae 'Blue Maid'* | Soil | S | Maryland |
| **N323** | *P. plurivora* | Nursery 7 | *I. meserveae 'Blue Maid'* | Soil | S | Maryland |
| **N324** | *P. plurivora* | Nursery 7 | *I. meserveae 'Blue Maid'* | Soil | AS | Maryland |
| **N325** | *P. plurivora* | Nursery 7 | *I. meserveae 'Blue Maid'* | Soil | S | Maryland |
| **N329** | *P. plurivora* | Nursery 7 | *I. meserveae 'Blue Maid'* | Soil | S | Maryland |
| **N330** | *P. plurivora* | Nursery 7 | *I. meserveae 'Blue Maid'* | Soil | S | Maryland |
| **N331** | *P. plurivora* | Nursery 7 | *I. meserveae 'Blue Maid'* | Soil | S | Maryland |
| **N338** | *P. plurivora* | Nursery 6 | *P. japonica 'Valley Valentine'* | Soil | AS | Maryland |
| **N339** | *P. plurivora* | Nursery 6 | *Rhododendron 'Eng. Roseum'* | Soil | S | Maryland |
| **N340** | *P. plurivora* | Nursery 6 | *P. japonica 'Valley Valentine'* | Soil | AS | Maryland |
| **N341** | *P. multivora* | Nursery 6 | *P. japonica 'Valley Valentine'* | Soil | AS | Maryland |
| **N345** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N346** | *P. plurivora* | Nursery 6 | *P. japonica 'Valley Valentine'* | Soil | AS | Maryland |
| **N348** | *P. multivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N349** | *P. plurivora* | Nursery 6 | *P. japonica 'Valley Valentine'* | Soil | AS | Maryland |

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| **N352** | *P. plurivora* | Nursery 6 | *Rhododendron 'Eng. Roseum'* | Soil | S | Maryland |
| **N353** | *P. plurivora* | Nursery 6 | *P. japonica 'Valley Valentine'* | Soil | AS | Maryland |
| **N354** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | S | Maryland |
| **N355** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | S | Maryland |
| **N357** | *P. plurivora* | Nursery 6 | *Rhododendron* | Stem | S | Maryland |
| **N358** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | S | Maryland |
| **N360** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | S | Maryland |
| **N361** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | S | Maryland |
| **N362** | *P. plurivora* | Nursery 6 | *Rhododendron* | Stem | S | Maryland |
| **N363** | *P. plurivora* | Nursery 6 | *Rhododendron* | Stem | S | Maryland |
| **N364** | *P. plurivora* | Nursery 6 | *Rhododendron* | Stem | S | Maryland |
| **N365** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | S | Maryland |
| **N368** | *P. plurivora* | Nursery 6 | *Rhododendron* | Stem | S | Maryland |
| **N369** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | S | Maryland |
| **N372** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N373** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N376** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N377** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | AS | Maryland |
| **N378** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | AS | Maryland |
| **N380** | *P. plurivora* | Nursery 6 | *B. sempervirens 'Duf. English'* | Soil | S | Maryland |
| **N381** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N383** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N384** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N386** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N387** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N388** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N389** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |

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| **N390** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | AS | Maryland |
| **N391** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N392** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N393** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | AS | Maryland |
| **N394** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N395** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N396** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N398** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N404** | *P. multivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | AS | Maryland |
| **N407** | *P. multivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | AS | Maryland |
| **N409** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N410** | *P. multivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N411** | *P. citrophthora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N413** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | AS | Maryland |
| **N416** | *P. citrophthora* | Nursery 6 | *Rhododendron* | Stem | S | Maryland |
| **N417** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Stem | AS | Maryland |
| **N418** | *P. pini* | Nursery 6 | *Rhododendron* | Stem | S | Maryland |
| **N419** | *P. plurivora* | Nursery 6 | *B. sempervirens 'Duf. English'* | Soil | S | Maryland |
| **N420** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N424** | *P. pini* | Nursery 6 | *B. sempervirens 'Duf. English'* | Soil | S | Maryland |
| **N425** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N426** | *P. multivora* | Nursery 6 | *Rhododendron* | Stem | S | Maryland |
| **N431** | *P. cinnamomi* | Nursery 6 | *Rhododendron 'Azaleas'* | Soil | S | Maryland |
| **N432** | *P. plurivora* | Nursery 6 | *Rhododendron* | Stem | S | Maryland |
| **N433** | *P. pini* | Nursery 6 | *Rhododendron* | Stem | S | Maryland |
| **N441** | *P. cinnamomi* | Nursery 6 | *Rhododendron 'Azaleas'* | Soil | S | Maryland |
| **N442** | *P. cinnamomi* | Nursery 6 | *Rhododendron 'Azaleas'* | Soil | S | Maryland |

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| **N443** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N444** | *P. citrophthora* | Nursery 2 | *I. crenata 'Centennial Girl'* | Soil | S | Maryland |
| **N445** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N446** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N447** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N448** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N449** | *P. citrophthora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N453** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N454** | *P. citrophthora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N458** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N459** | *P. citrophthora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N460** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N461** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N463** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N464** | *P. citrophthora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N465** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N466** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N467** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N468** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N469** | *P. citrophthora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N470** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N471** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N472** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N473** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N474** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |
| **N476** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N478** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |

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| **N490** | *P. pini* | Nursery 6 | *B. sempervirens 'Duf. English'* | Soil | S | Maryland |
| **N491** | *P. plurivora* | Nursery 6 | *B. sempervirens 'Duf. English'* | Soil | S | Maryland |
| **N493** | *P. citrophthora* | Nursery 1 | *Rhododendron 'Percy Wiseman'* | Soil | S | Maryland |
| **N494** | *P. cinnamomi* | Nursery 7 | *I. meserveae 'Blue Maid'* | Soil | S | Maryland |
| **N495** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N497** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N500** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N502** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N503** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N505** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N506** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N507** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N509** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N510** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N514** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N516** | *P. multivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N517** | *P. multivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N518** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N519** | *P. cinnamomi* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N521** | *P. citrophthora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N524** | *P. citrophthora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N525** | *P. citrophthora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N526** | *P. multivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N540** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N541** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N542** | *P. multivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N548** | *P. pini* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | AS | Maryland |

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| **N551** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |
| **N553** | *P. plurivora* | Nursery 6 | *P. japonica 'Mountain Fire'* | Soil | S | Maryland |

**Supplemental Table 1.** Isolate sources and origins.

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