OCCURRENCE OF PHYTOPHTHORA ON REFORESTED LOOSE-GRADED SPOILS IN EASTERN KENTUCKY[1]

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Abstract. Plant pathogens of the genus *Phytophthora* may pose a threat to trees on reclaimed mine lands. The presence of these pathogens in forest soils of Appalachia has been documented, but their presence and relative distribution in mine spoils is unknown. Soil samples from mine spoils at the Bent Mountain, KY reforestation site were tested for presence of *Phytophthora* spp., and, specifically, *P. cinnamomi*, using a soil-baiting method. Loose-graded mine spoils (brown sandstone, gray sandstone, mixed spoil, and shale) dumped in 2005 and 2007 at the Bent Mountain reforestation site were tested monthly from May to October 2007 for *Phytophthora* at the surface (top 0-10 cm) using a soil baiting method. Soils collected from two non-mined control sites within Robinson Forest and Berea Forest were also tested using the same method. Colonies obtained from the baiting method were isolated in PARP-H V8 *Phytophthora*- selective medium and incubated at 25°C. Colonies with *Phytophthora*-characteristic morphology were tested by PCR. PCR results, confirmed by DNA sequencing, indicate that *P. cinnamomi* was successfully isolated using the baiting method from forest soils. No *Phytophthora* spp. were detected by the baiting method from mine spoils. Future work will involve testing water that has infiltrated through mine spoils for presence of *Phytophthora* spp. Water collected from lysimeters will be filtered to capture mycelial fragments or reproductive structures of *Phytophthora species* through exclusion on the filter membrane and the membrane will be subjected to DNA extraction and PCR. The presence of *Phytophthora* spp. in spoils and water in comparison to media physiochemical characteristics, aqueous geochemistry of infiltrated waters, and tree growth with regards to reforestation efforts on different tailing media will be evaluated.

Additional Key Words: PCR, *P. cinnamomi*

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Introduction

Phytophthora species are significant crop and forest pathogens worldwide; pathogens in the genus are well documented throughout the forests of Appalachia, where they cause root rot and dieback of several hardwood species. *P. cinnamomi* is the most prevalent species of *Phytophthora* in eastern oak forests (Balci et al., 2007). Although the presence of *Phytophthora* spp. has been detected in soils and streams in areas of eastern Kentucky, its prevalence in newly reclaimed surface mine spoils is unknown (de Sá and Barton, 2007). The implications of determining the presence of *P. cinnamomi* on surface mine spoils are great as it poses a significant threat to reforestation efforts. In addition, efforts to reestablish American chestnut founder populations on surface mine lands may hinge on the presence/absence of this plant pathogen, which has proven problematic in experimental plantings elsewhere (Brosi 2001, French et al., 2007a).

As *Phytophthora* species have mycelial growth habit and method of nutrient acquisition similar to fungi, they were formerly classified in the kingdom Fungi and are commonly known as "water molds". The most current taxonomy places the genus *Phytophthora* in the kingdom Chromista, phylum Oomycota, order Peronosporales and family Peronosporaceae (Hardham 2005). The genus is closely related to the *Pythium* genus, and agar media selective for *Phytophthora* may also allow for growth of some *Pythium* species.

Phytophthora spp. may be introduced to a site by surface water, wind, and the movement of soils and host species. Site factors that govern the presence and aggressiveness of *Phytophthora* to susceptible species include infiltration rate, elevation and climate. Rhoades et al. (2003) found that compacted soils with high moisture contents were more conducive to root rot caused by *P. cinnamomi*. High soil compaction levels from excessive grading are a common problem on surface mine lands in Appalachia (Burger et al. 2005; Angel et al. 2006, 2007; Sweigard et al. 2007). Compaction not only impedes forest establishment by restricting root growth, infiltration and aeration, but highly graded sites may be ideally suited for *Phytophthora* infection. Loose-graded spoils as described in the Forestry Reclamation Approach (FRA) (Burger et al. 2005, Sweigard et al. 2007a, Sweigard et al. 2007b); however, provide conditions necessary for forest establishment and may be initially devoid of plant pathogens if the spoils are fresh and derived from deep geologic stratum.

At the Bent Mountain reforestation site, a long-term study is underway to determine the suitability of various mine spoils with respect to reforestation efforts. These spoils include weathered brown sandstone, gray sandstone, shale, and mixed mine-run spoil which differ in physiochemical properties and age of emplacement. In an effort to determine the presence of *Phytophthora* spp., in particular, *P. cinnamomi*, on various spoil types reclaimed using FRA, soil and water samples were analyzed to detect their occurrence as influenced by spoil physiochemical properties and age of emplacement.

Materials and Methods

Study area

The study site is located at the Bent Mountain strip mine in Pike County, Kentucky (latitude N 37º 35' 49", longitude W 82 º 24' 19") operated by Appalachian Fuels (Fig. 1). Bent Mountain is in the Cumberland Plateau physiographic region and the Hazard Coal Reserve District as defined by the U.S. Geological Survey (Huddle et al., 1963). At the site, the Lower and Middle Pennsylvanian Breathitt Group, consisting of shale, siltstone, argillaceous and lithic sandstone, and some thin limestone, is mined for coal. The land is classified as mixed mesophytic forest and Appalachian oak forest. The principal soil order is Ultisols (USDA, 1998) and at the study site, the Dekalb soil series is present on upper side slopes and ridges (Hayes, 1982).

Six research plots to evaluate the performance of trees on three loose-graded spoil types were created in 2005 (Fig. 2), consisting of two replicates of 1) brown weathered sandstone, 2) predominantly gray unweathered sandstone and 3) equally mixed brown weathered sandstone, gray unweathered sandstone, and shale material (mine-run spoil). Plots are approximately 63 meters square on each side and cover an approximate area of one acre or around 4,050 square meters. The spoil types were "end dumped" in large, parallel rows 2-3 meters deep. The parallel ridges were "struck-off" with one pass of a small bulldozer (D-5) down the length of the ridge, creating a rough, non-uniform surface with parallel valleys as specified in Reclamation Advisory Memorandum Number 124 issued by the Kentucky Department of Natural Resources (KDSMRE, 1997).

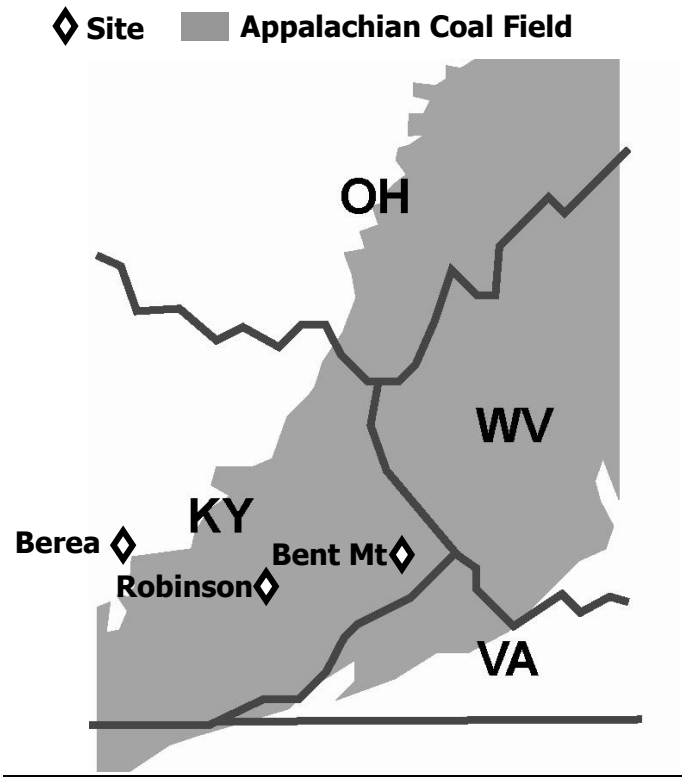

Figure 1. Location of study sites.

Figure 2. Aerial view of the 2005 loose-dumped plots at the Bent Mountain surface mine in Pike County, Kentucky. The size of each plot is approximately 0.4-hectare.

Figure 3. Aerial view of the 2007 loose-dumped plots. The size of each plot is approximately 0.2-hectare.

Each plot is isolated from the others by a 2.5 meter buffer zone and drains into its own sample monitoring station by means of lysimeters and PVC pipes. There are three 4.6 meter square lysimeters in each plot that are drained to the exit points by PVC pipes 2.5 centimeters in diameter.

Four species of trees, white oak (*Quercus alba*), red oak (*Quercus rubra*), yellow poplar (*Liriodendron tulipifera*) and green ash (*Fraxinus pennsylvanica*), were planted on a 1.8 m x 2.4 m spacing onto the six plots in April 2005. In the spring of 2006, American chestnut (*Castanea dentata*) seedlings were planted on each of the six plots on one sub-plot measuring 7 m x 7 m. Each sub-plot consists of approximately 23 container-grown seedlings with 1.5 m x 1.5 m spacing.

In addition to the above sites, 12 additional half-acre (0.2-ha) research plots consisting of end-dumped gray sandstone, brown sandstone, shale, and mixed sandstones and shale (three plots of each) were constructed in March 2007 (Fig. 3). All plots were planted with native hardwood species including: white ash (*Fraxinus americana*), white oak, northern red oak (*Quercus rubra*), chestnut oak (*Quercus prinus*), sugar maple (*Acer saccharum*), American sycamore (*Plantanus occidentalis*), white pine (*Pinus strobus*), black locust (*Robinia pseudoacacia*), dogwood (*Cornus florida*), redbud (*Cercis canadenis*) and a blight resistant hybrid of American Chestnut at a rate of 1,200 trees per hectare. These plots will be used as a chronosequential site for surface sampling of *Phytophthora* spp.

Phytophthora Detection

Detection methods for presence of *Phytophthora* spp. at Bent Mountain were adapted for soil and water samples for the 2005 plots and soils from the 2007 plots. A baiting method for detection of the presence of *Phytophthora* in soil was used.

Soil Baiting. Soil baiting occurred monthly from May-October 2007. Five sub-samples were composited from each site to create approximately 1 kg of sample. Sampling equipment (trowels, hands, tubs, etc.) were sterilized with a 10% bleach solution and thoroughly rinsed with de-ionized water (DI H_2O) between each sampling site, but not between sub-samples. The soil baiting procedure was adapted from previous methods to accommodate a larger, composite soil sample (Erwin and Ribeiro 1996, Tsao 1983). *Rhododendron maximum* leaves were collected the same day as the spoil was sampled and from the same site for each round of sampling. Leaves were cut from the branches, placed in Ziploc® bags and stored in a cooler on ice until

ready for baiting (within one day of collection). Each soil sample was weighed out to 1 kg and placed into a sterilized, thoroughly rinsed (10% bleach; DI H_2O) 2 gal. plastic tub. Enough distilled water was added to the tubs to cover the soil to a depth of about 2 inches. Rhododendron leaves were cut a sterile razor into pieces of approximately 1 cm^2 and 12 pieces were floated on the surface of the water in each tub. The tubs were covered with foil, and left at room temperature for 72 hrs. Leaf pieces were removed from the soil immersion and inspected for symptoms of *Phytophthora* infection, i.e., brownish-black discoloration and water soaking. These sections were excised and placed into PARP-H V8 agar plates and incubated at 20 °C for 1 week. Cultures grown on the PARP-H V8 agar plates were isolated and transferred to new PARP-H V8 agar plates; two transfers were made to ensure pure cultures.

The baiting included a positive control for *Phytophthora* using soils from known infected areas of the Robinson and Berea Forests (Fig. 3). A tub of DI water and rhododendron leaf pieces covered with foil served as negative control; these leaf pieces were placed into the agar. In addition, excisions from leaves collected from the site were plated directly in agar each month to ensure that the rhododendron was free of *Phytophthora* infection.

Isolate Identification by Morphology. Isolates obtained from soil baiting with morphology characteristic of *Phytophthora* spp. were transferred twice; isolates with *Pythium* spp. growth patterns i.e. rapid mycelial growth and fine flexuous hyphae were discarded. Colony morphology of isolates was observed for mycelium habit and pattern. At 40x magnification, structures present in the agar, such as hyphal swellings, chlamydospores, and sporangia were observed; these features are characteristic of *Phytophthora* spp., but not necessarily diagnostic. Colonies underwent a sporangium production test (Jeffers 2006) using a non-sterile soil extract solution (1.5%) (Jeffers and Aldwinckle 1987) and results were noted. Of the 34 second-transfer isolates obtained from soil baiting, 25 showed some structures characteristic of *Phytophthora* spp. at 40x magnification and were tested by PCR.

Molecular Identification of *Phytophthora* Isolates. The identification of the collected isolates was done by PCR amplification of DNA extracted from each isolate and by sequencing the PCR products. Mycelium was scraped from the surface of culture plates and an amount equivalent to a match head was used for DNA extraction with the UltraClean™ Microbial DNA Isolation Kit (MoBio Laboratories, Inc.) according to manufacturer's instructions. The purified DNA was used in PCR amplifications of internal transcribed spacer regions (ITS) using the primers ITS 6

and ITS 4 (Cooke et al., 2000) (Table 1). *P. cinnamomi* specific primers CIN 3A – CIN ITS 4 (Anderson, 2006) (Table 1) were also used for PCR amplifications of DNA from some of the isolates in separate reactions. PCR amplification reactions contained 25 µl GoTaq® Green Master Mix (Promega), 2 µl of the DNA template diluted 1/50, 2.5 µl of each primer, and 18 µl molecular grade water. PCR cycling parameters were according to Anderson (2006) and consisted of a 5 minute denaturation at 94 ºC followed by 30 cycles of 94 ºC for 1 minute, 60 ºC for 1 minute, 74 ºC for 1 minute, and a final extension of 74 ºC for 5 minutes. PCR products were visualized by electrophoresis in 0.7% agarose gels stained with ethidium bromide.

DNA from PCR products was quantities for sequencing using the SybrGold stain on a FluorImager 595. Direct sequencing of the PCR products was performed by the Sanger reaction using the ABI Big Dye terminator v 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on an ABI 3730xl DNA Analyzer at the University of Kentucky Advanced Genetics Technology Center (UK-AGTC). The primer pairs ITS 6 - ITS 4 and CIN 3A - CIN ITS 4 were used to sequence PCR products in the forward and reverse direction. DNA sequences were compiled, edited and analyzed using ChromasPro (Technelysium Pty, Ltd, Australia) and sequence homology searches were done using BLAST (www.ncbi.nlm.nih.gov).

Table 1 Sequence of primers used for PCR amplification of DNA and sequencing of PCR products cultures of *Phytophthora* spp isolated by soil baiting..

Primer Name	Primer Sequence
ITS 6	5'- GAA GGT GAA GTC GTA ACA AGG - 3'
ITS 4	5'- TCC TCC GCT TAT TGA TAT GC - 3'
CIN 3A	5' CAT TAG TTG GGG GCC TGC 3'
CIN ITS 4	5' TGC CAC CAC AAG CAC ACA 3'

Results

Phytophthora spp were consistently isolated from forest soils by the baiting method judging from characteristics of the cultures on PARP-H V8 plates and from the morphology of the isolates (Table 2). PCR amplification products were seen on agarose gels when ITS 6 and ITS 4 primers and *P. cinnamomi* specific primers were used. Sequence analysis of 21 PCR products of 16 samples indicate that *P. cinnamomi* was successfully baited from forest soils. It was possible

to amplify the ITS region of *P. cinnamomi* using the ITS 6 and ITS 4 primers from isolates obtained from seven forest soil samples, and sequence analysis showed that several PCR products had high percentages of identity (98 to 100%) with *P. cinnamomi*. Three of the samples from forest soils that yielded ITS sequences with high percentage of identity to *P. cinnamomi* were confirmed by PCR amplification of total DNA with *P. cinnamomi* specific primers CIN 3A and CIN ITS 4. Sequences of the ITS region with high percentage of identity with *Pythium sp* were also obtained from one isolate of a forest soil sample. No *Phytophthora* isolates were recovered from mine spoils dumped in 2005 and 2007 (Tables 2 and 3). Amplification of total DNA using ITS 6 and ITS 4 primers, from two isolates obtained by baiting mine spoils resulted in sequences with high identity to Mortierella spp. Four other isolates yielded sequences with high percentage of identity to sequences of unidentified fungi. Mortierella is a common soil zygomycete that can sometimes occur as a contaminant in isolation plates. Only two of the 21 sequenced samples yielded poor sequences.

Table 2. Soil baiting isolates collected monthly from May to October 2007 for 2005 plots and positive forest controls.

2005 Plots	Brown	Gray	Mixed	Forest Soils
Total isolates tested by PCR†	6	1	1	9
Phytophthora cinnamomi	0	0	0	8
Mortierella	1	0	0	0
Pythium	0	0	0	1
Unknown	1	1	0	0

†Some isolates did not yield PCR products.

Table 3. Soil baiting isolates collected monthly from May to October 2007 for 2007 plots.

2007 Plots	Brown	Gray	Mixed	Shale
Total isolates tested by PCR†	5	1	2	0
Phytophthora cinnamomi	0	0	0	0
Mortierella	1	0	0	0
Unknown	1	0	1	0

†Some isolates did not yield PCR products.

Phytophthora spp were consistently isolated from forest soils by the baiting method judging from characteristics of the cultures on PARP-H V8 plates and from the morphology of the isolates (Table 2). PCR amplification products were seen on agarose gels when ITS 6 and ITS 4 primers and *P. cinnamomi* specific primers were used. Sequence analysis of 21 PCR products of 16 samples indicate that *P. cinnamomi* was successfully baited from forest soils. It was possible to amplify the ITS region of *P. cinnamomi* using the ITS 6 and ITS 4 primers from isolates obtained from seven forest soil samples, and sequence analysis showed that several PCR products had high percentages of identity (98 to 100%) with *P. cinnamomi*. Three of the samples from forest soils that yielded ITS sequences with high percentage of identity to *P. cinnamomi* were confirmed by PCR amplification of total DNA with *P. cinnamomi* specific primers CIN 3A and CIN ITS 4. Sequences of the ITS region with high percentage of identity with *Pythium sp* were also obtained from one isolate of a forest soil sample. No *Phytophthora* isolates were recovered from mine spoils dumped in 2005 and 2007 (Tables 2 and 3). Amplification of total DNA using ITS 6 and ITS 4 primers, from two isolates obtained by baiting mine spoils resulted in sequences with high identity to Mortierella spp. Four other isolates yielded sequences with high percentage of identity to sequences of unidentified fungi. Mortierella is a common soil zygomycete that can sometimes occur as a contaminant in isolation plates. Only two of the 21 sequenced samples yielded poor sequences.

Media type seemed to influence the number of isolates obtained from each plot, as the 2005 brown sandstone plots had highest number of isolates followed by 2007 brown sandstone plots. Mixed sandstone plots followed in number of isolates with the most isolates acquired from the baiting method. Gray sandstone plots had one isolate for 2007 and one for 2005. No isolates were obtained from 2007 shale plots.

It was noted that after one month, as the inhibitory effects of the selective medium decreased, *Pythium* spp. and mortiella grew in medium from every plot type except for the 2007 shale plots. The presence of these microbial communities as acquired by the baiting method may be significant as colonizers of newly reclaimed a mine spoils. However, as these zygomycetes do not pose a great threat to hardwood trees, they were not analyzed by DNA methods in this study.

Discussion

Monthly testing of different types and ages of reforested mine spoils in Eastern Kentucky as well as forest soils by a baiting method for *Phytophthora* species was carried out from May to

October 2007. These results are encouraging as all mine spoils were negative for *Phytophthora* spp. by the baiting method, which proved consistent in isolating *Phytophthora cinnamomi* at two forest sites. Variation in number of non-*Phytophthora* isolates between types of mine spoils occurred however, with a greater number of isolates from brown sandstone.

The initial assessment is that soil content in brown sandstone spoil correlates with more isolates obtained from the spoil and with number of volunteer species colonizing the spoil. Brown sandstone spoil, as evident by woody debris and roots (Angel et al 2007), was derived from surface and near-surface strata. Mixed spoil contains some brown sandstone spoil and therefore a small amount of soil. Gray sandstone and shale spoil were derived from deeper, unexposed strata and therefore have no soil content. The isolate data demonstrates that the potential for colonization of the gray spoils is possible within one to three years of emplacement as isolates were obtained from the 2005 and 2007 gray sandstone plots. The presence of other microbial communities, including *Mortierella spp.*, is to be expected on the mine spoil plots as the plots were emplaced 1-3 years prior to testing. These microorganisms may have arrived by wind and trees planted at the site.

As measured by Angel et al (2007), the average percent ground cover of the volunteer vegetation during the second growing season were 42.3% on the brown spoil, 2.6% on the mixed spoil and less than 1% on the gray spoil. The number of volunteer species also varied; the brown sandstone had 40 species, 6 species occurred on the gray spoil and 21 species were on the mixed spoil. The majority of the volunteer vegetation on the brown spoil was: *Tussilago farfara* (Coltsfoot), *Chenopodium album* (Lambsquarter), and *Phytolacca americana* (Pokeweed).

Field evidence supports soil baiting for a *Phytophthora*-free prognosis at the Bent Mountain site. No host species of *Phytophthora* at Bent Mountain (i.e. red oak, white oak, and American Chestnuts) have yet shown symptoms of rot induced necrosis or dieback caused by *Phytophthora*. No volunteer species at Bent Mountain that are hosts to commonly occurring *Phytophthora* spp. have been reported and no tree mortality due to *Phytophthora* has yet to be observed (Angel et al 2007, French et al 2007). Although tree survival varied between plots, with lower survival on the brown spoil, competition from voluntary species (Angel et al 2007) rather than presence of microbial communities in soil is the likely correlating factor.

The outlook for planting American chestnut hybrids at the site is promising as *Phytophthora cinnamomi* is transferred mainly by soil and surface water. Current reforestation efforts at

surface mines in Western Australia, where *P. cinnamomi* threatens Jarrah (*Eucalyptus marginata*) forests, demonstrate that transport of the pathogen to pathogen-free areas may be avoided by restricting movement of infested soil and water to these sites (Colquhoun and Kerp 2007). The Bent Mountain research site does not have hydrologic surface or subsurface input other than rain water, and soil will not be moved onto it.

Future work

Soil sampling will be complemented by water testing for *Phytophthora* species which may be more readily detected or viable in infiltrated mine spoil waters. Water infiltrating from mine spoils will be filtered in April 2008 in attempts to capture potential propagules of *Phytophthora* spp. such as chlamydospores or clumps of mycelial fragments, and plated onto selective PARP-H V8 medium. In addition, DNA will be extracted from the filtrate and tested with the same PCR primers as for the isolates obtained by soil baiting. Another soil sampling will take place in April 2008. At this time, roots of pure American chestnuts that experienced mortality will be tested for presence of *Phytophthora*.

The presence of *Phytophthora* spp. in spoils and water in comparison to media physiochemical characteristics, aqueous geochemistry of infiltrated waters, and tree growth with regards to reforestation efforts on different tailing media will be evaluated.

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