ABSTRACT

GALLUP, COURTNEY ANNE. The Impacts of Black Shank Resistance Management on the Population Biology of *Phytophthora nicotianae* in Tobacco. (Under the direction of Howard David Shew.)

Black shank of tobacco, caused by the oomycete *Phytophthora nicotianae*, is an important disease of tobacco. Emergence of race 1 has led to loss of major gene resistance and to questions on the genetic diversity in the pathogen in North Carolina. Race 3 is reported for the first time in NC. Race 3 is virulent on plants with the *Phl* gene and not the *Php* gene and causes severe disease symptoms on mature plants. Isolates of race 3 were recovered from locations where the *Phl* gene was deployed and in fields characterized as the wild-type race, race 0, with no history of single-gene resistance. In order to determine whether races 1 and 3 can develop as natural variants from race 0, and to track loss of *Php* and *Phl* virulence in races 1 and race 3, soil was infested with one race of *P. nicotianae* and planted with tobacco varieties with multigenic resistance. Isolates were recovered after five months and screened for race. Additionally, zoospore isolates were derived from progenitor zoospore isolates representing the three races. Zoospore progeny were screened to identify changes in virulence during asexual sporulation. A subset of zoospore progeny was subjected to Fluorescent Amplified Fragment Length Polymorphism analysis to investigate genetic diversity generated through clonal sporulation. Results showed a gain and/or loss of virulence within all race progeny in soil and single-zoospore isolates. Race 1 was the most stable phenotype, with 91% in infested soil and 99.7% of the zoospore progeny retaining the virulence phenotype. The race structure in soil infested with races 0 and 3 were similar after five

months. Races were recovered in a 2:1 ratio (race 0: race 3) with a small percentage of race 1. Races 0 and 3 zoospore progeny also segregated. Race 0 progeny were 67% race 0 and 33% gained virulence to the *Phl* gene (race 3). Similarly, 68% of the race 3-derived progeny retained the parental virulent phenotype, 31% lost the virulent phenotype (race 0), and 1% gained virulence to the *Php* gene (race 1). Estimates of genetic diversity within each group of related zoospores ranged from 0.17013 to 0.44196. Phenotypic and genotypic investigations revealed that asexual sporulation may be a major source of variation in natural populations. A state-wide survey of *P. nicotianae* populations was conducted in NC tobacco-producing regions. Isolates were obtained from 76 tobacco fields in 23 counties and screened for race and mating type. Race 1 was predominant in most regions, with 59% of fields consisting of 90 to 100% race 1. The occurrence of race 1 within fields was positively correlated with the history of monogenic resistance deployment. Race 3 was identified in low frequency throughout the state, primarily in wild-type populations where no monogenic resistance was deployed. The A1 and A2 mating types were found throughout NC and were recovered concurrently from multiple fields. Pairings of isolates from within fields yielded viable oospores, indicating for the first time, the potential for sexual reproduction by *P. nicotianae*. A subset of the survey isolates were screened for sensitivity to the fungicide mefenoxam. All isolates were sensitive, with a mean EC_{50} value of 0.4 μg/ml mefenoxam, indicating fungicide applications are still a reliable method of black shank management. Results reveal a rapid state-wide shift toward race 1, correlating with the deployment of monogenic resistance and indicate that sexual recombination may be important in generating variation within the pathogen population.

The Impacts of Black Shank Resistance Management on the Population Biology of *Phytophthora nicotianae* in Tobacco

by
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DEDICATION

I dedicate my dissertation to my loving husband, Bill. I couldn't have made it through graduate school so happily without all of the love and support you have given me through these years in North Carolina. You're my favorite!

BIOGRAPHY

Courtney Gallup was born into a Marine Corps family on January 5, 1980 in Honolulu, Hawaii. She moved nationally and internationally throughout her childhood. She graduated from North Nash Senior High School in Rocky Mount, NC in 1998. As an undergraduate, Courtney attended North Carolina State University and received B.S. degrees in Horticultural Science and Botany. During these years, she interned at the J.C. Raulston Arboretum, assisted Dr. C. Lee Campbell in the NCSU Plant Pathology Department, and completed teaching assistantships for the laboratory sections of Introductory Botany, and later Introductory Plant Pathology. In her third year, she studied agroecology and ecology in Monteverde, Costa Rica. In her final year, she moved under the guidance of Dr. H. D. Shew as a student research assistant working with soilborne pathogens and assisting in the preparation of PP315 for distance education. In January of 2004, she followed her interests by pursuing graduate degree in the NCSU Plant Pathology Department under the direction of Dr. David Shew. Her research focused on clarifying the race structure and characterizing populations of *Phytophthora nicotianae* across North Carolina in order to enhance our understanding of pathogen races and how they may impact future management of the disease. The second major focus of her research aimed to describe pathogen variability arising from sexual and asexual recombination. Characterizing the level of genetic variability will help elucidate how this variability influences the development of pathogen races and how human activities drive pathogen evolution.

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Chapter 1

Occurrence of race 3 of *Phytophthora nicotianae* in North Carolina,

the causal agent of black shank of tobacco

Occurrence of race 3 of *Phytophthora nicotianae* in North Carolina, the causal agent of black shank of tobacco

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ABSTRACT

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Black shank, caused by the oomycete *Phytophthora nicotianae,* causes significant annual yield losses in tobacco. A new race, race 3, was identified from NC tobacco fields with a history of tobacco varieties with *Phl* gene resistance and field trials with no known history of selection pressure from single-gene resistance. Race 3 incites disease on burley tobacco varieties that utilize the *Phl* gene from *Nicotiana longiflora* but not on tobacco varieties that utilize the *Php* gene from *N. plumbaginifolia*. Race 3 was originally described from cigar-wrapper tobacco in the 1970s, but has not been reported in any other location since. A stem inoculation study was conducted to compare NC isolates to an original isolate of race 3 from CT. Stem inoculations were unable to distinguish between races 0 and 3 of *P. nicotianae* and are not a reliable method of identifying these virulence types. Race 1 did give a unique phenotype using stem inoculation. Root inoculations are the only reliable means of distinguishing races due to differences in *Phl* gene expression in the roots compared to the

stem. This is the first report of race 3 causing damage to seedlings from root inoculations and to field plants containing the *Phl* gene in naturally infested soil.

INTRODUCTION

Black shank of tobacco, caused by the oomycete *Phytophthora nicotianae*, occurs in most tobacco-producing regions worldwide (17, 24, 25). In North Carolina, annual losses to the disease have averaged over $10 million since 1995 (21). Black shank is primarily a root rot disease, but also causes severe above-ground symptoms characterized by black stem necrosis at and above the soil line, overall wilting and chlorosis, and plant death (9, 17, 24).

Planting resistant varieties is the most effective means of suppressing black shank disease pressure. Two types of host resistance are currently available to growers. Fla 301 resistance (30) is regulated by multiple genes and confers low to high levels of partial resistance against all races of *P. nicotianae*. (3, 13, 14, 23, 24, 33). Continuous exposure to high levels of this source of resistance results in increased aggressiveness in pathogen populations (8, 27). Complete resistance, regulated by single genes, is also available in commercial tobacco varieties. The *Php* gene from *Nicotiana plumbaginifolia* has been utilized primarily in flue-cured tobacco, but has recently been incorporated into burley varieties. The *Phl* gene from *N. longiflora* has been used only in burley tobacco (4, 12). Both genes confer complete resistance to race 0 and no resistance to race 1 of the black shank pathogen.

In the early 1960s, Chaplin successfully transferred the *Php* gene from *N. plumbaginifolia* to cultivated tobacco, *N. tabacum* (4). The gene was not released into

commercial varieties because race 1 of the pathogen developed in breeding nurseries before acceptable varieties could be produced (1). The breeding line NC 1071, which contains the *Php* gene but has no partial resistance, was developed at this time and is now used as an indicator line for presence of race 1 in field trials and greenhouse inoculations. The commercial flue-cured variety Coker 371-Gold, which also has a low level of Fla 301 resistance, was the first variety released to growers that contained the *Php* gene (12). Coker 371-Gold was not widely used by growers because of poor agronomic traits, but once the *Php* gene was incorporated into agronomically acceptable varieties in the 1990s, it was widely deployed and incidence of race 1 increased rapidly (5, 7, 28).

In 1953, the *Phl* gene was transferred from *N. longiflora* to a burley tobacco breeding line, L8 (31). L8, which is homozygous for the *Phl* resistance gene, but was not acceptable for commercial production because of a severe physiological leaf spotting associated with the gene (31). To correct for this problem, the resistance gene is used only in a commercial hybrid that is heterozygous for the *Phl* gene, KY14xL8. Similar to the *Php* gene, deployment of the *Phl* gene also resulted in the occurrence of race 1 (16, 17, 26).

Pathogen races are identified based on the inoculation of a set of host differentials. In the original descriptions of races 0 and 1, isolates avirulent on *N. plumbaginifolia* (*Php*) were designated race 0 and virulent isolates were designated as race 1 (1, 2). In many cases only one resistance gene, rather than both, was used to differentiate races 0 and 1. Since race 1 isolates overcome both the *Php* and *Phl* genes, pathogen isolates that could overcome only one of the resistance genes would have been misidentified if both resistance genes were used not used in race determination tests.

To date, only races 0 and 1 have been reported in North Carolina. Race 0 is considered the wild-type and occurs in all tobacco-growing regions. Race 2 was reported in South Africa based on a differential response of three varieties, KY 14xL8, Burley 21xL8, and Delcrest 202 (32). Since there is no known resistance gene in Delcrest 202 and race 2 has not been described elsewhere, it is not considered epidemiologically significant. Race 3 of *P. nicotianae* was reported in Connecticut (CT) by McIntyre and Taylor (20). They identified isolates from CT cigar-wrapper tobacco that differed from isolates of races 0 and 1 based on stem-inoculations of L8 (*Phl*) and NC 1071 (*Php*) plants. Their race 0 and 1 isolates gave expected results on resistant and susceptible genotypes. CT isolates caused a limited stem necrosis on L8 (*Phl* gene) but no disease on a line that had the *Php* gene (19). In a more detailed study, the CT isolates were named race 3 based primarily on physiological differences, and did not differ in virulence from race 0 except that they caused a limited stem necrosis (several cm from the inoculation site) on 56$\pm$4% of the L8 plants that were stem-inoculated (20). Limited stem necrosis also has been reported with race 0 isolates on varieties with the *Phl* gene (11, 32). No damage from root inoculations were reported with the CT race 3 isolates. Since pathogen races should be defined by their ability to overcome single-gene resistance, and the major difference described for this race was physiological, race 3 was not widely accepted.

Since 1999, isolates of *P. nicotianae* have been recovered from NC tobacco fields that do not fit descriptions of race 0 or 1. A study conducted from 2000 through 2003 explored the affects of variety rotation on race development in field microplots (27). Some plots were infested with race 0 and planted each year with varieties with only partial

resistance. As expected, most isolates recovered at the end of this study were race 0. However, some isolates caused disease on root-inoculated KY 14xL8 seedlings (*Phl*) and not on NC 1071 seedlings (*Php*) (Fig 1.1). Race 3 was described as causing limited stem necrosis on approximately 60% of inoculated KY 14xL8 plants. In contrast to race 3 descriptions, the NC isolates killed root-inoculated KY 14xL8 plants. Since root inoculations were never conducted, and the development of stem lesions was not severe in the initial reports of race 3, it was unclear if the isolates recovered from this infested microplot study were race 3 or a new race. Additionally, no tobacco varieties with the *Phl* gene are planted in CT, so no field losses on plants with this gene had been described. Field trials conducted in multiple NC counties also yielded an unexpected race phenotype. In some plots where single-gene resistance had not been deployed previously, adult plants of KY 14xL8 (*Phl)* were killed, yet adjacent rows of NC 1071 (*Php*) were healthy (Fig 1.2).

No race has yet been described that would cause field losses in varieties with the *Phl* gene but not varieties with the *Php* gene. The objective of this study was to characterize the isolates from multiple locations in NC that exhibited the new phenotype on a set of host differentials.

MATERIALS AND METHODS

Collection of isolates. Isolates included in this study were collected over numerous locations and years (Table 1). Briefly, isolate 129 was obtained from the microplot field study conducted by M.J. Sullivan (27) and was maintained in sterile water tubes at room temperature. Isolate 97 is a race 3 isolate identified and obtained in 1983 by our lab. The

isolate was from cigar-wrapper tobacco in Connecticut and had been maintained on agar plugs in sterile water tubes at room temperature and periodically transferred to maintain viability. All of the other isolates were obtained from a field in each of nine NC counties. Isolates were recovered from soil that was collected from the root zone of symptomatic plants.

Soil assays were conducted according to Sullivan (27) with some modifications. To recover isolates from soil, 1 g of infested soil was suspended into each of three beakers containing 25 ml deionized (DI) water. The suspension was poured in 5 ml aliquots over five plates of PARPH semi-selective medium using 5% V8 agar as the basal medium (14, 22). Briefly, 2 g of $CaCO_3$ was suspended in 250 ml of V8 juice (Campbell Soup Company, Camden, NJ) and 750 ml DI water. The suspension was autoclaved at 121°C for 10 minutes, clarified by vacuum filtration through Celite 545 (Fisher Scientific, Fair Lawn, NJ), brought to volume and autoclaved at 121°C for 30 min for 2 consecutive days. Broth was stored at room temperature until needed. PARPH was prepared by autoclaving 200 ml filtered V8 broth, 800 ml DI water, and 20 g of Bacto Agar (Difco, Detroit, MI) at 121°C for 30 min (5% V8). The medium was allowed to cool to 55°C before adding the following chemicals and pouring: 125 mg pentachloronitrobenzene (PCNB), 50 mg hymexazol, 250 mg ampicillin, 2 ml rifampicin stock (500 mg rifamycin SV sodium salt in 100 ml 95% EtOH), and 2 ml pimaricin stock (500 mg natamycin in 100 ml sterile DI water). Plates were incubated in the dark at room temperature for 2 days and then were washed with running tap water to remove the soil from the agar surface. Distinct colonies of *P. nicotianae* were transferred to a fresh plate of PARPH. Once obtained, isolates were grown on 5% clarified carrot agar (CA)

(Hollywood or The Hain Celestial Group Inc., Melville, NY) (28). For this, carrot juice was filtered through Celite 545 (Fisher Scientific) and stored at -20°C until needed. The CA medium was made by adding 50 ml of the filtered carrot juice to 950 ml of DI water and 20 g of Bacto Agar. The medium was autoclaved at 121°C for 30 min and allowed to cool to 55°C before pouring. For storage, colonized agar plugs were placed in sterile water in test tubes and maintained at room temperature.

Race determination. *Root inoculation*. Twelve isolates were selected for this study based on an initial race determination. Race was initially determined based on root inoculation of a set of host differentials. Genotypes consisted of cv. Hicks, with no resistance gene, the breeding line NC 1071 with only the *Php* gene, and the cv. KY 14xL8 with only the *Phl* gene.

Isolates were screened for race as described by Sullivan et al. (2005a, 2005b) except the variety K326 was not included because it is not necessary to identify races. Briefly, 3-wk-old seedlings were transplanted into cell packs (72 cells, 4 × 4 × 5 cm) containing 1:1:1 (v:v:v) mixture of steam pasteurized soil, potting mix, and course builder's sand. Inoculum was prepared by autoclaving 500 cc oat grains with 300 ml DI water for three consecutive days at 121°C. Sterile oat grains were poured onto the surface of pure cultures, wrapped with Parafilm®, and incubated at room temperature for 7 to 10 days. Plants were inoculated by placing two colonized oat grains into each cell containing one 4-wk-old seedling. Disease was rated 10-14 days after inoculation. If only seedlings of the variety Hicks were killed, then the isolate was designated as race 0. If the seedlings of variety Hicks, the breeding line NC 1071 (*Php*), and the variety KY 14xL8 (*Phl*) were killed, the isolate was designated as

race 1. If NC 1071 seedlings were asymptomatic, Hicks was killed, and at least one of the three KY 14xL8 seedlings was killed, then the isolate tentatively was designated as race 3. Root inoculations were repeated to confirm race designation.

Stem inoculation. In order to make comparisons consistent with the original description of race 3 and with previous studies on race determination that used a stem inoculation method (6, 11, 20, 32, 34), a stem inoculation study was conducted with isolates of races 0, 1, the CT race 3 isolate, and the set of NC isolates that gave a race 3 reaction in initial root inoculations (Table 1).

Inoculum was prepared for each isolate according to Csinos (6, 7) with slight modifications. Cultures of *P. nicotianae* were grown on 5% CA and toothpicks were sterilized in a clarified 5% V8 broth for 30 min prior to use. Each isolate was grown on CA medium for 7 days at room temperature, 22 to 25°C, then the sterile, broth-infused toothpicks were placed on the agar surface and incubated for 7 days in the dark at room temperature. Colonized toothpicks were removed from the agar surface and used immediately to inoculate plants.

Three tobacco varieties and a breeding line were used as host differentials: Hicks, NC 1071, KY 14xL8, and L8. Seed of each variety were sown into flats containing potting mix (Metro mix 200, The Scotts Company, Marysville, OH) and allowed to grow for 4 wks. After 4 wks, the seedlings were transplanted into 4-in pots containing a 1:1:1 (v:v:v) mixture of steam pasteurized (80°C for 30 min) soil, potting mix, and course builder's sand. Plants were allowed to grow for an additional 12 wks before inoculation. All plants were watered twice

daily and fertilized every 7 with 20-20-20 Miracle-Gro® (The Scotts Miracle-Gro Company, Marysville, OH).

Plants were inoculated by pushing a sterilized probe through the stem at approximately half the plant height. An infested toothpick was immediately inserted into the wound and the inoculation site was wrapped with several layers of Parafilm®. Wounded plants without inoculum served as controls. The experiment was arranged in a randomized complete block with five replicate plants per treatment and was conducted twice. Lesion lengths were measured using a digital caliper at 3, 5, and 7 days after inoculation. Area under the lesion expansion curve (AULEC) values for each plant was calculated using SAS (Version 9.2, SAS Institute, Cary, NC). Analysis of variance was performed on the AULEC values using the PROC MIXED procedure of SAS (Version 9.2, SAS Institute, Cary, NC).

RESULTS

Race determination. *Root inoculation.* Each isolate used in this study initially was characterized to race based on root inoculations of a set of standard host differentials (Table 1.1). Plants were rated qualitatively, dead or living, and no quantitative measure, such as percent root rot, was assessed. From these initial tests, several isolates of races 0 and 1 were selected to compare to the NC isolates that gave the race 3 reaction (Table 1.2). No intermediate symptoms were observed on the root-inoculated KY 14xL8 seedlings; seedlings either died or showed no disease symptoms.

Stem inoculation. There was a significant run effect ($P < 0.001$) in the experiment, so data from the two runs of the experiment were analyzed separately. All isolates caused

disease on the susceptible variety Hicks. AULEC values were highest on Hicks for all variety x isolate combinations except for the race 1 isolates on NC 1071, which is highly susceptible to race 1 (Fig. 1.3, Fig. 1.4). All three race 1 isolates produced extensive stem lesions on all varieties as predicted from the root inoculations, averaging between 163 and 416 mm in length over the experiment.

Within each run, lesion expansion on Hicks was not significantly different among races 0, 1, and the NC isolates. Isolate 97, which was the only race 3 isolate available for comparisons, was much less aggressive than all of the other isolates, was characterized by smaller stem lesions on all varieties in both runs of the experiment (P=0.0001) . The average AULEC for isolate 97 was only 56 compared to the overall average of 377 in the first run. In the second run, the average AULEC for isolate 97 was only 12 compared to the overall average of 207.7.

Although isolate 97, the CT race 3 isolate, caused very low levels of disease, the pattern of disease was very similar to the NC isolates that were tentatively identified as race 3 based on root inoculations. Isolate 97 resulted in no necrosis on NC 1071 with the *Php* gene and only limited stem necrosis on KY 14xL8 with the *Phl* gene, averaging 48 mm in run 1 and 2 mm in run 2. Similar to the CT isolates, the NC isolate caused no necrosis on NC 1071, the most extensive necrosis was on the susceptible Hicks, and intermediate levels of disease on KY 14xL8 and L8. All of the race 3 and NC isolates caused more extensive lesions KY 14xL8 than on L8 in both runs of the experiment (Fig 1.3, Fig. 1.4).

Race 0 is defined by its inability to incite disease on varieties utilizing single-gene resistance (1). The race 0 isolates used in this study did not kill either genotype in the initial

root inoculations. However, in the first run of the experiment, all three isolates caused stem lesions on KY 14xL8, which is heterozygous for the *Phl* gene, and two of the three isolates caused stem lesions on the L8 variety, which is homozygous for the *Phl* gene. Similarly, in the second run of the experiment, all race 0 isolates caused stem necrosis on KY 14xL8, and two of the three isolates caused stem lesions on the L8 variety. However, the two isolates that did not cause stem lesions on L8 in the two runs of the experiment are not the same. Overall, AULEC values were lower on the *Phl* varieties than on Hicks (Fig. 1.3, Fig. 1.4).

All of the race 0 and the race 3 NC isolates had statistically similar AULEC values on all stem-inoculated varieties in both runs of the experiment. All isolates that were characterized as race 0 from initial root inoculations were defined as race 3 based on stem inoculations because lesions formed on both varieties with the *Phl* gene (Table 1.2). The race 1 phenotype was distinct because of the clear qualitative response of NC 1071 plants to stem inoculation. However, the stem inoculation procedure was unable to differentiate between races 0 and 3, which had initially been clear from root inoculations, because the stems of varieties with the *Phl* gene did not respond qualitatively to inoculation.

Occurrence of race 3 in field populations of *P. nicotianae*. Isolate 31, an original isolate of a potential race 3 in NC, was obtained and characterized from a field of burley tobacco planted with KY 14xL8 in Buncombe County. Several other field isolates have also been collected from field plots planted with KY 14xL8 (Table 1.3). However, many of the race 3 isolates used in the stem inoculation study were obtained from plots that had no history of exposure to the *Phl* gene (Table 1.2). This is the first report of isolates of *P.*

nicotianae that kill tobacco plants with the *Phl* gene in the field but do not cause disease on varieties with the *Php* gene (Fig. 1.3).

DISCUSSION

Isolates of *P. nicotianae* that could overcome the *Phl* gene but not the *Php* gene in root inoculations were recovered from multiple tobacco fields in NC. Fields included locations with and without a history of tobacco varieties with *Phl* single-gene resistance. The only report of a race that was virulent on the *Phl* gene and avirulent on the *Php* gene was in the 1970s on cigar-wrapper tobacco in CT (19, 20). Pathogen races typically are defined based on the qualitative response of host differentials to inoculation. However, the initial description of race 3 was based on the quantitative susceptibility of the breeding line L8, which is homozygous for the *Phl* gene, to stem inoculation by isolates of the pathogen. Additional characterization of race 3 focused on multiple physiological differences compared to races 0 and 1 (10, 18, 20). No root inoculations were conducted in the original description, and since no varieties of tobacco grown in CT contain the *Phl* gene, it was unclear whether the isolates collected from NC were race 3 or a new race.

Since the original description of race 3 was based on stem inoculations, NC isolates that were tentatively defined as race 3 based on root inoculations were subsequently compared to the CT race 3 isolate according to the original description. NC isolates were selected for this study based on a clear qualitative response of host differentials to root inoculations (Table 1.1).

When these isolates were inoculated into the stems of the same tobacco varieties, the response was less clear. The variety NC 1071 (*Php*) maintained the same qualitative response to stem inoculations as was observed in root inoculations: no lesions developed in response to stem inoculations with the race 0 isolates, the CT race 3, or the NC race 3 isolates, and extensive lesions developed in response to stem inoculations with race 1. Unlike NC 1071, the two tobacco varieties with the *Phl* gene did not respond qualitatively to stem inoculation. All isolates, regardless of the race based on root inoculations, caused stem lesions on both the KY 14xL8 (*Phl* heterozygous) and L8 (*Phl* homozygous) varieties. Therefore, isolates initially defined as race 0 based on root inoculations would be defined as race 3 based on stem inoculation (Table 1.2).

It is likely that the apparent susceptibility of the stems of tobacco plants with the *Phl* gene is due to differences in gene expression in different parts of the plant. Differential expression of resistance to *P. nicotianae* in different tobacco tissues has been reported for other sources of resistance. Qualitative resistance from Fla 301 is expressed only in the roots, so stem inoculation is not a reliable technique to discriminate between levels of resistance (6, 17). In contrast, resistance conditioned by the *Php* gene is expressed in the stem and root tissues (6). In the original description of race 3, 56$\pm$4% of L8 plants developed limited lesions when stem-inoculated with CT isolates (20). The original description of race 3 may have been an indication of susceptibility of the stem tissue to race 0 rather than a new virulent type in *P. nicotianae*.

A previous study demonstrated that the leaves of KY 14xL8 are very susceptible to *P. nicotianae* when inoculated with race 0 isolates. The level of susceptibility in the leaves was

comparable to the susceptibility of varieties KY 14 and B 21, both of which lack any level of resistance (29). *Phl* gene expression in the stem has not been directly assessed. However, Hendrix and Apple (2) had findings similar to ours, concluding that the response of stem-inoculated L8 plants to race 0 was variable. Breeders have long recognized this single-gene source of resistance as a qualitative type of root resistance to the black shank pathogen (15, 26). If resistance is completely expressed in the roots and not expressed in the leaves, it is possible that the stems express resistance at some intermediate level.

Based on this work, it is clear that root inoculations are the only reliable means of differentiating all known races of the black shank pathogen. Race 1 can be identified from stem inoculations because qualitative resistance from the *Php* gene in NC 1071 is completely expressed in the stems as well as the roots. However, varieties with the *Phl* gene cannot differentiate races 0 and 3.

This investigation confirms the occurrence of the race 3 phenotype of *P. nicotianae* as virulent on the *Phl* gene but not the *Php* gene based on root inoculations where both genes are qualitatively expressed. Race 3 isolates were recovered from multiple locations throughout NC. Race 3 was recovered from the soil in fields where KY 14xL8 had been planted. It was also recovered from field trial plots with no prior history of the *Phl* gene (Fig. 1.2). In both the tobacco fields and trial plots, fully mature tobacco plants displayed typical black shank symptoms. This is the first report of race 3 in NC and the first report of damage to root inoculated plants and losses to mature plants in infested field soil due to race 3.

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Table 1.1. Differential response of root-inoculated tobacco varieties (*Nicotiana tabacum*) to races of *Phytophthora nicotianae*

	Variety [a]			
	Hicks	**NC 1071 (*Php*)**	**KY 14xL8 (*Phl* hetero.)**	**L8 (*Phl* homo.)**
Race 0	+	-	-	-
Race 1	+	+	+	+
Race 3	+	-	+	+

[a] Hicks is susceptible; NC 1071 is a flue-cured variety with the *Php* gene; KY 14xL8 is a hybrid burley variety, heterozygous for the *Phl* gene; L8 is a burley breeding line, homozygous for the *Phl* gene.

Table 1.2. Isolates included in the stem inoculation study to determine the race of 5 *Phytophthora nicotianae* isolates in NC

			Field History [a]				Race [b]		
Isolate	**Location**	**Year**	**2003**	**2004**	**2005**	**2006**	**Initial Race Root inoculation**	**Run 1 Stem inoculation**	**Run 2 Stem inoculation**
DT10	Duplin Co. NC	2006	NH	301	NH	301	0	3	3
EL2	Edgecombe Co. NC	2006	301	NH	301	*Php*	0	3	3
RB15	Rockingham Co. NC	2006	NH	VA116	NH	*Php*	0	3	3
DB12	Davie Co. NC	2006	NH	NH	TN90	*Php*	1	1	1
FL9	Forsyth Co. NC	2006	No Data				1	1	1
YB10	Yadkin Co. NC	2006	NH	NH	301	*Php*	1	1	1
97 [c]	CT	1983					3	3	3
31 [d]	Buncombe Co. NC	1999					3	3	3
129	Field study, NC	2003	301	301	301	301	3	3	3
H109	Hoke Co. NC	2004	301	301	NA	NA	3	3	3
SF2	Sampson Co. NC	2006	NH	*Php*	NH	301	3	3	3
WP10	Wayne Co. NC	2006	NH	NH	301	301	3	3	3

[a] History of tobacco resistance gene deployment from 2003 to 2006: *Phl* = Single-gene resistance from *N. longiflora*; *Php* = Single-gene resistance from *N. plumbaginifolia*; 301= Fla 301 resistance; NH = Nonhost crop.

[b] Races were initially defined based on root inoculations and were used for statistical analysis. Races were not adequately differentiated based on AULEC for stem inoculations.

[c] Isolate 97 was collected in the 1970s from cigar-wrapper tobacco in CT. Isolates collected from NC were compared against this isolate.

[d] Isolate 31 was isolated from a soil sample taken from the root zone of a KY 14xL8 plant during a survey of the burley tobacco regions for race.

Table 1.3. Race structure of *Phytophthora nicotianae* in NC plots where tobacco varieties with single-gene or partial resistance was deployed over a three year period

Plot number	NC County	Tobacco Variety [a]			Gene deployed [b]	Race (%) [c]		
		2002	2003	2004		R-0	R-1	R-3
1	Franklin	unknown	unknown	KY-14xL8	*Phl*	-	100	-
2	Franklin	unknown	unknown	KY-14xL8	*Phl*	-	100	-
3	Franklin	unknown	unknown	NC-1071	*Php*	-	100	-
4	Rockingham	K-346	K-346	K-346	Fla 301	-	80	20
8	Rockingham	SP-NF3	NC-71	K-346	*Php* / Fla 301	-	100	-
16	Rockingham	SP-168	K-346	K-346	*Php* / Fla 301	-	100	-
24	Rockingham	SP-NF3	NC-71	K-346	*Php* / Fla 301	-	100	-
28	Rockingham	SP-NF3	NC-71	K-346	*Php* / Fla 301	-	100	-
36	Rockingham	SP-168	K-346	K-346	*Php* / Fla 301	20	80	-
44	Rockingham	SP-168	K-346	K-346	*Php* / Fla 301	-	100	-
46	Rockingham	SP-NF3	NC-71	K-346	*Php* / Fla 301	-	100	-
1	Hoke	unknown	KY-14xL8	Mn-944	*Phl* / Fla 301	12	88	0
11	Hoke	unknown	K-394	KY-14xL8	*Phl* / Fla 301	-	100	-
34	Hoke	unknown	K-346	C371G	*Php* / Fla 301	20	40	40
42	Hoke	unknown	K-149	K-346	Fla 301	40	-	60
83	Hoke	unknown	SP-190	KY-14xL8	*Php* /*Phl*	-	90	10
97	Hoke	unknown	KY-14xL8	SP-218	*Php* /*Phl*	50	20	30
126	Hoke	unknown	C371G	K-346	*Php* / Fla 301	-	60	20
133	Hoke	unknown	KY-14xL8	GL-939	*Phl* / Fla 301	33	50	17
148	Hoke	unknown	GL-939	KY-14xL8	*Phl* / Fla 301	-	100	-

[a] Variety of tobacco that was planted from 2002 through 2004 in NC field trial plots

[b] Source(s) of resistance to the black shank pathogen based on variety records. The *Phl* and *Php* genes are sources of complete resistance from wild tobacco *Nicotiana longiflora* and *N. plumbaginifolia*, respectively. Fla 301 resistance is partial resistance effective against all races of the pathogen.

[c] Races were determined by inoculating a set of host differentials, Hicks, susceptible, NC 1071, with the *Php* gene, KY 14xL8, with the *Phl* gene. Race 0 killed only Hicks, race 1 killed all genotypes, and race 3 only Hicks and KY 14xL8.

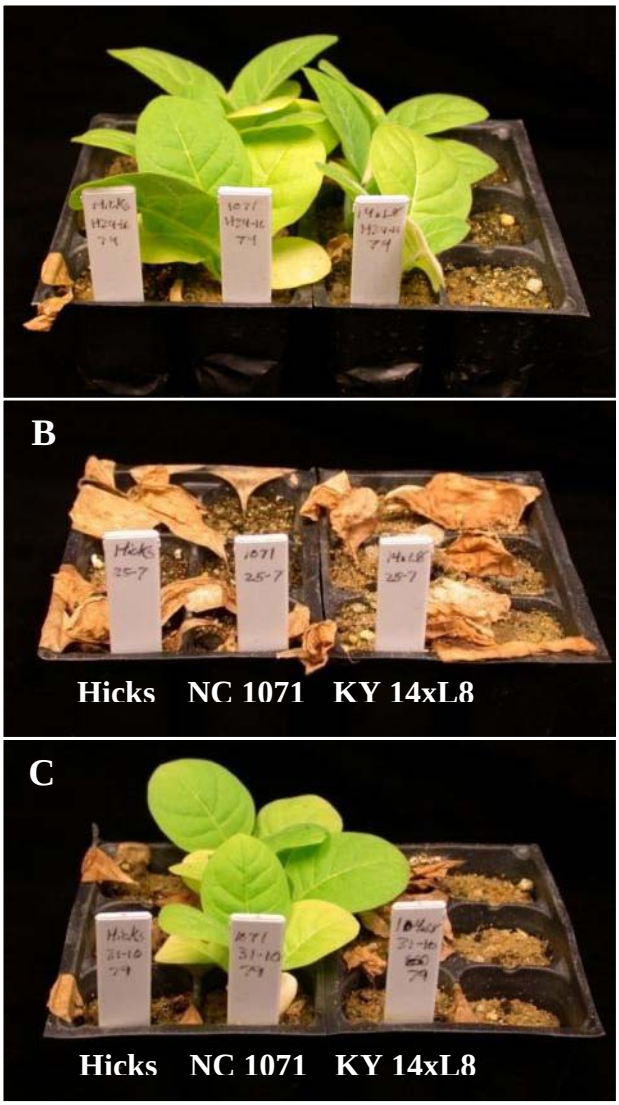

Figure 1.1. Differential tobacco (*Nicotiana tabacum*) varieties root-inoculated with each race of *Phytophthora nicotianae*. **A**. Race 0. **B**. Race 1. **C**. Race 3.

Figure 1.2. NC black shank field trial where burley tobacco plants of the variety KY 14xL8 (*Phl*) (right) were dying, yet plants of the flue-cured variety NC 71 (*Php*) (left) in the adjacent row were healthy

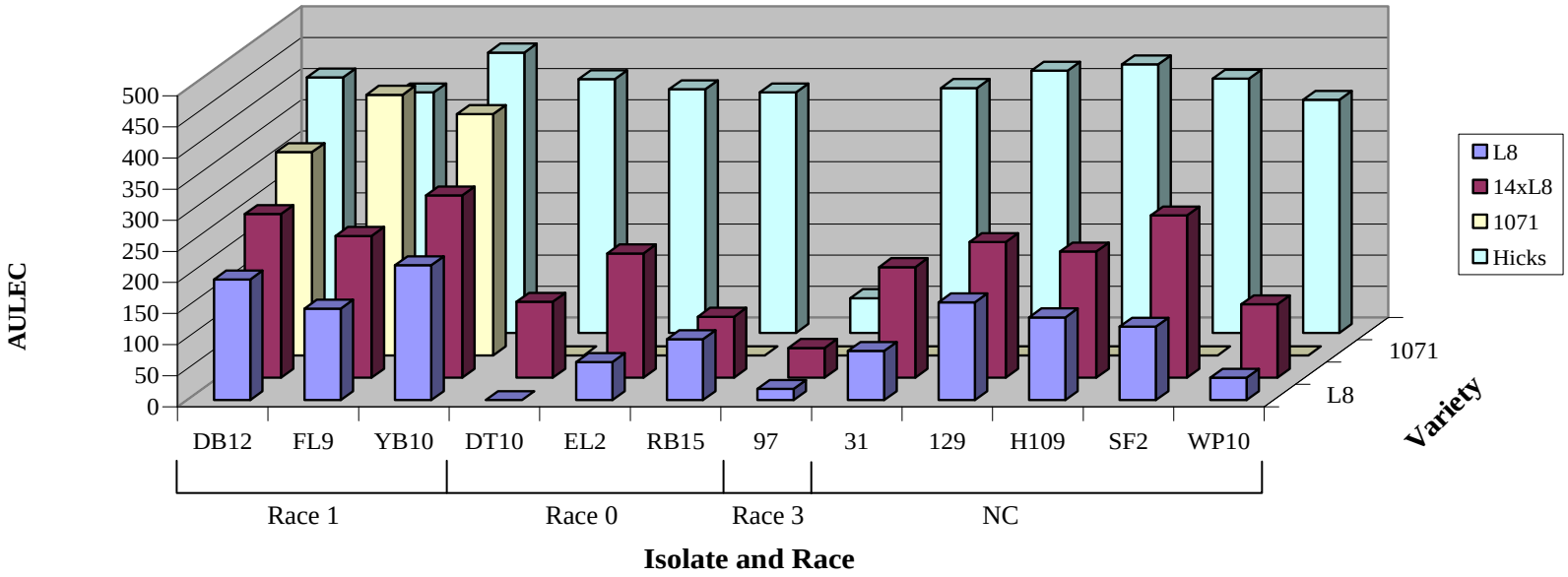

Figure 1.3. Run 1. Area Under the Lesion Expansion Curve (AULEC) comparing lesions resulting from stem inoculations with *Phytophthora nicotianae* isolates of known races 0, 1, 3, to isolates collected from NC tobacco fields. Tobacco varieties L8 (homozygous *Phl*), KY 14xL8 (heterozygous *Phl*), NC 1071 (*Php*), and Hicks (susceptible) were stem inoculated with colonized toothpicks. Races are based on *a priori* root inoculations using a set of host differentials, Hicks, susceptible, NC 1071, with the *Php* gene, KY 14xL8, with the *Phl* gene. Race 0 killed only Hicks, race 1 killed all genotypes, and race 3 only Hicks and KY 14xL8.

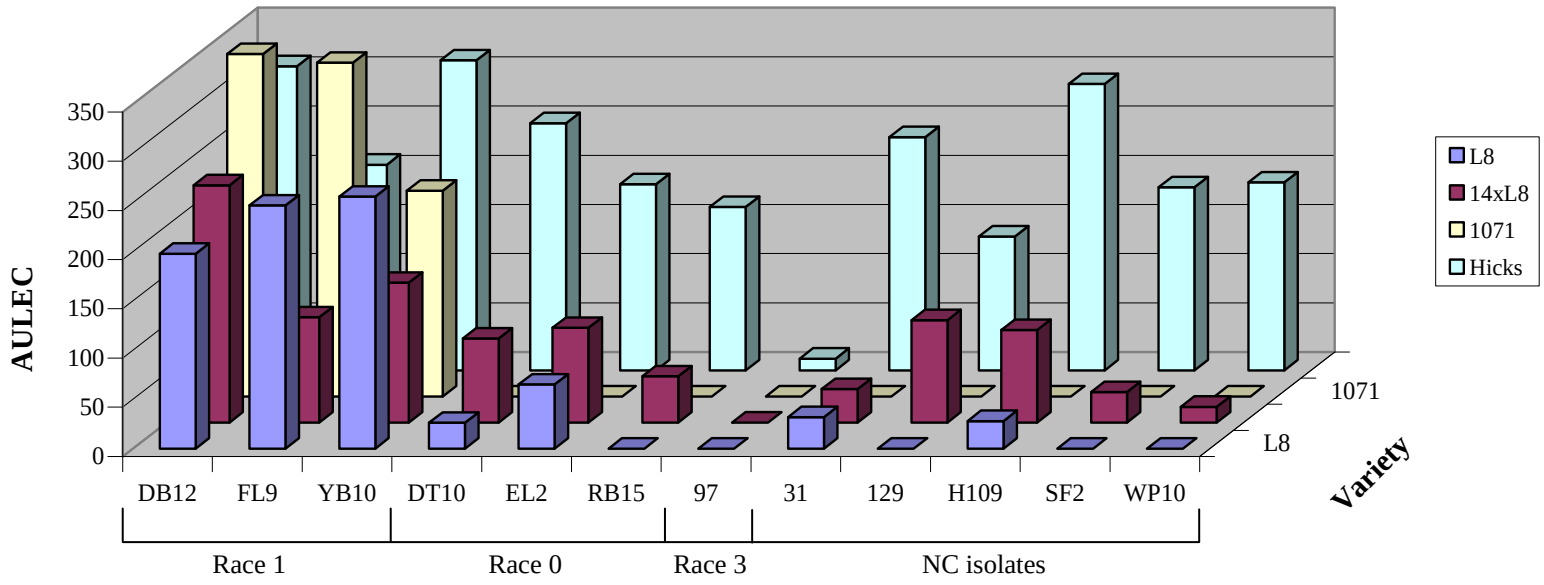

Figure 1.4. Run 2. Area Under the Lesion Expansion Curve (AULEC) comparing lesions resulting from stem inoculations with *Phytophthora nicotianae* isolates of known races 0, 1, 3, to isolates collected from NC tobacco fields. Tobacco varieties L8 (homozygous *Phl*), KY 14xL8 (heterozygous *Phl*), NC 1071 (*Php*), and Hicks (susceptible) were stem inoculated with colonized toothpicks. Races are based on *a priori* root inoculations using a set of host differentials, Hicks, susceptible, NC 1071, with the *Php* gene, KY 14xL8, with the *Phl* gene. Race 0 killed only Hicks, race 1 killed all genotypes, and race 3 only Hicks and KY 14xL8.

Chapter 2

Race development and genetic variability in asexual populations of *Phytophthora nicotianae*, the causal agent of black shank of tobacco

Race development and genetic variability in asexual populations of *Phytophthora nicotianae*, the causal agent of black shank of tobacco

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ABSTRACT

Gallup, C. A. and Shew, H. D. 2009. Development, stability, and genetic variability of asexual populations of *Phytophthora nicotianae* races 0, 1 and 3, the causal agent of black shank of tobacco. Plant Dis. 93:000-000.

Black shank of tobacco, caused by the oomycete *Phytophthora nicotianae*, is an important disease of tobacco. Three races (0, 1, 3) and four mating types (A0, A1, A2, A1/A2) of the pathogen occur in North Carolina. Soil in greenhouse flats was separately infested with an A2 isolate of each of race of *P. nicotianae* and planted with tobacco varieties with multigenic resistance to the black shank pathogen. Isolates were recovered from the infested soil after five months and screened for race and mating type to investigate stability of these traits. Race 1 was the most stable phenotype in infested soil, with 91% of race 1 progeny retaining virulence against both the *Php* and *Phl* genes; 3% were virulent only to the *Php* gene (race 3), and 6% of the progeny lost virulence to both genes (race 0). The race structure from the greenhouse study for the race 0 and 3 treatments were statistically the same after five months. Approximately 2/3 of the isolates gave the race 0 phenotype and 1/3 were the race 3 phenotype. Between 2 and 3% of the isolates recovered were race 1. To further characterize phenotypic variability, single-zoospore progeny were obtained from

progenitor isolates representing each race and screened to identify any changes in virulence during asexual reproduction. Among the zoospore progeny, the race 1 phenotype was most stable with 99.7% of the zoospore progeny retaining the race 1 virulent phenotype. Races 0 and 3 progeny were both more variable. Race 0 progeny consisted of 67% race 0 and 33% race 3. Among the race 3 progeny, 68% retained the parental virulent phenotype, 31% lost the virulent phenotype (race 0), and 1% gained virulence to the *Php* gene (race 1). A subset of 69 zoospore progeny were further subjected to fluorescent amplified fragment length polymorphism (FAFLP) analysis to investigate genetic diversity generated through asexual reproduction. Based on the FAFLP analysis, estimated total gene diversity (Ht) ranged from 0.1512 to 0.2278 within each race. Estimates of genetic diversity (Hj) within each group of related asexual zoospores ranged from 0.17013 to 0.44196. The progeny that were least phenotypically variable had the highest estimate of diversity. Phenotypic and genotypic investigations reveal that asexual reproduction may be a major source of variation in natural populations of *P. nicotianae*.

INTRODUCTION

Black shank of tobacco, caused by the soilborne oomycete *Phytophthora nicotianae*, occurs world-wide and causes yield losses on all tobacco types (13, 33). Infection can occur at any growth stage in the roots, stems, and leaves and leads to root and stem necrosis, wilting, chlorosis, disking of the pith, and death (13, 20).

Four races of the black shank pathogen (0, 1, 2, and 3) have been reported in tobacco production areas (4, 20, 23, 28). Race 0 is the wild-type race and occurs in all regions where

tobacco is grown (20). It does not incite disease on any tobacco varieties that have single-gene resistance.

Race 1 was first described in the 1960s in black shank nurseries based on its ability to cause disease on *Nicotiana plumbaginifolia* and breeding lines that incorporated the resistance gene from this species (4). The *Php* gene from *N. plumbaginifolia* confers complete resistance to the wild-type race 0 and is overcome by race 1. The *Phl* gene from *N. longiflora* is deployed only as a hybrid in the burley variety KY 14xL8. The *Phl* gene is also overcome by race 1. Incidence of race 1 has increased in all tobacco production areas since the 1990s when single-gene resistance from the *Php* gene first became widely deployed in agronomically acceptable varieties. Race 1 is now the dominant race of the black shank pathogen in NC (Gallup, Chapter 3) and Georgia (10). Sullivan (36) found that levels of race 1 can increase from undetectable to detectable levels after just one season of deploying *Php*-gene resistance. Race 1 levels continue to increase in *P. nicotianae* populations due to growers planting tobacco varieties with single-gene resistance (Gallup Chapter 3). However, Sullivan (36) reported that race 1 was less fit than race 0 and in multiple field locations diminished in prevalence compared to race 0 when selection pressure from single-gene resistance was removed. An objective of this study was to determine the stability of race 1 under controlled inoculations of varieties with no single-gene selection pressure.

Race 2 has been reported only from South Africa. It was defined based on a differential response on varieties KY 14xL8, Burley 21xL8, and Delcrest 202. According to the authors, Delcrest 202 confers resistance to race 2 via a single-dominant resistance gene

(28). In a recent survey of tobacco areas in South Africa, no race 2 was identified and the race is not known to occur elsewhere (39).

Race 3 of *P. nicotianae* was first reported in Connecticut (CT) on cigar-wrapper tobacco. In that study, isolates from CT tobacco differed in virulence from race 0 by causing limited stem necrosis on 56±4% of the stem-inoculated seedlings of the burley breeding line L8, which is homozygous for the *Phl* gene (24). Recently, race 3 also was described from NC. The race 3 phenotype was confirmed, noting the potential for root damage from infested field soil and root inoculations (Gallup Chapter 1). Unlike race 1, race 3 discriminates between the two sources of single-gene resistance. Race 3 is able to incite disease on root-inoculated tobacco varieties ultilizing the *Phl* gene, but not the *Php* gene.

Sullivan (35) recovered isolates with the race 3 phenotype from field microplots infested with race 0 and planted each year with varieties with only multigenic resistance (Gallup Chapter 1). Multigenic resistance confers low to high levels of partial resistance to the black shank pathogen and imposes no selection pressure on the pathogen for the development of races (7, 15, 17, 33, 42). Similarly, in a recent NC black shank survey, race 3 was identified most commonly and most abundantly in race 0 fields with no known history of selection pressure from single-gene resistance (Gallup Chapter 3). Therefore, an objective of this study was to determine if race 3 develops from race 0 in soil during pathogenesis and during asexual reproduction of race 0 parental isolates.

It is generally assumed that asexual zoospores are the primary infective propagule in primary and secondary infections by *P. nicotianae* (13). Variability in virulence among asexual spores has been documented in numerous other *Phytophthora* pathosystems (2, 8,

30). Zoospores may represent a major source of variability in *Phytophthora* spp. (1, 11, 22). In tobacco, it is hypothesized that *P. nicotianae* race 1 is present below detectable levels as a natural variant of field populations, then increases to detectable levels as a result of selection pressure from single-gene resistance (5, 36). The role of variability generated via asexual reproduction has not been investigated in this pathosystem.

Variability that gives rise to new races may also arise during sexual reproduction. Although sexual sporulation is documented widely *in vitro* (3, 9, 19), it has never been observed under field conditions for *P. nicotianae*. A recent NC black shank survey revealed that some populations of *P. nicotianae* from tobacco are entirely one mating type, while others have populations of mixed mating types (Gallup Chapter 3). Populations comprised of only a single mating type may be due to a founder effect from the introduction of a *P. nicotianae* isolate of that mating type (6). Populations with both mating types may be the result of multiple introductions, or a mating type shift may have enabled the establishment of the opposite mating type within the field. Mating type shifts have been documented *in vitro* in response to exposure to chemicals, long-term storage, and repeated hyphal tip transfers (6, 18, 27, 29, 38). However mating type shifts have never been reported under field conditions. Therefore, the stability of mating types was explored in infested soil to determine whether mating type shifts occur under field conditions.

In this study we investigated the stability of *P. nicotianae* race and mating type phenotypes when no selection pressure was applied through single-gene resistance. Populations of *P. nicotianae* were followed during infection cycles on roots of susceptible plants and through asexual sporulation of single-zoospore-derived progeny.

The ability of new virulent races to develop within asexual populations suggests that genetic and phenotypic variation can arise through asexual reproduction. In addition to phenotypic variability, fluorescent amplified fragment length polymorphism (FAFLP) was used to investigate genetic variability generated through asexual reproduction among 11 single-spore isolates of the pathogen representing the three races reported from NC. The generation of genetic variability through asexual reproduction would present significant potential for an important change in pathogen populations, such as shifts in race or resistance to fungicides.

MATERIALS AND METHODS

Development and stability of races in soil. The test was conducted as a factorial split-plot design with inoculum race as the main plot and host variety as the subplot. Greenhouse flats (51 x 36 x 9.5 cm) were filled with a 1:1:1 (vol:vol:vol) mixture of steam-pasteurized soil, Metro mix, and coarse builder's sand then each was planted with one variety of 5-wk-old tobacco seedlings. Varieties used varied in level of partial resistance and included: K326 (low), K149 (moderate), and K346 (high). Three wks after transplant, the soil in each flat was infested with oat grains colonized by an isolate of *P. nicotianae* previously identified as race 0, 1, or 3. One isolate of each race was used. The race 0 isolate originated from Edgecombe Co., NC; the race 1 isolate originated from Duplin Co., NC; and the race 3 isolate was collected from a field microplot study (35) from soil that was infested with a race 0 isolate and planted with varieties with partial resistance. All isolates were the A2 mating type. Flats were blocked by race and spatially separated by splash guards to reduce the

potential for splash contamination from flats infested with different races. There were three replicate flats for each race x variety combination. The test was repeated once.

Inoculum preparation. Inoculum of each isolate was prepared by placing sterile oat grains onto a 3- to 7-day-old culture grown on 5% clarified carrot agar (CA) (36) Sterile oat grains were prepared by autoclaving 500 cc with 300 ml deionized (DI) water for 3 consecutive days. The pathogen colonized the oat grains during a 7- to 10-day incubation at room temperature. To infest soil, 30 colonized oat grains were removed directly from each plate using sterile technique, and placed in the soil around the root zones of the 8-wk-old seedlings within a flat. Seedlings were continually replaced as they died over the subsequent 5-month period. Greenhouse temperatures were set for 29ºC day and 20ºC night, and high intensity lights were set to supplement natural lighting to provide a minimum of 12-hr of light per day.

Isolate recovery. After 5 months, isolates were recovered from the soil and screened for race. To recover isolates, 10 soil cores were taken from each flat and mixed in a plastic bag. Individual *P. nicotianae* isolates were collected from the soil according to Sullivan (35), with some modifications. Briefly, two 0.5-g subsamples were each suspended in 25 ml DI water and distributed over 5 plates of PARPH V8 semi-selective medium. The PARPH medium utilizes 5% clarified V8 juice as the basal medium and is amended with pentachloronitrobenzene, hymexazol, ampicillin, rifampcin, and pimiricin (17, 32). The plates were incubated at room temperature for 48 hrs then the soil was washed from the agar surface. Individual colonies were immediately transferred to fresh PARPH. Isolates were successively transferred to PARPH until devoid of bacterial contaminants. Pure cultures were

maintained by repeated transfer on CA until ready for race screenings. Twenty-five isolates were collected from each flat, resulting in 75 isolates per isolate x variety treatment for each run of the experiment.

Race determination. The race of each isolate obtained from infested soil was determined as previously reported (Gallup Chapter 1). Four-wk-old seedlings of each differential variety, Hicks (susceptible), NC 1071 (*Php*), and KY 14xL8 (*Phl*), were inoculated with colonized oat grains. Plants were scored for the presence or absence of symptoms. Isolates that only caused symptoms on the susceptible variety Hicks were designated as race 0. If all differential varieties were symptomatic, then the isolate was scored as race 1. If Hicks and at least one KY 14xL8 seedling were symptomatic and no NC 1071 seedlings developed symptoms, then the isolate was scored as race 3.

*Mating type analysis***.** Isolates also were screened for mating type to determine if changes in mating type occurred during the experiment. Mating type was identified by pairing each isolate separately with a known A1 and a known A2 tester isolate of *P. nicotianae* on CA amended with water-soluble cholesterol (CAS). CA was made according to Sullivan *et al.* (36). Before pouring the medium, 5 mg of water-soluble cholesterol (Sigma-Aldrich, Inc. St. Louis, MO) was suspended in 5 ml of DI water. The suspension was filter-sterilized directly into the medium using a needleless 5 cc syringe attached to a 0.2 µm syringe filter (NALGENE, NUNC, Thermo Scientific, Rochester, NY). The medium was stirred with a magnetic stir bar and poured into 9 cm diam Petri dishes. Pairings were conducted by placing a 1 cm diam colonized agar plug of an isolate on one side of each of two Petri dishes containing CAS. A similar colonized plug of the A1 tester was placed on the

opposite side of one Petri dish, and a plug of the A2 tester was placed on the opposite side of the other dish. The Petri dishes were wrapped in Parafilm® and allowed to incubate in the dark at room temperature for 4 wks. After 4 wks, the center region, where the hyphae of the two isolates grew together, was scanned for the presence of oospores under an inverted compound light microscope. If oospores were present when paired with the A1 tester, then the isolate was an A2, and vice versa. If no oospores were found, then the isolate was paired again using a different set of A1 and A2 testers. If oospores still did not form, then the isolate was designated as A0.

Single-zoospore progeny. *Isolation of single-zoospore isolates.* To obtain individual zoospore isolates of selected isolates, leaf disks were cut from cv. Hicks tobacco leaves using a no. 5 cork borer and autoclaved in DI water. Sterile leaf disks were placed on the surface of 7- to 9-day-old cultures of *P. nicotianae* races 0, 1, and 3. Plates were incubated in the dark at 25ºC for 2 days then colonized leaf disks were carefully lifted and transferred to Petri dishes containing 15-20 ml sterile DI water. The dishes were subsequently incubated for 2 days in the dark at 25ºC, during which copious sporangia formed along the cut edge of the leaf disks. Leaf disks were washed 3 times in sterile DI water then incubated in sterile DI water at 4ºC for 30 min. Zoospore release was observed after bringing the leaf disks back to room temperature. After zoospore release occurred, 100 µl of a 1:10 dilution of the zoospore suspension was pipetted onto the center of Petri dishes containing 5% CA amended with 250 mg filter-sterilized ampicillin. The zoospores were allowed to encyst for 5 min before gently spreading the zoospore suspension across the agar surface using a sterile bent-glass rod. Following 18-24 hr incubation in the dark at 25ºC, individual germinating zoospores were

transferred to fresh 5% CA. Isolates were maintained on 5% CA until needed for race or molecular characterization.

Thirty single-zoospore isolates were obtained and screened for race from each of three race 0, three race 1, and five race 3 isolates. These isolates were selected to represent a range of geographic locations in NC and tobacco types (Table 2.1). One zoospore isolate then was selected from each of the 11 isolates based on a clear race phenotype, and 100 zoospore isolates were then collected from each of the 11 selected parents. These zoospore progeny were examined for race and mating type as described above, and for FALFP genotype as described below.

Variability in FAFLP DNA Fingerprints. The FALFLP protocol is modified from Vos *et al.* (41), which did not utilize fluorescent probes for detecting amplified DNA fragments. This FALFP protocol is adapted from Desai (12). Reaction conditions were optimized for *P. nicotianae* and were modified if the source of reagents required different conditions than were described by Desai.

Isolate selection and DNA extraction. Zoospore isolates were chosen for FAFLP characterization to represent the range of phenotypes recovered within each parental grouping. For example, a representative sample of race 0 and race 3 zoospore isolates were selected from each race 0 progenitor isolate. Likewise, race 0 and race 3 zoospore isolates were selected from a race 3 progenitor. A total of 69 zoospore progeny were chosen. Between 4 and 9 zoospore progeny were selected from each parental grouping.

Three plugs of each isolate were transferred to each of four Petri dishes filled with 25 ml potato dextrose broth (Difco™). Plates were incubated for 7 days in the dark at 25ºC. The four plates were combined, agar plugs were removed, and the mycelia were rinsed with sterile DI water by vacuum filtration. Mycelia were lyophilized in 1.5 ml microcentrifuge tubes, and the freeze-dried mycelium of each isolate was ground in liquid nitrogen using a sterile microcentrifuge pestle. DNA was isolated by adding 500 µl of cetyl trimethyl ammonium bromide (CTAB) extraction buffer to each sample. The mycelial powder was suspended with a sterile dissecting needle. Samples were incubated at 65ºC for 60 min in a water bath. An equal volume (500 µl) of phenol-chloroform-isoamyl alcohol (25:24:1) was added and gently shaken for 10 min to maintain an emulsion. Tubes were centrifuged at 13,000 x g for 15 min. The upper aqueous phase was removed and transferred to a clean microcentrifuge tube. The phenol-chloroform-isoamyl alcohol step and centrifugation steps were repeated. The aqueous phase was again removed and transferred to a clean tube. Fifty µl RNAse A was added to each tube then incubated at 37ºC for 30 min. The phenol-chloroform step was then repeated for a third time using 250 µl of phenol-chloroform-isoamyl alcohol. Samples were centrifuged for 15 min at 13,000 x g and 150 µl of the upper aqueous was removed and transferred to a clean tube. An equal volume of 95% ethanol was added to precipitate the DNA overnight at 4ºC. The precipitate was then collected by centrifugation for 5 min at 13,000 x g. The supernatant was discarded and the pellets were washed briefly with 100 µl of cold 80% ethanol. After removing the alcohol, the pellets were dried completely in a DNA SpeedVac. The DNA was resuspended overnight in 30 µl of

sterile nuclease-free water. The quality and quantity of DNA was assessed by gel electrophoresis and via a NanoDrop1000 Spectrophotometer (Thermo Fisher Scientific Inc.).

Restriction Digest. DNA samples were standardized to 20 ng/µl. Ten µl of each standardized sample was added to a 96-well PCR plate, leaving the last three wells empty for controls. DNA was co-digested with restriction enzymes *Eco*RI (New England Biolabs, Inc.) and *Mse*I (New England Biolabs, Inc.). An aliquot of 10.25 µl sterile nuclease-free water, 0.25 µl Bovine Serium Albumin (BSA), 2.5 µl NEBuffer 2 10x (New England Biolabs, Inc.), 1 µl *Eco*RI (5U/µl), and 1 µl *Mse*I (1U/µl) was added to each well containing DNA template plus an additional well to serve as a control. The plate was incubated in a thermal cycler for 2 hrs at 37°C followed by 70°C for 5 min to inactivate the restriction enzymes. Contents were collected by centrifugation.

Ligation of adapters. Four single-stranded oligonucleotides were used to create an *Eco*RI and an *Mse*I adapter (Table 2.3). 25 µl (100 µM) of each single-stranded *Eco*RI oligo was added to 450 µl of TE buffer in a 1.5 ml microcentrifuge tube. 250 µl (100 µM) of each single-stranded *Mse*I oligo was combined in a 1.5 ml microcentrifuge tube. The *Eco*RI and *Mse*I adapter mixtures were incubated for 5 min at 95°C in a dry bath. The entire heat block containing the tubes was then removed and allowed to slowly cool to room temperature. The adapters (Table 2.4) were stored at -20°C until needed. Adapters were annealed to the digested template DNA by adding an aliquot of 7.4 µl nuclease-free water, 1.5 µl 10x T4 rxn buffer, 0.5 µl *Eco*RI adaptor, and 0.5 µl *Mse*I adaptor, and 0.1 µl T4 DNA ligase (New England Biolab, Inc.) to each well including the no-DNA control. The 96-well plate was incubated at 20°C for 3 hrs. Digestion was confirmed by gel electrophoresis. The prepared

template DNA was diluted 1:10 into a new 96-well plate, which was used in the preselective amplification step.

Primer selection. Twelve primer combinations were screened using a representative subsample of 11 single-zoospore progenitor and progeny isolates. Initial screenings included the combinations EcoRI-G + MseI-C; EcoRI-G + MseI-G; EcoRI-G + MseI-CA; EcoRI-GC + MseI-CC; EcoRI-GC + MseI-CA; EcoRI-GG + MseI-CC; EcoRI-GG + MseI-CA; EcoRI-A + MseI-C; EcoRI-A + MseI-G; EcoRI-A + MseI-CA; EcoRI-AC + MseI-CC; and EcoRI-AC + MseI-CA. Amplifications were performed as described below, except only the fluorescent probe 6FAM was used in the selective amplification. The primer combination EcoRI-G + MseI-CA was selected for further investigation because it gave many bright, easily resolvable bands that could be used to identify differences between related progeny. Primer combinations with additional selective nucleotides did not yield enough bands to identify differences between related progeny.

Preselective amplification. For the preselective amplification step, 1 µl of the diluted prepared template DNA was mixed with 1 µl of each preselective primer (10 µM) (Table 2.5), 1 µl dNTPs (10 mM each) (Qiagen), 0.25 µl Taq (5U/µl), 1 µl $MgCl_2$ (25 mM), 17.25 µl sterile nuclease-free water, and 2.5 µl 10x CoralLoad PCR Buffer. The 10x CoralLoad PCR Buffer, Taq, and $MgCl_2$ are all supplied in the Qiagen Taq DNA Polymerase kit. The components for the preselective amplification were added to the restriction/digestion control as well as a second negative control for the preselective amplification to ensure that the reagents were not contaminated at either step of the process. Amplification was accomplished by PCR in an Eppendorf Mastercycler Thermalcycler (Eppendorf Scientific,

Westbury, NY). The thermal cycling parameters were 94°C for 1 min; 35 cycles of 30 sec annealing at 56°C, 1 min of extension at 72°C, and 30 sec of denaturation at 94; followed by one extension cycle at 72°C for 10 min. Following amplification, the PCR product was visualized as a smear on a 1% agarose gel and photographed under shortwave UV using the UVP BioDoc-It System. Preselective amplicons were diluted 1:10.

Selective amplification. The selective amplification step was repeated four times for each primer set, each time using a different fluorescent probe: 6FAM (blue), NED (yellow), PET (red), or VIC (green) (Table 2.5). Fluorescently labeled primers were custom ordered from Applied Biosystems. The selective amplification was performed as described for the preselective amplification, except different primers were used (Table 2.5). The components for the selective amplification were added to the restriction/digestion control, the preselective amplification control, as well as a third negative control for the selective amplification to ensure that the reagents were not contaminated at any step of the process. Amplification was accomplished by PCR in an Eppendorf Mastercycler Thermalcycler (Eppendorf Scientific, Westbury, NY). The thermal cycling parameters were the same as that of the preselective amplification. Amplicons were visualized as described above. Selective amplicons were diluted 1:10.

Fragment separation and visualization. In a semi-skirted 96-well plate, 1 µl of each of the four diluted fluorescently labeled selective amplicons were combined with 5.5 µl Hi-Di Formamide (Applied Biosystems cat. no. 4311320) and 0.5 µl 600 LIZ size standard (Applied Biosystems cat. no. 4366589). Plates were heat sealed with an aluminum foil. DNA was denatured at 94°C for 30 sec then stored at 4°C until visualized. Fragments were

automatically separated and visualized on an Applied Biosystems 3730xl DNA Analyzer by the North Carolina State University Genomic Sciences Laboratory. The software program GeneMarker® (SoftGenetics, LLC. State College, PA) was used to assess the FAFLP fingerprints and generate a binomial table ("1" for present and "0" for absent). The binomial data were imported into AFLP Surv (40). Nei's estimate of genetic diversity within each population was quantified by calculating the percentage polymorphic loci (PLP) at the 5%level and expected heterozygosity (HE) according to the protocols of Lynch & Milligan (21) and Nei and Li (26). AFLP Surv was used to create 1000 bootstrapped datasets which were imported into MEGA version 4 (37) to do unweighted pairgroup arithmetic means (UPMGA)-based cluster analysis.

RESULTS

Development and stability of races in soil. There was no run effect of treatments, so data were combined for analysis and presentation. Likewise, there was no effect of variety on the race profile at the end of the 5-month period. Therefore, data across the subplots (variety) also were combined within the main plots (race) for subsequent analyses. Only the race of the initial inoculum influenced the final race profile observed.

Race determination. The final race structure from soil infested with race 1 was significantly different from the race structure in soil infested with races 0 and 3. Approximately 91% of the isolates recovered from the soil infested with race 1 were race 1, with 6% race 0 and 3% race 3 isolates recovered (Table 2.2).

The race structure among isolates recovered from soil infested with races 0 or 3 were not significantly different from each other. Both treatments resulted in approximately 67% percent race 0, 31% race 3, and a low percentage of race 1 (Table 2.2). Race 1 was recovered in both runs of the experiment and from multiple replications across treatments.

*Mating types***.** All recovered isolates retained the A2 mating type of the initial inoculum, except a few isolates that failed to produce oospores and were designated as the neutral mating type, A0. The percent of A0 isolates ranged between 0 and 6 percent for each inoculum x variety treatment.

Single-zoospore progeny. The race 1 phenotype was the most stable in the zoospore progeny of the three races studied. Between 99% and 100% of the progeny retained the virulence phenotype of the parental zoospore isolate. Only one isolate from one race 1 parent had the race 3 phenotype, losing virulence to the *Php* gene (Table 2.1).

The wild-type race, race 0, generated significant levels of phenotypic variation. Only 46-87% of the zoospore progeny retained the parental phenotype. The remaining zoospore progeny were race 3, gaining virulence to the *Phl* gene (Table 2.1).

The race 3 phenotype also generated significant variability in the single generation of zoospores. Four of the progenitor zoospore isolates yielded a ratio of approximately 2:1 race 3: race 0 zoospore progeny. No isolate gained virulence to the *Php* gene (race 1). One progenitor isolate, 129-7, yielded 80% race 3 and 19% race 0 progeny, and one nonpathogenic isolate (Table 2.1).

Variability in FAFLP DNA Fingerprints. In all, 69 *P. nicotianae* zoospore isolates were analyzed with one FAFLP primer set (Table 2.6). Seventy-eight loci were scored from

150 to 600 bp. Every locus was polymorphic, yielding a unique AFLP profile for each isolate.

No locus correlated to a specific race or group of zoospore progeny isolates. In fact, the UPGMA-based cluster analysis of the amplification patterns did not separate the *P. nicotianae* zoospore isolates by progeny group, by race of the progenitor isolate, nor by race of the progeny zoospores (Fig. 2.1).

The estimate of total genetic variability (Ht) across all isolates was 0.2724 ($\pm$0.023264), with 71.2% of the variability due to within population diversity (Hw) and 28.2% from between population diversity (Hb). Total genetic variability (Ht) of zoospore progeny derived from race 3 isolates was 0.2803$\pm$0.018572 with over 80% of the variability due to within-population differences (Table 2.7). Genetic variability (Ht) of race 1, which was the most phenotypically stable, was 0.3357, 84.8% of which was due to within-population differences. The variability of each zoospore population derived from race 1 zoospore isolates ranged from 0.33586 to the highest variability estimate of all, 0.44196. Zoospores derived from the race 1 isolate 25-25 were the most genotypically variable, yet only 1 of the 93 zoospore progeny from isolate 25-25 changed in virulence phenotype. In contrast, race 0 was highly phenotypically variable. One-third of isolates recovered from infested soil and zoospore progeny gained virulence to the *Phl* gene (race 3). However, within each population of zoospores, the progeny derived from race 0 isolates had the lowest genetic variability, ranging from the lowest of all, 0.17013 to 0.29587 (Table 2.8). The zoospore progeny from the race 0 isolate 167-21, which had the lowest estimate of diversity, were less phenotypically variable than other race 0 progeny. However, phenotypic variation

was great across all groups of race 0 progeny, and all were less genotypically variable (Tables 2.1 and 2.8).

DISCUSSION

Numerous changes in the virulence phenotype occurred in *P. nicotianae* after only five months of pathogenic activity in the soil and within a single generation of progeny produced during asexual reproduction. Changes observed in race phenotype included additions and losses of virulence to multiple resistance genes, especially in the race 0 and race 3 isolates. Variation in virulence in asexual spores also has been observed in *P. infestans* and *P. megasperma* f. sp. *glycinea* (2, 30).

Sources of variation have been hypothesized to include mutation, mitotic crossing over, and presence of extra-chromosomal elements (31). Mutation was excluded as a likely source of the asexual variability because it would be unlikely that mutations of chromosomal genes in a diploid organism could result in the high frequency of variability observed in oomycete pathogens. Mitotic recombination is a possible origin of the high level of variability because it can reveal recessive variation that was hidden in heterozygotes (2). Lastly, extra-chromosomal genes in zoospore mitochondria are a strong candidate for mutations. In terms of *P. nicotianae* race 3, the mechanism for virulence is unknown. If the gene(s) regulating avirulence and virulence in races 0 and 3 originate in the uniparentally inherited mitochondrial DNA, it is possible that extra-chromosomal mutations could explain this variability.

A spontaneous gain of virulence was reported in the pathogen *Magnaporthe grisea*, a fungal pathogen that causes rice blast (16). In this case, the mobile transposon Pot3 is inserted into the avirulence gene *AVR-Pita*. By interrupting the gene function of *AVR-Pita*, the pathogen became virulent to rice varieties with the resistance gene *Pi-ta*. *P. infestans* is known to harbor three transposons and numerous retrotransposons within an elicitin gene cluster (14). Although not identified yet, it is possible that *P. nicotianae* similarly harbors transposons and/or retrotransposons that may interrupt avirulence gene function in the race 0 wild-type and race 3.

Race 1 was the most stable phenotype both among asexual zoospore progeny and in the soil. Of the 300 total single-zoospore progeny screened for variations in virulence, only one zoospore yielded a change. However, in soil, only 91% of the 430 isolates recovered from flats initially infested with race 1 retained the race 1 phenotype. Of the remaining isolates, 5.6% were identified as race 0 and 3.3% as race 3. The higher percentage of progeny exhibiting a loss of virulence in soil compared to the zoospore progeny is probably due to multiple generations of progeny that occurred in the soil over the five month period.

This is the first report of race 3 as a natural variant of the wild-type race, race 0. Race 3 was recovered among asexual zoospore progeny of race 0 zoospore-progenitor isolates and among isolates recovered from soil initially infested with race 0. The high level of race 3 in the population was unexpected. The stability of race 3 phenotypes within the race 0 population requires further investigation. Race 3 was identified at a 2:1 ratio, race 0: race 3, both among the zoospore progeny of race 0 progenitors and from flats initially infested with race 0. Conversely, race 0 was identified among the zoospore progeny of race 3 progenitors

at a 2:1 ratio, race 3: race 0. In soil, the final ratio with the race 3 inoculum was 2:1, race 0: race 3.

It is difficult to determine from this data whether the 2:1 ratio (race 0: race 3) is an indicator of fitness or if it suggests some mechanism of asexual genetic variation or regulation of virulence gene expression. In the artificial scenario, 2/3 of the race 3 and race 0 zoospore progeny retained the virulence phenotype of the progenitor. This zoospore study represents the natural level of variation in virulence with no exposure to tobacco. When exposed to flue-cured tobacco, only 1/3 of the isolates recovered after 5 months retained the race phenotype of the initial inoculum, indicating that race 3 may be less fit than race 0 when exposed to flue-cured tobacco. The ratio of race 3: race 0 is slightly lower given more time of exposure in natural flue-cured systems. A NC black shank survey was conducted in 2006 (Gallup Chapter 3). Race 3 comprised 30% of *P. nicotianae* populations in fields that had only planted flue-cured tobacco with multigenic resistance.

This same 2:1 ratio was also observed in the first descriptions of race 3 in the 1970s. Race 3 of *P. nicotianae* was first reported from Connecticut (CT) by McIntyre and Taylor (24). They identified isolates collected from the tissue of cigar wrapper tobacco that differed from isolates of races 0 and 1 based on stem-inoculation of L8 (*Phl*) and NC 1071 (*Php*) plants. Their race 0 and 1 isolates gave expected results on resistant and susceptible genotypes, but the CT isolates differed in virulence from race 0 only in that they caused a limited stem necrosis (several cm from the inoculation site) on 56$\pm$4% of the L8 plants that were stem-inoculated (24). However, Gallup and Shew (Chapter 3) suggested that the susceptibility of L8 stems may be due to a difference in *Phl* gene expression in the stem

compared to the roots rather than virulence in the pathogen. This study illustrates that extensive levels of variation in virulence can exist among asexual populations of *P. nicotianae* in tobacco fields such as those that were identified in NC where only one mating type was present.

The FAFLP® technique can yield many genome-wide polymorphic loci that can be used to assess genetic variability among closely related individuals. The technique is useful for assessing variation in a system such as related *P. nicotianae* zoospores for which there is limited *a priori* sequence information, insufficient alternative marker types, closely related individuals, and the potential necessity to amplify many loci in order to locate polymorphisms (25).

FAFLP markers revealed that the overall total genetic variability was 0.2724, and diversity within groups of related zoospore-derived *P. nicotianae* zoospore progeny ranged between 0.17013 to 0.44196. Similar high levels of genetic variation derived from AFLP and RAPD markers were identified among zoospore isolates of *P. infestans* (1). These results indicate that substantial levels of genotypic variation can be generated in a very short generational time scale.

Variability in virulence was not correlated to genetic variability. Race 1, which was the least variable phenotype in infested soil and among zoospore progeny, was the most genetically variable. A previous study similarly found that variation in virulence of *P. infestans* is not indicative of levels of genetic variation derived from AFLP and RAPD markers (1). Alternatively, the race 0 isolate 167-21 was highly variable, yielding significant changes in virulence yet had the lowest estimate of genetic variation.

No FAFLP phenotype was linked to virulence phenotype. The genes that regulate virulence in the black shank pathogen are unknown and represent only a small fraction of the overall genome. Additionally, AFLP markers, which amplify fragments across the entire genome, are neutral, independent of host selection, and do not identify differences in gene expression (1). These results demonstrate that zoospores possess significant levels of variability not only in their genome, but also in their virulence phenotype.

Genetic variability was similarly assessed among single-zoospore progeny of *P. sojae* by Chang-Qing *et al.* (8). A moderate level of genetic variability was identified among related zoospore progeny using the sequence-related amplified polymorphism (SRAP) technique. Of the 98 and 94 amplified bands from two primer combinations, 19.4% and 10.7% were polymorphic. The SRAP technique preferentially targets open reading frames, and observed polymorphisms originate in the variation of the length of introns, promoters and spacers. Genotype was not correlated to observed phenotypes (8).

AFLP markers were applied to two populations of single-zoospore isolates of *P. infestans* (1). For the zoospore progeny of isolate PI-105, 1277 amplicons were scored, of which 22.6% were polymorphic. For the zoospore progeny of isolate PI-1, 743 amplicons were scored, of which 62.9% were polymorphic (1). Similar to observations of *P. sojae* (8), and *P. nicotianae*, no genotype was correlated to observed phenotypes. The authors conclude that a substantial level of genotypic variability exists among asexual progenies of *P. infestans*, regardless of the level of phenotypic variation (1).

This study similarly reveals that *P. nicotianae* generates significant levels of variability during asexual reproduction and that variability can be generated within a very

short generational time frame. Asexual reproduction can also create changes in virulence phenotype, indicated by the frequent variation between race 0 and race 3. Changes in virulence were also identified within asexual populations derived from race 1 isolates. These changes were less prolific, but potentially significant given the polycyclic nature of the disease (13, 33). The variation observed in the genetics and virulence phenotype were not correlated, exemplified by the rapid change in virulence among race 0 zoospore progeny with low levels of genotypic variation as well as the phenotypic stability of race 1 with high levels of genotypic variation. Because of the rapid generation of genetic differences and virulence phenotypes as well as the sheer number of zoospores within a field, there is significant potential for an important change, such as race or resistance to fungicides, and must be taken into account when considering disease management options.

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Table 2.1. Percentage of races of single-zoospore-derived progeny from each *Phytophthora nicotianae* parental zoospore isolate of races 0, 1, and 3.

Parental Isolate[a]	Geographic Origin[b]	Tobacco Source	Race of parental zoospore isolate[c]	No. of zoospore progeny	Percentage of races among zoospore progeny			
					Race 1	Race 0	Race 3	NP[a]
25-25	Yancy Co., NC	Flue-cured	Race 1	93	98.9	0	1.1	0
CS2-9	Caswell Co., NC	Flue-cured	Race 1	100	100	0	0	0
SJ7-15	Surry Co., NC	Flue-cured	Race 1	100	100	0	0	0
75-27	Field Test, NC	Flue-cured	Race 0	100	0	49	51	0
103-18	KY	Burley	Race 0	100	0	64	36	0
167-21	Duplin Co., NC	Flue-cured	Race 0	100	0	87	13	0
31-10	Buncombe Co., NC	Burley	Race 3	92	0	32.1	66.7	1.2
H34-16	Hoke Co., NC	Flue-cured	Race 3	96	3.1	32.3	64.6	0
65-5	Field Test, NC	Flue-cured	Race 3	100	0	36	64	0
82-7	Field Test, NC	Flue-cured	Race 3	100	0	36	64	0
129-7	Field Test, NC	Flue-cured	Race 3	100	0	19	80	1

[a] Parental zoospore isolates selected based on a clear race phenotype during root inoculations of differential tobacco varieties

[b] Location where isolates were first collected from the soil within the root zone of symptomatic tobacco plants. Field test isolates were collected from a microplot study in Clayton, NC from plots infested with race 0 and planted with tobacco varieties with multigenic resistance (Sullivan 2005a).

[c] Races were determined by inoculating a set of host differentials, Hicks, susceptible, NC 1071, with the *Php* gene, KY 14xL8, with the *Phl* gene. Race 0 killed only Hicks, race 1 killed all genotypes, and race 3 only Hicks and KY 14xL8.

Table 2.2. Percentage of each race recovered after five months from infested soil planted with partially resistant tobacco varieties and initially infested with *Phytophthora nicotianae* races 1, 0, and 3.

Inoculum race	N [a]	Races of recovered isolates [b]		
		Race 0	Race 1	Race 3
R-1	439	5.6%	91.1%	3.3%
R-0	431	67.2%	1.6%	31.2%
R-3	429	64.9%	3.1%	32.0%

[a] Number of isolates recovered from flats after five months and screened for race

[b] Races were determined by inoculating a set of host differentials, Hicks, susceptible, NC 1071, with the *Php* gene, KY 14 x L8, with the *Phl* gene. Race 0 killed only Hicks, race 1 killed all genotypes, and race 3 only Hicks and KY 14xL8.

Table 2.3. Single-stranded oligos used to create *Eco*RI and *Mse*I adapters for FALFP investigation of *Phytophthora nicotianae.*

Adapter	**Sequence 5'-3'**
*Eco*RI adapter 1	5’ - CTC GTA GAC TGC GTA CC - 3’
*Eco*RI adapter 2	5’ - AAT TGG TAC GCA GTC TAC - 3’
*Mse*I adapter 1	5’ - GAC GAT GAG TCC TGA G - 3’
*Mse*I adapter 2	5’ - TAC TCA GGA CTC AT - 3’

Table 2.4. Double-stranded *Eco*RI and *Mse*I adapters ligated to digested DNA template in FAFLP investigation of *Phytophthora nicotianae.*

Adapter	**Sequence 5'-3'**
*Eco*RI adapter	5’ - CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA - 3’
*Mse*I adapter	5’ - GACGATGAGTCCTGAG TACTCAGGACTCAT - 3’

Table 2.5. Preselective and selective primers used FALFP amplifications of *Phytophthora nicotianae*.

Primer Name	**Sequence 5'-3'**
*Mse*I+C preselective	5’ - GAT GAG TCC TGA GTA AC - 3’
EcoRI+0 preselective	5’ - GAC TGC GTA CCA ATT C - 3’
*Mse*I+CA selective	5’ - GAT GAG TCC TGA GTA ACA - 3’
EcoRI+G selective, fluorescently labeled	EcoRI+G 6FAM 5’ - 6FAM - GAC TGC GTA CCA ATT CG - 3’
	EcoRI+G NED 5’ - NED - GAC TGC GTA CCA ATT CG - 3’
	EcoRI+G PET 5’ - PET - GAC TGC GTA CCA ATT CG - 3’
	EcoRI+G VIC 5’ - VIC - GAC TGC GTA CCA ATT CG - 3’

Table 2.6. Single-zoospore isolates of Phytophthora nicotianae used for FAFLP investigations.

Isolate	Race	Isolate	Race	Isolate	Race
Race 1 zoospore progeny		75-27-40	3	31-10-75	3
25-25-22	1	75-27-44	3	31-10-79	0
25-25-24	1	75-27-76	0	H34-16-15	0
25-25-25	1	75-27-85	3	H34-16-45	3
25-25-28	1	75-27-90	3	H34-16-68	0
25-25-63	1	75-27-97	0	H34-16-72	0
CS2-9-2	1	103-18-37	3	65-5-24	3
CS2-9-6	1	103-18-60	3	65-5-29	3
CS2-9-7	1	103-18-66	3	65-5-53	3
CS2-9-33	1	103-18-83	0	65-5-56	3
CS2-9-38	1	103-18-98	0	65-5-72	0
CS2-9-54	1	167-21-9	0	82-7-4	0
CS2-9-96	1	167-21-14	0	82-7-37	3
SJ7-15-21	1	167-21-19	0	82-7-47	3
SJ7-15-22	1	167-21-26	0	82-7-50	3
SJ7-15-78	1	167-21-46	0	82-7-78	0
SJ7-15-89	1	167-21-51	0	82-7-102	3
SJ7-15-90	1	167-21-59	0	129-7-24	3
SJ7-15-99	1	*Race 3 zoospore progeny*		129-7-32	0
SJ7-15-101	1	31-10-35	3	129-7-50	3
Race 0 zoospore progeny		31-10-42	3	129-7-65	3
75-27-23	0	31-10-59	3	129-7-67	3
75-27-36	3	31-10-71	3	129-7-88	0
75-27-38	3	31-10-72	3	129-7-94	3

Table 2.7. Population genetic structure of *Phytophthora nicotianae* zoospore populations.

Popn [a]	**n** [b]	**Ht** [c]	**Hw** [d]	**Hb** [e]
All	11	0.2724 (+0.023264)	0.1956 (+0.026666)	0.0769 (+0.290231)
Race 1	3	0.3357 (+0.019207)	0.2848 (+0.000000)	0.0509 (+0.019194)
Race 0	3	0.3654 (+0.062117)	0.1512 (+0.000000)	0.2142 (+0.211234)
Race 3	5	0.2803 (+0.018572)	0.2276 (+0.007596)	0.0527 (+0.138144)

[a] Popn: groups of zoospore progeny included in determining genetic structure (Lynch and Milligan 1994). 'All' includes all groups of zoospore progeny as detailed in table 2.6. The race 1 population includes zoospore progeny from isolates 25-25, CS2-9, and SJ7-15; the race 0 population includes zoospore progeny from isolates 75-27, 103-18, and 167-21; and the race 3 population includes zoospore progeny from isolates 31-10, H34-16, 65-5, 82-7, and 129-7.

[b] n: number of subpopulations. For example, three race 0 progenitor isolates were included in this study.

[c] Ht: the total gene diversity

[d] Hw: mean gene diversity within populations

[e] Hb: mean gene diversity between populations

Table 2.8. Estimates of genetic diversity within each group of zoospore progeny derived from a single-zoospore progenitor isolate (Lynch and Milligan 1994).

Progenitor	Race [a]	n [b]	#loc. [c]	#loc_P [d]	Hj [e]	S.E. (Hj) [f]	VarI% [g]	VarL% [h]
25-25	1	5	53	47	0.44196	0.02512	61.5	38.5
CS2-9	1	7	67	52	0.33976	0.02554	35.7	64.3
SJ7-15	1	7	55	42	0.33586	0.02850	34.5	65.5
75-27	0	9	51	41	0.29587	0.02671	40.7	59.3
103-18	0	5	43	17	0.18307	0.03612	21.3	78.7
167-21	0	7	17	8	0.17013	0.04882	32.6	67.4
31-10	3	7	45	35	0.31927	0.03016	43.2	56.8
H34-16	3	4	45	19	0.32639	0.03743	36.3	63.7
65-5	3	5	43	30	0.31702	0.03434	44.0	56.0
82-7	3	6	39	31	0.38723	0.03552	27.9	72.1
129-7	3	6	55	45	0.35354	0.02626	56.8	43.2

[a] Races were determined by inoculating a set of host differentials, Hicks, susceptible, NC 1071, with the *Php* gene, KY 14xL8, with the *Phl* gene. Race 0 killed only Hicks, race 1 killed all genotypes, and race 3 only Hicks and KY 14xL8.

[b] *n* : average number of scored individuals (n)

[c] *#loc* : number of loci scored

[d] *#loc_P* : number of polymorphic loci at the 5% level, i.e. loci with allelic frequencies lying within the range 0.05 to 0.95

[e] *Hj* : Nei's gene diversity (analogous to H or He)

[f] *S.E. (Hj)* : standard error of Hj

[g] *VarI%* : proportion of Var(Hj) due to sampling of individuals

[h] *VarL%* : proportion of Var(Hj) due to sampling of loci

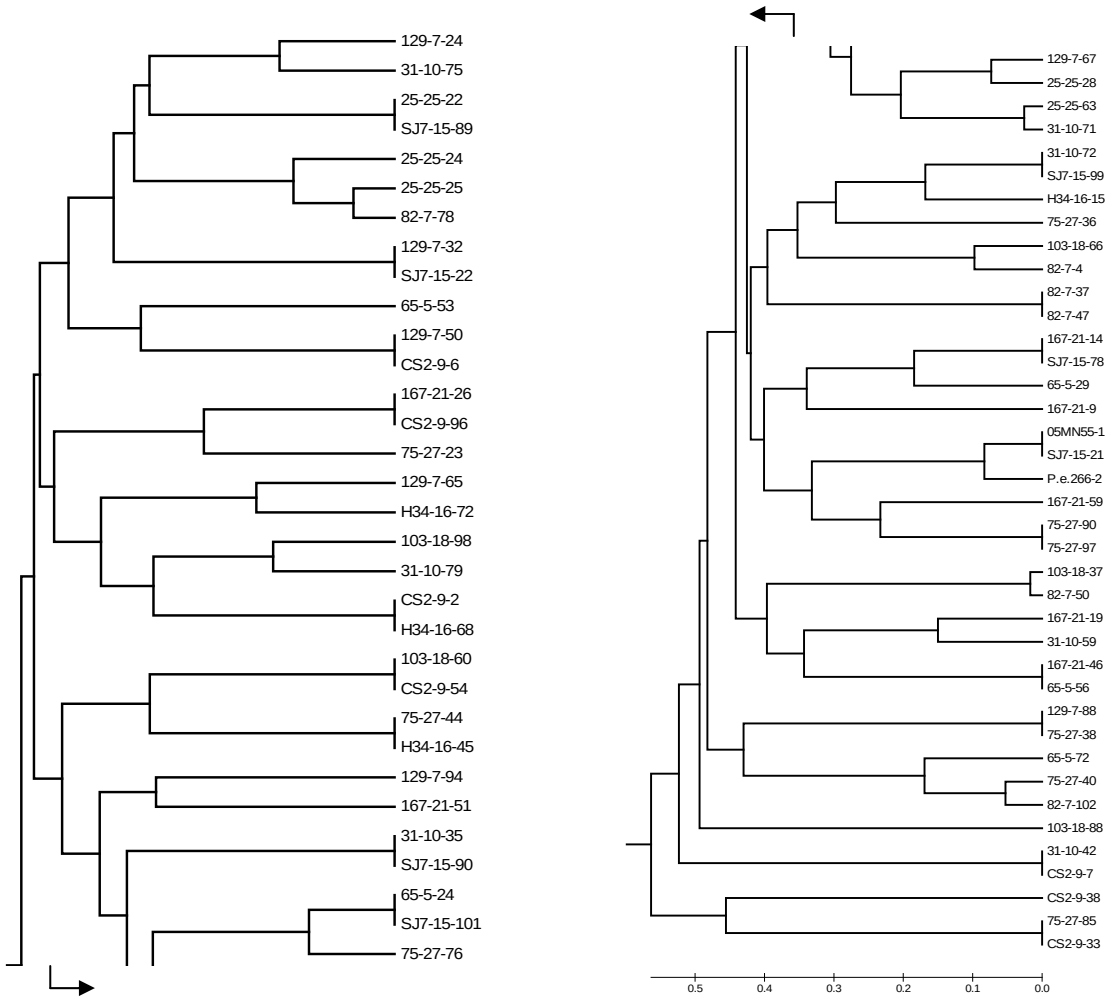

Figure 2.1. Dendogram generated by 1000 bootstrap reiterations using the AFLP Surv and MEGA applications to analyze FAFLP data.of single-zoospore isolates derived from zoospore progenitor isolates. Genetic distance was determined by cluster analysis using the Unweighted Pair-Group Mean Analysis. The scale is the genetic distance calculated using simple matching coefficients. The arrows indicate the separation of the dendogram.

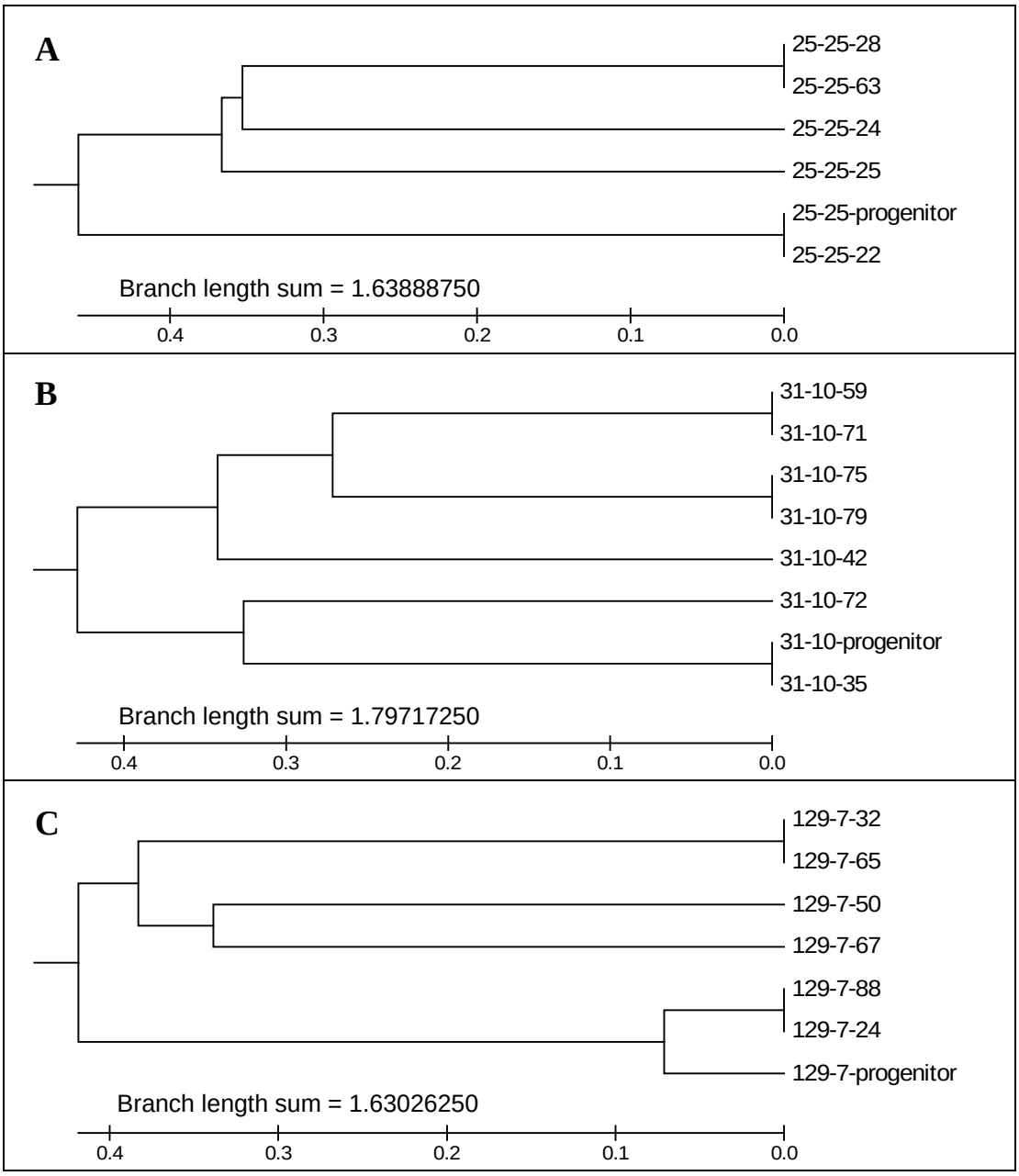

Figure 2.2. Dendograms of representative single-zoospore isolates derived from zoospore progenitors 25-25 (A), 31-10 (B), and 129-7 (C), of races 1, 3, and 0, respectively. Dendograms are inferred based on 1000 bootstrap reiterations using AFLP Surv and the Unweighted Pair-Group Mean Analysis (UPGMA) method (Sneath 1973) in MEGA (Tamura 2007). The scale is the genetic distance calculated using similarity coefficients. Optimal trees with the sum of branch lengths are shown. Trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Chapter 3

Population structure of *Phytophthora nicotianae*, the causal agent of black shank, in North Carolina tobacco fields

Population structure of *Phytophthora nicotianae*, the causal agent of black shank, in North Carolina tobacco fields

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ABSTRACT

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The black shank disease, caused by the oomycete *Phytophthora nicotianae*, is a major threat to tobacco production. Concerns regarding the development of race 1 and resistance gene breakdown have arisen since the widespread deployment of monogenic resistance in agriculturally acceptable cultivars. A state-wide survey of *P. nicotianae* populations was conducted in the major tobacco-producing regions of North Carolina. Isolates were obtained from soil samples collected from 76 tobacco fields in 23 counties and screened for race and mating type. Race 1 was predominant in most tobacco regions, with 59% of fields consisting of 90 to 100% race 1. The coastal plain region in eastern NC had the lowest incidence of race 1. The occurrence of race 1 within fields was positively correlated with the history of monogenic resistance deployment. Race 3 was identified in low frequency throughout the state, primarily in wild-type populations where no monogenic resistance was deployed. A1

and A2 mating types were found throughout the state, and both were recovered concurrently from multiple fields. Pairings of isolates from within fields yielded viable oospores, indicating for the first time the potential for sexual sporulation by *P. nicotianae* in field populations. The loss of complete resistance as an option for disease control will result in a greater demand for chemical control measures. A subset of the survey isolates were screened for sensitivity to the compound mefenoxam. All isolates were sensitive, with a mean EC_{50} value of 0.4 μg/ml mefenoxam. Results reveal a rapid state-wide shift toward race 1, correlating with the deployment of monogenic resistance, and indicate that sexual recombination may be important in generating variation within the pathogen population.

INTRODUCTION

Black shank of tobacco, caused by the soilborne pathogen *Phytophthora nicotianae*, occurs world-wide and affects all tobacco types (17, 40). Infection can occur in the roots, stems, and leaves, and leads to root and stem necrosis, wilting, chlorosis, disking of the pith, and death (17). In North Carolina, black shank caused the highest losses in crop value due to disease in 2008, with losses reported at 4.66% (31).

Disease management relies upon an integration of practices including crop rotation, planting resistant cultivars, and fungicide applications (30, 40). Two types of host resistance are currently available in commercially acceptable cultivars. Fla301 resistance is multigenic and confers low to high levels of partial resistance against all races of *P. nicotianae*. (8, 23, 25, 40, 48). Conversely, complete resistance is monogenically regulated and confers complete resistance to race 0 and no resistance to race 1 of the black shank pathogen. *Php*

and *Phl* are the only genes available that confer complete resistance. The *Php* gene from *Nicotiana plumbaginifolia* is incorporated extensively into flue-cured tobacco and is also used in a few recent burley cultivars. The *Phl* gene from *N. longiflora* is utilized commercially only in the burley tobacco cultivar KY 14xL8 (9, 22). Burley cultivars are grown primarily in the mountain regions of NC, whereas flue-cured tobacco is grown primarily in the central piedmont and the coastal plains in the east. Incidence of race 1 has increased since the widespread deployment of complete resistance in agriculturally acceptable cultivars in the 1990s. Generation of pathogen races is widely reported in other species of *Phytophthora*, but not in *P. nicotianae* (13, 34, 47, 49). The first objective of this study was to elucidate the distribution of races of *P. nicotianae* in North Carolina. A second objective of this study was to describe the mating type distribution of *P. nicotianae* in the state, and determine if genetic diversity in the pathogen population could be enhanced by sexual reproduction. To date, sexual sporulation has been considered unimportant in the epidemiology of the pathogen, but it also has not been thoroughly explored. Sexual sporulation has been documented and investigated in several *in vitro* studies with *P. nicotianae* (10, 20, 21), but it has never been demonstrated in agroecosystems.

Mefenoxam is the primary active ingredient in fungicides used for black shank control (31, 46). Resistance to mefenoxam has not yet been reported in tobacco populations of *P. nicotianae*, but it has been documented in other hosts (18, 19). As monogenic resistance continues to fail, many growers will rely on chemical application as part of a comprehensive control program. Because the most recent sensitivity survey of *P. nicotianae* in tobacco was conducted in 1985 (39), we investigated the current sensitivity of the pathogen population in

NC, and established a baseline sensitivity for race 1 isolates. Current sensitivity levels were compared to previously established baselines for the pathogen (39).

MATERIALS AND METHODS

Isolate recovery. In the summer of 2006, soil was collected from 76 tobacco fields in 23 NC counties, representing the major tobacco-producing regions of the state. Fields were selected based on occurrence of the disease in the summer of 2006 and previous years. Using a 3-cm soil probe, 2 to 4 soil cores were collected 15-20 cm deep and 5-8 cm from the stem of tobacco plants displaying typical black shank symptoms. At least 10 plants were sampled from each field. The soil probe was rinsed with 70% ethanol or 0.5% NaClO between fields and allowed to dry. Samples were stored in a dry, dark location until ready to assay.

Soil assays were conducted according to Sullivan *et al.* (43), with some modifications, in order to collect individual *P. nicotianae* isolates from the soil around each plant. Briefly, three 1-g subsamples were each suspended in 25 ml deionized (DI) water and distributed over 5 dishes of PARPH V8 semi-selective medium. The PARPH medium contained 5% clarified V8 juice as the basal medium and was amended with pentachloronitrobenzene, hymexazol, ampicillin, rifampcin, and pimiricin (25, 37). The dishes were incubated at room temperature for 48 hrs, then the soil was washed from the agar surface. If present, individual colonies were immediately transferred to fresh PARPH. If no colonies were visible, the dishes were allowed to incubate for an additional 24 hrs. If no colonies were present following 72 hrs, the procedure was repeated using 1.5 g of soil per subsample.

If no isolates were recovered from the soil assays, the soil sample was mixed with sterile greenhouse soil and placed in a 10 cm diam clay pot. A 4-wk-old susceptible tobacco seedling, cv. Hicks, was planted into the soil. When a plant developed typical black shank symptoms, a *P. nicotianae* isolate was obtained from the symptomatic stem tissue. Stem tissue was rinsed in 0.5% NaClO for 30 sec, rinsed in sterile DI water for 30 sec, blotted on a sterile paper towel, then placed on the surface of semi-selective PARPH medium.

Isolates were successively transferred to PARPH until free of contaminants, primarily bacteria. Pure cultures were maintained by repeated transfers on 5% clarified carrot agar (CA) (44) until ready for race, mating type, or fungicide sensitivity screenings. Whenever possible, one isolate was collected from each soil sample, resulting in at least 10 isolates from each field infested with *P. nicotianae*.

Cropping history. When possible, cropping histories for the 2001 through 2006 field seasons and GPS coordinates were collected. Cropping histories were examined for use of cultivars with the *Php*-gene. *Php*-gene cultivars were tallied for presence/absence in the 2001-2006 time period and the total number of years deployed.

Race survey. The race of each isolate was identified according to Sullivan *et al*. (44) with some modifications. Briefly, Three 4-wk-old seedlings of each differential cultivar, Hicks (susceptible), NC 1071 (*Php*), and KY 14xL8 (*Phl*), were transplanted into cell packs (72 cells, 4 by 4 by 5 cm) containing a 1:1:1 (vol:vol:vol) mixture of steam-pasteurized soil, Metro mix, and coarse builder's sand. Seedlings of each differential were inoculated 1 wk later with oat grains colonized by a single pathogen isolate. Inoculum was prepared by placing sterile oat grains onto a 3- to 7-day-old CA culture of each isolate. Sterile oat grains

were prepared by autoclaving 500 cc with 300 ml DI water for 3 consecutive days. The pathogen colonized the oat grains during a 7- to 10-day incubation at room temperature. To inoculate the 5-wk-old plants in the greenhouse, oat grains were removed directly from each plate using sterile technique. Two colonized oat grains were placed into each 4x4x5-cm cell, one on either side of the seedling stem. After 14 days, plants were scored for the presence or absence of symptoms. Isolates that only caused symptoms on the susceptible cultivar Hicks were designated as race 0. If all differential cultivars were symptomatic, then the isolate was scored as race 1. If Hicks and at least one KY 14xL8 seedling were symptomatic and no NC 1071 seedlings developed symptoms, then the isolate was scored as race 3.

Correlation between occurrence of race 1 and *Php*-gene deployment. Data from fields with complete cropping histories were used to identify correlations between the deployment of monogenic resistance and the composition of race 1 populations. Data were analyzed using the PROC CORR procedure of SAS (version 9.1, SAS Institute, Cary, NC) in order to obtain Pearson correlations.

Mating type survey and isolate compatibility. The mating type of each isolate was identified by pairing each isolate separately with a known A1 and a known A2 tester isolate of *P. nicotianae* on CA amended with water-soluble cholesterol (CAS). CA was made according to Sullivan (44). Before pouring the medium, 5 mg of water-soluble cholesterol (Sigma-Aldrich, Inc. St. Louis, MO) was suspended in 5 ml of DI water. The suspension was filter-sterilized directly into the medium using a needleless 5 cc syringe attached to a 0.2 µm syringe filter (NALGENE, NUNC, Thermo Scientific, Rochester, NY). The medium was stirred with a magnetic stir bar and poured into 9 cm diam Petri dishes. Pairings were

conducted by placing a 1 cm diam colonized agar plug of an isolate on one side of each of two Petri dishes containing CAS. A similar colonized plug of the A1 tester was placed on the opposite side of one Petri dish, and a plug of the A2 tester was placed on the opposite side of the other dish. The Petri dishes were wrapped in Parafilm® and allowed to incubate in the dark at room temperature for 4 wks. After 4 wks, the center region, where the hyphae of the two isolates joined, was scanned for the presence of oospores under an inverted compound light microscope. If oospores were present when paired with the A1 tester, then the isolate was an A2, and vice versa. If oospores formed with both testers, then the isolate was designated as A1/A2. However, if no oospores were found, then the isolate was paired again using a different set of A1 and A2 testers. If oospores still did not form, then the isolate was designated as A0.

NC fields were tallied for mating type distribution. If the distribution was mixed, then isolates from within a field were paired to each other. Mixed distributions were identified as having isolates of one of the following combinations: A1 and A2; A1 and A1/A2; A2 and A1/A2; or A1, A2, and A1/A2. In cases where both mating types were identified within a field, representative A1, A2, and/or A1/A2 isolates were paired on CAS as described above. A subset of pairings that formed oospores were subjected to a secondary analysis to evaluate percent oospore viability.

To evaluate percent oospore viability, plugs were taken from the growing edge of 3-day-old cultures and paired to each other in five 6-cm Petri dishes containing CAS, except only 3g of Bacto agar was used per liter of medium to give a soft agar medium. Plates were wrapped in Parafilm® and incubated in the dark for 4 wks at 25°C.

Oospores were extracted and stained according to Sutherland (45) with some modifications. To extract oospores from agar, the entire contents of the five dishes were poured into a sterile blender cylinder containing 10 ml sterile DI water and blended until smooth. The suspension was pipetted 1 ml at a time into 1.7 ml microcentrifuge tubes. The tubes were centrifuged for 5 mins at 10,000 rpm and the supernatant was discarded. Sterile DI water, 0.5 ml, was added to each tube, vortexed, centrifuged at 12,000 rpm for 6 min, and the supernatant was discarded again. The wash process was repeated.

To determine viability, oospores were stained with thaizolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich). For this, 0.5 ml MTT was added to each tube and vortexed. The oospore-MTT suspension was incubated in the dark at 35°C for 48 hrs. A droplet of the suspension was placed on a slide with a coverslip and examined at 40x-100x. One-hundred oospores were assessed for viability, using multiple slides if necessary. Viable oospores were red to rose in color, while spores that were black or unstained oospores were considered nonviable.

Mefenoxam Sensitivity. A subset of 120 isolates was screened for sensitivity to mefenoxam (Ridomil Gold® SL, 45.3% a.i). A 1-cm plug was taken from the growing margin of a 3-day-old culture of each isolate and placed in the center of Petri dishes containing 18 ml of 5% CA amended with 0, 0.1, 1, or 10 µg/ml mefenoxam. Plates were incubated in the dark at 25°C for 7 days. Following the 7-day incubation, the largest radius of mycelial growth was measured to the closest millimeter. Treatments were replicated three times, and the experiment was conducted twice. Analysis of variance was performed on percent inhibition and EC_{50} data using the PROC GLM procedure of SAS (version 9.1, SAS

Institute, Cary, NC). Means separation was conducted using the Waller-Duncan K ratio test (k=100). If phenotypic data were missing for an isolate, such as race or mating type, then those isolates were excluded from the comparisons for which the data were missing.

RESULTS

Isolate recovery. When possible, one isolate was recovered from each of at least 10 soil samples per field. However, soil samples were excluded from the study if no isolates were recovered after two soil assays and recovery with the bioassay also was unsuccessful. Therefore, some fields were represented by fewer than 10 pathogen isolates. A total of 581 isolates were included in tests for race and mating type distribution.

Cropping history. In some cases, growers either did not have complete records, did not recall cultivars planted in a field over the preceding 6 yrs, or the farm had recently been acquired and there were no prior records. When analyzing data based on years of resistance gene deployment, only fields with complete records were used. Of the 76 fields surveyed, 30 (40%) had complete records.

Of the fields with complete records, 26 (87%) had been planted in a cultivar with *Php*- or *Phl*-gene resistance at least once from 2001-2006. Cultivars with single-gene resistance were deployed most frequently in the piedmont of NC, averaging 2 seasons from 2001 through 2006 compared to 1.2 and 0.5 seasons for fields in the coastal plain and mountains, respectively. In individual fields, deployment of single-gene resistance ranged from 0 to 4 seasons of the 6 seasons surveyed (Table 3.1).

Across all fields, including those with incomplete cropping histories, 68% of them had a history of deploying single-gene resistance at least once from 2001 to 2006. More specifically, 87% of the fields in the piedmont and 70% of the fields in the coastal plain had a record of planting tobacco cultivars with resistance from the *Php*-gene. Of the fields surveyed in the mountains, only 35% of them had record of planting tobacco cultivars with resistance from the *Php*-gene.

Crop rotation practices also varied by region (Table 3.1). In the coastal plain, growers typically grew tobacco only every third or fourth year, with little use of *Php*-gene resistance, except for Edgecombe and Sampson Counties which were more similar to the piedmont. In the piedmont, growers typically grew tobacco every other year. The cultivars planted often utilized *Php*-gene resistance. The cropping history obtained for fields in the mountain area was less complete, with only 2 fields having complete records from 2001-2006. Continuous planting of tobacco was common in the mountain area, with no history of the *Phl*-gene and limited deployment of *Php*-gene resistance. The tobacco cultivar KT204 was the only source of single-gene resistance utilized in burley tobacco in the mountains (Table 3.1).

Race survey. Four isolates of *P. nicotianae*, 0.5%, were nonpathogenic on all three cultivars (Hicks, NC 1071, and KY 14xL8) following two inoculation attempts. These nonpathogenic isolates were excluded from further evaluation. All other isolates were pathogenic on one or more of the host differentials and were characterized as one of the three races described in NC (Gallup Chapter 1).

Ninety-five percent of all fields, including those with incomplete histories, had race 1 as a component of the pathogen population. Across the state, 75% of the isolates recovered

were race 1, 21% race 0, and 4% race 3 (Table 3.1). The wild-type race, race 0, was recovered from only 33 of the 76 fields surveyed and was not recovered from any fields in six of the counties surveyed. In contrast, race 1 was identified from every county surveyed. The percent of race 1 was greatest in the piedmont, at 89% of the pathogen population, followed by 67% in the mountains, and 50% in the coastal plain (Table 3.2). The race structure between fields varied greatly. Only 4% of the fields had populations comprised entirely of race 0; 20% had populations >50% race 0; 20% had populations >50% race 1; and 56% had populations consisting of only race 1.

Race 3 was identified in 14 of the 76 fields surveyed. This race was most frequently found in populations that were dominated by the wild-type race 0, whereas race 3 was identified in only 5 fields that were comprised predominantly of race 1. The proportion of race 3 isolates within a population also was greater in wild-type populations than in race 1 populations. On average, the percent of race 3 within a field was 30% in wild-type fields, compared to 12% in race 1 fields.

Correlation between occurrence of race 1 and deployment of single-gene resistance. In fields for which complete cropping histories were available, the *P. nicotianae* populations were comprised of 10 to 100% race 1 (Fig. 3.1). Twenty-two fields yielded only race 1 isolates, with the history of single-gene resistance deployment ranging from 0 to 3 years from 2001 to 2006. Five of the fields had no known history of single-gene resistance deployment, yet race 1 levels ranged from 20 to 100%. Most of the race 1 fields utilized single-gene resistance 3 of the 6 years surveyed. The Pearson correlation between the percent

race 1 in the population and years of *Php*-gene deployment was 0.44161, with a p-value of 0.0129.

Forty percent of all the fields with race 1 as a component of the pathogen population, including those with incomplete field histories, had no history of single-gene resistance. In the mountains, race 1 was recovered from 90% of the fields, yet most fields had no history of single-gene resistance. In the piedmont, 87% of the fields with race 1 had a history of *Php*-gene deployment. In the coastal plain, 73% of the fields with race 1 had a history of *Php*-gene deployment.

Four fields were characterized as 100% races 0 and 3, three of which had no known history of deploying single-gene resistance. Field 48 in Sampson County in the NC coastal plain was characterized 100% races 0 and 3 and planted with the cultivar NC71 (*Php*) once between 2002 and 2006.

Mating type survey and isolate compatibility. In total, 23 fields were sampled from six counties in the mountains. *P. nicotianae* populations were all A1 in seven fields, and all A2 in two fields. Fourteen fields had a mixed mating type distribution, 12 of which had sexually compatible isolates (Table 3.3). The A1 and A2 mating types were also found in flue-cured tobacco regions of the state. Several fields in the piedmont regions contained isolates of both mating types that yielded oospore progeny.

Viable oospores were observed in pairings of isolates from the same field. Compatible isolates from a single location in Guilford County were paired to each other, and oospores were stained to assess viability. The percentage of oospores that were viable varied depending upon the isolate combination (Table 3.4). For example, isolate GC-11 yielded

20% and 48% viable oospores when paired with isolate GC-4 and GC-9, respectively. Each set of pairings from different fields in Surry County yielded similar levels of viable oospores, between 44 and 48%.

Mefenoxam sensitivity. Analysis of percent inhibition revealed no run effect (P=.5557), so data from both runs were combined for analysis. Percent inhibition was calculated by averaging the radius of the mycelial growth across all replications and dividing it by the average mycelial growth on non-amended medium. Mean percent inhibition increased logarithmically (R = 0.82) with increasing concentrations of mefenoxam (Table 3.5).

The range of responses among isolates was great. For example, isolate 129 from Haywood County was inhibited by 85% at the lowest concentration and 100% at the highest concentration. Whereas other isolates, such as isolate 96, had increased growth at the lowest concentration and was inhibited by 85% at the highest concentration. Individual responses varied most at 0.1 μg/ml of mefenoxam. At 0.1 μg/ml mefenoxam, 31% of the isolates had a larger growth radius than the fungicide-free control. However, growth at this concentration was sparse and generally weak.

The mean EC_{50} for all isolates was 0.4 μg/ml mefenoxam. One isolate from Sampson County, isolate 59, was treated as an outlier and excluded from the data set. This isolate was inhibited by only 36.6 and 66.3% at 1 and 10 μg/ml, respectively. Isolate 41 from Guilford County was excluded from EC_{50} analyses because inhibition was 72.1, 82.7, and 76.0% at 0.1, 1, and 10 μg/ml, respectively. Therefore, the regression equation has an R^2 value of only 0.14 and could not be used to accurately calculate an EC_{50} value.

The mean EC_{50} differed among counties (Table 3.6). The most sensitive isolates were found in Stokes, Craven, Rockingham, and Alamance counties, where the EC_{50} values were less than 0.0086 µg/ml. The least sensitive isolates were found in Sampson and Watauga counties, where the mean EC_{50} ranged from 0.7621 to 0.7967 µg/ml mefenoxam.

Within a county, fields typically had statistically similar EC_{50} values (P=.2215 - .9624), with the exception of Rockingham County (P<0.0001). In Rockingham County, one field was statistically less sensitive than the other two fields, although all fields were highly sensitive to the fungicide.

Sensitivity to mefenoxam was similar among the A1, A2, and A1/A2 mating types. The neutral mating type, A0, was less sensitive than the A1 and A1/A2 mating types, but not the A2 (Table 3.7). There were no differences in sensitivity to mefenoxam among races of *P. nicotianae*. Mean EC_{50} values for each race ranged from 0.3747 to 0.6388 µg/ml mefenoxam (Table 3.8).

DISCUSSION

In 2006, race 1 was the most prevalent race of *P. nicotianae* in NC tobacco fields, comprising 79% of all isolates recovered. Race 1 of *P. nicotianae* was first reported in NC in the 1960s (3, 4). These first observations of race 1 were in black shank nurseries where breeding material that contained the resistance factor from *N. plumbaginifolia* was being screened. Apple (4) concluded that race 1 would rapidly become prevalent in the wild type population of the pathogen, so this single-gene source of resistance was not introduced into tobacco varieties for release to growers. Race 1 was not a concern in the flue-cured regions

of the US until the release of Coker 371-Gold in the 1980s. This variety was not popular with growers in some areas, but race 1 was reported across Georgia following the deployment of this variety (11). The source of complete resistance in Coker 371-Gold was unknown at first (8), but later was identified as the *Php* gene from *N. plumbaginifolia* (22). Once the resistance gene was incorporated into other more agronomically acceptable varieties, for example NC 71, race 1 was confirmed from many other areas.

This survey, conducted 40 years after Apple's initial race 1 report and only 10 years following the release of NC 71, represents the first survey of *P. nicotianae* races across all NC tobacco production regions. The *Php* gene from *N. plumbaginifolia* is widely deployed in flue-cured tobacco and released recently in burley tobacco. Race 1 was recovered in very high proportion from all counties in all tobacco-producing regions of the state. The prevalence of race 1 in *P. nicotianae* populations substantiates Apple's (4) concerns regarding the rapid shift to race 1 following deployment of *N. plumbaginifolia*-type resistance.

As part of this survey, cropping histories were collected from growers for the 2001 through 2006 field seasons. In fields where complete records were obtained, the Pearson Correlation for number of years that the *Php* gene was deployed and the proportion of *P. nicotianae* race 1 was moderately positive, 0.44161, with a P-value of 0.0129. This correlation was not as strong. However, two data points had a strong effect on this correlation, and if removed as outliers, the correlation increased to 0.68677 with a P-value of <0.0001.

The first data point, field 20 in Edgecombe County, had a population of only 30% race 1 despite a 4-year history of *Php*-gene deployment. However, only 3 isolates were successfully obtained from this field, so the data point may not be representative of the field population. Based on the long history of *Php* gene deployment, a higher sampling rate probably would have revealed a greater proportion of race 1 in the population.

The second data point, field 34 in Madison County, was characterized as 100% race 1 despite the absence of selection pressure from single-gene resistance in the 6 yrs surveyed. Madison County is located in the mountainous region of NC where burley tobacco is typically grown. Only the most current burley varieties incorporate single-gene resistance from the *Php* gene. However, one burley cultivar, KY 14xL8, utilizes the *Phl* gene from *N. longiflora*. Like the *Php* gene, the *Phl* gene leads to an increase in race 1 in *P. nicotianae* populations (6, 22). Race 1 of *P. nicotianae* is not known to occur at the levels observed in field 34 without selection pressure from single-gene resistance. In fact, race 1 was found in high frequency throughout the mountains despite the apparent lack of selection pressure. This level of race 1 indicates that the pathogen was probably introduced into multiple areas on transplants that were infected with race 1, or possibly, that single-gene resistance from the *Phl* gene had been used in years prior to 2001.

Greenhouse inoculation studies have demonstrated the potential for race 1 to remain stable in the absence of selection pressure (Gallup Chapter 2). Flats planted with increasing levels of Fla301 resistance were inoculated with a race 1 isolate, and after five months, 299 of 300 recovered isolates retained the race phenotype of the initial inoculum. Sullivan *et al.* (43) demonstrated a fitness penalty imposed on the pathogen to overcome the *Php* gene in

tobacco. The authors demonstrated that race 1 isolates were less aggressive on cultivars with partial resistance from Fla301, with a longer incubation period on all cultivars. Race 1 was also less able to overwinter. In populations mixed with races 0 and 1, only 43% of the race 1 population survived compared to 60% of the race 0 population. However, in populations of only race 1, the absence of competition from race 0 and the stability of race 1 may result in race 1 persisting even in the absence of the *Php* or *Phl* genes, as was observed in the mountainous regions of NC where burley tobacco is grown.

The widespread presence of race 1 in *P. nicotianae* populations has major implications for black shank control. Growers must be aware of the source of resistance and the concerns at the time of cultivar selection. Previous work has shown that race 1 populations can be recovered after just one season of *Php*-gene deployment (4, 44). The rate of change toward race 1 is dependent on the initial race structure of the population. Race 1 increases in abundance more quickly in fields where race 1 is initially detected (44). Therefore, if race 1 is already present in a field, then race 1 can increase extremely quickly unless the variety also has a high level of partial resistance to race 1.

Because race 1 can increase to detectable levels after just a single year of planting cultivars with the *Php* gene, Apple speculated that race 1 is a natural mutational variant of race 0 that occurs in very low frequency due to a competitive disadvantage in the absence of single-gene resistance (4, 43). The deployment of single-gene resistance selects for its success over race 0 (4). Previous work identified very high levels of AFLP polymorphisms among asexual zoospore progeny (Gallup Chapter 2). Using two AFLP markers, 100% of the loci were polymorphic, yielding a diversity index between 0.17013 to 0.44196 for each group

of related asexual zoospore progeny. Not all differences are necessarily significant, but such high levels of asexual variability increases the potential for a significant change, such as a change in race. Other work by Sullivan *et al*. (Appendix B) found a unique AFLP (Amplified Fragment Length Polymorphism) fingerprint for every new occurrence of *P. nicotianae* race 1 in a field that was initially race 0 and planted over years with *Php* gene resistance. A unique fingerprint for each occurrence suggests that race 1 developed through multiple independent events. According to Apple's speculations, each occurrence of race 1 could have been a random mutation already present below detectable levels in the initial population that was selected for by planting a cultivar with single-gene resistance.

Race 3, recently described in NC (Gallup Chapter 1), was recovered at low levels throughout NC, with greatest numbers in fields that were primarily race 0. This survey further substantiates previous work indicating that race 3 is a natural variant of race 0 (Gallup Chapter 1). In greenhouse inoculation experiments, soil infested with race 0 or race 3 was planted with tobacco varieties with various levels of Fla301 resistance. After five months, all flats had mixed populations of races 0 and 3 in similar proportion (Gallup Chapter 2).

Race 3 is virulent on tobacco cultivars utilizing the *Phl* gene but not on cultivars utilizing the *Php* gene. The *Phl* gene is incorporated into only one burley cultivar, KY 14xL8 and is not likely to be used in new varieties because of an undesirable linked trait. Therefore, race 3 poses little risk to burley or flue-cured tobacco growers, as the *Php* gene and the Fla301 resistance genes control this race of the pathogen.

This was the first widespread survey of mating types in *P. nicotianae* in NC. Isolates originating from the piedmont and coastal plain of NC are still under evaluation. Therefore,

only general comments can be made about the isolates from these two areas. All other data presented pertain only to isolates originating from the 24 fields sampled in the NC mountains where burley tobacco is grown.

Fourteen fields had populations with mixed mating types (Table 3.3). Natural mixed mating type populations have not been reported previously in this pathosystem. The only investigation of mating type in tobacco areas was in 1959 (2). Based on a small sample set, one mating type was limited to the flue-cured regions and the other mating type was limited to the mountainous burley regions. Based on this information and other studies with *P. nicotianae*, populations of the pathogen in tobacco fields have been regarded as asexual, with zoospores exclusively driving the onset and spread of disease (17).

Of the 14 fields with a mixed mating type population, 12 fields had isolates that produced oospores when paired in culture. This is the first report of the potential for sexually compatible field populations of *P. nicotianae*. Oospores stained with tetrazolium bromide revealed a low to moderate (20-48%) level of viable oospores. Germination attempts have been unsuccessful. Similarly, 30-36% of oospores originating from hybrid alder *Phytophthora spp* were viable, yet no germination attempts were successful (14).

Further investigations are necessary to confirm that the observed oosporogenesis was the result of sexual recombination rather than hormonal heterothallism (1, 27, 28). Hormonal heterothallism, observed only in *in vitro* studies, results in functionally homothallic oospores (1, 27, 28). Evidence for sexual recombination has been illustrated in other species of *Phytophthora* using hyphal tracings, differential staining, drug resistant markers, and segregation of pathogenicity factors (6, 26, 35, 36, 41). Förster and Coffey (16) exhibited

direct evidence for sexual recombination in *P. nicotianae* using molecular restriction fragment length polymorphism markers. In that study, most single-oospore isolates carried markers from both parental isolates. In a few cases, single-oospore isolates had a recombinant phenotype at one locus and a homozygous phenotype at another locus.

The proportion of race 1 was correlated to *Php* gene deployment rather than the presence of both mating types. However, the presence of compatible mating types within a field is indicative of the potential for sexual recombination to enhance the genetic variability of the population. McDonald (29) hypothesized that populations such as these, with mixed mating systems from sexual reproduction (oospores) and asexual reproduction (zoospores), pose the highest risk of evolution and ultimately the risk of overcoming selection pressure imposed by single-gene resistance in the host.

In addition to the 14 fields with mixed mating type populations, 10 fields consisted of only one mating type: 7 fields were all A1, and 3 fields were all A2. The presence of a single mating type may indicate a founder effect resulting from only a single mating type being introduced into the field (7). Once the pathogen established, the mating type was maintained through asexual sporulation. Alternatively, one mating type may be better fit to the selection pressures of the agroecosystem, and so became the predominant mating type (7). The fixing of mating types from either founder effects or fitness differences cannot be distinguished based on the data described here.

Of the single-mating type populations, field 39 was 100% race 0 and fields 73, 74, and 75 were 100% race 1. Field 3 was 60% race 0 and field 60 was 90% race 1. Based on the

mating type distribution within each of these fields, it is likely that a single isolate of the pathogen was introduced and then maintained through clonal reproduction in these fields.

Evolutionary potential of a pathogen is a predictor of the durability of resistance genes (29). Pathogens pose the greatest risk of breaking resistance genes when they have a mixed sexual and asexual reproductive system, a high mutation rate, and large population sizes. Pathogens pose the least risk when they rely on strictly asexual reproduction, have a low mutation rate, and small population sizes (29). Based on these parameters, the asexual populations identified in NC appear to pose some level of moderate risk. The exclusively A1 or A2 populations may be highly variable as indicated by previous AFLP work (Gallup Chapter 2, Sullivan App. B) with large population sizes attributed to multiple cycles of asexual zoospores. Based on this survey, variation arising through strictly asexual means provides a genetic structure capable of quickly circumventing single-gene resistance in the host.

In this study, all isolates were sensitive to mefenoxam, except isolate 59 from Sampson County, which had a reduced sensitivity to the fungicide. The overall mean EC_{50} was 0.4 μg/ml mefenoxam, similar to the previously reported EC_{50} of 0.4 μg/ml metalaxyl on 5% V-8 agar (38). Staub and Young (42) reported an EC_{50} for mycelial growth of 0.2 μg/ml metalaxyl for one isolate tested on 10% V-8 agar. Shew (38) demonstrated that a higher nutrient concentration in the basal medium decreases isolate sensitivity to metalaxyl. The basal medium used in this study, 5% CA, was not included in the previous study. Therefore, it is unclear how previous reports of fungicide sensitivity on 5% and 10% V-8 agar would compare to this study.

Isolates were screened against mefenoxam rather than metalaxyl because mefenoxam is now only used by growers. Mefenoxam is a more recent R-enantiomer developed as an alternative to metalaxyl. It was introduced in 1996, providing the same level of control as metalxyl, but at half the application rate. The reduced rate is believed to reduce the environmental risk of fungicide application (32).

Mefenoxam and metalaxyl share the same chemistry and mode of action. They are phenylamides that selectively inhibit ribosomal RNA synthesis, affecting the activity of the RNA polymerases, thus leading to reduced spore production and mycelial growth (12, 33). Due to site-specificity, mefenoxam and metalaxyl have a high risk of resistance development in target pathogens. Resistance to mefenoxam was recently reported in high levels among *P. nicotianae* isolates collected from ornamental nurseries (18). Shew (39) reported that *P. nicotianae* isolates collected from tobacco decreased in sensitivity to metalaxyl after just 2 to 3 years of exposure.

Isolate 59, which was inhibited by only 36.6% when exposed to 1 µg/ml mefenoxam, and was treated as an outlier and excluded from the overall EC_{50} analyses. This isolate may be a natural variant within the population. However, it could also be an early indication of the development of mefenoxam insensitivity in NC tobacco populations. Fungicide application histories were not collected from growers, although the grower from this field in Sampson County happened to provide the information. The field history is unknown in 2001 and 2002; Ridomil®, a mefenoxam formulation, was used in 2003.

Even though all isolates were characterized as sensitive to mefenoxam, the level of sensitivity varied among counties, and sometimes between fields within a county. *P.*

nicotianae is a soilborne pathogen with limited movement between fields. Differences in sensitivity to mefenoxam may be related the original introduction of the pathogen to each field. If the isolate(s) that was first introduced to a field was more sensitive, then subsequent asexual generations would most likely be similarly sensitive. Genetic studies of other *Phytophthora* spp. indicate that insensitivity to metalaxyl is linked to two major linked loci (*MEX1* and *MEX2*) that are responsible for the majority of the phenotype, with additive effects of additional minor loci, similar to quantitative trait loci (15, 24).

P. nicotianae is a diploid pathogen and may be heterozygous or homozygous for the mefenoxam insensitivity. Field populations with sexually compatible isolates may provide new opportunity for the development of resistance to phenylamide fungicides. Heterozygosity for the major loci conferring metalaxyl resistance was identified in *P. infestans*, and homozygosity was identified in *P. sojae* (5, 15). Like *P. nicotianae*, *P. infestans* is heterothallic, requiring opposite and compatible mating types for the production of sexual spores. DNA markers linked to a major metalaxyl resistance locus revealed segregation in *P. infestans* crosses consistent with the existence of heterogeneous mechanism for conferring resistance (15).

When monogenic resistance from the *Php* gene was first introduced to the tobacco industry in the 1990s, growers relied upon it as the only method for disease control, not realizing the potential for race development. During this time, growers commonly reduced the chemical aspect of their management program. Therefore, chemical applications for the control of black shank became less common. As race 1 populations develop, growers are re-introducing chemical applications into their disease management strategies. This study

provides a baseline for future comparisons as mefenoxam is more widely used. As long as growers rotate crops and chemistries, the risk of resistance development is low. However, field populations with mixed mating types may be at higher risk.

This study illustrated that race 1 of *P. nicotianae* is widespread and often predominant in NC tobacco fields. In many cases, tobacco cultivars that utilize the *Php* gene as the only resistance source are no longer viable options for disease control. In order to achieve control of the black shank disease as well as restrict the success of *P. nicotianae* race 1, it is crucial to integrate control practices. Many control practices effectively reduce the overall population numbers, including crop rotation, fungicide application, and rouging out tobacco stalks. Rotating between types of disease resistance (Fla301 and *Php*-gene) reduces the rate of race 1 development in fields where it is not already present.

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Table 3.1. Race composition of *Phytophthora nicotianae* populations and cropping history in all NC tobacco fields surveyed in 2006

County	Farm	Region	Tot R [a]	R-0 [b]	R-1 [c]	R-3 [d]	*Ph* gene [e]	Yrs *Ph* gene [f]	Complete history [g]
Buncombe	2	mountains	8	0.25	0.62	0.13	yes	1	no
Buncombe	3	mountains	8	0.62	0.38	0.00	no	0	no
Buncombe	4	mountains	1	1.00	0.00	0.00	no	0	no
Buncombe	5	mountains	9	0.89	0.11	0.00	yes	1	no
Buncombe	6	mountains	10	0.00	1.00	0.00	no	0	no
Haywood	30	mountains	10	0.90	0.10	0.00	yes	1	no
Haywood	31	mountains	9	0.56	0.44	0.00	no	0	no
Haywood	32	mountains	7	0.29	0.71	0.00	no	0	no
Madison	33	mountains	10	0.20	0.70	0.10	yes	1	yes
Madison	34	mountains	7	0.00	1.00	0.00	no	0	yes
Madison	35	mountains	4	0.25	0.75	0.00	yes	2	no
Madison	36	mountains	10	0.10	0.80	0.10	ND	ND	no
Mitchell	39	mountains	9	1.00	0.00	0.00	no	0	no
Mitchell	40	mountains	9	0.00	1.00	0.00	ND	ND	no
Mitchell	41	mountains	10	0.80	0.20	0.00	no	0	no
Watauga	60	mountains	10	0.10	0.90	0.00	ND	ND	no
Watauga	61	mountains	10	0.00	1.00	0.00	ND	ND	no
Yancey	70	mountains	4	0.25	0.75	0.00	no	0	no
Yancey	71	mountains	8	0.00	1.00	0.00	no	0	no
Yancey	72	mountains	4	0.75	0.25	0.00	yes	2	no
Yancey	73	mountains	4	0.00	1.00	0.00	no	0	no

Table 3.1 Continued.

Yancey	74	mountains	7	0.00	1.00	0.00	yes	3	no
Yancey	75	mountains	7	0.00	1.00	0.00	no	0	no
Yancey	76	mountains	9	0.00	1.00	0.00	no	0	no
Alamance	1	piedmont	10	0.00	1.00	0.00	yes	2	no
Caswell	7	piedmont	10	0.00	1.00	0.00	yes	2	yes
Caswell	8	piedmont	7	0.00	1.00	0.00	yes	3	yes
Caswell	9	piedmont	11	0.00	1.00	0.00	yes	3	yes
Davie	12	piedmont	12	0.00	1.00	0.00	yes	1	yes
Davie	13	piedmont	10	0.30	0.40	0.30	no	0	no
Davie	14	piedmont	5	0.00	1.00	0.00	yes	2	no
Davie	15	piedmont	5	0.20	0.60	0.20	no	0	yes
Davie	16	piedmont	5	0.60	0.20	0.20	no	0	yes
Forsyth	23	piedmont	19	0.00	1.00	0.00	ND	ND	no
Forsyth	24	piedmont	12	0.08	0.92	0.00	yes	3	yes
Granville	25	piedmont	1	0.00	1.00	0.00	yes	3	yes
Granville	26	piedmont	10	0.00	1.00	0.00	yes	3	yes
Guilford	27	piedmont	10	0.10	0.90	0.00	yes	2	yes
Guilford	28	piedmont	10	0.00	1.00	0.00	yes	2	yes
Guilford	29	piedmont	3	0.00	1.00	0.00	no	0	no
Rockingham	42	piedmont	10	0.50	0.20	0.30	ND	ND	no
Rockingham	43	piedmont	9	0.67	0.33	0.00	ND	ND	no
Rockingham	44	piedmont	10	0.00	1.00	0.00	yes	1	yes
Rockingham	45	piedmont	9	0.00	1.00	0.00	yes	3	yes
Rockingham	46	piedmont	6	0.00	1.00	0.00	yes	3	yes
Stokes	51	piedmont	10	0.00	1.00	0.00	yes	3	yes

Table 3.1 Continued.

Stokes	52	piedmont	5	0.00	1.00	0.00	yes	3	no
Surry	53	piedmont	9	0.00	1.00	0.00	yes	3	yes
Surry	54	piedmont	8	0.00	1.00	0.00	yes	3	yes
Surry	55	piedmont	5	0.00	1.00	0.00	yes	3	yes
Surry	56	piedmont	10	0.00	1.00	0.00	yes	3	yes
Surry	57	piedmont	10	0.00	1.00	0.00	yes	2	yes
Surry	58	piedmont	9	0.00	1.00	0.00	yes	2	yes
Surry	59	piedmont	7	0.00	1.00	0.00	ND	ND	no
Yadkin	65	piedmont	10	0.00	1.00	0.00	yes	2	no
Yadkin	66	piedmont	2	0.00	1.00	0.00	yes	1	no
Yadkin	67	piedmont	3	0.33	0.67	0.00	yes	2	no
Yadkin	68	piedmont	5	0.00	1.00	0.00	yes	1	no
Yadkin	69	piedmont	10	0.00	1.00	0.00	yes	1	no
Craven	10	coastal plain	2	0.50	0.50	0.00	no	0	no
Craven	11	coastal plain	5	1.00	0.00	0.00	no	0	no
Duplin	17	coastal plain	5	0.80	0.20	0.00	no	0	yes
Duplin	18	coastal plain	9	0.00	0.89	0.11	yes	2	no
Duplin	19	coastal plain	10	0.80	0.10	0.10	no	0	no
Edgecombe	20	coastal plain	3	0.00	0.33	0.67	yes	4	yes
Edgecombe	21	coastal plain	7	0.43	0.14	0.43	yes	1	yes
Edgecombe	22	coastal plain	10	0.50	0.30	0.20	yes	1	yes
Martin	37	coastal plain	1	0.00	1.00	0.00	yes	1	yes
Martin	38	coastal plain	1	0.00	1.00	0.00	yes	1	yes
Sampson	47	coastal plain	10	0.00	1.00	0.00	yes	3	yes
Sampson	48	coastal plain	8	0.62	0.00	0.38	yes	1	no

Table 3.1 Continued.

Sampson	49	coastal plain	10	0.10	0.90	0.00	yes	2	no
Sampson	50	coastal plain	11	0.00	1.00	0.00	yes	2	no
Wayne	62	coastal plain	11	0.82	0.09	0.09	no	0	no
Wilson	63	coastal plain	1	0.00	1.00	0.00	yes	1	no
Wilson	64	coastal plain	6	0.17	0.83	0.00	yes	1	no
TOTAL			**581**	**0.21**	**0.75**	**0.04**			

[a] Total number of isolates screened for race
[b] Proportion of isolates designated race 0, 1=100%
[c] Proportion of isolates designated race 1, 1=100%
[d] Proportion of isolates designated race 3, 1=100%
[e] *Ph* gene deployment at least once 2001 – 2006
[f] Number of seasons Ph gene cultivars deployed
[g] Complete field history records 2001 – 2006

Table 3.2. Race structure of *Phytophthora nicotianae* in NC tobacco regions

Region[y]	Proportion Race 0	Proportion Race1	Proportion Race 3
Mountains (Western NC)	31.52%	66.85%	1.63%
Piedmont (Central NC)	7.32%	89.89%	2.79%
Coastal Plain (Eastern NC)	38.18%	50.00%	11.82%

[y] Fields with active black shack disease were sampled from each geographic region of NC. Isolates were obtained from soil collected from around symptomatic plants and a single was obtained per plant. A total of 581 isolates were characterized in the study.

[z] Races were determined by inoculating a set of host differentials, Hicks, susceptible, NC 1071, with the *Php* gene, KY 14 x L8, with the *Phl* gene. Race 0 killed only Hicks, race 1 killed all genotypes, and race 3 only Hicks and KY 14xL8.

Table 3.3. Mating type composition of *Phytophthora nicotianae* populations in the burley tobacco regions of the NC mountains

County	Farm	Tot MT	Mating Type [a]				Sexually Compatible [b]
			A0	A1	A2	A1/A2	
Buncombe	2	9	0.00	0.56	0.44	0.00	+
Buncombe	3	9	0.00	1.00	0.00	0.00	
Buncombe	4	1	0.00	0.00	1.00	0.00	
Buncombe	5	8	0.00	0.75	0.25	0.00	+
Buncombe	6	4	0.00	1.00	0.00	0.00	
Haywood	30	8	0.00	0.75	0.63	0.00	+
Haywood	31	7	0.00	0.29	0.71	0.00	+
Haywood	32	7	0.00	0.14	0.86	0.00	-
Madison	33	10	0.00	0.80	0.20	0.00	+
Madison	34	7	0.00	0.57	0.43	0.00	+
Madison	35	2	0.00	0.50	0.50	0.00	+
Madison	36	9	0.00	0.56	0.44	0.00	+
Mitchell	39	10	0.00	1.00	0.00	0.00	
Mitchell	40	9	0.00	0.89	0.11	0.00	+
Mitchell	41	10	0.00	0.40	0.60	0.00	+
Watauga	60	8	0.00	1.00	0.00	0.00	
Watauga	61	10	0.00	0.00	1.00	0.00	
Yancey	70	4	0.00	1.00	0.00	0.00	
Yancey	71	8	0.00	0.10	0.90	0.00	+
Yancey	72	4	0.00	0.25	0.75	0.00	-
Yancey	73	4	0.00	0.00	1.00	0.00	
Yancey	74	6	0.00	1.00	0.00	0.00	
Yancey	75	4	0.00	1.00	0.00	0.00	
Yancey	76	9	0.00	0.33	0.33	0.33	+
		167	**0.00**	**0.60**	**0.38**	**0.02**	

[a] Proportion of isolates designated A1, A2, or A1/A2

[b] Presence (+) or absence (-) of oospores produced when opposite mating types were paired on 5% carrot agar amended with 5 µg/ml cholesterol. No pairings when all isolates in a field were the same mating type

Table 3.4. Percent of viable oospores from pairings of *Phytophthora nicotianae* isolates collected in NC tobacco fields

County	Field[a]	A1 isolate	A2 isolate	% oospore viability[b]
Guilford	27-28	GC-11	GC-9	48
Guilford	27-28	GC-11	GC-4	20
Guilford	27-28	GC-15	GC-2	26
Surrey	56	SJ-7	SJ-2	48
Surrey	53	SC-1	SC-2	44

[a] Arbitrary field designation based on cropping histories. For example, fields 27 through 28 share location, but differ in cropping history.

[b] Oospores formed were rated as viable based on a staining technique using tetrazolium bromide.

Table 3.5. Percent growth inhibition of *Phytophthora nicotianae* in response to increasing mefenoxam concentrations

Mefenoxam conc. (µg/ml)[a]	Mean % Inhibition[b]	Min-Max % Inhibition
0.1	29.2 (±36.8)	-37.7 [c] - 89.9
1.0	85.7 (±8.1)	55.3 - 100
10.0	91.7 (±6.6)	60.5 - 100

[a] Concentration of mefenoxam in 5% carrot agar medium.

[b] Percent inhibition of isolates after 7 days of growth in the dark at room temperature, determined by comparing treatments to growth in 5% carrot without mefenoxam.

[c] Negative value indicates mycelial growth was greater than non-amended control for some isolates.

Table 3.6. Inhibition of *Phytophthora nicotianae* tobacco isolates at each concentration of mefenoxam in 21 NC counties surveyed

County	No. of isolates	No. of fields	Percent inhibition at each mefenoxam concentration (µg/ml)			Mean EC_{50}	Means Separation[a]		
			0.1	1	10				
Sampson	8	3	14 ± 32.3	85.0 ± 8.7	93.4 ± 4.9	0.7967	A		
Watauga	2	2	-7.2 ± 4.4	77.8 ± 5.0	88.1 ± 2.1	0.7621	A	B	
Duplin	5	2	6.8 ± 20.6	75.9 ± 13.0	83.5 ± 11.6	0.719	A	B	
Mitchell	3	2	2.4 ± 8.4	77.1 ± 6.5	88.7 ± 5.2	0.7116	A	B	
Wayne	4	1	-4.1 ± 14.4	80.1 ± 7.5	91.1 ± 4.7	0.7079	A	B	
Buncombe	5	4	-2.6 ± 18.1	80.4 ± 4.6	88.7 ± 3.5	0.6612	A	B	
Davie	9	4	-3.8 ± 20.6	84.6 ± 5.7	91.1 ± 4.7	0.6213	A	B	
Granville	4	1	-.04 ± 15.1	84.4 ± 5.4	92.8 ± 2.7	0.5751	A	B	
Madison	6	3	14.8 ± 22.3	84.9 ± 6.2	90.1 ± 6.5	0.4644	A	B	C
Caswell	5	3	22.0 ± 36.5	85.0 ± 8.2	92.1 ± 5.3	0.4579	A	B	C
Yancey	16	4	-27.0 ± 33.7	87.6 ± 8.1	93.3 ± 5.6	0.4375	A	B	C
Surrey	8	3	17.6 ± 25.5	85.7 ± 5.1	91.1 ± 4.9	0.4142	A	B	C
Forsyth	2	1	17.5 ± 29.9	91.6 ± 7.0	95.2 ± 3.8	0.3616	A	B	C
Edgecombe	5	3	26.6 ± 28.2	84.2 ± 8.8	90.0 ± 10.4	0.3498	A	B	C
Yadkin	4	1	-27.7 ± 28.3	88.7 ± 6.0	94.4 ± 3.5	0.2971	A	B	C
Haywood	8	3	52.3 ± 35.3	89.2 ± 8.5	94.7 ± 5.9	0.258		B	C
Guilford	9	1	69.9 ± 13.5	85.6 ± 6.4	87.2 ± 8.8	0.0133			C
Alamance	4	1	67.5 ± 10.9	85.7 ± 5.7	91.2 ± 5.9	0.0086			C
Rockingham	10	3	81.8 ± 6.2	94.6 ± 3.7	96.9 ± 3.6	3.8E-5			C
Craven	1	1	82.3 ± 2.9	93.8 ± 0.1	97.7 ± 1.0	4.2E-6			C
Stokes	1	1	83.0 ± 3.8	87.5 ± 2.5	93.3 ± 0.6	3.7E-8			C

[a] Mean EC_{50} values with the same letter do not differ significantly (Waller-Duncan k=100)

Table 3.7. Mean EC_{50} for mefenoxam sensitivity of each mating type of *Phytophthora nicotianae* collected from NC tobacco fields

Mating Type	N	Mean EC_{50}	Means Separation[a]	
A1	32	0.4600	A	
A2	69	0.4507	A	
A1/A2	12	0.4134	A	B
A0	4	0.0086		B

[a] Mean EC_{50} values with the same letter do not differ significantly (Waller-Duncan k=100)

Table 3.8. Mean EC_{50} for mefenoxam sensitivity of each race of *Phytophthora nicotianae* collected from NC tobacco fields

Race	N	Mean EC_{50}	Means Separation[a]
Race 0	28	0.4131	A
Race 1	78	0.3996	A
Race 3	8	0.3747	A
Nonpathogenic	3	0.6388	A

[a] Mean EC_{50} values with the same letter do not differ significantly (Waller-Duncan k=100)

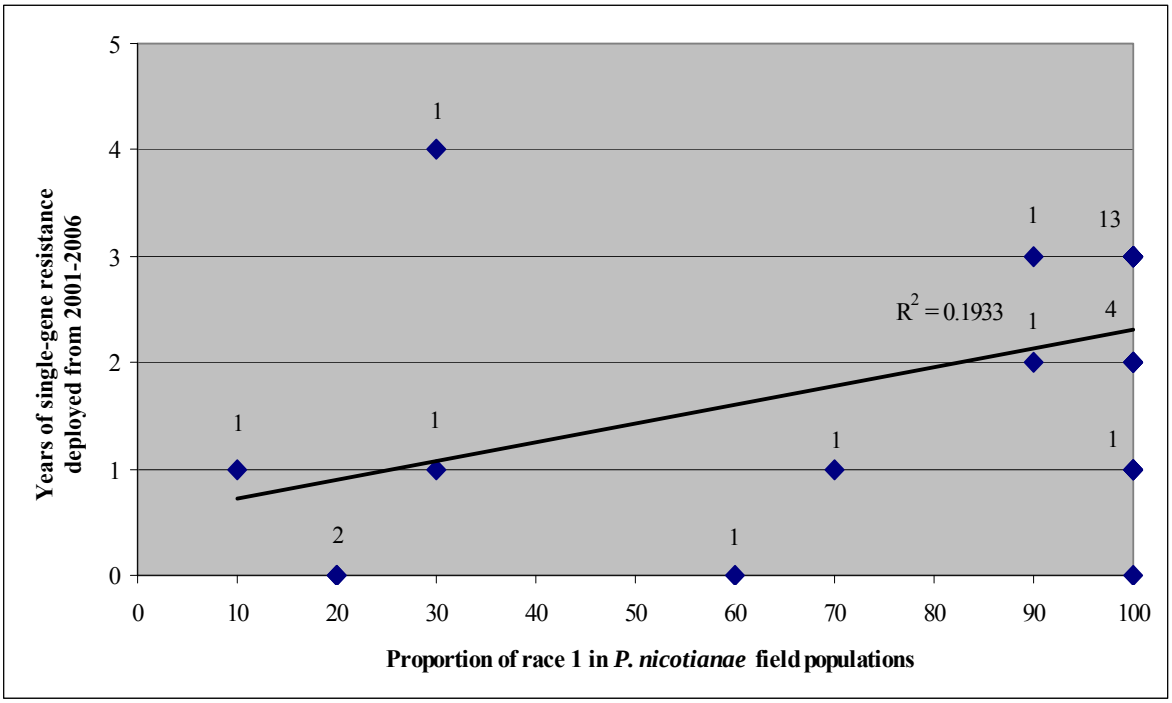

Figure 3.1. Scatter plot of race 1 proportions of *Phytophthora nicotianae* in relation to the number of years that tobacco cultivars with single-gene resistance were deployed. Data are compiled from fields for which complete cropping histories are available from 2001 through 2006. Number above ◆ represents the number of fields with the same correlation.

APPENDICES

Appendix A

Gallup, C.A., M.J. Sullivan, and H.D. Shew. 2006. Black Shank of Tobacco. *The Plant Health Instructor*. DOI: 10.1094/PHI-I-2006-0717-01

DISEASE: Black Shank of Tobacco

PATHOGEN: *Phytophthora nicotianae* Breda de Haan (synonyms: *Phytophthora parasitica* var. *nicotianae*, *Phytophthora nicotianae* var. *nicotianae*).

HOSTS: Tobacco: *Nicotiana tabacum* and *N. rustica*

Cultivated tobaccos are primarily in the species *N. tabacum*, with a small amount of commercial tobacco coming from *N. rustica*. Within *N. tabacum*, multiple types of tobacco are grown, including flue-cured, light air-cured (primarily burley and Maryland types), oriental, dark air-cured (includes cigar types), and dark fire-cured. Infection of the roots of numerous other plants has been reported, but tobacco isolates appear to be host specific.

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Black shank can be a devastating root and crown rot disease of all types of tobacco, with losses in individual fields reaching 100%. The disease was first described from Indonesia in 1896, but has since spread to most major tobacco growing areas worldwide. Management requires an integrated approach based on cultural practices, host resistance, and soil applied fungicides.

Symptoms of black shank on flue-cured tobacco with characteristic yellowing and wilting of leaves.

Symptoms and Signs

Black shank affects tobacco plants at all growth stages. Disease begins on young seedlings or transplants (Figure 2) once soil temperatures rise above 20°C. The most common symptom of the disease is a root and crown rot (Figure 3), but the pathogen may also infect leaves if they come in contact with infested soil during rainy periods (Figure 4). Signs of the pathogen are infrequently observed on plant stems and around leaf lesions, but hyphae often are readily observed in pith tissues upon splitting of the stem (Figure 5). Since other fungi, especially species of *Fusarium*, are often present in these necrotic pith tissues, microscopic observation of pith cells may help confirm the presence of characteristic hyphae of *Phytophthora* (Figure 6). In the field, diseased plants are often associated with wet soil and losses may reach 100% in susceptible cultivars in years favorable for disease development (Figure 7).

Figure 2

Figure 3

Figure 4

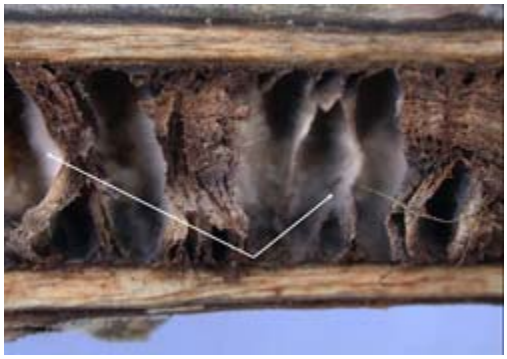


Figure 5

Figure 6

Figure 7

Seedlings

Once infected, disease develops rapidly in young, succulent seedlings (Figure 8). In the field, symptoms begin as wilting, followed by yellowing of leaves, development of stem lesions, and plant death (Figures 2, 9). Depending on the size of the seedling, the plant may wilt and die within a few days or a few weeks. Infection of seedlings may occur directly on the stem as well as on the roots.

Figure 8

Figure 9

Roots

Root tips and wounds are the primary sites of infection by the pathogen (Figures 10, 11). Infected roots are initially water-soaked, then rapidly become necrotic (Figure 12). On susceptible varieties, lesion expansion progresses rapidly into larger roots until most or all of the root system is destroyed (Figures 3, 13). Colonization of root tissues in susceptible varieties proceeds rapidly and ultimately reaches the stem, resulting in the characteristic black shank symptom (Figure 14) and plant death.

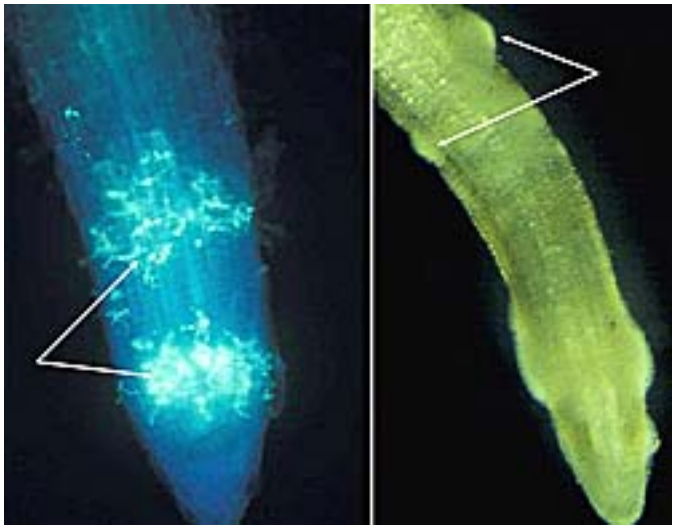


Figure 10

Figure 11

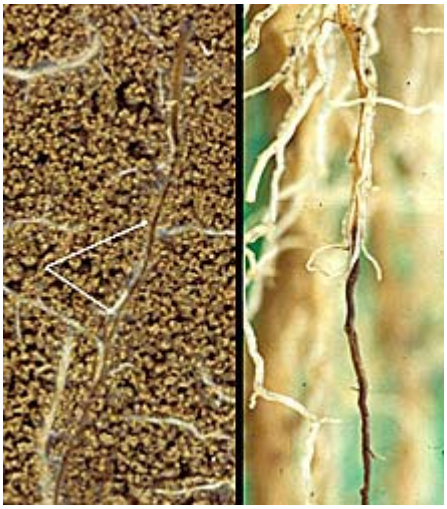


Figure 12

Figure 13

Figure 14

Stems

Stem lesions may continue up the stem 30 cm or more above the soil surface. A longitudinal cut through the stem reveals necrotic pith that is often separated into disks (Figure 15). The disking of the pith does not extend beyond the necrosis observed on the outside of the stem (Figure 16). Disking of the pith should not be used as a sole diagnostic feature of black shank, as other pathogens (Figure 17) and lightning injury (Figure 18) may also cause disking of the pith. The disking caused by lightning injury is not associated with necrosis initially and may extend the entire length of the stem.

Figure 15

Figure 16

Figure 17

Figure 18

Figure 19

In addition to stem symptoms that develop as a result of root infection, infection also can occur directly on the stem. Infection of the stem occurs in old leaf traces that are buried during cultivation, which results in a stem lesion with little or no root necrosis (Figure 19). This symptom is sometimes referred to as stem black shank and may also result in plant death.

Leaves

Above ground symptoms of the stem and root rot phases of the disease begin as a temporary wilt that progresses into chlorosis of leaves and permanent wilting. The extent of chlorosis varies with the level of host resistance and the type of tobacco, with burley tobacco characteristically becoming a brighter yellow than flue-cured plants as the disease develops (Figures 1, 20).

Figure 20

Figure 21

Direct infection of leaves also may occur. Leaf infections may result from zoospores splashing onto the leaf surface or leaves coming into direct contact with infested soil. Lesions

may remain brown to black or develop into circular, yellowish-to-brown spots that may reach up to 8 cm in diameter (Figure 4). The pathogen may grow down the petiole and into the stem (Figure 21), resulting in a stem lesion and plant death.

Pathogen Biology

Phytophthora nicotianae is a fungus-like organism in the Kingdom Straminipila (sometimes written as Stramenopila), phylum Oomycota, class Oomycetes. All stages of the organism are diploid; whereas, most true fungi are haploid. The organism can grow vegetatively at temperatures between 5°C and 37°C, with optimal growth occurring between 26°C and 32°C. Extended exposure to temperatures above 40°C is lethal to the organism.

Hyphae
Hyphae are hyaline (colorless, transparent), aseptate or coenocytic, and typically irregular in width (3-11µm) with few to numerous hyphal swellings (Figure 22). With age, hyphae acquire pseudosepta and colonies become light yellow. There is considerable morphological variation in colony type among isolates of *P. nicotianae* (Figure 23). The growth habit or colony morphology of an individual isolate may vary when grown on different culture media (Figure 24).

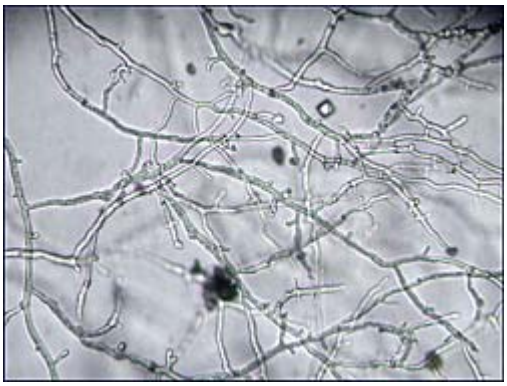


Figure 22

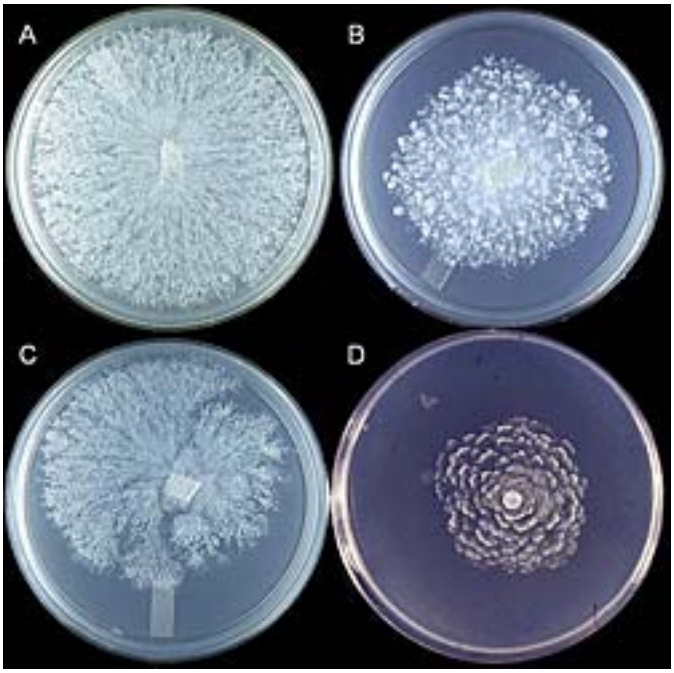


Figure 23

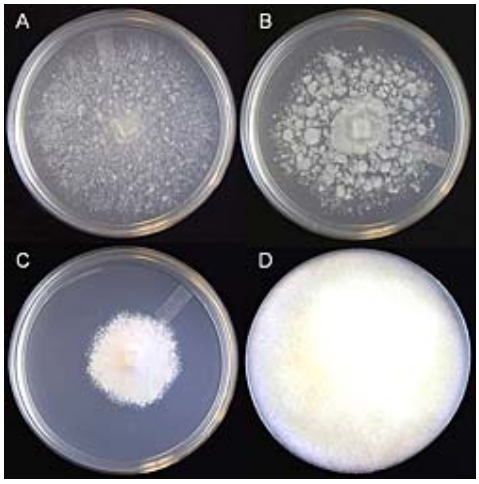


Figure 24

Asexual reproduction

Sporangia are ovoid, pear-shaped, or spherical, and have very conspicuous papillae (Figure 25). Sizes of sporangia vary (18-70 x 14-39 µm) with isolate and the growth medium. Germination is either direct by production of hyphae or indirect by the production of five to 30 zoospores (Figure 26).

The zoospore is typically kidney-shaped, with a ventral groove from which two flagella emerge (Figure 27a). The posterior flagellum is whip-like and the anterior flagellum, a tinsel type, is shorter with hairs along its length. After settling down on a plat surface, zoospores encyst (produce a cell wall), and a single germ tube emerges from the spore (Figure 27b,c).

Chlamydospores are asexual, thick-walled spores produced at the tips (terminal) or in the middle (intercalary) of hyphae, and range from 13 to 60 µm in diameter, with walls approximately 1.5 µm thick (Figure 28). Chlamydospores serve as the primary survival propagule and as the primary inoculum that initiates epidemics. Survival for 4 to 6 years in soil has been reported.

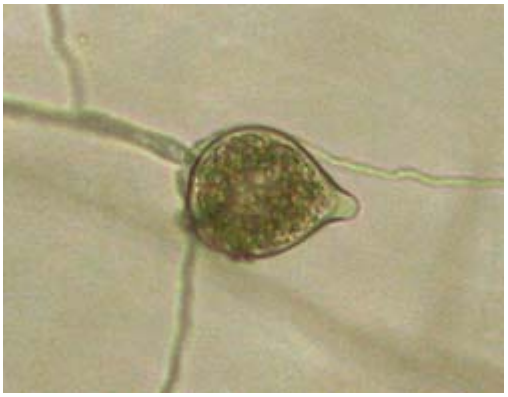


Figure 25

Figure 26

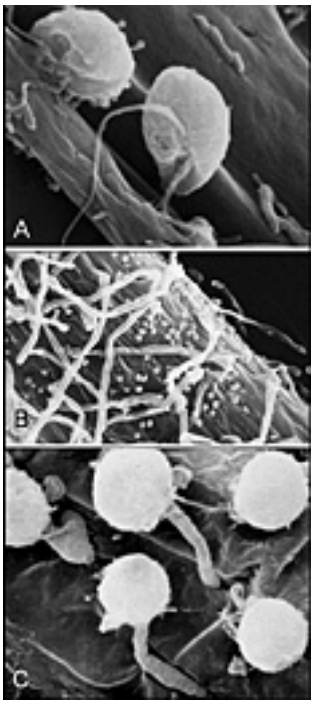


Figure 27

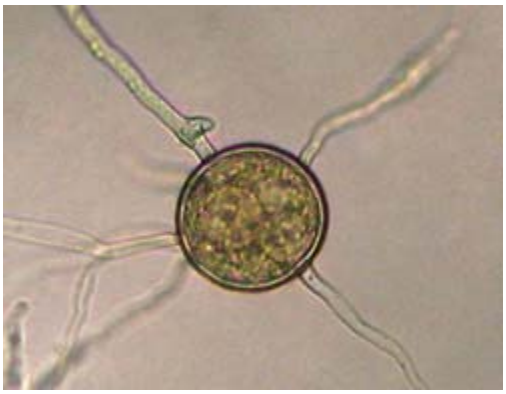


Figure 28

Sexual reproduction

Phytophthora nicotianae is heterothallic, requiring two mating types (A1 and A2) for the production of oospores. Some single-strain cultures may develop oospores with age. Oospores are thick-walled, between 13 and 35 µm in diameter, almost filling the spherical oogonium cavity (between 15 and 64 µm in diameter). The antheridium is amphigynous (the oogonium grows through the antheridium at mating), spherical and remains permanently attached to the oogonium (Figure 29). It functions to contribute a nucleus during fertilization of the oogonium. Since many fields contain only one mating type and the oospores rarely germinate in culture, they are not thought to serve as a primary survival propagule or initiate infections that lead to epidemic development.

Figure 29

Nomenclature
Currently, the most frequently used name for the black shank pathogen is *Phytophthora nicotianae*. *Phytophthora parasitica* var. *nicotianae* and *Phytophthora nicotianae* var. *nicotianae* have also been used, but their use is declining. See the Appendix below and Erwin and Ribeiro (1996) for a more detailed description of the taxonomy of this organism.

Host Range
The species *P. nicotianae* (synonym *P. parasitica*) has a very wide host range. However, there is much evidence of host specialization within isolates obtained from various hosts. Isolates from tobacco are generally considered to be important pathogens only on tobacco. Isolates of *P. nicotianae* from other hosts also have shown some level of host specialization as well. However, current understanding of the level of specialization in the species has not resulted in development of forma specialis, which is a sub-specific level of taxonomy based on host specialization used with some other plant pathogens. See Erwin and Ribeiro (1996) for a comprehensive list of hosts for this species.

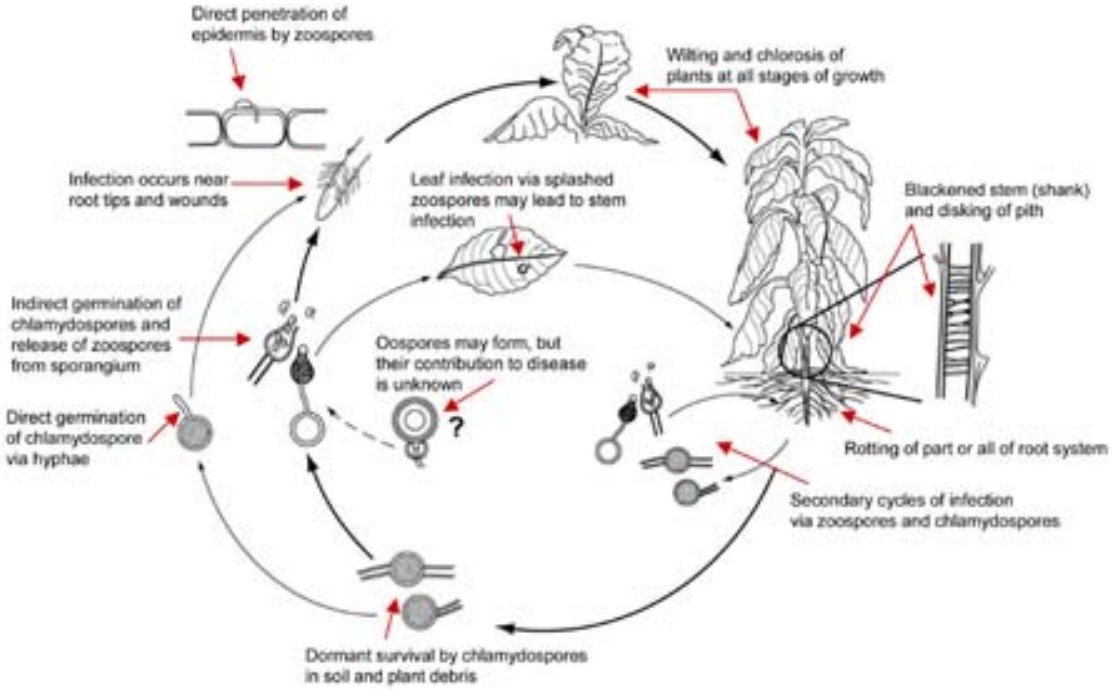
Other Phytophthora spp.
In addition to *P. nicotianae*, other species of *Phytophthora* also can cause root rot of tobacco. Although black shank has not been reported from Brazil, the primary causal agent of the yellow stunt disease has been identified as *P. glovera*, a previously unreported *Phytophthora* spp. Other unidentified species of *Phytophthora* spp. have been isolated from diseased tobacco from Kentucky and North Carolina, but their contribution to disease losses has not yet been determined.

APPENDIX: Additional Information on Pathogen Nomenclature
J. van Breda de Haan first described the pathogen as *Phytophthora nicotianae* in 1896 from Indonesia, but failed to submit a Latin description of the species. Additionally, his original drawings of the organism were inaccurate due to the presence of a contaminant, probably a *Pythium* species. Dastur also described the species from India in 1913, but gave it the name *P. parasitica*. In 1928, Ashby proposed that the name of all strains of this pathogen from tobacco be merged under the properly described name *P. parasitica*. Tucker in 1931 chose the name *P. parasitica* var. *nicotianae* to designate isolates based on pathogenicity, but pathogenicity should not have been used as a basis for variety because variety status is based on differences in morphology. Since isolates from tobacco display host specific pathogenicity, the use of *forma specialis* (f. sp.) would have been more appropriate, but again, studies have not been conducted to confirm the validity of this subspecific classification for tobacco isolates compared to isolates from other hosts. In fact, some isolates from tobacco will infect other plants.

It is generally agreed that *P. parasitica* is the more appropriate name, but the International Code of Botanical Nomenclature restricts its use. In 1963, Waterhouse replaced the name *P. parasitica* with *P. nicotianae* because it had priority despite errors in the original description. She further separated the species into *P. nicotianae* var. *parasitica* and *P. nicotianae* var. *nicotianae* based on morphological differences. Since 1963, multiple studies have shown that

the morphology of these two varieties overlaps greatly and is not a valid basis for separating the species into varieties. Use of *Phytophthora nicotianae* is slowly gaining acceptance in the United States, including by tobacco pathologists; most pathologists in other countries have used this nomenclature for a number of years. We have used the name *P. nicotianae* in this disease lesson as this is the most widely recognized name for this organism at the current time.

Disease Cycle and Epidemiology



Black shank is a polycyclic disease. The number of cycles of infection per growing season and the extent of spread within a field are related to environmental conditions and the level and type of resistance in the cultivar planted. Chlamydospores in the soil and infested crop debris serve as the primary inoculum that initiates epidemics. Chlamydospores germinate in warm moist soil to produce one or several germ tubes that either directly infect the plant or produce a sporangium (Figure 30, 31).

Figure 30

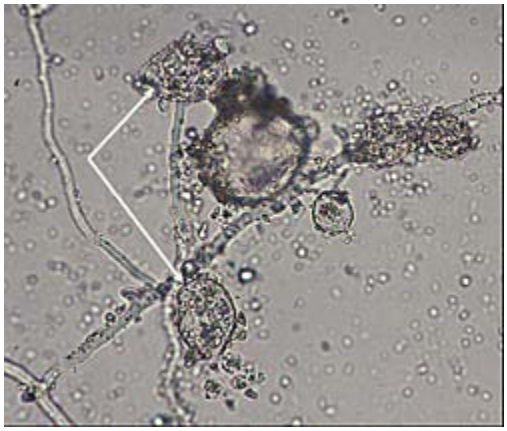


Figure 31

Saturated soil stimulates the release of motile zoospores from sporangia, and these are the primary infective propagules. Zoospores swim through saturated soil pores or move in surface water over greater distances (Figure 32). Active movement is directed toward nutrient gradients that occur around the root tips and wounds on the host plant (Figures 10, 11). Once the zoospore contacts the root surface, it encysts (produces a cell wall), loses the flagella, then germinates to form a germ tube that directly penetrates the host epidermis (Figure 27). The pathogen continues to colonize the roots and stem via hyphae (Figure 33). Sporangia serve as secondary inoculum, and can form within 24 hours of inoculation under moderate temperature and moist soil conditions (Figure 34). Root and stem colonization results in typical root rot and black shank symptoms (Figures 3, 13, 14). New chlamydospores form on and in the root as disease progresses (Figure 35) and can either germinate to initiate new infections or serve as a survival structure until the next tobacco crop is planted.

Figure 32

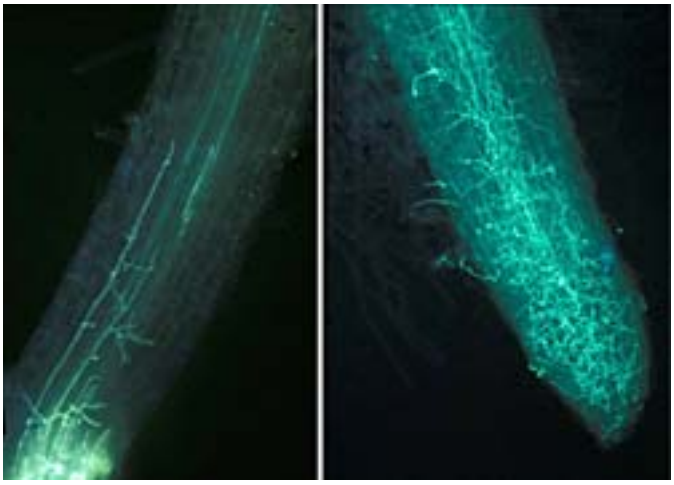


Figure 33

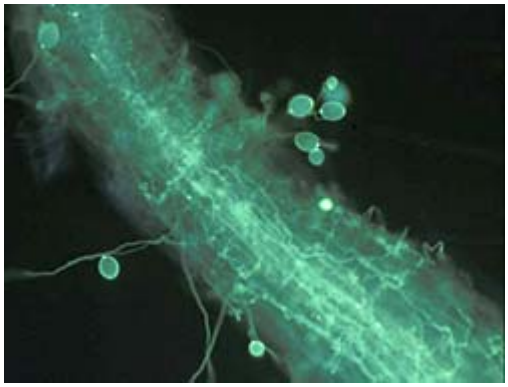


Figure 34

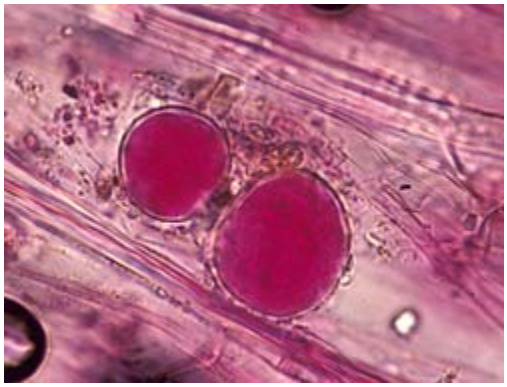


Figure 35

Disease Management

An effective disease management program for black shank requires an integration of cultural and chemical practices and planting resistant cultivars. The goal is to reduce pathogen populations in the soil that initiate epidemics of black shank. Since initial inoculum level is related to the development and severity of black shank epidemics, practices that reduce initial inoculum may greatly affect yield and quality of a tobacco crop.

Cultural practices

Cultural practices are vital to the long term management of black shank. Effective cultural practices include: planting on raised beds, which prevents or reduces duration of soil saturation required for zoospore movement in the root zone; crop rotation, which removes potential sources of nutrition for reproductive growth of the pathogen; and stalk and root destruction after harvest, which effectively removes the infected root system from the soil and suppresses inoculum buildup. Planting on raised beds (Figure 36) is not a common practice for many tobacco types and production regions, but it is widely used in flue-cured tobacco production.

Strains of *P. nicotianae* that cause black shank are host-specific, so rotation with any other crop plant is effective in reducing pathogen populations. Preliminary studies, however, indicate that long term survival in the absence of tobacco may also be related to limited colonization of certain weed hosts (Figure 37). The duration of the rotation should be at least two years, but even long rotations of four or more years will not totally eliminate the pathogen from many soils.

Figure 36

Figure 37

Figure 38

Stalk and root destruction is the physical removal of the root system after the final harvest. Plants are plowed up and inverted to expose the root system to the sun. This practice is not used for all tobacco and soil types, but is effective in reducing late-season buildup of the pathogen on roots that remain alive long after leaves are harvested and stalks are cut. Root and stalk destruction also reduces populations of other pests and pathogens, especially nematodes such as root knot, *Meloidogyne* spp., that can greatly exacerbate black shank severity (Figure 38).

Soil chemistry greatly affects black shank development. The disease is favored by pH values greater than 6.2 and is suppressed at lower pH values. The suppression of the disease at low pH values has been related to the increased activity of aluminum (Al^{3+}) that is present at low pH values, and which is highly toxic to *Phytophthora* spp. Since Al also is toxic to tobacco at pH values below 5.5, acidification of soil to reduce black shank severity, if attempted, should be done carefully so that phytotoxicity associated with high Al or Mn concentrations does not occur. Soil pH values between 5.5 and 6.0 provide favorable growing conditions for tobacco without providing highly conducive conditions for *P. nicotianae*.

Host Resistance

The most widely used method of control for black shank is planting of resistant varieties (Figure 39). Multiple sources and types of resistance are available to growers. The most

widely deployed type is partial resistance. This type of resistance is present at levels that range from low to high. Continuous deployment of a high level of partial resistance results in the selection of more aggressive isolates of the pathogen.

Figure 39

Figure 40

Two single-gene sources of resistance have been incorporated into tobacco cultivars. Both of these genes provide complete resistance to race 0 of *P. nicotianae,* and no resistance to race 1 of the pathogen. Deployment of these two genes has resulted in race 1 of *P. nicotianae* becoming the dominant race of the pathogen present in many areas. Identification of races is based on the inoculation of a set of host differentials (Figure 40). Rotation of partial resistance and single-gene resistance is effective in slowing race shifts and prolonging the effectiveness of single gene resistance.

Chemical control

Chemical control can be effective when used in combination with other practices, but it is not highly effective when used in conjunction with a susceptible variety. The options for chemical control are limited to a single chemistry, with either metalaxyl or its near-identical twin mefenoxam used in single or multiple applications. Mefenoxam is twice as active as metalaxyl, but has the same mode of action. Application rates and time of application (preplant, at first cultivation, or four weeks after transplanting) vary with the level and type of host resistance used and the history of disease in the field. The use of soil fumigants such as chloropicrin may reduce pathogen populations, and fumigant nematicides such as 1,3-dichloropropene reduce nematode populations that enhance black shank incidence and severity.

Significance

The Dutch scientist J. van Breda de Haan first described black shank on cigar wrapper tobacco in Java (Indonesia) in 1896, two years after arriving in Sumatra as the chief of the laboratory for Deli tobacco. Since tobacco was introduced to Northern Sumatra in the 1860s by the Dutch, it is likely that black shank had been occurring for years prior to the report in 1896. The native hosts of *P. nicotianae* in Sumatra are unknown, but it also is likely that the pathogen was surviving (causing disease?) on other plants prior to encountering its new tobacco host. Van Breda de Haan also reported *P. nicotianae* from *Amaranthus* in Sumatra, and other researchers have reported that tobacco isolates attack multiple plants. However, tobacco appears to be the only economically important host for tobacco isolates of this species.

The disease was observed in the western hemisphere in Florida and Georgia around 1915, but it is thought that it took multiple introductions before it became established in the United States and Puerto Rico by 1925. The disease is now present in all tobacco-producing continents and in most major production areas with the notable exception of Brazil. In the 1930s and 1940s, the pathogen was introduced and became established in the major tobacco growing areas of North Carolina and Kentucky, and is now in all tobacco producing states of the US. Losses to the disease were often so high that tobacco could no longer be grown on a farm. This was the case until resistant varieties were introduced in the 1950s. A single source of partial resistance was used for many years in the management of this disease and is still the basis for resistance worldwide. Today, single-gene resistance also is widely deployed, but races of the pathogen have quickly appeared where single-gene resistance has been used. Chemical control was based on soil fumigants until the 1980s, when the fungicide metalaxyl was labeled for use on tobacco.

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Figure Legend

Figure 2. Symptoms of black shank on a young tobacco transplant. Note the wilting and yellowing of lower leaves.

Figure 3. Root and stem symptoms of black shank. Note the total loss of the root system and the necrotic stem lesion.

Figure 4. Leaf lesions caused by *Phytophthora nicotianae* on a leaf of flue-cured tobacco.

Figure 5. Hyphae of *Phytophthora nicotianae* in the pith of an infected tobacco stem.

Figure 6. Hyphae of *Phytophthora nicotianae* in a parenchyma cell from the pith of an infected tobacco plant. Note the irregular width and lack of septations typical of hyphae of *P. nicotianae*.

Figure 7. Total loss of a flue-cured tobacco crop to black shank.

Figure 8. Symptoms of black shank on a young tobacco seedling (left) and growth of *Phytophthora nicotianae* from an infected seedling (right).

Figure 9. Early stages of the black shank disease on a tobacco seedling. Note the wilting of leaves and the development of a stem lesion below the soil level (arrow).

Figure 10. Sites of zoospore accumulation on tobacco roots. Note clusters of zoospores at the root tip (both roots) and the zones of accumulation away from the root tip on the root on the right (arrows). Fluorescent microscopy (left), with pathogen structures bright in appearance, and dark field microscopy (right) of whole mounts following exposure of root to a zoospore suspension.

Figure 11. Zoospore germination and direct penetration (left) and penetration through a wound (right) on a tobacco root. Fluorescent microscopy (left), showing zoospore (arrow) and hyphae in epidermal cell, and bright field microscopy (right) of longitudinal section through root showing infection occurring between epidermal cells and colonization of root tissue.

Figure 12. Necrosis of small tobacco roots caused by *Phytophthora nicotianae*. This is the primary site of infection and the first stage of this disease.

Figure 13. Extensive root necrosis caused by *Phytophthora nicotianae*.

Figure 14. Typical stem lesion (black shank) late in the growing season.

Figure 15. Disking of the pith typical of the black shank disease. The symptom is characteristic of black shank, but it should not be considered as diagnostic for the disease as other diseases may also cause disking.

Figure 16. Exterior and interior appearance of a stem lesion of black shank. Note that the disking does not extend beyond the stem lesion visible on the outside of the plant.

Figure 17. Disking of the pith caused by infection by *Rhizoctonia solani*. Notice the collapse of the stem that is not typical of black shank.

Figure 18. Symptoms of lightning injury on tobacco seedlings. Notice that the disking is not necrotic and it extends along the entire length of the stem.

Figure 19. Stem infection by *Phytophthora nicotianae*. Note that roots remain healthy.

Figure 20. Wilting and chlorosis of flue-cured (left) and burley (right) tobacco plants.

Figure 21. Leaf lesions of the black shank disease. Note the colonization of the midrib (left) and growth from the leaf into the stem (right).

Figure 22. Typical hyphae of *Phytophthora nicotianae*. Note the absence of septations, irregular width, and presence of hyphal swellings.

Figure 23. Varied colony morphologies of *Phytophthora nicotianae* grown on 5% carrot agar.

Figure 24. Colony morphology of an individual *Phytophthora nicotianae* isolate on different media. A. 5% V-8 juice agar. B. 5% carrot agar. C. nutrient agar. D. oatmeal agar.

Figure 25. Ovoid *Phytophthora nicotianae* sporangium with conspicuous papillae.

Figure 26. A. Direct germination of a *Phytophthora nicotianae* sporangium by means of hyphae. B. Indirect germination of *Phytophthora nicotianae* sporangia by means of motile zoospores.

Figure 27. *Phytophthora nicotianae* zoospores attach to a host root, encyst, and directly penetrate the host epidermis. A. Motile zoospores. B. Zoospores on root surface. C. Encysted zoospores germinating and infecting root.

Figure 28. Asexual *Phytophthora nicotianae* chlamydospore; note thick wall that aids in survival.

Figure 29. Sexual *Phytophthora nicotianae* oospores. The antheridium is amphigynous and remains permanently attached. The oospore is spherical and almost fills the oogonial cavity.

Figure 30. *Phytophthora Nicotianae* chlamydospore germinating by the production of hyphae. Note the characteristic hyphae as show in a pith cell in Figure 5.

Figure 31. *Phytophthora nicotianae* chlamydospore (center) germinating to produce sporangia (arrows).

Figure 32. In surface water, zoospores of *Phytophthora nicotianae* may move long distances within a field and to adjacent fields.

Figure 33. Hyphae of *Phytophthora nicotianae* colonizing tobacco roots.

Figure 34. Sporangia of *Phytophthora nicotianae* on root surface.

Figure 35. Chlamydospores of *Phytophthora nicotianae* in root tissue.

Figure 36. Planting in raised beds controls soil saturation and may help to limit zoospore movement and the spread of the black shank disease.

Figure 37. Investigation of various weeds and crop plants as hosts for *Phytophthora nicotianae*.

Figure 38. Nematode damage on roots creates wounds for infection by *Phytophthora nicotianae*. In the presence of root-knot nematodes, black shank severity will increase greatly.

Figure 39. Resistant tobacco cultivar (left) and susceptible tobacco cultivar (right) in a field infested with *Phytophthora nicotianae*.

Figure 40. Races of *Phytophthora nicotianae* present in tobacco-growing regions of the US, can be identified based on a reaction on a set of host differentials. A. Healthy plants, prior to inoculatio*N*. B. Race 0, the wild-type, can cause disease only on the susceptible cultivar Hicks. C. Race 1 can cause disease on Hicks (susceptible), NC 1071 (single-gene resistance from *N. plumbaginifolia*), and KY 14xL8 (single-gene resistance from *N. longiflora*). D. Race 3 can cause disease only on Hicks (susceptible) and KY 14xL8 (single-gene resistance from *N. longiflora*).

Appendix B

An assessment of the genetic diversity in a field population of **Phytophthora nicotianae** *with a changing race structure.*

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ABSTRACT

Sullivan, M. J., Parks, E. J., Cubeta, M. A., Gallup, C. A., Melton, T. A., Moyer, J.W., and Shew, H. D. 2009. An assessment of the genetic diversity in a field population of Phytophthora nicotianae *with a changing race structure. Plant Dis. 94:000-000.*

One hundred fifty-three isolates of *Phytophthora nicotianae* collected over a 4-year period from a single tobacco field were subjected to amplified fragment length polymorphism (AFLP) analysis to investigate the effects of deployment of different types of host resistance on genetic diversity in the pathogen population. Seventy-six of the 153 isolates had a unique AFLP profile, while the remaining 77 isolates were represented by 27 AFLP profiles, each shared by at least two isolates. There were 102 race 0 isolates (wild type) and 51 race 1 isolates, with isolates of both races found in unique and shared AFLP

profile groups. Race 1 isolates that were detected multiple years were always obtained from the same plot each year. Three race 0 AFLP profiles were recovered from noncontiguous plots, and in each case, the isolates were recovered from plots planted with the same variety. Cluster analysis provided a high level of bootstrap support for 41 isolates in 19 clusters that grouped primarily by race and treatment. In only one case did a race 0 and a race 1 isolate have an identical AFLP profile; the race 0 profile was recovered in year one, and the race 1 profile was recovered in each of the last three years of the study from the same plot. Estimates of genetic diversity ranged from 0.365 to 0.717 and varied depending on tobacco cultivar planted and race. Type of resistance deployed affected genetic diversity of the population and the incidence of race 1 isolates of *P. nicotianae* in each year of the study.
Additional keywords: oomycete, *Nicotiana tabacum*, black shank, *Php* gene

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INTRODUCTION

Black shank, caused by the soilborne pathogen *Phytophthora nicotianae*, is an economically important disease of cultivated tobacco (*Nicotiana tabacum*) throughout the southeastern United States and most production areas of the world (10, 31). Infection of roots, stems, and leaves can occur at any stage of plant growth, resulting in root necrosis, wilting, chlorosis, stem lesions, and stunting of plants. The disease is managed using an integrated approach, but the most practical and economical method of control is the use of resistant cultivars (10, 31, 35). Cultivated *N. tabacum* and the wild species *N. plumbaginifolia* and *N. longiflora* have been used as sources of resistance, with both partial and complete resistance incorporated into available cultivars. Partial resistance is effective

against all races of *P. nicotianae* and ranges from a low to a high level (33). Single-gene resistance introgressed from both wild species confer complete resistance to race 0 but no resistance to race 1 of *P. nicotianae* (2, 6, 7, 34).

Race 0 has been the predominant race of *P. nicotianae* in the flue-cured tobacco areas of North Carolina (NC) since the pathogen was introduced in 1931 **(**31**).** Although race 1 was observed in breeding nurseries in the 1960s, it was not observed widely in production areas until cultivars containing the *Php* gene, from the species *N. plumbaginifolia*, were widely deployed in the 1990s (11, 31). The rapid emergence of race 1 prompted studies on ways to preserve the effectiveness of the *Php* gene. A four-year cultivar rotation study was conducted to investigate the short and long-term effects of *Php* gene deployment on the race structure of the tobacco black shank pathogen (33**)**. Two partially resistant cultivars ('K 326' and 'K 346') and one completely resistant cultivar ('NC 71') were cropped continuously or in various combinations of complete and partial resistance. In a field where no race 1 was initially detected, race 1 was detected after a single growing season of deploying the *Php* gene and continued to increase over space and time during the study. At the end of the study, race 1 isolates had been recovered from 14 non-contiguous plots throughout the field. The authors speculated that the occurrence of race 1 was due to multiple independent selections of race 1 from the race 0 population (32**).**

In this study, amplified fragment length polymorphism (AFLP)-based genetic markers were used to characterize the genetic diversity within the population of *P. nicotianae* obtained during the four-year rotation study. Isolates were selected across all treatments and years for further investigation. Analyses of AFLP data were conducted to monitor changes in

the genetic diversity of race 0 and race 1 over the course of the study and to determine the association of AFLP profile with race and host variety.

MATERIALS AND METHODS

Isolates. A sample of 153 isolates of *P. nicotianae* was selected for use in this study (32). All isolates were collected from soil assays taken from a single tobacco field in Duplin County, NC between 2000 and 2003 in a 4-year cultivar rotation experiment. Nineteen of the 153 isolates were selected from isolates collected in14 different plots prior to initiating the study to provide a baseline estimate on the race structure and genetic diversity of the population. The remaining 134 isolates for which AFLP analysis was completed were collected following experimental planting of the field and were chosen to represent isolates collected across years and treatments in the study. Briefly, the rotation experiment involved the planting of three flue-cured tobacco cultivars ('NC 71' with complete single-gene resistance to race 0; 'K 326' with a low level of partial resistance to race 0 and 1; and 'K 346' with a high level of partial resistance to race 0 and 1) continuously or alternately over the duration of the 4-year experiment (Table 1). The origin and race of each isolate for which AFLP data were determined (Table 2). Each isolate was identified as *P. nicotianae* based upon morphological characteristics and results using one universal (28) and five different sets of species-specific primers (12, 18, 22, 24, 26).

DNA extraction. Total genomic DNA was extracted from lyophilized mycelium of *P. nicotianae* grown in 15 ml of pea broth at 22°C for 7 to 10 days. DNA for AFLP analysis was prepared by extraction with cetyl trimethyl ammonium bromide (CTAB) and

phenol/chloroform, followed by precipitation in 95% EtOH and 3M sodium acetate as previously described by Gonzalez et al. (17).

AFLP Analysis. The AFLP protocol was a modification of Vos, et al. (36) using the AFLP Core Reagent Kit (Invitrogen) and procedure of Ceresini et al. (8). Approximately 250 ng of genomic DNA was digested overnight at 37°C with *Eco*RI and *Mse*I. Double-stranded adaptor sequences were ligated to the digested samples for 6 hr with the addition of T4 DNA ligase. The PCR pre-amplification step employed primers containing the adaptor sequence, restriction endonuclease recognition sequences, plus one selective nucleotide as provided in the AFLP Pre-Amplification Primer Mix (Invitrogen). Each sample included 2.5 µl of DNA template, 20.0 µl of pre-amplification primer mix, 2.5 µl enzyme specific PCR buffer, and 0.5 µl *Taq* DNA polymerase (Qiagen). PCR was performed for 20 cycles of 94°C for 30s, 56°C for 1 min, and 72°C for 1 min. The pre-amplification reaction mixture was diluted 10-fold with sterile deionized water and stored at –20°C until needed for the selective amplification step.

Three *Eco*RI (5′-GACTGCGTACCAATTC*AC*-3′; 5′-GACTGCGTACCAATTC*GC*-3′; and 5′-GACTGCGTACCAATTC*GG*-3′) and two *Mse*I (5′-GATGAGTCCTGAGTAA*CA*-3′ and 5′-GATGAGTCCTGAGTAA*CC*-3′) primers with two selective nucleotides were used for selective amplification of the secondary template. The *Eco*RI primers were 5′ labeled with the fluorescent dye carboxyfluorescein (6-*FAM*) and screened for polymorphisms in all six possible combinations (*Eco*RI-*AC* and *Mse*I-*CC*, *Eco*RI-*AC* and *Mse*I-*CA*, *Eco*RI-*GC* and *Mse*I-*CC*, *Eco*RI-*GC* and *Mse*I-*CA*, *Eco*RI-*GG* and *Mse*I-*CC*, and *Eco*RI-*GG* and *Mse*I-*CA*) on a sample of 24 isolates of *P. nicotianae* over

multiple years and locations, including Indonesia, Virginia, Kentucky, Connecticut, and multiple counties within NC that represented different races, mating types, and different tobacco types. The primer pair *Eco*RI-GG/*Mse*I-CC generated easily resolvable and reproducible fragments, and was subsequently used in the selective amplification step for characterizing the 153 isolates used in this study. The *Eco*RI primers were synthesized by MWG Biotech (High Point, NC) and all other primers were synthesized by Applied Biosystems.

Each selective PCR mixture included 10 µl of reaction mixture, 1.5 µl of diluted secondary DNA template obtained from the pre-amplification, 1 µm of *Mse*I selective primer, 1 µm of *Eco*RI selective primer, 2.0 mM dNTPs, enzyme-specific buffer, and 0.5 ul Taq DNA polymerase (Qiagen). The thermal cycling parameters were an initial denaturation at 94°C for 2 min; 13 cycles of 94°C for 30 sec, 65°C for 1 min, 72° C for 2 min; this was followed by 23 cycles of 94°C for 30 sec, 56°C for 1 min, and 72°C for 2 min. Completed reactions were stored at 4°C.

Following selective PCR amplification, products were diluted 10-fold with sterile distilled H_2O, and 0.8 µl of the resulting diluted samples was mixed with 0.5 µl of the fluorescent sequencing dye Genescan ROX-500 containing formamide and an internal molecular marker standard (Applied Biosystems). The mixture was denatured at 90°C for 3 min and placed on ice. One µl of each reaction mixture was loaded on a 6% denaturing polyacrylamide gel in an ABI model 377 sequencing electrophoresis system (Applied Biosystems). The running buffer was 1X TBE and electrophoresis was performed at 60 W for 3.0 h. The AFLP fragments were sized using GeneScan 2.02 (ABIPRISM, Applied

Biosystems) software. The polymorphic AFLP fragments were scored with the software Genographer (Montana State University) and coded as present (1) or absent (0) to determine the AFLP profile. The reproducibility of the AFLP analysis was determined by scoring fragments from two independent extractions of each isolate.

Data Analysis. Phylogenetic analysis was conducted with Phylip 3.65 package (13). The program Seqboot was initially used to create 1000 bootstrapped datasets. Restdist was then used to calculate distance using the modified restriction sites distance method of Nei and Li (29) using 14 as the number of sites. The Neighbor Joining program was used to estimate Unweighted Pair-Group Mean Analysis (UPGMA) clustering. Consense was used to determine the Majority Rule dendrogram. Isolates with identical AFLP profiles were removed for clustering analysis. Genetic diversity was calculated as Weir's gene diversity using $D=1-(1/m)\Sigma p_{lu}^2$, where p is the frequency of the *u*th allele at the *l*th locus averaged over *m* loci (37). Diversity was calculated on both the entire and clone-corrected data sets.

RESULTS

AFLP analysis. The primer pair *Eco*RI-*GG* and *Mse*I-*CC* provided 302 fragments (loci) that could be scored reliably across the 153 isolates of *P. nicotianae*. These data are available upon request to the authors. Of these loci, 257 (85%) were polymorphic, which produced 84 unique AFLP profiles (Table 2). Nineteen of the unique AFLP profiles were observed from the 19 isolates of race 0 collected in the spring of 2000 and were used to represent the pathogen population present in plots prior to initiating the study (data not shown). None of these original profiles were subsequently recovered in the fall of 2000, 2001, 2002, or 2003.

The AFLP profiles identified varied with host cultivar planted. Experimental plots continuously planted with 'K 326' (treatment 1, Table 1), a tobacco cultivar with a low level of partial resistance to races 0 and 1, resulted in the identification of one, five, 12, and three, different AFLP profiles in 2000, 2001, 2002, and 2003, respectively, with eight profiles recovered multiple years (Table 2). Five, six, and one AFLP profiles were identified in 2001, 2002, and 2003, respectively, from experimental plots continuously planted with 'K 346' (treatment 3, Table 1), a tobacco cultivar with a high level of partial resistance to race 0 and race 1 (Table 2); six profiles from this treatment were found in multiple years. Continuous planting of the experimental plots with a tobacco cultivar with single gene resistance to race 0 ('NC 71', treatment 2, Table 1) resulted in the identification of one AFLP profile in 2000, two in 2001, nine in 2002, and nine in 2003, with seven profiles recovered in multiple years (Table 2). The only rotationally planted treatment where profiles were recovered multiple years was from treatment 5 (Table 1), alternately planted with 'K 326' and 'NC 71', where two of the 16 profiles were recovered multiple years.

Diversity. Relative differences among treatments were similar when genetic diversity was calculated with the entire or clone-corrected set of data (*data not shown*). Therefore, estimates of genetic diversity were calculated on the entire data set. The overall diversity in the field population of *P. nicotianae* prior to initiating the experiment was 0.629 (n=19, Table 3). The overall diversity for each treatment ranged from 0.349 (treatment 5, Table 2) to 0.717 (treatment 4, Table 2). Isolates of *P. nicotianae* from plots planted with the tobacco cultivar 'K 326' (treatment 1, Table 2) was 0.526 (n= 5) in 2001, and 0.563 (n=21) in 2002 and 0.70 (n=10) in 2003. Continuous planting of 'K 346' (treatment 3, Table 2) had the

lowest level of diversity, 0.254 (n=6) in 2001, and it remained low 0.386 (n=11) in 2002 and 0.383 (n=10) in 2003. The diversity of isolates of *P. nicotianae* from plots continuously planted with 'NC 71' (treatment 2, Table 2) was low following the second year of planting 0.40 (n=4) in 2001, and was higher in 2002 (0.466, n=13) and 2003 (0.55,n=17) in 2003. The diversity observed from isolates sampled from rotated plots planted with 'NC 71' in years 1 and 3 and with 'K 326' in years 2 and 4 (treatment 5, Table 2) was 0.463 (n=9) in 2003. Because of the limited number of isolates (<3) of *P. nicotianae* sampled from experimental plots for treatment 5 in 2001 and 2002 and from treatments 4, 6, 7, and 8 from 2001-2003, no estimates of diversity could be calculated for these treatments.

Estimates for genetic diversity for race 0 ranged from 0.439 to 0.705 and for race 1 ranged from 0.394 and 0.547, during the 4 year study (Table 4). The genetic diversity of race 0 isolates at the end of 2000 was 0.564 (n=6), after 2001 (0.439, n=11), and then increased to 0.539 (n=35) in 2002 and 0.709 (n=31) in 2003. Diversity of race 1 was not calculated at the end of 2000, as only one isolate of race 1 was recovered; however, diversity increased each year of the study, with a value of 0.394 (n=3) in 2001, 0.414 (n=19) in 2002, and 0.547 (n= 28) in 2003.

UPGMA Clustering. Isolates with identical AFLP profiles were removed for clustering analysis, leaving 84 isolates with a unique profile. Forty-one isolates formed 19 clusters with a high level of bootstrap support (Fig. 1). Isolates clustered primarily by race and treatment. All clusters only contained isolates of a single race, except for three instances. The isolates in clusters that contained isolates of both race 0 and race 1 were recovered from the same plot

in two cases and from an adjacent plot in another case. Thirty-one isolates originating from all treatments were unresolved.

DISCUSSION

The AFLP technique can reveal polymorphisms in a genome and in this study were used to identify differences among closely related isolates of *P. nicotianae* in order to elucidate small changes within a field population over time in response to selection pressures imposed by host resistance. The primers used in this study produced a large number of reproducible and unambiguous fragments and were developed based on screening multiple sets of primers on a diverse subsample of isolates representing different races and mating types of *P. nicotianae*. The isolates selected used were collected from different host tobacco types across diverse geographic locations including Indonesia, Kentucky, Virginia, Connecticut, and multiple counties in North Carolina. AFLP-based genetic markers have been used extensively to assess the genetic diversity and structure of populations of species of *Phytophthora* (1, 3**,** 5, 20, 21, 23, 27**)**. However, except for the molecular characterization of natural hybrids of *P. nicotianae* and *P. cactorum* (4), these genetic markers have not been used previously to examine a large sample of isolates from a single-field population of *P. nicotianae*.

Previous studies have examined the genetic diversity in *P. nicotianae* with isozyme and restriction fragment length polymorphism analyses (RFLP) of nuclear and mitochondrial DNA (14, 15, 25, 30). In these studies, a small sample of isolates of *P. nicotianae* collected from a range of hosts other than tobacco was examined. Colas et al. (9) were able to distinguish a genetically distinct group of isolates causing black shank from other isolates of

P. nicotianae in a sample from tobacco (n=57) and other hosts (n=16) by examining the association of virulence with RFLP-based genetic markers. However, no estimates of genetic diversity within tobacco-specific isolates or races of *P. nicotianae* were conducted.

The field site in Duplin County provided a unique opportunity to study the spatial and temporal dynamics of a field population of *P. nicotianae* with a changing race structure. Prior to initiating the experiment, a diverse sample of race 0 isolates with unique AFLP profiles was recovered from the experimental plots and no isolates of race 1 were detected (33). These results suggest that race 1 was not present, or was present in a frequency below the threshold for detection in the soil assay. In the fall of 2000 (year 1 of the study), additional AFLP profiles of race 0 isolates were recovered. Also by the end of the first growing season, one isolate of race 1 with a unique AFLP profile was detected in a plot planted with 'NC 71'. In subsequent years, numerous race 1 isolates were recovered from 'NC 71' plots, either in continuous or rotational planting. Three of the race 1 isolates recovered from a single 'NC 71' plot in 2001, 2002, and 2003 had an identical AFLP profile to a race 0 isolate recovered from the same plot in 2000. This was the only occurrence of isolates from the two races having an identical profile. There were numerous cases of repeated recovery isolates of either race with identical AFLP profiles from a single plot across multiple years. There were only a few isolated cases of recovery of an AFLP profile from non-contiguous plots, and all of these were found in plots planted with the same variety. These patterns of recovery of AFLP profiles only within small areas of a field suggest that there is a clonal component to the population structure of *P. nicotianae*, and that spread is limited due to irrigation water or on farm machinery. Shew (32) reported limited movement

of the pathogen in soil, especially during dry years and with the planting of cultivars with high levels of partial resistance. The moderate level of recovery of isolates sampled in the population with an AFLP profile not shared by another isolate (~55%) provided additional ancillary evidence for a mixed population structure. Although beyond the scope of this study, further investigation with a larger sample is needed to better determine the processes (mutation, recombination, and selection) and aspects of reproductive biology (asexual versus sexual) that contribute to the mixed population structure in this pathosystem.

The small sample of isolates of *P. nicotianae* included in this study from the rotation plots limited our ability to compare estimates of genetic diversity across the rotation treatments. However, sample size was taken into account in the diversity calculation to allow for comparisons between sets of isolates with differing sample sizes. The data suggest that there was a decrease in genetic diversity in the experimental plots in 2001 (second year of the study), followed by a gradual increase in genetic diversity in subsequent years (2002 and 2003), although the diversity of the race 0 and race 1 isolates never reached baseline levels. It was interesting that the diversity within the race 1 isolates increased each year, and were approaching the diversity of the initial race 0 population by the end of year 4. A comparison of the gene diversity of the isolates sampled from plots continuously planted with a tobacco cultivar with single gene ('NC 71') to race 0 and a low (‘K 326’) or high ‘K 346’ level of partial resistance race 0 and race 1, indicated that isolates sampled from plots planted continuously with 'K346' had the lowest genetic diversity over the course of the study. In this study, we also calculated genotypic diversity as recommended by Grunwald et al. (19). The

results for isolates from the continuously planted treatments were similar. However, the rotationally planted treatments did not have sufficient numbers to utilize this method.

Race and treatment were somewhat confounded in this study since treatments with continuous planting of a type of resistance led to either the maintenance of race 0 (continuous planting of either 'K 326' or 'K 346', treatments 1 and 3, respectively) or the development of race 1 (continuous planting of 'NC 71', treatment 2). The clustering of isolates in the dendrogram was largely related to treatment and race. The clustering of multiple plots of the same treatment provides support for the presence of a treatment signature in the AFLP profiles and not of the microcosm of the isolates. The clusters consisting of isolates of the same race, from the same treatment but different plots, highlight the inseparability of treatment and race, but illustrate the signature of either race or treatment contained within the AFLP profile. However, the 19 clusters of mixed treatments were largely based on race, with only three of these being of mixed race; these data suggests that the clustering is primarily a result of race.

The AFLP profiles within a race were very similar and often differed by only two to four fragments. However, no unique fragments were identified that had a one-to-one correspondence with race. Similar results were observed from a RFLP analysis of *P. sojae* with 48 isolates representing 25 physiological races of the pathogen (16). One group of isolates, representing seven races, had nearly identical RFLP profiles to race 1 and probably arose clonally by mutation. All other races seem to have arisen from rare outcrosses between four progenitor lines. However, in this study, the evolutionary relatedness of isolates of race

0 and race 1 was not determined because of the difficulty in inferring common descent from common state when comparing co-migrating fragments in the AFLP profiles.

This study illustrates the dynamic nature of a field population of a soilborne pathogen undergoing severe selection pressure due to the introduction of complete resistance. In the case of the black shank pathogen, the race structure of the population can adapt relatively quickly in response to the deployment of this type of resistance. A comparable level of genetic diversity was observed both within and between races of *P. nicotianae*, but the genetic composition and diversity of the population changed in response to the deployment of both partial and complete resistance. Current recommendations to use an integrated approach to disease management will continue to be necessary. By rotating types of resistance, populations of race 0 and race 1 can be better managed over time (33), regardless of their inherent genetic diversity in field populations of *P. nicotianae*. The biological and ecological characteristics (e.g., mating type, zoospore production, virulence) of isolates of race 1 and race 0 belonging to different AFLP profiles are currently in progress and needs further study with additional co-dominant genetic markers.

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Table 1. Treatments in the 4-year tobacco cultivar rotation experiment.

Treatment[a]	Tobacco Cultivar Planted, by Year [b]			
	2000	**2001**	**2002**	**2003**
1	K 326	K 326	K 326	K 326
2	NC 71	NC 71	NC 71	NC 71
3	K 346	K 346	K 346	K 346
4	K 346	NC 71	K 346	NC 71
5	K 326	NC 71	K 326	NC 71
6	K 346	NC 71	K 346	K 346
7	K 326	NC 71	K 326	K 326
8	NC 71	K 326	NC 71	K 326

[a] Cultivars with varying levels and types of resistance: 'K326' = low level of partial resistance to race 0 and race 1, 'K346' = high level of partial resistance, to race 0 and race 1and 'NC71' = complete (*Ph*) single gene resistance to race 0 and a low level of partial resistance (32).

[b] Tobacco planted in Duplin County North Carolina with the rotation treatments from 2000-2003.

Table 2. Source code, number and AFLP designation of isolates of *Phytophthora nicotianae* sampled from soil in the four-year crop rotation study. Baseline isolates collected prior to year one are not shown.

Isolate Code[a]	No. of isolates[b]	Years Collected[c]	Isolate Code[a]	No. of isolates[b]	Years Collected[c]
T1-Y0-17A-R0	1		T3-Y1-12B-R0	5	2001, 2002 (2), 2003 (2)
T1-Y1-17A-R0	3	2001, 2002, 2003	T3-Y1-24A-R0	2	2001, 2002
T1-Y1-21A-R0	2	2001, 2002	T3-Y1-24B-R0	4	2001, 2002, 2003 (2)
T1-Y1-21B-R0	3	2001, 2002, 2003	T3-Y1-30A-R0	3	2001, 2003 (2)
T1-Y1-39A-R0	4	2001, 2002 (2), 2003	T3-Y1-7A-R0	3	2001, 2002, 2003
T1-Y1-5A-R0	3	2001, 2002 (2)	T3-Y2-12A-R0	1	
T1-Y2-17A-R0	1		T3-Y2-12C-R0	2	2002, 2003
T1-Y2-17D-R0	1		T3-Y2-24C-R0	1	
T1-Y2-17E-R0	1		T3-Y2-30A-R0	1	
T1-Y2-17F-R0	1		T3-Y2-30B-R0	2	2002, 2003
T1-Y2-21C-R0	1		T3-Y2-7A-R0	1	
T1-Y2-21D-R0	1		T3-Y3-42A-R0	2	2003 (2)
T1-Y2-21E-R0	1		T4-Y0-32A-R0	1	
T1-Y2-36A-R0	2	2002, 2003	T4-Y2-44A-R0	1	
T1-Y2-36D-R0	1		T4-Y3-26A-R1	1	
T1-Y2-39B-R0	3	2002 (2), 2003	T4-Y3-26B-R0	1	
T1-Y2-39C-R0	1		T4-Y3-26C-R0	1	
T1-Y2-5B-R0	3	2002 (2), 2003	T4-Y3-9A-R1	1	
T1-Y3-39A-R0	1		T4-Y3-9B-R0	1	
T1-Y3-39C-R0	1		T5-Y0-20A-R0	1	
T1-Y3-5A-R0	2	2003 (2)	T5-Y0-2A-R0	1	
T2-Y0-43A-R0[d]	4	2000, 2001, 2002, 2003	T5-Y2-34A-R1	1	
T2-Y1-6A-R1	5	2001 (2), 2002, 2003 (2)	T5-Y2-34B-R1	5	2002, 2003 (4)
T2-Y1-6B-R0	1		T5-Y2-34C-R1	1	
T2-Y2-18A-R1	3	2002 (2), 2003	T5-Y3-20A-R1	1	
T2-Y2-22A-R1	1		T5-Y3-34A-R1	1	
T2-Y2-22B-R1	2	2002 (2)	T5-Y3-34B-R1	2	2003 (2)
T2-Y2-28A-R1	2	2002, 2003	T5-Y3-34C-R1	2	2002, 2003
T2-Y2-43A-R1	1		T5-Y3-34E-R1	1	
T2-Y2-43C-R1	2	2002, 2003	T6-Y0-15A-R0	1	
T2-Y2-43D-R1	1		T6-Y2-45A-R0	1	
T2-Y2-6B-R1	1		T6-Y3-15A-R1	1	
T2-Y2-6C-R1	2	2002, 2003	T7-Y2-29A-R1	1	
T2-Y3-22B-R1	1		T7-Y2-41A-R0	1	
T2-Y3-28B-R0	1		T7-Y3-25A-R0	1	
T2-Y3-43A-R1	1		T7-Y3-29B-R0	1	
T2-Y3-43B-R0	1		T7-Y3-29C-R0	1	
T2-Y3-6A-R1	1		T8-Y0-40A-R1	1	
T2-Y3-6C-R1	1		T8-Y2-40C-R1	1	
T2-Y3-6E-R1	1		T8-Y3-11B-R0	1	
T2-Y3-6I-R1	1		T8-Y3-35A-R0	1	
T2-Y3-6P-R1	1		T8-Y3-40A-R1	1	

Table 2 Continued.

[a] Isolate code indicates treatment (T), year (Y, 2000-2003), experimental plot, isolate (alpha-numeric), and race (R) of *P. nicotianae*. AFLP profiles are named for the isolate in which they first occurred.

[b] Number of isolates with that AFLP profile.

[c] Years collected are the years of the study in which that AFLP profile was detected. For profiles that were detected multiple times in a year the number of occurrences are given in parentheses.

[d]Isolate from 2000 was race 0; isolates having this AFLP profile in subsequent years were race 1.

Table 3. Estimates of genetic diversity for isolates sampled a field population of *Phytophthora nicotianae* from each treatment.

Treatment	Genetic Diversity[a]
1	0.595 (n=37)
2	0.519 (n=34)
3	0.365 (n=27)
4	0.717 (n=7)
5	0.349 (n=16)
6	0.575 (n=3)
7	0.612 (n=5)
8	0.623 (n=5)

[a] Baseline estimate of genetic diversity prior to establishing treatments was 0.629 (n=19). Genetic diversity was calculated using $D=1-(1/m)\Sigma p_{lu}^2$, where p is the frequency of the *u*th allele at the *l*th locus averaged over *m* loci (36). Treatments are given in Table 1. Numbers in parenthesis indicate the number of samples for each treatment.

Table 4. Estimates of genetic diversity of race 0 and race 1 sampled from a field population of *Phytophthora nicotianae* in experimental plots cropped with tobacco cultivars with different levels of resistance from 2000-2003.

Year	Genetic diversity[a] Race 0	Genetic diversity Race 1
2000	0.564 (n=6)	N/A (n=1)
2001	0.439 (n=11)	0.394 (n=3)
2002	0.539 (n=35)	0.414 (n=19)
2003	0.705 (n=31)	0.547 (n=28)

[a] Genetic diversity was calculated using $D=1-(1/m)\Sigma p_{lu}^2$, where p is the frequency of the *u*th allele at the *l*th locus averaged over *m* loci (36).

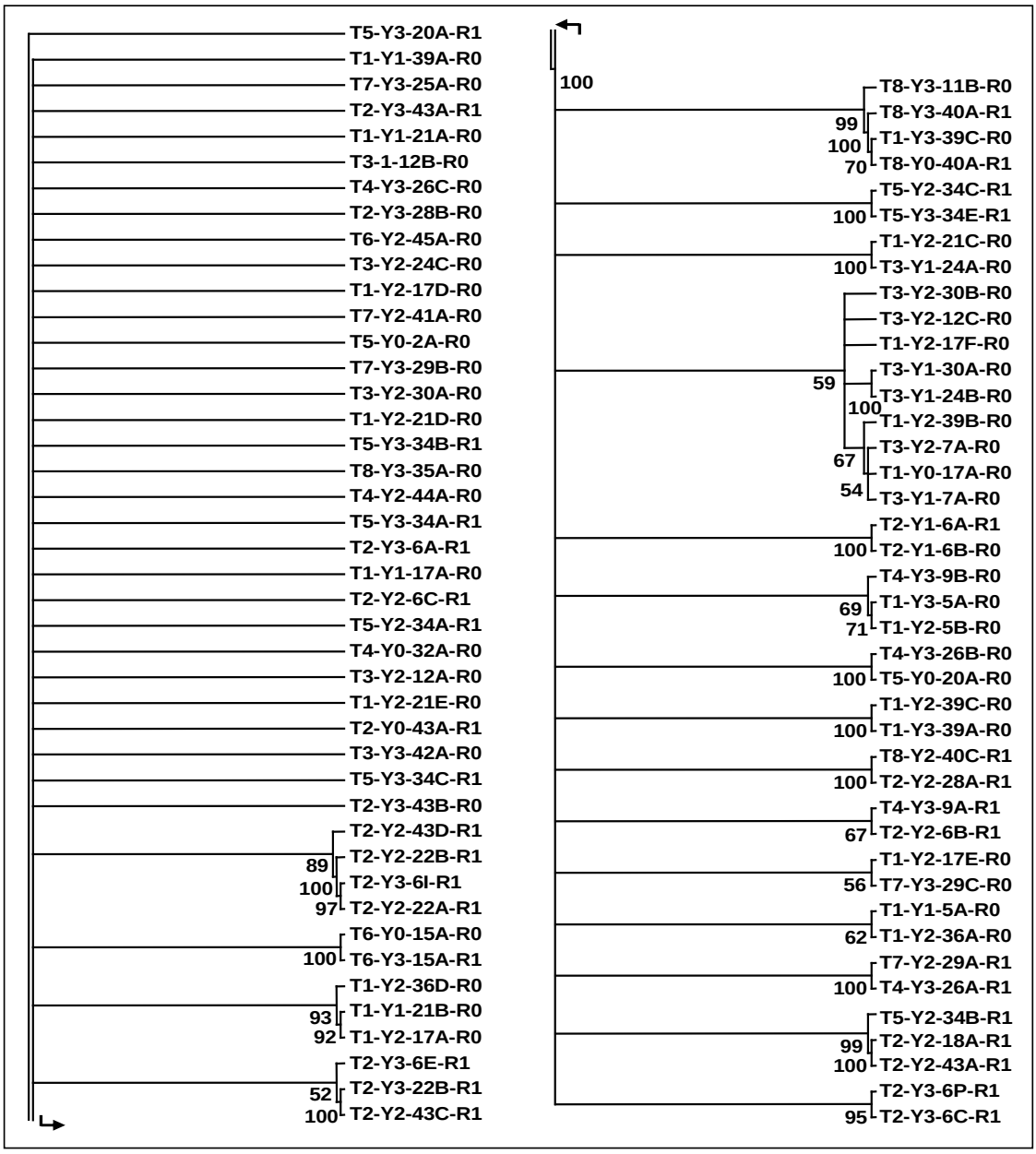

Figure 1. Majority-Rule UPGMA bootstrap consensus dendrogram of *Phytophthora nicotianae* isolates analyzed in this study. Bootstrap values less than 50% were collapsed and values >50% are shown below the corresponding node. Arrows indicate the separation of the dendrogram. Abbreviations are given in Table 2.

Appendix C

Special Report

A Foliar Blight and Tuber Rot of Potato Caused by *Phytophthora nicotianae*: New Occurrences and Characterization of Isolates

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ABSTRACT

Taylor, R. J., Pasche, J. S., Gallup, C. A., Shew, H. D., and Gudmestad, N. C. 2008. A foliar blight and tuber rot of potato caused by *Phytophthora nicotianae*: New occurrences and characterization of isolates. Plant Dis. 92:492-503.

Phytophthora spp. are pathogenic to many plant species worldwide, and late blight, caused by *Phytophthora infestans*, and pink rot, caused by *P. erythroseptica*, are two important diseases of potato. Another *Phytophthora* sp., *P. nicotianae*, was recovered from pink-rot-symptomatic tubers collected from commercial fields in Nebraska, Florida, and Missouri in 2005, 2006, and 2007, respectively. *P. nicotianae* also was recovered from foliage obtained from commercial potato fields in Nebraska and Texas exhibiting symptoms very similar to those of late blight. Isolates of *P. cactorum* also were recovered from foliar infections in a commercial potato field in Minnesota in 2005. Natural infection of potato foliage by *P. cactorum* and infection of wounded potato tuber tissue via inoculation with zoospores of *P. capsici* are reported here for the first time. Isolates of *P. nicotianae*, regardless of origin, were primarily of the A1 mating type. All isolates of *P. nicotianae* and *P. cactorum* were sensitive to the fungicide mefenoxam. Optimum growth of *P. nicotianae*, *P. erythroseptica*, and *P. cactorum* in vitro occurred at 25°C; however, only *P. nicotianae* sustained growth at 35°C. Regardless of the tissue of origin, all isolates of *P. nicotianae* and *P. cactorum* were capable of infecting potato tubers and leaves. However, isolates of *P. nicotianae* were less aggressive than *P. erythroseptica* isolates only when tubers were not wounded prior to inoculation. Pink rot incidence varied significantly among potato cultivars following inoculation of nonwounded tubers with zoospores of *P. nicotianae*, ranging from 51% in Red Norland to 19% in Atlantic. *Phytophthora* spp. also differed significantly in their ability to infect potato leaves. Highest infection frequencies were obtained with *P. infestans* and levels of infection varied significantly among *P. nicotianae* isolates. The rate of foliar lesion expansion was similar among isolates of *P. nicotianae* and *P. infestans*. Whereas *P. infestans* infections yielded profuse sporulation, no sporulation was observed with foliar infections of *P. nicotianae*.

Additional keywords: *Solanum tuberosum*, water rot

A number of plant pathogens cause foliar diseases of potato (*Solanum tuberosum*). Brown spot, caused by *Alternaria alternata* (4), and early blight, caused by *A. solani* (9), occur worldwide and generally are associated with senescing foliage on early-maturing cultivars. *Colletotrichum coccodes* also is capable of infecting potato foliage and reducing yield (13,14). Perhaps the most well-known foliar disease of potato is late blight, caused by *Phytophthora infestans* (10). *P. infestans* is the type species of the genus *Phytophthora* and may be the most important disease of potato. All of the above-mentioned foliar pathogens also can infect tubers, causing various rots or blemishes.

Other *Phytophthora* spp. also infect potato tubers. *P. erythroseptica* is the primary pathogen causing pink rot (15). Pink rot is an important soilborne "water rot" of potato tubers in the United States (25) and most potato-growing regions throughout the world (15,28). As the name implies, the diagnostic characteristic of this disease is a watery, pink discoloration of infected tissue that develops and intensifies after exposure to the air for a short period of time (11). Pink rot first was described as a potato tuber disease in Ireland (21) and subsequently was reported in North America (1). Although *P. erythroseptica* is the principal pathogen associated with the disease, several other *Phytophthora* spp. have been reported to produce pink rot symptoms (8). Species isolated from infected tubers include *P. drechsleri* (6), *P. megasperma* (2), *P. cryptogea* (12,16,22), and *P. nicotianae* (12,18). Prior to the latter reports, *P. nicotianae* (synonym = *P. parasitica*) had not been isolated from naturally infected potato tissue but was shown to be able to cause both tuber rot and foliar and stem blights by artificial inoculation under controlled environments (8). Although it has an extensive and diverse host range including over 300 plant species, *P. nicotianae* has not been considered to be a significant potato pathogen. Results obtained in a multiyear survey consisting of 2,277 potato tubers with water rot symptoms collected from 16 states and 2 Canadian provinces support the view that *P. erythroseptica* is the primary pathogen responsible for causing pink rot in North America (31).

During extensive travels to many potato production areas in the United States, one of the authors (N. C. Gudmestad) has encountered late-blight-like symptoms on the foliage in a number of potato fields. The symptoms vary but lesions generally are roughly circular in shape, surrounded by a light-green or chlorotic halo (Fig. 1A). In some instances, the lesions extend into the petiole and stem, causing lesions similar to the newer immigrant genotypes of *P. infestans* (Fig. 1B). Unlike typical late blight, foliar lesions of the disease lacked the "downy mildew" appearance characteristic of late blight. However, under environmental conditions conducive to disease development, large areas of potato fields can be affected (Fig. 1C), with stems becoming girdled and plants completely defoliated (Fig. 1D). Our research group has undertaken studies to determine the cause of this disease.

This article reports the occurrence of *P. nicotianae* isolates capable of causing foliar symptoms similar to late blight caused by *P. infestans* as well as tuber rot symptoms typical of *P. erythroseptica*-induced pink rot. In contrast to *P. erythroseptica* infections, tuber tissue infected by *P. nicotianae* generally becomes tan to brown in color when exposed to air, and the intensity of the infection varies with cultivar. Additionally, aggressiveness of *P. nicotianae* isolates was compared with other *Phytophthora* spp. when inoculated

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onto tubers and leaves of commonly grown potato cultivars.

MATERIALS AND METHODS

Isolate collection. Isolates evaluated in this study were obtained from naturally occurring field infections (Table 1) by transferring small pieces of infected potato leaf or tuber tissue, approximately 25 mm^3 in size, to culture dishes containing water agar. Following incubation in the dark at 17 to 20°C for 3 to 5 days, colonies with mycelia resembling that of *Phytophthora* spp. were selected and isolated utilizing hyphal tip methods.

Morphological characterization. Cultures were grown on 5% clarified carrot agar (CA) (50 ml of Hollywood carrot juice, 950 ml of deionized water, and 20 g of Difco Bacto agar) and maintained with regular transfers. Vegetative and reproductive stages were examined in cultures grown for 1 to 4 weeks at 20 to 25°C in light using a Nikon TMS Inverted Phase Contrast Microscope. Isolates were scored for appearance of hyphae, size and shape of sporangia and chlamydospores, and presence or absence of oospores. Observations were compared to published descriptions for *Phytophthora* spp. (8).

DNA extraction. Agar plugs were placed in 25-ml sterile pea broth and allowed to grow for 7 days. Pea broth was prepared by autoclaving 120 g of frozen store-bought peas in 1 liter of deionized water for 5 min. The broth was filtered through a double layer of cheese cloth and then sterilized by autoclaving for 30 min. After 7 days of growth at 20 to 24°C, mycelium was vacuum filtrated, rinsed with sterile deionized water, and lyophilized. DNA was extracted from frozen mycelia using a cetyl trimethyl ammonium bromide (CTAB) procedure. Samples were ground in liquid nitrogen, and genomic DNA was isolated by suspending the mycelial powder in 500 µl of CTAB. Samples were incubated at 65°C for 60 min; then, 500 µl of phenol-chloroform-isoamyl alcohol (25:24:1) was added. Samples were shaken gently for 10 min, then centrifuged at 13,000 × *g* for 15 min. The upper aqueous phase was transferred to a clean microcentrifuge tube before the phenol-chloroform-isoamyl alcohol and centrifugation steps were repeated. After the upper aqueous phase was collected a second time, 50 µl of RNAse A was added, and samples were incubated at 37°C for 30 min. The phenol-chloroform-isoamyl alcohol and centrifugation steps were repeated a third time using only 250 µl of phenol-chloroform-isoamyl alcohol. After collect-

Fig. 1. Symptoms of naturally occurring *Phytophthora nicotianae* infections of **A,** potato leaf tissue, **B,** stem tissue, **C,** infection in a commercial potato field, and **D,** complete defoliation of a plant.

ing the upper aqueous suspension a third time, an equal volume of 95% EtOH was added. The samples were incubated at 4°C overnight. DNA precipitate was collected by centrifugation at 13,000 × *g* for 5 min. The supernatant was discarded, and the pellets were washed with 100 µl of cold 80% EtOH for 5 min. The pellets were allowed to dry completely and DNA was suspended in 30 µl of nuclease-free water and stored at 4°C. Extracted DNA was electrophoresed in a 1% agarose gel to confirm the quality of the DNA. DNA concentrations were determined using a NanoDrop 1000. DNA was diluted to 10 ng/µl for subsequent use in polymerase chain reaction (PCR).

PCR amplification. DNA samples were amplified using species-specific primers previously described for *P. nicotianae* (7). Primer no. 1 (5′-CTGACGATCCAGATC CTCTGCACG-3′) was used as the forward primer and primer no. 2 (5′-CTTGCGAGG CTTGACCGCTTCCTA-3′) was used as the reverse primer. Amplification reactions were done in 25-µl reactions using an Eppendorf Mastercycler Thermalcycler (Eppendorf Scientific, Westbury, NY). Each reaction tube contained 2 µl of 10 ng/µl template DNA, 19 µl of sterile nuclease-free water, 0.25 µl each of 10 µM (2.5 µM) forward and reverse primers, 2.5 µl of 10× PCR buffer, 50 µM each dNTP, and 2.5 units (0.5 µl) of *Taq* DNA polymerase (Qiagen). The thermal cycling parameters were one cycle at 94°C for one min; 30

Table 1. Identity, source, mating type, and mefenoxam sensitivity of *Phytophthora* spp. isolates

Isolate	State of origin	Source tissue	Mating type[a]	EC_{50} (µg/ml)[b]	Growth temperature experiments	Preliminary pathogenicity experiments	Tuber and foliar cultivar susceptibility experiments
Phytophthora erythroseptica							
266-2	Washington	Potato tuber	SF	0.02	X	X	X
364-5	Minnesota	Potato tuber	SF	0.04	X	X	X
05NE1-3	Nebraska	Potato tuber	SF	0.04	X	…	…
E7[c]	Maine	Potato tuber	SF	0.07	X	…	…
06CO1-2	Colorado	Potato tuber	SF	0.08	X	…	…
PE-89	Maine	Potato tuber	SF	>100	X	…	…
217-1	Idaho	Potato tuber	SF	>100	X	…	…
05MN7-2	Minnesota	Potato tuber	SF	>100	X	…	…
06NE2-1	Nebraska	Potato tuber	SF	>100	X	…	…
06CO1-1	Colorado	Potato tuber	SF	>100	X	…	…
P. nicotianae							
05NE1-1	Nebraska	Potato tuber	A2	0.13	…	X	X
05NE1-2	Nebraska	Potato tuber	A2	0.09	X	…	X
06NE1-1	Nebraska	potato leaf	A2	0.18	…	…	…
06NE1-5	Nebraska	Potato leaf	A1	0.09	X	…	…
06FL1-7	Florida	Potato tuber	A1	0.24	X	X	…
06FL1-8	Florida	Potato tuber	A1	0.15	…	…	…
06FL1-9	Florida	Potato tuber	A1	0.25	X	…	…
N3[c]	Delaware	Potato tuber	A1	0.32	…	X	X
N5[c]	Delaware	Potato tuber	A1	0.29	…	…	X
06TX1-1	Texas	Potato leaf	A1/A2	0.12	…	…	…
06TX1-3	Texas	Potato leaf	A1	0.14	X	X	X
06TX1-4	Texas	Potato leaf	A1	0.14	…	…	…
06TX1-5	Texas	Potato leaf	A1	0.08	…	…	…
06TX1-6	Texas	Potato leaf	A1	0.08	X	…	X
07TX1-1	Texas	Potato leaf	A1	0.08	X	…	…
07TX1-2	Texas	Potato leaf	A1	0.12	…	…	…
07TX1-3	Texas	Potato leaf	A1	0.36	…	…	…
07TX1-4	Texas	Potato leaf	A1	0.10	…	…	…
07TX1-5	Texas	Potato leaf	A1	0.12	…	…	…
07TX1-6	Texas	Potato leaf	A1	0.07	…	…	…
07TX1-7	Texas	Potato leaf	A1	0.09	…	…	…
07TX1-8	Texas	Potato leaf	A1	0.09	…	…	…
07TX1-9	Texas	Potato leaf	A1	0.39	…	…	…
07TX2-1	Texas	Potato leaf	A1	0.42	…	…	…
07TX2-2	Texas	Potato petiole	A1	0.32	X	…	…
07MO1-1	Missouri	Potato tuber	A1	0.37	X	…	…
07MO1-2	Missouri	Potato tuber	A1	0.07	X	…	…
07MO1-3	Missouri	Potato tuber	A1	0.07	X	…	…
07MO1-4	Missouri	Potato tuber	A1	0.07	…	…	…
07MO1-5	Missouri	Potato tuber	A1	0.07	…	…	…
07MO1-6	Missouri	Potato tuber	A1	0.07	…	…	…
07MO1-7	Missouri	Potato tuber	A0	0.09	…	…	…
P. cactorum							
05MN55-1	Minnesota	Potato leaf	SF	0.05	X	…	X
05MN55-2[d]	Minnesota	Potato leaf	NT	0.05	X	…	X
05MN55-3[d]	Minnesota	Potato leaf	NT	0.06	…	…	X
P. capsici							
C19[c]	Delaware	Unknown	NT	83.5	…	…	X
P. infestans							
02-007-1[e]	Michigan	Potato leaf	A2	NT	…	…	X
02-007-2[e]	Michigan	Potato leaf	A2	NT	…	…	X

[a] SF = self fertile/homothallic, AO = oospores not produced with either A1 or A2 tester isolates, and NT = not tested.
[b] EC_{50} = concentration resulting in 50% reduction of mycelial growth.
[c] From the collection of Robert Mulrooney, University of Delaware.
[d] Isolate identification based upon morphological and physiological characteristics only.
[e] From the collection of William Kirk, Michigan State University.

cycles of 2 min of annealing at 65°C, 3 min of extension at 72°C, and 1 min of denaturation at 94°C; followed by one extension cycle at 72°C for 10 min. A negative control (without template DNA) was included in the amplification to ensure that the reagents were not contaminated. Following amplification, the amplicons were visualized on a 1% agarose gel and photographed under shortwave UV using the UVP BioDoc-It System.

DNA from each isolate also was amplified using universal internal transcribed spacer (ITS) primers for the purpose of sequencing for species determination. Protocols, primers, and reaction conditions were those described in detail for the identification of plant-pathogenic *Phytophthora* spp. (http://www.phytid.org/methods.htm). After amplification, the amplicons were visualized on a 1% agarose gel to check DNA concentration and purity. However, instead of fingerprinting, as described in the website, PCR products were purified using the QIAquick PCR Purification Kit Protocol (QIAquick Gel Extraction Kit; Qiagen). Purified PCR products then were sent to the DNA Sequencing and Genotyping facility in the Life Sciences Core Laboratory Center at Cornell University for sequencing. The same primers were used for sequencing as those used for amplification.

Mating type. The mating type of *P. nicotianae* isolates was determined by pairing each isolate with a known A1 and a known A2 isolate. The known mating types were isolates of *P. nicotianae* recovered from tobacco fields in 2006 from Surry County (A1) and Duplin County (A2), NC. Isolates were paired on CA supplemented with water-soluble cholesterol at 5 ppm and then incubated in the dark for 21 days at 20 to 24°C. Each pairing was observed microscopically for the presence of oospores. If oospores formed when paired with the known A1, the isolate was considered to be an A2 mating type, and vice versa. Isolates that formed oospores with both testers were scored as A1/A2 and isolates that failed to form oospores with either isolate were designated AO.

Reference isolates of *Phytophthora* spp. Isolates of *P. nicotianae* (N3 and N5), and *P. capsici* (C19) received from the collection of Robert Mulrooney, University of Delaware, and *P. infestans* isolates 02-007-1 and 02-007-2 obtained from William Kirk, Michigan State University, were included in the inoculation studies as reference controls for comparison (Table 1). Isolates of *P. infestans* were maintained on rye B medium and all other isolates were maintained on clarified 10% V8 juice agar medium (CV8; 100 ml of clarified V8 juice, 15 g of agar, and 900 ml of deionized H_2O) in the dark at 20 ± 1°C prior to testing. Isolates of *P. erythroseptica* used in these experiments were obtained from an extensive culture collection held by the authors at North Dakota State University (Table 1). All isolates were held in long-term storage and grown as previously described (23,24,29–32).

Mefenoxam sensitivity. Mefenoxam (Ridomil Gold 4EC; Syngenta Crop Protection, Greensboro, NC) sensitivity of the isolates was determined using an in vitro screening method similar to a technique described previously (29–31). Tests were conducted on modified V8 juice agar (5% V8 juice filtered through four layers of cheesecloth and 2% agar) amended with fungicide in a 10-fold dilution series ranging from 0.01 to 100 µg/ml. Plates not amended with mefenoxam served as controls. A cork borer was used to excise 5-mm-diameter disks containing mycelium and agar from the margin of actively growing colonies of 4- to 6-day-old cultures. Each disk was positioned in the center of a 9-cm culture dish with the mycelium in contact with the test medium. The dishes contained 17 ml of either amended or nonamended medium and testing was performed in duplicate at each concentration.

Isolate growth was determined by measuring colony diameters in two perpendicular directions after 6 days of incubation in the dark at 20 ± 1°C. Measurements were averaged, the diameter of the mycelial plug was subtracted, and relative growth reduction for each rate of fungicide was calculated as follows: 100 – (growth with fungicide/growth in control plate) × 100. The concentration resulting in 50% reduction of mycelial growth (EC_{50}) relative to the control was estimated by plotting the percent inhibition against the log-scale of fungicide concentration. Two independent trials were conducted to determine the EC_{50} values for each sensitivity test.

Temperature-dependent growth. The effect of temperature on in vitro growth was conducted on *P. erythroseptica* isolates 266-2, 364-5, 05NE1-3, E7, 06CO1-2, PE-89, 217-1, 05MN7-2, 06NE2-1, and 06CO1-1; *P. nicotianae* isolates 05NE1-2, 05NE1-5, 06FL1-7, 06FL1-9, 06TX1-3, 06TX1-6, 07TX1-1, 07TX2-2, 07MO1-1, 07MO1-2, and 07MO1-3; and *P. cactorum* isolates 05MN55-1 and 05MN55-2 (Table 1). Isolates were grown in the dark on CV8 agar medium at 20°C. A cork borer was used to excise 5-mm-diameter disks containing mycelium and agar from the margin of actively growing colonies of 72-h cultures. Each disk was positioned in the center of a 9-cm culture dish containing CV8 agar medium, with the mycelium in contact with the medium. Culture dishes were incubated in the dark at 5, 15, 25, and 35°C. Three culture dishes (replications) were prepared for each isolate at each temperature. Treatments (isolates grown at a specific temperature) were arranged in a completely random design in each incubator. Isolate growth was assessed daily by measuring colony diameters in two perpendicular directions. Measurements were averaged and the diameter of the original mycelial plug was subtracted. Growth evaluations continued until the fastest-growing isolate reached the margin of the culture dish. The experiment was performed twice.

Pathogenicity evaluations. *Inoculum preparation.* All test isolates were grown on culture plates containing CV8 juice agar in an environmentally controlled incubator at 20 ± 1°C in the dark. After 3 days, 5-mm-diameter disks containing mycelium and agar were removed from the margin of the colonies and placed in culture plates (three disks per plate) containing autoclaved clarified V8 broth (100 ml of clarified V8 juice and 900 ml of deionized H_2O). After incubation for 3 days at 20 ± 1°C in the dark, the clarified V8 juice broth subsequently was removed from the plates, and the mycelial mats were rinsed twice with 10 ml of sterile deionized H_2O and resuspended in 10 ml of autoclaved, filtered soil extract from a potato field (10% soil). Sporangial formation occurred after 36 to 48 h of incubation under constant illumination (eight Sylvania F20T12/CW lamps) in an environmentally controlled incubator (20 ± 1°C). *P. nicotianae* isolates spontaneously released abundant zoospores within 24 to 36 h under these conditions without further treatment. For other species, zoospore release was stimulated by chilling cultures at 10 ± 1°C for 1 h followed by a warming period at ambient temperature (20 to 25°C). Inoculum concentration was adjusted to 2×10^4 zoospores/ml using a hemacytometer. Zoospore suspensions were held in the dark at 8 to 10°C until inoculations were carried out, generally within 10 to 60 min.

Preliminary tuber inoculations. The ability of *P. nicotianae* isolates from Florida (06FL1-7), Nebraska (05NE1-1), and Texas (06TX1-3) to infect tubers of four potato cultivars was assessed in laboratory challenge inoculation studies. Aggressiveness of these isolates was compared with a previously identified isolate of *P. nicotianae* (N3) collected in Delaware and two isolates of *P. erythroseptica* (266-2 and 364-5) used in earlier pink rot studies (29,30,32). Certified seed of potato cultivars (Atlantic, Red Norland, Russet Norkotah, and Snowden) displaying varying levels of susceptibility to *P. erythroseptica*-induced pink rot (23) were planted in irrigated research plots in central Minnesota. Each cultivar was grown in a block of four rows by 75 m and managed using agronomic practices typical of those recommended for potato production in that area. Plants were killed by mechanical flailing 2 to 3 weeks prior to maturity to insure the availability of a sufficient quantity of tubers of the desired size and adequate skin set. After harvest, tubers were held for 2 weeks at 15°C and 90% relative humidity to facilitate wound healing, then

stored at 10°C until inoculation studies were conducted.

Disease-free tubers (140 to 190 g) with apical eyes free of soil and having an intact periderm were hand selected for inoculation trials. Tubers were acclimated for 1 to 2 days at ambient temperature (20 to 25°C) and placed in plastic moist chamber boxes (33 by 24 by 12 cm) lined at the bottom with moistened paper towels and inoculated with 10 µl of the zoospore suspension (approximately 200 zoospores) on each of three apical eyes. Inoculated tubers were covered with four layers of paper towels moistened to saturation with deionized water. Chamber boxes were covered to establish high humidity to promote infection and placed in the dark at an ambient temperature of 20 to 22°C for 10 days. Inoculations were carried out as two separate trials, each consisting of four replications of 10 tubers per cultivar.

Cultivar susceptibility of tubers and foliage. Based on the results of preliminary pathogenicity experiments conducted on tubers, further studies were performed to determine the pathogenicity and aggressiveness of *P. nicotianae* and other *Phytophthora* spp. on foliage and in wounded and nonwounded tubers. Aggressiveness of isolates of *P. nicotianae* from Nebraska (05NE1-1 and 05NE1-2) and Texas (06TX1-3 and 06TX1-6) was compared with *P. cactorum* isolates (05MN55-1, 05MN55-2, and 05MN55-3) collected in Minnesota and known reference isolates of *P. nicotianae* (N3 and N5), *P. erythroseptica* (266-2 and 364-5), *P. capsici* (C19), and *P. infestans* (02-007-1 and 02-007-2) via foliar and tuber inoculations. Tubers selected from certified seed of potato cvs. Atlantic, Red Norland, Russet Burbank, Russet Norkotah, and Snowden were utilized in tuber inoculation experiments as well as to produce plant material for foliar inoculations. Tuber eyes (nonwounded) as well as tubers that had been wounded artificially by removing the periderm (1 by 1 cm) with a no. 96 general-purpose, commercially available abrasive pad were inoculated with zoospore suspensions as previously described (30). Inoculations were carried out as two separate trials consisting of five replications, with one tuber per replication per cultivar.

Source plants for foliar inoculations were grown in the greenhouse from the same certified seed source as was used in the tuber inoculations. Seed tubers were planted in square, 13.5-cm plastic pots containing Sunshine Mix Number 1 (Sun-Gro Horticulture Canada Ltd., Seba Beach, AB, Canada). Each pot received a single 9.86 cm^3 application of Multicote 4 (14-14-16+minors) control release fertilizer (Haifa NutriTech Inc., Altamnote Springs, FL) at planting. Plants were grown under 16 h of natural sunlight per day and ambient temperatures of 24 to 29°C. Three leaves, having at least five fully expanded leaflets, were collected from each of the five cultivars prior to flowering and placed into plastic boxes containing moistened paper towels. A 20-µl drop of the zoospore suspension was deposited near the center of the long axis of each leaflet, in an area midway between the midvein and the margin of the leaflet. Five leaflets of each of three leaves were inoculated per isolate. Plastic boxes were sealed with Press'n Seal (The Glad Products Co., Oakland, CA) and leaves were incubated at ambient temperature (20 to 25°C) under constant fluorescent illumination for 7 days. Leaves were inverted with the abaxial side down to ensure the proper microclimate for infection 24 h after inoculation. Inoculations were carried out as two separate trials.

Disease assessment. Disease development in infected tubers was quantified using techniques similar to those described in earlier studies involving pink rot and leak (*23*,*29*,*30*). Tubers inoculated in the apical eyes were removed from the moist chambers and infection was determined by cutting each tuber in half through the axis from the sites of inoculation on the apical bud end to the basal stem end. Tubers inoculated following wounding were bi-

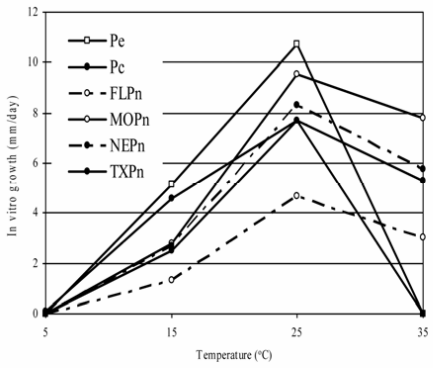

Fig. 3. Effect of temperature upon in vitro growth of *Phytophthora erythroseptica* (isolates 266-2, 364-5, 05NE1-3, E7, 06CO1-2, PE-89, 217-1, 05MN7-2, 06NE2-1, and 06CO1-1), *P. cactorum* (isolates (05MN55-1 and 05MN55-2), and *P. nicotianae* isolates from Florida (06FL1-7 and 06FL1-9), Missouri (07MO1-1, 07MO1-2, and 07MO1-3), Nebraska (05NE1-2 and 06NE1-5), and Texas (06TX1-3, 06TX1-6, 07TX1-1, and 07TX2-2).

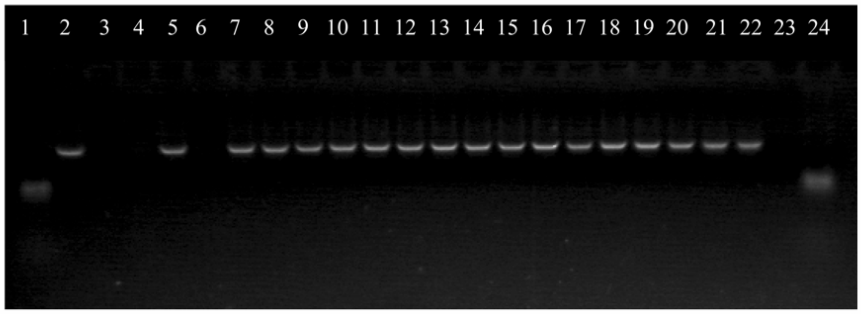

Fig. 2. Polymerase chain reaction amplification using *Phytophthora nicotianae* species-specific primers. *P. nicotianae* positive control (lane 2), *P. infestans* negative control (lane 3), *P. erythroseptica* negative control (lane 4), PnN3 (lane 5), 05MN55-1 (lane 6), 05NE1-2 (lane 7), 06GA1-7 (lane 8), 06GA1-9 (lane 9), 06NE1-1 (lane 10), 06TX1-1 (lane 11), 07TX1-1 (lane 12), 07TX1-2 (lane 13), 07TX1-3 (lane 14), 07TX1-4 (lane 15), 07TX1-5 (lane 16), 07TX1-6 (lane 17), 07TX1-7 (lane 18), 07TX1-8 (lane 19), 07TX1-9 (lane 20), 07TX2-1 (lane 21), and 07TX2-2 (lane 22). Lanes 1 and 24 contain a 1-kb ladder, and lane 23 contains a no-template control.

sected through the point of inoculation, perpendicular to the longitudinal axis. Split tubers were covered with moist paper towels and incubated at ambient temperatures of 20 to 24°C for approximately 30 min to enhance the development of the discoloration diagnostic of pink rot infection. Infected tubers were counted and disease incidence was calculated as follows: (number of diseased tubers/number of inoculated tubers) × 100. To determine disease severity, the maximum depth (D) of rotted tissue was measured from the point of inoculation and penetration rate (P) was calculated as P = D/T, where T is time in days after inoculation.

Disease development in infected leaves was quantified by scoring leaflets for lesion expansion from 2 to 7 days after inoculation and incidence of sporulation and infection at 7 days after inoculation. Severity of infection was rated on a 0-to-5 scale according to the size of the lesion, where 0 = no visible lesion, 1 = necrotic flecking <1 cm in diameter, 2 = necrotic spots between 1 and 2 cm in diameter, 3 = 2 to 3 cm in diameter, 4 = 3 to 4 cm in diameter, and 5 = >4 cm in diameter. The development of small, necrotic, hypersensitive spots was not considered true pathogen infection and was not used to calculate infection incidence or lesion expansion. The ability of the pathogen to sporulate was rated as: no mycelial growth or sporulation observed, mycelial growth with little or no sporulation, and dense sporulation. Incidence was determined by counting the number of visible lesions greater than 1 cm in diameter and calculating the frequency of infection out of 15 total leaflets inoculated per isolate–cultivar combination. Those lesions remaining <1 cm and scored as 1 after 7 days were considered to be the result of a hypersensitive response and were not considered to be the result of active pathogen growth. The area under the lesion expansion curve (AULEC) was calculated according to a modified formula based upon the formula of Shaner and Finney (26) for area under the disease progress curve (AUDPC):

$$\text{AULEC} = \sum_{i=1}^{n} \left[\left(W_{i+1} + W_i \right) / 2 \right] \left(t_{i+1} - t_i \right)$$

where W_i = lesion diameter rating at the ith observation, t_i = time in days at the ith observation, and n = total number of observations.

Data analysis. Levene's test for homogeneity of variance was conducted on all tuber and foliar inoculation trials as well as temperature-dependent growth experiments to ensure that data could be combined for further analysis (17). Data for individual *P. erythroseptica, P. cactorum*, and *P. infestans* isolates were pooled by species. *P. nicotianae* isolates were subdivided by location in foliar and tuber cultivar susceptibility experiments as well as temperature-dependent growth experiments. A one-way analyses of variance (ANOVA) was performed using PROC GLM (SAS, version 9.0; SAS Institute Inc., Cary, NC) among isolate groups for in vitro growth at each temperature for temperature-dependent growth data and for disease incidence and severity for pathogenicity and cultivar-susceptibility experiments. To compare susceptibility across cultivars to *P. nicotianae*, disease incidence data from tuber pathogenicity and cultivar-susceptibility experiments were combined using Levene's method for all isolates of *P. nicotianae* and a one-way

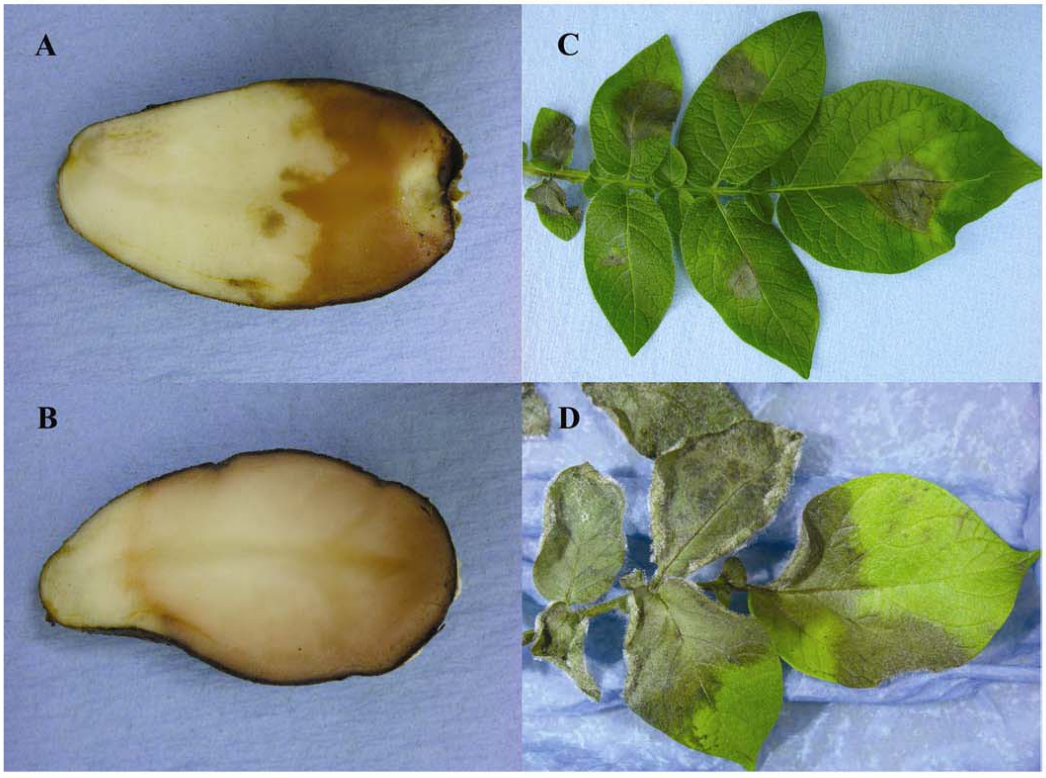

Fig. 4. Symptoms of *Phytophthora nicotianae* infections of potato tuber tissue (cv. Red Norland) inoculated with **A,** *P. nicotianae* isolate 06TX1-6 and **B,** *P. erythroseptica* isolate 364-5 and foliar tissue (cv. Atlantic) inoculated with **C,** *P. nicotianae* and **D,** *P. infestans*. Note sporulation on leaf infected with *P. infestans* but absent on leaf infected with *P. nicotianae*.

ANOVA was performed. Differences in mean fungal lesion expansion and in vitro growth, disease incidence, and severity of infection of inoculated tubers, as well as mean disease incidence of foliar infection and AULEC, were determined using Fisher's protected least significant difference (LSD) test ($\alpha = 0.05$). Pearson's correlation coefficient was utilized to compare disease incidence of tuber infection by *P. nicotianae* and *P. erythroseptica* among cultivars inoculated in the preliminary, nonwounded, and wounded pathogenicity experiments.

RESULTS

Isolate identification and characterization. *Morphological characterization.* Isolates recovered from infected tubers collected in Nebraska in 2005 and leaves in 2006, tubers from Florida in 2006, leaves from Texas in 2006 and 2007, and tubers from Missouri in 2007 all were identified as *P. nicotianae*. One isolate (05MN55-1) recovered from an infected leaf collected in Minnesota in 2005 was identified as *P. cactorum* (Table 1). Two other isolates (05MN55-2 and 05MN55-3) were designated as *P. cactorum* based on morphological characteristics identical to isolate 05MN55-1. All isolates had hyphae typical of *Phytophthora* spp., with slight to prominent hyphal swellings in CA. Colony type varied slightly among isolates, with all but one isolate having a rosette to arachnoid colony type typical of *P. nicotianae*. Isolate 05MN55-1 had a slightly petaloid to very diffuse colony type described for *P. cactorum*. All 32 *P. nicotianae* isolates produced typical sporangia and chlamydospores in CA, but the intensity of sporulation varied with isolate. Sporangia were variable in size and shape; most sporangia were ellipsoid, ovoid, obpyriform, or spherical, and all had prominent papilla. Isolates of *P. cactorum* produced typical sporangia for the species, but chlamydospores were not observed in CA. Oospores were abundant in the isolates of *P. cactorum*, but no oospores were produced in the isolates of *P. nicotianae*.

Species-specific amplification. DNA samples were assayed using PCR primers specific to *P. nicotianae*. A known isolate of *P. nicotianae* was included as a positive control, and *P. infestans* and *P. erythroseptica*, both important potato pathogens, were included as negative controls. Negative controls from the collections of Neil Gudmestad (*P. erythroseptica*) and Jean Ristaino (*P. infestans*) were identified previously based upon morphological characteristics and pathogenicity or DNA characterization. The species-specific primers amplified the positive control but neither of the negative controls. All of the isolates identified as *P. nicotianae* based on morphological features were amplified using the species-specific primers (Fig. 2). The isolate of *P. cactorum* also was not amplified.

ITS sequencing was completed to confirm identification of the *P. nicotianae* isolates and one isolate of *P. cactorum*. Sequences were blasted against GenBank accessions through National Center for Biotechnology Information blastn. Isolate 05MN55-1 was 99% identical to *P. cactorum*, and all other isolates were identified as *P. nicotianae* based on similarity to reported *P. nicotianae* isolates.

Mating type. Mating type was determined successfully for all isolates (Table 1). All isolates of *P. nicotianae* were the A1 mating type except for three of the Nebraska isolates (05NE1-1, 05NE1-2, and 06NE1-1), which were A2, and one of the Texas isolates (06TX1-1), which produced oospores with both testers and was scored as an A1/A2. Missouri isolate 07MO1-7 did not produce oospores with either tester and subsequently was scored AO.

Mefenoxam sensitivity. Isolates of *P. nicotianae* from Florida, Missouri, Nebraska, and Texas all were sensitive to mefenoxam, with EC_{50} values ranging from 0.07 to 0.42 µg/ml (Table 1). *P. cactorum* isolates 05MN55-1, 05MN55-2, and 05MN55-3 also were sensitive, as were reference *P. nicotianae* isolates N3 (0.32 µg/ml) and N5 (0.29 µg/ml) and *P. erythroseptica* isolates 266-2 (0.02 µg/ml), 364-5 (0.04 µg/ml), 05NE1-3 (0.04 µg/ml), E7 (0.07 µg/ml), and 06CO1-2 (0.08 µg/ml). Other *P. erythroseptica* isolates included for comparison in the temperature-dependent growth study were resistant to mefenoxam (>100 µg/ml).

Temperature-dependent growth. Optimum growth of *P. nicotianae, P. cactorum*, and *P. erythroseptica* isolates occurred at 25°C. Growth of *P. erythroseptica* was significantly greater than either *P. nicotianae* or *P. cactorum* isolates at that temperature (Fig. 3). Growth of *P. erythroseptica* and *P. cactorum* isolates at 15°C was significantly greater and nearly double that recorded for isolates of *P. nicotianae*. All *P. nicotianae* isolates sustained active growth at 35°C, whereas isolates of *P. erythroseptica* and *P. cactorum* did not. Significant differences in growth among *P. nicotianae* isolates obtained from different locations were observed at 15, 25, and 35°C and among *P. nicotianae* isolates. Isolates from Missouri grew most rapidly, whereas isolates from Florida consistently had the least growth at each temperature. Growth of *P. nicotianae* isolates from Missouri at 35°C was 82% of that observed at 25°C. Likewise, growth of *P. nicotianae*

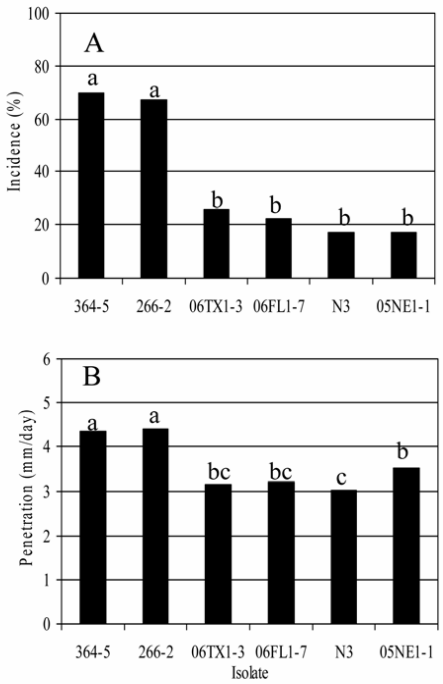

Fig. 5. A, Incidence and **B,** penetration of tubers of four potato cultivars following inoculation of tuber eyes by zoospore suspensions of *Phytophthora erythroseptica* isolates (364-5 and 266-2) and isolates of *P. nicotianae* obtained from Texas (06TX1-3), Florida (06FL1-7), Delaware (N3), and Nebraska (05NE1-1). Columns with the same letter are not statistically different based on Fisher's protected least significant difference ($P = 0.05$).

isolates from Nebraska, Texas, and Florida ranged from 65 to 70% of the maximum observed at 25°C.

Pathogenicity evaluations. *Preliminary tuber inoculations.* Tuber symptoms caused by *P. nicotianae* were typical of pink rot symptoms resulting from *P. erythroseptica* infections; however, the pink discoloration was not as intense with *P. nicotianae* and the infected tissue became tan to pale brown after extended exposure to air (Fig. 4A and B). A significant difference in disease incidence and disease severity among isolates was observed. Isolates of *P. nicotianae* from Texas (06TX1-3), Florida (06FL1-7), Nebraska (05NE1-1), and reference isolate N3 from Delaware were pathogenic, infecting tubers following eye inoculation of nonwounded tubers with zoospore suspensions (Fig. 5A). Across cultivars, disease incidence was highest with *P. erythroseptica* isolates 364-5 (70%) and 266-2 (67%). *P. nicotianae* isolates were significantly less aggressive, with disease incidences values between 17 and 26%. Disease severity followed a similar pattern. Tissue penetration by *P. erythroseptica* isolates 364-5 and 266-2 was significantly greater than colonization by any of the *P. nicotianae* isolates (Fig. 5B). Across *P. nicotianae* isolates, cv. Red Norland was the most susceptible cultivar, but only significantly so when compared with cv. Snowden. Colonization of tuber tissue was significantly slower in cv. Atlantic than in cvs. Red Norland and Snowden (*data not shown*).

Cultivar susceptibility of tubers and foliage. A significant difference was observed in disease incidence and disease severity between wounded and nonwounded tubers; therefore, these data were analyzed separately. Following tuber eye inoculation of nonwounded tubers, significant differences in disease incidence among isolate groups were observed across the five cultivars used in this study (Fig. 6A). Control isolates of *P. erythroseptica* (364-5, 266-2) *P. nicotianae* (N3 and N5), and *P. infestans* (02-007-1 and 02-007-2) infected nonwounded potato tubers via eye inoculation with zoospore suspensions, as did *P. nicotianae* isolates from Nebraska (05NE1-1 and 05NE1-2) and Texas (06TX1-3 and 06TX1-6) (Fig. 6A). The highest infection frequencies were obtained with isolates of *P. erythroseptica* and disease incidence was significantly lower following inoculation with *P. nicotianae* and *P. infestans* isolates. Disease incidence did not differ among *P. nicotianae* isolates from Delaware, Nebraska, and Texas, and was similar to the infection frequency observed with isolates of *P. infestans*. In contrast, isolates of *P. cactorum* (05MN55-1, 05MN55-2, and 05MN55-3) obtained from naturally occurring potato leaf infections in Minnesota, and the *P. capsici* control isolate C19 from pepper, did not infect nonwounded tubers. Significant differences in colonization of nonwounded tuber tissue also were noted among *Phytophthora* spp. (Fig. 5B). The highest level of penetration was observed with isolates of *P. erythroseptica* (5.2 mm/day), which was significantly greater than penetration by *P. nicotianae* isolates from Texas. Penetration by *P. nicotianae* isolates from Delaware and Nebraska was similar to isolates of *P. erythroseptica*. Penetration rates by *P. nicotianae* isolates from Delaware, Nebraska, and Texas ranged from 3.7 to 4.5 mm/day. The penetration rate of *P. infestans* isolates (0.7 mm/day) was significantly lower than rates observed for isolates of *P. erythroseptica* and *P. nicotianae*.

Wounding also affected infection efficiency. Disease incidence increased significantly following abrasive removal of

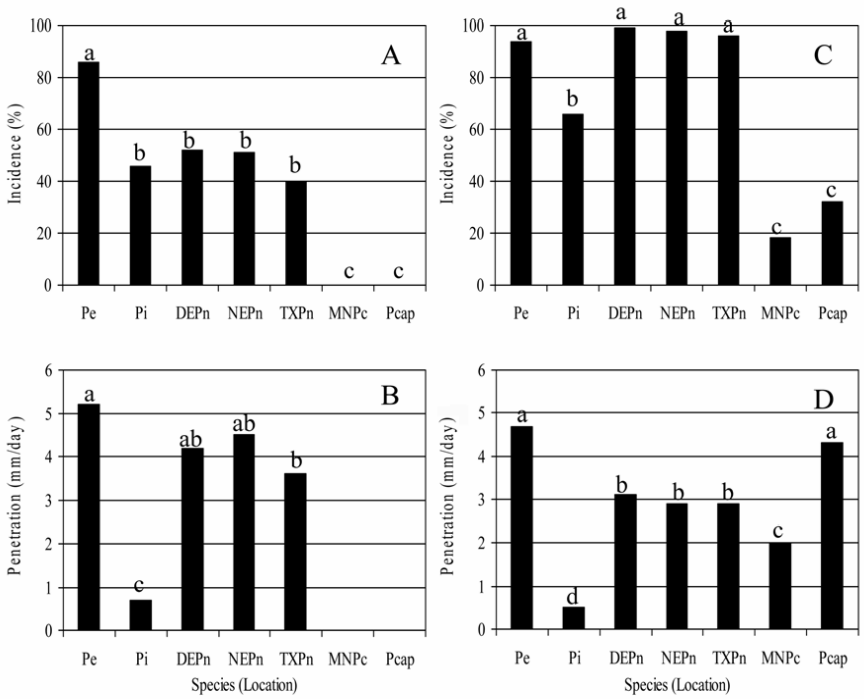

Fig. 6. A and **C,** Incidence and **B** and **D,** penetration of potato tubers following inoculation of **A** and **B,** tuber eyes and **C** and **D,** wounds with zoospore suspensions of *Phytophthora erythroseptica* isolates (364-5 and 266-2; Pe), *P. infestans* (02-007-1 and 02007-2; Pi), *P. capsici* (C19; Pcap), and isolates of *P. nicotianae* obtained from Texas (06TX1-3 and 06TX1-6; TXPn), Delaware (N3 and N5; DEPn), Nebraska (05NE1-1 and 05NE1-2; NEPn) and Minnesota (05MN55-1, 05MN55-2, and 05MN55-3; MNPc). Columns with the same letter are not statistically different based on Fisher's protected least significant difference ($P = 0.05$).

the periderm compared with nonwounded eye inoculations. Additionally, in contrast to the results obtained in nonwounded tubers, all *Phytophthora* spp. were able to infect wounded tubers, and significant differences in aggressiveness were observed among groups (Fig. 6A and C). This was most notable with *P. capsici* and *P. cactorum*, which were not able to infect nonwounded tuber tissue, and *P. nicotianae*, where wounding nearly doubled infection frequencies. Infection frequencies obtained with *P. erythroseptica* and *P. nicotianae* were similar, ranging from 94 to 99% following wounding. Infection frequencies observed for *P. infestans* (66%), *P. capsici* (32%), and *P. cactorum* (18%) were significantly lower than observed with the other species. Although infection frequency was low, tissue penetration by *P. capsici* was similar to the rate of penetration observed with *P. erythroseptica* and significantly greater than that of *P. infestans*, *P. nicotianae*, and *P. cactorum* (Fig. 6D). *P. erythroseptica* and *P. capsici* had the highest rates of tissue colonization at 4.7 and 4.3 mm/day, respectively. Penetration rates of *P. nicotianae* from Delaware, Nebraska, and Texas differed significantly from those isolates, ranging from 2.9 to 3.1 mm/day. *P. infestans* and *P. cactorum* had significantly lower rates of tissue colonization at 0.5 and 2.0 mm/day, respectively.

Across *P. nicotianae* isolates, nonwounded tubers of cv. Red Norland were most susceptible, supporting the preliminary tuber inoculation experiments, and were significantly more susceptible than cvs. Russet Burbank and Atlantic (Fig. 7A). Tuber tissue colonization was greatest in cvs. Atlantic, Red Norland, and Russet Norkotah, ranging from 4.7 to 5.5 mm/day, and was significantly lower in cvs. Snowden and Russet Burbank, penetrating 3.3 and 3.2 mm/day, respectively (Fig. 7B). Percent disease incidence ranged from 96 to 100% among cultivars wounded and inoculated with *P. nicotianae* isolates; therefore, there was no difference observed in the level of susceptibility to this pathogen when wounding has occurred (Fig. 7C). However, rates of penetration of wounded tissue did differ among the cultivars (Fig. 7D). As with nonwounded tissue, cvs. Atlantic, Red Norland, and Russet Norkotah suffered the highest rate of pathogen penetration. A significant correlation (r = 0.72, P = 0.0038) existed for pink rot incidence caused by *P. erythroseptica* and *P. nicotianae* among cultivars used in all tuber inoculation experiments (Fig. 8).

As was observed with nonwounded tuber tissue, isolate groups of *Phytophthora* spp. differed significantly in foliar infection frequency (Fig. 9A). *P. infestans* isolates infected foliage with 100% efficiency and isolates of *P. nicotianae* from Delaware (88%), Nebraska (73%), and Texas (50%) also infected foliage more frequently than isolates of *P. erythroseptica* (38%). Control *P. capsici* isolate C19 and *P. cactorum* isolates from Minnesota were the least aggressive. Although *P. capsici* was able to infect nearly 10% of the time, isolates of *P. cactorum* produced only necrotic hypersensitive reactions on the foliage.

Lesion expansion in infected leaf tissue also differed significantly among isolate groups (Fig. 9B). AULEC was similar for *P. infestans* and *P. nicotianae* isolates (Fig. 9B). *P. erythroseptica* isolates and *P. cactorum* isolates from Minnesota did infect foliar tissue, but at a significantly slower rate. *P. capsici* was incapable of infecting potato foliage. Foliar symptoms caused by *P. nicotianae* isolates were nearly indistinguishable from that of *P. infestans* (Fig. 4C and D). However, *P. infestans* produced abundant sporulation under conditions of high relative humidity (>90%) provided in this experiment, in contrast to the isolates

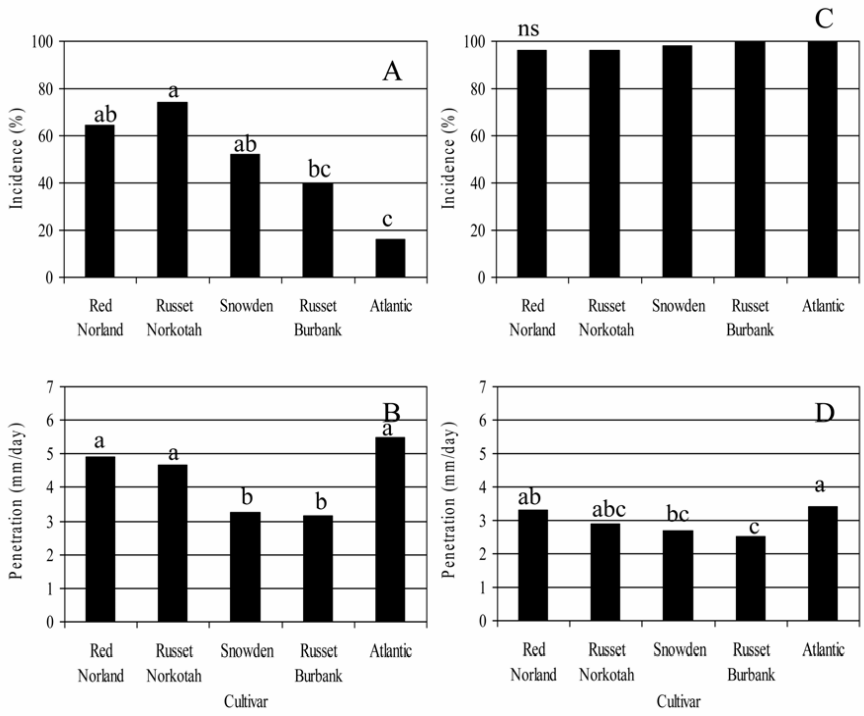

Fig. 7. A and **C,** Incidence and **B** and **D,** penetration of tubers of five potato cultivars following inoculation of **A** and **B,** tuber eyes and **C** and **D,** wounds by zoospore suspensions of *Phytophthora nicotianae* isolates N3, 06TX1-3, 05NE1-1, and 06FL1-7. Columns with the same letter are not statistically different based on Fisher's protected least significant difference (P = 0.05).

of *P. nicotianae* evaluated, where mycelial growth and sporangiophores were observed but sporangia were not produced (*data not shown*). Additionally, no sporulation was observed for isolates of *P. erythroseptica*, *P. capsici*, or *P. cactorum* on inoculated foliage of any cultivar.

DISCUSSION

Late blight is a disease of potato that elicits an immediate and dramatic response by most potato producers. Because the disease can progress very rapidly and destroy potato foliage in a very short time under conducive weather conditions, potato producers generally are inclined to reduce risk by the application of preventative fungicides such as mancozeb or chlorothalonil. However, if late blight is found in a field, the grower response is likely to be more drastic and will include the applications of fungicide chemistries more efficacious and more expensive. In the presence of mefenoxam-resistant *P. infestans*, these fungicides include cymoxanil, cyazofamid, dimethomorph, famoxadone, fenamidone, propamocarb, or zoxamide. These fungicides individually represent an increase in cost of late blight control two to three times that of preventative fungicides such as mancozeb or chlorothalonil.

It is because of the importance of late blight as a potato disease that we became aware of foliar infections of *P. nicotianae* on potato. Under wet weather conditions in a number of Southern potato production states, *P. nicotianae* was able to infect potato foliage. However, the disease frequently is confined to low areas in fields and along irrigation tracks (Fig. 1C) where standing water persists because of soil compaction. These observations suggested that the foliar disease does not spread via the production of secondary inoculum originating from primary lesions. However, continued wet weather can cause additional primary infections to occur, giving the appearance of a secondary pathogen cycle. Controlled inoculations under conducive environmental conditions support our field observations because we failed to find evidence of sporangial production from foliar lesions as we did with *P. infestans*. Nonetheless, in southern Texas in early 2007, which was characterized by unusually wet weather over a prolonged period, several *P. nicotianae* infection events occurred and a number of growers expressed concern about the severity the disease. Because the data collected at the time suggested that the *P. nicotianae* isolates were mefenoxam sensitive, we recommended that they use this fungicide chemistry, which they did successfully. *P. nicotianae* isolates recovered from the South Texas and Missouri epidemics were included in the studies reported here and subsequently found to be mefenoxam sensitive.

P. nicotianae is known to cause foliar and stem blights in numerous plant species (8), including potato (34), where foliar infections where observed via artificial inoculation. Person and Nielsen (20) described symptoms of foliar and stem infections occurring in North Carolina and also were able to produce leaf infections by inoculation under laboratory conditions. Our observations provide additional insights into *P. nicotianae*-induced blight of potato. Disease incidence did not differ significantly among the five cultivars used to evaluate foliar infection, suggesting that

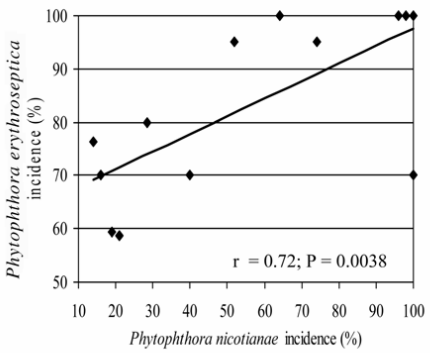

Fig. 8. Pearson's correlation coefficient comparing the percent disease incidence of tubers from cvs. Atlantic, Russet Norkotah, Red Norland, Russet Burbank, and Snowden inoculated with *Phytophthora erythroseptica* and *P. nicotianae* in the preliminary, nonwounded, and wound inoculation experiments.

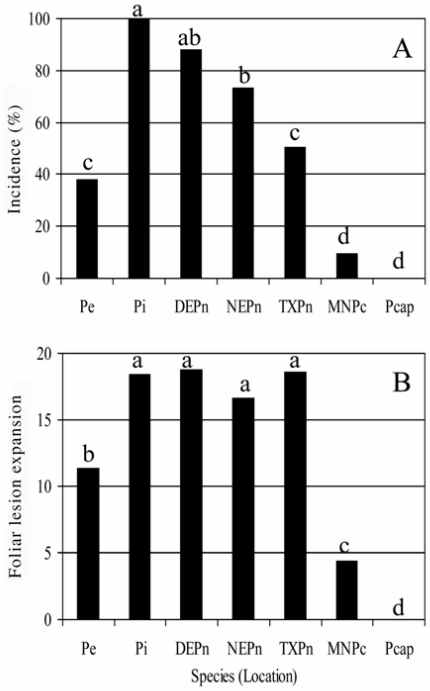

Fig. 9. A, Incidence of foliar infection and **B,** foliar lesion expansion illustrated by area under the lesion expansion curve following inoculation of leaflets of five potato cultivars by zoospore suspensions of *Phytophthora erythroseptica* isolates (364-5 and 266-2; Pe), *P. infestans* (02-007-1 and 02007-2; Pi), *P. capsici* (C19; Pcap), and isolates of *P. nicotianae* obtained from Texas (06TX1-3 and 06TX1-6; TXPn), Delaware (N3 and N5; DEPn), Nebraska (05NE1-1 and 05NE1-2; NEPn), and Minnesota (05MN55-1, 05MN55-2, and 05MN55-3; MNPc). Columns with the same letter are not statistically different based on Fisher's protected least significant difference ($P = 0.05$).

current genotypes may be equally susceptible to attack by *P. nicotianae*. Additionally, incidence and extent of lesion development with *P. nicotianae* isolates 05NE1-1 and 05NE1-2 did not differ from levels attained with *P. infestans*. Therefore, it appears that some *P. nicotianae* isolates are capable of causing foliar damage comparable in aggressiveness to *P. infestans*. Based upon our observations and those of earlier workers (20), the symptoms of foliar and stem infections caused by *P. nicotianae* are indistinguishable from potato late blight in the field. Lesion shape and size are typical of those caused by *P. infestans*, lesions generally are found on lower plant parts, and mycelium often can be seen within the infected lesion area. The primary difference is that sporangia are rarely, if ever, produced with blight caused by *P. nicotianae* and lesions devoid of sporangia may be found on infected plants for extended periods of time. This obviously impacts the epidemiology of *P. nicotianae* versus *P. infestans*, making the former less explosive as a foliar disease of potato. The lack of true sporulation of *P. nicotianae* also provides a means to distinguish infections of this pathogen from *P. infestans* and can be utilized in the field by crop consultants and field scouts working in the potato industry.

Although numerous *Phytophthora* spp. have been reported to infect tuber tissue resulting in pink-rot-like symptoms (8), *P. erythroseptica* is considered to be the primary cause of pink rot in most potato-growing regions. Results obtained by our research group in an extensive sampling of tubers exhibiting typical pink rot symptoms are consistent with these observations (31). Only *P. erythroseptica* was isolated from infected tuber tissue during this study, which involved >2,000 tubers from various potato-growing areas in the United States spanning multiple years. Other *Phytophthora* spp. rarely are associated with the pink rot, particularly in North America. However, in 2005, we isolated *P. nicotianae* from tubers originating in Nebraska. Foliar samples also were received from Nebraska that year displaying late-blight-like symptoms; however, the pathogen was not recovered at that time. Isolates were recovered from foliage collected in Nebraska in 2006. That same year, *P. nicotianae* was isolated from tubers collected in Florida and leaves obtained from potato plants growing in a commercial field in Texas. In 2007, the pathogen was isolated again from leaves collected in Texas as well as tubers from Missouri. It is interesting to note that the only isolates of *P. nicotianae* of the A2 mating type have been recovered from Nebraska, where the A1 mating type also was recovered. Thus, it would appear at this time that Nebraska is the only state in which both mating types of this pathogen that affect potato exist. Although we presume that *P. nicotianae* is surviving as chlamydospores in the soil, it is intriguing that, if both mating types are present, oospores may be forming in some locations

Since the first report of *P. nicotianae* (*P. parasitica*) infecting potato tubers in Oklahoma and Kentucky under field conditions (6), the pathogen has been reported to cause tuber pink rot in Texas (12) and has been isolated from symptomatic tubers in Delaware (18). The current study supplements this information by reporting the first occurrences of pink rot in Nebraska, Florida, and Missouri caused by *P. nicotianae*. From the studies reported here, it is apparent that *P. nicotianae* is not as aggressive as *P. erythroseptica* in causing pink rot; thus, it is likely that its occurrence and distribution in southern states most probably is due to a greater tolerance to higher temperatures as evidenced by our vitro growth experiments.

Lambert and Salas (15) asserted that *P. nicotianae* is likely to be more prevalent than *P. erythroseptica* under hot growing conditions, thus implying that *P. nicotianae* could be a major cause of pink rot under those conditions. This view is supported by the fact that most early observations of foliar and tuber infections were made in potato-growing areas generally having warm growing seasons, such as those in Delaware and New Jersey (18), Kentucky and Oklahoma (6), North Carolina (20), and Texas (12). Erwin and Ribeiro (8) reported maximum growth temperatures of 33 to 40°C for *P. nicotianae* isolates from a variety of host species, and Wijers (36) recorded maximum growth temperatures of 33 to 35°C for isolates from carnation obtained from various locations. This association is further supported by results obtained by Grisham and coworkers (12), who reported optimum growth temperatures of 32 to 33°C and a maximum growth temperature of 36°C for an isolate of *P. parasitica* from Texas, as well as by our recovery of isolates from Florida, Missouri, and Texas. Results obtained in the current study indicated that *P. nicotianae* from these areas was able to sustain nearly optimum growth rates at 35°C whereas growth of *P. erythroseptica* and *P. cactorum* was completely inhibited at that temperature. Pathogenicity tests conducted by Grisham and co-workers (12) demonstrated that *P. parasitica* caused the greatest amount of damage at 34 to 36°C; therefore, the ability of *P. nicotianae* to sustain growth at these high temperatures is likely to play a significant role in disease development and severity. The warmer summers experienced by growers in the upper Great Plains in recent years may at least partially explain the emergence of *P. nicotianae* as a potato pathogen in Nebraska reported here. Additional cases of *P. nicotianae* infections are likely to be documented in the northern potato crop, and the pathogen may compose a greater proportion of the overall *Phytophthora* population if seasonal temperatures continue to remain above average. It is also possible that *P. nicotianae* could become a more important foliar pathogen of potato because mefenoxam is no longer being used as a foliar fungicide due to the presence of resistance to this fungicide in the *P. infestans* (3,5) and *P. erythroseptica* populations (31).

Interestingly, a single case of an outbreak of a foliar disease in southern Minnesota caused by *P. cactorum* also was observed during the current study. As with *P. nicotianae*, infections of this fungus also were limited to irrigation wheel tracks where soil was compacted and there was standing water in the field. Early work (36) demonstrated that *P. cactorum* could cause a tuber rot of potato; however, these infections were obtained artificially by inoculation. In an extensive review of the host range of *P. cactorum*, Nienhaus (19) referenced numerous studies involving inoculations of potato resulting in pink rot symptoms. Results obtained in the current study are consistent with these observations and, for the first time, demonstrate that *P. cactorum* also is capable of infecting potato leaf tissue and that such infections can occur naturally under field conditions. Additionally, a number of other *Phytophthora* spp. have been reported to cause pink rot of potato. Previous mycelium-based inoculation studies showed that *P. capsici* can cause pink rot in wounded tubers (34,35) but will not infect intact tuber tissue (33–35). Despite these data, *S. tuberosum* is not considered to be a host of *P. capsici* (8). Results of the present study confirm these earlier observations and, for the first time, demonstrate that zoospores of *P. capsici* will initiate such infections. The pathogen was unable to infect tubers through eye buds but readily infected and grew within tuber tissue wounded by abrasion. In light of these findings, supplemental studies to examine pathogenicity and aggressiveness of other isolates of *P. capsici* and *P. cactorum* should be performed.

The development and spread of a new disease, or the resurgence of an older one, should prompt growers and researchers alike to take notice. Although currently localized and not widespread within or among potato-growing regions in the United States, the appearance of *P. nicotianae* as a potato pathogen should prompt such a reaction. Sparse sporulation and low infection efficiencies of some isolates suggest that the pathogen may not be an immediate threat to potato production. However, results reported here demonstrate that isolate aggressiveness can be highly variable and, in some instances, infection frequencies of *P. nicotianae* were comparable to those of *P. erythroseptica* or *P. infestans*. Isolates of *P. nicotianae* obtained from multiple samples of potato leaves from south Texas in May 2007 were confirmed

to be pathogenic and highly aggressive on both foliage and tuber tissue. The growers providing these samples reported very high incidence of infection and expressed concern that their crop could become completely defoliated. Severe losses due to *P. nicotianae*-induced pink rot have been reported to occur in the high plains of Texas in the past (12).

Strategies used to manage *P. erythroseptica*, such as planting in well-drained soils, avoiding excessive irrigation at the end of the growing season, allowing sufficient time between vine killing and harvest to promote proper periderm development, and modifying tuber-handling procedures to reduce wounding, should provide effective control of *P. nicotianae*. Our results demonstrate that limiting tuber damage would be of utmost importance because *P. nicotianae* is most aggressive on wounded tuber tissue. This importance is compounded because wounds destroy the protective barrier provided by mefenoxam, thus reducing the fungicide's effectiveness (30). All of the isolates reported here are sensitive to mefenoxam, with sensitivities similar to isolates that have no previous exposure to the fungicide (27). Mefenoxam currently is used to control *P. erythroseptica* and, therefore, should provide effective control of *P. nicotianae* if tuber damage is kept at a minimum because wounding has been demonstrated previously to negate the tuber protection provided by mefenoxam against invasion by water rot pathogens (30).

Cultivar resistance also may be an alternative to effectively manage pink rot caused by *P. nicotianae*. It has been demonstrated previously that susceptibility to *P. erythroseptica* varies widely among commercially acceptable cultivars (23). From the studies reported here, it appears that the susceptibility of cultivars to *P. nicotianae* does not differ from that of *P. erythroseptica*. However, further studies are needed involving a greater range of cultivar susceptibilities to fully investigate the relationship of resistance to *P. nicotianae* and *P. erythroseptica*.

Results reported and discussed in this article should increase awareness that foliar and tuber infections caused by *P. nicotianae* can occur and that these diseases potentially could become more common. The foliar phase also may become more important because it can be confused with late blight, leading to misdiagnosis of *P. nicotianae* infections. That problem could be compounded further because both pathogens can cause foliar blight in the same field at the same time.

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Appendix D

Morphological and molecular characterization of *Phytophthora glovera* sp. nov. from tobacco in Brazil.

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Abstract: A root rot disease of cultivated tobacco called yellow stunt has been observed in the burley production areas of Brazil since the early 1990s. Numerous root infecting fungi were isolated from the roots of diseased tobacco plants, including a semi-papillate, homothallic, slow growing *Phytophthora* species. Pathogenicity trials confirmed that the *Phytophthora* sp. caused root rot and stunting of burley and flue-cured tobaccos.

Morphological characteristics of the asexual and sexual stages of this organism did not match any reported *Phytophthora* species and were very different from the widely known tobacco black shank pathogen *P. nicotianae*. Phylogenetic analysis based on sequences of the internal transcribed spacer rDNA, cytochrome oxidase I and II, ß-tubulin, and translation elongation factor 1-alpha regions indicated that this organism represents a previously unreported *Phytophthora* species that is significantly supported in Clade 2 and most closely related to *P. capsici*. However, *P. glovera* differs from *P. capsici* in a number of morphological characters, most significantly, *P. glovera* is homothallic and produces both paragynous and amphigynous antheridia, while *P. capsici* is heterothallic and produces only amphigynous antheridia. In this paper we confirm pathogenicity of this species on tobacco and describe the morphological and molecular characteristics of *Phytophthora glovera* sp. nov.

Key Words: ITS, Amarelão, morphology, taxonomy, Straminipiles, Oomycetes.

INTRODUCTION

The Genus *Phytophthora* in the recently established Kingdom Straminipila (= Straminipiles, Straminipilous) (Dick, 2001) represents a very important group of plant pathogens affecting many important crops around the world. Multiple species of *Phytophthora* are known to attack tobacco including: *P. cinnamomi* Rands, *P. fragariae* Hickman var. *fragariae* and *P. nicotianae* Breda de Haan (= *P. parasitica* Dastur., *P. parasitica* Dast. var. *nicotianae* (Breda de Haan) Tucker) (Erwin and Ribeiro 1996). *Phytophthora nicotiane* is the most destructive *Phytophthora* sp. on tobacco and is widely distributed in tobacco regions causing the blank shank disease (Shew and Lucas 1991). *Phytophthora cinnamomi* was reported in Russia in 1974 (Novatel'nova 1974) and *P.*

fragariae var. *fragariae*, reported in Canada in 1955 (Anonymous 1955) are apparently of no economic importance and very narrow distribution.

A yellow stunt disease, also known as 'Amarelâo', was observed in southern Brazil, (Santa Catarina state) affecting burley tobacco production areas in the early 1990s, and more recently was reported from flue-cured production areas in the country. It is not known when or where (one or many fields) the disease first occurred in Brazil, and the etiology of the disease has remained a matter of uncertainty for many years. Isolates of *Fusarium*, *Rhizoctonia, Pythium*, and *Phytophthora* have been recovered from roots of diseased plants and associated with the disease and is the subject of another investigation (Shew, unpublished). During studies of the yellow stunt disease of tobacco, isolates of an atypical *Phytophthora* sp. were obtained from multiple locations in burley tobacco fields in Brazil. Morphological characters of the eight isolates collected did not fit with any of the reported species in the genus.

Validation of new *Phytophthora* taxa in recent years has been supported with phenologies generated by sequence analysis of the internal transcribed spacer region (ITS) of the ribosomal DNA gene repeat, translation elongation factor 1 alpha, β-tubulin, and mitochondrial encoded cytochrome oxidase (cox) I and II regions (Cooke et al 2000, Kroon et al 2004, Martin and Tooley 2003). A multi-locus phylogeny for 82 *Phytophthora* species using seven molecular markers has been published recently (Blair et al 2008) and confirms the presence of the 10 well-supported clades for the genus previously observed by Cook et al (2000). At present there are at least 95 valid *Phytophthora* taxa recognized including the recently described *Phytophthora morindae* Abad ZG & S Nelson sp. nov. (Nelson and Abad 2009), with thirty five of the species described using integration of morphological and molecular characters. In this publication we integrate morphological and molecular characterization for the description of *Phytophthora glovera* sp. nov.

Preliminary reports on this new species have been published (Shew et al 1999, Abad ZG and Abad JA, 2001).

MATERIALS AND METHODS

Isolation, culture, and morphological characterization.— Tobacco plants showing symptoms of yellowing, stunting and root rot were collected in burley tobacco production areas in Brazil. Symptomatic roots were thoroughly washed free of soil, suspended in deionized water, and secondary roots showing necrotic areas were excised. The individual roots were blotted in sterile paper towels, sectioned into small pieces (2-5 mm), and floated in sterile deionized water in 6-cm-diam Petri dishes. After 24-48 hr at 22 to 25°C, obpyriform sporangia were observed on numerous root sections. Isolations from these root sections were consistently negative on a standard *Phytophthora* semi-selective medium PARPH (Kannwischer and Mitchell 1978). Attempted isolation for *Pythium* spp. from these roots on the above medium without hymexazol frequently yielded a slow growing organism with morphological features of *Phytophthora* (Erwin and Ribeiro 1996).

Further isolations from diseased roots were completed on 5% clarified V8 P_{10}ARP selective medium without hymexazol (Kannwischer and Mitchell 1978, Shew 1983). Briefly, 2 g of $CaCO_3$ was suspended in 250 ml V8 juice (Campbell Soup Company, Camden, NJ) and 750 ml DI water. The suspension was autoclaved at 121°C for 10 minutes and then clarified by vacuum filtration through Celite 545 (Fisher Scientific, Fair Lawn, NJ). The clarified V8 broth was divided into 500 ml bottles then autoclaved at 121°C for thirty minutes for two consecutive days. V8 medium contained 50 ml of the V8 broth, 950 ml deionized (DI) water and 20 g Bacto agar (Difco, Detroit, MI). Root sections (1 cm) were embedded in the agar medium and dishes were incubated at room temperature (ca. 25°C) in the dark for 14 d. Clarified carrot agar (CA), clarified V8, corn meal

agar (CMA, Difco) and clarified CA/V8 agar media (100 ml V8 juice plus 25 ml of carrot juice and 2 g calcium carbonate) were used for the evaluation of colony morphology and minimum, optimum and maximum cardinal temperatures. For the CA medium, carrot juice (Hollywood or The Hain Celestial Group Inc., Melville, NY) was filtered through Celite 545 and then stored at -20°C until needed. The CA medium was made by adding 50 ml filtered carrot juice to 950 ml DI water and 20 g Bacto Agar.

For sporangia production, isolates were grown for 7-15 days in CMA, small plugs were transferred from the border of the colony into sterile distilled water (ca. 20 ml) or 10% soil solution and incubated under continuous fluorescent light at room temperature for ca. 48 hrs. Oogonia, antheridia and oospores were produced by all isolates on multiple culture media, including CMA, CA, CA/V8 and water cultures, indicating the species is homothallic. Fifty sporangia and fifty oogonia, oopores, and antheridia were measured for each isolate. Stock fungal cultures were maintained on slants of CMA and water blank cultures (glass tubes with 15 ml sterile DI water). Isolates were characterized morphologically using published keys for the genus *Phytophthora* (Waterhouse 1963, Stamps et al 1990, Erwin and Ribeiro 1996) and a Morphological/Molecular key (© Pictorial/Phylogenetic Oomycetes Lucid key) (Abad ZG, unpublished).

Molecular characterization.— Isolates growing on CMA (7-15 days) were transferred from the colony edge into 5 ml pea broth (120 g frozen peas/1 L of distilled H_20) in 50 ml Falcon tubes (Fisher Scientific) and incubated at room temperature for 7-10 days. Mycelium was removed and ground in microcentrifuge tubes using liquid nitrogen. Genomic DNA was isolated from 20 mg of mycelium using the PUREGENE DNA isolation kit and protocol (Qiagen; Germantown, MD) and eluted in 50 µl ultra-pure water. DNA extracts were stored at -20°C.

To determine phylogenetic placement of this putative new *Phytophthora* species, multiple

regions of four of the isolates were amplified and sequenced as listed in TABLE I. Amplification protocols for each locus were followed as described in the corresponding reference. Sequences were compared with those of other *Phytophthora* species downloaded from GenBank (if available) and with other putative new species in progress of official description (TABLE II). Alignments were produced using Sequencher 4.1.2., edited manually, and phylogenetic trees were constructed for each locus using neighbor-joining (Saitou and Nei 1987) with a Kimura two parameter nucleotide substitution model as implemented in MEGA version 4 (Tamura et al 2007). Robustness of the resulting phylogenies was evaluated by bootstrap analyses of the datasets using 1000 replicates (Felsenstein 1985). Individual gene datasets and neighbor-joining topologies have been deposited in TreeBase (Accession No. X).

Pathogenicity tests.— Five species of *Nicotiana* and six cultivars of *N. tabacum* were used in glasshouse pathogenicity tests. Seed of each genotype were sown into flats containing potting mix (Metro mix 200, The Scotts Company, Marysville, OH) and allowed to grow for 4 weeks. Seedlings were then transplanted into cell packs containing a 1:1:1 (v:v:v) mixture of steam pasteurized (80°C for 30 min) soil, potting mix, and course builder's sand, and allowed to grow for an additional 2 weeks before inoculation. Each cell was 4 x 4 x 5 cm and there were nine single plant replicates of each genotype. The experiment was repeated once. All plants were watered twice daily and fertilized as needed to maintain normal growth of control plants.

Inoculum of seven isolates of the pathogen was prepared by growing the isolates on CA for 14 days at 22 to 25°C. Eight 9-cm-diam dishes were blended in sterile DI water to give a final volume of approximately 5 L of suspension. Each cell was drenched with 50 ml of the inoculum suspension and then plants were watered. Disease was rated 14 days after inoculation using a severity scale of 1 to 7 with 1 = no disease and 7 = plant death (Table 3).

TAXONOMY

***Phytophthora glovera* Z.G. Abad & H.D. Shew sp. nov.** **MycoBank:** 514015 FIGS. 1-49

Etym.: Named to honor Mr. Derald Glover who worked in David Shew's laboratory at the Dept. of Plant Pathology-NCSU during the years 1966 to 1999. Mr. Glover's initial observation of *Phytophthora*-like sporangia on the symptomatic roots of tobacco in water led to the initial isolation of the pathogen and the discovery of this new species.

Coloniae in agaro farina maydis (CMA) sparso, submerse, temperature crecentiae minima 13, optima 25, maxima 30 C, crecentibus ad optima temperature 1.8 mmd^{-1}. Hyphae primariae 3-6 μm latae. Chlamidosporae ignotae. Sporangiophora simplicia vel interdum ramosa, simpodalia. Sporangia nonpedicellata, persistentia, papillae semi conspicuae; sporangiis ovoidea, ellipsoidea, obclavate, vel irregular; 43-61 (medio 50) um longum; 26-36 (medio 32) um latae. Zoosporae encystatae 8.6 um diametro. Oogonia sphaerica, plerumque terminalia raro intercalaria, 26-41 (medio 34) um diametro. Antheridia sic diclina, paragyna (c. 50%), amphigyna (c. 30%), paragyna ad amphigyna per one single oogonia, digitalis saepe, pargyna 7-11(medio 9.5) um, amphigyna 10-11 (medio 10.7) um. Hyphae antheridiferae longae. Oosporae valde apleroticae, 23-30 (medio 27) μm diam., parietibus 4-6 (medio 5) crassis.

Phytophthora glovera Z.G. Abad & H.D. Shew Figs. 1-49

Phytophthora glovera has moderately slow growth at 25°C on CA with a radial growth about 5.4 mmd^{-1}, on C-CA about 5.2 mmd^{-1}, on V8A about 3.6 mmd^{-1}, slow growth on CMA with about 1.8 mmd^{-1} and on PDA about 0.8 mmd^{-1}. On CMA, isolates grew between 13 and 30°C with an optimum at 25°C. Colonies on CA, V8 (clarified and non-clarified) all adhered to the substrate, and growth with slight stellate pattern at the center and uniform pattern at the border of the colony. This pattern was less obvious on CMA. Main hyphae in CMA measure 3-6 μm wide.

Chlamydospores and hyphal swellings are not produced in culture media or in water cultures. Sporangia are readily produced on sections of infected root pieces or agar plugs under water culture, 10% soil solution and on solid media. Shapes of sporangia range from ovoid, obpyriform, obclavate, sub-globose, to irregular distorted shapes (Figs. 1-10, 23-30), measuring from 43-61 (av. 50) μm on length x 26-36 (av. 32) μm width. Sporangia are persistent, and frequently laterally attached to the sporangiophore, and are produced in unbranched simple sporangiophores or in simple sympodial pattern in sporangiophores originated laterally or apically in a previous sporangium (Fig. 23). Typical sporangia produced under water blank cultures are semi-papillate, with apical thickening ranging from 7 to 9 μm (av. 8 μm), and an exit pore of about 7 to 9 (av. 7 μm). Occasionally sporangia with two (bilobed) or three apices (trilobed) are produced. Some sporangia show a prominent papilla previous to liberation of zoospores. Sporangia are mostly terminal, and germinate either directly or indirectly. Encysted zoospores measure ca. 8.6 μm diam. *Phytophthora glovera* is homothallic, and oospores are produced abundantly in single-strain culture in all media, and occasionally in diseased host tissues. Oospores with a single antheridium are most frequently paragynous (ca. 50%) (Figs. 11-14, 16, 19, 20, 31-40), although many are amphigynous (ca. 30%) (Figs. 15, 17, 18, 41-46). Multiple antheridia per oogonium are frequently produced (ca.20 %) in forms of double paragynous (Figs. 48), and paragynous-amphigynous (Figs. 21, 22, 49). Some unusual tooth-shaped or digitated projections from paragynous or amphigynous antheridia are frequently observed (Figs. 14, 18, 46). Both types of antheridia in could frequently be seen occurring in close proximity (Fig. 47). Paragynous antheridia measuring 7-11 (av. 9.5) μm wide, amphigynous antheridia 10-11 (av. 10.7) μm wide. Oogonia are smooth-walled and hyaline (when young) to dark yellow to orange (when old) measuring 26-41(av. 34) μm in diameter. Oospores are markedly aplerotic (do not fill the oogonial content) (Figs. 38, 45), measuring 23-30

(av. 27) μm diam with a wall thickness of 4-6 (av. 5) μm. Some crescent forms of aplerotic oospores are observed (Figs. 17, 43, 44). Germination of oospores by production of sporangia attached to the oogonial wall has been observed. Hyphal rings intercalary in the mycelium or connected with sporangia are sometimes observed.

Holotype. BRAZIL, Santa Catarina state close to Chapeco in southern part of Brazil from *Nicotiana tabacum* (tobacco 'burley' field) showing symptoms of 'Amarelâo', 10 Aug. 1995, collector David Shew. Isolate 11099 #23. BPI 878720 (Dried culture on baby carrot agar). *Ex-type: CBS* 121969. WPC 11685. GenBank accession ITS No. AF279124, EF-1 α FJ008029, ß-Tub FJ008021.

DNA amplification and sequence data analysis

The PCR product of the ITS-rDNA regions yielded a single band of approximately 900 bp for all 4 isolates of the undescribed *Phytophthora* sp. (Table I). ITS sequences of other *Phytophthora* species representing Clades 1 – 9 were downloaded from GenBank and used in phylogenetic tree construction; due to the limited number of nucleotides in some downloaded sequences, 670 characters were included in the analysis. Likewise for the other three loci, with 536, 671, and 837 nucleotides of sequence for the Cox I and II, Tef-1α, and ß-tubulin regions (respectively) included in the individual analyses.

Phylogeny. — Phylogenetic analysis of the ITS rDNA region positions *P. glovera* in Clade 2 of Cooke et at (2000) that contains other semi-papillate species including: *P. botryosa*, *P. citrophthora*, *P. meadii*, *P. colocasiae*, *P. tropicalis*, *P. inflata*, *P. citricola*, *P. capsici*, *P. multivesiculata*, the recently described *P. frigida*, *P. bisheria* and *P. siskiyouensis* and

Phytophthora sp. WA2 (FIG. 51). *P. glovera* was most closely related to *P. capsici*. Phylograms of the cytochrome oxidase I and II, ß-tubulin, and Translation Elongation Factor 1-alpha regions were also constructed and showed similar topologies, all indicating that *P. glovera* is a distinct species with moderate to strong support regarding close evolutionary relationships with other Clade 2 species. Sequences of the four *P. glovera* isolates were identical at all loci except for the Translation Elongation Factor 1-alpha region, where three of the four isolates shared 11 identical heterozygous nucleotides. All *P. glovera* sequences generated in this study are available at GenBank.

RESULTS

Pathogenicity tests. All isolates of the new species produced typical symptoms of the yellow stunt disease, root rot and stunting on susceptible genotypes of tobacco in glasshouse inoculations (Table 3). All species and cultivars of tobacco were susceptible to the pathogen except for *N. plumbaginifolia*, which had no symptoms of root rot after 21 days. The pathogen was consistently reisolated from diseased tissues, thus completing Koch's Postulates for this new pathogen on tobacco.

DISCUSSION

A new species, *Phytophthora glovera*, was isolated consistently from roots of cultivated tobacco affected with a yellow stunt disease known as 'Amarelâo' in Brazil. Pathogenicity tests show that *P. glovera* causes root rot and yellowing of tobacco under greenhouse conditions. Reisolation of *P. glovera* from symptomatic tissue and the morphological and molecular characterization confirmed the identity of the organism as a new pathogen of tobacco and a new species of *Phytophthora*. *Phytophthora glovera* is a homothallic species with semi-papillate sporangia, which can be classified in Group III of the Morphological Classification System of

Waterhouse (1963) and Stamps et al (1990). Molecular analysis of the ITS-rDNA sequences indicate that *P. glovera* belongs to Clade 2 of Cooke et al (2000). Morphologically, *Phytophthora glovera* is very close to *P. citricola* in the sporangial forms that include ovoid, obpyriform, and irregular shapes, and produces semi-papillate sporangia with two or three papillae. However, both species differ in their sexual stages; *P. citricola* produces predominantly paragynous and rarely amphigynous antheridia, while *P. glovera* produces predominantly paragynous antheridia in addition to some percentage of amphigynous antheridia (ca. 30%). Additionally both types of antheridia are frequently observed on a single oogonium in *P. glovera*; also *P. citricola* produces mostly plerotic oospores, while *P. glovera* produces mostly aplerotic oospores. Phylogenetic analysis of the four sequenced regions clearly shows the genetic distance between both species.

All four phylogenies indicate that *P. glovera* is a distinct species most closely related to *P. capsici*. However, there are clear differences in their asexual and sexual stages; for example, *P. capsici* is a heterothallic species with amphigynous antheridia, while *P. glovera* is a homothallic species that produces predominantly paragynous antheridia. The placement of *P. glovera* among other Clade 2 species is also unambiguously supported by studies conducted by Blair et al (2008). *P. glovera* sequences were useful to delimit the molecular distinctions between *P. capsici* and *P. tropicalis* after phylogenetic analysis with different molecular markers (Donahoo and Lamour, 2008). Aragaki and Uchida (2001) presented the morphological differences between both species in the official description of *P. tropicalis*.

Pathogenicity studies confirmed that this species is a primary causal agent in yellow stunt disease. Results from the glasshouse inoculations also indicated that *N. plumbaginifolia* may be a good source of resistance to this pathogen. This species also provides a source of single gene resistance to *P. nicotianae* (Shew and Lucas, 1991).

After integration of morphological and molecular data for the characterization of *Phytophthora glovera,* we conclude that this organism is a solid nova species in the genus.

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TABLE I. Regions of *P. glovera* amplified and sequenced in this study

Target DNA	Primers	Approx. size of fragment (bp)	Reference(s)
ITS	ITS6 and ITS4	900	White et al 1990; Cooke et al 2000
Portions of the *cox* I and II gene cluster	FM 79 and FM 80	981	Martin and Tooley 2003
Translation elongation factor 1-alpha	ELONGF1 and ELONGR1	972	Kroon et al 2004
ß-tubulin	TUBUF2 and TUBUR1	989	Kroon et al 2004

Table II. Accession numbers and origins of *Phytophthora* isolates included in phylogenetic analyses.

		Isolate Codes					Isolate origins		
		nDNA genes			mtDNA gene				
Species	Clade	ITS	ß-tubulin	TEF-1α	cox I-II	International	Local	Host	Location
P. glovera[9] (Type) 11099	2	AF279124[1]	FJ008018[1]	FJ008026[1]	FJ008022[1]	CBS 121969	11099.23C	*Nicotiana tabacum*	Brazil
P. glovera[9] 11085	2	AF279126[1]	FJ008019[1]	FJ008027[1]	FJ008023[1]	CBS 122201	11085.C	*Nicotiana tabacum*	Brazil
P. glovera[9] 11095	2	AF279128[1]	FJ008020[1]	FJ008028[1]	FJ008024[1]	CBS 122083	11095.C	*Nicotiana tabacum*	Brazil
P. glovera[9] 11091	2	AF279127[1]	FJ008021[1]	FJ008029[1]	FJ008025[1]		11091.1	*Nicotiana tabacum*	Brazil
P. bisheria[9]	2	AY241924[2]				CBS 122081	Cg 2.3.3	*Fragaria* x *ananassa*	North Carolina, USA
			EU080742[4]	EU080743[4]			P10117	*Fragaria* sp.	USA
P. botryosa	2	AF266784[9]				ATCC 52221		*Hevea brasiliensis*	Malaysia
			EU079935[4]	EU079936[4]		IMI 130422	P6945	*Hevea brasiliensis*	Malaysia
P. cactorum	1	AF266772[9]				IMI 296524	CAC2	*Rubus idaeus*	Wales
			AY564052[14]	AY564108[14]			P6183	*Rubus idaeus*	USA
					DQ365737[11]	CBS 279.37		*Prunus armeniaca*	India
P. capsici	2	DQ464056[5]				CBS 128.23		*Capsicum annuum*	New Mexico, USA
			EU080852[4]	EU080853[4]		ATCC 46012	P0253	*Theobroma cacao*	Mexico
					EF623880[10]		Is. 77	*Capsicum annuum*	California, USA
P. citricola	2	AF266788[9]				IMI 031372	CIT2	*Rubus idaeus*	Ireland
			AY564055[14]	AY564111[14]			P1817	*Medicago sativa*	South Africa
					DQ071410[20]		CH95PHE31	*Estoma gradiflorum*	Chiba
P. citrophthora	2	AF266785[9]				IMI 332632		*Actidinia chinensis*	Chile
			AY564056[14]	AY564112[14]		CBS 274.33	PD94/353	*Citrus limonium*	Cyprus
					N/A[b]		PD352		
P. colocasiae	2	AF266786[9]	AY564058[14]	AY564114[14]		IMI 368918		*Colocasia esculenta*	Malaysia
					AY129187[15]	ATCC 52233	Is. 347	*Colocasia esculenta*	India
P. fragariae var. *fragariae*	7	AF266762[9]				IMI 330736	FVF12	*Fragaria* x *ananassa*	Scotland
			AY564062[14]				A2	*Fragaria* x *ananassa*	
				AY564119[14]			NS4	*Fragaria* x *ananassa*	
					AY129195[15]		Is. 398	*Fragaria* x *ananassa*	Oregon, USA
P. frigida	2	DQ988182[17]					CMW 20311	*Eucalyptus smithii*	South Africa
P. heveae	5	AF266770[9]	AY564067[14]	AY564123[14]		IMI180616		*Hevea brasiliensis*	Malaysia
					DQ365742[11]	CBS 296.29	MG 25-8	*Hevea brasiliensis*	Malaysia
P. ilicis	3	AJ131990[9]					ILI1 (P590)	*Ilex* sp.	UK
			EU080137[4]				PD00178 (P6860)		
				AY564127[14]			PD91/595	*Ilex aquifolium*	The Netherlands
					AY423311[13]		4175a	*Ilex aquifolium*	Oregon, USA
P. inflata	2	AF266789[9]	AY564072[14]	AY564128[14]		IMI 342898		*Syringa* sp.	UK
P. insolita	9		AY564073[14]	AY564129[14]		IMI 288805		soil	Taiwan
					DQ365744[11]	CBS 691.79	MG 33-8	soil	Taiwan

P. kernoviae	10	AY940661[7]					P1571; CAE4	*Fagus sylvatica* bark	UK
			EU080054[4]	EU080055[4]			PD00165 (P10958)		
P. lateralis	8	AF266804[9]	AY564076[14]	AY564132[14]		IMI 040503		*Chamaecyparis lawsoniana*	USA
					DQ365745[11]	CBS 168.42	MG 33-7	*Chamaecyparis lawsoniana*	Oregon, USA
P. meadii	2	AY251649[12]						*Zantedeschia aethiopica*	Taiwan
			AY564077[14]	AY564133[14]		IMI 129185		*Hevea brasiliensis*	India
P. megakarya	4	AF266782[9]				IMI 337104	UQ2822	*Theobroma cacao*	Ghana
			AY564078[14]	AY564134[14]		IMI 337098		*Theobroma cacao*	Equatorial Guinea
					AY129208[15]		P132; Is. 327	*Theobroma cacao*	Nigeria
P. megasperma	6	AF541897[6]				P476	29-4-1	*Prunus armeniaca*	California, USA
			AY564079[14]	AY564135[14]		IMI 133317	MEG23	*Malus sylvestris*	Australia
					DQ365747[11]	CBS 402.72	MG 42-1	*Althaea rosea*	USA
P. multivesiculata	2	AF266790[9]		AY564136[14]		CBS 545.96	PD95/8679	*Cymbidium* sp.	The Netherlands
P. siskiyouensis	2	EF523386[18]				ATCC MYA-4187	WA5-030403	forest stream	Oregon, USA
P. tropicalis	2	EF617388[10]	EF617446[10]				Is. 32	*Macadamia integrifolia*	Hawaii
				AY564103[14]			PD97/11132; AN97/86	*Rosa* sp.	The Netherlands
					EF623889[10]		Is. 722	*Rhododendron* sp.	Tennessee, USA
Phytophthora sp. nov. WA2	2	EF121961[19]				VHSC 13615	WAC 13038	*Eucalyptus marginata*	Western Australia
Pythium vexans		AM701798[16]					IFAPA-CH837	avocado soil	Spain
			EF426556[3]		EF426547[3]	CBS 119.80		soil	Iran
				EU080485[4]		ATCC 12194	PD00391 (P3980)		

[a] Isolates from Z. G. Abad collection at the USDA/APHIS/PPQ/PHP/RIPPS/MDL, USA.

References from sequences in GenBank: [1] Present publication; [2] Abad et al. 2008; [3] Belbahri et al direct submission; [4] Blair et al 2008; [5] Bowers et al 2007; [6] Brasier et al 2003; [7] Brasier et al 2005; [8] Cooke et al 1999; [9] Cooke et al 2000; [10] Donahoo and Lamour 2008; [11] Goker et al 2007; [12] Huang and Liou direct submission; [13] Ivors et al 2004; [14] Kroon et al 2004; [15] Martin and Tooley 2003; [16] Martin-Sanchez et al direct submission; [17] Maseko et al 2007; [18] Reeser et al 2007; [19] Stukely et al 2007; [20] Villa et al 2006.

TABLE III. Disease severity on species and cultivars of wild and cultivated tobaccos by seven isolates of *Phytophthora glovera*.

Genotype[a]	Isolate Designation/Mean Disease Severity[b]						
	11083	**11085**	**11095**	**11099-1**	**11099-3**	**11099-23**	**11099-51**
N. africana	3	7	6	7	3	2	6
N. plumbaginifolia	1	1	1	1	1	1	1
N. repanda	1	6	5	5	5	2	5
N. rustica	1	1	1	1	3	1	1
N. sylvestris	3	5	6	6	5	3	5
K-326 - FC	2	3	2	3	3	2	3
K-346 - FC	2	2	2	2	2	2	2
NC 71 - FC	3	3	3	3	3	2	3
B21 x Ky 10 - B	3	6	3	3	3	2	3
L8 - B	3	4	3	4	3	2	4
TN 86 - B	2	3	2	2	3	2	2

[a]Species of *Nicotiana* and cultivars of *N. tabacum* used in pathogenicity tests. In the cultivars, FC indicates a flue-cured type of tobacco and B indicates a Burley type.

[b]Seven isolates of P. glovera used to complete Koch's Postulates for the organism on tobacco.

Disease was rated 14 days after inoculating 6-wk old seedlings with a mycelia suspension of a given isolate. Severity was rated on a 1 to 7 scale with 1 = healthy root system, 2 = trace of root rot (<1%), 3 = 1-5% root rot, 4 = 6-25% root rot, 5 = 26-50% root rot, and 7 = >50% root rot or stem lesion. No disease was observed in control seedlings.

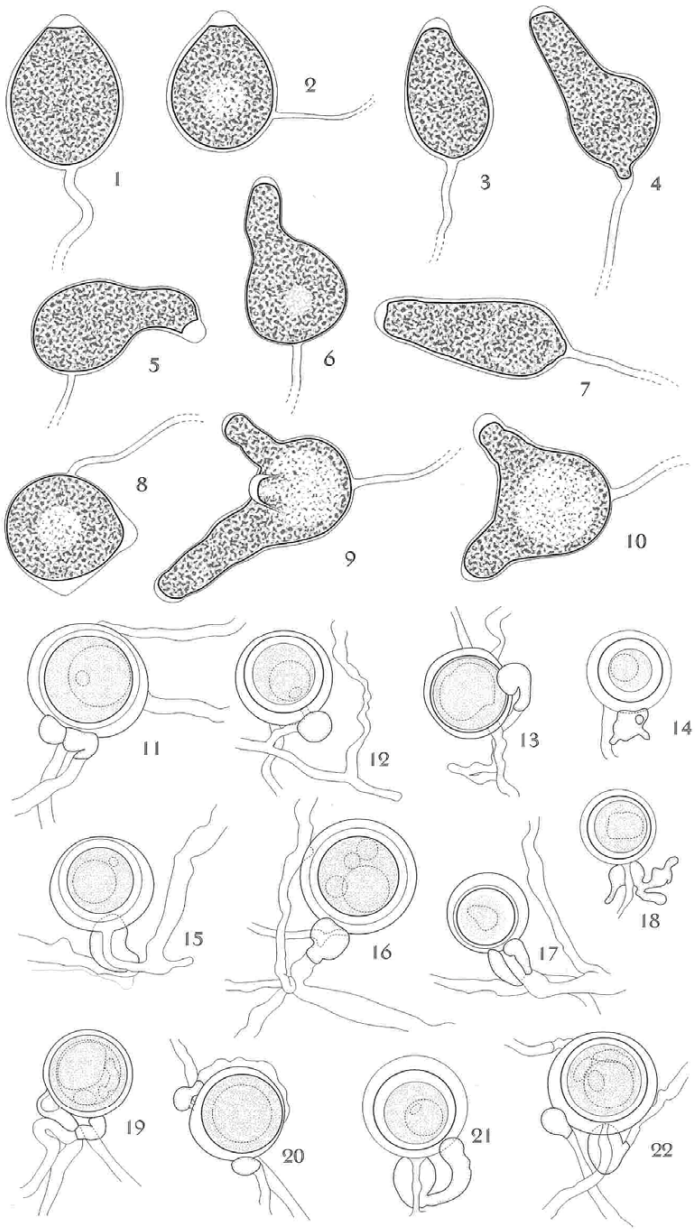
FIGURE LEGENDS

FIGS. 1-49. *Phytophthora glovera.* Morphology of the asexual and sexual stages showing the variation in morphological characters.

FIG. 50. Phylogenies of *P. glovera* obtained using the Neighbor joining method with 670 bp, 536 bp, 671 bp, and 837 bp of sequence of the ITS, Cox I and II, Tef-1α, and ß-tubulin regions (respectively), including representative *Phytophthora* species in Clades 1 through 9 (Cooke et al 2000). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Evolutionary distances were computed using the Kimura two parameter nucleotide substitution model with scale bar displaying the number of nucleotide substitutions per site.

FIG. 51. Sporangia and hyphae of *Phytophthora glovera* in tobacco roots after pathogenicity tests.

FIG. 52. Oospore and hyphae of *Phytophthora glovera* in tobacco roots after pathogenicity tests.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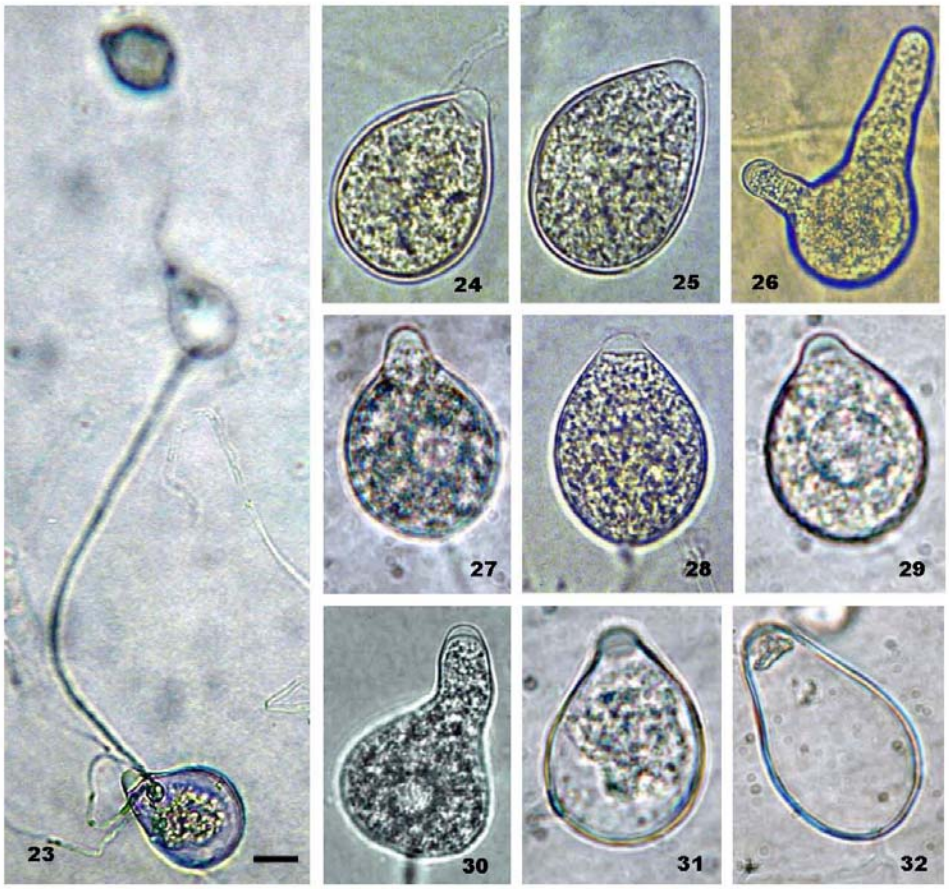
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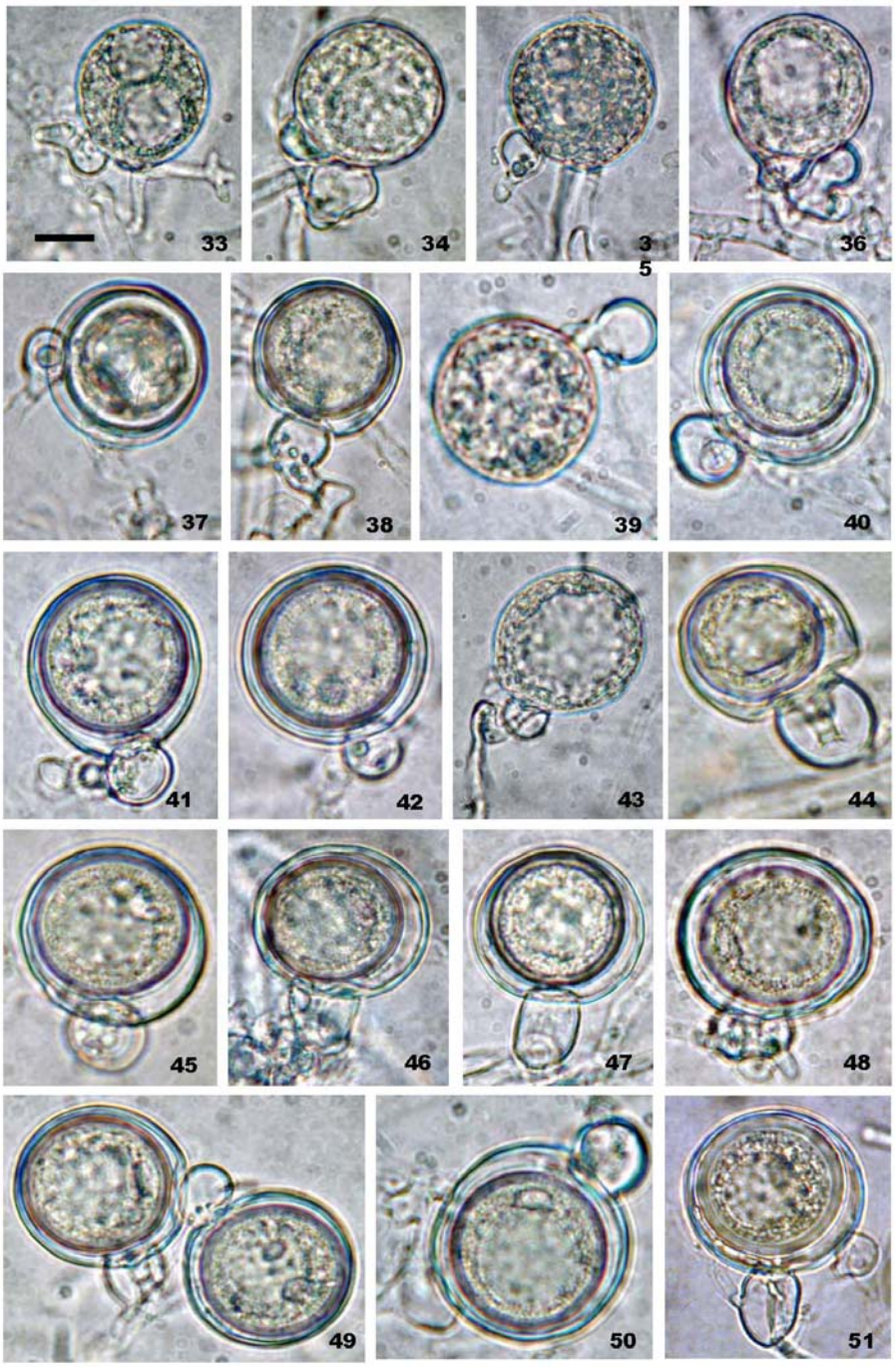
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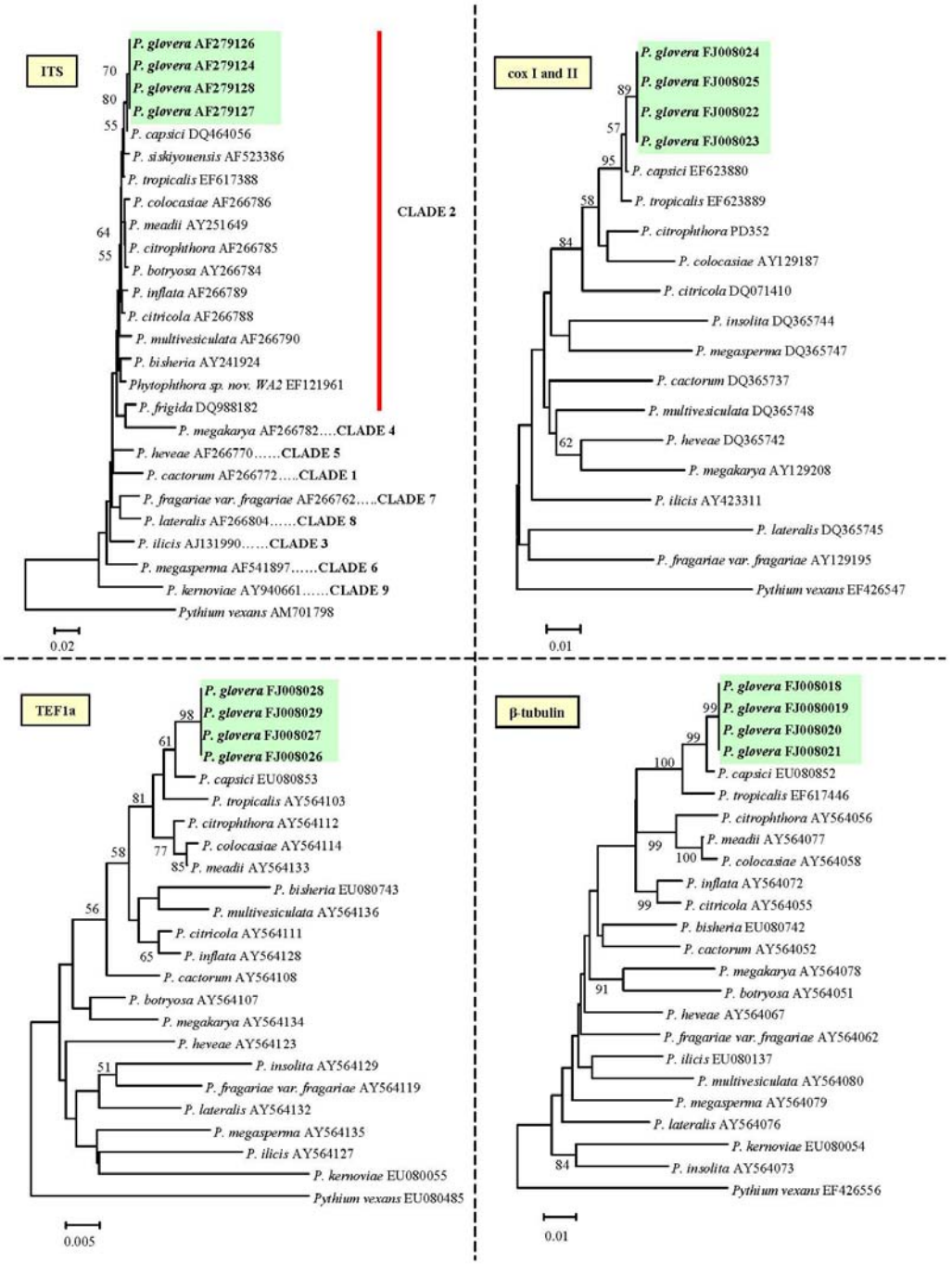
ITS
P. glovera AF279126
P. glovera AF279124
P. glovera AF279128
P. glovera AF279127
P. capsici DQ464056
P. siskiyouensis AF523386
P. tropicalis EF617388
P. colocasiae AF266786
P. meadii AY251649
P. citrophthora AF266785
P. botryosa AY266784
P. inflata AF266789
P. citricola AF266788
P. multivesiculata AF266790
P. bisheria AY241924
Phytophthora sp. nov. WA2 EF121961
P. frigida DQ988182
P. megakarya AF266782....CLADE 4
P. heveae AF266770.....CLADE 5
P. cactorum AF266772.....CLADE 1
P. fragariae var. fragariae AF266762.....CLADE 7
P. lateralis AF266804.....CLADE 8
P. ilicis AJ131990.....CLADE 3
P. megasperma AF541897.....CLADE 6
P. kernoviae AY940661.....CLADE 9
Pythium vexans AM701798
CLADE 2
0.02
cox I and II
P. glovera FJ008024
P. glovera FJ008025
P. glovera FJ008022
P. glovera FJ008023
P. capsici EF623880
P. tropicalis EF623889
P. citrophthora PD352
P. colocasiae AY129187
P. citricola DQ071410
P. insolita DQ365744
P. megasperma DQ365747
P. cactorum DQ365737
P. multivesiculata DQ365748
P. heveae DQ365742
P. megakarya AY129208
P. ilicis AY423311
P. lateralis DQ365745
P. fragariae var. fragariae AY129195
Pythium vexans EF426547
0.01
TEF1a
P. glovera FJ008028
P. glovera FJ008029
P. glovera FJ008027
P. glovera FJ008026
P. capsici EU080853
P. tropicalis AY564103
P. citrophthora AY564112
P. colocasiae AY564114
P. meadii AY564133
P. bisheria EU080743
P. multivesiculata AY564136
P. citricola AY564111
P. inflata AY564128
P. cactorum AY564108
P. botryosa AY564107
P. megakarya AY564134
P. heveae AY564123
P. insolita AY564129
P. fragariae var. fragariae AY564119
P. lateralis AY564132
P. megasperma AY564135
P. ilicis AY564127
P. kernoviae EU080055
Pythium vexans EU080485
0.005
β-tubulin
P. glovera FJ008018
P. glovera FJ0080019
P. glovera FJ008020
P. glovera FJ008021
P. capsici EU080852
P. tropicalis EF617446
P. citrophthora AY564056
P. meadii AY564077
P. colocasiae AY564058
P. inflata AY564072
P. citricola AY564055
P. bisheria EU080742
P. cactorum AY564052
P. megakarya AY564078
P. botryosa AY564051
P. heveae AY564067
P. fragariae var. fragariae AY564062
P. ilicis EU080137
P. multivesiculata AY564080
P. megasperma AY564079
P. lateralis AY564076
P. kernoviae EU080054
P. insolita AY564073
Pythium vexans EF426556
0.01


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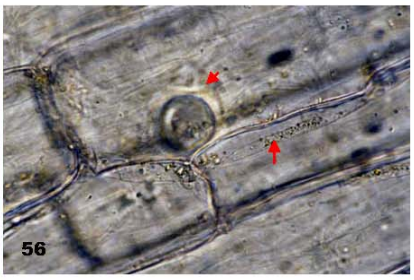
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