ABSTRACT

BITTNER, RICHARD JOHN. *In Vitro* and Field Efficacy of Oxathiapiprolin Against *Phytophthora nicotianae* and the Potential for Resistance Development. (Under the direction of Dr. Asimina L. Mila, and Dr. Michael Benson).

The soilborne oomycete *Phytophthora nicotianae*, causal agent of black shank of tobacco, is one of the most destructive plant pathogens of tobacco throughout the world. The use of fungicides is one of the most effective practices used by tobacco growers to manage this disease. Oxathiapiprolin, a newly discovered chemistry that has shown high efficacy against multiple oomycetes. The efficacy of oxathiapiprolin against *P. nicotianae* was examined in *in vitro* assays using four *P. nicotianae* isolates originating from tobacco. Four life cycle stages of the pathogen were examined: mycelial growth, sporangia production, zoospore germination, and zoospore motility. All life cycle stages were sensitive to oxathiapiprolin, with EC_{50} values ranging from 0.0039 to 0.0049 µg a.i./ml for mycelial growth, 0.00052 to 0.00081 µg a.i./ml for sporangia production, 0.0035 to 0.0051 µg a.i./ml for zoospore germination, and 0.0055 to 0.0166 µg a.i./ml for zoospore motility. Sixty tobacco and six ornamental *P. nicotianae* isolates, five resistant to mefenoxam at 100 µg a.i./ml, were tested for their mycelial growth sensitivity to the discriminating dose of oxathiapiprolin at 1 µg a.i./ml. A single isolate from tobacco was found to be insensitive to oxathiapiprolin at 1 µg a.i./ml.

The efficacy of oxathiapiprolin against the black shank disease was further examined from 2012 to 2014 in four naturally infested fields in North Carolina. In 2012, the efficacy of different rates, application methods, and timing of applications of oxathiapiprolin against black shank was examined. Fungicides were applied to the soil on the day of tobacco transplanting (transplant), 2 to 4 weeks after transplant (first cultivation), and/or 7 to 9 weeks
after transplant (layby). In 2012, treatments with three applications of oxathiapiprolin significantly reduced the black shank disease index (DI) regardless of the rate examined when compared to the non-treated control and the treatment of three applications of mefenoxam. Single applications of oxathiapiprolin via the transplant water at 0.07, 0.14, and 0.28 kg a.i./ha caused a significant decrease in DI when compared to the non-treated control. In contrast, single applications of oxathiapiprolin at first cultivation and layby at 0.07 kg a.i./ha did not significantly reduce DI when compared to non-treated controls.

The efficacy of alternating applications of oxathiapiprolin with mefenoxam was further examined in 2013 and 2014 field studies. Treatments with applications of oxathiapiprolin at transplant, mefenoxam at first cultivation, and oxathiapiprolin at layby had a significantly lower DI than the non-treated control. Treatments that alternated applications of oxathiapiprolin at transplant and mefenoxam at layby also significantly reduced DI when compared to the non-treated control. Initiation of disease was delayed in every oxathiapiprolin treatment in both 2013 and 2014 studies. These field results indicate that oxathiapiprolin, alone or in alteration with mefenoxam, is efficacious for the reduction of the black shank disease.

Effective resistance management strategies are critical to the longevity of a fungicide. The potential for resistance development to oxathiapiprolin in *P. nicotianae* populations was also examined. The production of resistant isolates to oxathiapiprolin was attempted using three methods: mass selection of zoospores, UV light mutagenesis, and mycelial adaptation through repeated culturing on oxathiapiprolin-amended medium. No resistant *P. nicotianae* isolates were produced from 46.2 million zoospores spread on selective oxathiapiprolin-amended medium. UV light mutagenesis generated two isolates, EdgeB7-M1 and YadA28-
M1, with stable mycelial resistance to oxathiapiprolin. In addition, sporangia of mutants were produced at oxathiapiprolin concentrations completely inhibitory to the wild-type isolates. Mutant isolate EdgeB7-M1 remained pathogenic to tobacco, while YadA28-M1 was found to be nonpathogenic. For the mycelial adaptation experiment, 48 mycelial colonies, twelve replicate colonies from each of four isolates, were transferred fifteen times on oxathiapiprolin-amended medium. After the 15th transfer, twelve colonies from the initial 48 had a significant increase in mycelial insensitivity to oxathiapiprolin. Number of sporangia produced by the adapted isolates was significantly reduced when compared to the wild-type isolates. Oxathiapiprolin insensitivity from mycelial adaptation was not stable in sixty six percent of the isolates. Of the twelve isolates, one was pathogenic on tobacco. The generation of oxathiapiprolin resistant *P. nicotianae* isolates was possible through artificial methods, however fitness costs associated with increased insensitivity to oxathiapiprolin were observed. Overall, oxathiapiprolin is highly efficacious on *P. nicotianae* and should be an effective tool that tobacco growers can use for the control of black shank.
In Vitro and Field Efficacy of Oxathiapiprolin Against *Phytophthora nicotianae* and the Potential for Resistance Development

by
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DEDICATION

I dedicate this work to my family, friends, and dog. I would not be where I am today without your constant love, encouragement, and support.
BIOGRAPHY

Richard John Bittner grew up on a farm in Kempton, PA raised by his mother Millyann and father Richard. His interests in science began in his first high school biology and chemistry classes. After completing high school he attended Susquehanna University in Selinsgrove, PA, where he pursued an undergraduate degree in biology. His interests in plant pathology were due to his experiences on his family’s farm, as well as his research at Susquehanna University, under the direction of Dr. Alissa A. Packer. After graduation, with a degree in biology, he decided to pursue a master’s degree in plant pathology. Once accepted to North Carolina State University, under the direction of Dr. Asimina Mila he began his new career in Plant Pathology. Under the direction of Dr. Mila, he studied many aspects of the Granville wilt disease, caused by *Ralstonia solanacearum*, in tobacco and received his Master’s degree in 2011. Richard loved working with his advisor, Dr. Mila, so much that he decided to stay at North Carolina State University to work on his Ph.D. in Plant Pathology. His Ph.D. research examined the efficacy of a new fungicide, oxathiapiprolin, on black shank, caused by the soilborne oomycete *Phytophthora nicotianae*, of tobacco.
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CHAPTER I.

Review of the literature

Black Shank History and Nomenclature of Phytophthora nicotianae

Black shank, caused by the soilborne oomycete Phytophthora nicotianae, is a destructive root and stem disease of all types of cultivated tobacco throughout the world. In 1896, Jacob van Breda de Haan, a Dutch botanist, was the first to describe this disease in Indonesia, formerly the Dutch East Indies (van Breda de Haan, 1896). Shortly after finishing his Ph.D. thesis, van Breda de Haan moved to the Dutch East Indies in 1891, where he was stationed at the Botanical Garden in Bogor (Zadoks, 2014). With no formal training in plant pathology, van Breda de Haan was a self-made plant pathologist through the publications and books he had read. In 1893, van Breda de Haan was named the botanist for tobacco research in the Dutch East Indies (Zadoks, 2014). One of his first responsibilities was to investigate the “bibit disease”, meaning young plant disease, which was causing many serious problems for tobacco growers in Deli. After numerous field studies and the two preliminary reports, van Breda de Haan published his final report in 1896, where he described the pathogen and disease in detail (Zadoks, 2014). In this report, van Breda de Haan (1896) names the pathogen Phytophthora nicotianae nov. spec., basing the genus name on the pathogen morphology and the species name after the host (van Breda de Haan, 1896). However, he did not include a Latin description of the species, and it is believed that he described a mixed culture of Phytophthora and Pythium (Shew, 1991; Zadoks, 2014). Van Breda de Haan
(1896) also reported the disease symptoms, spread of disease, environmental factors affecting disease, and possible control of the disease.

Black shank of tobacco was not observed in the United States (US) until 1915, where it was confirmed to be located specifically in a southern region of Georgia (Lucas, 1975; Shew, 1991). In 1922, Tisdale (1922) reported that the disease had spread to the Florida-Georgia cigar tobacco district. Tisdale (1922) hypothesized that the disease was caused by the same species of *Phytophthora* that caused disease on tobacco in the Dutch East Indies. A few years after its discovery, Tisdale and Kelley (1926) reported that the black shank pathogen isolated in Florida was virtually identical in morphology to the pathogen isolated from tobacco in Java, and was confirmed to be *P. nicotianae*. In North Carolina (NC), the disease was first observed in Forsyth County in 1931, while black shank was not observed in either Tennessee and Kentucky until 1935 (Lucas, 1975; Shew, 1991). Upon its introduction and discovery, the pathogen quickly spread throughout all the tobacco growing regions of each state. Today, the black shank disease is a widespread problem for most tobacco growers of the southeastern US.

Since the pathogen’s discovery, there has been much disagreement on the pathogen’s correct scientific name. In 1913, J. F. Dastur, a pathologist from India, described the pathogen as the cause of disease on castor bean (*Ricinus communis* L.) and named it *P. parasitica* (Dastur, 1913; Zadoks, 2014). *P. parasitica* was more accepted than *P. nicotianae* because of the findings of Ashby (1928) at the time, who noted that the pathogen described by van Breda de Haan was most likely a mixed culture of *Phytophthora* and *Pythium*. Ashby
(1928) came to these conclusions based on van Breda de Haan’s illustrations of the pathogen, which showed the antheridium as paragynous in nature, which is not a character of Phytophthora isolates from tobacco which are amphigynous. In 1931, the tobacco pathogen was renamed as P. parasitica Dastur var. nicotianae (Breda de Haan) Tucker based on host preference to tobacco (Tucker, 1931; Shew, 1991; Erwin and Ribeiro, 1996). This name was used and accepted for many years by tobacco pathologists. In botanical nomenclature the first description of an organism has priority over later descriptions, thus Waterhouse (1963), stated that P. nicotianae Breda de Haan should be the current specific epithet. The lack of a Latin description of the species by van Breda de Haan (1896) was acceptable, because before 1908 publications were not required by the International Code of Botanical Nomenclature (Waterhouse, 1963). Although it is agreed by most tobacco pathologists that P. parasitica is the correct name of the pathogen, the International Code of Botanical Nomenclature gives priority to P. nicotianae, which has frequently been used in current publications in the US.

**Signs and Symptoms**

All parts of the tobacco plant, at all stages of growth, are susceptible to infection by the black shank pathogen, although black shank is primarily a root and stem disease (Dukes et al., 1965; Lucas, 1975; Shew, 1991; Shoemaker and Shew, 1999; Erwin and Ribeiro, 1996; Gallup et al., 2006). In the field, leaves of infected plants become chlorotic and wilt as roots are parasitized. Wilting is exacerbated by high temperatures because of the lack of water transpiration to the leaves (van Breda de Haan, 1896). When soil temperatures are cool, early wilting symptoms may be absent, making it very difficult to identify the disease (Shew,
However, once wilting and chlorosis of the leaves occur, the entire plant will soon turn necrotic, especially under stressful conditions. Stem infections from soilborne inoculum also form on the lower portion of the stalk or shank of the tobacco plant at the soil line, contributing to the “black shank” name (Lucas, 1975; Shew, 1991). If the stem or shank of an infected plant is cut vertically from the healthy tissue, through the lesion, and to the root zone, the lesion area of the plant will be separated into necrotic plate-like disks of the pith, which will not extend past the necrotic tissue (Lucas, 1975; Shew, 1991; Mila and Radcliff, 2014). Mycelium of the pathogen can also often be observed on the necrotic plate-like disks of the stem lesion. Observations of these necrotic plate-like disks can be a key to help identify the black shank disease; however, one should not diagnose black shank based on this observation alone, as other factors, such as damage from lighting can cause disking of the pith in tobacco as well (Shew, 1991). The sore shin disease, caused by the fungus *Rhizoctonia solani*, can also be confused with black shank because it can cause separation of the pith into necrotic plate-like disks in some instances (Gallup et al., 2006). It is also important to note that the disking of the pith symptom is not always observed in plants infected with *P. nicotianae* (Mila and Radcliff, 2014). Below ground symptoms of the disease include necrosis of roots, where lesions expand rapidly and eventually move to the stem (Shew, 1991; Lucas, 1975). This soilborne oomycete also can directly infect tobacco leaves through splash dispersal of zoospores during periods of heavy rainfall (Shoemaker and Shew, 1999). The lowest leaves of the tobacco plant are most affected and lesions can easily be confused with the brown spot disease, caused by *Alternaria alternata*. Lesions due to *P.*
nicotianae are necrotic, circular in shape, and expand overtime (Lucas, 1975; Shew, 1991; Gallup et al., 2006). In very infrequent instances, the leaf lesions expand to the extent that the pathogen colonizes the stem of the tobacco plant that results in severe infection (Shew, 1991).

Causal Organism

Phytophthora nicotianae is a soilborne oomycete belonging to the Kingdom Stramenopila (Meng, et al., 2014). Unlike true fungi, all life stages of this fungal-like pathogen are diploid (Shew, 1991; Erwin and Ribeiro, 1996). The hyphae formed by this organism are hyaline and aseptate, with distinctive hyphal swellings (Shew, 1991; Erwin and Ribeiro, 1996; Meng, et al., 2014). This pathogen reproduces both asexually and sexually, although the role of sexual reproduction in the disease cycle is not understood. Types of asexual spores produced by this organism include: chlamydospores, sporangia, and zoospores. Chlamydospores are thick-walled spores produced at the middle (intercalary) or tip (terminal) of the hyphae (Shew, 1991; Erwin and Ribeiro, 1996). Sporangia are light brown to brown in color and can be ovoid, ellipsoid, pyriform, or spherical with a distinct papilla (Shew, 1991; Erwin and Ribeiro, 1996). Sporangia can germinate either indirectly, by production of zoospores, or directly, by production of hyphae. Motile zoospores are bean shaped with a tinsel and whiplash flagellum for motility in films of water within soil pores for several hours (Shew, 1991; Erwin and Ribeiro, 1996). Phytophthora nicotianae is heterothallic with oospores formed through sexual reproduction of an A1 and A2 mating type. Oospores are thick-walled, and the amphigynous antheridium remains permenantly
fixed to the oogonium (Erwin and Ribeiro, 1996). Four races (0, 1, 2, and 3) of *P. nicotianae* have been found throughout the world, and can be identified by interaction with different resistance genes in tobacco (Apple, 1962; Shew, 1991; Erwin and Ribeiro, 1996; Gallup et al., 2006). Race 1 of the pathogen has the ability to overcome the *Php* and *Phl* complete resistance genes, which control race 0 (Gallup et al., 2006). Race 2 of the pathogen has been reported in South Africa based on the response of the isolate to three different varieties; however this is not considered an epidemiologically significant race (Gallup and Shew, 2010). Race 3 of the pathogen was first reported by McIntyre and Taylor (1978) in Connecticut, and can overcome the *Phl* gene, but not the *Php* gene. Presently, only races 0, 1, and 3 of *P. nicotianae* have been reported in North Carolina (Gallup and Shew, 2010).

**Disease Cycle**

Chlamydospores are the major survival structure for *P. nicotianae* and serve as the primary inoculum in the soil, surviving for multiple years. Under favorable conditions chlamydospores germinate to form hyphae, which can infect tobacco roots either directly or indirectly. Direct infection involves penetration of the tobacco root by a germ tube produced by a chlamydospore, while indirect infection involves the production of a sporangium that subsequently releases zoospores for root penetration (Lucas, 1975; Shew, 1991; Gallup et al., 2006). Sporangia also serve as secondary inocula in this cycle, and can either germinate by producing a hyphae or motile zoospores (Shew, 1991). Black shank of tobacco is a polycyclic disease, constantly producing chlamydospores and sporangia on infected roots (Shew, 1991; Gallup et al., 2006). Zoospore release from sporangia is triggered by water
saturated soils, where zoospores can swim through soil pores along nutrient gradients in the rhizosphere to tobacco roots (Shew, 1991; Gallup et al., 2006). Since the zone of elongation behind the root tip is a major contributor of root exudates, motile zoospores encyst in this zone, lose the flagella, and produce a cell wall (Erwin and Ribeiro, 1996). Encysted zoospores produce a germ tube with a bulbous appressorium that forms a penetration peg for direct penetration of host tissue (Shew, 1991; Meng et al., 2014). The pathogen colonizes root tissues establishing a parasitic relationship first as a biotroph then as a necrotroph that leads to colonization of the lower stem tissues (Shew, 1991; Meng et al., 2014). As root colonization progresses, secondary inoculum in the form of sporangia develop in the rhizoplane and release zoospore into soil water films for secondary infection that results in a polycyclic disease (Shew, 1991; Gallup et al., 2006). Finally, chlamydospores, formed in diseased tissues, are released into the soil as plant material decays for either germination and new infections or dormancy until the next growing season (Lucas, 1975; Shew, 1991; Meng et al., 2014).

**Disease Management**

Effective long-term management of the black shank disease involves an integrated approach, focusing on the use of various cultural practices, resistant varieties, and chemical application (Lucas, 1975; Shew, 1991; Gallup et al., 2006; Mila and Radcliff, 2014). The use of only a single management practice will lead to ineffective management, reduced crop yield, and increases in the population of the pathogen. Black shank management should
focus on the prevention of the disease epidemics rather than the cure, which has been suggested since its discovery by Breda de Haan (1896).

Cultural Practices

The use of effective cultural practices is critical for effective management of this disease. These practices include: crop rotation, avoidance of root wounding, planting on raised beds, pre-plant destruction of tobacco roots and stalks, and efficient nematode control (Lucas, 1975; Mila and Radcliff, 2014). Crop rotation is the most important measure that can be utilized for black shank management. Because of the pathogen’s high specialization to infect only tobacco in the field, rotation with non-host crops can dramatically reduce the population of the black shank pathogen in infested agronomic soils (Clayton et al., 1944; Clayton et al., 1944; Matthews et al., 1959; Kincaid, 1960; Nusbaum and Todd, 1970; Lucas, 1975; Mila and Radcliff, 2014). In fields with a known history of black shank, a three-year rotation with non-host crops resulted in a significant reduction in black shank incidence because of the reduction in carry-over of chlamydospore inoculum of the pathogen from year to year (Matthews et al., 1959). A highly susceptible flue-cured variety, Virginia Gold, planted in fields with three and four year rotations away from tobacco developed little black shank incidence (Matthews et al., 1959). Nusbaum and Todd (1970) also showed that a two-year rotation with non-hosts resulted in a change in disease incidence from 80 to 41% for the highly-susceptible tobacco variety, Hicks. However, it is important to remember that the pathogen has the ability to survive in the soil longer than four years, thus one should use
other management strategies when planting a highly susceptible variety in a field with a history of black shank (Matthews et al., 1959; Lucas, 1975).

The destruction of tobacco roots and stalks immediately after harvest is also an important management practice for limiting the overwintering population of the pathogen from infected plants infest and reproduce on the plant parts (Lucas, 1975; Shew, 1991; Mila and Radcliff, 2014). In addition to helping manage black shank, root and stalk destruction also helps manage other soilborne pathogens such as root knot nematodes and \textit{Ralstonia solanacearum}, the Granville wilt pathogen (Lucas, 1975; Mila and Radcliff, 2014). Effective management programs of black shank, should also consider the effect of soil chemistry on the pathogen. Black shank is favored in basic soils with pH higher than 6.0 because of the rapid growth and reproduction of the pathogen, thus pH below 6.0 is recommended when growing tobacco in a field with a previous history of black shank (Shoemaker and Shew, 1999). In some soils, the suppression of multiple plant pathogens, including \textit{P. nicotianae}, has been connected to the toxicity of aluminum (Al), (Sidebottom et al., 1985; DeLuca and Shew, 1988; Fichttnern et al., 2006). In black shank studies on suppressive soils, the soil pH was low and had high levels of exchangeable Al (Sidebottom et al., 1985; DeLuca and Shew, 1988; Fichttnern et al., 2006). At low soil pH, the activity and availability of free ionic Al (Al$^{3+}$) increases, although this is not the only species of Al found in soils. \textit{P. nicotianae} has been found to be sensitive to multiple monomeric Al species, while other plant pathogens, such as \textit{Thielaviopsis basicola}, are pH dependent and only certain Al species are effective (Fichttnern et al., 2006). A major setback to manipulating pH and Al in tobacco soils for
black shank control, is that Al can also be phytotoxic to tobacco at pH values below 5.5 (Gallup et al., 2006).

Black shank severity can increase with infestations of root-knot nematodes (*Meloidogyne* spp.) (Lucas, 1975; Shoemaker and Shew, 1999). Severe root infections by root-knot nematodes create entry points in the root for the pathogen, which can cause black shank resistant tobacco varieties to become susceptible (Shoemaker and Shew, 1999). *Phytophthora nicotianae* and root-knot nematodes (*Meloidogyne* spp.) may coexist in the same field and cause a devastating loss of tobacco. In tobacco, the use of fumigants, nematicides, and fungicides within the season has been studied to control the black shank/root-knot complex (Nusbaum and Todd, 1970; Csinos and Minton, 1983; Csinos et al., 1986; Johnson et al., 1992). Nusbaum and Todd (1970) demonstrated that the application of 1, 3-dichloropropene at 187 L/ha as a preplant fumigant reduced both black shank and root-knot disease index (i.e. a modified area under the disease progress curve) when compared to the nontreated control. Specifically, in tobacco variety Speight G-5, black shank index was reduced from 89 to 17 and root-knot index was reduced from 70 to 12 (Nusbaum and Todd, 1970). In studies by Csinos et al., (1986) and Johnson et al., (1992), the application of the nematicide fenamiphos and the fungicide metalaxyl was examined for control of the root-knot/black shank complex. When fenamiphos and metalaxyl were applied together, yield was increased and a significant reduction in black shank index, and root-gall index were observed for treated plants compared to the nontreated control was observed (Csinos et al., 1986; Johnson et al., 1992).
Host Resistance

The deployment of resistant varieties of tobacco is one of the most popular and effective black shank management practices used by growers. Currently, partial (polygenic) and complete (monogenic) resistant varieties are readily available to tobacco growers (Mila and Radcliff, 2014). Complete resistance is only conferred to race 0 of *P. nicotianae*. The *Php* and *Phl* genes, which confer the complete resistance, in tobacco originate from *Nicotiana plumbaginifolia* Viv. and *Nicotiana longiflora* Cav., respectively (Valleau et al., 1960; Apple, 1962a; Chaplin, 1962; Carlson et al., 1997; Johnson et al., 2002). Csinos (1999) found that the complete resistance to race 0 with the *Ph* gene is conveyed in both the roots and the stem of the plant. While both *Ph* genes confer complete resistance to *P. nicotianae* race 0, the *Php* is incorporated into many flue-cured and burley varieties and the *Phl* gene has only been deployed in a few select burley varieties (McCorkle et al., 2013).

Chaplin (1962) was able to successfully transfer the *Php* gene to *N. tabacum*, but it was never commercially released. In 1987, the first commercial variety released to growers that contained the *Php* gene was Coker 371-Gold (Johnson et al., 2002). At the time of release the origin of the resistance was not known to be from *N. plumbaginifolia*. At the time of deployment, Coker 371-Gold was not widely used by growers because of poor leaf quality (Gallup and Shew, 2010). In the 1990s, the *Php* gene was inserted into more acceptable agronomic varieties, causing a dramatic increase in the deployment of the *Php* gene by growers (Gallup and Shew, 2010). Overuse of the *Php* gene then led to an increase in the prevalence of *P. nicotiane* race 1, which was not widely observed prior to *Php*
deployment (Sullivan et al., 2005). Race 1 was first reported in 1954 in Kentucky where breeding lines carrying the \( Phl \) gene were used (Valleau et al., 1960; Apple, 1962b). In the 1960’s, Apple (1967) observed race 1 isolates in breeding nurseries where varieties with the \( Php \) gene were being evaluated, but this race was not considered a major threat to tobacco growers at the time. With the constant use of varieties with the \( Php \) gene by tobacco growers, a rapid increase in race 1 of the pathogen occurred in the 1990’s. In field studies by Sullivan et al., (2005) where only race 0 isolates were detected initially, race 1 was detected in that same field the following growing season after a variety (NC 71) with the \( Php \) gene was planted. After four years, race 1 was the predominant race in the field after continuous deployment of variety NC 71 (Sullivan et al., 2005; Sullivan et al., 2010). Sullivan et al. (2005) concluded that a rotation between cultivars with complete and partial resistance should provide adequate control, while minimizing the risk of race shifts in the pathogen.

Tisdale (1931) described partial black shank resistant cigar tobacco variety Florida 301 (Fla. 301) that originated from a cross between selections from ‘Little Cuba’ and ‘Big Cuba’ varieties. Fla. 301 partial resistance is regulated by multiple genes and confers varying levels of resistance to \( P. \) nicotianae (Wernsman et al., 1974; Shew,1991; Jones and Shew, 1995; Gallup, 2009; Xiao et al., 2013). Resistance associated with Fla. 301 is effective against all races of the pathogen and is only expressed in the roots of tobacco, where varieties with moderate-high levels of resistance seem to produce the smallest root systems when compared to varieties with less resistance (Jones and Shew, 1995; Antonopoulos et al., 2010). In contrast, varieties with the \( Php \) gene convey resistance in both
the roots and stem (Csinos, 1999). Xiao et al. (2013) determined that resistance in Fla. 301 was polygenic and controlled by 11 quantitative trait loci (QTL) with large, intermediate, and small effects.

Beinhart 1000 (BH) is another cigar tobacco type that has partial resistance to \textit{P. nicotianae}, having the highest level of partial resistance of any cultivar noted to date (Chaplin, 1966; Nielsen, 1992). Unlike Fla. 301, BH has been observed to have resistance not only in the roots, but also in the stem and leaf tissue (McCorkle et al., 2013). Vontimitta and Lewis (2012) identified six QTL located on linkage groups 2, 4, 8, 9, 11, and 14, that are thought to contribute to the resistance in BH. Two QTL on linkage groups 4 and 8 were most significant to plant survival, explaining 20.4 and 25.4\% of phenotypic variation respectively (Vontimitta and Lewis, 2012). The QTL found on linkage group 8 is also present in Fla. 301, while the QTL on linkage group 4 is not found in Fla. 301 (McCorkle et al., 2013). McCorkle et al. (2013) demonstrated that in greenhouse studies, the QTL from BH on linkage groups 4 and 8 acted in an additive fashion. Overall, the continuous deployment of tobacco varieties with partial resistance actually has been shown to result in increased aggressiveness in \textit{P. nicotianae} isolates (Dukes and Apple, 1961; Sullivan et al., 2005). Specifically, Dukes and Apple (1961) demonstrated that when \textit{P. nicotianae} isolates were inoculated and re-isolated from partially resistant varieties, aggressiveness of the isolates increased when compared to isolates transferred through susceptible varieties.

\textit{Chemical Control}
The use of soil applied fungicides is one of the most utilized methods to manage black shank for non-organic tobacco growers. Attempts to control black shank with fungicides date back to the 1890’s when van Breda de Haan first described the pathogen causing the disease. In his original publication that described the pathogen *P. nicotianae*, van Breda de Haan (1896) reported numerous field experiments conducted in Deli, focused on the control of the disease. One of the main management tools for effective disease control in his studies was the use of chemical control, specifically the Bordeaux mixture (van Breda de Haan, 1896). The Bordeaux mixture was one of the first successful fungicides discovered by the French botanist Millardet (1882), a few years prior to the discovery of the black shank disease. Millardet (1882) observed grape vines with no downy mildew symptoms, but leaves were covered in what he reported as a “bluish white substance”. After questioning the vineyard manager, he learned that it was a mixture of copper sulfate and lime, used to deter people from stealing the fruit (Millardet, 1882). Millardet (1882) later conducted trials with the Bordeaux mixture and concluded that it could be used for effective control of downy mildew on grapes. The Bordeaux mixture also has been used successfully for control of diseases caused by *Phytophthora*, such as late blight of potato (Erwin and Ribeiro, 1996).

In Deli, van Breda de Haan (1896) was most likely aware of the Bordeaux mixture’s potential to control diseases caused by fungal-like organisms, which would explain why he included the treatments in his field experiments. Not only did van Breda de Haan test to see if this fungicide was efficacious, but he also examined time and frequency of application of the fungicide as other factors contributing to efficacy (van Breda de Haan, 1896). Having
observed no phytotoxicity of the fungicide to tobacco seedlings from preliminary experiments, van Breda de Haan designed treatments with the Bordeaux mixture made at either three days after seed sow, two days after seed germination, or four days after seed germination. Applications of the fungicide were then made every seven days after the initial application (van Breda de Haan, 1896). Overall van Breda de Haan (1896) concluded that applications of the Bordeaux mixture provided adequate black shank control. It was recommended that the fungicide be applied to the soil once every five days or after periods of heavy rainfall. In Deli in 1895, tobacco growing regions were exposed to a season of heavy rainfall, despite these unfavorable conditions disease pressure remained low, which he attributed to the use of the Bordeaux mixture on every estate. These field studies were the very beginning of research for the management of black shank with soil applied chemicals.

The use of soil applied fumigants for control of numerous soilborne pathogens and nematodes is well documented (Erwin and Ribeiro, 1996). Soil applied fumigants can be phytotoxic, which is why they must be applied at least three weeks prior to transplant of tobacco (Mila and Radcliff, 2014). One, 3-dichloropropene (1,3D) is a liquid fumigant that has been shown highly toxic to plant parasitic nematodes and soilborne oomycetes (Zentmyer and Klotz, 1949; Baines et al., 1977). In moist soil, 1,3D can become hydrolyzed into chloroallyl alcohol, which is highly toxic (Baines et al., 1977). Nusbaum and Todd (1970) showed that applications of 1,3D at 187 L/ha significantly decreased black shank when compared to the untreated control.
Chloropicrin is another highly effective and widely used pre-plant gaseous fumigant for control of soilborne pathogens (Erwin and Ribeiro, 1996). Control of black shank disease by pre-plant applications of chloropicrin was observed by Taylor et al. (1978). Mixtures of fumigants have also been shown to be effective against soilborne pathogens. In a study by Reilly (1980), application of Telone C-17, a mixture of 1,3D and chloropicrin, at 136 L/ha was efficacious for control of black shank when compared with the untreated control. While fumigants are effective at reducing soilborne pathogen populations, their environmental and potential human health risks can outweigh their agronomic benefits.

Numerous fungicides have been tested for control of black shank of tobacco. Fungicides that are not currently registered on tobacco for black shank, but that have been previously studied include maneb and propamocarb. Maneb, manganese ethylene bisdithiocarbamate, is a member of the dithiocarbamates that has been shown to be effective against black shank (Nusbaum and Todd, 1970). The dithiocarbamates are a group of broad spectrum contact fungicides that are non-systemic (Erwin and Ribeiro, 1996). In a North Carolina field study, with either susceptible or resistant tobacco varieties, the application of maneb at 80.7 kg/ha was reduced black shank incidence (Nusbaum and Todd, 1970). Propamocarb is a systemic fungicide of the carbamates class, with efficacy against Phytophthora spp. (Erwin and Ribeiro, 1996). Reilly (1980) tested the efficacy of propamocarb against black shank in field studies and found that it decreased black shank index and increased yield when compared to the non-treated control. Transplant water applications of propamocarb at 3.4 kg a.i./ha were most effective. However, phytotoxicity
was observed in most of the propamocarb treatments, but plants were able to recover four weeks later (Reilly, 1980). However, treatments of propamocarb in the transplant water were not significantly different from the control when comparing black shank incidence and yield in Georgia (Csinos and Minton, 1983).

Currently, there are two fungicides registered for use in North Carolina for control of black shank of tobacco, fluopicolide (Presidio®) and mefenoxam (Ridomil Gold SL® formerly an pair of enantiomers known as metalaxyl). Fluopicolide is a systemic fungicide that belongs to the benzamides group. While previously registered in other crops for oomycete control, fluopicolide was not registered for use on tobacco until the 2015 growing season. Mefenoxam is translocated apoplastically in the plant and belongs to a systemic class of fungicides known as the phenylamides (Erwin and Ribeiro, 1996). Fungicides from the phenylamide class are active against many different oomycete plant pathogens, including the black shank pathogen, \textit{P. nicotianae} (Kannwischer and Mitchell, 1978). The mode of action of phenylamides involves the inhibition of the biosynthesis of rRNA (Davidse et al., 1983; Gisi and Cohen, 1996). Specifically, phenylamides interact with RNA polymerase I, which transcribes the rRNA (Davidse et al., 1983). Significant loss of rRNAs can deprive the fungal cells of ribosomes and ultimately halt protein synthesis resulting in death of the hyphal cell (Erwin and Ribeiro, 1996). Sporangia and zoospores contain a reserve of ribosomes, which explains the in-activity of phenylamides on sporangia or zoospore germination (Erwin and Ribeiro, 1996).
One of the first released phenylamide compounds was metalaxyl, which was introduced in 1977 with studies showing it to be efficacious against oomycetes (Kannwischer and Mitchell, 1978; Erwin and Ribeiro, 1996). Interestingly, metalaxyl when released was an unresolved isomeric mixture with a pair of enantiomers, S(+) and R(-) (Zadra et al., 2002). Metalaxyl was replaced in 1996 with the release of mefenoxam, the more active R(-) enantiomer of metalaxyl, also known as metalaxyl-m (Zadra et al., 2002, Randall et al., 2014).

When first released, many studies in the lab and the field confirmed the efficacy of metalaxyl against the *P. nicotianae* (Kannwischer and Mitchell, 1978; Reilly, 1980; Staub and Young, 1980; Shew, 1984; Shew, 1985). Staub and Young (1980) were the first to assess the efficacy of metalaxyl against one isolate of *P. nicotianae* in culture, determining that the mycelial growth, sporangia production, and chlamydospore production were sensitive to the fungicide at rates of 0.2 to 1 µg/ml depending on structure tested. Germination of sporangia and zoospores were not found to be sensitive to metalaxyl (Staub and Young, 1980). Staub and Young (1980) also found that development of the pathogen in tobacco roots treated with the fungicide at 1 µg a.i./ml was entirely stopped. Shew (1984) later screened a collection of 317 isolates of *P. nicotianae* from multiple locations across NC for sensitivity to metalaxyl and found the mean EC$_{50}$ for mycelial growth to be 0.4 µg a.i./ml.

Studies conducted by Kannwischer and Mitchell (1978) demonstrated efficacy of metalaxyl against black shank in the greenhouse and field. When metalaxyl was applied to greenhouse soil at greater than or equal to 1 µg a.i./ml, no black shank symptoms were
observed in the experiments. Transplant applications of the fungicide resulted in significant increases in yield of resistant and susceptible tobacco plants when compared to the nontreated control (Kannwischer and Mitchell, 1978;). Reilly (1980) also demonstrated the effectiveness of metalaxyl against black shank in the field studies from 1976 to 1978, applying the fungicide as either a transplant water or band application. Pre-plant treatments of metalaxyl were evaluated in a study by Csinos and Minton (1983) and were found to be highly efficacious against black shank. In general, early applications of metalaxyl or mefenoxam have been found to be critical for black shank management (Reilly, 1980; Csinos and Minton, 1983; Csinos et al., 1986; Antonopoulos et al., 2010; Mila and Radcliff, 2014). Antonopoulos et al. (2010) demonstrated that transplant water and first cultivation treatments with mefenoxam significantly reduced black shank incidence compared to the non-treated control or applications of the fungicide at the last cultivation of tobacco (layby).

Mefenoxam has remained efficacious for use against black shank since its introduction in 1996, with no true fungicide resistance observed in this pathosystem; however continuous use in the field has been shown to decrease the sensitivity of *P. nicotianae* isolates to the fungicide over time without causing the development of a true resistant phenotype (Shew, 1985; Csinos and Bertrand, 1994; Parkunan et al., 2008; Wang et al., 2013). Despite the lack of resistance to mefenoxam in the black shank system, resistance development to the fungicide has been observed in many other oomycete pathosystems. Resistance was first observed by Davidse et al. (1981) in *P. infestans* strains obtained from a Dutch potato field subjected to multiple sprays of metalaxyl. The mechanism behind the
mefenoxam resistance phenotype is complex and is still subject to discussion, with multiple loci possibly playing a role in the resistance (Judelson and Roberts, 1999; Matson et al., 2015; Randall et al., 2014). Previous studies with *P. infestans* has proposed that the resistant phenotype is possibly due to two *MEX* loci and minor effects are also contributed from other genes as well (Judelson and Roberts, 1999). Studies by Randall et al. (2014) specifically sequenced the subunits of RNA polymerase I from sensitive and insensitive *P. infestans* isolates. Single nucleotide polymorphisms (SNPs) were identified in the gene that encoded the largest subunit of RNA polymerase I, RPA190 (Randall et al., 2014). After a survey of sensitive and resistant isolates, Randall et al. (2014) identified SNPT1145A, as a SNP that was associated with the resistant phenotype, an 86% association with mefenoxam resistance. To further validate findings, resistance alleles were transferred into mefenoxam sensitive isolates, and yielded mefenoxam resistant isolates (Randall et al., 2014). Interestingly, this work was recently challenged by Matson et al. (2015), who demonstrated that resistant *P. infestans* isolates do not always contain SNPT11145A, and that multiple mefenoxam sensitive isolates did contain SNPT11145A. Based on these findings by Matson et al. (2015), the mechanism of resistance to phenylamides is still a subject for further discussion and research.

Oxathiapiprolin is a new fungicide of the piperdinyl-thiazole-isoxazoline class and has been shown to be efficacious against diseases caused by oomycetes, including black shank (Ji et al., 2014; Pasteris et al., 2016). This new fungicide class has a novel mode of action site, with oxathiapiprolin binding tightly to an oxysterol binding protein, which still
has no known cellular function across oomycetes (Pasteris et al., 2016). Oxathiapiprolin was registered for use in tobacco against black shank in 2016. Previous field studies in tobacco by Ji et al. (2014) have shown that treatments of oxathiapiprolin were efficacious against black shank in Georgia. Oxathiapiprolin applications were made to the soil at transplant, first cultivation, and last cultivation (layby). In general, a majority of treatments of oxathiapiprolin significantly reduced black shank incidence and increased crop yield when compared to the nontreated control (Ji et al., 2014). Treatments of oxathiapiprolin also had less disease incidence than the standard treatment of three applications of mefenoxam (Ji et al., 2014). However, Ji et al (2014) did not examine the rotation of oxathiapiprolin and other MOA fungicides within a season or the efficacy of oxathiapiprolin on the life cycle stages of *P. nicotianae*. The Fungicide Resistance Action Committee (FRAC) considers oxathiapiprolin as a medium to high risk potential for pathogen resistance. To combat the risk of resistance of any pathogen to oxathiapiprolin, a chemical program with rotations of fungicides with different mode of actions (MOAs) should be further examined.
Literature Cited


CHAPTER II. Effects of oxathiapiprolin on *Phytophthora nicotianae*, the causal agent of black shank of tobacco

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Effects of oxathiapiprolin on *Phytophthora nicotianae*, the causal agent of black shank of tobacco

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**Abstract**

Oxathiapiprolin is a new fungicide active against oomycetes. In vitro assays and field studies were conducted to examine the effect of oxathiapiprolin on *Phytophthora nicotianae* (Pn), the causal agent of black shank. The efficacy of oxathiapiprolin on mycelial growth, sporangia production, zoospore motility, and zoospore germination was assessed with four Pn isolates. EC₅₀ values were low ranging from 0.0039 to 0.0049 μg a.i./ml for mycelial growth, 0.00052–0.00081 μg a.i./ml for sporangia production, 0.0005–0.0006 μg a.i./ml for encysted zoospore germination, and 0.0055–0.0066 μg a.i./ml for zoospore motility. Sixty-six Pn isolates, 60 from tobacco and six from ornamental plants were examined for sensitivity to oxathiapiprolin at 1 μg a.i./ml. Mycelial growth at 1 μg a.i./ml was observed in only one isolate from tobacco whereas the six Pn isolates from ornamental plants five of which were resistant to mefenoxam at 100 μg a.i./ml were found to be sensitive to oxathiapiprolin. The efficacy of oxathiapiprolin against black shank was assessed in three field studies in North Carolina. Treatments of oxathiapiprolin were equal or superior to mefenoxam against black shank in each field study. Our results suggest that oxathiapiprolin is a highly efficacious fungicide against *P. nicotianae* and will be a useful tool in controlling black shank of tobacco.

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1. Introduction

*Phytophthora nicotianae* van Breda de Haan (= *P. nicotianae* var. *nicotianae*) is a devastating soilborne pathogen to many plant species including tobacco (*Nicotiana tabacum* L.). In tobacco, the pathogen causes black shank, a disease that results in millions of dollars of loss in flue-cured and burley tobacco each year in the southeastern United States and throughout the world (Lucas, 1975; Shoemaker and Shew, 1999). In North Carolina alone, over $10 million in annual losses of tobacco from black shank have been reported (Mila and Radcliff, 2014).

*P. nicotianae* was first introduced into the United States in Georgia in 1915. It was reported in North Carolina on tobacco in 1931 (Lucas, 1975). Currently, three physiological races (race 0, 1, and 3) of *P. nicotianae* have been described on tobacco in North Carolina, with race 0 and 1 being the most prevalent races (Gallup and Shew, 2010). Race 1 is distinguished from race 0 based on its ability to infect cultivars of tobacco that carry a single-gene (known as the *Ph* gene) source of resistance to race 0 (Mila and Radcliff, 2014). In North Carolina, race 0 was the predominant race in all tobacco-growing areas until cultivars containing the *Ph* gene were introduced in the mid 1990s (Sullivan et al., 2005).

Effective black shank management involves an integrated approach using cultural practices, crop rotation, host resistance, and chemical control (Lucas, 1975; Shew and Lucas, 1991; Mila and Radcliff, 2014). Presently, mefenoxam is the only fungicide registered for use on tobacco for black shank control, which was introduced to the market in 1996. Mefenoxam is the more active isomer of metalaxyl, which was introduced to the market in 1977. This systemic fungicide belongs to the phenylamides and has been shown to be highly active against oomycetes, including *P. nicotianae* (Staub and Young, 1980; Shew, 1983). Specifically, mycelial growth and sporulation are both inhibited by mefenoxam and metalaxyl (Hu et al., 2008). The mode of action (MOA) of mefenoxam and metalaxyl involves the inhibition of ribosomal RNA synthesis, specifically interacting with RNA polymerase (Davies et al., 1983). However, due to the site-specificity of mefenoxam and its repeated use by growers, there is a high risk of resistance development in its target pathogens (Hu et al., 2008).

Mefenoxam and metalaxyl resistance has been identified in
many different Phytophthora spp. covering a wide range of hosts throughout the world (Davidsen et al., 1981; Lambert and Salas, 1994; Gusi and Cohen, 1996; Parra and Ristaino, 2001; Jeffers et al., 2004; Taylor et al., 2006; Hu et al., 2008). Over the past few years, a large number of P. nicotianae isolates collected from hosts in ornamental nurseries have been resistant to mefenoxam (Hu et al., 2008; Olson et al., 2013) whereas no isolates of P. nicotianae from tobacco have ever been resistant to it (Parkman et al., 2010). Nevertheless, repeated use of metalaxyl in a single field has been demonstrated to reduce the sensitivity of the native population of P. nicotianae to metalaxyl (Shew, 1985). A rotation of fungicides with different MOAs should prevent loss of fungicidal sensitivity from occurring.

Oxathiapiprilin is the first fungicide of the new piperidinyl thiazole oxazoline class of fungicides with a novel MOA that targets oxysterol binding protein, it is currently not registered for use in any crop. Ji et al. (2014) have shown that application of oxathiapiprilin is efficacious against the black shank disease of tobacco in field trials. In their study, oxathiapiprilin was investigated in field trials over a three-year span. Oxathiapiprilin applications were made in a similar manner to applications of Ridomil Gold SL (i.e. mefenoxam), and at the same time, that is at the time of transplanting tobacco, and with first and last cultivation during the tobacco growing season. In this study, three applications of oxathiapiprilin significantly increased tobacco yield and decreased disease incidence when compared to the untreated plots. Oxathiaprilin treatments were also comparable to treatments with mefenoxam in yield and disease incidence. The direct effects of oxathiapiprilin on the causal agent of the black shank disease, P. nicotianae, remains to be investigated.

Here we investigate the efficacy of oxathiapiprilin: (i) on mycelial growth, sporangia production, zoospore germination, and zoospore motility in in vitro assays using four isolates of P. nicotianae. (ii) in controlling black shank disease in field studies and (iii) the sensitivity of a collection of P. nicotianae isolates from different hosts to oxathiapiprilin by measuring mycelial growth.

2. Materials and methods

2.1. Isolates

Thirty race 0 and thirty race 1, for a total of 60, isolates of P. nicotianae from tobacco were used in this study. Of these isolates, 21 were obtained from the D. Shew collection and 39 were from the A. L. Milia collection, North Carolina State University. P. nicotianae isolates from tobacco were collected in North Carolina and Virginia (Table 1). Of the 60 tobacco isolates, two race 0 (Edge07 and Edgea12) and two race 1 (YadA3 and YadA28) isolates were selected for use in the four life cycle stage studies because of their prolific spore production.

Six isolates of P. nicotianae from ornamental plants were also used. Three isolates were from Dusty Miller, two from Calibrachoa, and one from Gerbera Daisy. Five isolates were obtained from H. Olson and a single isolate from D. Shew, North Carolina State University. Of these isolates, five were resistant to mefenoxam at 100 µg a.i./ml (Dr. Benson, personal communication).

2.2. Fungicides

Oxathiapiprilin (Zorvec®) was provided by DuPont, Wilmington, DE and mefenoxam (Ridomil Gold SL® 4 lb a.i./gallon) was supplied by Syngenta Crop Protection, Greensboro, NC. Stock solutions of oxathiapiprilin and mefenoxam were prepared with sterile deionized water and stored at 4°C to preserve fungicidal activity. For amended medium, stock solutions of the fungicides were added to medium after it was cooled to approximately 55°C. Amended medium was stored in the dark at 4°C for up to two weeks.

2.3. Mycelial growth

Mycelial plugs (6 mm in diameter) of the four isolates were cut with a sterile cork borer from the edge of 4–5 day-old cultures grown on V8 agar (V8A; V8 juice, 200 mL; CaCO3; 3 g; agar, 17 g; and deionized water, 800 mL) at 28°C in the dark. Plugs were transferred to 100 mm x 15 mm Petri plates with V8A medium amended with oxathiapiprilin at the following concentrations: 0.001, 0.002, 0.003, 0.005, and 0.015 µg a.i./mL. A non-amended with oxathiapiprilin control was also included. A single mycelia plug was placed in the center of each Petri dish. There were three replicates Petri dishes for each treatment per isolate. Petri dishes were then incubated in a growth chamber at 28°C in darkness until the mycelium reached the edge of the dish in the untreated dishes, which occurred 4–5 days after the initiation of the experiment. Colony diameters were then measured in two perpendicular directions for all dishes. The two colony diameters were averaged after mycelial plug diameters were subtracted. Experiments were conducted three times.

2.4. Sporangia production

Sporangia production was induced with the method described by Hu et al. (2007). Sterile soil extract (SSE) was used as a liquid medium to induce sporangia formation. SSE was produced by mixing 50 g of sterile soil with 1 L of deionized water, and letting it incubate at room temperature (23–25°C) for 72 h, after which it was sterilized, filtered through Fisher Brand Quantitative 5 grade filter paper (Pittsburgh, PA), and sterilized again. Three mycelial plugs, 5 mm in diameter, were cut from the edge of 4–5 day-old cultures grown on V8A and placed into 60 mm x 15 mm Petri dishes with the mycelia side facing up. Dishes were then filled with 8 mL of SSE amended with or without oxathiapiprilin at the following concentrations: 0.0005, 0.001, 0.002, 0.003, and 0.005 µg a.i./mL. In each Petri dish, the medium level just reached the top of the mycelia plug. Three replicate Petri dishes were used for each treatment per isolate. Petri dishes were incubated under constant light at room temperature for 10h, after which the number of sporangia produced along the edges of each mycelia disk was counted using a microscope. Experiments were conducted twice.

\begin{table}
\centering
\begin{tabular}{|l|l|l|l|l|}
\hline
Host & County & State & Race & Number of isolates \\
\hline
Tobacco & Edgecombe & NC & 0 & 8 \\
Tobacco & Edge & NC & 0 & 2 \\
Tobacco & Duplin & NC & 0 & 6 \\
Tobacco & Johnston & NC & 0 & 8 \\
Tobacco & Dimock & VA & 0 & 1 \\
Tobacco & Halifax & VA & 0 & 2 \\
Tobacco & Newton & VA & 0 & 1 \\
Tobacco & Pittsylvania & VA & 0 & 2 \\
Tobacco & Edgecombe & NC & 1 & 2 \\
Tobacco & Forsyth & NC & 1 & 5 \\
Tobacco & Hoke & NC & 1 & 6 \\
Tobacco & Yadkin & NC & 1 & 17 \\
Dusty Miller & N/A & NC & N/A & 3 \\
Calibrachoa & N/A & NC & N/A & 2 \\
Gerbera Daisy & N/A & NC & N/A & 1 \\
\hline
\end{tabular}
\caption{Host and geographic location of Phytophthora nicotianae isolates.}
\end{table}
2.5. Zoospore germination

Zoospore germination was evaluated using the method described by Von Brownsee and Deacon, 1997 with modification. Mycelial plugs, 6 mm in diameter, were cut with a cork borer from the edge of cultures grown on V8A and were transferred to the center of oatmeal agar (Oatmeal agar, 72.5 g; deionized water, 1 L). Petri dishes. Petri dishes were then incubated in darkness at 28 °C until mycelia growth filled the plate, about 2-4 weeks later. Mycelial mats were then peeled off the surface of the medium and placed into a Petri dish with 20 mL of SSE and incubated at 28 °C until mats had produced numerous sporangia, approximately 1-2 weeks after they were placed in SSE. Zoospores were released by incubating Petri plates with mycelia mats at 4 °C for 30 min, followed by incubation at 28 °C for an additional 30 min. Then zoospores were vortexed for 70s to induce encystment and the concentration was adjusted to 1.5 × 10^5 by dilution based on hemocytometer counts. The zoospore suspensions were transferred to 1.5 mL Eppendorf tubes in 0.9 mL aliquots. A tenth of a milliliter of oxathiapipril stock solutions were added to each Eppendorf tube to achieve final concentrations of 0.001, 0.003, 0.005, 0.007, and 0.01 μg a.i./mL. A non- amended control was also used by adding 0.1 mL of non-amended SSE to the Eppendorf tube. Three replicate tubes were used for each concentration per isolate. Eppendorf tubes were then incubated at room temperature for 8 h. After incubation, 50 μL of the zoospore suspension was removed from the Eppendorf tubes and placed on a microscope slide for observation. One hundred zoospores were counted at random as either germinated or non-germinated. Zoospores were counted as germinated if the germ tube was longer than the diameter of the encysted zoospore. Experiments were conducted twice.

2.6. Zoospore motility

Motility was assessed using the method described by Hu et al. (2007). Zoospores were produced as previously described and were adjusted to a concentration of 1.5 × 10^4 using a hemocytometer. Three point six mL of zoospore suspensions were transferred to 60 mm × 15 mm petri dishes and amended with an oxathiapipril stock solution to obtain the following concentrations: 0.001, 0.002, 0.003, 0.005, and 0.01 μg a.i./mL. Non-amended zoospore suspensions received an equal volume of SSE only. Three replicate Petri dishes were used for each treatment per isolate. Zoospore suspensions in the Petri plates were observed microscopically to determine the maximum elapsed time for complete cessation of motility. Observations were made at 15 min intervals for the first hour and then every 30 min until no zoospore movement was detected. Total time of motility was recorded.

2.7. Sensitivity assay

The sub-lethal concentration for mycelial inhibition of the four isolates tested was 0.01 μg a.i./mL, based on the results from the mycelial growth experiments. A collection of 69 P. nicotianae isolates from tobacco and 6 P. nicotianae isolates from ornamental plants were tested for sensitivity to oxathiapipril at a rate of 1 μg a.i./mL. Actively growing cultures of each isolate were established on 10% clarified V8 juice agar (CV8A; 100 mL of buffered and clarified V8 juice, 900 mL deionized water, and 15 g agar) in 60-mm × 15-mm Petri dishes. Clarified and buffered V8 juice was prepared by filtering 1 g of CaCO₃ with 100 mL of V8 juice, and then centrifuging at 7000 rpm for 10 min. Petri dishes were incubated in the dark at 28 °C for 3-4 days. Oxathiapipril sensitivity was determined with an assay previously reported (Olson et al., 2013).

The assay used 48-well tissue culture plates (VWR International, Inc.) with 0.5 mL of 3% CV8A (30 mL buffer and clarified V8 juice, 950 mL deionized water, and 15 g agar) added to each individual well. In each plate, rows A through C contained non-amended 3% CV8A and wells D through F contained 5% CV8A amended with oxathiapipril at 1 μg a.i./mL each. A column in a plate was used for a single isolate, allowing for three replicate wells of non-amended (A-C) and amended (D-F) agar for each isolate (Fig. 1). Agar plugs, 1 mm in diameter, were cut from the edge of cultures grown on 10% CV8A using a sterilized glass Pasteur pipette. Individual plugs were then transferred to each well in the specific column of the isolate. Tissue cultures plates were then covered and incubated for 3-4 days at 28 °C in the dark. Wells were then evaluated for mycelial growth as described in Olson et al. (2013) on a 0-5 scale, where 0 = no growth; 1 = a few hyphae growing from the plug visible only microscopically; 2 = hyphae growing uniformly around the plug visible microscopically; 3 = hyphae growing uniformly around the plug, visible macroscopically; 4 = substantial mycelial growth visible macroscopically, without complete covering surface of agar; and 5 = mycelial growth equal to that of non-amended wells. Isolates were tested twice and their sensitivity ratings were averaged. Isolates with an average score of less than 4 were sensitive and isolates that had an average score of greater than or equal to 4 were deemed insensitive (Olson et al., 2013).

2.8. Isolate response to EC₅₀ values of oxathiapipril and 100 μg a.i./mL of mefenoxam

Based on the results from the mycelial growth sensitivity assay, the 66 isolates of P. nicotianae were tested for mycelial growth sensitivity to oxathiapipril at 0.004 μg a.i./mL, determined as the average EC₅₀ concentration for the 4 isolates used in the experiments, and mefenoxam at 100 μg a.i./mL. Fresh cultures of each isolate were produced by placing an agar plug in the center of a new V8A dish and incubated at 28 °C for 3-4 days. Mycelial plugs, 6-mm diameter, were taken from the edge of each fresh culture using a sterile cork borer, and placed at the center of three replicate V8A dishes amended with either oxathiapipril at 0.004 μg a.i./mL or mefenoxam at 100 μg a.i./mL. The Petri dishes were then incubated in the dark at 28 °C until the growth on the non-amended dishes had reached the edge of the plate. The diameter of mycelial growth was measured in two perpendicular directions for all dishes. Colony diameters were averaged after the mycelial plug diameters were subtracted. Experiments were conducted twice.

2.9. Efficacy of oxathiapipril against black shank in field studies

The efficacy of oxathiapipril to manage P. nicotianae was...
investigated in tobacco fields in North Carolina with a history of the black shank disease. Field studies were conducted in 2009 and 2012 at the Upper Coastal Plain Research Station (UCPRS), in Edgecombe County, and in an on-farm site in Yadkin County in 2012. The soil type at the UCPRS was a Goldthwaite Fine Sandy Loam at the Yadkin County field study. Blue-cured tobacco cultivar NC71 was used in each field study and seedlings were transplanted on 29 April at the UCPRS in 2009, 17 April at the UCPRS in 2012, and 5 May in Yadkin County in 2012.

In 2009, two treatments using oxathiapiprolin and one treatment using methenoxam were tested (Table 5). Treatments included three applications: at transplant (29 April), at first cultivation (13 May), and at last cultivation (15 June). At transplant oxathiapiprolin was applied as a tray drench at a rate of 0.08 kg a.i./ha. The fungicide was sprayed onto the seedlings using a CO2-powered sprayer operated at 206.84 kPa and then soil drenched immediately after by lightly sprinkling the treated seedlings with water to rinse the product from the foliage and into the soil. Drench applications were conducted at the greenhouse before the tobacco seedlings were transplanted into the field. The methenoxam treatment at transplant was applied as a 61-cm band over the top of the row using a CO2-powered backpack sprayer operated at 206.84 kPa At first cultivation and layby, fungicides were applied at their respective rates as a 61-cm band on both sides of the row of plants with a CO2-powered backpack sprayer immediately prior to cultivation in order to successfully incorporate the fungicide into the soil.

In 2012, treatments with three applications of oxathiapiprolin, and one treatment of methenoxam with three applications were tested (Table 5). Treatments were at transplant, conducted on 17 April in UCPRS and 2 May in Yadkin, at first cultivation on 7 May in UCPRS and 30 May in Yadkin, and at layby conducted on 18 June in UCPRS and 21 June in Yadkin. In contrast to 2009, transplant applications of oxathiapiprolin were conducted to mimic fungicide amended transplant water. To mimic application with transplant water, 3 fl oz. of a fungicide solution was poured over each plant in their respective plots. The rate of application of oxathiapiprolin at transplant was 0.07 kg a.i./ha instead of 0.14 kg a.i./ha used in 2009 (Table 5). At first cultivation and layby, fungicides were applied as a 61-cm band on both sides of the row immediately prior to cultivation, in order to successfully incorporate the fungicide into the soil.

Field studies were arranged in a randomized complete block design, with four replicated plots of 1.17 m wide rows, 15.24 m in length for each treatment. Plants were 56 cm apart in each row. A nurseplant control was used in each study. Treated rows of tobacco were separated by a nontreated row of tobacco plants. Plant stand count was obtained three weeks after tobacco was transplanted. All recommended cultural practices were followed throughout the tobacco growing season.

Beginning five to six weeks after transplant, black shank disease incidence was evaluated by counting plants with visual characteristic symptoms of black shank. Disease evaluations were conducted once every two weeks for a total of five evaluations for each study throughout the season. A plant was counted as diseased if the leaves were permanently wilted and had a black lesion at the base of the stem. Disease index (DI) was calculated for all treatments in each field study based on the five disease incidence evaluations, with earlier evaluations being weighted more heavily than later evaluations according to Cianchin et al. (1986). For five disease evaluations, DI was calculated as:

\[ DI = \frac{1}{n} \sum_{i=1}^{n} X_i \times (100 - i \times 10) \]

where i is the ordinal evaluation number, n is the number of disease evaluations, X is the number of diseased tobacco plants since the previous count, and N is the total number of plants.

2.10. Statistical Analysis

Data from experiments were combined together according to homogeneity of variances based on Levene's Test (SAS 8.2; SAS Institute, Cary, NC). The percentage of inhibition for each life cycle stage was calculated relative to the non-amended control and converted to a probability scale using a probit table. The fungicide concentration was expressed on a logarithmic scale. A log-probit regression analysis was conducted for each isolate and each life cycle stage (SAS 9.2; SAS Institute, Cary, NC). EC50 values were calculated using the regression equations from the analysis.

Data from the experiments evaluating the effect of 0.004 μg a.i./ml oxathiapiprolin and 100 μg a.i./ml methenoxam were analyzed with analysis of variance (PROC ANOVA; SAS, version 9.2) and means were separated according to Fisher's least significant difference at P ≤ 0.05. For the efficacy field studies, DI data were logarithmically transformed and then analyzed using PROC GLM of the Statistical Analysis System (SAS, version 9.2). Treatment means were further separated according to Fisher's least significant difference test (FSLD) at P ≤ 0.05.

3. Results

3.1. Mycelial Growth

The estimated EC50 values for inhibition of mycelial growth ranged from 0.0039 to 0.0049 μg a.i./ml (Fig. 3A, Table 2). All isolates tested were sensitive to oxathiapiprolin, with no significant differences in sensitivity observed among the isolates. As the concentration of oxathiapiprolin in the media increased, the inhibition of mycelial growth increased, as well (Fig. 2A). EdgeA12 had the lowest numerically estimated EC50 value of the four isolates (Table 2). All isolates were inhibited by over 80% when tested against oxathiapiprolin at 0.01 μg a.i./ml (Fig. 2).

3.2. Sporangia Production

Production of sporangia was the most sensitive life cycle stage tested, with EC50 estimates ranging from 0.00056 to 0.00081 μg a.i./ml (Fig. 3B, Table 2). The estimated EC50 values for sporangia production for isolate EdgeA12 was significantly lower than that of isolates Edge B7 and YadA28, while EdgeB7 was intermediate (Table 2). EdgeA12 was also the only isolate inhibited at 100% at the 2 highest concentrations of the fungicide that is 0.003 and 0.005 μg a.i./ml (Fig. 2B).

3.3. Zoospore Germination

Germination of the zoospores in all isolates was sensitive to oxathiapiprolin at varying concentrations, with the highest concentration, 0.01 μg a.i./ml inhibiting germination the most (Fig. 4C). Estimated EC50 values for inhibition of zoospore germination ranged from 0.0035 to 0.0051 μg a.i./ml (Fig. 3C, Table 2). Isolate EdgeB7 had a significantly larger EC50 value than the other three isolates (Table 2).

3.4. Zoospore motility

Untreated zoospores remained motile on the average for 600 min. The total time of zoospore motility was also sensitive to oxathiapiprolin at varying concentrations (Fig. 2D). Isolate EdgeB7 was the most sensitive to the fungicide isolate at each tested concentration (Fig. 2D). The estimated EC50 values for inhibition of zoospore motility for the four isolates ranged from 0.0055 to 0.0166 μg a.i./ml (Fig. 3D, Table 2). Isolate YadA3 had largest
estimated EC\textsubscript{50} value of all the isolates tested (Table 2).

3.5. Oxathiapiprolin sensitivity assay

A single P. nicotianae isolate, YadA18, from tobacco was insensitive to oxathiapiprolin at 1 µg a.i./ml with a consistent rating of 4 whereas all other tobacco isolates were sensitive with ratings of 0 (Table 3). The five P. nicotianae isolates that were resistant to fenoxaprop at 100 µg a.i./ml were found to be sensitive to oxathiapiprolin.

3.6. Isolate response to EC\textsubscript{50} values of oxathiapiprolin and 100 µg a.i./ml of fenoxaprop

Range of mycelial growth inhibition of race 0 and race 1 tobacco isolates was 87.63 ± 0.68 and 89.51 ± 0.39 respectively. In general, the mycelial growth of race 0 isolates was inhibited at a greater rate than the race 1 isolates when tested against the 0.004 µg a.i./ml rate of oxathiapiprolin with values of 71.4% ± 0.69 and 59.8% ± 1.1 respectively. Sensitivity of isolates to 0.004 µg a.i./ml oxathiapiprolin differed between the two races (Fig. 4). Inhibition of race 0 isolates did not fall below 51% (Fig. 4A) while some race 1 isolates were below 51% inhibition and as low as 16.9% inhibition (Fig. 4B). Isolates of P. nicotianae collected from ornamental plants that were resistant to fenoxaprop at 100 µg a.i./ml, were not resistant to oxathiapiprolin when tested at the EC\textsubscript{50} rate of 0.004 µg a.i./ml (Table 4).

3.7. Efficacy of oxathiapiprolin against black shank in field studies

In 2009, black shank disease index was significantly lower for all treatments of oxathiapiprolin and fenoxaprop when compared to the nontreated control (Table 5). There was no significant difference between treatments with oxathiapiprolin and fenoxaprop. Oxathiapiprolin used as a tray drench application at 0.28 kg a.i./ha was not significantly different than the treatment with the lower rate of 0.07 kg a.i./ha (Table 5). In 2012, black shank disease index was significantly lower for all treatments of oxathiapiprolin in comparison to the nontreated control in both field studies (Table 6). The fenoxaprop treatment in both field studies was not significantly different than the nontreated control. In UCPRS both treatments of oxathiapiprolin were significantly more efficacious in controlling black shank than the standard three applications of fenoxaprop whereas in Yadkin County no significant difference was found between treatments using oxathiapiprolin or fenoxaprop (Table 5). In both studies no significant differences were observed between the treatments of oxathiapiprolin using varying rates at the transplant application (Table 5).

4. Discussion

This is the first in vitro study examining the effect of oxathiapiprolin, a new MOA, on P. nicotianae, the pathogen that causes black shank of tobacco. Oxathiapiprolin was efficacious against several stages of P. nicotianae life cycle, such as mycelial growth, sporangia production, zoospore germination, and zoospore motility, with sporangia production being the most sensitive stage tested. The EC\textsubscript{50} values of oxathiapiprolin against mycelial growth ranged from 0.0039 to 0.0046 µg a.i./ml, lower than the EC\textsubscript{50} values reported for metalaxyl that ranged between 0.14 and 0.4 µg a.i./ml (Staub and Young, 1989; Fair et al., 1989), and fenoxaprop that have been reported at 0.4 µg a.i./ml (Gallup, 2009). The mycelia growth of Phytophthora capsici isolates has also been shown to be
Fig. 3. Dose-response curves of mycelial growth inhibition (A), sporangia production (B), zoospore germination (C), and zoospore motility (D) of four isolates of Phytophthora nicotianae. Percent inhibition was transformed to a probability scale plotted against the log concentration of oxathiapiprolin. The vertical bars at each point represent the standard error of the mean of the experiments.

Table 2: Range of effective concentration of oxathiapiprolin for 50% reduction in mycelial growth, sporangia production, zoospore germination, and zoospore motility of four isolates of Phytophthora nicotianae.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Race</th>
<th>EC50 (µg a.i./mL)</th>
<th>Mycelial growth</th>
<th>Sporangia production</th>
<th>Zoospore germination</th>
<th>Zoospore motility</th>
</tr>
</thead>
<tbody>
<tr>
<td>EdgeA12</td>
<td>0</td>
<td>0.0103 a</td>
<td>0.00056 b</td>
<td>0.004 b</td>
<td>0.0104 b</td>
<td></td>
</tr>
<tr>
<td>EdgeB7</td>
<td>0</td>
<td>0.0049 a</td>
<td>0.00091 a</td>
<td>0.0051 a</td>
<td>0.0055 b</td>
<td></td>
</tr>
<tr>
<td>YadA3</td>
<td>1</td>
<td>0.0046 a</td>
<td>0.00062 ab</td>
<td>0.0035 b</td>
<td>0.0160 a</td>
<td></td>
</tr>
<tr>
<td>YadA28</td>
<td>1</td>
<td>0.0046 a</td>
<td>0.00031 a</td>
<td>0.0037 b</td>
<td>0.0060 b</td>
<td></td>
</tr>
</tbody>
</table>

* Concentration of oxathiapiprolin providing 50% inhibition of growth (EC50). Values within a column followed by the same letter are not significantly different (P ≤ 0.05) according to Fisher’s least significant difference test.

Table 3: Sensitivity of a collection of Phytophthora nicotianae isolates to oxathiapiprolin at 1 µg a.i./mL.

<table>
<thead>
<tr>
<th>Host</th>
<th>State</th>
<th>Oxathiapiprolin sensitivity rating</th>
<th>Insensitive</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco</td>
<td>NC, VA</td>
<td>1</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Dusty Miller</td>
<td>NC</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Gerbera Daisy</td>
<td>NC</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Calligraphica</td>
<td>NC</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

* Isolates were rated on a 0 to 5 scale, where 0 = no growth; 1 = a few hyphae growing from the plug but visible microscopically; 2 = hyphae growing uniformly around plug visible microscopically; 3 = hyphae growing uniformly around the plug, visible macroscopically; 4 = mycelial growth visible macroscopically, but not completely covering surface of agar; and 5 = mycelial growth equal to that of non-affected wells. Isolates with scores less than 4 were considered sensitive, and isolates with scores greater than or equal to 4 were insensitive.

Sensitive to oxathiapiprolin, with EC50 values averaging 0.001 µg a.i./mL. (5) and (6), 2013). Our in vitro studies suggest that P. nicotianae is more sensitive to oxathiapiprolin, than metalaxyl or its more active isomer mefenoxam. In this pathosystem the motility and germination of zoospores is critical for infection and disease (Lucas, 1975; Shew and Lucas, 1991). In this pathosystem the motility and germination of zoospores is critical for infection and disease (Lucas, 1975; Shew and Lucas, 1991). EC50 values of oxathiapiprolin for zoospore germination and zoospore motility ranged from 0.0035 to 0.0052 and 0.0050–0.0166 µg a.i./mL, respectively. Farish et al. (1981) found that metalaxyl did not greatly inhibit zoospore germination whereas zoospore motility was not tested. Our results indicate that low concentrations of oxathiapiprolin can be efficacious at inhibiting zoospore germination and motility that may inhibit the pathogen from not only swimming towards the roots, but germinating and infecting tobacco roots as well. This is an important
finding because frequently fungicide application programs are designed so that timing of an application is aligned with its effect on the pathogen disease cycle.

Sporangia are just as important as zoospores in the black shank disease cycle, serving as the secondary inoculum for the disease (Lucas, 1975; Shew and Lucas, 1991). Sporangia production was the most sensitive life cycle stage to oxathiapiprolin, with EC₅₀ values ranging from 0.00296 to 0.00818 µg a.i./mL. Ji and Cinosis (2015) found that sporangia production in P.ropicoides was sensitive to oxathiapiprolin with EC₅₀ values averaging 0.0003 µg a.i./mL. Furthermore, Ji and Cinosis (2015) also demonstrated that sporangia production was the most sensitive stage of the pathogen. Our findings, in combination with the results from P.ropicoides, suggest that sporangia production may be the most sensitive stage across the genus of Phytophthora. Fath et al. (1981) found that in the presence of 0.1 µg a.i./mL metalaxyl, sporangia production by P. nicotianae was reduced by 85%. Based on these findings sporangia formation in the black shank pathogen is more sensitive to oxathiapiprolin than metalaxyl.

In our study, 66 isolates of P. nicotianae originating from tobacco and ornamental plant species, were found to be sensitive to oxathiapiprolin at 1 µg a.i./mL. However, a single race 1 isolate from tobacco, Yada19, could grow at a restricted rate with oxathiapiprolin at 1 µg a.i./mL when examining mycelial growth. Interestingly, however, mycelial growth of Yada19 was inhibited by 69.5% when isolates were tested at 0.004 µg a.i./mL of oxathiapiprolin. We hypothesise that Yada19 is not truly resistant to oxathiapiprolin, but it rather appears to have the ability to grow at a limited rate on high concentrations. All mefenoxam resistant isolates were identified as being sensitive to 0.004 µg a.i./mL of oxathiapiprolin. Although only five mefenoxam resistant isolates were tested our results suggest that there is no multiple resistance between mefenoxam and oxathiapiprolin.

The two major races of P. nicotianae on tobacco are races 0 and 1. The present study indicated that race 0 isolates were more sensitive to oxathiapiprolin than race 1 isolates, with 71.4 and 59.8% mean percent inhibition respectively when tested against 0.004 µg a.i./mL. When looking at the distribution of the mycelial growth response of the isolates, none of the race 0 isolates were inhibited less than 51%, while more than 13% of race 1 isolates fell between 11% and 50% inhibition. No other studies to our knowledge have examined the effect of this fungicide against races of P. nicotianae. Shew (1984) found that inhibition of mycelial growth by metalaxyl at 1 µg a.i./mL varied among isolates of P. nicotianae, although the race of the isolates tested was not determined. There is no clear

Table 4

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Host</th>
<th>Percent inhibition of mycelial growth for Phytophthora nicotianae isolates from ornamental plants.</th>
<th>Oxathiapiprolin</th>
<th>Mefenoxam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pe-2</td>
<td>Dusy Miller</td>
<td>48.7</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>80025</td>
<td>Dusy Miller</td>
<td>61.5</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>80005</td>
<td>Dusy Miller</td>
<td>51.0</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td>8046</td>
<td>Calimachae</td>
<td>70.2</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>8103</td>
<td>Calimachae</td>
<td>78.2</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>S1103</td>
<td>Gerbera Daisy</td>
<td>73.4</td>
<td>89.7</td>
<td></td>
</tr>
</tbody>
</table>

* Percent inhibition of mycelial growth when compared to non-amended media.
* Oxathiapiprolin at a rate of 0.004 µg a.i./mL.
* Mefenoxam at a rate of 100 µg a.i./mL.

Table 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2009 UCPRS</th>
<th>2012 UCPRS</th>
<th>2012 York County</th>
</tr>
</thead>
<tbody>
<tr>
<td>Application</td>
<td>Rate (µg a.i./ha)</td>
<td>DI</td>
<td>Application</td>
</tr>
<tr>
<td>Non-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mefenoxam</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* DI was calculated for each treatment in a field study from disease incidence evaluations. Earlier evaluations were weighted more heavily than the later evaluations. A disease index of 0 indicates no detectable levels of black shank whereas that of 100 indicates that all plants had died within the period of evaluation.

* Data are means of four replications. DI means in a column followed by the same letters are not significantly different according to Fisher’s least significant difference test (P < 0.05).
explanation of why there is a difference between the two races or the significance of the variation of oxathiapiprolel sensitivity between races. The results indicate that P. nicotianae isolates are diverse with respect to oxathiapiprol sensitivity. The difference in results observed between the two races may suggest that race is a contributing factor to this variation but likely there are more reasons, unknown at this point.

In field studies oxathiapiprol was observed to be highly efficacious against the black shank disease. Interestingly, compared to the nontreated control, treatments of oxathiapiprol were as, or significantly more effective than the 3 applications of mefenoxam, the only registered fungicide for black shank at the current time of these studies. Ji et al. (2014) found similar results in field studies conducted in Georgia. In their study they determined that soil directed treatments of oxathiapiprol were as effective at controlling the black shank disease as the standard three application treatment of mefenoxam (Ji et al. 2014). The two rates of oxathiapiprol used at transplant produced no statistically different results. Thus the rate of 0.07 kg a.i./ha oxathiapiprol at transplant may be sufficiently efficacious to control black shank when followed by two more applications at first cultivation and layby.

In summary, our study suggests that oxathiapiprol could provide comparable black shank control to the widely used fungicide, mefenoxam. However, chemical control of black shank should not rely on a single fungicide due to the potential development of resistance to mefenoxam, which has been observed in numerous Phytophthora species (Daskalou et al. 1981; Lamberti and Salas, 1994; Cesi and Cohen, 1998; Parra and Ristaino, 2001; Jeffers et al., 2004; Taylor et al., 2006; Hu et al., 2008), including P. nicotianae isolates from ornamental plants (Hu et al., 2008; Olson et al., 2013). Rotation of fungicides with different MOEs such as oxathiapiprol and mefenoxam, may help reduce the selection pressure of the pathogen to develop fungicide resistance.

Acknowledgments

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References


CHAPTER III. Efficacy of oxathiapiprolin against black shank of flue-cured tobacco in field trials in North Carolina

To be submitted to *Crop Protection*.

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Abstract

*Phytophthora nicotianae*, the causal agent of black shank, is one of the most important soilborne pathogens of tobacco (*Nicotiana tabacum* L.) in North Carolina. The use of soil applied fungicides has been a significant component of effective black shank management for tobacco growers. Oxathiapiprolin is a new fungicide with a novel mode of action that has shown efficacy against many oomycetes, including *P. nicotianae*. Studies were conducted in 2012 in fields naturally infested with *P. nicotianae* to determine the efficacy of different rates, methods, and timing of applications of oxathiapiprolin against black shank. In 2013 and 2014 field trials were conducted to examine the efficacy of alternating applications of oxathiapiprolin and mefenoxam against black shank. Fungicide treatments were applied to the soil on the day of transplanting tobacco to the field (transplant), 2 to 4 weeks after transplant (first cultivation), and 7 to 9 weeks after transplant (last cultivation). A single application of oxathiapiprolin in the transplant water at 0.07, 0.14, and 0.28 kg a.i./ha had a significant decrease in the area under the disease progress curve (AUDPC) when compared to the non-treated control in the 2012 field trials. In addition, treatments with three applications of oxathiapiprolin at all tested rates significantly reduced AUDPC. In field trials from 2013 and 2014, treatments alternating fungicide applications of oxathiapiprolin at transplant, mefenoxam at first cultivation, and oxathiapiprolin at last cultivation had a significantly lower AUDPC than the non-treated control. In contrast, the AUDPC of three applications of mefenoxam at transplant, first cultivation, and last cultivation did not differ significantly from the non-treated control. Overall, our results
indicate that soil directed applications of oxathiapiprolin, alone or in alternation with mefenoxam, are efficacious for the reduction of black shank of tobacco.

1. Introduction

Black shank of tobacco, caused by the soilborne oomycete pathogen *Phytophthora nicotianae* van Breda de Haan (=*P. nicotianae* var. *nicotianae*), is one of the most destructive diseases on flue-cured and burley tobacco (*Nicotiana tabacum* L.) in North Carolina and throughout the world (Lucas 1975; Shew and Lucas 1991; Shoemaker and Shew 1999). *Phytophthora nicotianae* was first introduced into North Carolina tobacco in 1931 and then quickly spread across the tobacco growing regions of the state (Lucas 1975). North Carolina is the largest flue-cured tobacco producing state in the USA, representing over 75% of the market (Brown and Snell 2015). In North Carolina, annual tobacco yield losses due to black shank may range between 1 and 3%, resulting in the lost of millions of USA dollars for tobacco growers (Mila and Radcliff 2014).

*Phytophthora nicotianae* directly infects the tobacco plant roots and lower stems, and can occasionally infect the leaves through splash dispersal (Shew and Lucas 1991; Shoemaker and Shew 1999). Infections happen at any growth stage when environmental conditions are favorable. Wet and humid environmental conditions, which are prevalent during the growing season, favor the pathogen and make the disease one of the most difficult to manage in tobacco (Lucas 1975; Mila and Radcliff 2014; Shew and Lucas 1991). Black shank symptoms include a black lesion on the base of the stem, wilting and chlorosis of the plant leaves (especially under dry conditions), necrosis of the roots, and separation of the pith
into necrotic plate like disks (Lucas 1975; Mila and Radcliff 2014; Shew and Lucas 1991). Effective management of this disease requires an integrated strategy involving several measures such as: two or more years of rotation with non-host crops, destruction of plant material at the end of a growing season, use of resistant cultivars, and application of fungicides (Lucas 1975; Mila and Radcliff 2014; Shew and Lucas 1991).

Cultural practices such as crop rotation can be difficult to implement because of insufficient land availability that reduces the length of the rotation and the long-term survival of chlamydospores formed by the pathogen, which are known to persist in the soil for several years (Shew and Lucas 1991). The use of resistant cultivars is one of the most effective management practices, however because of risk for development of new pathogen races in North Carolina, resistant cultivars should not be the only control method implemented. For instance, in North Carolina, there are currently three physiological races (0, 1, and 3) of *P. nicotianae* on tobacco (Apple 1962; Gallup and Shew 2010; Lucas 1975). Race 0 was the most predominant race of *P. nicotianae* in tobacco growing areas until the 1990’s, when tobacco cultivars expressing the *Ph* gene, a qualitative resistance gene, were introduced (Sullivan et al 2005). Race 0 is controlled by the *Ph* gene, whereas race 1 overcomes this type of resistance. After the introduction of the *Ph* gene, a rapid change in the pathogen population occurred with the emergence and dominance of the race 1 because of the extensive use of cultivars containing the *Ph* gene (Gallup and Shew 2010).

The use of fungicides is one of the most utilized methods to manage black shank. Currently two fungicides, fluopicolide and mefenoxam (Presidio® and Ridomil Gold SL®)
are registered for control of black shank of tobacco in the USA. Fluopicolide is a systemic fungicide in the benzamides group previously registered in other crops for oomycete control. Fluopicolide was not registered for tobacco until the 2015 growing season. Mefenoxam, also known as metalaxyl-m, was registered in 1996 and is the more active isomer of the original fungicide metalaxyl that was introduced in 1977. Metalaxyl and mefenoxam belong to a systemic class of fungicides known as the phenylamides, and have shown efficacy against black shank in numerous studies (Antonopoulos et al 2010; Cohen and Coffey 1986; Csinos et al 1986; Csinos and Minton 1983; Ferrin and Kabashima 1991; Shew 1984; Staub and Young 1980). Kannwischer and Mitchell (1978) were the first to demonstrate efficacy of metalaxyl in the field, showing that it increased tobacco yield especially when resistant cultivars were also used. Mefenoxam and metalaxyl are most efficacious against black shank when applied early in the growing season, specifically at transplant and first cultivation (Antonopoulos et al 2010; Mila and Radcliff 2014; Reilly 1980). Mefenoxam has remained efficacious for use since its introduction in 1996, with no fungicide resistance observed in the field. Nevertheless, continuous use has decreased the sensitivity of *P. nicotianae* isolates to the fungicide over time (Shew 1985; Parkunan et al 2010).

Oxathiapiprolin (Orondis®), of the new fungicide class piperdinyl-thiazole-isoxazoline, is efficacious against oomycetes, and will be available for the 2016 tobacco growing season. Three applications of oxathiapiprolin applied to the soil at transplant, first, and last cultivation (layby) were as efficacious against black shank as mefenoxam in three field studies in Georgia (Ji et al. 2014). At transplant, the two fungicides were applied
independently in the transplant water, while at first and last cultivation fungicides were applied to the raised tobacco bed and incorporated into the soil through immediate cultivation. In general, black shank incidence was significantly reduced with oxathiapiprolin and crop yield increased when compared to the non-treated control (Ji et al 2014). In all three field studies disease incidence of plants in treatments with three applications of oxathiapiprolin also was significantly lower than or equal to the standard treatment of three applications of mefenoxam (Ji et al 2014). The Fungicide Resistance Action Committee (FRAC) lists oxathiapiprolin as a medium to high risk for pathogen resistance development (Anonymous, 2016). One potential strategy to combat the risk of fungicide resistance is the use of alternate applications of oxathiapiprolin with fungicides of different modes of action.

In this field study we investigated the efficacy of: (i) different rates, number, and methods of oxathiapiprolin applications and (ii) alternation of oxathiapiprolin and mefenoxam applications for management of black shank caused by *P. nicotianae* in North Carolina tobacco fields.

2. Materials and Methods

2.1 Field locations. Field trials were conducted at the black shank nursery in the Upper Coastal Plain Research Station (UCPRS), in Edgecombe County, North Carolina, in 2012, 2013 and 2014, and at an on-farm site in Yadkin County, North Carolina, in 2012 (for a total of four different field environments). The soil types were a Goldsboro Fine Sandy Loam at the UCPRS and a Poplar Forest Fine Sandy Loam at the Yadkin County field site.
The field location in Yadkin County had a history of severe black shank incidence in tobacco in previous years. Race 1 of *P. nicotianae* was the predominant race present at both locations.

### 2.2 Experimental design.
Flue-cured tobacco cultivar NC71, which has the *Ph* gene that is effective against race 0 of *P. nicotianae*, was used in each field study. Plants were grown from seed in 288-cell styrofoam trays with 2.5 x 2.5 cm cells containing potting mix (Carolinas Choice, Carolina Soil Company, Kinston, North Carolina). Tobacco seedlings were maintained in a greenhouse until the transplant date. Seedlings were transplanted on 17 April in 2012, 23 April in 2013, 24 April in 2014 at UCPRS; and on 5 May 2012 at the on-farm location in Yadkin County. Seedlings were transplanted 56 cm apart in each row. Field plots were arranged in a randomized complete block design, with four replicate one-row plots that were 1.17 m wide and 15.24 m long. A non-treated, control plot was used in each year. To separate treated rows of tobacco plants, a non-treated row was planted between two treated rows. Plots were separated from each other with a 3 m alley. The plant stand count was obtained approximately three weeks after tobacco was transplanted in each location each year.

### 2.3 Application of fungicides.
In 2012, treatments at both locations were identical (Table 1). Applications of fungicides were made to seedlings in the tray immediately prior to transplanting and at transplant conducted 17 April in UCPRS and 5 May in Yadkin County, at first cultivation conducted on 7 May in UCPRS and 30 May in Yadkin County, and at last cultivation conducted on 18 June in UCPRS and on 21 June in Yadkin County. Tray applications were conducted at the greenhouse the same day tobacco seedlings were
transplanted to the field. Seedlings in the 288-cell tray were sprayed with oxathiapiprolin (Experimental Material, DPX-QGU42) at a rate of 0.035 kg a.i./ha with a CO₂-powered sprayer operated at 207 kPa, immediately followed by lightly sprinkling with water to rinse the product from the foliage and into the root ball. No further fungicide application was made to seedlings treated in the tray until the first cultivation application. At transplanting, seedlings in other treatments were given 88.7 ml of fungicide applications of oxathiapiprolin at a rate of 0.07, 0.14, or 0.28 kg a.i./ha or mefenoxam at a rate of 0.28 kg a.i./ha into the furrow at each plant to mimic application of fungicide by the water system of a commercial tobacco transplanter (Table 1). At first and last cultivation, fungicide sprays of oxathiapiprolin at a rate of 0.07 or 0.14 a.i./ha and sprays of mefenoxam at a rate 0.56 kg a.i./ha were applied to the soil surface as a 61-cm band on both sides of the row of plants with a CO₂-powered back sprayer at 207 kPa (Table 1). Plots were immediately cultivated in order to incorporate the fungicide into the soil.

The 2013 and 2014 field trials included the same fungicides as in 2012, although treatments focused on alternate applications of oxathiapiprolin and mefenoxam (Table 2). Applications were made at transplant on 23 April in 2013 and 24 April 2014, at first cultivation on 15 May in 2013 and 8 May in 2014, and at last cultivation on 28 June in 2013 and 27 June in 2014. Application of fungicides at transplant was performed in the transplant water only, and first and last cultivation applications were completed as described above. Oxathiapiprolin was applied through transplant water at 0.07 or 0.14 kg a.i./ha and at last
cultivation at 0.07 kg a.i./ha (Table 2). Mefenoxam was applied at transplant at 0.28 kg a.i./ha, and at first and last cultivation at 0.56 kg a.i./ha as described above (Table 2).

2.4 Data collection and statistical analysis. Black shank incidence was evaluated once every 2 weeks starting 5 to 6 weeks after transplanting tobacco plants to the field for a total of five disease evaluations. During each disease evaluation, each plant was assessed for disease and the number of diseased plants was counted in each plot. A plant was counted as diseased if the leaves were permanently wilted and had a black lesion at the base of the stem. The area under the disease progress curve (AUDPC) was calculated for all treatments in each field trial based on the five disease incidence evaluations. Levene’s test for homogeneity of variances was conducted to determine the possibility to combine AUDPC data from different field trials. AUDPC data were analyzed with PROC GLM of the Statistical Analysis System (SAS, version 9.2) and significant differences among treatment means were further separated according to Fisher’s least significant difference test (FLSD) at $P \leq 0.05$. Single-degree-of-freedom linear contrasts were calculated to compare the AUDPC of specific fungicide treatments.

3. Results

3.1 2012 field trials. Plant stand count per plot ranged from 17 to 27 plants per plot in Yadkin County and was exactly 22 plants per plot in the UCPRS. AUDPC values from each location were combined due to homogeneity of variance based on Levene’s Test. For field trials in the UCPRS and Yadkin County in 2012, fungicide treatment ($P<0.0001$) and field environment ($P<0.0001$) had a significant effect, while the interaction between
environment and fungicide treatment did not have a significant effect (P=0.6143) on AUDPC. Treatments of a single application of oxathiapiprolin at transplant, regardless of the rate used, significantly reduced the AUDPC when compared to the non-treated control (Fig. 1). Interestingly, no significant differences in AUDPC were observed between the oxathiapiprolin treatments with a single transplant water application at three different rates (Table 3). At UCPRS, disease development was delayed up to 93 days after transplant for at transplant only applications of oxathiapiprolin at 0.14 and 0.28 kg a.i./ha (Fig. 2A). In both 2012 field trials, transplant water applications of oxathiapiprolin at the highest rate of 0.28 kg a.i./ha, resulted in the lowest disease incidence at the final observation date (Fig. 2A and 3A). A single application of oxathiapiprolin in the transplant water at 0.14 or 0.28 kg a.i./ha caused a significantly reduction in AUDPC when compared to the three consecutive applications of mefenoxam (Fig. 1). In Yadkin County, black shank incidence in all transplant application treatments was reduced when compared to the non-treated control at each date of observation, with disease incidence at less than 20% 124 days after transplant (Fig. 3A).

Single applications of oxathiapiprolin at first and last cultivation, at a rate of 0.07 kg a.i./ha, significantly reduced AUDPC when compared to the non-treated control (Fig. 1). No significant difference in AUDPC was observed between first and last cultivation applications (Table 3). In both 2012 field trials, oxathiapiprolin applied as a single transplant-water application at 0.07 kg a.i./ha, resulted in the lowest black shank incidence at the final date of evaluation (Fig. 2B and 3B). At 77 days after transplant at the UCPRS, single 0.07 kg a.i./ha
applications of oxathiapiprolin held disease incidence below 10%, compared to the non-treated control where disease incidence climbed to 19.3% (Fig. 2B). At 93 days after transplant at the UCPRS, single 0.07 kg a.i./ha applications of oxathiapiprolin at transplant, first cultivation, or last cultivation held disease incidence to 23.8% or less depending on timing of application, while disease incidence of the non-treated control reached 52.3% (Fig. 2B). Black shank development was slower for single 0.07 kg a.i./ha applications of oxathiapiprolin at transplant, than first cultivation, and last cultivation (Fig. 2B). The AUDPC of the 0.07 kg a.i./ha transplant application was significantly lower (P=0.0056) than the last cultivation application (Table 3). In Yadkin County, a single application at last cultivation resulted in the highest disease incidence at each observation point (Fig. 3B).

Black shank incidence was highest in the fungicide treatment with three consecutive applications of mefenoxam at all observation dates in both field trials (Fig. 2C and 3C). At UCPRS, black shank incidence for the three application mefenoxam treatment increased dramatically between 93 and 122 days after transplant (Fig. 2C). Fungicide treatments with three consecutive applications of oxathiapiprolin at transplant, first cultivation, and last cultivation significantly reduced AUDPC when compared to the non-treated control (Fig. 1). Disease incidence for treatments with three applications of oxathiapiprolin was lower than the non-treated control at each observation date throughout the season (Fig. 2C and 3C). AUDPC values for the three treatments with three consecutive applications of oxathiapiprolin were significantly lower than the treatment of three consecutive applications of mefenoxam (Table 3). No significant difference was observed in AUDPC between the
treatments with three applications of oxathiapiprolin, regardless of method or rate of application at transplant (Table 3). At UCPRS, three applications of oxathiapiprolin delayed the initiation of disease until 93 days after transplant (Fig. 2C). Application of oxathiapiprolin via the transplant water at 0.14 kg a.i./ha, followed by two consecutive applications of mefenoxam at 0.56 kg a.i./ha at first and last cultivation, significantly lowered (P=0.0003) AUDPC in comparison to three consecutive applications of mefenoxam (Table 3).

3.2 2013 and 2014 field trials. Plant stand count per plot was exactly 22 plants in 2013 and ranged from 17 to 27 plants in 2014 at the UCPRS. AUDPC values from each year were combined due to homogeneity of variance based on Levene’s Test. In 2013 and 2014, fungicide treatment (P=0.0003) had a significant effect on black shank index, while field environment (P=0.4886) and the interaction between field environment and fungicide treatment (P=0.7690) did not have a significant effect. AUDPC values produced by three consecutive applications of mefenoxam at 0.28 kg a.i./ha at transplant, and at 0.56 kg a.i./ha at first and last cultivation were not significantly different from the non-treated control (Fig. 4). Treatments of oxathiapiprolin applied at transplant and last cultivation were also found to significantly decrease AUDPC when compared to the non-treated control (Fig. 4). Alternation of oxathiapiprolin and mefenoxam in all treatments significantly decreased AUDPC compared to the non-treated control (Fig. 4). Alternating treatments of applications of oxathiapiprolin at 0.07 or 0.14 kg a.i./ha at transplant, mefenoxam at 0.56 kg a.i./ha at first cultivation, and oxathiapiprolin at 0.07 kg a.i./ha at last cultivation significantly reduced
AUDPC (P=0.0022 and P=0.0031) when compared to the treatment with three consecutive applications of mefenoxam (Table 4).

In 2013, the fungicide treatment with oxathiapiprolin at 0.14 kg a.i./ha at transplant and at 0.07 kg a.i./ha at last cultivation, and mefenoxam at 0.56 kg a.i./ha at first cultivation was the only treatment that did not develop black shank symptoms until 141 days after transplant (Fig. 5A). In contrast, black shank was observed 114 days after transplant on tobacco treated with oxathiapiprolin at 0.07 kg a.i./ha at both transplant and last cultivation, and mefenoxam at 0.56 kg a.i./ha at first cultivation (Fig. 5A). In 2014, all fungicide treatments with oxathiapiprolin reduced disease incidence in each evaluation date when compared to the non-treated control and treatment with three applications of mefenoxam (Fig. 6A and 6B). In contrast to 2013, tobacco treated in 2014 with oxathiapiprolin at 0.14 kg a.i./ha at transplant and at 0.07 kg a.i./ha at last cultivation, and mefenoxam at 0.56 kg a.i./ha first cultivation, developed disease 92 days after transplant, whereas in all other treatments disease began 106 days after application (Fig. 6A).

All fungicide treatments with two applications made at transplant and last cultivation significantly reduced disease compared to the non-treated control (Fig. 4). No significant differences in AUDPC were observed between fungicide treatments with two applications of oxathiapiprolin and treatments alternating oxathiapiprolin at transplant and mefenoxam at last cultivation (Table 4). In 2013, tobacco treated with oxathiapiprolin applied at 0.07 kg a.i./ha at transplant and mefenoxam at 0.56 kg a.i./ha at layby, developed the highest disease incidence 141 days after transplant among all treatments that included applications only at
transplant and last cultivation (Fig. 5B). In contrast to 2013, in 2014 all treatments with applications only at transplant and last cultivation showed a decrease in black shank incidence, with initiation of disease not occurring until 106 days after transplanting, while disease initiation on non-treated control began 79 days after transplanting (Fig. 6B).

4. Discussion

The use of soil applied fungicides remains a significant tool for flue-cured tobacco growers to manage the black shank disease (Mila and Radcliff 2014). Unfortunately, prior to the 2015 growing season, only one fungicide, mefenoxam, was registered for use against black shank. Thus, there is great need for discovery and investigation of new fungicides active against the black shank pathogen, *P. nicotianae*. In NC field trials conducted over 3 years, oxathiapiprolin was a very effective fungicide for prevention of black shank of tobacco, caused by *P. nicotianae*. In 2012, applications of oxathiapiprolin to tobacco at transplant, first cultivation, and last cultivation were highly effective at control of black shank when compared to the non-treated control. Interestingly, method of fungicide application at transplant, either through a pre-plant tray application or a soil drench with the transplant water, resulted in the same level of disease control, suggesting that either method of application would be appropriate at tobacco transplant. Our results confirm those by Ji et al. (2014), who demonstrated no significant difference in black shank incidence between either a tray application or a transplant water application of oxathiapiprolin at the time of transplant.
Rate of fungicide application is another important factor to examine when evaluating a new fungicide. Oxathiapiprolin applied at 0.28 kg a.i./ha at transplant produced a significantly lower AUDPC value than when it was applied at 0.07 kg a.i./ha. Ji et al. (2014) had comparable results, with transplant applications of oxathiapiprolin at larger rates being more efficacious against black shank than lower ones. Timing of fungicide applications is also critical for effective control of a disease. The importance of early season fungicide applications at transplant and first cultivation for black shank control has been documented in a previous study with mefenoxam (Antonopoulos et al 2010). Antonopoulos et al., (2010) found that single layby (last cultivation) applications of mefenoxam were not adequate for black shank control, while applications at pre-plant, transplant, or at first cultivation provided significant control when compared to non-treated. We compared single applications of oxathiapiprolin in the transplant water, at first cultivation, and at last cultivation. Similar to results reported by Antonopoulos et al. (2010) on mefenoxam, in our field trials oxathiapiprolin applied at 0.07 kg a.i./ha at transplant significantly reduced AUDPC compared to the non-treated control and the application of oxathiapiprolin at 0.07 kg a.i./ha at last cultivation. Our findings suggest that like mefenoxam, application of oxathiapiprolin at transplant is the most efficacious timing of application for control of the black shank disease, although subsequent applications may be required to keep incidence below economic loss levels by harvest.

Since oxathiapiprolin has an extended half life and low leaching potential in soil profiles (Anonymous, 2015), we tested application strategies designed to minimize total
fungicide applications in the growing season. Thus, treatments in 2013 and 2014 investigated the efficacy of oxathiapiprolin applications at transplant and at last cultivation, with no fungicide application at first cultivation. Oxathiapiprolin applied at transplant and at last cultivation significantly reduced AUDPC when compared to the non-treated control, suggesting that application at first cultivation may not be necessary for black shank control. Given the low leaching potential and extended half-life of oxathiapiprolin, application at first cultivation may not be necessary in a fungicide management program for black shank and would eliminate any unnecessary oxathiapiprolin application, while saving producers on operation costs.

The decrease in efficacy of mefenoxam against black shank in our studies is troublesome. Mefenoxam at rates recommended for black shank management in NC, and applied at transplant, at first and last cultivation did not prevent disease development compared to the non-treated control or treatment with oxathiapiprolin, which suggests the possibility of fungicide resistance. Although, resistance to mefenoxam and metalaxyl has been reported in many pathosystems, resistance has never been reported for isolates of *P. nicotianae* from tobacco. Shew (1985) found a decrease in sensitivity of *P. nicotianae* isolates to metalaxyl after the fungicide was repeatedly used in tobacco fields, but no isolates were insensitive to the fungicide.

Lack of efficacy of mefenoxam also may be linked to repeated use of metalaxyl or mefenoxam in soils that can lead to increases in biodegradation of the fungicide by microorganisms present in the soil (Al-Sa’di et al 2008; Bailey and Coffey 1986; Droby and
Coffey 1991; Farrar et al 2002). In a study conducted by Droby and Coffey (1991), tobacco soils in NC with a history of metalaxyl use had greater microbial activity toward fungicide degradation when compared to tobacco soils with no previous use of metalaxyl.

Consequently, in NC tobacco soils with repeated metalaxyl use, 100% degradation of metalaxyl occurred 21 days after incubation, compared to 30% degradation of metalaxyl in NC tobacco soils without a history of repeated metalaxyl use (Droby and Coffee 1991). The decrease in efficacy of mefenoxam against black shank in our studies is troublesome, hence the identification and deployment of new efficacious fungicides against the black shank disease is well needed.

The probability of populations of *P. nicotianae* in tobacco fields to develop resistance to oxathiapiprolin is unknown. However, oxathiapiprolin is a fungicide with a single mode of action site in oomycetes which is a higher risk than a fungicide with multiple modes of action. The FRAC has classified oxathiapiprolin in medium to high risk for resistance development (Anonymous, 2016). To reduce the risk of selection pressure for resistant pathogen populations, fungicides with a different mode of action can be alternated throughout the growing season of a particular crop (Staub 1991). In 2012, oxathiapiprolin was applied at 0.14 kg a.i./ha at transplant, followed by applications of mefenoxam at 0.56 kg a.i./ha at first and last cultivation. This treatment scheme was not significantly different from use of three consecutive applications of oxathiapiprolin, leading us to conclude that alternation of oxathiapiprolin with mefenoxam would be highly effective for disease control. Thus, in our 2013 and 2014 field trials we examined efficacy of alternating oxathiapiprolin
with mefenoxam within a single growing season. Treatments alternating these two fungicides during the tobacco growing season reduced black shank significantly, when compared to the non-treated control. Based on our results, alternation of oxathiapiprolin with mefenoxam should be recommended to prolong efficacy, while reducing the resistance selection pressure on both fungicides.

In summary, results from our field trials suggest that treatments of oxathiapiprolin alone or alternating with mefenoxam are efficacious against black shank of tobacco and can be more effective than the standard mefenoxam treatment. However, more work is needed to determine the possible risk of resistance to oxathiapiprolin in the field. In addition, future tobacco field trials should be conducted that focus on the in season alternation of all currently registered fungicides for black shank use, that is mefenoxam, fluopicolide, and oxathiapiprolin. An effective black shank fungicide alternating program will help reduce resistance selection pressure in the pathogen population to each fungicide, helping to prolong and retain the efficacy of each individual fungicide.

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Literature Cited


Table 1 Fungicide treatments from tobacco field trials conducted in 2012 at the Upper Coastal Plain Research Station and in Yadkin County.

<table>
<thead>
<tr>
<th>Fungicide</th>
<th>Application</th>
<th>Rate (kg a.i./ha)</th>
<th>AUDPC&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>…</td>
<td>3024.8 &lt;sup&gt;a&lt;/sup&gt;</td>
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<td>1683.1 &lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Last Cultivation</td>
<td>0.07</td>
<td>2050.1 &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Tray Drench</td>
<td>0.035</td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>First Cultivation</td>
<td>0.07</td>
<td>389.9 &lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Last Cultivation</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Transplant Water</td>
<td>0.14</td>
<td>520.4 &lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>First Cultivation</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Last Cultivation</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Transplant Water</td>
<td>0.07</td>
<td>485.6 &lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>First Cultivation</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Last Cultivation</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>Transplant Water</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>First Cultivation</td>
<td>0.56</td>
<td>385.0 &lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>First Cultivation</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Last Cultivation</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Transplant Water</td>
<td>0.28</td>
<td></td>
</tr>
</tbody>
</table>

1580.6 **bc**

*Area under the disease progress curve (AUDPC) data in the columns are the mean of the two field studies. Means followed by the same letters are not significantly different according to Fisher’s least significant difference test (P ≤ 0.05).*
**Table 2** Fungicide treatments from tobacco field trials conducted at the Upper Coastal Plain Research Station in 2013 and 2014.

<table>
<thead>
<tr>
<th>Fungicide</th>
<th>Application</th>
<th>Rate (kg a.i./ha)</th>
<th>AUDPC&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-treated</td>
<td>...</td>
<td>...</td>
<td>788.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>Transplant Water</td>
<td>0.28</td>
<td>624.4&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>First Cultivation</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Last Cultivation</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Transplant Water</td>
<td>0.14</td>
<td>112.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>First Cultivation</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Last Cultivation</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Transplant Water</td>
<td>0.07</td>
<td>94.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>First Cultivation</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Last Cultivation</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Transplant Water</td>
<td>0.14</td>
<td>195.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Last Cultivation</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Transplant Water</td>
<td>0.07</td>
<td>105.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Last Cultivation</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Transplant Water</td>
<td>0.14</td>
<td>137.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>Last Cultivation</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>Oxathiapiprolin</td>
<td>Transplant Water</td>
<td>0.07</td>
<td>337.2&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mefenoxam</td>
<td>Last Cultivation</td>
<td>0.56</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Area under the disease progress curve (AUDPC) data in the columns are the mean of the two field studies. Means followed by the same letters are not significantly different according to Fisher’s least significant difference test ($P \leq 0.05$).
Table 3. Linear contrasts for area under the disease progress curve of specific fungicide treatments in 2012 field trials at the Upper Coastal Plain Research Station and in Yadkin County.

<table>
<thead>
<tr>
<th>Contrasts abc</th>
<th>AUDPC</th>
<th>F Value</th>
<th>P&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single Transplant Applications</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXA TPW 0.28 vs. OXA TPW 0.14 3</td>
<td>599.4 vs. 836.6</td>
<td>0.55</td>
<td>0.4597</td>
</tr>
<tr>
<td>OXA TPW 0.14 vs. OXA TPW 0.07</td>
<td>836.6 vs. 1140.0</td>
<td>0.90</td>
<td>0.3447</td>
</tr>
<tr>
<td>OXA TPW 0.28 vs. OXA TPW 0.07</td>
<td>599.4 vs. 1140.0</td>
<td>2.87</td>
<td>0.0943</td>
</tr>
<tr>
<td><strong>Timing of Application</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXA TPW 0.07 vs. OXA FC 0.07</td>
<td>1140.0 vs. 1683.1</td>
<td>2.90</td>
<td>0.0929</td>
</tr>
<tr>
<td>OXA FC 0.07 vs. OXA LC 0.07</td>
<td>1683.1 vs. 2050.1</td>
<td>1.32</td>
<td>0.2538</td>
</tr>
<tr>
<td>OXA TPW 0.07 vs. OXA LC 0.07</td>
<td>1140.0 vs. 2050.1</td>
<td>8.13</td>
<td>0.0056</td>
</tr>
<tr>
<td><strong>Three Consecutive Fungicide Applications</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXA TD 0.035, FC 0.07, LC 0.07 vs. OXA TPW 0.14, FC 0.07, LC 0.07</td>
<td>389.9 vs. 520.4</td>
<td>0.14</td>
<td>0.7046</td>
</tr>
<tr>
<td>OXA TD 0.035, FC 0.07, LC 0.07 vs. OXA TPW 0.07, FC 0.07, LC 0.07</td>
<td>389.9 vs. 485.6</td>
<td>0.07</td>
<td>0.7876</td>
</tr>
<tr>
<td>OXA TD 0.035, FC 0.07, LC 0.07 vs. OXA TPW 0.14; MEF FC 0.56, LC 0.56</td>
<td>389.9 vs. 385.0</td>
<td>0.02</td>
<td>0.8989</td>
</tr>
<tr>
<td>OXA TD 0.035, FC 0.07, LC 0.07 vs. MEF TPW 0.28, FC 0.56, LC 0.56</td>
<td>389.9 vs. 1580.6</td>
<td>13.71</td>
<td>0.0004</td>
</tr>
<tr>
<td>OXA TPW 0.14, FC 0.07, LC 0.07 vs. OXA TPW 0.07, FC 0.07, LC 0.07</td>
<td>520.4 vs. 485.6</td>
<td>0.01</td>
<td>0.9136</td>
</tr>
<tr>
<td>OXA TPW 0.14, FC 0.07, LC 0.07 vs. OXA TPW 0.14; MEF FC 0.56, LC 0.56</td>
<td>520.4 vs. 385.0</td>
<td>0.18</td>
<td>0.6726</td>
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<tr>
<td>Treatment Comparison</td>
<td>Area Under Disease Progress Curve</td>
<td>Significance</td>
<td>p-value</td>
</tr>
<tr>
<td>------------------------------------------------------------</td>
<td>-----------------------------------</td>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>OXA TPW 0.14, FC 0.07, LC 0.07 vs. MEF TPW 0.28, FC 0.56, LC 0.56</td>
<td>520.4 vs. 1580.6</td>
<td>11.03</td>
<td>0.0014</td>
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<tr>
<td>OXA TPW 0.07, FC 0.07, LC 0.07 vs. OXA TPW 0.14; MEF FC 0.56, LC 0.56</td>
<td>485.6 vs. 385.0</td>
<td>0.10</td>
<td>0.7534</td>
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<tr>
<td>OXA TPW 0.07, FC 0.07, LC 0.07 vs. MEF TPW 0.28, FC 0.56, LC 0.56</td>
<td>485.6 vs. 1580.6</td>
<td>11.77</td>
<td>0.0010</td>
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<tr>
<td>OXA TPW 0.14; MEF FC 0.56, LC 0.56 vs. MEF TPW 0.28, FC 0.56, LC 0.56</td>
<td>385.0 vs. 1580.6</td>
<td>14.03</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

\( ^a \) Contrasts were made between the area under the disease progress curve values of fungicide treatments (Trt) from Table 1. 
\( ^b \) Data from UCPRS and Yadkin County were analyzed together based on homogeneity of variance. 
\( ^c \) OXA = Oxathiapiprolin, MEF = Mefenoxam, TPW = Transplant Water, TD = Tray Drench, FC = First Cultivation, LC = Last Cultivation. Fungicide rates in kg a.i./ha.
Table 4. Linear contrasts for area under the disease progress curve of specific fungicide treatments in 2013 and 2014 at the Upper Coastal Plain Research Station.

<table>
<thead>
<tr>
<th>Contrastsabc</th>
<th>AUDPC</th>
<th>F Value</th>
<th>P&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three Fungicide Applications</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEF TPW 0.28, FC 0.56, LC 0.56 vs. OXA TPW 0.14, LC 0.07; MEF FC 0.56</td>
<td>624.4 vs. 112.3</td>
<td>9.58</td>
<td>0.0031</td>
</tr>
<tr>
<td>MEF TPW 0.28, FC 0.56, LC 0.56 vs. OXA TPW 0.07, LC 0.07; MEF FC 0.56</td>
<td>624.4 vs. 94.3</td>
<td>10.27</td>
<td>0.0022</td>
</tr>
<tr>
<td>OXA TPW 0.14, LC 0.07; MEF FC 0.56 vs. OXA TPW 0.07, LC 0.07; MEF FC 0.56</td>
<td>112.3 vs. 94.3</td>
<td>0.01</td>
<td>0.9135</td>
</tr>
<tr>
<td>Two Fungicide Applications</td>
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<td></td>
<td></td>
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<tr>
<td>OXA TPW 0.14, LC 0.07 vs. OXA TPW 0.07, LC 0.07</td>
<td>195.8 vs. 105.2</td>
<td>0.30</td>
<td>0.5861</td>
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<td>OXA TPW 0.14, LC 0.07 vs. OXA TPW 0.14; MEF LC 0.56</td>
<td>195.8 vs.137.2</td>
<td>0.13</td>
<td>0.7247</td>
</tr>
<tr>
<td>OXA TPW 0.14, LC 0.07 vs. OXA TPW 0.07; MEF LC 0.56</td>
<td>195.8 vs. 337.2</td>
<td>0.74</td>
<td>0.3963</td>
</tr>
<tr>
<td>OXA TPW 0.07, LC 0.07 vs. OXA TPW 0.14; MEF LC 0.56</td>
<td>105.2 vs. 137.2</td>
<td>0.04</td>
<td>0.8470</td>
</tr>
<tr>
<td>OXA TPW 0.07, LC 0.07 vs. OXA TPW 0.07; MEF LC 0.56</td>
<td>105.2 vs.337.2</td>
<td>1.97</td>
<td>0.1663</td>
</tr>
<tr>
<td>OXA TPW 0.14; MEF LC 0.56 vs. OXA TPW 0.07; MEF LC 0.56</td>
<td>137.2 vs. 337.2</td>
<td>1.46</td>
<td>0.2318</td>
</tr>
</tbody>
</table>

a Contrasts were made between the area under the disease progress curve values of fungicide treatments (Trt) from Table 1.
b Data from UCPRS and Yadkin County were analyzed together based on homogeneity of variance.
c OXA = Oxathiapiprolin, MEF = Mefenoxam, TPW = Transplant Water, TD = Tray Drench, FC = First Cultivation, LC = Last Cultivation. Fungicide rates in kg a.i./ha.
Fig. 1. Progression of black shank incidence throughout the season in 2012 at a field trial at the Upper Coastal Plain Research Station with (A) applications of oxathiapiprolin (Oxa) in
the transplant water at different rates, (B) single application of oxathiapiprolin at 0.007 kg a.i. /ha at three different growth stages, and (C) tobacco treated at three growth stages with oxathiapiprolin and mefenoxam (Mef) at different rates. Non-treated data repeated in each plot for comparison. Each point represents the mean of disease incidence of four replicate plots. The vertical bars represent the standard error of the mean.
Fig. 2. Progression of black shank incidence throughout the season in 2012 in Yadkin County with (A) applications of oxathiapiprolin (Oxa) in the transplant water at different rates, (B) single applications of oxathiapiprolin at 0.007 kg a.i./ha at three different growth stages, and
(C) treatments with three applications of oxathiapiprolin and mefenoxam (Mef) at different rates. Non-treated data repeated in each plot for comparison. Each point represents the mean of disease incidence of four replicate plots. The vertical bars represent the standard error of the mean.
Fig. 3. Progression of black shank incidence at the Upper Coastal Plain Research in 2013. Treatments were (A) three or (B) two applications of oxathiapiprolin (Oxa) and mefenoxam (Mef) at different rates and timing. Each point represents the mean of disease incidence of four replicate plots. The vertical bars represent the standard error of the mean.
Fig. 4. Progression of black shank incidence at the Upper Coastal Plain Research in 2014. Treatments were (A) three or (B) two applications of oxathiapiprolin (Oxa) and mefenoxam (Mef) at different rates and timing. Each point represents the mean of disease incidence of four replicate plots. The vertical bars represent the standard error of the mean.
CHAPTER IV. Assessing the resistance potential of *Phytophthora nicotianae* to oxathiapiprolin with laboratory mutants

Submitted to *Pest Management Science*.


First and third authors: Department of Plant Pathology, North Carolina State University, Campus Box 7616, Raleigh, NC 27612; and second author: DuPont Crop Protection, Discovery Research, Stine-Haskell Research Center, PO Box 30, Newark, DE 19714.
ABSTRACT

Black shank, caused by *Phytophthora nicotianae*, is a devastating disease of tobacco throughout the world. The newly-discovered fungicide, oxathiapiprolin has shown efficacy against oomycetes including *P. nicotianae*, but the potential for fungicide resistance is unknown. Production of oxathiapiprolin resistant *P. nicotianae* isolates was attempted through mass selection of zoospores, UV light mutagenesis, and mycelial adaptation through repeated culturing on oxathiapiprolin-amended medium. No resistant isolates were detected from mass selection of zoospores plated on selective oxathiapiprolin-amended medium. UV light mutagenesis of mycelium generated two isolates from tobacco plants, EdgeB7-M1 and YadA28-M1, with stable mycelial and sporangial resistance to oxathiapiprolin. Isolate EdgeB7-M1 remained pathogenic to tobacco, while YadA28-M1 was nonpathogenic. For mycelial adaptation, 48 colonies, 12 replicate colonies from four isolates, were transferred 15 times on oxathiapiprolin-amended medium. Twelve of the 48 colonies had a significant increase in mycelial insensitivity to oxathiapiprolin; however, the mycelial adaptation was not stable. Mycelial growth and sporangia production of the adapted isolates was significantly less compared to the wild-type. One isolate of the original four remained pathogenic to tobacco. In summary, the generation of isolates of *P. nicotianae* resistant to oxathiapiprolin was possible *in vitro*. In addition, constant exposure to oxathiapiprolin increased insensitivity, however fitness costs associated with insensitivity reduced the probability of isolate reproduction and survival.
1 INTRODUCTION

Black shank, caused by the devastating soil-inhabiting oomycete *Phytophthora nicotianae* van Breda de Hann (=*P. nicotianae* var. *nicotianae*), is a major disease of tobacco (*Nicotiana tabacum* L.) throughout the world and results in millions of dollars in annual loses each year (Lucas 1975; Shoemaker and Shew 1999; Mila and Radcliff 2014). In North Carolina alone, tobacco losses from black shank can total over $10 million annually (Mila and Radcliff 2014). Black shank was first introduced to the United States in Georgia in 1915. The pathogen eventually spread to North Carolina by 1931 possibly from out-of-state tobacco transplants infected with the pathogen (Lucas 1975; Shew and Lucas 1991).

Presently, there are three physiological races (0, 1, and 3) of *P. nicotianae* on tobacco in North Carolina, with race 0 and 1 the most frequently found across all tobacco growing areas of the state (Gallup and Shew 2010). Race 1 of *P. nicotianae* has the ability to infect and cause disease on tobacco cultivars that have the single resistance gene, *Ph* gene, which confirms immunity against race 0 (Mila and Radcliff 2014).

Management of the black shank disease is dependent upon an integrated approach with resistant tobacco cultivars, crop rotation, standard cultural practices, nematode control, and the use of fungicides (Lucas 1975; Shew and Lucas 1991; Mila and Radcliff 2014). Since the increase in prevalence of race 1 of *P. nicotianae* in North Carolina due to the release of the *Ph* resistance gene, the use of fungicides has become an important tool for black shank management. Currently, there are three systemic fungicides (mefenoxam,
fluopicolide, and oxathiapiprolin) registered for use on tobacco for black shank control in North Carolina.

Mefenoxam, released to the market in 1996, is the more active isomer of the fungicide metalaxyl, which was first introduced in 1977 (Kannwischer and Mitchell 1978; Erwin and Ribeiro 1996). Metalaxyl and mefenoxam are phenylamides, with activity against oomycetes, specifically against mycelial growth and sporangia production (Staub and Young 1980; Erwin and Ribeiro 1996; Hu et al. 2008). The mode of action (MOA) of mefenoxam and metalaxyl is the inhibition of ribosomal RNA synthesis, caused by an interaction of the fungicide with RNA polymerase I (Davidse et al. 1983). Overall, both metalaxyl and mefenoxam have proved to be effective fungicides against the black shank disease since their introduction (Kannwischer and Mitchell 1978; Reilly 1980; Staub and Young 1980; Csinos and Minton 1983; Antonopoulos et al. 2010). Resistance to these fungicides has been well documented in multiple pathosystems. Metalaxyl resistance was first observed by Davidse et al. (1981) in *P. infestans* strains from a Dutch potato field that was subjected to multiple applications of the fungicide. Shew (1985) demonstrated that in tobacco fields after repeated applications of metalaxyl a decrease in mycelial sensitivity of *P. nicotianae* to metalaxyl occurred in field isolates. Despite the development of phenylamide resistance in multiple oomycetes, including *P. nicotianae* from non-tobacco hosts, metalaxyl and mefenoxam have remained efficacious for use against black shank since their introduction, with no fungicide resistance reported.
Fluopicolide, a systemic fungicide belonging to the benzamides, has shown efficacy against several oomycetes, including *P. nicotianae*. The MOA is different from the phenylamide fungicides, and has been suggested to cause the perturbation of spectrin-like proteins (Toquin et al. 2006). In *P. capsici*, fluopicolide has shown efficacy against all growth stages, including mycelial growth, sporangia production, and zoospore germination and motility (Jackson et al. 2010). The risk of resistance to fluopicolide has been reported in a number of systems. Lu et al (2011) demonstrated that fluopicolide-resistant mutants of *P. capsici* could be produced with mass spore selection, a method that simulates the resistance naturally occurring in a field. All fluopicolide resistant *P. capsici* isolates had similar fitness levels to the wild type isolates, suggesting that no fitness cost was associated with the resistance mutation (Lu et al. 2011). Fluopicolide was registered for black shank control in the USA in 2015.

Oxathiapiproolin, released for use in the USA in 2016, is an example of the first chemistry of the new piperidinyl thiazole isoxazoline class of fungicides that has shown efficacy against multiple oomycete pathogens, including *P. nicotianae* (Ji et al. 2014; Pasteris et al. 2016; Bittner and Mila 2016). Bittner and Mila (2016) found that oxathiapiproolin was able to inhibit the mycelial growth, sporangia production, zoospore germination, and motility of zoospores of *P. nicotianae* from tobacco. Similar results were also reported with *P. capsici* from pepper (Ji and Csinos 2015). In both studies, sporangia production was the most sensitive life-cycle stage (Ji and Csinos 2015; Bittner and Mila 2016). Pasteris et al. (2016), found that oxathiapiproolin binds strongly to the oxysterol
binding protein (OSBP). This is a novel site of action different from all other previously discovered fungicides. The function of OSBP in oomycetes has yet to be determined. Isolates of *P. capsici* resistant to oxathiapiprolin were produced by irradiating zoospores of *P. capsici* with ultraviolet light and then selecting on oxathiapiprolin-amended media (Pasteris et al., 2016). Oxathiapiprolin-resistant isolates of *P. capsici* then were sequenced to determine mutations within their genome. Pasteris et al. (2016) found that all resistant mutants had a single nucleotide polymorphism in the OSBP gene. Although the possible mechanism of resistance to oxathiapiprolin in *P. capsici* was described, resistant isolates were not further characterized to determine the possible fitness effects of resistance (Pasteris et al., 2016). Currently, it is unknown if *P. nicotianae* isolates from tobacco are at risk for resistance to oxathiapiprolin.

The objectives of this study were to (i) produce isolates of *P. nicotianae* resistant to oxathiapiprolin through mass zoospore selection, UV light mutagenesis, and mycelial adaptation, (ii) determine stability of resistance to oxathiapiprolin, (iii) determine fitness costs associated with resistance to oxathiapiprolin, and (iv) determine pathogenicity of any resistant isolates to flue-cured tobacco.

2 MATERIALS AND METHODS

2.1 Isolates

For these studies, two race 0 (EdgeB7 and EdgeA12) and two race 1 (YadA3 and YadA28) isolates of *P. nicotianae* from tobacco were selected from the A. L. Mila collection,
North Carolina State University. All isolates were sensitive to oxathiapiprolin as previously determined (Bittner and Mila 2016).

2.2 Fungicide

Oxathiapiprolin (DPX-QGU42, DuPont™ Zorvec™ Enicide™) formulated as DPX-QGU42, was provided by DuPont, Wilmington, DE. Stock solutions of oxathiapiprolin were prepared in sterilized deionized water and stored at 4°C in the dark to preserve fungicidal activity. For amended medium, stock solutions of the fungicide were added to the medium when it had cooled to roughly 55°C. Media amended with oxathiapiprolin was stored in the dark at 4°C.

2.3 Production of resistant isolates through mass selection from zoospores

P. nicotianae isolates EdgeB7 and YadA3, with prolific sporangia production, were selected. Zoospores were produced by a method described by McCorkle et al. (2013). The concentration of zoospore suspensions was adjusted by dilution to 1.5 x 10^5 ml with the aid of a hemacytometer. Zoospores were screened for resistance by plating 0.5 ml of the zoospore suspension onto V8 agar (V8A; V8 juice, 200 ml; CaCO₃, 3 g; agar, 17 g; and deionized water, 800 ml) in Petri plates (90 mm x 15 mm) amended with oxathiapiprolin at 0.2 µg a.i./ml. This rate was determined to be ten times the minimum inhibition rate for mycelial growth of P. nicotianae (data not shown). A 0.5 ml volume of the zoospore suspensions, 1.5 x 10^5 ml concentration, were spread evenly over each Petri dish evenly with a sterile L-shaped glass rod. Non-amended Petri plates of V8A were used as a control. After the addition of zoospore suspensions, Petri plates were incubated in the dark at 28°C for 14
days. After the 14-day incubation period, Petri plates were examined for *P. nicotianae* colony formation.

2.4 Production of resistant isolates through UV mutagenesis

*P. nicotianae* isolates EdgeA12, EdgeB7, YadA3, and YadA28 were grown on V8 agar at 28°C until the medium in the Petri plate (90 mm x 15 mm) became completely colonized. Ultraviolet (UV) light mutagenesis was performed under a portable UV lamp (Mineralight Lamp; San Gabriel, CA) at a wavelength of 254 nm. A total of eleven Petri plates of each isolate were exposed to UV light without lids at a 10 cm distance for 4 (five plates) and 5 (six plates) min. After irradiation, cultures were immediately transferred to an incubator at 28°C for 1 hr in the dark to minimize potential photorepair. Forty agar disks (6-mm-diameter) were excised with a sterile cork borer from each of the forty-four total irradiated plates, and five disks were placed inverted on each Petri plate of V8A amended with oxathiapiprolin at 0.2 µg a.i./ml. A single agar disk (6-mm-diameter) also was excised from each irradiated culture and placed on a non-amended V8A plate. Plates were then incubated in the dark at 28°C and examined at 5-day intervals for colony growth over a period of 30 days. Mycelial colonies that formed on oxathiapiprolin amended V8A were excised and transferred to V8A amended with oxathiapiprolin at 0.2 µg a.i./ml. Isolates were then again excised from the actively growing region and transferred onto non-amended V8A medium for future use.

UV-mutant and corresponding wild-type (WT) isolates were transferred to oxathiapiprolin amended V8A at 0.2, 2.0, 20, 200, and 400 µg a.i./ml. A non-amended V8A
control also was included. Three replicate plates per isolate were used for each concentration. Petri dishes were incubated in the dark at 28°C for 96 hrs, which was the time required for mycelia to reach the edge of the Petri dish for the non-amended control. Colony diameters were then measured in two perpendicular directions along the axis of greatest growth and averaged after the diameter of the original disk was subtracted. The experiment was conducted twice.

Sporangia production of *P. nicotianae* isolates was examined for sensitivity to oxathiapiprolin with a method similar to Hu et al., (2007). Sporangia were produced in a liquid medium of 5% sterile soil extract (SSE). The medium was prepared by mixing 50 grams of sterile sandy-loam soil with 1 L of deionized water. The solution was incubated at room temperature (23-25°C) for 72 h. After incubation, SSE was sterilized in an autoclave, filtered through filter paper (Fisher Brand Quantitative 5 grade, Pittsburgh, PA), and sterilized a final time before use. Three mycelial disks, 5 mm in diameter, were cut from the edge of 4 to 5 day-old cultures grown on V8A and placed into a 60mm x 15mm Petri dish with the mycelia side facing up. Petri dishes were then filled with 8 ml of SSE with or without oxathiapiprolin at the following concentrations: 0, 0.005, 0.05, 0.5, and 1 µg a.i./ml, with just enough SSE to reach the top of each mycelia plug. Petri dishes were incubated under a constant fluorescent light source at room temperature for a period of 10 h, after which the number of sporangia produced along the outside edge of each mycelia disk was counted under a microscope at 100X. Three replicate Petri dishes were used for each treatment, for a total of nine mycelial disks. The experiments were repeated once.
2.5 Stability of UV-mutant isolates

Mutant isolates of *P. nicotianae* recovered from the UV mutagenesis procedure were transferred to V8A Petri plates. Mycelial disks (6 mm in diameter) were cut with a sterile cork borer from the edge of 4 to 5 day-old cultures grown on V8A at in the dark at 28°C. Disks were transferred to non-amended V8A medium. A total of five successive transfers at 4-day intervals were made for each isolate onto non-amended V8A medium. After the fifth transfer, 6-mm-diameter mycelial disks were cut with a sterile cork borer from the edge of the cultures and transferred to V8A medium amended with oxathiapiprolin at 0.2 µg a.i./ml. Three replicate Petri dishes were used for each isolate. The mycelial colony diameter was then measured in two perpendicular directions in each Petri dish after 4 days. The two mycelial colony diameters for each dish were averaged after the diameter of the original 6-mm-diameter disk was subtracted. The experiments were repeated once.

2.6 Screening of the OSBP gene in UV-mutant isolates

Whole genomic DNA of *P. nicotianae* from hyphal mats of isolates EdgeB7-WT, EdgeB7-M1, YadA28-WT, YadA28-M1 was obtained. Hyphal mats were produced by placing 6-mm-diameter mycelial disks from stock V8A cultures in Petri dishes containing potato dextrose broth (PDB; 24 g PDB and 1 L deionized water [Becton, Dickinson and Company, Franklin Lakes, NJ) for 7 days in an incubator in the dark at 28°C. The original agar disk was removed aseptically then the hyphal mat was collected by pouring the broth culture into a sterile Buchner funnel with filter paper (Fisher Brand Quantitative 5 grade, Pittsburgh, PA) to separate the hyphal mat from the liquid by vacuum filtration. Mats were
collected aseptically and placed into sterile 1.5 ml microcentrifuge tubes for storage at -20°C for 24 hrs. Tubes were lyophilized at -50°C for 96 hrs in a FreeZone 4.5 lyophilizer (Labconco, Kansas City, MO). Lyophilized tissue was then ground using a sterile pestle and DNA was extracted with a PUREGENE DNA Isolation Kit (Qiagen, Valencia, CA) per a protocol optimized for Phytophthora (Lookabaugh, et al., 2015).

PCR amplification of the OSBP gene was performed with primer pair S4633 (5’ GCGCTTCAAGTATGTCATATC) and S4635 (5’ GTGTCTTCGAGCTTGAG) and Q5 High-Fidelity Polymerase (New England BioLabs, Ipswich, MA). Thermal cycling conditions consisted of an initial denaturing of 98°C for 30sec; 36 cycles of denaturation at 98°C for 10 s, annealing at 62°C for 15 s, and extension at 72°C for 15 s and a final cycle of extension at 72°C for 2 min. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and submitted for Sanger sequencing at the DuPont Industrial Biosciences sequencing center (Wilmington, DE).

2.7 Production of resistant isolates through mycelial adaptation

Twelve colonized agar disks, 6 mm in diameter, were excised from the margin of an actively growing culture of each isolate of P. nicotianae. Three mycelial disks were transferred to each of four V8A Petri dishes (90 mm x 15 mm) amended with oxathiapiprolin at either 0.006 (isolates EdgeB7 and EdgeA12) or 0.007 (isolates YadA3 and YadA28) μg a.i./ml. The concentration of oxathiapiprolin used in the experiment was the effective 70% inhibition concentration (EC70) of the mycelial growth for each isolate as determined previously (Bittner and Mila, 2016). A single, 6-mm-diameter mycelial disk of each isolate
also was placed, mycelial side down, in the center of a non-amended V8A Petri dish. When the mycelial growth on the non-amended V8A dish was just about to reach the edge of the dish, the mycelial diameter for each of the twelve colonies on amended media was measured in two perpendicular directions, subtracting the diameter of the plug. The twelve replicate colonies from each of the four isolates were labeled A to L.

A 6-mm-diameter disk was cut arbitrarily from the edge of each of the twelve mycelial colonies of each isolate and transferred inverted onto V8A amended with the same concentration of oxathiapiprolin, for a total of 15 subcultures. Following 15 subcultures, the colony diameters of the first and fifteenth subculture were compared for each of the 12 replicate colonies. Colonies were selected for further analysis if the diameter of the mycelial growth increased by two-fold or greater between the first and fifteenth subculture. The effective concentration to inhibit 50% (EC$_{50}$) of the mycelial growth was calculated for each selected isolate, wild-type, and isolate sub-cultured on non-amended V8A.

To calculate the EC$_{50}$, mycelial disks were cut with a sterile cork borer from the edge of 4 to 5 day-old cultures grown on V8A medium at 28°C in the dark. Disks were transferred to the center of a V8A medium Petri dish (90 mm x 15 mm) amended with oxathiapiprolin at the following concentrations: 0.001, 0.002, 0.003, 0.005, and 0.01 µg a.i./ml. A non-amended V8A medium was also included as a control. There were three replicate Petri dishes per treatment for each selected isolate. Petri dishes were incubated at 28°C in darkness until the mycelium reached the edge of the dish in the non-amended media, which occurred 4 to 5 days after the initiation of the experiment. Colony diameters were then
measured in two perpendicular directions for each dish. The two measurements were averaged, after the mycelial disk diameter was subtracted. Experiments were conducted twice. Isolates with a significant larger EC50 value than the wild-type based on Fisher’s LSD Test were selected for further study.

2.8 Stability of Mycelial Adaptation

Mycelial disks (6 mm in diameter) were cut with a sterile cork borer from the edge of 4 to 5 day-old cultures of selected adapted isolates grown on V8A in the dark at 28°C. Disks then were transferred successively 15 times to the center of non-amended V8A Petri dishes. Each successive transfer to the next dish of non-amended medium was made after 4 to 5 days when the mycelial colony was about to reach the edge of the Petri dish. After 15 transfers, each isolate was tested to determine EC50 for mycelial growth, as previously described. Experiments were repeated once.

2.9 Fitness of UV-mutated and mycelial-adapted isolates of P. nicotianae

Mycelial growth and sporangia production on non-amended media was measured for the UV-mutated, selected-mycelial adapted, WT, and WT/V8A isolates. Mycelial disks (6 mm in diameter) were cut with a sterile cork borer from the edge of 4 to 5 day-old cultures grown on V8A in the dark at 28°C. Disks were transferred to non-amended V8A Petri dishes. Three replicate Petri dishes were used for each isolate. Again cultures were incubated at 28°C in darkness for 4 to 5 days until the mycelia of the WT isolates had just reached the edge of the Petri dish. Colony diameters of each isolate were then measured in
two perpendicular directions for each dish, mycelial disk diameters were subtracted, and the
two measurements were averaged. Experiments were conducted twice.

Sporangia production was assessed as described previously. Three 5-mm-diameter
mycelial disks were cut from the edge of 4 to 5 day-old cultures grown on V8A and placed
into 60mm x 15mm Petri dishes with the mycelia side up. Dishes were then filled with 8 ml
of SSE medium until the liquid just reached the top of the mycelia plug. Petri dishes were
incubated under constant light at room temperature for 10 h, after which the number of
sporangia produced along the edges of each mycelia disk were counted with a microscope.
There were three replicate Petri dishes and experiments were conducted twice.

2.10 Pathogenicity of UV-mutated and mycelial-adapted isolates of *P. nicotianae*

Pathogenicity of UV-mutated, mycelial-adapted WT, and WT/T15 isolates of *P. nicotianae* was examined in greenhouse trials with a stem inoculation method (Csinos, 1999).
In the greenhouse, seeds of flue-cured cultivar K326 susceptible to black shank were sown
directly onto a potting mix (Fafard 2 Mix, Conrad Fafard, Inc., Agawam, MA) in 15-cm-
diameter plastic pots. Tobacco seedlings were transferred after 4 weeks into 10- cm-diameter
clay pots filled with a 1:1:1 mixture of the potting mix, steam pasteurized sandy-loam soil,
and coarse builder’s sand. Tobacco plants were watered daily and fertilized with a liquid
solution once a week with 24-8-16 Miracle-Gro (The Scotts Miracle Gro Company,
Marysville, OH) at 100 mg/L N. Greenhouse temperatures were maintained between 28°C
during the day and 25°C during the night. Plants were inoculated at a height of at least 20
to 25 cm, approximately 8 weeks after transplanting. Rounded wooden toothpicks, 65 mm
by 2 mm in length, were autoclaved in a 5% V8 broth for 30 min at 121°C once a day for three consecutive days. Toothpicks then were halved aseptically and placed on the surface of fresh V8A plates. A mycelial disk (6 mm in diameter) of each isolate was placed in the center of a separate V8A plate and incubated at 28°C in the dark. After 7 days, the colonized toothpicks were removed from the Petri dishes and inserted into the tobacco stems, 3 to 4 cm above the soil line. As a control, non-colonized toothpicks were inserted into the stem of control plants. Plants were examined for the presence of a lesion over 10 consecutive days. Four replicate plants were used for each isolate, and experiments were repeated once. Plants were arranged in a randomized complete block design. Isolates were determined to be pathogenic if a necrotic lesion was produced around the location where the toothpick was inserted into the tobacco stem.

2.11 Statistical Analysis

Percentage of mycelial growth inhibition was calculated relative to the non-amended control and converted to a probability scale from a probit table. Concentration of oxathiapiprolin was expressed on a logarithmic scale. A log-probit regression analysis was conducted for each isolate (SAS 9.2; SAS Institute, Cary, NC). EC$_{50}$ values were interpolated from the regression equations. Data from the experiments comparing mycelial growth diameter and sporangia production between wild-type and mutant isolates were analyzed with analysis of variance (PROC ANOVA; SAS, version 9.2, Cary, NC) and means were separated according to Fisher’s least significant difference at $P \leq 0.05$. Comparison between EC$_{50}$ values of isolates was analyzed by analysis of variance (PROC ANOVA; SAS,
version 9.2), where the mean of the six replicate EC_{50} values for each isolate were separated according to Fisher’s least significant difference at P ≤ 0.05.

3 RESULTS

3.1 Production of resistant isolates through mass selection from zoospores

No colonies of *P. nicotianae* were produced on V8A medium amended at 0.2 µg a.i./ml oxathiapiprolin when 27.8 x 10^6 motile zoospores of isolate EdgeB7 (race 0), or 18.4 x 10^6 motile zoospores of isolate YadA3 (race 1) were cultured. Colonies readily developed when zoospores of isolates, EdgeB7 and YadA3, were cultured on non-amended V8A.

3.2 Production of resistant isolates through UV Mutagenesis

Following UV mutagenesis, two mycelial colonies, one from EdgeB7 (EdgeB7-M1) and one from YadA28 (YadA28-M1), were found from a total of 1760 mycelial disks that were exposed to UV light at 254 nm. The two colonies were not apparent until at least 10 days after incubation. However, subsequent growth of the mutant isolates on non-amended V8A was normal with no evidence of disrupted colony morphology or reduced growth rates compared to non-exposed cultures.

Mycelial growth of isolate EdgeB7-M1 was inhibited 54.7% on V8A media amended with 0.2 µg a.i./ml oxathiapiprolin, ten times the minimum inhibition concentration for the wild-type isolate (Table 1). EdgeB7-M1 was inhibited at 92.8 and 90.0% respectively on V8A media amended with 2 and 20 µg a.i./ml oxathiapiprolin (Table 1) and did not grow on V8A medium amended with oxathiapiprolin at 200 and 400 µg a.i./ml. In comparison,
mycelial growth of isolate YadA28-M1 was inhibited only 10.5% on media with 0.2 µg a.i./ml oxathiapiprolin (Table 1). Mycelial growth of YadA28-M1 was inhibited 32.3% at 400 µg a.i./ml oxathiapiprolin a concentration 20,000 times greater than the minimal inhibition concentration for the wild-type isolate (Table 1). EdgeB7-WT and YadA28-WT, the wild-type isolates, were inhibited 100% at 0.2 µg a.i./ml and concentrations of oxathiapiprolin (Table 1).

Sporangia production of UV-mutant isolates of EdgeB7-M1 and YadA28-M1 were less sensitivity to oxathiapiprolin when compared to the WT isolates (Table 2). For isolate EdgeB7-M1, sporangia were not produced at oxathiapiprolin concentrations of 0.5 µg a.i./ml and higher, while sporangia production for YadA28-M1 occurred at all concentrations of oxathiapiprolin tested (Table 2). Sporangia were not produced at any concentration of oxathiapiprolin tested for both wild-type isolates (Table 2).

3.3 Stability of UV mutant isolates

After five consecutive transfers on non-amended V8A media, there was no significant decrease in mycelial colony diameter between the original UV mutant isolates and the transferred isolates when grown on V8A amended with oxathiapiprolin at 0.2 µg a.i./ml (Table 3). Furthermore, storage of the mutant isolates in test tubes caused no decrease in insensitivity to oxathiapiprolin (data not shown).

3.4 Screening of the OSBP gene in UV-mutant isolates

The OSBP gene was sequenced in UV-mutants YadA28-M1 and EdgeB7-M1, as well as their corresponding WT isolates. Mutant isolate YadA28-M1 showed a single G/T single
nucleotide polymorphism (SNP) in the OSBP domain, which corresponds to G686V amino acid change from glycine (GGG) to valine (GTG) (Fig. 1A). The mutation in EdgeB7-M1 was downstream of the mutation in YadA28 with a six base pair deletion in the OSBP gene, corresponding to a two amino acid deletion, glycine (734) and phenylalanine (735) respectively (Fig. 1B).

3.5 Mycelial adaptation to oxathiapiprolin

Overall, mycelial growth of all isolates significantly increased from the first to the fifteenth transfer on oxathiapiprolin-amended media (Fig. 2). Replicate mycelial colonies of each isolate (A-L) were chosen for further analysis if a twofold or greater increase in mycelial colony diameter was observed between first and fifteenth subculture (Data not shown). Of the twelve replicate colonies for each isolate, three were selected from EdgeA12, four from EdgeB7, five from YadA3, and eight from YadA28 (Table 4). The effective concentration of oxathiapiprolin to inhibit 50% (EC$_{50}$) of the mycelial growth was determined for the selected mycelial colonies (A-L) from each isolate, the original WT isolates, and the wild-type isolates transferred fifteen consecutive times on non-amended V8A (WT/T15) (Table 4).

Of the twenty total selected adapted mycelial colonies, twelve were found to have a significantly larger EC$_{50}$ value for mycelial growth than the original wild-type isolate (Table 4). Significant adaptation of mycelial growth to oxathiapiprolin occurred most frequently with isolate YadA28, with seven of the twelve replicate mycelial colonies having significantly increased EC$_{50}$ values. The EC$_{50}$ values of mycelial growth for EdgeA12-
WT/T15 and YadA3-WT/T15 were found to be significantly smaller than the original wild-type values, however no significant difference was observed between values of the other two isolates (Table 4).

3.6 Stability of mycelial adaptation

The stability of the mycelial adaptation to oxathiapiprolin was examined for the twelve colonies with a significant increase in EC\textsubscript{50} mycelial growth values (Table 5). After fifteen repeated transfers of each selected isolate on non-amended V8A, eight of the twelve adapted isolates (66.6\%) had a significant decrease in mycelial insensitivity to oxathiapiprolin when compared to the original EC\textsubscript{50} values of the adapted isolates (Table 5). The largest change in EC\textsubscript{50} values for mycelial growth was isolate YadA3-L, which was decreased from 0.0112 to 0.0031 μg a.i./ml (Table 5). Adapted isolates EdgeB7-I, YadA28-B, YadA28-E, YadA28-J had no significant change in mycelial insensitivity to oxathiapiprolin after repeated transfer on non-amended V8A.

3.7 Fitness of P. nicotianae UV-mutated and mycelial-adapted isolates

No significant differences were observed between the mycelial growth of EdgeB7-WT and EdgeB7-M1, or the growth of YadA28-WT and YadA28-M1 (Table 6). A significant difference between EdgeB7-WT and EdgeB7-M1 in the number of sporangia produced was observed, with the WT isolate producing significantly more sporangia than the UV mutant (Table 6). In contrast, no significant difference in sporangia production was observed between YadA28-WT and YadA28-M1 (Table 6).
Overall, mycelial growth and sporangia production were reduced in multiple oxathiapiprolin adapted isolates when compared to the WT isolates. Sporangia production in EdgeA12-WT was significantly greater than in EdgeA12-WT/T15 and EdgeA12-L, while no significant difference in mycelial colony diameter was observed between these three isolates (Table 6). No significant difference in mycelial growth or sporangia production was observed between EdgeB7-WT and EdgeB7-WT/T15. Mycelial growth and sporangia production of all adapted EdgeB7 isolates was significantly lower than EdgeB7-WT and EdgeB7-WT/T15 (Table 6). No sporangia were produced by EdgeB7-A (Table 6).

The mycelial growth and sporangia production of YadA3-L was reduced significantly when compared to YadA3-WT (Table 6). Sporangia production of YadA3-WT/T15 was also lower than YadA3-WT, but significantly higher than YadA3-L, which did not produce sporangia (Table 6). Mycelial growth and sporangia production were not statistically different from each other for YadA28-WT and YadA28-WT/T15. Mycelial colony diameter of all oxathiapiprolin-adapted colonies, except for YadA28-J, were significantly less than the mycelial colony diameter of YadA28-WT (Table 6). YadA28-WT and YadA28-WT/T15 produced significantly more sporangia than all oxathiapiprolin-adapted colonies (Table 6). The ability to produce sporangia was lost in four out of the seven oxathiapiprolin-adapted YadA28 colonies (Table 6).

3.8 Pathogenicity of *P. nicotianae* UV-mutated and mycelial-adapted isolates

The four WT isolates of *P. nicotianae* were pathogenic to tobacco (Table 6). After 15 culture transfers, the four WT/T15 isolates still were pathogenic to tobacco. EdgeA12-E was
the only mycelial-adapted isolate found to produce a lesion on the tobacco stems (Table 6). UV-mutated isolate EdgeB7-M1, was, whereas YadA28-M1 was non pathogenic on tobacco (Table 6).

4 DISCUSSION

Oxathiapiprolin, a novel single MOA fungicide, has shown an extremely high efficacy against multiple oomycetes (Ji and Csinos 2015; Pasteris et al. 2016; Bittner and Mila 2016). Since the release of systemic, single MOA fungicides, field resistance to fungicides in oomycete and fungal plant pathogens has become increasingly common. Recently, Pasteris et al (2016) found that single base pair changes in the binding site of the oxysterol binding protein can confer resistance, making oxathiapiprolin a prime candidate for a high risk of resistance development. We report the first comprehensive study of the potential for oxathiapiprolin resistance for any oomycete pathogen. Resistance to oxathiapiprolin was produced through UV mutagenesis and mycelial adaptation. Interestingly, mass selection of zoospores on a fungicide-amended medium, a technique that represents naturally-occurring resistance in the field (Lu et al. 2010; Lu et al. 2011), did not produce resistant isolates. We speculate that the probability of generation of a zoospore with natural oxathiapiprolin resistance from P. nicotianae is low. Although it is unknown what the actual frequency of resistance to the fungicide is under field conditions, our results suggest a frequency of less than 1 in 20 x 10^6 zoosores.

Our UV-mutagenesis experiments demonstrated that P. nicotianae can develop resistance to oxathiapiprolin after mycelium irradiation. UV-mutants had different
sensitivity to oxathiapiprolin. Mycelial growth and sporangia production of isolate YadA28-M1 was more insensitive to oxathiapiprolin at much higher concentrations than isolate EdgeB7-M1. Interestingly, sporangia production of YadA28-M1, like mycelial growth, was not completely inhibited by the highest oxathiapiprolin concentrations tested.

Our results on the mycelial growth and sporangia production of both UV-mutants suggested two different, resistant phenotypes, which led us to further investigate the OSBP gene in the two mutants. Pasteris et al. (2016) identified the OSBP gene as target site of oxathiapiprolin activity in oomycetes. Based on our findings from sequencing the OSBP gene, the resistance phenotypes observed appear to result from two unique mutation events in the OSBP gene of *P. nicotianae*. YadA28-M1 was determined to have a SNP in the OSBP gene that resulted in a single amino acid change. A similar pattern with nine different, single amino acid changes, due to SNPs, in the OSBP gene was found in mutant isolates of *P. capsici* (Pasteris et al., 2016). The development of fungicide resistance from point mutations in the target gene has been observed in many different systems, including carboxylic acid amide (CAA) fungicides. Resistance to CAA fungicides has been associated with amino acid substitutions in cellulose synthase (CesA3) gene in pathogens such as *P. infestans* and *P. melonis* (Blum et al. 2010; Chen et al. 2012). Interestingly, *P. nicotianae* mutant EdgeB7-M1 produced a resistance genotype, a two amino acid deletion, corresponding to a six base pair deletion that has not been observed in oxathiapiprolin previously (Pasteris et al., 2016). The two amino acid deletion conferring fungicide resistance is not a phenomenon unique to isolate EdgeB7-M1. A two amino acid deletion, ΔY459/G460, which confers resistance to
DMI fungicides has also been found in the Cyp51 gene in *Mycosphaerella graminicola* (Leroux and Walker 2010). The unique mutations observed in the OSBP gene, enforce the observed phenotypic differences of the resistant mutants in the presence of oxathiapiprolin. Our study suggests that the single amino acid change based on the SNP, results in greater insensitivity to oxathiapiprolin than the two amino acid deletion event.

The use of mycelial adaptation techniques to produce fungicide resistant isolates has been studied in numerous plant pathogens with varying rates of success (Bruin and Edgington 1981; Young et al. 2001; Stein and Kirk 2004; Luo and Schnabel 2007). The mycelial adaptation of multiple *Phytophthora* and *Pythium* species to metalaxyl has been successful, yielding resistant isolates in several studies (Bruin and Edgington 1981; Young et al. 2001). Repeated transfer of mycelial colonies of *P. nicotianae* on oxathiapiprolin amended V8A resulted in isolates with an increased insensitivity to the fungicide. However, oxathiapiprolin insensitivity of mycelial adapted isolates was much less than our UV-mutated isolates.

Investigation of the stability of oxathiapiprolin resistance in UV mutants revealed that resistance was stable. Our results confirm findings in other systems where fungicide resistance produced from either UV light or a chemical mutagen is stable (Joseph and Coffey 1984; Yuan et al. 2006; Zhu et al. 2008). The stability of the oxathiapiprolin resistance in the UV-mutant isolates complements our findings of specific mutations that occurred in the OSBP gene. In contrast, the increased oxathiapiprolin insensitivity of mycelial adapted colonies was not stable after repeated mycelial transfers on non-amended media. Bruin and
Edgington (1981) also observed a loss of metalaxyl resistance (obtained through mycelial adaptation) in *Pythium* species and *Phytophthora capsici* after 12 transfers on non-amended media. Based on our results, we hypothesize that increased insensitive to oxathiapiprolin in mycelial adapted isolates was not the result of mutation in the OBSP gene. Instead we suggest that increased insensitivity may be related to reduced uptake of the fungicide, detoxification of the fungicide, or over-production of the target protein (Young et al. 2001).

The fitness of resistant isolates is an important factor when evaluating the risk a fungicides resistance, with selection of resistant isolates key to development of resistant populations in a pathogen. UV-generated, resistant mutants of *P. nicotianae* showed no significant difference in mycelial growth, when compared to the WT isolates. Sporangia production was significantly reduced in the UV-mutant EdgeB7-M1, but not in YadA28-M1. These results, in combination with sequencing data, suggest that a reduction of sporangia production in isolate EdgeB7-M1 may be related to a different resistance mutation when compared to YadA28-M1. However, we hypothesize that less sporangia production in EdgeB7-M1 is not the result of the unique OSBP mutation, but more likely the result of other non-OSBP mutations in the genome caused by UV-light irradiation. In our mycelial adapted isolates, we believe that the increase in oxathiapiprolin insensitivity may have caused the reduction in mycelial growth and sporangia production.

The ability of fungicide resistant isolates to infect and cause disease on host plants is critical to survival and establishment in nature. Our results suggest that oxathiapiprolin resistance, produced from the single amino acid change, may cause a loss of pathogenicity in
*P. nicotianae.* However, because of the random nature of UV mutagenesis, other mutations may have occurred in the isolate, causing a loss of pathogenicity. More isolates with this specific mutation to the OSBP must be screened before a determination can be made. In contrast, no loss in pathogenicity of flumorph-resistant, UV-mutant isolates of *P. infestans* was found (Yuan et al., 2006). In mycelial adapted isolates, all but one isolate were found to be non-pathogenic to tobacco. In contrast, WT isolates transferred on non-amended V8A (WT/T15) were still pathogenic on tobacco. Studies by Bruin and Edgington (1981), suggest that mycelial adaptation to fungicides does not cause a loss in pathogenicity, although the results were produced with a different *Phytophthora* species and fungicide. In their study, mycelial adapted, metalaxyl-resistant isolates of *P. capsici* did not lose pathogenicity to pepper plants. The combination of our pathogenicity, mycelial growth, and sporangia production results suggest that the repeated transferring of isolates on oxathiapiprolin amended V8A results in the loss of fitness, which may be a result of the cost of increased fungicide insensitivity or stress on the isolates caused by constant exposure to sub-lethal doses of oxathiapiprolin.

Currently, the risk of development of field resistance to oxathiapiprolin in *P. nicotianae* is unknown; however the lack of resistance in *P. nicotianae* isolates from tobacco to mefenoxam shows promise for reduced risk of resistance development in other fungicides. Further work is needed to determine the probability of mutations in the OSBP gene in nature. In addition, with the recent registration of fluopicolide in tobacco for black shank control, tobacco growers will have three different single MOA fungicides (mefenoxam, fluopicolide,
and oxathiapiprolin) to use in alternation within a growing season. The flexibility for
tobacco growers to have multiple different MOA fungicides for use against *P. nicotianae* will
aid in prolonging the life of all three fungicides, if properly rotated for resistance
management.

**ACKNOWLEDGEMENTS**

The authors would like to thank DuPont Crop Protection (Wilmington, Delaware, USA) for providing oxathiapiprolin for use in this study and for their suggestions and input with this research. We would also like to thank Phillip Morris International and RJ Reynolds Tobacco Company for their financial support.
LITERATURE CITED


2 Bittner RJ and Mila AL, Effects of oxathiapiprolin on *Phytophthora nicotianae*, the causal agent of black shank of tobacco. *Crop Protection* **81**:57-64 (2016).


Table 1. Percent inhibition of mycelial growth of UV-mutant and wild-type *Phytophthora nicotianae* isolates on V8A media amended with oxathiapiprolin

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Inhibition of Mycelial Growth (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2 µg a.i./ml</td>
</tr>
<tr>
<td>EdgeB7-WT</td>
<td>100.0 a</td>
</tr>
<tr>
<td>EdgeB7-M1</td>
<td>54.7 b</td>
</tr>
<tr>
<td>YadA28-WT</td>
<td>100.0 a</td>
</tr>
<tr>
<td>YadA28-M1</td>
<td>10.5 b</td>
</tr>
</tbody>
</table>

<sup>a</sup> *Phytophthora nicotianae* race 1 isolate YadA28 and race 0 isolate EdgeB7. WT = wild-type, and M1 = mutant.<br><sup>b</sup> Average percent inhibition of mycelial growth when compared to mycelial growth on non-amended medium over six total replicates. Values for each isolate within a column followed by the same letter are not significantly different (P≤0.05) according to Fisher’s least significant difference test.
Table 2. Sporangia production of UV-mutant isolates of *Phytophthora nicotianae* in oxathiapiprin-amended and non-amended sterile soil extract

<table>
<thead>
<tr>
<th>Isolatea</th>
<th>Non-Amended</th>
<th>0.005 µg a.i./ml</th>
<th>0.05 µg a.i./ml</th>
<th>0.5 µg a.i./ml</th>
<th>1 µg a.i./ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>EdgeB7-WT</td>
<td>74.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
</tr>
<tr>
<td>EdgeB7-M1</td>
<td>48.0 b</td>
<td>38.7 b</td>
<td>14.4 b</td>
<td>0.0 a</td>
<td>0.0 a</td>
</tr>
<tr>
<td>YadA28-WT</td>
<td>46.1 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
<td>0.0 a</td>
</tr>
<tr>
<td>YadA28-M1</td>
<td>38.6 a</td>
<td>35.6 b</td>
<td>36.9 b</td>
<td>37.2 b</td>
<td>33.4 b</td>
</tr>
</tbody>
</table>

*a* *Phytophthora nicotianae* race 1 isolate YadA28 and race 0 isolate EdgeB7. WT = wild-type, and M1 = mutant.

b Average number of sporangia produced on 18 mycelial disks incubated in oxathiapiprin amended and non-amended sterile soil extract. Values for each isolate within a column followed by the same letter are not significantly different (P≤0.05) according to Fisher’s least significant difference test.
**Table 3.** Stability of UV-light generated, oxathiapiprolin-resistant isolates of *Phytophthora nicotianae* on V8A amended with oxathiapiprolin at 0.2 µg a.i./ml after five successive transfers on non-amended V8A media

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mycelial Colony Diameter (cm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>T0&lt;sup&gt;b&lt;/sup&gt;</th>
<th>T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>EdgeB7-M1</td>
<td></td>
<td>2.4 a</td>
<td>2.2 a</td>
</tr>
<tr>
<td>YadA28-M1</td>
<td></td>
<td>5.8 a</td>
<td>6.0 a</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean mycelial colony diameter of six replications after 96 hrs of incubation. Values for each isolate within a row followed by the same letter are not significantly different (P≤0.05) according to Fisher’s least significant difference test.

<sup>b</sup> Mycelial colony diameter directly after UV light mutation (T0), and after five successive transfers on non-amended V8A (T5).
Table 4. Mean calculated effective concentration of oxathiapiprolin for 50% reduction in mycelial growth for four wild-type isolates, selected oxathiapiprolin-adapted mycelial colonies, and the wild-type isolates of *Phytophthora nicotianae* transferred fifteen times on non-amended media

<table>
<thead>
<tr>
<th>Isolate(^a)</th>
<th>EC(_{50}) (µg a.i./ml) of Mycelial Growth(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EdgeA12-WT</td>
<td>0.0039 b</td>
</tr>
<tr>
<td>EdgeA12-WT/T15</td>
<td>0.0030 c</td>
</tr>
<tr>
<td>EdgeA12-E</td>
<td>0.0045 a</td>
</tr>
<tr>
<td>EdgeA12-K</td>
<td>0.0043 ab</td>
</tr>
<tr>
<td>EdgeA12-L</td>
<td>0.0042 ab</td>
</tr>
<tr>
<td>EdgeB7-WT</td>
<td>0.0041 d</td>
</tr>
<tr>
<td>EdgeB7-WT/T15</td>
<td>0.0038 d</td>
</tr>
<tr>
<td>EdgeB7-A</td>
<td>0.0069 b</td>
</tr>
<tr>
<td>EdgeB7-G</td>
<td>0.0044 cd</td>
</tr>
<tr>
<td>EdgeB7-H</td>
<td>0.0078 a</td>
</tr>
<tr>
<td>EdgeB7-I</td>
<td>0.0051 c</td>
</tr>
<tr>
<td>EdgeB7-K</td>
<td>0.0037 d</td>
</tr>
<tr>
<td>YadA3-WT</td>
<td>0.0049 b</td>
</tr>
<tr>
<td>YadA3-WT/T15</td>
<td>0.0032 cd</td>
</tr>
<tr>
<td>YadA3-C</td>
<td>0.0041 bc</td>
</tr>
<tr>
<td>YadA3-D</td>
<td>0.0044 b</td>
</tr>
<tr>
<td>YadA3-F</td>
<td>0.0035 cd</td>
</tr>
<tr>
<td>YadA3-H</td>
<td>0.0032 d</td>
</tr>
<tr>
<td>YadA3-L</td>
<td>0.0110 a</td>
</tr>
<tr>
<td>YadA28-WT</td>
<td>0.0046 de</td>
</tr>
<tr>
<td>YadA28-WT/T15</td>
<td>0.0037 e</td>
</tr>
<tr>
<td>YadA28-A</td>
<td>0.0082 a</td>
</tr>
<tr>
<td>YadA28-B</td>
<td>0.0076 a</td>
</tr>
<tr>
<td>YadA28-E</td>
<td>0.0052 c</td>
</tr>
<tr>
<td>YadA28-G</td>
<td>0.0063 b</td>
</tr>
<tr>
<td>YadA28-H</td>
<td>0.0083 a</td>
</tr>
<tr>
<td>YadA28-J</td>
<td>0.0051 c</td>
</tr>
<tr>
<td>YadA28-K</td>
<td>0.0050 cd</td>
</tr>
<tr>
<td>YadA28-L</td>
<td>0.0062 b</td>
</tr>
</tbody>
</table>
Adapted colonies chosen for calculation of EC$_{50}$ of mycelial growth (A-L), WT = wild-type, and WT/T15 = transferred 15 times on non-amended V8A medium.

Effective concentration of oxathiapiprolin providing 50% inhibition of mycelial growth. Values for each isolate within a row followed by the same letter are not significantly different (P≤0.05) according to Fisher’s least significant difference test.
Table 5. Stability of oxathiapiprolin insensitivity of mycelium adapted isolates of *Phytophthora nicotianae* after fifteen successive transfers on non-amended V8A medium

<table>
<thead>
<tr>
<th>Isolate</th>
<th>EC$_{50}$ (µg a.i./ml) of Mycelial Growth$^a$</th>
<th>T0$^c$</th>
<th>T15</th>
</tr>
</thead>
<tbody>
<tr>
<td>EdgeA12-E</td>
<td></td>
<td>0.0045 a</td>
<td>0.0033 b</td>
</tr>
<tr>
<td>EdgeB7-A</td>
<td></td>
<td>0.0069 a</td>
<td>0.0050 b</td>
</tr>
<tr>
<td>EdgeB7-H</td>
<td></td>
<td>0.0078 a</td>
<td>0.0044 b</td>
</tr>
<tr>
<td>EdgeB7-I</td>
<td></td>
<td>0.0051 a</td>
<td>0.0048 a</td>
</tr>
<tr>
<td>YadA3-L</td>
<td></td>
<td>0.0112 a</td>
<td>0.0031 b</td>
</tr>
<tr>
<td>YadA28-A</td>
<td></td>
<td>0.0082 a</td>
<td>0.0057 b</td>
</tr>
<tr>
<td>YadA28-B</td>
<td></td>
<td>0.0076 a</td>
<td>0.0080 a</td>
</tr>
<tr>
<td>YadA28-E</td>
<td></td>
<td>0.0052 a</td>
<td>0.0042 a</td>
</tr>
<tr>
<td>YadA28-G</td>
<td></td>
<td>0.0063 a</td>
<td>0.0033 b</td>
</tr>
<tr>
<td>YadA28-H</td>
<td></td>
<td>0.0083 a</td>
<td>0.0051 b</td>
</tr>
<tr>
<td>YadA28-J</td>
<td></td>
<td>0.0051 a</td>
<td>0.0056 a</td>
</tr>
<tr>
<td>YadA28-L</td>
<td></td>
<td>0.0062 a</td>
<td>0.0044 b</td>
</tr>
</tbody>
</table>

$^a$ Concentration of oxathiapiprolin providing 50% inhibition of mycelial growth (EC$_{50}$). Values for each isolate within a row followed by the same letter are not significantly different (P≤0.05) according to Fisher’s least significant difference test.

$^b$ Adapted colonies (A-L) were selected based on twice as much mycelial growth on V8A after 15 successive transfers as compared to growth on initial transfer.

$^c$ Calculated EC$_{50}$ value after adaptation (T0) and after fifteenth successive transfer (T15) of isolate onto non-amended V8A media.
Table 6. The mean mycelial colony diameter and sporangia production of selected oxathiapiprolin-adapted, corresponding wild type, and UV-mutant isolates of *Phytophthora nicotianae*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mycelial Colony Diameter (cm)</th>
<th>Sporangia Production</th>
<th>Pathogenicity on tobacco</th>
</tr>
</thead>
<tbody>
<tr>
<td>EdgeA12-WT</td>
<td>7.5 a</td>
<td>91.1 a</td>
<td>+</td>
</tr>
<tr>
<td>EdgeA12-WT/T15</td>
<td>7.4 a</td>
<td>60.1 b</td>
<td>+</td>
</tr>
<tr>
<td>EdgeA12-E</td>
<td>7.2 a</td>
<td>4.3 c</td>
<td>+</td>
</tr>
<tr>
<td>EdgeB7-WT</td>
<td>7.4 a</td>
<td>74.0 a</td>
<td>+</td>
</tr>
<tr>
<td>EdgeB7-WT/T15</td>
<td>7.3 a</td>
<td>77.7 a</td>
<td>+</td>
</tr>
<tr>
<td>EdgeB7-M1</td>
<td>7.5 a</td>
<td>48.0 b</td>
<td>+</td>
</tr>
<tr>
<td>EdgeB7-A</td>
<td>6.6 b</td>
<td>0.0 c</td>
<td>-</td>
</tr>
<tr>
<td>EdgeB7-H</td>
<td>6.3 b</td>
<td>53.2 b</td>
<td>-</td>
</tr>
<tr>
<td>EdgeB7-I</td>
<td>4.5 c</td>
<td>0.9 c</td>
<td>-</td>
</tr>
<tr>
<td>YadA3-WT</td>
<td>7.3 a</td>
<td>59.8 a</td>
<td>+</td>
</tr>
<tr>
<td>YadA3-WT/T15</td>
<td>7.3 a</td>
<td>25.4 b</td>
<td>+</td>
</tr>
<tr>
<td>YadA3-L</td>
<td>4.3 b</td>
<td>0.0 c</td>
<td>-</td>
</tr>
<tr>
<td>YadA28-WT</td>
<td>7.3 a</td>
<td>45.8 a</td>
<td>+</td>
</tr>
<tr>
<td>YadA28-WT/T15</td>
<td>7.4 a</td>
<td>46.1 a</td>
<td>+</td>
</tr>
<tr>
<td>YadA28-M1</td>
<td>7.6 a</td>
<td>38.6 a</td>
<td>-</td>
</tr>
<tr>
<td>YadA28-A</td>
<td>4.4 d</td>
<td>9.8 b</td>
<td>-</td>
</tr>
<tr>
<td>YadA28-B</td>
<td>4.3 d</td>
<td>1.8 c</td>
<td>-</td>
</tr>
<tr>
<td>YadA28-E</td>
<td>5.3 c</td>
<td>0.0 c</td>
<td>-</td>
</tr>
<tr>
<td>YadA28-G</td>
<td>5.4 c</td>
<td>12.8 b</td>
<td>-</td>
</tr>
<tr>
<td>YadA28-H</td>
<td>4.1 d</td>
<td>0.0 c</td>
<td>-</td>
</tr>
<tr>
<td>YadA28-J</td>
<td>7.1 a</td>
<td>0.0 c</td>
<td>-</td>
</tr>
<tr>
<td>YadA28-L</td>
<td>6.2 b</td>
<td>0.0 c</td>
<td>-</td>
</tr>
</tbody>
</table>

*a* Adapted mycelium colonies (A-L), WT = wild-type, WT/T15 = transferred 15 times on non-amended medium, and M1 = UV-mutant.

*b* Mycelial growth was measured at 96 hrs for EdgeB7 and at 120 hrs for YadA28 isolates. Values for each pair of wild type and mutant isolate within a column followed by the same letter are not significantly different (P≤0.05) according to Fisher’s least significant difference test.

*c* The average number of sporangia produced from 18 mycelial disks grown in sterile soil extract. Values for each pair of wild type and mutant isolate within a column followed by the
same letter are not significantly different (P≤0.05) according to Fisher’s least significant difference test.

\textsuperscript{d} Pathogenic (+) and non pathogenic(-). Isolate considered pathogenic if number of tobacco plants with a stem lesion was >0.
Fig. 1. Alignment of partial amino acid sequence of oxysterol binding protein (OSBP) in two isolates of *Phytophthora nicotianae*. **A**, isolate YadA28-M1 with rectangle at location of mutation. **B**, isolate EdgeB7-M1 with rectangle at location of mutation.
Fig. 2. Mean mycelial colony diameter of four *Phytophthora nicotianae* isolates on oxathiapiprolin-amended (0.006 and 0.007 µg a.i./ml) media for fifteen successive transfers. Bars represent the standard error of the mean from twelve replicate colonies.