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Alternative Methods of Control for Phytophthora nicotianae of Tobacco

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ALTERNATIVE METHODS OF CONTROL FOR *PHYTOPHTHORA NICOTIANAE* OF TOBACCO

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science
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ABSTRACT OF THESIS

ALTERNATIVE METHODS OF CONTROL
FOR *PHYTOPHTHORA NICOTIANAE* OF TOBACCO

Kentucky is the nation's leading producer of burley tobacco and the crop's most economically important disease is black shank, caused by *Phytophthora nicotianae* (Pn). Current management is effective, however, problems with expense and pathogen persistence are issues. Two alternative methods for control of Pn were examined: biofumigation and soil application of an organic, yeast fermentation-derived product (Soil-Set). Field studies in 2009 and 2010 found no effect on populations of fungi, disease severity of Pn, and yield between mustard- and wheat-amended plots. Experiments in the greenhouse suggested that survival of Pn was impacted by biomass rather than biofumigation. Biofumigation is not a viable option for controlling black shank in tobacco production. Soil-Set was inhibitory against mycelial growth of Pn on corn meal agar rather than V8 juice. Results from a greenhouse study indicated that increasing the dose of Soil-Set by four times what is recommended held the most potential for suppression of Pn in a burley variety with no resistance. A field study in 2012 found no differences among treatments in reducing severity of Pn in a variety with high resistance. More field and greenhouse studies need to be conducted to examine the potential of Soil-Set in tobacco production.

KEYWORDS: *Phytophthora nicotianae*, black shank, tobacco, biofumigation, Soil-Set

Maria Holdcroft

February 8th 2013

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FOR *PHYTOPHTHORA NICOTIANAE* OF TOBACCO

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To my mother, Ann Holdcroft. END ALZ.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE:
LITERATURE REVIEW

I. Tobacco Production in Kentucky

In 2009, the world's top five tobacco producers were (in order of highest to lowest, metric tons) China, Brazil, India, the United States of America (USA), and Malawi (36). In the USA, there were 134,275 hectares of tobacco harvested from ten states in 2011 (94). Out of the ten states still producing tobacco, North Carolina had the highest total hectares of tobacco harvested (65) while Kentucky was second with 31,363 total hectares of tobacco harvested. However, the number of tobacco farms in Kentucky surpassed North Carolina by as much as three times (94). The 2007 Census of Agriculture found that 106 out of 120 counties in Kentucky produced tobacco (94).

In Kentucky, the two types of tobacco grown are dark and burley (94). Kentucky is the nation's leading producer of dark tobacco. Dark tobacco production is concentrated in western Kentucky. Dark tobacco can either be air-cured like burley or fire-cured. In Kentucky, approximately two-thirds of dark tobacco is fire-cured. In 2010, the total number of hectares harvested of dark fire-cured tobacco was 3,561, while production was valued at $71,148,000. Likewise, in 2010, the total number of hectares of harvested dark air-cured tobacco was 1,781, while production was valued at $27,720,000 (94). Dark tobacco is most often used in pipe tobacco, chewing tobacco, and snuff. Dark tobacco has large, heavy leaves that are dark green in color as the name implies. Fire-curing is achieved by using the smoke from burning wood to dry the leaves, which gives a smoky flavor and adds color for some smokeless tobacco products. On average, the yield for dark tobacco growers in Kentucky is around 7,000 kg/ha (9,10).

Kentucky is the nation's leading producer of burley tobacco. In 2010, the total number of hectares harvested of burley tobacco was 29,138. Additionally, burley tobacco production in 2010 for the entire state of Kentucky was valued at $210,600,000 (94). Burley tobacco is most often used in cigarettes. Burley tobacco has broad leaves that are light green in color as well as a light green to creamy white stalk and leaf midrib. Burley tobacco is harvested by cutting the stalk and spearing it on wooden stakes, which are then hung in a ventilated barn to cure with no added heat. After curing, leaves are stripped from the stalk and sold. On average, the yield for burley tobacco growers in Kentucky is around 2,352 kg/ha (100).

Disease management programs are vital to tobacco production. Challenges faced by tobacco growers include economic factors (loss of federal price supports and labor costs), weather, and pests (insects, weeds and disease) (118,119). Effective management of diseases impacts the yield and ultimately sales of tobacco.

II. Black Shank

The most destructive and economically important disease for tobacco growers not only in Kentucky, but also all over the U.S., is black shank. Belonging to a genus that literally translates as "plant destroyer", *Phytophthora nicotianae* is the causal agent of black shank. *P. nicotianae* is classified under the Kingdom Straminopila and it belongs to the class of fungus-like organisms, the oomycetes. Major characteristics that separate oomycetes like *P. nicotianae* from true Fungi are a cellulose-composed cell wall, bi-flagellated zoospores, and diploid vegetative hyphae.

Breda de Haan, a Dutch scientist, first described in 1896 a disease affecting cigar wrapper tobacco in Indonesia, and he named the causal agent *Phytophthora nicotianae* (15). It is understood that *P. nicotianae* was introduced in the United States multiple times through trade before it was first observed in the U.S. in 1915 in southern Georgia (80). By 1922, it had spread to Florida (141). In 1935, black shank was first observed in Guthrie, Kentucky, which is located in the southwestern part of the state, close to the Tennessee border (134). *P. nicotianae* continued to spread most likely due to the movement of soil from small farms sharing labor and farm equipment. In 1940, black shank had moved from Logan County to the north and east, appearing on several Georgetown (Scott County) farms and one farm each in Owen and Nicholas County. After this, drainage from farms infested with *P. nicotianae* in Georgetown flowed into the nearby Elkhorn Creek, which flowed into the Kentucky River and eventually the Ohio River, spreading the pathogen along the river bottoms. By 1952, black shank was reported on hundreds of farms in over 60 counties (134).

Historically, the causal agent of black shank has been referred to as both *Phytophthora nicotianae* and *Phytophthora parasitica*. The reason for the debate on taxonomy and nomenclature stems from the fact that Breda de Haan never put forth a Latin description when he described *P. nicotianae*. Furthermore, Breda de Haan's depiction of the organism in his description was inaccurate. It is now believed that his illustrations depicted a mixed culture of *P. nicotianae* and a *Pythium* species. All structures were consistent with *P. nicotianae* except for the paragynous antheridium which most likely belonged to a *Pythium* spp. (49). Dastur described the same pathogen

on castor bean in 1913 in India, but he called the organism *Phytophthora parasitica* because he observed the antheridium in a different position from that described by Breda de Haan (28).

In 1931, Tucker differentiated between isolates pathogenic only on tobacco from those that were not as *Phytophthora parasitica* var. *nicotianae* and *Phytophthora parasitica* var. *parasitica,* respectively (133). Waterhouse in 1963 described morphological differences between what she called *Phytophthora nicotianae* var. *nicotianae* and var. *parasitica* (138). However, several biochemical, serological, mitochondrial and chromosomal DNA analyses provide strong evidence that *P. nicotianae* should not be separated into the two varieties Waterhouse described(24,33,40,84,91,97).

According to the priority rule of the International Code of Botanical Nomenclature (ICBN), *P. nicotianae* is the valid name of the black shank pathogen. In 1993, Hall neotypified the name *P. nicotianae* since Breda de Haan also failed to provide a holotype (49). The accepted neotype now links this pathogen back to both plant and cultural specimens preserved at the International Mycological Institute in the United Kingdom and the University of California at Riverside. In keeping with the ICBN code, *P. nicotianae* will be used in this thesis.

Strong evidence shows that the host range for *P. nicotianae* is limited to tobacco. Tobacco isolates are not pathogenic on other solanaceous crops such as tomato, potato and peppers (32,77). Elena published a study in which he examined the pathogenicity of 61 *P. nicotianae* isolates from Greece, the United States, and the Netherlands on

tobacco and tomato. None of the tobacco isolates were able to cause disease on tomato after stem inoculation (32). Two isolates colonized the stem with no disease symptoms.

P. nicotianae is extremely persistent in soil where it survives as spherical, non-papillate, hyaline to brown, thick walled chlamydospores averaging 25 µm in diameter (80). The diploid mycelium of *P. nicotianae* is hyaline, coenocytic and in culture it forms a distinctive rosette pattern in the presence of V8 vegetable juice (49,80). Sporangia are typically papillate and vary in size (18-61 µm x 14-39 µm). The explosive spread of this pathogen is due in part to the number of zoospores that are contained in one sporangium, which can range anywhere from 5-30. The kidney shaped zoospores range in size from 7 to 11 µm in length (80). The zoospores have two flagellae (anterior and posterior) of different sizes that enable them to swim. *Phytophthora nicotianae* is heterothallic, thus requiring two mating types (A1 and A2) for sexual reproduction. However, oospores have rarely been found in nature or produced in culture so the consensus is that these spores are not necessary for pathogen survival or epidemic development. Oospores, when formed, are hyaline, spherical (25 µm average diameter) with amphigynous antheridia (66,80).

The pathogen is polycyclic, meaning it undergoes multiple reproductive cycles in one growing season. The polycyclic nature of *P. nicotianae* makes black shank a difficult disease to manage. *P. nicotianae* infection is most favored by soil temperatures above 20 °C which triggers direct germination of chlamydospores or indirect germination to form lemon-shaped sporangia. Saturated soils favor the release and spread of the zoospores. Once released from the sporangium, zoospores find neighboring tobacco

roots by means of chemotaxis, electrotaxis, or thigmotaxis, and then encyst (50). Finally, the encysted zoospores germinate and penetrate the root epidermis. *P. nicotianae* is hemibiotrophic so once the infection hypha enters the tobacco root cell, the mycelium grows intercellularly and eventually spreads throughout the root cortical cells (50,68). Kincaid et al. found a positive linear relationship between soil pH and incidence of black shank (69). The most favorable soil pH for disease development is between 6 and 7, while incidence of disease was found to decrease dramatically in a soil pH as low as 4.2 (62).

Once infection has taken place, common above-ground symptoms include wilting and chlorosis of leaves. Below ground symptoms include root and stem necrosis. The very characteristic blackened lower stalk and occasional disking of the pith can be seen both above and below ground (114).

III. Management of Black Shank

Cultural Practices

There are several cultural practices that can be used to manage black shank. One of the most important practices is rotation with a non-host crop. Rotating to a crop on which *P. nicotianae* cannot survive and reproduce helps to lower pathogen populations (chlamydospore survival) over time and limit build-up of the pathogen (7,34,114,115). The best rotation involves wheat or grass in a three- to five-year rotation with tobacco (7,99,114). Proper sanitation is another important cultural control practice (34,114). Since *P. nicotianae* is a soil-borne pathogen, it is important to sanitize shoes and equipment that have been used in an infested field before moving to another

because pathogen propagules can be carried in this manner via soil. Inoculum such as zoospores or sporangia can also be carried via water so sources such as rivers or creeks should not be used for irrigation. Field location and history is important in managing black shank. Since the disease is favored by wet conditions, a low-lying field where water collects would not be an ideal spot for planting tobacco especially if it is located below a field that is infested with black shank. Avoidance of fields with a history of black shank is recommended. Adequate field drainage is also important as saturated soil favors pathogen growth and infection.

Resistance

When used in conjunction with cultural practices and chemicals, resistant varieties can be an effective tool for managing black shank. The different races of *P. nicotianae* confound breeding for resistance to black shank. Four races (0, 1, 2, and 3) of *P. nicotianae* have been described (34,42,63,114). From the time when black shank was first reported in 1935 through the 1970s, race 0 was the predominant race in Kentucky. The use of varieties with single-gene resistance (described below) to race 0 eventually led to selection for race 1 (6,20,135). In the late 1950s, Valleau described highly virulent strains of *P. nicotianae* in Kentucky that were pathogenic on hybrid varieties containing single-gene resistance to race 0 (135). In 1962, Apple was the first to delineate two races of *P. nicotianae* (race 0 and 1). The race 1 isolate used in Apple's study was from Kentucky (6). Currently, race 0 and race 1 are found in Kentucky. In 2007, the race structure of *P. nicotianae* was analyzed after collecting soil samples from ten counties across eastern and western Kentucky with a previous history of black shank (K. Ivors,

unpublished data). Race 0 and 1 were present together in a majority of the counties. Race 3 was discovered in one county and 9 isolates of *P. nicotianae* for which race could not be determined were found in 6 counties. Race 2 has only been reported in South Africa (63), while race 3 has also been reported in Connecticut and North Carolina (42,85).

There are two types of resistance to black shank found in tobacco cultivars: vertical (syn: complete, single-gene) and horizontal (syn: partial, multi-gene) resistance. Since the 1930s, resistance to black shank was derived from the cigar cultivar Florida 301 (Fla 301). Tisdale transferred horizontal resistance into 'Fla 301' by crossing 'Big Cuba' with 'Little Cuba' (67,123,125). It is not know what specific genes or loci control the resistance in 'Fla 301', but it is hypothesized that the horizontal resistance which confers varying levels of resistance to race 0 and 1 of *P. nicotianae* is due to different levels of gene expression (128). 'Fla 301' was the only source of black shank resistance available for the development of new burley tobacco varieties for approximately twenty years and nearly thirty years for flue-cured tobacco (65,135). Available tobacco varieties with horizontal resistance include KT 204, TN 86, and TN 90, which have a moderate level of resistance to race 0 and 1 of black shank (Table 1.1). Examples of dark tobacco varieties include KT D4, KT D6, and KT D8, which all confer a moderate level of resistance to race 0 and race 1 (Table 1.2).

With respect to vertical resistance to black shank, a single, dominant gene (*Ph* gene) (18) from *Nicotiana longiflora* (*Phl*) or *N. plumbaginifolia* (*Php*) can be incorporated into field tobacco cultivars via interspecific crosses thus conferring

complete resistance to race 0. However, these lines are susceptible to race 1 (113). Results of root inoculations both in the greenhouse and the field have shown that race 3 of *P. nicotianae* is pathogenic on varieties with the *Phl* gene, while those with the *Php* gene are resistant (42). In 1953, Valleau introgressed the *Phl* gene from *N. longiflora* into the burley tobacco line L8 (135). However, 'L8' exhibited severe leaf spotting, which made it unsuitable for commercial use necessitating the development of 'L8' hybrids (e.g. 'KY 14 x L8') that are heterozygous for the *Phl* gene (23,79,116,122). Chaplin transferred the *Php* gene into flue-cured tobacco in the early 1960s. The *Php* gene has been transferred mostly into flue-cured lines, but recently burley lines such as KT 206, 209 and 210 now have both the *Php* and *Phl* gene (R.D. Miller, personal communication). The KT 206, 209, and 210 lines also contain horizontal resistance to 1 thus providing burley tobacco growers with the best resistance to race 0 and 1 of black shank (Table 1.1). The *Ph* gene has been incorporated into dark tobacco, which gives it complete or vertical resistance to race 0. Some examples of dark tobacco varieties with the *Ph* gene include PD 7302, PD 7309, PD 7318, and PD 7305 (which also has horizontal resistance to race 1) (Table 1.2).

Chemical Control

Fungicides for black shank suppression are restricted to just one group, the phenylamides. Metalaxyl (Meta Star) or its R-enantiomer, mefenoxam (Ridomil Gold or Ultra Flourish) are systemic phenylamide fungicides (99). They have a very specific mode of action, namely inhibition of RNA synthesis by interfering with a nuclear, α-amanitin insensitive RNA polymerase-template complex, thus impacting mycelial growth and

sporulation (22,29). Despite the specific mode of action of phenylamides, resistance to these products has not been found in isolates of *P. nicotianae* from tobacco fields (41). Resistance has been found in other oomycetes, such as the causal agent of blue mold of tobacco (*Peronospora tabacina*) (17).

Chemical control, resistant varieties, and cultural practices used together can be effective for managing black shank. It is recommended that mefenoxam/metalaxyl products be used with varieties that have a resistance level of 4 or higher for the best economic returns. This is because disease incidence can be high on varieties with little or no resistance even after using the maximum amount of fungicide approved by the label, cutting into profits (112,121). One major disadvantage to using fungicides for control of black shank is that they are expensive, generally costing growers around $247/ha for a single application. To reduce production costs and the potential release of chemicals to the environment, there is a desire to find affordable, alternative control methods that can be integrated into a comprehensive management plan for black shank.

IV. Biofumigation

Plants in the family Brassicaceae are known to produce an important class of secondary metabolites known as the glucosinolates, which can function in plant defense against insects, nematodes, and microbial pests (4,71,81,87,107). Glucosinolate production is primarily found in the order Capparales, which contains 15 families including Capparacease, Caricacease and Brassicaceae (105). Glucosinolate production

has also been reported in the genus *Drypetes*, which belongs to the family Euphorbiaceae and order Euphorbiales (106).

Approximately 120 glucosinolates have been identified and they all share the same core structure, in which a β-thiolglucosyl residue is attached to a central carbon atom to form a sulfated ketoxime. The structural variability stems from the amino acid side chain elongation, thus glucosinolates can be classified into three groups based on their amino acid precursors. The three groups include: 1) the aliphatic glucosinolates which are derived from alanine, leucine, isoleucine, methionine, and valine; 2) the aromatic glucosinolates which are derived from phenylalanine and tyrosine; and 3) the indole glucosinolates which are derived from tryptophan (35,140).

Glucosinolates vary in both concentration and structure among different *Brassica* spp. (37,71). Differences in concentration of glucosinolates can be due to factors such as environmental stress (e.g. temperature, water, ultraviolet light), which increases glucosinolate content (5,19); developmental stage, with glucosinolate content highest right before flowering (38); and the tissue type with aliphatic glucosinolates occuring primarily in shoots and aromatic glucosinolates were found mostly in roots (71).

Plants that produce glucosinolates also contain a thiolglucoside glucohydrolase also known as myrosinase, which is produced in specialized myrosin cells, and hydrolyzes the glucose moiety found in the glucosinolate core structure (72,103). Once plant tissue is damaged and exposed to air, glucosinolates in the plant vacuole are hydrolyzed by myrosinase released from the damaged myrosin cells (16,45,48). The

hydrolysis products include isothiocyanates, thiocyanates, nitriles, oxazolidine-2-thiones, and epithionitriles. The most important compound produced is the volatile isothiocyanate which has been shown to have antimicrobial activity (44,71). The synthetic fumigants metam-sodium and metam-potassium, which produce volatilized methyl isothiocyanate, are sold commercially as pesticides in the U.S. (Vapam, Sectagon, and K-Pam). In Vapam, the concentration of metam-sodium is 0.5 kg a.i./l. However, they are all costly and extremely hazardous to human and animal health.

J. A. Kirkegaard was the first to coin the term biofumigation (70). Kirkegaard's conceptualization of biofumigation encompassed the suppressive effects of *Brassica* spp. on soilborne organisms due to *Brassica*-released isothiocyanates during glucosinolate hydrolysis (70). Understanding how to utilize the unique glucosinolate hydrolysis reaction of *Brassica* plants for biofumigation can provide growers with a non-toxic, cost-effective method for managing soilborne pathogens.

Several factors dictate the efficiency of the glucosinolate hydrolysis reaction and the release of isothiocyanates in the soil. The efficiency of isothiocyanate release improves when choosing a high-glucosinolate producing variety such as 'Pacific Gold' mustard (*Brassica juncea*), adding proper moisture to drive the glucosinolate to isothiocyanate reaction, and increasing tissue maceration (90). In the field, isothiocyanates have been recovered in the soil up to 4 days post-incorporation (90).

Biofumigation has been studied in several different cropping systems and against a range of pathogens including nematodes, bacteria, and fungi (4,14,21,51,71,76,82,88,107,117). Zasada et al. found that soilborne pathogens of cut-

flower in California responded differently to incorporation of various Brassicas (Brassicaceae) (142). Incorporation of broccoli did not consistently reduce populations of *Fusarium* spp. and weeds, but increases in broccoli biomass resulted in proportionate decreases in citrus nematode populations. The incorporation of brussels sprouts and horseradish with higher sinigrin (allyl glucosinolate) concentrations than broccoli (73) significantly reduced nematode populations, but no effect from incorporation of Brassicas was found on *Fusarium* spp. Based on these studies, Brassicas which produced high levels of biomass and also contained high concentrations of glucosinolate appear to have the most potential for pest suppression.

Monfort et al. examined the incorporation of different Brassicas for managing the root-knot nematode, *Meloidogyne incognita*, in Georgia vegetable production (89). After incorporation of Brassicas into the soil, the plots were covered with black plastic mulch, which in addition to suppressing weeds can contain the volatiles released from Brassica tissues in the soil. Similar to Zasada et al., they found that Brassicas high in glucosinolate content and yield were the most effective at increasing crop yield and weight while decreasing *M. incognita* populations and root gall formation. The increase in yield and weight also shared correlation with one major challenge found in this study. Before vegetables were planted, Monfort et al. found that some Brassicas supported higher reproduction of *M. incognita* and thus resulted in a lower vegetable yield than Brassica plots (e.g. radish and turnip) with significantly lower populations of nematodes at planting.

Dunne et al. used an *in-vitro* screen to examine the suppressive effect of *Brassica juncea* and two varieties of *Brassica napus* on ten isolates of *Phytophthora* spp (31). Their results showed that *Brassica juncea* provided the best suppression of mycelial growth over all ten isolates and that *P. cinnamomi* was the most sensitive isolate in the biofumigation assay. In 2008, Mattner et al. published a study that examined the effect of biofumigation in strawberry production. Their *in-vitro* bioassays indicated that volatiles from *Brassica rapa* and *Brassica napus* roots suppressed the growth of seven soilborne pathogens including *Alternaria alternata*, *Colletotrichum dematium*, *Cylindrocarpon destructans*, *Fusarium oxysporum*, *Pythium ultimatum*, *Phytophthora cactorum*, and *Rhizoctonia fragariae*. However, incorporation of these crops into the field soil did not reduce the survival of *P. cactorum* and *C. destructans* (83).

Csinos et al. in Georgia examined the effect of the mustard variety 'Florida Broadleaf' on *P. nicotianae* populations in flue-cured tobacco fields. They examined the incorporation of Brassica cover crops with and without treatment of mefenoxam to reduce incidence of disease from 2004 to 2007. Treatments were arranged in a split-plot, randomized complete-block design with four replications, in which the subplots treatments included Ridomil Gold and a non-treated control. In the spring of 2004, flue-cured variety K-236 (low resistance to both race 0 and 1 of black shank), rye, and peanut were planted and in the fall, rye and 'Florida Broadleaf' mustard were planted and tilled into the soil the following spring. In the spring of 2005, variety K-236 was planted in all of the plots and in the fall, wheat and mustard were planted and tilled into the soil the following spring. In the spring of 2006 and 2007, variety K-236 was planted in all of the

plots and in the fall, wheat and mustard were planted and tilled into the soil the following spring. The number of tobacco plants with symptoms of black shank was collected during each year and used to calculate the percent disease (25,26,27).

The incidence of black shank in plots with incorporated 'Florida Broadleaf' (16.2%) incorporation was not significantly lower than plots with incorporated rye and wheat (30.2%) incorporation. These results indicated that there was no suppression of black shank resulting from incorporation of mustard. As compared to the cover crop control with no application of mefenoxam, there was a significant decrease in disease incidence and increase in yield when biofumigation was used in conjunction with mefenoxam applications at transplant and lay-by. Overall, these field studies suggested that there was potential for biofumigation with Brassicas in Georgia, but more research was needed due to variable results (25,26,27).

In the fall of 2008, Brassica cover crops including arugula and 'Caliente' mustard were planted in Daviess county, Kentucky to evaluate the effect of incorporation of different Brassica cover crops on incidence of black shank. The experiment failed due to below freezing temperatures during the winter, which resulted in no plant growth in the spring. Prior to the initiation of this experiment, 'Caliente' mustard was promoted to farmers in Kentucky as a tool that would reduce severity of black shank in the field. However, this product was sold without data to support the supplier's claims of disease control following biofumigation with 'Caliente' mustard. Further research is needed to address whether or not biofumigation is a viable method of control for black shank in Kentucky.

Table 1.1. Burley tobacco varieties and their resistance to black shank, as listed in the 2011-2012 Kentucky & Tennessee Burley Tobacco Production Guide. Rating scale 0-10, with 10 having the highest resistance and 0 with no resistance. *=Based on a limited number of field tests.

	Resistance to black shank (0-10)	
Variety	**Race 0**	**Race 1**
KY 14 x L8LC	10	0
KY 907LC	2	2
KY 200LC	6	6
KY 204LC	7	7
KY 206LC	10	7
KY 209LC	10	8
KY 210LC	10	7
NC BH 129	1	1
NC 3	2	2
NC 4	2	2
NC 5	10	4
NC 6	10	3
NC 7	10	3
NC 2000	0	0
NC 2002	0	0
TN 86LC	4	4
TN 90LC	4	4
TN 97LC	4	4
HYBRID 403LC	0	0
HYBRID 404LC	0	0
HYBRID 503LC	5	5
N 126	0	0
N 777LC	2	2
N 7371LC	4*	4*
NBH 98	2	2
HB04PLC	0	0
HB3307PLC	10*	4*
R 610LC	4	4
R 630LC	3	3
R7-11	0	0
R7-12LC	0	0

Table 1.2. Dark tobacco varieties and their resistance to black shank, as listed in the 2011-2012 Kentucky & Tennessee Burley Tobacco Production Guide. Scale 0-10, with 10 having the highest resistance and 0 with no resistance.

	Black Shank	
Variety	**Race 0**	**Race 1**
NL MadLC	0	0
TR Madole	0	0
Lit Crit	0	0
DF 911	0	0
KY 160	0	0
KY 171	0	0
VA 309	2	2
VA 359	1	1
TN D950	3	3
KT D4LC	4	4
KT D6LC	3	3
KT D8LC	4	4
DT 538LC	4	4
PD 7312LC	0	0
PD 7302LC	10	0
PD 7309LC	10	0
PD 7318LC	10	0
PD 7305LC	10	3

CHAPTER TWO:
THE BIOFUMIGATION POTENTIAL OF *BRASSICA JUNCEA* AGAINST *PHYTOPHTHORA NICOTIANAE*

INTRODUCTION

Kentucky is the nation's leading producer of burley tobacco and the most economically important disease of the crop is black shank, caused by *Phytophthora nicotianae*. Current recommendations for control include cultural practices (crop rotation, sanitation), use of resistant varieties, and fungicides. When disease pressure in the field is high, varieties with little or no resistance still experience high disease incidence even after using the maximum amount of fungicide approved by the label (112,121). This cuts into profits because fungicides for black shank control are expensive (ca. $247/ha per application) and restricted to one class, the phenylamides (mefenoxam and metalaxyl). There is a need to find affordable, alternative control methods that can be integrated into the cultural practices mentioned above for better management of black shank.

Plants belonging to the Brassicaceae (Brassica) family produce a group of secondary metabolites known as glucosinolates (48). When tissues of Brassicas are disrupted, an endogenous enzyme, myrosinase, hydrolyzes glucosinolates and volatile compounds are liberated from crop residues. The most important antimicrobial agent released from this reaction is isothiocyanate (44,90). The suppressive effect of Brassicas on soilborne organisms due to Brassica-released isothiocyanate is called biofumigation (70,107).

There have been *in-vitro* studies that have shown the potential of biofumigation against *Phytophthora* spp. Dunne et al. found that *Brassica juncea* was most effective at suppressing the mycelial growth of ten *Phytophthora* spp (31). Mattner et al. found that volatiles from the roots of *B. rapa* and *B. napus* suppressed mycelial growth of *P. cactorum* (83). Biofumigation with brassica cover crops has not been well-studied in tobacco. Csinos et al. conducted a field experiment over four years to examine the potential of biofumigation for control of black shank on flue-cured tobacco in Georgia (27). Incorporation of mustard was no different than rotation with wheat or rye. However, when compared to the control with no fungicide, there was a significant decrease in disease incidence and increase in yield when biofumigaton was used in conjunction with two applications of mefenoxam. More research was needed to determine the applicability of biofumigation in tobacco production. The purposes of this study were: to evaluate the effect of biofumigation on populations of *P. nicotianae*, *Fusarium* spp., *Rhizoctonia* spp., and saprophytic fungi in the soil, progression of black shank through the growing season, and yield in the field; to evaluate the effect of biofumigation on populations of *P. nicotianae* in the greenhouse; and to compare concentration of glucosinolates from mustard grown in the field and in the greenhouse.

MATERIALS AND METHODS

Field site. Field studies were conducted in Clark County, KY at Anderson Brothers Farm during 2008-2010. The soil type was a Bluegrass-Maury silt loam. The field had been under continuous tobacco production for ten years and had a history of severe

black shank. Average temperature and rainfall data were collected from NOAA National Climatic Data Center (Lexington, KY; http://www.ncdc.noaa.gov/).

Pilot field experiment 2008. Treatments were arranged in a randomized complete-block design with four replications. Plots consisted of two rows and measured 2 m by 15.24 m. Two cover crop regimes were evaluated: bare ground (no cover crop) and 'Pacific Gold' mustard (*Brassica juncea*), sown on 2 April at a seeding rate of 13.45 kg/ha. On 30 May, the mustard was in full bloom and was incorporated into the soil to a depth of 15-20 cm using a tractor-driven rotary tiller. On 16 June, one row each of 'KT 204' and 'KT 206' were transplanted into plots. Both varieties have a high level of partial resistance to race 1 of *P. nicotianae*, while 'KT 206' offers complete or vertical resistance to race 0 and 'KT 204' offers a high level of partial resistance to race 0. Between-plant spacing was 0.51 m, resulting in a population of 30 plants per variety in each plot. Fertility, insect, and weed management was carried out according to guidelines from the University of Kentucky Cooperative Extension Service (110).

Soil samples for enumeration of soil fungi were collected from each plot by removing the top 7 cm of soil with a 3-cm diameter probe. Ten cores were collected per plot in locations determined by following a zig-zag course through the plot area. Soil was collected four times on 28 May (two days before mustard was incorporated), 19 June, 6 August, and 28 August. Soil collected from each plot was thoroughly mixed by hand and maintained in the dark at 4˚C until analysis. Soil was dried on the lab bench at room temperature approximately 24 hours before analysis. To examine populations of *P. nicotianae*, 1 g of dried soil was added to 25 ml of 0.3% water agar (amended with 250

mg of ampicillin sodium salt/L) and stirred for approximately 2 minutes. Five-ml of the soil dilution was distributed over five PARPH V8 (64) plates using a cell spreader and incubated at room temperature in the dark for five days. This was repeated a total of ten times for each plot. After five days, all colonies were counted. From the first soil sampling, a subset of isolates that were suspected to be *P. nicotianae* in each plot were confirmed by Koch's postulates; infected tobacco roots were floated in tap water and then examined under the microscope to confirm identity. Additionally, mycelial growth patterns on 10% V8 medium were observed and compared to illustrations of *P. nicotianae* in Erwin and Roberio (34) as an additional confirmatory step. Data were analyzed using the GLM procedure of the SAS (Cary, NC).

Plant survival was assessed during the growing season by subtracting the number of dead or dying plants from the total number of healthy plants at the beginning of the season in each plot five times on 16 June, 1 July, 22 July, 20 August, and 23 September. The mean plant survival was calculated by combining the number of healthy plants from 'KT 204' and 'KT 206' for mustard and fallow (bare ground) plots, and data were analyzed using the GLM procedure of the SAS (Cary, NC).

Field experiments 2009 and 2010. Treatments were arranged in a split-plot, randomized complete-block design with four replications in 2009 and 2010. Plots consisted of four rows and measured 4.2 by 12.19 m with a 3.05-m fallow buffer between plots. Two cover crop regimes were evaluated: winter wheat, sown on 2 April 2009 and 14 April 2010 at a seeding rate of 56.04 kg/ha; and 'Pacific Gold' mustard (*Brassica juncea*), sown on 2 April 2009 and 14 April 2010 at a seeding rate of 112.06

kg/ha. Cover crops were incorporated into the soil to a depth of 15.24-20.34 m on 20 May in 2009 and 2 June in 2010. The following four burley tobacco varieties were used in this experiment: 'KY 14 x L8', 'TN 90', 'NC 7', 'KT 206' (10/0, 4/4, 10/3, 10/7 levels of resistance to races 0 and 1 of *P. nicotianae*, respectively, with 1 being the lowest and 10 being the highest level of resistance). Tobacco was transplanted on 16 June in 2009 and 19 June in 2010.

For enumeration of soil fungi, soil was collected as described for the 2008 pilot study. In 2009, soil samples were collected before cover crop emergence on 4 April (2 days after planting), the day of cover crop incorporation on 20 May, and 23 May. In 2010, soil samples were collected 19 days after planting of cover crops on 3 May, three days after cover crop incorporation on 5 June, and 7 June.

Quantification of *P. nicotianae* in the soil was performed as described for the 2008 study, with modifications. For each plot, 10 grams of soil was diluted in 100 ml of 0.3% water agar (amended with 250 mg of ampicillin sodium salt/L) and mixed for approximately 2 minutes. One-ml of the soil dilution was spread onto 5 plates of PARPH V8. This was repeated ten times for each plot. Peptone pentachloronitrobenzene (PCNB) (93), tannic acid benomyl agar (TABA) (39), and Ohio State media (OSU) (139) were used for isolation of *Fusarium* spp., *Rhizoctonia* spp., and saprophytic fungi, respectively. To examine populations of *Fusarium* spp. and saprophytic fungi, 10 grams of dried soil was added to 100 ml of 0.3% water agar (amended with 250 of mg ampicillin sodium salt/L) and stirred for approximately 2 minutes. One-ml of the original soil dilution was further diluted in 20 ml of 0.3% water agar (amended with 250 mg of

ampicillin sodium salt/L) and stirred for approximately two minutes. From this dilution, 1 ml was spread on PCNB and OSU plates using a cell spreader. PCNB plates were incubated at room temperature, in the light for five days and OSU plates were incubated at room temperature, in the dark for five days. After five days, all colonies were counted and genera were identified by reference to Barnett and Hunter (12).

To examine populations of *Rhizoctonia* spp. in soil, 45 grams of dried soil was wetted to field capacity. Using a mechanical soil pelleter, 15 pellets were placed on five TABA plates for each plot sample (56). TABA plates were incubated in the dark at room temperature for five days. After five days, all colonies that produced a brown pigment (126) were counted and confirmed to be *Rhizoctonia* spp. by microscopic examination. The number of colony forming units per gram of soil (cfu/g) was calculated using the following equation: ln [1/1-y]*15*correction factor, where y=proportion of pellets confirmed to have *Rhizoctonia* spp. and the correction factor is the percent moisture and soil dilution factor (56). Data were analyzed using the GLM procedure of the SAS (Cary, NC).

Incidence of black shank was evaluated at two-week intervals, from transplant to harvest, and recorded as the number of plants in each plot with symptoms of disease. The area under the disease progress curve (AUDPC) (136) was calculated from incidence data and analyzed using the GLM procedure of the SAS (Cary, NC). Tobacco was harvested on 17 September in 2009 and 30 August in 2010 and leaves were air-cured for approximately three to four months; yield data was analyzed using the GLM procedure of the SAS (Cary, NC).

In 2009, tobacco roots from every plot were collected to examine the effect of mustard and wheat incorporation on below-ground symptoms. Each root was assigned a disease severity rating based on the Horsfall-Barratt scale (58). Horsfall-Barratt ratings were converted to percentages with the ELANCO formula (104) before analysis using the GLM procedure of the SAS (Cary, NC).

To estimate the biomass of wheat and mustard incorporated into the field, a 0.09 m^2 area was randomly chosen from each plot and plants were dug up on the day of incorporation in 2009 and 2010. The roots were washed and the whole plants were dried in an oven at 50˚C for one week. The dry weight of all plants was measured for each plot and a biomass rate (kg/ha) was calculated based on 90% plant water content (8). In 2010, roots and shoots of six randomly chosen mustard plants from each plot were lyophilized, ground in a Wiley mill (1-mm screen), and stored at -20°C until analyzed for concentration of glucosinolates.

Greenhouse experiments. The first experiment in the greenhouse was conducted to evaluate the effect of different amounts of mustard biomass resulting from different seeding rates on the survival of *P. nicotianae* chlamydospores in soil. Treatments were arranged in a completely randomized design. The experiment was not repeated. Treatments were replicated six times and included: 'Pacific Gold' mustard sown at 13.46 kg/ha (recommended seeding rate for field), 1.35 kg/ha, 6.73 kg/ha, and 26.90 kg/ha; wheat sown at 112.08 kg/ha; and negative control (peat-based medium).

'Pacific Gold' mustard and winter wheat in the greenhouse were seeded into 20.32 cm x 14.29 cm round plastic pots containing a peat-based medium (Pro Mix BX;

Premier Horticulture, Inc.; Quakertown, PA). Plants were fertilized at approximately 3 weeks, 5 weeks, and 7 weeks after seeding with 20-10-20 Peters Peat-Lite (150 ppm; WR Grace and Co., Fogelsville, PA). Plants were grown at 23°C and 1000 watt high pressure sodium bulbs (Eye Hortilux; Mentor, OH) were used to provide 12 hours of daylight. Once mustard was in bloom (ca. 8 weeks after seeding), all plant biomass above the soil line from each round pot was harvested and chopped with a stainless steel knife. The plant biomass was added to a polyethylene bag containing 2,966 cm^3 dry volume of the peat-based medium. Nitex mesh bag (10 micron nylon mesh; Sefar America, Depew, NY) were filled with 1 g of peat-based medium (1 g) infested with chlamydospores of *P. nicotianae* (ca. 100 chlamydospores/g of soil as determined with a hemacytometer) were placed in the center of each bag of peat/mustard mixture. Chlamydospore inoculum was prepared as described by Tsao (132). In 10% liquid V8 culture (200 ml), 3 mm plugs from 7 day-old cultures of *P. nicotianae* growing on corn meal agar were grown horizontally for 7 days in an amber medicine glass bottle (1 L) at room temperature in the dark. All liquid was drained and mycelium was resuspended in sterile deionized water (200 ml) then incubated at 18°C for 6-8 weeks.

Sealed bags containing the peat/mustard mixture and inoculum were stored in the dark at approximately 18°C/32°C (max/min) for seven days. After seven days, the Nitex bags were removed and the soil plus inoculum inside was diluted in 25 ml of 0.3% water agar amended with ampicillin (250 mg/L), and stirred for approximately 2 minutes. Five-ml of the dilution was plated on to 5 PARPH V8 plates and incubated in

the dark for five days. *P. nicotianae* colonies were counted and the data was analyzed using the GLM procedure of the SAS (Cary, NC).

Amounts of biomass were roughly equal for all seeding rates in the first experiment. A second experiment was conducted using harvested mustard to achieve different rates of biomass (g of fresh biomass/324 cm^2 area of a round pot) for assessment of effects on the viability of *P. nicotianae* chlamydospores in soil. Treatments were arranged in a completely randomized design and the experiment was carried out twice (run 1 and run 2). Treatments were replicated three to four times within an experiment and included: 'Pacific Gold' mustard at 10 g/324 cm^2 (equivalent to 3,086 kg/ha), 50 g/324 cm^2 (15,432 kg/ha), 100 g/324 cm^2 (30,864 kg/ha), 200 g/324 cm^2 (61,726 kg/ha), and 490 g/324 cm^2 (151,234 kg/ha); wheat at 75 g/324 cm^2 (23,148 kg/ha); and non-amended soil, using steam sterilized field soil (Fayette County, Maury silt loam).

Growing conditions for the plants, the treatment bag assembly, quantification of *P. nicotianae*, and data analysis was the same as described for the first experiment with two modifications. Gas-impermeable plastic bags (Saranex Bitran specimen bags, 60.96 cm x 60. 96 cm, Com-Pac International, Carbondale, IL) were substituted to contain volatile isothiocyanates and they were filled with steam sterilized field soil (2,966 cm^3) rather than the peat-based medium. Roots and shoots of six randomly chosen mustard plants from one experiment were lyophilized and stored as described above for analysis of glucosinolate concentration.

A third experiment in the greenhouse was conducted to compare the effects of incorporated mustard versus wheat at equivalent rates of biomass. Treatments were arranged in a completely randomized design and the experiment was repeated twice. Treatments were replicated three times within an experiment. Run 1 included: 'Pacific Gold' mustard at 10 g/324 cm^2 (equivalent to 3,086 kg/ha), 50 g/324 cm^2 (15,432 kg/ha), 100 g/324 cm^2 (30,864 kg/ha), 200 g/324 cm^2 (61,726 kg/ha), and 400 g/324 cm^2 (133,333); wheat at 50 g/324 cm^2 (15,432 kg/ha), 100 g/324 cm^2 (30,864 kg/ha), and 200 g/324 cm^2 (61,726 kg/ha); unplanted, non-sterilized field soil (Fayette County, Maury silt loam). Run 2 included the same 'Pacific Gold' mustard and unplanted, non-sterilized field soil treatments as in run 1 and wheat at 300 g/324 cm^2 (92,593 kg/ha);

Growing conditions for the plants, the treatment bag assembly, inoculation procedure, quantification of *P. nicotianae*, and data analysis was the same as described for the second experiment. Roots and shoots of six randomly chosen mustard plants were analyzed the day of harvest for concentration of glucosinolates.

Quantification of glucosinolates. The extraction of glucosinolates from fresh and lyophilized plant material was performed according to the method of Radovitch et al. previously described (5,102). For lyophilized plant tissue, a 200 mg subsample was heated in 5 ml of 90% boiling methanol in a capped, 20 ml glass vial for 15 minutes then vacuum-filtered through filter paper (Whatman no. 1; Piscataway, NJ). The remaining plant material was reheated twice with 5 ml of 70% boiling methanol, each for 3 minutes. The filtrates were combined, the methanol was evaporated under vacuum (IKA® RV 10 Basic; IKA® Works Inc., Wilmington, NC), and reconstituted in 10 ml of 70%

methanol. A homogeneous extract (10 ml) was prepared by filtration through a glass pasteur pipette (22.9 cm) containing a glass wool plug and 1 g of celite. Glucosinolates from fresh plant tissue were extracted as described above except that a 1 g subsample was heated in 10 ml of 90% boiling methanol and reheated twice in 10 ml of 70% boiling methanol.

The separation procedure described by Antonious et al. was used to quantify total glucosinolates with a few modifications (5). Pasteur pipettes (22.9 cm) with a glass wool plug were filled with preswelled (overnight in 2 M ammonium acetate) DEAE-Sephadex A-25 (2-[diethylamino] ethyl ether) resin (Sigma Chemical Co., St. Louis, MO) to give a settled height of 6 cm. The column was washed twice with 2 ml of deionized water. One-ml of the plant extract described above was added to column, allowed to completely drain, and washed with 2 mL of deionized water. A new 20 ml glass vial was placed under the column and 0.25 ml of thioglucosidase (dissolved in 5 mM phosphate buffer, pH 7; Sigma Chemical Co., St. Louis, MO) was added. The columns were covered with aluminum foil and incubated at room temperature for 18 h, then eluted with 1 ml of deionized water. Glucose (HK) assay kits (Sigma Chemical Co., St. Louis, MO) and UV-Vis spectrophotometry (340 nm) were used to determine the concentration of glucosinolates in the eluate (Spectronic BioMate 5, Thermo Electron Corporation, Waltham, MA). A calibration curve was created using 0.15, 0.10, and 0.05 mg/ml of glucose standard (Sigma Chemical Co., St. Louis, MO). Glucosinolate concentration was calculated as described by VanEtten et al. in which moles of glucose and glucosinolate were equimolar (137). Extracts without addition of thioglucosidase were used as a

control. Sinigrin (2-propenyl glucosinolate; 6 µmol, Sigma Chemical Co., St. Louis, MO) was used as an internal standard to estimate percent recovery.

RESULTS

Pilot field experiment 2008. Plots with the incorporated mustard had lower populations of *P. nicotianae* than non-amended (fallow) plots ($p \leq 0.1$, Table 2.1). Plant survival at the end of the growing season was significantly higher in plots incorporated with mustard relative to the fallow control ($p \leq 0.1$; Table 2.2). Average rainfall data showed no precipitation on the day of mustard incorporation and a total of ca. 2.66 cm in the 5-day period post-incorporation (Table 2.13).

Field experiments 2009 and 2010. The field biomass rate calculated for mustard was 11,679 kg/ha in 2009 and 36,770 kg/ha in 2010. The field biomass rate calculated for wheat was 41,753 kg/ha in 2009 and 14,628 kg/ha in 2010. In 2009, no significant differences in populations of *P. nicotianae, Fusarium* spp., *Rhizoctonia* spp., and saprophytic fungi were observed in the mustard- and wheat-amended plots ($p \leq 0.05$; Table 2.3). No significant differences were found between incorporated mustard or wheat for severity (AUDPC) of black shank ($p \leq 0.05$ NS; Table 2.4). Differences in disease severity between varieties were observed in the trial. Overall, severity of black shank was highest on 'KY 14 x L8', compared with 'TN 90', 'NC 7', and 'KT 206' ($p \leq 0.05$; Table 2.4). No significant differences were found between mustard and wheat for severity of disease on tobacco roots ($p \leq 0.05$; Table 2.4). No significant differences were found in yield between plots with mustard or wheat cover ($p \leq 0.05$; Table 2.4). Average rainfall

data before and after incorporation of mustard was less than rainfall averages reported in 2008 (Table 2.13).

In 2010, no differences were found in populations of *P. nicotianae* and *Fusarium* spp. in plots amended with wheat or mustard ($p \leq 0.05$; Table 2.5) For soils sampled 24 hours post-incorporation, populations of *Rhizoctonia* spp. were greater in the mustard-amended plots than the wheat ($p \leq 0.05$; Table 2.5). Populations of saprophytic fungi were significantly lower in plots amended with mustard than with wheat at the first sampling and 72 hours post-incorporation ($p \leq 0.05$; Table 2.5). Only three replications were included in the analysis of disease incidence because of damage due to animal feeding in the fourth replication. Incidence of black shank was similar in mustard- and wheat-amended plots ($p \leq 0.05$; Table 2.6). As in 2009, disease incidence was greater overall in 'KY 14 x L8' than in 'TN 90', 'NC 7', and 'KT 206' ($p \leq 0.05$; Table 2.6). Yield of cured leaf was not different between plots with mustard and wheat cover ($p \leq 0.05$; Table 2.6). Average rainfall data before and after incorporation of mustard was less than rainfall averages reported in 2008 (Table 2.13).

Greenhouse experiments. In the first greenhouse experiment, the amount of harvested biomass did not differ between mustard seeding rates (Table 2.7). All four mustard treatments significantly reduced chlamydospore survival in soil as compared to the non-amended control ($p \leq 0.05$; Fig. 2.8). Wheat-amended soil had no effect on chlamydospore survival ($p \leq 0.05$; Fig. 2.8).

In the second greenhouse experiment, the highest amount (490 g/324 cm^2) of mustard significantly reduced populations of *P. nicotianae* in soil compared to the wheat

and non-amended control ($p \leq 0.05$; Tables 2.9 and 2.10). In the first run (Table 2.9), the control had a lower survival of chlamydospores than all but the high mustard (490 g/324 cm^2). In the second run of the study, all four of the mustard treatments had lower survival of chlamydospores as compared to the non-amended control and the wheat was no different than the non-amended control ($p \leq 0.05$; Table 2.10).

In the third greenhouse experiment, when the rate of wheat and mustard biomass was increased to 200 g/324 cm^2 or higher, survival of chlamydospores was reduced (Tables 2.11 and 2.12).

Quantification of glucosinolates. There were no significant differences in mean glucosinolate content between field and greenhouse grown plants ($p \leq 0.05$; Table 2.14). In the field, glucosinolate content was higher in the shoots (17.4 µmol/g) than in the roots (8.8 µmol /g) (data not shown). Efficiency of the system was calculated to be 95% when using sinigrin as an internal standard.

DISCUSSION

The pilot field study in 2008 showed potential for using biofumigation to control black shank in Kentucky, because populations of *P. nicotianae* were lower after incorporation and survival at the end of the season was higher in mustard-amended plots. More experiments were carried out to evaluate the applicability of biofumigation in tobacco production. However, field studies in 2009 and 2010 did not show that Brassica amendments could lower populations of *P. nicotianae* in the soil, incidence of black shank, or increase yield. Additionally, there was not enough space at the site of our field studies to include a fallow control. Although in the same field, the space we

used to start our large-scale biofumigation study in 2009 did not overlap with the portion used in 2008. Therefore, our first soil sample collected in 2009 was comparable to fallow ground since no cover crops had been previously planted in these plots. Additionally, due to herbivory and dry weather, the wheat did not grow very well and many of the wheat plots were filled with weeds. Under these conditions, it is possible that the wheat plots we examined in 2009 were similar to a fallow control.

When examining populations of other fungi in the soil, there were differences in how each one responded (population increase or decrease) due to Brassica amendments which is similar to results observed by Zasada et al. (142). In their study, *Fusarium* spp. were not significantly reduced between different Brassica amendments, while citrus nematode populations were only reduced with specific Brassicas. The differences reported by Zasada et al. in pathogen response to biofumigation can be influenced by several factors, such as Brassica-specific toxicity, soil moisture, and glucosinolate content of different Brassicas (31,90).

With regards to soil moisture, rainfall varied between 2008, 2009, and 2010. On the day of mustard incorporation, there was no rainfall in 2008 and 2009, while trace amounts of rainfall were recorded in 2010. Even with no rainfall on the day of mustard incorporation, populations of *P. nicotianae* were still significantly lower in the 2008 field study. Rainfall (total ca. 2.66 cm) occurred every day for 6 days post mustard incorporation in 2008. Gimsing and Kirkegaard found that both isothiocyanate and glucosinolate concentrations were highest in the soil 30 minutes after Brassica incorporation with detectable levels for up to 12 and 8 days, respectively (43). If

glucosinolates were present in the soil for up to 8 days, the additional soil moisture in 2008 might explain why there were lower populations of *P. nicotianae* in mustard-amended plots than observed in 2009 or 2010.

Our greenhouse experiments revealed an unexpected factor that could explain the lack of *P. nicotianae* suppression in the field. Incorporation of a high rate of mustard or wheat biomass (≥200 g/324 cm^2) reduced chlamydospore survival of *P. nicotianae* in the soil. The high rate of mustard and wheat biomass incorporation (as much as 133,333 kg/ha in a field setting) used in the greenhouse was as much as three to ten times more than obtained in our field studies (11,679-41,753 kg/ha). These results suggested that the reduction in chlamydospore survival had more to do with biomass than biofumigants. These results are similar to those reported by Larkin and Griffin (74). Larkin and Griffin found that a non-Brassica crop (barley) reduced *in-vitro* growth of *Rhizoctonia solani* and soil populations of *R. solani* in the greenhouse were significantly lower after barley was incorporated. Additionally, they observed in the field that another non-Brassica, ryegrass, was also effective at reducing incidence and severity of powdery scab on potato. As discussed by Larkin and Griffin, the suppression of soilborne pathogens by non-Brassicas suggests that there must be an alternative mechanism, which is not related to volatile isothiocyanates released from Brassica tissues. One possible explanation includes changes in microbial populations that occur when using rotation crops. It has been documented that rotation crops may enhance moisture content of soil, reduce erosion, support diverse populations of soil microbes that are

beneficial for disease suppression, and increase availability and plant uptake of nutrients such as nitrogen (1,11).

At the time of our 2008 field study, companies were communicating to farmers that incorporating a spring mustard crop into the soil would control black shank in the field. Based on our studies, we do not recommend biofumigation to tobacco growers in Kentucky as a viable management practice for black shank in the short-term. One additional benefit beyond disease suppression that can be provided by Brassica cover crops is the increase of beneficial soil microbes. Larkin et al. documented that the long-term (3 years or longer) use of Brassica cover crops increased populations of bacteria and other microbial populations that were beneficial for disease suppression of soilborne potato diseases, decreased soil erosion, increased yield, and enhanced soil quality (75).

Table 2.1. Effect of incorporation 'Pacific Gold' mustard on populations of *P. nicotianae*.

	cfu/g of soil[1]		
Soil Sampling Date	**Mustard[2]**	**Bare Ground[3]**	***p*-value[5]**
28 May 2008	5.33b[4]	10.48a	0.08
19 June 2008	1.43b	2.93a	0.02
6 August 2008	0.75a	3.03a	0.18
28 August 2008	0.65a	1.00a	0.51

[1]Average colony forming units (cfu) per g of soil, determined by dilution plating on a selective medium.
[2]Mustard (*Brassica juncea* 'Pacific Gold') was mowed and incorporated on 30 May in 2008.
[3]Bare ground = fallow ground with no cover crop.
[4]Means in the same row followed by the same letter are not statistically different as determined by Fisher's LSD test ($p \leq 0.1$).
[5]Calculated *p*-values for pairwise comparisons of *P. nicotianae* populations in mustard-amended versus bare ground plots.

Table 2.2. Effect of incorporated mustard versus bare ground on the incidence of black shank on burley tobacco survival field study in 2008.

	Survival[1]		
Date	**Mustard[2]**	**Bare Ground[3]**	***p*-value[5]**
16 June 2008	57.5a[4]	57a	0.72
1 July 2008	57.5a	56.5a	0.53
22 July 2008	56.75a	55.25a	0.55
20 August 2008	49a	48.75a	0.95
23 September 2008	47.5a	41.5b	0.07

[1]Combined mean plant survival (total number of healthy plants) for burley tobacco varieties KT 204 and KT 206.

[2]Mustard (*Brassica juncea* 'Pacific Gold') was mowed and incorporated on 30 May in 2008.

[3]Bare ground = fallow ground with no cover crop.

[4]Means in the same row followed by the same letter are not statistically different as determined by Fisher's LSD test ($p \leq 0.1$).

[5]Calculated *p*-values for pairwise comparisons of incidence of black shank in mustard-amended versus bare ground plots.

Table 2.3. Effect of incorporated mustard versus wheat cover crops on populations of *Phytophthora nicotianae*, *Fusarium* spp., saprophytic fungi, and *Rhizoctonia* spp. in soil for field study in 2009.

	Populations (cfu/g of soil)[1]											
	P. nicotianae			***Fusarium* spp.**			**Saprophytic fungi**			***Rhizoctonia* spp.**		
Cover	**3 Apr**	**19 May**	**22 May**	**3 Apr**	**19 May**	**22 May**	**3 Apr**	**19 May**	**22 May**	**3 Apr**	**19 May**	**22 May**
Mustard[2]	1.50a[4]	0.88a	0.13a	59,350a	50,925a	76,350a	64,250a	98,575a	66,275a	31.69a	38.88a	36.44a
Wheat[3]	2.50a	1.00a	0.63a	63,150a	49,650a	80,825a	65,750a	100,125a	75,925a	37.25a	36.44a	37.06a
***p*-value[5]**	0.28	0.89	0.25	0.39	0.86	0.32	0.82	0.86	0.28	0.74	0.57	0.28

[1]Average colony forming units (cfu) per g of soil, determined by dilution plating on selective media.

[2]Mustard (*Brassica juncea* 'Pacific Gold') was mowed and incorporated on 20 May in 2009.

[3]Winter wheat was mowed and incorporated on 20 May in 2009.

[4]Means in the same column followed by the same letter are not statistically different as determined by Fisher's LSD test ($p \leq 0.05$).

[5]Calculated *p*-values for pairwise comparisons of populations of fungi in mustard-amended versus bare ground plots.

Table 2.4. Mean AUDPC, root disease severity, and yield for whole plot (cover crop) and sub plot (variety) for field study in 2009 examining the potential of biofumigation for control of black shank of burley tobacco.

Cover Crop	AUDPC[1]	Root Disease Severity[2]	Yield (kg/ha)
Mustard[3]	13.4a[5]	26.97a	2128a
Wheat[4]	13.1a	28.04a	2157a
***p*-value[6]**	0.72	0.09	0.91
Variety			
'KY 14 x L8'	48.7a	100.00a	0a
'TN 90'	1.7b	3.45b	2617b
'NC 7'	1.3b	3.50b	2777b
'KT 206'	1.4b	3.06b	3175b
***p*-value[6]**	<0.0001	<0.0001	<0.0001

[1]Mean area under the disease progress curve (AUDPC) was calculated using 7 ratings of disease incidence taken at 2-week intervals.

[2] Percent root damage was determined using the Horsfall-Barratt scale and values were converted to percentages using the ELANCO formula.

[3]Mustard (*Brassica juncea* 'Pacific Gold') was mowed and incorporated on 20 May in 2009.

[4] Winter wheat was mowed and incorporated on 20 May in 2009.

[5]Means in the same column followed by the same letter are not statistically different as determined by Fisher's LSD test ($p \leq 0.05$).

[6]Calculated *p*-values for pairwise comparisons of populations of fungi in mustard-amended versus bare-ground plots.

Table 2.5. Effect of incorporated mustard versus wheat cover crops on populations of *Phytophthora nicotianae*, *Fusarium* spp., saprophytic fungi, and *Rhizoctonia* spp. in soil for field study in 2010.

	Populations (cfu/g of soil)[1]											
	P. nicotianae			***Fusarium* spp.**			**Saprophytic fungi**			***Rhizoctonia* spp.**		
Cover	**3 Apr**	**19 May**	**22 May**	**3 Apr**	**19 May**	**22 May**	**3 Apr**	**19 May**	**22 May**	**3 Apr**	**19 May**	**22 May**
Mustard[2]	3.00a[4]	2.35a	0.75a	14,479a	15,785a	12,080a	12,530a	20,020a	18,680a	5.68a	2.15a	3.64a
Wheat[3]	4.00a	5.75a	1.00a	14,300a	12,560a	16,595a	14,615b	21,780a	27,875b	5.36a	1.25b	2.79a
***p*-value[5]**	0.46	0.18	0.77	0.87	0.06	0.10	0.03	0.32	0.003	0.75	0.04	0.15

[1]Average colony forming units (cfu) per g of soil, determined by dilution plating on selective media.
[2]Mustard (*Brassica juncea* 'Pacific Gold') was mowed and incorporated on 2 June in 2010.
[3]Winter wheat was mowed and incorporated on 2 June in 2010.
[4]Means in the same column followed by the same letter are not statistically different as determined by Fisher's LSD test ($p \leq 0.05$).
[5]Calculated *p*-values for pairwise comparisons of populations of fungi in mustard-amended versus bare ground plots.

Table 2.6. Mean AUDPC and yield for whole plot (cover crop) and sub plot (variety) for field study in 2010 examining the potential of biofumigation for control of black shank of burley tobacco.

Cover Crop	AUDPC[1]	Yield (kg/ha)
Mustard[2]	26.32a[4]	1182a
Wheat[3]	25.25a	1123a
***p*-value[5]**	0.83	0.85
Variety		
'KY 14 x L8'	45.44a	205b
'TN 90'	12.33b	1049ab
'NC 7'	20.84b	1408a
'KT 206'	12.33b	1947a
***p*-value[5]**	<0.0001	0.01

[1]Mean area under the disease progress curve (AUDPC) was calculated using 7 ratings of disease incidence taken at 2-week intervals.

[2]Mustard (*Brassica juncea* 'Pacific Gold') was mowed and incorporated on 2 June in 2010.

[3]Winter wheat was mowed and incorporated on 2 June in 2010.

[4]Means in the same column followed by the same letter are not statistically different as determined by Fisher's LSD test ($p \leq 0.05$).

[5]Calculated *p*-values for pairwise comparisons of populations of fungi in mustard-amended versus bare-ground plots.

Table 2.7. Fresh biomass weights of wheat and mustard grown in the greenhouse at different seeding rates.

Cover crop	Seeding rate (kg/ha)	Biomass (g)
Wheat	111.98	138.83a[1]
Mustard	1.35	572.33b
Mustard	6.73	516.22b
Mustard	13.46	587.72b
Mustard	26.90	567.84b

[1]Means in the same column followed by the same letter are not statistically different as determined by Fisher's LSD (p<0.0001).

Table 2.8. Greenhouse study examining the effect of different amounts of mustard biomass resulting from different seeding rates on populations of *Phytophthora nicotianae* in soil, 2009.

Treatments	Seeding rate (kg/ha)	cfu/g of soil[1]	*p*-value[2]
Non-amended control	--	215.00a[3]	--
Wheat	111.98	144.67ab	0.14
Mustard	26.90	94.00bc	0.02
Mustard	13.46	20.00c	0.0004
Mustard	6.73	36.00c	0.0009
Mustard	1.35	50.00c	0.002

[1]Mean colony forming units (cfu) per g of soil, determined by dilution plating on selective medium.

[2]Calculated *p*-values for pairwise comparisons of populations of *P. nicotianae* in mustard- and wheat-amended plots versus the non-amended control.

[3]Means in the same column followed by the same letter are not statistically different as determined by Fisher's LSD test ($p \leq 0.05$).

Table 2.9. Run 1 of greenhouse study examining the effect of mustard versus wheat amendments on populations of *Phytophthora nicotianae* in the soil, 2010.

Treatment	Biomass (g)	cfu/g of soil[1]	*p*-value[2]
Non-amended Control	--	35.00c[3]	--
Wheat	75	43.00bc	0.45
Mustard	10	72.00a	0.0006
Mustard	50	71.00a	0.0008
Mustard	100	60.00ab	0.02
Mustard	200	62.55ab	0.01
Mustard	490	11.00d	0.02

[1]Mean colony forming units (cfu) per g of soil, determined by dilution plating on selective medium.

[2]Calculated *p*-values for pairwise comparisons of populations of *P. nicotianae* in mustard- and wheat-amended plots versus the non-amended control.

[3]Means in the same column followed by the same letter are not statistically different as determined by Fisher's LSD test ($p \leq 0.05$).

Table 2.10. Run 2 of greenhouse study examining the effect of mustard versus wheat amendments on populations of *Phytophthora nicotianae* in the soil, 2010.

Treatment	Biomass (g)	cfu/g of soil[1]	*p*-value[2]
Non-amended Control	--	262.20a[3]	--
Wheat	75	259.00ab	0.90
Mustard	10	166.00cd	0.0002
Mustard	50	211.00bc	0.04
Mustard	100	210.00bc	0.04
Mustard	200	207.00c	0.03
Mustard	490	132.00d	<0.001

[1]Mean colony forming units (cfu) per g of soil, determined by dilution plating on selective medium.

[2]Calculated *p*-values for pairwise comparisons of populations of *P. nicotianae* in mustard- and wheat-amended plots versus the non-amended control.

[3]Means in the same column followed by the same letter are not statistically different as determined by Fisher's LSD test ($p \leq 0.05$).

Table 2.11. Run 1 of greenhouse study examining the effect of equivalent rates of mustard and wheat amendments on populations of *Phytophthora nicotianae* in the soil, 2012.

Treatment	Biomass (g)	cfu/g of soil[1]	*p*-value[2]
Non-amended Control	--	24.00abc[3]	--
Wheat	50	16.67abc	0.16
Wheat	100	18.67bcd	0.30
Wheat	200	14.00cd	0.06
Mustard	10	32.00a	0.12
Mustard	50	24.00abc	0.53
Mustard	100	28.00ab	0.44
Mustard	200	28.00ab	0.44
Mustard	400	9.33d	0.005

[1]Mean colony forming units (cfu) per g of soil, determined by dilution plating on selective medium.

[2]Calculated *p*-values for pairwise comparisons of populations of *P. nicotianae* in mustard- and wheat-amended plots versus the non-amended control.

[3]Means in the same column followed by the same letter are not statistically different as determined by Fisher's LSD test ($p \leq 0.05$).

Table 2.12. Run 2 of greenhouse study examining the effect of equivalent rates of mustard and wheat amendments on populations of *Phytophthora nicotianae* in the soil, 2012.

Treatment	Biomass (g)	cfu/g of soil[1]	*p*-value[2]
Non-amended Control	--	12.67a	--
Wheat	300	4.67bc	0.005
Mustard	10	6.00b	0.01
Mustard	50	3.33bc	0.002
Mustard	100	5.33b	0.008
Mustard	200	4.67bc	0.005
Mustard	400	0.00c	0.0001

[1]Mean colony forming units (cfu) per g of soil, determined by dilution plating on selective medium.

[2]Calculated *p*-values for pairwise comparisons of populations of *P. nicotianae* in mustard- and wheat-amended plots versus the non-amended control.

[3]Means in the same column followed by the same letter are not statistically different as determined by Fisher's LSD test ($p \leq 0.05$).

Table 2.13. Average monthly temperature (max/min) and rainfall (cm) data for Lexington, KY from 2008-2010.[1]

2008			2009			2010		
	Temperature (max/min°C)	**Rainfall (cm)**		**Temperature (max/min°C)**	**Rainfall (cm)**		**Temperature (max/min°C)**	**Rainfall (cm)**
27 May	27/13	Trace	**17 May**	16/8	0	**30 May**	30/19	0
28 May	22/10	0.05	**18 May**	18/4	0	**31 May**	28/20	0
29 May	25/10	0	**19 May**	23/4	0	**1 June**	29/19	0
30 May[2]	27/14	0	**20 May**[2]	26/9	0	**2 June**[2]	31/20	Trace
31 May	29/21	Trace	**21 May**	28/13	0	**3 June**	30/19	0
1 June	28/18	0.13	**22 May**	29/17	0	**4 June**	28/18	Trace
2 June	29/16	0.03	**23 May**	29/16	0.25	**5 June**	31/21	0
3 June	27/18	2.5	**24 May**	27/18	Trace	**6 June**	26/17	0.13
4 June	30/22	Trace	**25 May**	26/19	1.85	**7 June**	27/15	0

[1]Average temperature and rainfall data was obtained from NOAA National Climatic Data Center.

[2]Day of mustard incorporation for field studies in 2008, 2009, and 2010.

Table 2.14. Average glucosinolate content of *Brassica juncea* 'Pacific Gold' grown in field and greenhouse studies during 2010 and 2012.

	Glucosinolate content (µmol g^{-1})
Field[1]	26.0±17.0a[2]
Greenhouse[1]	45.0±25.8a
***p*-value**	0.20

[1]Mean root and shoot combined content from six subsamples and three replications.
[2]Means in the same column followed by the same letter are not significantly different as determined by Fisher's LSD ($p \leq 0.05$).

CHAPTER THREE:
EFFECT OF AN ORGANIC, YEAST FERMENTATION-DERIVED PRODUCT ON *PHYTOPHTHORA NICOTIANAE*, THE CAUSAL AGENT OF BLACK SHANK OF TOBACCO

INTRODUCTION

Black shank is caused by *Phytophthora nicotianae*, a soilborne oomycete pathogen of tobacco that is classified in the Kingdom Straminopila. Worldwide, *P. nicotianae* causes significant economic losses in tobacco production. In 2010, burley tobacco production in Kentucky was valued at $210,600,000. Annual losses to black shank around the state of Kentucky have been estimated at between 1 to 5%, which is worth approximately $10 million (95). Black shank is difficult to control due to the persistence of the *P. nicotianae* survival structures, chlamydospores, which persist in the soil for 5 or more years (7). Current recommendations for control include cultural methods (sanitation and rotation), resistance, and chemical (111). Fungicides for control of black shank are restricted to one class, the phenylamides, which include mefenoxam and metalaxyl. Although no resistance has been observed in *P. nicotianae* isolates from the field, the continued use of these fungicides that target a single site (29) could lead to resistance. Mefenoxam resistance has been observed in other oomycetes such as *Pernospora tabacina*, the causal agent of blue mold of tobacco (17). Alternative pesticides with different modes of action against *P. nicotianae* would help to improve the sustainability of chemical control in integrated plant management programs for black shank.

Soil-Set (Stubble Aid and Stubble Aid Plus; Alltech Crop Science, Nicholasville, KY) is commercially sold as a soil additive. Soil-Set is derived from Alltech's patented yeast

fermentation technology. It contains enzymes, nutrients, and bacterial metabolites to enhance decomposition of plant material and improve soil composition for plant growth (2,3). In 2011, Soil-Set was added to the OMRI (Organic Materials Research Institute) products list for restricted use as a fertilizer under conditions when plants or the soil are deficient in iron, copper, manganese and zinc (96).

Soil-Set also has potential as an anti-microbial pesticide. Soil-Set's complex repertoire of enzymes includes cellulases, which degrade cellulose. Cellulose is an important structural component in the cell walls of plants and oomycetes including *P. nicotianae* (52,86). A limited amount of information has been published on the effectiveness of Soil-Set in the control of plant diseases (13,131). Bellotte et al. found that Soil-Set + Compostaid (Alltech Crop Science, Nicholasville, KY) increased the rate of decomposition of citrus leaves, which serve as a source of inoculum for the fungus *Guignardia citricarpa* (13). Tosun et al. observed that four soil applications of Soil-Set (2 L of Soil-Set/100 L of water) in the greenhouse was effective at suppressing disease symptoms on tomato and pepper plants caused by *Rhizoctonia solani*, *Pythium spp.,* and *Pseudomonas syringae* pv. *tomato* (131). The purpose of the current study was to examine the effect of Soil-Set on *P. nicotianae in vitro*, and on the incidence of black shank on tobacco in the field and greenhouse.

MATERIALS AND METHODS

Two isolates of *P. nicotianae* (New 202 and 301 C4) collected from the tobacco field that was described in Chapter 2 of this thesis, were used for the *in vitro* and greenhouse experiments described below. Both isolates were determined to be race 1

(data not shown) using the laboratory assay described by Gutiérrez and Mila (47). Cultures were maintained on corn meal agar (CMA; Difco Laboratories, Detroit, MI) and CMA amended with 10% clarified V8 juice at 25°C in the dark.

Fungicide sensitivity. Isolate 301 C4 of *P. nicotianae* was tested for sensitivity to mefenoxam. V8 medium was amended with mefenoxam (Ridomil Gold EC, Syngenta Crop Protection, Greensboro, NC) at 1 ppm, 5 ppm, 10 ppm, and 100 ppm (active ingredient). Non-amended V8 plates were used as a control. Mycelial plugs (5 mm diameter), taken from the edge of 4-day-old cultures of *P. nicotianae* maintained on V8 medium, were placed in the centers of six replicates each of amended and non-amended Petri plates (100 x 15 mm). All plates were incubated at 25°C in the dark. When mycelial growth reached the edge (ca. 5-6 days) of the Petri plate, perpendicular measurements of the colony diameter were averaged and the plug diameter was subtracted. The experiment was conducted twice with similar results. Differences between mean diameters were analyzed using the GLM procedure of the SAS (SAS institute, Cary, NC).

The percent inhibition was calculated using the following formula: [(average diameter of untreated – average diameter of treated)/average diameter of treated] X 100. The concentration of fungicide effective at inhibiting 50% of mycelial growth (EC_{50}) was estimated by plotting the percent inhibition against the $\log_{10}$ of mefenoxam concentration (ppm). Analysis using nonlinear regression models found that the mechanistic growth curve best fit the data (R^2=1; JMP statistical software, SAS institute, Cary, NC). With the mechanistic growth curve formula, $a(1-b\text{Exp}(-cx))$ where a relates to

the asymptote, *b* to the scale, *c* the growth rate, and x the concentration (ppm), the EC_{50} was calculated using the data parameters obtained.

Soil-Set bioassay. The effect of Soil-Set on the mycelial growth of *P. nicotianae* was first examined by amending CMA with Soil-Set (% v/v). The active ingredient of Soil-Set is unknown therefore the concentration is not expressed in ppm but as percent volume (volume of Soil-Set/total volume of solution). Final concentrations included: 0.1% (1 ml/L), 0.05% (0.5 ml/L), 0.01% (0.1 ml/L), and 0.001% (0.01 ml/L). Non-amended CMA was used as a control. Mycelial plugs (5 mm), taken from the edge of 7-day-old cultures of *P. nicotianae* (isolates 202 and 301 C4) growing on CMA, were placed in the centers of six replicate plates of amended and non-amended Petri plates. All plates were incubated at 25°C in the dark. Once mycelium on control plates grew to the edge (ca. 10-14 days) of the Petri plate, perpendicular measurements of the colony diameter were taken on all plates, averaged, and the plug diameter was subtracted. The experiment was repeated once.

Due to poor growth on CMA, mycelial growth of *P. nicotianae* was also examined by amending V8 medium with Soil-Set® (% v/v) as described above. Non-amended V8 medium (containing no Soil-Set) and V8 amended with mefenoxam at 1 ppm were used as controls. Mycelial plugs (5 mm), taken from the edge of 4-day-old cultures of *P. nicotianae* (301 C4) growing on V8 medium, were placed in the centers of amended and non-amended Petri plates. All plates were incubated at 25°C in the dark. Once mycelial growth of the non-amended controls grew to the edge (ca. 5-6 days) of the Petri plate, perpendicular measurements of the colony diameter were taken on all plates, averaged,

and the plug diameter was subtracted. The experiment was repeated once. Differences between mean diameters were analyzed using the GLM procedure of the SAS (SAS institute, Cary, NC).

The percent inhibition was calculated as described above for the mefenoxam sensitivity assay. The percent volume of Soil-Set effective at inhibiting 50% of mycelial growth (EC_{50}) was estimated by plotting percent inhibition against the $\log_{10}$ of the percent volume (% v/v). Analysis using nonlinear regression models found that the Gompertz 3 parameter sigmoidal function best fit the data (R^2=0.98; JMP statistical software, SAS institute, Cary, NC). The Gompertz formula has been used to estimate the EC_{50} of mefenoxam against *Phytophthora erythroseptica* and *Pythium ultimum* isolates from North America (127). With the Gompertz formula, $a\text{Exp}(-\text{Exp}(-b(x-c)))$ where *a* related to the asymptote, *b* the growth rate, *c* the inflection point, and x the concentration (% v/v), the EC_{50} was calculated using the data parameters obtained.

Greenhouse experiment. Inoculum for greenhouse experiments was produced by infusing oat grains (500 g) with 10% V8 juice (filtered through cheesecloth) and autoclaving once for 30 minutes at 23 psi and 122°C. *P. nicotianae* was grown in liquid culture (5% V8 juice) for seven days and was used to inoculate the V8-infused oat grains. The inoculated oat grains were grown at 18°C for 4-6 weeks and air-dried in the hood overnight. Grain was ground into a fine powder using a Wiley mill and stored at 18°C until used to inoculate soil.

The burley varieties 'KY 14 x L8', 'TN 90', and 'Cross Creek 812' (10/0, 4/4, and 9/9 levels of resistance to races 0 and 1 of *P. nicotianae*, respectively) were used in the

greenhouse to examine the effect of Soil-Set on incidence of black shank, root disease severity, and plant dry weight. All burley tobacco varieties were grown under the same conditions in the greenhouse. Each variety was seeded into trays filled with Pro Mix BX (Premier Horticulture, Inc., Quakertown, PA). Four weeks after germination, each variety was transplanted into a 32-cell Styrofoam tray, floated in a vessel containing water, and grown for 3-4 additional weeks at 23°C. All plants in the greenhouse were grown under 1000 watt high pressure sodium bulbs (Eye Hortilux, Mentor, OH) to provide 12 hours of daylight.

Steam-sterilized soil (531 cm^3) was added to square pots (89 mm x 89 mm) inoculated with 5 cm^3 of powdered inoculum of *P. nicotianae* and thoroughly mixed. Tobacco seedlings (ca. 7-8 weeks old) were transplanted individually into inoculated soil and solutions of mefenoxam or Soil-Set (ca. 118 ml per pot) were added directly to the soil. Five mycelial culture (isolate 301 C4) plugs (5-mm dia.) were added to the soil ca. 3 days post-transplant.

Incidence of black shank was assessed in the greenhouse by counting the number of symptomatic plants every 3-4 days for 4 weeks. The area under the disease progress curve (AUDPC) was calculated using the incidence data (136). The Horsfall-Barratt scale was used to score root rot severity and the values were converted to percentages using the ELANCO formula (58,104). Plants were dried in an oven at 71°C for 7 days and dry weight was recorded.

A 3 x 5 factorial design was used and treatments were replicated six times. Factors were tobacco variety ('KY 14 x L8', 'TN 90', and 'CC 812') and soil treatment

(non-infested soil control, untreated *P. nicotianae* control, Soil-Set 1.2 L/ha, Soil-Set 4.8 L/ha, and mefenoxam) resulting in 15 treatment combinations (not randomized). The experiment was conducted once.

Factor interactions were analyzed using a two-way ANOVA and differences between means were analyzed using Tukey's honestly significant difference (HSD) test (JMP statistical software, SAS institute, Cary NC).

Field experiment. In 2012, a field experiment was conducted at the same location described in Chapter 2. The burley variety Cross Creek 812 was used in this experiment. Treatments were: Soil-Set (1.2 L/ha and 4.8 L/ha), mefenoxam (Ridomil Gold SL, 1.2 L/ha) and a water control. Each treatment was replicated 3 times in a randomized complete-block design. Tobacco was transplanted on 21 May. Treatments were applied three days after transplant on 24 May by drenching each plant with 118 ml of Soil-Set or fungicide solution. A funnel attached to a 3-m section of PVC tubing was used to direct the solution to the base of the plant. The number of plants with symptoms of black shank (incidence) was assessed every two weeks through the growing season. Data were analyzed using the GLM procedure of the SAS (SAS institute, Cary, NC).

RESULTS

***In vitro* experiment.** Rates of mefenoxam at least 1 ppm or higher completely inhibited the mycelial growth of *P. nicotianae* (Fig. 3.1, Table 3.1). The EC_{50} value calculated was 0.08 ppm. Growth of *P. nicotianae* on both amended and non-amended

CMA was slow and sparse. The colony diameter was difficult to measure accurately due to uneven growth, which included runner hyphae (Fig. 3.2).

On CMA, no mycelial growth was observed on the 0.1% Soil-Set treatment plates and very little mycelial growth with a few runner hyphae was observed on the 0.05% Soil-Set treatment plates. On V8 medium, *P. nicotianae* produced the characteristic rosette pattern (34) and the diameters were easier to measure due to more uniform growth than seen on the CMA (Fig. 3.3, Table 3.1). Mefenoxam inhibited mycelial growth of *P. nicotianae* more effectively than Soil-Set (Table 3.1). However, all but the lowest treatment of Soil-Set did significantly inhibit mycelial growth of *P. nicotianae* as compared to the non-amended control (Table 3.1). The EC_{50} calculated for Soil-Set was 0.38% v/v.

Greenhouse experiment. A two-way ANOVA (α=0.05) analysis showed significant interaction between factors (treatment and variety). Due to the interaction between factors (Table 3.2), data were analyzed separately for each variety (Tables 3.3-3.5). For 'KY 14 x L8', which has no resistance to race 1 of *P. nicotianae*, mefenoxam was the best treatment for control of black shank symptoms. When compared to the *P. nicotianae*-infested control, the severity of black shank (AUDPC) was less for Soil-Set at 4.8 L/ha than at the recommended rate of Soil-Set at 1.2 L/ha (Table 3.3). Mefenoxam and Soil-Set at 4.8 L/ha were the best treatments for reducing the percent root damage of 'KY 14 x L8'. The Soil-Set 1.2 L/ha treatment was no different than the *P. nicotianae*-infested control for percent root damage on 'KY 14 x L8'. Un-inoculated controls of 'KY

14 x L8' had the highest dry weights. When comparing all of the pesticide treatments, 'KY 14 x L8' plants treated with Soil-Set at 4.8 L/ha were higher in dry weight (Table 3.3).

For 'TN 90', which has moderate resistance to race 1 of *P. nicotianae*, no treatment was better at reducing disease severity (AUDPC) of black shank or percent root damage. Plants in the non-infested control had the highest dry weights as compared to all of the other treatments. When compared to all of the pesticide treatments, 'TN 90' plants treated with Soil-Set at 4.8 L/ha had greater dry weight, but they were no different in weight than the *P. nicotianae*-infested control (Table 3.4).

For 'CC 812', which has high resistance to race 1 of *P. nicotianae*, no treatment was better at reducing disease severity (AUDPC) of black shank or percent root damage. Plants in the non-infested control had the highest dry weights as compared to all of the other treatments. When compared to all of the pesticide treatments, 'CC 812' plants treated with Soil-Set at 4.8 L/ha had greater dry weight, but they were lower in weight than the *P. nicotianae*-infested control (Table 3.5).

Field experiment. The mean AUDPC and standard deviation for each treatment was calculated and no treatment was better than any of the others at reducing disease severity (Table 3.6).

DISCUSSION

Tosun reported inhibition of growth of *Phytophthora infestans* on CMA amended with Stubble Aid (130). Therefore, in this study, we also used CMA as our base medium for the *in-vitro* assay. Soil-Set appeared to be more inhibitory to *P. nicotianae* growing on CMA than V8. *Phytophthora* spp. do not produce their own sterols (53).

Common membrane sterols, phosphatidylcholine and phosphatidylethanol-amine, found in fungi such as *Neurospora crassa* or *Sacchromyces pasterianus* are not found in *P. nicotianae* (55). It has been observed that the addition of sterols to medium from sources such as vegetable juice promotes sporulation and mycelial growth (53,54). The poor mycelial growth of *P. nicotianae* on CMA was most likely caused by a lack of sterols. The complete inhibition of *P. nicotianae* on CMA with 0.1% v/v Soil-Set and minimal inhibition of *P. nicotianae* on V8 medium with 0.1% v/v Soil-Set, may be explained by the presence of sterols. Papavizas et al. reported that the addition of sterols reversed the fungitoxicity of a carbamate fungicide to eight *Pythium* spp. *in vitro* (98). *P. nicotianae* isolate 301 C4 was sensitive to mefenoxam with as little as 1 ppm inhibiting mycelial growth and therefore serving as a good positive control for the greenhouse experiment.

In the greenhouse experiment, pots were grouped into trays based on treatment to prevent chemicals from mixing and *P. nicotianae* from spreading to the non-infested controls. All plants grown in the sterilized non-inoculated soil were healthy with no symptoms of disease, which indicated that the observations in this experiment were due to inoculation with *P. nicotianae*.

The interaction between treatment and variety suggested that the effect of soil-applied chemicals was influenced by the amount of resistance to black shank in the varieties. For, 'KY 14 x L8', with no resistance to *P. nicotianae* (race 1), mefenoxam was the most effective treatment for control of black shank. Although not as effective as mefenoxam, the high dose of Soil-Set at 4.8 L/ha did lower incidence of black shank and

it was just as effective as mefenoxam in reducing root disease severity when used in combination with 'KY 14 x L8'. The recommended dose of Soil-Set at 1.2 L/ha had no effect on *P. nicotianae*. Soil-Set was approved for the use as a fertilizer in organic production and the plant dry weight for the high dose was greater than all the other treatments except the non-infested control. The differences in dry weight observed between the non-infested controls and the other treatments can be explained by infection of roots by *P. nicotianae*. Sullivan et al. (124) observed significant stunting in plants that were root inoculated with race 1 isolates but stunting was more severe on flue-cured varieties with moderate to high resistance to *P. nicotianae*. Plants treated with mefenoxam (Ridomil Gold SL) in our greenhouse study were stunted, yellow, and lower in weight due to phytotoxicity.

No treatment was more effective than another when used in combination with varieties containing moderate to high levels of resistance to *P. nicotianae*. Control of black shank in 'TN 90' and 'CC 812' was due to resistance rather than treatment with Soil-Set or mefenoxam and further supported that the effect of the treatment was dependent on variety. As with 'KY 14 x L8', the increase in plant weight of the non-infested control can be explained by stunting due to race 1 of *P. nicotianae*.

It is recommended that mefenoxam be used with varieties that have a resistance level of 4 or higher for the best economic returns. This is due to the fact that disease incidence can be high on varieties with little or no resistance even after using the maximum amount of fungicide approved by the label (112,121). Further greenhouse

experiments should focus on more replications to improve accuracy and reproducibility as well as randomization of treatments.

Soil-Set had no effect on disease severity in the field for a variety with high levels of resistance to race 0 and 1 of *P. nicotianae*. Severe storms and flooding affected the field experiment in 2012 in the month of July. Three plots (control, Soil-Set at 1.2 L/ha, and Soil-Set at 4.8 L/ha) in one block were affected by both wind and flood damage. However, the disease pressure was low in this field study. This was most likely due to the high resistance of 'CC 812' to both races of *P. nicotianae*, which further supported our results from the greenhouse study where the effect of the treatment was dependent on the variety.

Future field experiments should focus on more replications to improve the accuracy and reproducibility of these experiments. Additionally, our greenhouse experiments indicated the potential of Soil-Set and it should be tested alongside Ridomil in the field on low- and high-resistance varieties. Since there was a three-day gap between transplant of tobacco and treatments, future field experiments should aim to treat plants the day of transplanting. This will reduce the likelihood of roots being infected by *P. nicotianae* before treatment and clearly address preventative activity of Soil-Set.

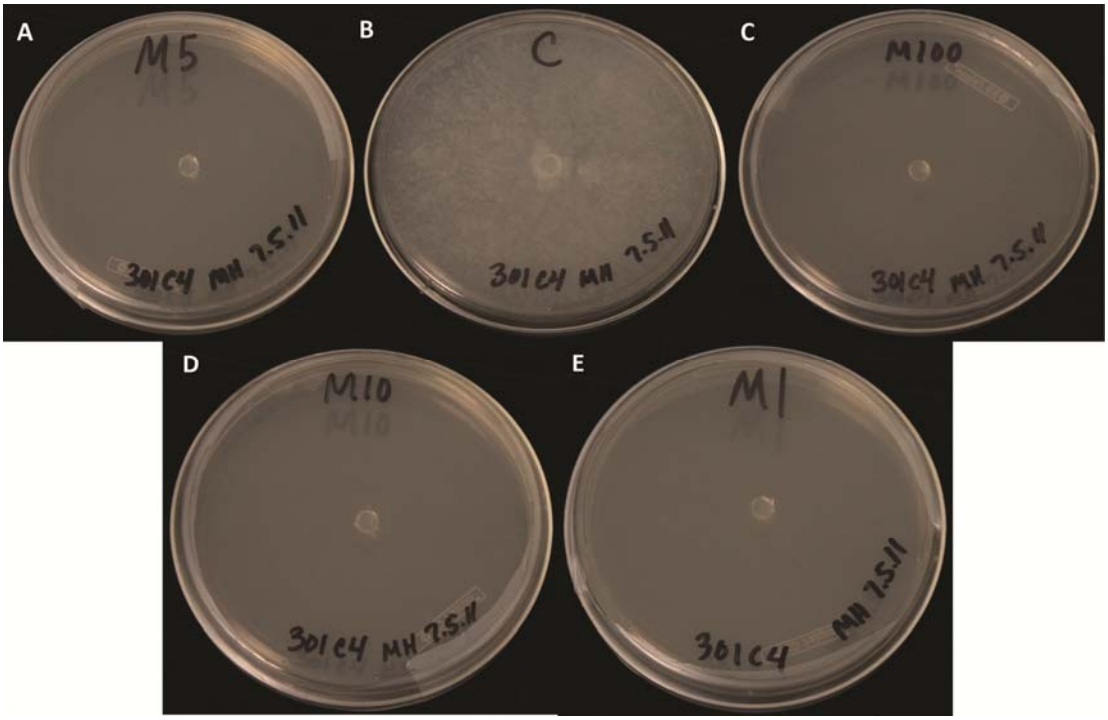

Figure 3.1. *in-vitro* inhibition of *P. nicotianae* isolate 304 (race 1) growing on V8 medium amended mefenoxam (Ridomil Gold EC). A) V8 medium amended with 5 ppm mefenoxam; B) V8 medium non-amended control; C) V8 medium amended with 100 ppm mefenoxam; D) V8 medium amended with 10 ppm mefenoxam; E) V8 medium amended with 1 ppm mefenoxam.

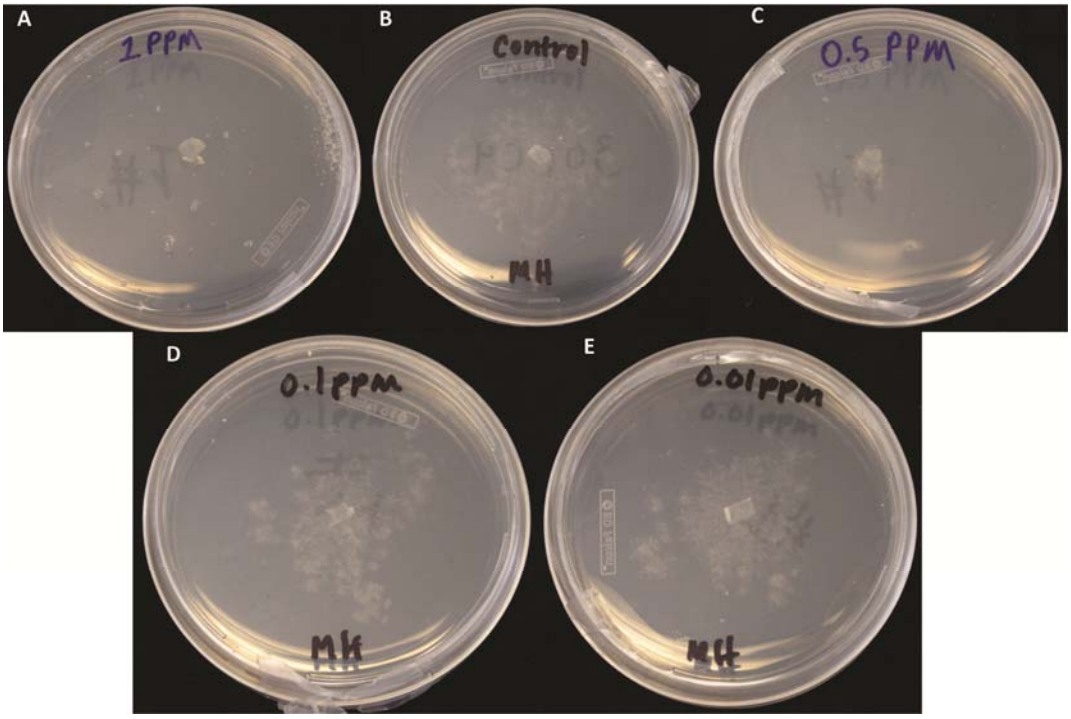

Figure 3.2. Sparse and spindly *in-vitro* growth of *P. nicotianae* isolate 301 C4 (race 1) on corn meal agar (CMA) amended with Soil-Set®. A) CMA amended with (0.1% v/v) Soil-Set® (diameter=0 cm); B) CMA non-amended control (sparse growth and runner hyphae); C) CMA amended with (0.5% v/v) Soil-Set® (few mycelial strands); D) CMA amended with (0.01% v/v) Soil-Set® (sparse growth and runner hyphae); E) CMA amended with (0.001% v/v) Soil-Set® (sparse growth and runner hyphae).

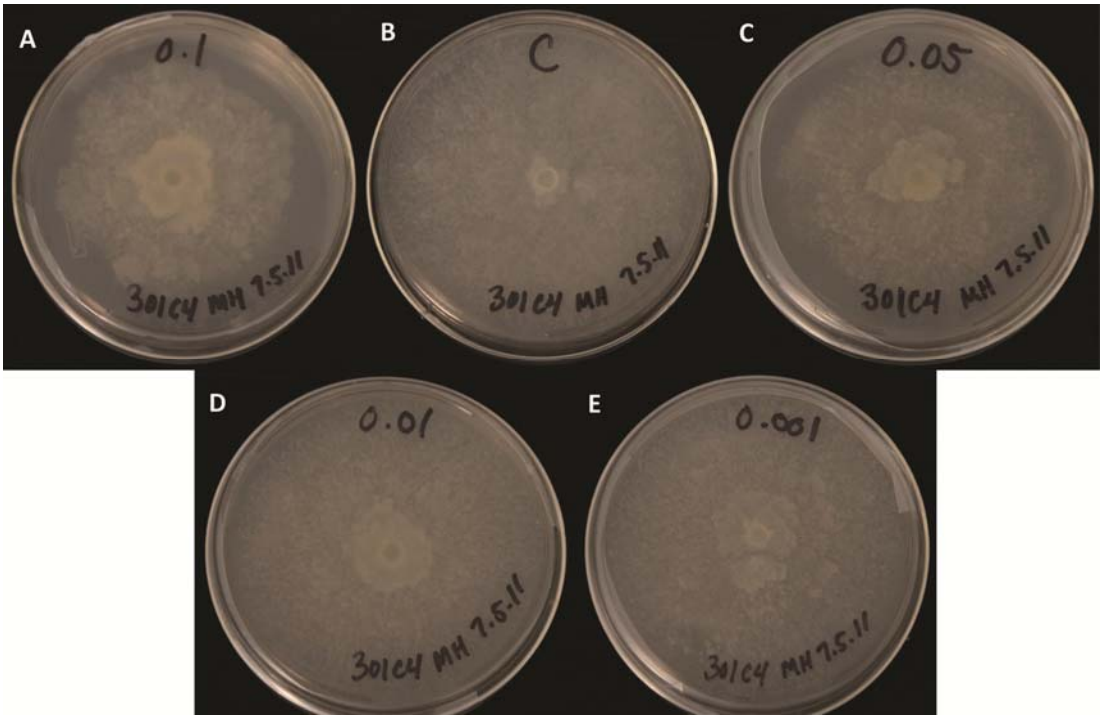

Figure 3.3. *in-vitro* inhibition of *P. nicotianae* isolate 301 C4 (race 1) growing on V8 medium amended with Soil-Set®. A) V8 medium amended with (0.1% v/v) Soil-Set®; B) V8 medium non-amended control; C) V8 medium amended with (0.5% v/v) Soil-Set®; D) V8 medium amended with (0.01% v/v) Soil-Set®; E) V8 medium amended with (0.001% v/v) Soil-Set®.

Table 3.1. Colony diameter of *P. nicotianae* isolate 301 C4 (race 1) growing on V8 medium amended with Soil-Set or mefenoxam.

Treatment	Dose[1]	Colony Diameter (cm)[2]
Control	--	7.00a
Soil-Set	0.001	7.00a
Soil-Set	0.01	6.04b
Soil-Set	0.05	5.23c
Soil-Set	0.1	4.47d
Mefenoxam	0.1	1.33e
Mefenoxam	1	0f
Mefenoxam	5	0f
Mefenoxam	10	0f
Mefenoxam	100	0f

[1]Soil-Set is reported as % v/v and mefenoxam is reported as ppm.

[2]Means in the same column followed by the same letter are not significantly different as determined by Fisher's LSD ($p \leq 0.05$).

Table 3.2. *p*-values from a two-way ANOVA analysis examining the effects of factors (replication, treatment, and variety) and their interactions for AUDPC of black shank, % root damage, and plant dry weight.

	Pr > F		
Source	**AUDPC[1]**	**% Root Damage[2]**	**Plant Dry Weight (g)**
Rep	0.0010	0.13	<0.0001
Treatment	<0.0001	<0.0001	<0.0001
Rep*Treatment	0.32	0.39	0.31
Variety	<0.0001	<0.0001	<0.0001
Treatment*Variety	<0.0001	<0.0001	<0.0001

[1] Mean area under the disease progress curve (AUDPC) was calculated using 6 assessments of disease incidence.

[2] Percent root damage was determined using the Horsfall-Barratt scale and values were converted to percentages using the ELANCO formula.

Table 3.3. Effect of Soil-set and mefenoxam on the severity of black shank on burley variety KY 14 x L8.

Treatment[1]	Dose (L/ha)	AUDPC[2]	% Root Damage[3]	Plant Dry Weight (g)
Control	--	0c[4]	0b	1.05a
PN	--	14.2a	83.3a	0.17c
Soil-Set	1.2	8.2ab	100a	0.22c
Soil-Set	4.8	2.0bc	16.7b	0.55b
Mefenoxam	1.2	0c	0b	0.22c

[1]Control=steam sterilized field soil; PN=*P. nicotianae* infested field soil (race 1); Soil-Set=*P. nicotianae* infested soil (race 1) treated with Soil-Set at 1.2 L/ha or Soil-Set at 4.8 L/ha; Ridomil=*P. nicotianae* infested soil (race 1) treated with Ridomil Gold SL at 1.2 L/ha.

[2,]Mean area under the disease progress curve (AUDPC) was calculated using 6 assessments of disease incidence.

[3]Percent root damage was determined using the Horsfall-Barratt scale and values were converted to percentages using the ELANCO formula.

[4]Means the same column followed by the same letter are not significantly different as determined by Tukey's HSD (α=0.05).

Table 3.4. Effect of Soil-set and mefenoxam on the severity of black shank on burley variety TN 90.

Treatment[1]	Dose (L/ha)	AUDPC[2]	% Root Damage[3]	Plant Dry Weight (g)
Control	--	0a[4]	0a	1.53a
PN	--	2.8a	6.3a	0.67b
Soil-Set	1.2	1.2a	19a	0.28c
Soil-Set	4.8	0.5a	16.7a	0.77b
Mefenoxam	1.2	0a	0a	0.12c

[1]Control=steam sterilized field soil; PN=*P. nicotianae* infested field soil (race 1); Soil-Set=*P. nicotianae* infested soil (race 1) treated with Soil-Set at 1.2 L/ha or Soil-Set at 4.8 L/ha; Ridomil=*P. nicotianae* infested soil (race 1) treated with Ridomil Gold SL at 1.2 L/ha.

[2,]Mean area under the disease progress curve (AUDPC) was calculated using 6 assessments of disease incidence.

[3]Percent root damage was determined using the Horsfall-Barratt scale and values were converted to percentages using the ELANCO formula.

[4]Means in the same column followed by the same letter are not significantly different as determined by Tukey's HSD (α=0.05).

Table 3.5. Effect of Soil-set and mefenoxam on the severity of black shank on burley variety CC 812.

Treatment[1]	Dose (L/ha)	AUDPC[2]	% Root Damage[3]	Plant Dry Weight (g)
Control	--	0a[4]	0a	2.12a
PN	--	0a	1.6a	0.65b
Soil-Set	1.2	1.5a	20.6a	0.23d
Soil-Set	4.8	0a	0a	0.52c
Mefenoxam	1.2	0a	0a	0.18d

[1]Control=steam sterilized field soil; PN=*P. nicotianae* infested field soil (race 1); Soil-Set=*P. nicotianae* infested soil (race 1) treated with Soil-Set at 1.2 L/ha or Soil-Set at 4.8 L/ha; Ridomil=*P. nicotianae* infested soil (race 1) treated with Ridomil Gold SL at 1.2 L/ha.

[2,]Mean area under the disease progress curve (AUDPC) was calculated using 6 assessments of disease incidence.

[3]Percent root damage was determined using the Horsfall-Barratt scale and values were converted to percentages using the ELANCO formula.

[4]Means in the same column followed by the same letter are not significantly different as determined by Tukey's HSD (α=0.05).

Table 3.6. Pairwise comparisons (Fisher's LSD) for disease incidence (AUDPC) data collected in 2012 from a field experiment examining the effect of Soil-Set treatments to the soil around tobacco transplants, Cross Creek 812, as compared to Ridomil Gold SL.

		***p*-value for pairwise comparisons**			
Treatment	**Mean AUDPC[1]**	**Control (Water)**	**Ridomil (1.2 L/ha)**	**Soil-Set (1.2 L/ha)**	**Soil-Set (4.8 L/ha)**
Control (Water)	5.83±6.25	--	0.97	0.40	0.81
Ridomil (1.2 L/ha)	5.64±4.90	0.97	--	0.38	0.84
Soil-Set (1.2 L/ha)	10.68±9.73	0.40	0.38	--	0.29
Soil-Set (4.8 L/ha)	4.48±7.38	0.81	0.84	0.29	--

[1]Mean area under the disease progress curve (AUDPC) and standard deviation for each treatment.

APPENDIX ONE:
A DUPLEX SCORPION ASSAY FOR QUANTIFICATION OF *PHYTOPHTHORA NICOTIANAE* IN THE SOIL

INTRODUCTION

The traditional technique used for quantifying populations of fungi in the soil is soil-dilution plating on various types of growth media that are semi-selective for different groups of fungi. While relatively reliable, results from this assay take several days (ca. 5-7 days). Kary Mullis invented conventional PCR in 1984, which provided a qualitative method for amplification and detection of DNA (92). With the invention of real-time PCR in the early 1990s, conventional PCR was coupled with non-specific dyes (SYBR green I) or fluorescent probes (TaqMan, molecular beacon, scorpion) for detection and quantification of DNA (57,92). Results appear in 'real-time' and assays can take as little as one hour to complete.

Real-time PCR has been utilized in plant disease diagnosis (46,108,129). The most commonly used real-time PCR probe chemistries used to detect and quantify plant pathogenic fungi include TaqMan, molecular beacon, and scorpion (109). The 'stem-loop' scorpion probe technology was used by Ippolito et al. to quantify *Phytophthora nicotianae* from citrus roots and soil (60). In a 'stem-loop' configuration, one oligonucleotide strand in the shape of a hairpin loop contains both the quencher and fluorophore. The primers used by Ippolito et al. in the design of the 'stem-loop' scorpion were only tested on *P. nicotianae* isolates from the following: citrus, olive, tomato, and an unknown (60). Solinas et al. reported that an improvement to the 'stem-loop', known

as the duplex scorpion, reduced false negatives due to the separation of the quencher and fluorophore on two complementary oligonucleotide strands (120).

For growers with black shank, once symptoms appear in the field, a majority of the recommendations focus on preventative measures for the following season. A duplex scorpion probe real-time PCR assay for black shank will be a useful tool for developing preventative recommendations that start at the beginning of the season before a problem arises and not having to wait until the following season. For example, *P. nicotianae* populations can be examined in the spring before tobacco is transplanted and a specific pathogen threshold density could be used to make better, cost-effective recommendations about whether or not to use Ridomil in the transplant water. The objectives of this study were to determine if the primers described by Ippolito et al. (60) would amplify *P. nicotianae* isolates from tobacco, and to develop a real-time PCR assay using a duplex scorpion probe to quantify *P. nicotianae* from soil.

MATERIALS AND METHODS

***Phytophthora nicotianae* isolates.** Seven isolates of *P. nicotianae* were collected from the same tobacco field described in Chapter 2. All seven isolates were determined to be race 1 (data not shown) using the laboratory assay described by Gutiérrez and Mila (47). The differential cultivars used for the laboratory assay included: KY 14 (burley variety, no resistance to black shank), KY 14 x L8 (burley variety, heterozygous *Phl* gene, high race 0 resistance, susceptible to race 1 and 3), TN 90 (burley variety, heterozygous *Phl* gene, medium resistance to race 0 and race 1), NC 1071 (flue-cured variety, *Php* gene, high race 1 resistance, resistant to race 3, susceptible to race 0), and KT 212 (KTH

2901, burley variety, heterozygous *Phl* gene, high resistance to race 0, moderate resistance to race 1). Mycelia were grown on potato dextrose broth (PDB, Difco) at room temperature for seven days and DNA was extracted using a modified CTAB (cetyltrimethylammonium bromide) protocol (78).

Modified CTAB extraction of nucleic acids. Mycelium (100 mg) was isolated from PDB and added to a 2.0-ml screw cap microcentrifuge tube containing two 4-6 mm beads. The microcentrifuge tube with mycelium and beads was dropped into liquid nitrogen for 30 seconds, bead-beated at 2500 rpm for 30 seconds in a Mini-BeadBeater (Biospec Products 3110BX), and repeated two more times. CTAB buffer (1 ml; 2% CTAB, 2% PVP-40, 100 mM Tris-HCL, 1.4 M NaCl, 20 mM EDTA, and pH 8.0) and 2-mercaptoethanol were added and mixed under the chemical fume hood. The homogenate was incubated at 65°C for 15 minutes and centrifuged at 10,000*g* (ca. 10,400 rpm) for 10 minutes. The supernatant was transferred to a 1.5-ml microcentrifuge tube and mixed with an equal volume of chloroform/isoamyl alcohol (24:1) then centrifuged at 15,000*g* (ca. 12,500 rpm) for 10 minutes. The supernatant (500 µl) was transferred to a 1.5-ml microcentrifuge tube containing isopropanol (350 µl), mixed, and centrifuged at 15,000*g* for 10 minutes. The pellet was washed by adding ice-cold ethanol (500 µl; 70%) followed by centrifugation at 15,000*g* for 5 minutes. The pellet was air-dried in the hood. Once the ethanol evaporated, the pellet was dissolved in Tris-HCl (100 µl, 20 mM, pH 8.0) and stored at -20°C.

***Phytophthora nicotianae* specific probe and primers design.** PCR primers (Pn5B-Pn6) (Figure A1.1 and Table A1.1) designed by Ippolito et al. were used to amplify a

portion of the ITS2 region from the seven *P. nicotianae* tobacco isolates (60). The amplicons from all seven isolates were sequenced by the Advanced Genetics Technology Center at the University of Kentucky (Lexington, Kentucky) and compared by BLAST analysis (blastn algorithm) to sequences in the NCBI GenBank database.

The duplex scorpion probe (Figure A1.1) was designed by modifying the 'stem-loop' Pn6-scorpion from Ippolito et al.(61). The quencher strand (QS; Sigma-Aldrich, St. Louis, Mo) was labeled with the Black Hole Quencher-1 (BHQ1), while the probe-primer strand (PPS; Sigma-Aldrich) was labeled with 6-carboxyfluorescein (6FAM) (Figure A1.2 and Table A1.1). The PPS also contained hexaethylene glycol (HEG), which functions to block *Taq* polymerase from copying the probe sequence.

Optimization of real-time PCR amplification. All real-time PCR experiments were carried out on the SmartCycler® system (Cepheid, Sunnyvale, CA). Cycling conditions for amplification included an initial denaturation at 95°C for 10 minutes followed by 40 cycles consisting of denaturing at 95°C for 15 seconds and annealing/elongation at 55°C for 55 seconds. All reaction mixtures (20 µl) contained: 10 µl of Bioline SensiFAST probe No ROX kit (Bioline Reagents Ltd., Taunton, MA), 2 µl of Pn5B primer (5 µM), 2 µl of PPS (0.02 µM), 2 µl QS (0.5 µM), 2 µl extracted *P. nicotianae* mycelial DNA (ca. 3-5 ng), and 2 µl of sterile nanopure water. To examine the sensitivity and efficiency of the assay, DNA extracted from *P. nicotianae* mycelium was serially diluted tenfold from 3-5 ng/µl to 300-500 fg/µl. Sterile nanopure water was used as a negative control in every experiment.

Different concentration ratios of QS to PPS were examined. Concentration combinations tested included QS 0.5 µM/PPS 0.1 µM, QS 0.5 µM/PPS 0.125 µM, QS 0.5 µM/PPS 0.25 µM, and QS 0.5 µM/PPS 0.5 µM. For both annealing phase and QS/PPS ratios, the cycling conditions and reaction mixture described above were used.

RESULTS AND DISCUSSION

Primer design. Using the Pn5B-Pn6 primers, the sequenced amplicons from all seven isolates shared strong similarity to *P. nicotianae* sequences in the GenBank database. This result supported our hypothesis that the primers (Pn5B-Pn6) designed by Ippolito et al. would amplify *P. nicotianae* isolates from tobacco. Therefore, the duplex scorpion was designed and implemented into further study as discussed below.

Optimization of real-time PCR amplification. Much of the optimization work focused on improving reaction efficiency to a target range of 90-100%. The reaction efficiency was calculated by graphing the log value of the serial dilution DNA concentrations vs. the Ct values and using the slope in the following equation: $E=10^{(-1/slope)}-1) \times 100$.

One step to increase reaction efficiency involved improvement of pipetting technique. Simple changes were made such as not pipetting extremely small amounts (<2 µl) when measuring DNA for the serial dilutions or other reagents in the reaction mixture (Table 2). Changing the annealing phase was another step in trying to improve the reaction efficiency. If the annealing time is too long there can be non-specific amplification (e.g. repeat sequences in DNA) and if too short, there is not enough time for primers to hybridize to the target. Annealing phase was analyzed by looking at Ct

values obtained when varying the time from 34 to 61 seconds. No differences in Ct value were observed and thus the original annealing/elongation time (55 seconds) was kept (data not shown).

All of the serial dilution experiments used a ratio of QS at 0.5 µM to PPS at 0.02 µM (Table 2). At best, reaction efficiency was 80%. More work should be done to optimize this assay so that the reaction efficiency reaches at least 90%. A step in that direction was examining the ratio of QS to PPS concentration. De-Ming et al. (30) reported that a 1.6:1 QS to PPS concentration ratio gave a 97% reaction efficiency while Ioos et al. (59) reported that a 5:1 QS to PPS concentration ratio gave a 99% reaction efficiency. Results for the QS/PPS concentration ratio experiments can be found in Table 3. Future work should include tenfold serial dilution experiments under the cycling conditions described above using the different QS/PPS concentrations. If the reaction efficiency is still low, the next step to optimize this assay should involve making a master mix instead of using a premade one from Bioline. Making the master mix in the laboratory would allow for optimization of individual PCR reagents. For example, magnesium is a co-factor of *Taq* polymerase so different concentrations can significantly impact its efficiency in synthesizing DNA. Once the assay has been optimized for mycelial DNA, experiments to detect and quantify *P. nicotianae* in the soil using the duplex scorpion probe should follow.

Based on the genome sequence released by the Broad Institute (101), the amount of DNA in one chlamydospore of *P. nicotianae* is approximately 0.16 pg. The duplex scorpion assay used in this study was sensitive enough to detect concentrations

of *P. nicotianae* DNA as low as 300-500 fg/µl. Since the assay can detect concentrations of DNA that are lower than one chlamydospore, it is feasible that once the real-time PCR protocol has been optimized it will be suitable for application in the field.

Figure A1.1. Illustration of the forward (Pn5B) and reverse (Pn6) primers designed to amplify from the ITS2 region of *Phytophthora nicotianae*. The expected amplicon length when using Pn5B-Pn6 is 120 bp. ITS1=internal transcribed spacer 1; ITS2=internal transcribed spacer 2; 18S, 5.8S, and 28S=nuclear encoded ribosomal RNA genes. Adapted from Ippolito et al. (60).

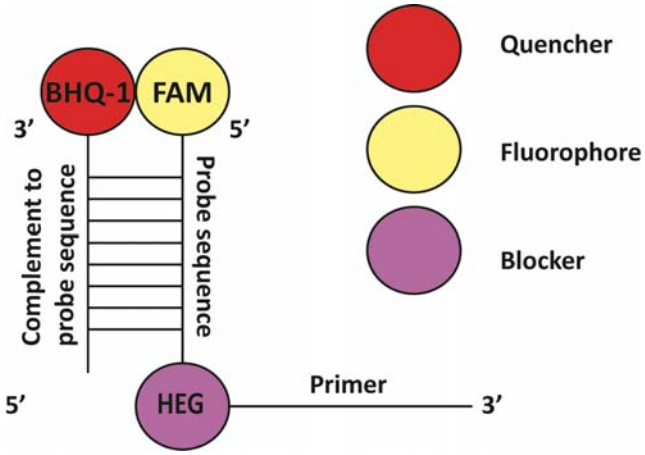

Figure A1.2. Diagram of the duplex scorpion probe used to amplify *Phytophthora nicotianae* mycelium. BHQ-1=Black Hole Quencher-1; FAM=6-carboxyfluorescein; HEG=hexaethylene glycol.

Table A1.1. The sequences for the forward (Pn5B) primer, reverse (Pn6) primer, quencher strand (QS), and probe-primer strand (PPS) used for the amplification and quantification of *Phytophthora nicotianae*. BHQ1=Black Hole Quencher-1; 6FAM= 6-carboxyfluorescein; HEG=hexaethylene glycol.

Name	**Sequence**
Pn5B	5'-GAACAATGCAACTTATTGGACGTTT-3'
Pn6	5'-AACCGAAGCTGCCACCCTAC-3'
Quencher strand (QS)	5'-CAACAGCAAACGGAATT-BHQ1-3'
Probe-primer strand (PPS)	5'-6FAM-AATTGGCTTTGCTGTTG-HEG-AACCGAAGCTGCCACCCTAC-3'

Table A1.2. Real-time PCR reaction efficiencies calculated for optimization experiments. QS=quencher strand; PPS=probe primer strand.

Experiment	Slope	Reaction Efficiency
QS 0.5 µM/PPS 0.02 µM Poor pipetting	-5.80	48%
QS 0.5 µM/PPS 0.02 µM Poor pipetting	-4.83	62%
QS 0.5 µM/PPS 0.02 µM Improved pipetting	-3.90	80%
QS 0.5 µM/PPS 0.02 µM Improved pipetting	-4.22	73%

Table A1.3. Threhold values (Ct) calculated for different QS (quencher strand) to PPS (probe primer strand) concentrations.

QS/PPS concentration	Ct value
0.5 µM/0.02 µM	14.9±0.2
0.5 µM/0.125 µM	15.0±0.2
0.5 µM/0.25 µM	15.0±0.1
0.5 µM/0.5 µM	16.4±0.3

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PROFESSIONAL PRESENTATIONS
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What a plant destroyer, a tobacco farm, and a buckeye have in common: alternative methods of control for *Phytophthora nicotianae* of tobacco, The University of Kentucky Department of Plant Pathology, 10 December 2012.

The biofumigation potential of *Brassica* spp. against *Phytophthora nicotianae* of tobacco, The University of Kentucky Department of Plant Pathology, 22 February 2010.

Chemical alternative control of *Phytophthora nicotianae* of tobacco, Alltech Headquarters, Nicholasville, KY, 23 August 2010.

"Fun with Fungi" Interview. WUKY Radio 91.3 FM Lexington, KY, 27 October 2009.

What it takes to be a plant destroyer: *Phyophthora kernoviae*, The University of Kentucky Department of Plant Pathology, 22 September 2008.

Poster Presentations

The biofumigation potential of *Brassica juncea* against black shank of tobacco. American Phytopathological Society-International Plant Protection Convention Joint Meeting, Honolulu, HI, 6-10 August, 2011.

Effect of biofumigation with brassica cover crops on black shank of burley tobacco, Tobacco Workers' Conference, Lexington, KY, 18-20 January, 2010.

PROFESSIONAL PUBLICATIONS

Extension Publications

Seebold, K. W., Holdcroft, A., and Dixon, E. 2008. Effect of potassium phosphite and fungicides on Phytophthora crown and fruit rot of summer squash, 2007. Plant Disease Management Reports 2:V005.

de Sá, P. B., Lensing, J., Leesutthiphonchai, W., Hartman, J., Dymarski, P.-Y., Holdcroft, A. M., Collins, J. and Harper, C. 2006. National Nursery Survey for *Phytophthora ramorum* in Kentucky, 2006. Nursery and Landscape Program 2006 Research Report. Agricultural Experiment Station. The University of Kentucky, College of Agriculture, pp. 28-29.

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Abstracts

Holdcroft, A. M. and Seebold, K. W. 2011. The biofumigation potential of *Brassica juncea* against black shank of tobacco. Phytopathology 101:S73.

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