12-2011

POTENTIAL OF THREE BRASSICA COVER CROPS FOR BIOFUMIGATION IN THE FIELD AND THE RELATIONSHIP BETWEEN SOIL MYROSINASE AND BIOFUMIGATION EFFICACY

Zachariah Hansen
Clemson University, zachariahrhansen@gmail.com

POTENTIAL OF THREE BRASSICA COVER CROPS FOR BIOFUMIGATION IN THE FIELD AND THE RELATIONSHIP BETWEEN SOIL MYROSINASE AND BIOFUMIGATION EFFICACY

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Plant and Environmental Sciences

---

by
Zachariah Robert Hansen
December 2011

Accepted by:
Dr. A.P. Keinath, Committee Chair
Dr. M.B. Riley
Dr. G.W. Zehnder

ABSTRACT

A greenhouse study was set up in January 2011 to investigate the effect of soil myrosinase concentration and *Bacillus* spp. on the release efficiency of isothiocyanates (ITCs) from freeze-dried 'Pacific Gold' mustard leaf tissue after incorporation into soil. Seventeen diverse soil samples collected from various regions of South Carolina were used in the study. A correlation analysis was conducted to determine if soil myrosinase was positively correlated with ITC concentrations after mustard incorporation. ITC concentrations were then analyzed with soilborne *Bacillus* spp. populations, bean emergence, disease incidence, and shoot weight to identify significant correlations. Finally, the effect of freeze-dried 'Pacific Gold' mustard leaf tissue on populations of soilborne *Rhizoctonia solani, Sclerotium rolfsii,* and *Pythium* spp., as well as the effect on bean emergence, disease incidence, and shoot weight was investigated using two different soil samples. Soil myrosinase activity ranged from -0.71 μg glucose g^{-1} soil 4 h^{-1} to 5.87 μg glucose g^{-1} soil 4 h^{-1} and was not significantly correlated with ITC concentrations following mustard incorporation. ITC concentrations ranged from 1.8 μg g^{-1} soil to 13.4 μg g^{-1} soil and were not significantly correlated with soilborne *Bacillus* spp. populations, bean emergence, disease incidence, or shoot weight. Incorporation of freeze-dried 'Pacific Gold' mustard leaf tissue into soil did not significantly affect populations of soilborne *R. solani, S. rolfsii,* or *Pythium* spp., nor did it significantly affect bean emergence, disease incidence, or shoot weight.

A three-season field experiment was set up to determine if biofumigation with oilseed radish, 'Pacific Gold' mustard, and winter rapeseed would reduce populations of the soilborne plant pathogens *R. solani*, *S. rolfsii*, and *Pythium* spp. Soil ITC

concentrations were quantified following incorporation of each brassica treatment at 4 hours and two days during the summer 2011 pepper experiment, and 4 hours, 2 days, and 4 days during the fall 2011 pepper experiment. The effect of brassica cover crops on pepper vigor and yield, and incidences of pepper mortality caused by root rot and Southern blight were also investigated. Additionally, the relative pathogenicity of five *Pythium* spp. isolates on pepper was determined in a greenhouse experiment. After incorporation of the three brassica cover crops, soil was covered with virtually impenetrable film (VIF). The two control treatments included weedy fallow (+) VIF and weedy fallow (-) VIF. Brassica cover crops were planted in Spring 2010, Fall 2010, and Spring 2011 with pepper crops planted Summer 2010, Spring 2011, and Summer 2011, respectively. ITCs concentrations were highest following incorporation of 'Pacific Gold' mustard. Winter rapeseed yielded the second highest ITC concentrations. Oilseed radish yielded very low ITC concentrations in spring 2011, and none during summer 2011. ITCs were detected in low concentrations in fallow (+) VIF treatments. All (+) VIF treatments reduced populations of *R. solani* compared to (-) VIF, with no differences between brassica treatments and fallow (+) VIF. Brassica treatments generally did not reduce populations of *Pythium* spp. compared to fallow (+) VIF, although results were inconsistent across repetitions. Population estimates of *S. rolfsii* were generally low with few significant differences among treatments. Pepper stunting was generally significantly lower in all (+) VIF treatments compared to fallow (-) VIF. In general, there were no significant differences in pepper stunting between brassica treatments and fallow (+) VIF. Brassica treatments did not consistently affect pepper death due to root rot or Southern blight compared to either control. Pepper yields were consistently highest in brassica

treatments compared to fallow (+) VIF, and (+) VIF yields were higher than (-) VIF yields. Only one of five *Pythium* spp. isolates recovered from soil in the pepper field used in the third repetition was pathogenic on pepper in the greenhouse, and it was identified as *P. aphanidermatum*.

DEDICATION

To everyone that has encouraged and supported me from the beginning.

ACKNOWLEDGMENTS

Dr. Keinath, my major advisor, has been an invaluable resource throughout my graduate school experience. His eagerness to teach, patience when doing so, and depth of scientific knowledge have helped me exceed my own expectations. I believe he has positively altered the direction of my life, and for that I am thankful.

Dr. Zehnder sparked my interest in agriculture when he hired me to work on the Clemson University Student Organic Farm during the summer of 2009. My positive experiences on the farm prompted me to pursue a graduate degree in Plant and Environmental Science.

Dr. Riley's plant pathology class introduced me to the subject and provided me with fundamental knowledge which I have used throughout my research. She also exposed me to analytical techniques in the classroom, and never hesitated to provide one-one-one instruction in the lab when it came time for me to incorporate those techniques into my research.

Ginny Dubose's contributions to my work are too numerous to mention. Her consistent help with day-to-day tasks, along with that of Jason Dufault and the rest of the farm crew, has allowed me to complete this research in a timely manner. They also provided casual conversation and much needed laughs throughout my time in Charleston.

I am thankful for the generous financial support provided by the Agricultural Society of South Carolina. I enjoyed meeting the members and discussing my work with them at the annual corn boils.

Finally, I thank my family. Without their love and support none of this would have been possible. Lindsay's confidence in me has never wavered, and that confidence has helped me persevere through the inevitable bumps in the road.

TABLE OF CONTENTS

LIST OF TABLES

List of Tables (Continued)

LIST OF FIGURES

CHAPTER ONE

LITERATURE REVIEW

Biofumigation

Introduction

Biofumigation is the use of *Brassica* species and their natural chemicals to control soilborne pathogens, nematodes, or weeds. This is accomplished by a reaction between two isolated compounds that occur naturally within the plant: the enzyme myrosinase (thioglucoside glucohydrolase), located in myrosin cells, and the secondary metabolites glucosinolates, which are contained in cell vacuoles. Upon tissue disruption the myrosinase catalyzes a hydrolysis reaction which converts glucosinolates into isothiocyanates (ITC), the compounds primarily responsible for the plant's pesticidal properties, as well as a variety of other breakdown products such as glucose, nitriles, thiocyanates, oxazolidine-2-thiones, hydroxynitriles, and epithionitriles (Al-Turki and Dick 2003, Morra et al. 2002, Fenwick et al. 1983). Biofumigation has been the subject of research since the early 1990's with varying levels of success, and remains an active area of research.

Management of soilborne pathogens can be particularly challenging as a result of both their biology and physical location. Many soilborne pathogens, such as fungi, bacteria, and nematodes, are microscopic. As a result, scouting for and monitoring their populations is often not feasible. Furthermore, once crops have been planted there are few control agents available to manage soilborne pathogens because of the physical disruption caused by fumigant application and the potential damage caused by phytotoxic

fumigants (Matthiessen et al. 2006). For this reason there is a need for an effective pesticide which can be applied to the soil prior to planting in areas where soilborne pathogen populations are consistently at economic threshold levels. Methyl bromide successfully filled this niche until 2005, by which time the EPA had phased out the chemical except for critical use exemptions due to its role in ozone depletion (USEPA, 2011). This phase out of methyl bromide has brought biofumigation back into the spotlight as one potential replacement.

Brassica selection

There is a great deal of variation among different species and cultivars of brassicas with regard to their biofumigation potential. *Brassicaceae* species should be selected based on the amount of glucosinolate that they contain, the type of resulting isothiocyanate that will be produced, as well as the amount of biomass they are capable of producing. For example, *Brassica juncea* cv. Pacific Gold (Pacific Gold mustard) was shown by Antonious et al. (2009) to have the highest above ground biomass and glucosinolate content (5.7 ± 1.7 kg m^{-2} and 50 ± 2 μmoles g^{-1} respectively) of seven *B. juncea* species tested. Mayton et al. (1996) demonstrated that allyl isothiocyanate released from macerated *Brassica juncea* cv. Cutlass tissue completely suppressed in vitro growth of five common plant pathogens; *Pythium ultimum*, *Rhizoctonia solani*, *Verticillium dahliae*, *Verticillium albo-atrum*, and *Colletotrichum coccodes*. In addition, *Brassica napus* (winter rapeseed) had the highest above ground biomass (7.7 ± 3.4 kg m^{-2}) of ten *Brassica* species studied. Although its glucosinolate content was found to be relatively low at 27 ± 14 μmoles g^{-1}, its high biomass gave it high glucosinolate yields per

unit area relative to the other ten species tested (Antonious et al. 2009). Furthermore, winter rapeseed requires a vernalization period to flower. As a result it will continue to produce vegetative growth and fail to seed when sown in the spring, as demonstrated by Sarwar et al. (1998). Thus winter rapeseed overcomes the limitation of early flowering, which affects many other brassica species, making it an excellent candidate for a biofumigant crop. *Raphanus sativus* (oilseed radish), according to Ohio State University Extension (Sundermeier 2008), has the potential to produce approximately 10 kg m^{-2} biomass. In addition to high biomass and subsequent high quantities of glucosinolates, Weil et al. at the University of Maryland's Cooperative Extension found that oilseed radish alleviated soil compaction and the need for deep tillage through the growth of a long taproot, which can penetrate six feet or more into the soil. Such characteristics are important to consider when selecting a potential biofumigant cover crop.

Effect of incorporation environment and technique

In addition to brassica variety there are several factors relating to incorporation which can affect how well biofumigation works. Two such factors are the level of tissue disruption prior to incorporation and the soil moisture level at incorporation. Morra et al. (2002) showed that ITC release efficiency could be increased from 1% to 26.4% when switching from whole plant incorporation to freezing and thawing plants before incorporation. They also found that extractable ITC concentrations were higher in waterlogged vs. non-waterlogged soil. Thus the level of tissue disruption and soil moisture play key roles in the percentage of glucosinolates that will be converted to ITC. Soil temperature, soil solarization, and soil type have all been shown to be influential

factors affecting the percentage of ITC measured after incorporation of *B. juncea* (Price et al. 2005). Increasing soil temperature from 15°C to 45°C increased ITC concentration by 81%. The covered treatment had 56% higher ITC concentrations than the non-covered control. Finally a sandy loam soil had 38% more ITC than a clay loam soil. Time of planting as well as growth stage at incorporation are also important considerations. Glucosinolate concentration has been shown to be highest at the bud-raised growth stage, prior to flowering, and higher in spring-seeded vs. fall-seeded brassicas (Sarwar et al. 1998). Although spring-seeded brassicas were shown to have higher concentrations of glucosinolates, it is important to note that a key to the success of biofumigation is the total amount of glucosinolate present in the field at the time of incorporation. As a result, unless spring-seeded brassicas can accumulate comparable biomass to fall-seeded brassicas by the time of incorporation, it may still be beneficial to seed in the fall.

Differential pathogen suppression by isothiocyanate type

Not all brassicas produce the same glucosinolates, nor are they produced at equal concentrations. As a result the type and concentration of ITC released upon tissue disruption varies among brassica species and cultivars, which can in turn affect their biofumigation potential. Thirty-five genotypes of *Brassica juncea* and 28 genotypes of *Brassica nigra* were screened for ITC production and suppression of the plant pathogens *Helminthosporium solani* and *Verticillium dahliae* (Olivier et al. 1999). A correlation between the concentration of ally-isothiocyanate (AITC) and pathogen suppression was observed. Genotypes of both brassica species were consistently 100% fungicidal to both pathogens at AITC concentrations >1.2 mg AITC g^{-1} dry plant tissue. Although

genotypes that released low concentrations of AITC were generally less suppressive, a few resulted in moderate to high suppression. Such genotypes released other ITCs, such as 2-phenylethyl and benzyl, at sufficient concentrations to suppress the pathogens. These results suggest that concentrations of AITC released from various brassicas can be used as indicators of relative pathogen suppressiveness. Additionally, it is important to consider that brassicas with low levels of sinigrin, the glucosinolate precursor to AITC, may also be suppressive to pathogens if they contain the precursors to other ITCs, such as 2-phenylethyl ITC and benzyl ITC. Smith and Kirkegaard (2002) demonstrated the suppressive effects of 2-phenylethyl ITC on a variety of pathogenic and non-pathogenic microorganisms in vitro. The primary hydrolysis product released from canola roots, 2-phenylethyl ITC, was examined because canola has been widely adopted as a rotation crop in Australian wheat production systems. There was a large range in the ITC concentration required for complete suppression in vitro, both interspecific and intraspecific. Bacteria were found to be generally more tolerant of 2-phenylethyl ITC than the eukaryotic organisms tested, and *Trichoderma* spp. were found to be more tolerant than the other eukaryotes tested, including *Aphanomyces, Gaeumannomyces*, *Phytophthora* spp., *Thielaviopsis*, *Rhizoctonia solani,* and *Pythium* spp. Despite variations in lethal doses, 2-phenylethyl ITC was found to be suppressive to all microorganisms tested, indicating its potential in biofumigation.

Plastic mulch selection

The type of plastic used to cover fumigated areas is also an important consideration. Virtually impenetrable film, or VIF, greatly reduces the quantity of volatile

chemicals allowed to escape from the soil into the atmosphere compared to conventional low density polyethylene mulch film (LDPE) (Noling et al. 2010). This both reduces the rate at which volatile chemicals must be applied to the soil to obtain adequate fumigation results and any harmful effects that may result from releasing toxic volatile chemicals into the atmosphere. Hanson et al. (2010) found that a reduced rate of methyl bromide with VIF offered comparable control of plant parasitic nematodes and reduced weed seed viability to that of the standard methyl bromide rate when high-density polyethylene film (HDPE) was used. Efficient volatile chemical containment is an important component to increasing the likelihood of successful biofumigation.

Effect of anaerobiosis and organic matter incorporation

Although ITCs are generally considered to be the primary factor behind pathogen suppression, other factors may be involved. Pathogen suppression, disease suppression, and improved plant health have all been associated with biofumigation, although these outcomes are not always apparently related to ITC release. The effects of tarping following incorporation of cover crops may reach beyond simply containing the volatile ITCs released from brassica tissues. The creation of an anaerobic environment as well as the concentration of fermentation by-products may play a role in the observed effects of biofumigation. Blok et al. (2000) demonstrated that both broccoli and grass amended into soil and covered with plastic (0.135 mm thick three-layered polyethylene strengthened with ethylene vinyl acetate) created a strongly anaerobic environment for up to 80 days. The covered and non-amended control resulted in an anaerobic environment lasting only 25% of that time. The amended plus plastic treatments consistently resulted in reductions

in populations of *Fusarium oxysporum* f.sp. *asparagi*, *R. solani*, and *V. dahliae* compared to no-plastic controls. The effect of broccoli was not significantly different from that of grass, suggesting that the pathogen inhibition was not related to exposure to ITCs released from the broccoli tissue, and more closely resembled general suppression. The non-amended plus plastic treatment, although resulting in an anaerobic environment for up to 20 days, did not significantly reduce pathogen populations. This result may indicate that the role played by anaerobiosis in pathogen suppression is limited. However, the relationship between length of anaerobic exposure time and resulting pathogen suppression has yet to be determined. Researchers with USDA ARS are currently investigating the effects of carbon sources, irrigation technique, plastic mulches, and length of tarping on the efficacy of anaerobic soil disinfestations in a project titled "Anaerobic Soil Disinfestation as an Alternative to Methyl Bromide Fumigation" (http://www.ushrl.saa.ars.usda.gov/research/projects/projects.htm?ACCN_NO=412624&fy=2009). Kasuya et al. (2006) demonstrated that dried *Brassica rapa* plant residue was suppressive to *R. solani* in pot assays and mycelial growth assays in vitro. Dried plant residues of peanut and clover were also suppressive to *R. solani*, further supporting the hypothesis that ITC exposure is not the sole factor contributing to pathogen suppression during biofumigation.

Proliferation of anaerobic bacteria

The proliferation of anaerobic bacteria in the soil, such as *Bacillus subtilis*, may also contribute to pathogen suppression in anaerobic soils. Chung et al. (2008) isolated a strain of *Bacillus*, *Bacillus subtilis* ME488, which inhibited the growth of 39 of 42 plant

pathogens tested in vitro. When applied to soil in pot assays *B. subtilis* ME488 suppressed disease caused by *F. oxysporum* f. sp. *cucumerinum* on cucumber and *Phytophthora capsici* on pepper.

Effect of organic acids produced by decomposing organic matter

Momma et al. (2006) found that acetic acid and butyric acid were both produced upon incorporation of wheat bran into soil. These organic acids were suppressive to *F. oxysporum* f. sp. *lycopersici* and *Ralstonia solanacearum*, the causal organisms of Fusarium wilt and bacterial wilt of tomato, respectively. The suppressive effect was observed in both pathogens in an organic acid suspension. However, when the organic acids were added to pathogen-infested soil samples, only *R. solanacearum* was suppressed at acid levels achieved through wheat bran incorporation. Such results indicate that organic acids may be involved in pathogen suppression through organic soil amendments, but such an effect may be differential among pathogen species.

Rhizoctonia solani

Introduction

R. solani is a soilborne fungal plant pathogen within the phylum Basidiomycota that has a wide host range (Sneh et al. 1996). It contributes to damping off, a common condition resulting in either pre-emergence or post-emergence seedling death. *R. solani* can injure plants at any growth stage, but younger plants often experience the most severe damage. *R. solani*, often in association with *Pythium* spp., can result in seed destruction,

seedling death, or reduced vigor of mature plants. Symptoms of infection by *R. solani* generally include dark brown, sunken stem lesions just above the soil line and stunting, wilting, and chlorosis characteristic of a damaged root system. *R. solani* can survive in the soil for long periods of time through the colonization of soil organic matter and the production of sclerotia (Roberts, 2003).

R. solani survival is sensitive to soil depth. Papavizas et al. (1975) studied the ecology and epidemiology of *R. solani* in field soil over a two year period. Nearly all of the *R. solani* activity was confined to the upper 0-5 cm of soil, with a small amount of activity from 5-10 cm, and no activity below 10 cm soil depth. This finding highlights the importance of consistent sampling depths when working with this pathogen in the field.

Control of *R. solani* using biofumigation

Control of *R. solani* through biofumigation has had varying success, most likely due to the conditions discussed previously. Njoroge et al. (2008) found no significant difference in *R. solani* population densities between brassica-amended and non-amended treatments in a field experiment. However, Larkin et al. (2006) showed significant *R. solani* population reductions in greenhouse assays, and also disease reductions in field tests following incorporation of brassica plant tissue, with *B. juncea* leading to the highest disease reductions. Dhingra et al. (2004) also found that essential oil extracted from mustard seeds (*Brassica rapa*) significantly reduced the saprophytic growth of *R. solani* both *in vitro* and in field soils, although significantly higher mustard oil concentrations were required for field soil. A key factor contributing to the observed variability in biofumigation's effectiveness is related to the balance between added

carbon as a nutrient source for pathogens and the resulting ITCs, which act as pesticides on those pathogens. Charron et al. (1999) clearly demonstrated the suppressive effect of allyl-isothiocyanate on *R. solani* and *Pythium ultimum* in a controlled laboratory study. Allyl-isothiocyanate had a fungicidal effect on *P. ultimum* at a rate of 2.2 µmol L^{-1} and 3.3 µmol L^{-1}, and reduced the growth of *R. solani* by 27% and 55% at the same rates, respectively. These results were obtained by separating the pathogens and the source of the ITC in space, thus precluding the pathogens from gaining an advantage from an added nutrient source.

Variability and classification of *R. solani*

The characterization of distinct but related strains of *R. solani* by anastomosis reaction, or fusion of fungal hyphae, has been used, and updated, by plant pathologists since the 1920's. It remains a useful way of classifying an extremely diverse plant pathogenic fungus. Papavizas et al. (1975) recovered 13 separate isolates from their research field, 11 of which belonged to anastomosis group 4 (AG-4). The other 2 isolates belonged to AG-3. These isolates ranged from highly pathogenic to nonpathogenic, even within AG-4. Relative pathogenicity is not the only notable variation among different isolates and AG groups of *Rhizoctonia*. Smith and Kirkegaard (2002) reported significant variations in growth rate response to 2-phenylethyl ITC among different isolates of *R. solani*. Among 11 isolates from 6 different AG groups tested, the dose required to inhibit in vitro radial growth by 90% ranged from 0.094 mM to 1.576 mM.

Sclerotium rolfsii

Introduction to *Sclerotium rolfsii*

S. rolfsii is another soilborne fungal plant pathogen within the phylum Basidiomycota with a wide host range that can cause the economically important plant disease Southern blight in coastal South Carolina. Mycelial growth of the pathogen is inhibited by high soil moisture, which also reduces survival of sclerotia, the organism's hardy overwintering structures, at temperatures above 20°C. Mycelia are capable of surviving for at least 6 months in dry soil (Beute & Rodriguez-Kabana, 1981). Germination of sclerotia is stimulated by alcohols released from decaying plant material (Roberts, 2003).

Southern blight

Development of Southern blight is favored by high temperature and low soil pH. Southern blight, as indicated by its name, is primarily a warm climate plant disease and rarely occurs where daily winter temperatures are below freezing. It has been reported to survive in cold climates, such as Illinois, Iowa, and Michigan under cover of snow or mulch (Love & Beckerman, 2002). Infection by *S. rolfsii* occurs at the soil line, and symptoms on peppers include yellowing and wilting, and eventually plant death. Often prior to infection, and certainly after infection, a mass of white mycelia can be seen on the base of the plant and the surrounding soil. Dark brown, round sclerotia can be seen within the mycelial mass. The sclerotia are the primary source of inoculum for the

disease, and also act as overwintering structures which can survive in the soil for extended periods of time without a viable host.

Effect of brassica incorporation on suppression of *S. rolfsii*

S. rolfsii has been shown by Gamliel et al. (1993) to be susceptible to population reduction in soil amended with dried cabbage residue (*Brassica oleracea* Capitata Group). Stapleton and Duncan (1998) reduced germination of *S. rolfsii* by 38% to 55% through the incorporation of cabbage residue into soil at a rate of 2%. They also examined a variety of other cruciferous plants for their pathogen suppressiveness including bok choy (*B. oleracea* var. *chinensis*), broccoli (*B. oleracea* var. *italiensis*), cauliflower (*B. oleracea* var. *compacta*), black mustard (*Brassica nigra*), and radish (*Raphanus sativus*). Bok choy, broccoli, and cabbage all significantly suppressed sclerotia germination compared to the unamended control.

Stapleton and Duncan (1998) also investigated the effect of temperature on the ability of the brassicas to suppress sclerotia germination. They used a diurnal temperature treatment with a maximum and minimum of 38°C and 27°C. Without the diurnal treatment, germination suppression ranged from 2% to 65%. With the diurnal treatment, suppression ranged from 86.5% to 99.9%. The non-amended control plus diurnal temperature treatment suppressed germination by 5.8%, suggesting that the difference in suppression between amended and non-amended can be attributed to the brassica amendments. Ristaino et al. (1991) also demonstrated that *S. rolfsii* is sensitive to prolonged heat exposure. Using solarization, a technique which utilizes clear plastic mulch to trap the sun’s energy close to the soil surface, they successfully reduced the

populations of *S. rolfsii* by 62% under favorable solarization conditions. During that experiment prolonged temperatures above 35°C were observed, with maximum temperatures exceeding 50°C. Similar reductions were not observed during years in which temperatures were consistently lower. Black plastic mulch has been shown to reduce both the disease incidence and disease severity of Southern blight in bell pepper in the field compared to a bare soil treatment (Brown et al. 1989). Increased yield in the black plastic mulch treatment compared to bare soil was also reported.

Pythium spp.

Introduction

Pythium spp. are commonly occurring fungus-like soilborne plant pathogens that are now classified in the Kingdom Straminopila and the phylum Oomycota. They can cause damping off and root rot in a variety of vegetable crops. Common symptoms on peppers include tan, water soaked stem lesions which can extend up the length of the stem. *Pythium* spp. overwinter on hosts left in the field, including weeds, and as oospores, which can survive in the soil for long periods without a host. Oospores can be easily spread through irrigation water, and their germination is stimulated by host root exudates (Roberts, 2003).

Species diversity, relative pathogenicity, and sensitivity to isothiocyanates

There are many species of *Pythium*, with varying degrees of pathogenicity, capable of colonizing pepper roots. The diversity in *Pythium* spp. also leads to

differential sensitivity to control strategies. Smith and Kirkegaard (2002) reported significant variations in growth rate response to 2-phenylethyl ITC among 13 different isolates across 3 species of *Pythium* (*P. sulcatum*, *P. ultimatum*, and *P. violae*). The ITC concentration required to reduce in vitro radial growth by 90% ranged from 0.005 mM to 0.862 mM. The least sensitive isolate was also the only isolate of *P. ultimatum*. There was little intraspecific variation in sensitivity among isolates. These results indicate that species of *Pythium* may vary significantly in sensitivity to toxins. Njoroge et al. (2008) and Collins et al. (2006) both found that the incorporation of brassica tissue did not significantly reduce, and in some cases increased, the population densities of *Pythium* spp. In contrast, Stapleton and Duncan (1998) reported significant reductions in survival of *P. ultimum* from incorporating 2% brassica tissue into soil in a controlled experiment. This suggests that under field conditions there is more involved in *Pythium* suppression than simply ITC exposure. To better understand the variation in pathogenicity among species of *Pythium*, Chellemi et al. (2000) conducted a study analyzing ten different species of *Pythium* collected from fresh market pepper plants in Florida. A large range in pathogenicity was found among the species. *P. aphanidermatum* and *P. myriotylum* were the most aggressive pathogens on pepper, leading to approximately 50% mortality in inoculated pepper plants. *P. periplocum* and *P. spinosum*, although capable of colonizing pepper roots, resulted in no adverse affects on root system weight or plant growth.

Effect of temperature and non-brassica composts on *Pythium* spp.

Chellemi et al. (2000) also indicated temperature was a factor affecting disease severity caused by *Pythium* spp., with 34°C as the optimal temperature for high disease

severity in pepper. Significant disease severity persisted down to 20°C. Stranghellini et al. (1983) also found temperature to be an important factor affecting colonization of sugar beet tap roots by *Pythium aphanidermatum*. Maximum colonization occurred between 27°C and 34°C with 0% colonization at 20°C. This low level of colonization at low soil temperature may be the result of competition with rhizobacteria. Tedla et al. (1992) found that rhizobacteria, particulary *Bacillus* spp., were able to out-compete *P. aphanidermatum* at low soil temperatures due to the relatively low rate of oospore germination and germ tube elongation. At higher soil temperatures, the competitive advantage was with the *P. aphanidermatum* due to the increased rates of oospore germination and germ tube elongation. Ben-Yephet et al. (1999) studied the effects of growth temperature on suppression of damping off in *P. aphanidermatum*, *P. irregulare*, and *P. myriotylum.* They found significant differences in suppression of Pythium damping off in cucumber as a result of the *Pythium* species responsible as well as growth temperature. *P. aphanidermatum* was the most aggressive pathogen, causing 100% damping off at all temperature levels (20°C, 24°C, 28°C, 32°C). *P. irregulare* and *P. myriotylum* were both sensitive to temperature. *P. irregulare* caused 100% damping off at 20°C, approximately 80% damping off at 24°C, and no damping off at the two highest temperature levels. *P. myriotylum* caused nearly 100% damping off at 20°C and 32°C, with damping off between 70% and 85% at the middle two temperatures. The Ben-Yephet study illustrates the complexity of the suppressive soil-amendment system. *Pythium* spp. are sensitive to abiotic and biotic factors, with each *Pythium* species reacting differently to its environment. This characteristic makes the development of non-chemical control strategies challenging.

Effect of soil solarization and timing of plastic mulch application on effectiveness of biofumigation

In a greenhouse study, Deadman et al. (2006) found that populations of *Pythium aphanidermatum* were significantly reduced after soil solarization and biofumigation + solarization, with post-harvest cabbage residue used as the brassica amendment. Population reductions for solarization alone were similar to those observed after the biofumigation + solarization treatment. As a result it is difficult to separate the role of the solarization from the role of the biofumigation in the observed reduction in pathogen populations. In a similar experiment Stapleton and Duncan (1998) investigated the effect of 6 different brassica soil amendments, in addition to a controlled temperature treatment, on pathogen suppression. They reported reductions in survival of *Pythium ultimum* ranging from 96.5% to 100%. To do so they used a diurnal temperature treatment with a maximum and minimum of 38°C and 27°C. When the temperature was held constant at 23°C, *P. ultimum* survival ranged from 6% to 39%. With the heat treatment, survival ranged from 0% to 3.5%. The non-amended control plus heat treatment allowed for 95% *P. ultimum* survival, suggesting that the difference in survival between amended and non-amended can be attributed to the brassica amendments. In their summer greenhouse study, Deadman et al. observed solarized soil temperatures of 46.6°C and 48.1°C, temperatures significantly higher than those used in the Stapleton heat treatment. The higher temperatures observed in the Deadman study could account for the disparity in the effect of the brassica incorporation. In a field solarization and biofumigation study conducted in 1999, Coelho et al. indicated that a soil temperature of 47°C was as

effective as methyl bromide at reducing soilborne populations of *Phytophthora* spp., another oomycete, to a depth of 10 cm. However, the addition of cabbage into the soil in concert with solarization did not enhance the effects of the solarization. This result was most likely related to the timing of the plastic application and the amount of brassica tissue incorporated. In the Coelho study the cabbage was harvested, and only the residue was incorporated. This reduced the amount of incorporated biomass, thereby reducing the amount of ITC that was released. Furthermore, the incorporation date and the plastic application date were separated by 9 days, which would be enough time for a large percentage of the volatile ITCs to escape from the soil surface. Njoroge et al. (2008) reported that ITCs were no longer detectable in brassica-incorporated plastic-covered soils after 12 days. This indicates that the results observed by Coelho et al. regarding brassica incorporation may have been the result of the biofumigation practices themselves, and not necessarily the ineffectiveness of the brassica incorporation.

Soil Microorganisms

Effect of exogenous myrosinase on glucosinolate hydrolysis efficiency

Naturally occurring soil microorganisms may affect the efficiency of the hydrolysis reaction which converts glucosinolates to isothiocyanates, and the subsequent biodegradation of the isothiocyanates. Gimsing et al. (2006) reported myrosinase activity between 6 and 24 nmol glucose h^{-1} g^{-1} soil (4.32 – 18 μg glucose g^{-1} soil 4 h^{-1}) in soil that had not been planted in a brassicaceous crop for at least 5 years, suggesting microbial myrosinase production. They also demonstrated that autoclaved soil had almost no

myrosinase activity, whereas the same soil samples without autoclaving had myrosinase activity. These findings further support the hypothesis that soil microorganisms are involved in the production of myrosinase and may play a role in the overall biofumigation system. Myrosinase has been shown to be produced by *Aspergillus* spp., commonly occurring soil fungi (Sakorn et al. 2002, Rakariyatham et al. 2006). Through mutagenesis Rakariyatham successfully increased the myrosinase production of a particular isolate of *Aspergillus*, *Aspergillus* sp. NR4617, by 71%. The efficient production of myrosinase by *Aspergillus* could have significant implications for the future of biofumigation. If the level of soil myrosinase could be increased at the time of brassica incorporation to a level that optimizes conversion efficiency of glucosinolate to isothiocyanate, then biofumigation could become significantly more efficacious. A study by Omirou et al. (2011) illustrated the effect that myrosinase had on biofumigation. In that study they compared the effects of fresh broccoli with and without added myrosinase on fungal community structure. Using phospholipid fatty acid detection (PLFA) to quantify the size of the ascomycete community in the soil, they found that both broccoli alone and broccoli with added myrosinase increased PLFA. Interestingly, the effect of broccoli with added myrosinase was delayed by approximately 60 days while the effect of broccoli alone was observed within 3 days. This result is most likely explained by an increase in ITC release efficiency due to the added myrosinase. The ascomycete community in the broccoli without myrosinase treatment was apparently able to overcome any fungicidal effects from the ITCs by utilizing the fresh broccoli tissue as a nutrient source. Upon addition of myrosinase the balance was tipped in favor of the ITCs, thereby creating a fungistatic effect. The effect of the ITCs lasted approximately 60 days,

at which time the ascomycete community had recovered and was then able to utilize the previously incorporated broccoli as a carbon source which lead to the delayed increase in the community size.

Bangarwa et al. (2011) tested conversion efficiencies of 3 types of glucosinolates to their subsequent isothiocyanates from 7 different brassicas. The ranges of conversion efficiencies were reported as follows: 2-propenyl ITC 3%-39%, benzyl ITC 1%-11%, 2-phenylethyl 1%-11%. Low ITC release efficiency is a significant obstacle which, if overcome, could significantly improve the results of biofumigation. Even if the commercial production of a highly efficient myrosinase producer such as *Aspergillus* sp. NR4617 proves not to be feasible, the improved understanding of the fungus as it relates to biofumigation could help to clarify why there is such a disparity in the results of previous biofumigation studies.

Factors influencing isothiocyanate biodegradation

Warton et al. (2001) linked the commonly occurring soil bacteria *Bacillus* spp. and *Rhodococcus* spp. to the enhanced biodegradation of methyl isothiocyanate (MITC) in soil. Enhanced biodegradation is the process by which soil microorganisms capable of catabolizing a specific pesticide proliferate as a result of repeated application of that pesticide, resulting in its rapid degradation and decreased effectiveness (Matthiessen & Kirkegaard, 2006). Warton demonstrated that the soil fumigant metam sodium produced less than half of the pesticidal MITC in a certain soil, demonstrating enhanced biodegradation compared to a control soil. Eleven isolates resembling *Rhodococcus* spp. and 4 isolates resembling *Bacillus* spp. were recovered from agar with MITC added as a

selective carbon source. They found that the introduction of the *Bacillus* and *Rhodococcus* isolates induced enhanced biodegradation. These results indicate that enhanced biodegradation of ITCs in soil is another factor that must be considered in the planning and implementation of biofumigation. As with any pesticide, repeated application of a biofumigant cover crop could result in its reduced efficacy. Even with natural management strategies or alternatives to synthetic chemical management, principles of integrated pest management should still be followed, specifically the rotation of chemical classes. As growers who previously relied on methyl bromide for control of soilborne diseases begin to implement methyl bromide alternatives, enhanced biodegradation may become a more widespread and serious issue. This is because methyl bromide is not susceptible to enhanced biodegradation. As a result growers could apply it year after year and not see a decrease in its efficacy. If growers adopt a methyl bromide alternative, such as an ITC based fumigant, without considering enhanced biodegradation then it is likely to develop over time. (Matthiessen & Kirkegaard, 2006).

Peppers (*Capsicum annuum*)

Overview of host plant

Peppers (*Capsicum annuum*), members of the plant family Solanaceae, are a warm-season crop that grow best at temperatures between 21°C to 24°C. Rows should be spaced 4 to 5 feet apart, with plants in double rows spaced 12 to 18 inches apart (Kemble et al. 2011). Peppers are susceptible to a variety of bacterial, viral, and fungal diseases. Genetic resistance and tolerance is available in some cultivars, however, all are

susceptible to the pathogens *R. solani*, *S. rolfsii*, and *Pythium* spp. *Capsicum annuum* cv. Hunter, the green bell pepper variety chosen for this study, has resistance to tobacco etch virus (TEV), tomato mosaic virus (TMV), and bacterial spot (Xcv: 1-5). It produces large to extra large, four-lobed, blocky fruit.

In 2007, 55,400 acres of bell peppers were planted in the United States yielding 270,000 lbs of peppers with a farm value of $468 million. Florida was the country's leading producer, generating $183 million in pepper production. California was second with production valued at $176 million. Florida and California consistently trade positions as the country's leading pepper producers. They were followed, in order of 2007 production value, by Georgia, Ohio, New Jersey, North Carolina, Michigan, and New York. Together those states generated $113 million. Bell pepper production in the Southeastern US was valued at over half of the country's total production value, $239 million, making bell peppers an important crop for Southeastern vegetable growers (USDA ERS, 2011).

Literature Cited

Al-Turki, A.I., & Dick, W.A. 2003. Myrosinase activity in soil. Soil Science Society of America Journal 67: 139-145.

Antonious, G F., Bomford, M., & Vincelli, P. 2009. Screening brassica species for glucosinolate content. Journal of Environmental Science and Health Part B 44: 3, 311-316.

Bangarwa, S.K., Norsworthy, J.K., Mattice, J.D., & Gbur, E.E. 2011. Glucosinolate and isothiocyanate production from Brassicaceae cover crops in a plasticulture production system. Weed Science 59: 247-254.

Ben-Yephet, Y., & Nelson, E. B. 1999. Differential suppression of damping-off caused by *Pythium aphanidermatum*, *P. irregulare*, and *P. myriotylum* in composts at different temperatures. Plant Disease. 83:356-360.

Blok, W. J., Lamers, J. G., Termorshuizen, A. J., & Bollen, G. J. 2000. Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. Phytopathology 90:253-259.

Borek, V., Morra, M.J., & McCaffrey, J.P. 1996. Myrosinase activity in soil extracts. Soil Science Society of America Journal 60: 1792-1797.

Beute, M.K., & Rodriguez-Kabana, R. 1981. Effects of soil moisture, temperature, and field environment on survival of *Sclerotium rolfsii* in Alabama and North Carolina. Phytopathology 71: 1293-1296.

Brown, J.E., Stevens, C., Osborn, M.C., & Bryce, H.M. 1989. Black plastic mulch and spunbonded polyester row cover as method of Southern blight control in bell pepper. Plant Disease 73: 931-932.

Charron, C. S., & Sams, C. E. 1999. Inhibition of *Pythium ultimum* and *Rhizoctonia solani* by shredded leaves of brassica species. Journal of the American Society of Horticultural Science 124(5): 462–467.

Chellemi, D. O., Mitchell, D. J., Kannwischer-Mitchell, M. E., Rayside, P. A., & Rosskopf, E. N. 2000. *Pythium* spp. associated with bell pepper production in Florida. Plant Disease 84: 1271-1274.

Coelho, L., Chellemi, D. O., & Mitchell, D. J. 1999. Efficacy of solarization and cabbage amendment for the control of *Phytophthora* spp. in North Florida. Plant Disease 83: 293-299.

Collins, H.P., Alva, A., Boydston, R.A., Cochran, R.L., Hamm, P.B., McGuire, A., & Riga, E. 2006. Soil microbial, fungal, and nematode responses to soil fumigation and cover crops under potato production. Biology and Fertility of Soils 42: 247–257.

Deadman, M., Al Hasani, H., & Al Sa'di, A. 2006. Solarization and biofumigation reduce *Pythium aphanidermatum* induced damping-off and enhance vegetative growth of greenhouse cucumber in Oman. Journal of Plant Pathology 88: 3, 335-337.

Dhingra, O.D., Costa, M.L.N., Silva Jr, G.J., & Mizubuti, E.S.G. 2004. Essential oil of mustard to control *Rhizoctonia solani* causing seedling damping off and seedling blight in nursery. Fitopatol. bras 29: 6.

Fenwick, G.R., Heaney, R.K., Mullin, W.J. 1983. Glucosinolates and their breakdown products in food and food plants. CRC Critical Reviews in Food Science and Nutrition 18:123–201.

Gamliel, A., & Stapleton, J. J. 1993. Characterization of antifungal volatile compounds evolved from solarized soil amended with cabbage residues. Phytopathology 83: 899-905.

Gardiner, J. B., Morra, M. J., Eberlein, C. V., Brown, P. D., & Borek, V. 1999. Allelochemicals released in soil following incorporation of rapeseed (*Brassica napus*) green manures. Journal of Agricultural and Food Chemistry 47: 3837-3842.

Gimsing, A.L., & Kirkegaard, J.A. 2006. Glucosinolate and isothiocyanate concentration in soil following incorporation of brassica biofumigants. Soil Biology and Biochemistry 38: 2255-2264.

Gimsing, A.L., Sorensen, J.C., Tovgaard, L., Jorgensen, A.M.F., & Hansen, H.C.B. 2006. Degradation kinetics of glucosinolates in Soil. Environmental Toxicology and Chemistry 25: 8, 2038–2044.

Hanson, B.D., J.S. Gerik, & S.M. Schneider. 2010. Effects of reduced rate methyl bromide applications under conventional and virtually impermeable plastic film in perennial crop field nurseries. Pest Management Science 66: 892-899.

Kasuya, M., Olivier, A. R., Ota, Y., Tojo, M., Honjo, H., & Fukui, R. 2006. Induction of soil suppressiveness against *Rhizoctonia solani* by incorporation of dried plant residues into soil. Phytopathology 96: 1372-1379.

Keinath, A. P., & DuBose, V. B. 2004. Evaluation of fungicides for prevention and management of powdery mildew on watermelon. Crop Protection 23: 35-42.

Kemble, J.M., Louws, F.J., Jennings, K.M., & Walgenbach, J.F. 2011. Southeastern U.S. 2011 vegetable crop handbook: 74-75.

Larkin, R.P., & Griffin, T.S. 2006. Control of soilborne potato diseases using brassica green manures. Crop Protection 26: 1067-107.

Love, J., & Beckerman, J. 2002. Southern blight of vegetables and herbaceous plants. University of Minnesota Extension Service P152S/P254S.

Matthiessen, J.N., & Kirkegaard, J.A. 2006. Biofumigation and enhanced biodegradation: opportunity and challenge in soilborne pest and disease management. Critical Reviews in Plant Sciences 25: 235-265.

Mayton, H. S., Olivier, C., Vaughn, S. F., & Loria, R. 1996. Correlation of fungicidal activity of brassica species with allyl isothiocyanate production in macerated leaf tissue. Phytopathology 86: 267-271.

Momma, N., Yamamoto, K., Simandi, P., & Shishido, M. 2006. Role of organic acids in the mechanisms of biological soil disinfestation (BSD). Journal of General Plant Pathology 72: 247–252.

Morra, M.J., & J.A. Kirkegaard. 2002. Isothiocyanate release from soil-incorporated brassica tissues. Soil Biology and Biochemistry 34: 1683-1690.

Njoroge, S. M. C., Riley, M. B., & Keinath, A. P. 2008. Effect of incorporation of *Brassica* spp. residues on population densities of soilborne microorganisms and on damping-off and Fusarium wilt of watermelon. Plant Disease 92: 287-294.

Noling, J.W., Botts, D.A., & MacRae, A.W. 2010. Alternatives to methyl bromide soil fumigation for Florida vegetable production. University of Florida IFAS extension vegetable production handbook: 47-54.

Olivier, C., Vaughn, S.V., Mizubuti, E.S.G., & Loria, R. 1999. Variation in allyl isothiocyanate production within brassica species and correlation with fungicidal activity. Journal of Chemical Ecology 25: 2687-2701.

Omirou, M., Rousidou, C., Bekris, F., Papadopoulou, K. K., Menkissoglou-Spiroudi, U., Ehaliotis, C., & Karpouzas, D. G. 2010. The impact of biofumigation and chemical fumigation methods on the structure and function of the soil microbial community. Microbial Ecology 61: 201-213.

Papavizas, G.C., Adams, P.B., Lumsden, R.D., Lewis, J.A., Dow, R.L., Ayers, W.A., & Kantzes, J.G. 1975. Ecology and epidemiology of *Rhizoctonia solani* in field soil. Phytopathology 65: 871-877.

Price, A.J., Charron, C.S., Saxton, A.M., & Sams, C.E. 2005. Allyl isothiocyanate and carbon dioxide produced during degradation of *Brassica juncea* tissue in different soil conditions. Hortscience 40: 1734-1739.

Rakariyatham, N., Butr-Indr, B., Niamsup, H., & Shank, L. 2006. Improvement of myrosinase activity of *Aspergillus* sp. NR4617 by chemical mutagenesis. Electronic Journal of Biotechnology 9 (4).

Ristaino, J.B., Perry, K.B., & Lumsden, R.D. 1991. Effect of solarization and *Gliocladium virens* on sclerotia of *Sclerotium rolfsii*, soil microbiota, and the incidence of southern blight of tomato. Phytopathology 81: 1117-1124.

Roberts, P.D. 2003. Damping-off and root rot. In Compendium of Pepper Diseases (pp. 12-13). The American Phytopathological Society.

Roberts, P.D. 2003. Southern blight. In Compendium of Pepper Diseases (pp. 20-21). The American Phytopathological Society.

Rodriguez-Kabana, R., Beute, M. K., & Backman, P. A. 1980. A method for estimating numbers of viable sclerotia of *Sclerotium rolfsii* in soil. Phytopathology 70: 917-919.

Sakorn, P., Rakariyatham, N., Niamsup, H., & Nongkunsarn, P. 2002. Rapid detection of myrosinase-producing fungi: a plate method based on opaque barium sulphate formation. World Journal of Microbiology & Biotechnology 18: 73-74.

Sarwar, M., & Kirkegaard, J.A. 1998. Biofumigation potential of brassicas. II. Effect of environment and ontogeny on glucosinolate production and implications for screening. Plant and Soil 201: 91-101.

Smith, B.J., & Kirkegaard, J.A. 2002. In vitro inhibition of soil microorganisms by 2-phenylethyl isothiocyanate. Plant Pathology 51: 585–593.

Sneh, B., Jabaji-Hare, S., Neate, S. & Dijst, G. 1996. *Rhizoctonia* species: taxonomy, molecular biology, ecology, pathology, and disease control. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Stapleton, J.J., & Duncan, R.A. 1998. Soil disinfestation with cruciferous amendments and sublethal heating: effects on *Meloidogyne incognita, Sclerotium rolfsii* and *Pythium ultimum*. Plant Pathology 47: 737-742.

Stranghellini, M.E., Stowell, L.J., Kronland, W.C., & von Bretzel, P. 1983. Distribution of *Pythium aphanidermatum* in rhizosphere soils and factors affecting expression of the absolute inoculum potential. Phytopathology 73: 1463-1466.

Sundermeier, A. 2008. Oilseed radish cover crop. Ohio State University Extension SAG-5-08.

Tedla, T., & Stranghellini, M.E. 1992. Bacterial population dynamics and interactions with *Pythium aphanidermatum* in intact rhizosphere soil. Phytopathology 82: 652-656.

[USDA ARS] U.S. Department of Agriculture Agricultural Research Service. 2011. Anaerobic soil disinfestation as an alternative to methyl bromide fumigation. http://www.ushrl.saa.ars.usda.gov/research/projects/projects.htm?ACCN_NO=412624&fy=2009. Accessed: July 21, 2011.

[USDA ERS] U.S. Department of Agriculture Economic Research Service. 2011. US bell & chili pepper statistics. http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1659. Accessed: June 20, 2011.

[USEPA] U.S. Environmental Protection Agency. 2011. Ozone layer depletion-regulatory programs: the phaseout of methyl bromide. http://www.epa.gov/ozone/mbr/index.html. Accessed: June 10, 2011.

van Bruggen, A. H. C., & Arneson, P. A. 1986. Quantitative recovery of *Rhizoctonia solani* from soil. Plant Disease 70: 320-323.

Warton, B. Matthiessen, J.N., & Roper, M.M. 2001. The soil organisms responsible for the enhanced biodegradation of metham sodium. Biology and Fertility of Soils 34:264–269.

Weil, R., White, C., & Lawley, Y. Forage radish: new multi-purpose cover crop for the Mid-Atlantic. University of Maryland Cooperative Extension Fact Sheet 824.

CHAPTER TWO

GREENHOUSE STUDY

Introduction

Biofumigation is the use of *Brassica* species and their natural chemicals to control soilborne pathogens, nematodes, or weeds. This is accomplished by a reaction between two isolated compounds that occur naturally within the plant: the enzyme myrosinase (thioglucoside glucohydrolase), located in myrosin cells, and the secondary metabolites glucosinolates (GSLs), which are contained in cell vacuoles. Upon tissue disruption the myrosinase catalyzes a hydrolysis reaction which converts GSLs into isothiocyanates (ITCs), the compounds primarily responsible for the plant's pesticidal properties, as well as a variety of other breakdown products including glucose, nitriles, thiocyanates, oxazolidine-2-thiones, hydroxynitriles, and epithionitriles (Al-Turki and Dick 2003, Gimsing and Kirkegaard 2009, Morra et al. 2002, Fenwick et al. 1983) (Figure 2.1).

A significant factor influencing the efficacy of biofumigation is the efficiency with which GSLs are converted into ITCs. Bangarwa et al. (2011) tested conversion efficiencies of three types of GSLs to their subsequent ITCs from seven different brassicas. The ranges of conversion efficiencies were: 2-propenyl ITC 3%-39%, benzyl ITC 1%-11%, and 2-phenylethyl 1%-11%. Morra et al. (2002) demonstrated that ITC conversion efficiency could be increased from 1% to 26.4% by freezing and thawing plants before incorporation rather than incorporating whole plants. Such a practice is obviously impractical for adaptation by growers and still fails to utilize the majority of the plant's biofumigation potential. Low ITC release efficiency is a significant obstacle

which, if overcome, could improve the results of biofumigation. Although GSL production can be used as a parameter by which to screen potential brassica biofumigants, it is ultimately the amount of ITCs released into the soil that determines whether or not the desired pathogen or weed suppression is achieved. This is because GSLs are inert and only become pesticidal once they have been converted into ITCs. For this reason it is important to understand the factors that affect GSL hydrolysis. It is known that GSLs and myrosinase are separated spatially within the tissues of brassicas. Thorough disruption of those tissues is therefore necessary to maximize contact between GSLs and myrosinase, thus achieving the plant's highest biofumigation potential. Although the amount of GSLs that are added to the soil at the time of incorporation is dependent on the chosen brassica cover crop, its biomass, and its growth stage, the amount of myrosinase available to hydrolyze those GSLs may be influenced by the soil microbial community. Gimsing et al. (2006) reported myrosinase activity between 6 and 24 nmol glucose h^{-1} g^{-1} soil (4.32 – 18 μg glucose g^{-1} soil 4 h^{-1}) in soil that had not been planted in a brassicaceous crop for at least 5 years, suggesting microbial myrosinase production. They also demonstrated that autoclaved soil had almost no myrosinase activity, whereas the same soil samples without autoclaving had myrosinase activity. These findings further support the hypothesis that soil microorganisms are involved in the production of myrosinase and may play a role in the overall biofumigation system. Myrosinase has been shown to be produced by *Aspergillus* spp., commonly occurring soil fungi (Sakorn et al. 2002, Rakariyatham et al. 2006). Omirou et al. (2011) illustrated the effect that myrosinase has on biofumigation. They compared the effects of fresh broccoli with and without added myrosinase on fungal community structure. Using phospholipid

fatty acid (PLFA) detection to quantify the size of the ascomycete community in the soil, they found that both broccoli alone and broccoli with added myrosinase increased PLFA. Interestingly, the effect of broccoli with added myrosinase was delayed by approximately 60 days while the effect of broccoli alone was observed within 3 days. This result is most likely explained by an increase in ITC release efficiency due to the added myrosinase. The ascomycete community in the broccoli without myrosinase treatment was probably able to overcome any fungicidal effects from the ITCs by utilizing the fresh broccoli tissue as a nutrient source. Upon addition of myrosinase the balance was tipped in favor of the ITCs, thereby creating a fungistatic effect. The effect of the ITCs lasted approximately 60 days, at which time the ascomycete community recovered and utilized the previously incorporated broccoli as a carbon source leading to the delayed increase in the community size. Understanding how exogenous soil myrosinase influences the conversion of GSLs into ITCs in field soil could help to clarify why there is such a disparity in the results of previous biofumigation studies (Collins et al. 2006; Larkin et al. 2006; Njoroge et al. 2008; Stapleton and Duncan 1998).

Another factor that could influence the efficacy of biofumigation is the rate at which ITCs are broken down in the soil. Warton et al. (2001) linked the commonly occurring soil bacteria *Bacillus* spp. and *Rhodococcus* spp. to the enhanced biodegradation of methyl isothiocyanate (MITC) in soil. Enhanced biodegradation is the process by which soil microorganisms capable of catabolizing a specific pesticide proliferate as a result of repeated application of that pesticide, resulting in its rapid degradation and decreased effectiveness (Matthiessen & Kirkegaard, 2006). Warton demonstrated that the soil fumigant metam sodium produced less than half of the

pesticidal MITC in soil demonstrating enhanced biodegradation compared to the control soil. Eleven isolates resembling *Rhodococcus* spp. and four isolates resembling *Bacillus* spp. were recovered from soil on agar with MITC added as a selective carbon source. They found that subsequent introduction of the *Bacillus* and *Rhodococcus* isolates induced enhanced biodegradation. These results indicate that enhanced biodegradation of ITCs in soil is another factor that must be considered in the planning and implementation of biofumigation.

Rhizoctonia solani is a commonly occurring soilborne fungal plant pathogen with a wide host range which contributes to damping-off and root rot (Sneh et al. 1996). *R. solani* has demonstrated sensitivity to brassica amendments in several studies. Larkin et al. (2006) showed significant *R. solani* population reduction in greenhouse assays and disease reduction in field tests following incorporation of brassica plant tissue, with *B. juncea* incorporation showing the highest disease reduction. Dhingra et al. (2004) also found that essential oil extracted from mustard seeds (*B. rapa*) significantly reduced the saprophytic growth of *R. solani* both in vitro and in field soils, although significantly higher mustard oil concentrations were required for field soil compared to in vitro studies.

Sclerotium rolfsii, the causal organism of Southern blight, is another commonly occurring soilborne fungal plant pathogen with a wide host range (Roberts, 2003). It also has shown sensitivity to brassica amendments. Gamliel et al. (1993) demonstrated *S. rolfsii* population reduction in soil amended with dried cabbage residue (*B. oleracea* Capitata group). Stapleton and Duncan (1998) reduced germination of *S. rolfsii* by 38% to 55% through the incorporation of cabbage residue into soil at a rate of 2%. They also

found that bok choy (*B. oleracea* var. *chinensis*) and broccoli (*B. oleracea* var. *italica*) suppressed sclerotia germination.

Pythium spp. are commonly occurring soilborne fungus-like plant pathogens that can cause damping-off and root rot in a variety of vegetable crops (Roberts, 2003). Smith and Kirkegaard (2002) reported reductions in growth rates of 13 different isolates and 3 species of *Pythium* in vitro following exposure to 2-phenylethyl ITC. Stapleton and Duncan (1998) reported significant reductions in survival of *P. ultimum* after incorporating 2% brassica tissue into soil in a controlled covered-container experiment.

The first objective of this study was to determine if naturally occurring background levels of soil myrosinase or soilborne *Bacillus* spp. were correlated with ITC concentrations following incorporation of freeze-dried 'Pacific Gold' mustard leaf tissue in diverse soil samples. The second objective was to determine the effect of the mustard amendment on populations of *R. solani, S. rolfsii*, and *Pythium* spp. and the rates of emergence and disease incidence in beans in the greenhouse. The third objective was to determine if levels of myrosinase and *Bacillus* spp. were correlated with populations of *R. solani, S. rolfsii*, and *Pythium* spp., and rates of emergence and disease incidence in beans.

Materials and Methods

Soil Sample Collection

From 31 October to 9 November 2010 15 soil samples were collected from various agricultural regions of South Carolina. Two additional samples were collected on

25 January 2011. Soil samples were collected from the base of plants in those fields which still had crops or crop residues in them. Samples were stored through winter in a non-heated storage room in 30 gallon plastic bins with lids. General characteristics of each soil sample are listed in Table 2.1.

Determination of soil infestation rate for *R. solani* and *S. rolfsii*

On 3 December 2010 an experiment was set up to determine the infestation rate required to induce 50% disease in beans for both *Rhizoctonia solani* and *Pythium* spp. Field soil collected from the Clemson University CREC was used in the experiment. Seventy-two-cell flats were used, with 18 cells per experimental unit and an empty row of cells between experimental units. For each pathogen three inoculum densities were tested along with an uninfested control. Experimental inoculum densities for *R. solani* were 25 sclerotia kg^{-1} soil, 50 sclerotia kg^{-1} soil, and 100 sclerotia kg^{-1} soil. *S. rolfsii* experimental inoculum densities were 41 sclerotia kg^{-1} soil, 82 sclerotia kg^{-1} soil, and 144 sclerotia kg^{-1} soil. Emergence, pre-emergence damping off, and post-emergence damping off counts were taken 12 days after seeding. One seed from each treatment that failed to emerge was plated on pimaricin-ampicilin-rifampicin-pentachloronitrobenzene medium prepared with pimaricin at 5 mg L^{-1} (P_5ARP) to determine if *Pythium* contributed to pre-emergence damping off (Jeffers and Martin, 1986). Seeds were rinsed with DDI water, soaked in 5% bleach for 30 seconds, rinsed again with DDI water, and blotted dry prior to plating for surface sterilization.

Determination of optimal soil conditions for bean emergence in the greenhouse

High levels of *Pythium* damping-off were observed during the preliminary experiment to determine soil infestation rates for *R. solani* and *S. rolfsii*. As a result it was necessary to amend the field soil for the greenhouse study in order to optimize bean emergence in the non-infested control plots. Field soil was again taken from the Clemson University CREC, and an additional preliminary greenhouse experiment was conducted. Treatments were designed to reduce moisture retention and *Pythium* spp. population densities in the field soil. The experimental design was a 2 X 2 X 3 factorial split-plot, with 2 whole plot factors and 3 sub-plot factors. The whole plot factors included 2 seed treatments, direct-seeded and pre-germinated, and 2 fungicide treatments, (+) Ridomil Gold EC (mefenoxam) and (-) Ridomil. Ridomil is labeled for the control of Pythium damping-off of beans. Seeds for the pre-germinated treatment were kept on a temperature gradient table at 26.7°C for one week prior to planting. Ridomil was applied at 1.054 μL 50 ml^{-1} H_20 (approximately 1 pint $acre^{-1}$) to each (+) Ridomil treatment. Ridomil application was done by placing 1 kg field soil in a large Ziploc bag, adding the Ridomil solution, and mixing thoroughly. The treated soil was then spread evenly over the entire 72-cell flat. The sub-plot factors included the following: mixed treatment of 75% field soil and 25% perlite; layered treatment of 50% field soil above 50% potting soil; and 100% field soil control. Whole plot factors were applied to 72 cell flats, which were divided into 3 18-cell sub plots with an empty row of cells between each. On 7 January 2011 beans were planted, 1 bean per cell. Two weeks after planting emergence and disease counts were taken. Plants were considered emerged if 2 true leaves had opened,

and diseased if a stem or leaf lesion was present or if there was no sign of emergence. All cells fit one of the two categories. On 24 January a representative sampling of stem lesions and roots were taken from diseased plants to determine whether *Pythium* spp. and *Rhizoctonia solani* were present. Samples were soaked in 5% bleach and 10% bleach for 30 seconds for *Pythium* and *Rhizoctonia*, respectively, for surface sterilization. They were then plated on water agar (15 g L^{-1}) with streptomycin (100 μg L^{-1}) (WAS) and identified morphologically.

Preliminary microorganism assays to determine background levels

From 10 January to 17 January 2011 pathogen populations in each of the regional soil samples were quantified. To estimate the inoculum density of *Pythium* spp. in the soil, 10 g of soil from each sample was added to 200 ml of 0.3% water agar and vortexed for 30 seconds. Five aliquots from each sample, 0.5 ml each, were then spread on plates of P_5ARP using a wide-mouth glass pipette. The plates were then incubated at 20°C for 20 h and then the adhering soil was washed away using DDI water. Plates then continued to incubate at 20°C, and colonies of *Pythium* > 0.5 cm in diameter were counted 48 h after plating. Colony counts were expressed as colony forming units (CFU) per gram of soil dried for 24 hours at 100°C.

To estimate the inoculum density of *R. solani* in the soil, 400 g of soil from each sample were placed in a #18 mesh sieve (1-mm openings) and rinsed thoroughly with tap water (Keinath, 1995). The organic matter trapped by the sieve was air dried on paper towels at ambient temperature overnight. The organic matter was then weighed and plated, 10 small heaps of equal size per plate (van Bruggen et al. 1986), on ethanol-

potassium nitrate (EPN_2) medium with prochloraz (Trujillo et al. 1987), prepared with 2% ethanol (Vincelli et al. 1989). Plates were stored in the dark at 23°C-25°C. After 3 days the total number of heaps with and without colonies of *Rhizoctonia* growing from them was recorded.

To estimate the inoculum density of *Sclerotium rolfsii* in the soil, 300 g of soil from each sample were air dried over night in aluminum pans (30 x 24 x 4 cm) at ambient temperatures. Soil was then crushed in the aluminum pans to break apart soil aggregates using a glass beaker. The dried soil was then spread evenly in the pans and moistened with 75 ml 1.33% methanol (Rodriguez-Kabana et al., 1980). Pans were then placed in large Ziploc plastic bags and incubated at 30°C for 3 days. Colonies of *S. rolfsii* were then counted by visually examining the soil surface.

Assays were conducted on 26 January 2011, and again on 17 March 2011, to quantify populations of spore-forming *Bacillus* spp. in the greenhouse soil samples. Using the methods described by Keinath (1996), 10 g of soil from each sample was added to 90 ml DDI water. Each sample was then diluted twice using a 10-fold serial dilution to obtain a concentration of 10^{-3}. Samples were then incubated at 80°C for 10 min, followed by an additional 10-fold serial dilution to obtain a concentration of 10^{-4}. Each dilution was then spread evenly on plates of one-tenth-strength tryptic-soy agar, 0.1 ml per plate, and 3 plates per dilution. On 17 March only 10^{-4} dilutions were plated. Plates were then stored in the dark at ambient temperature, and after 4 days colonies of thermotolerant *Bacillus* spp. were counted.

Soil myrosinase assay

Soil myrosinase activity was quantified using the methods described by Al-Turki and Dick (2003). Two separate sub-samples were collected from each regional soil sample, with two replications analyzed from each sub-sample (a total of four samples analyzed per soil sample). One gram of soil for each replication was placed in a 50 ml plastic centrifuge tube. Each tube then received 0.2 ml toluene, 2.2 ml 0.1M TES buffer (adjusted to pH 7 with 0.1 N NaOH), and 0.5 ml sinigrin prepared in 0.1M TES buffer (pH 7) for a final concentration of 20mM. Each tube was then vortexed and incubated for 4 h at 37°C. Control tubes for each sub-sample were prepared the same way without the addition of sinigrin, which was added after the 4 h incubation. Tubes were then centrifuged at 8000 x g for 10 min, and the supernatant filtered through a 0.45 μm MF-Millipore membrane filter. One ml of each filtered supernatant was then added to a glass test tube. The remaining steps were followed according to the glucose assay kit technical bulletin (Sigma-Aldrich, product code GAGO-20). To begin the reaction, 2 ml of assay reagent was added to each tube in 1 min intervals and allowed to react for exactly 30 min. Each reaction was stopped by adding 2 ml 12 N H_2SO_4. Absorbance of each sample and control was then measured against the reagent blank at 540 nm using a Molecular Devices Spectra Max 384 Plus spectrophotometer. Absorbance values were then converted into glucose concentrations using a standard curve (Figure 2.2). Glucose concentrations used to generate the standard curve were 1 μg mL^{-1}, 5 μg mL^{-1}, 20 μg mL^{-1}, 40 μg mL^{-1}, 60 μg mL^{-1}, and 80 μg mL^{-1}. Sample glucose concentrations were then compared directly to their control glucose concentrations. Higher glucose concentrations

in the samples indicated that myrosinase had converted sinigrin into glucose, among other hydrolysis products, during the 4 h incubation.

Mustard amendment preparation

'Pacific Gold' mustard plants were harvested at the bud-raised growth stage on 15 November 2010, 52 days after seeding, and immediately brought into the laboratory. Leaves were sliced to remove the mid-rib and placed in Ziploc bags, 50 g per bag, which were then immediately stored in a -80°C freezer prior to freeze-drying. The freeze-dryer shelf temperature was allowed to cool to -25°C before samples were loaded. Four 50 g sample bags were then quickly loaded onto each of the freeze-dryer's 5 shelves. The freeze-dryer was then sealed. The temperature was then reduced to -40°C, at which time the freezer was turned off and the heater and condenser were turned on. After 2 days the shelf temperature was increased to -15°C. On the morning of the 6^{th} day the shelf temperature was increased to 0°C, followed by 12°C at 12:00 pm and 25°C at 2:00 pm. Once the shelf temperature reached 25°C samples were removed and stored in large Ziploc bags at ambient temperature until all of the mustard tissue was freeze-dried. The mustard leaves were then completely homogenized into a powder in a Waring® commercial laboratory blender and placed in a sealed plastic container. The powder was then stored in a refrigerator.

Greenhouse study

An experiment was set up in the greenhouse to determine the effect of soil myrosinase and soilborne *Bacillus* spp. on ITC release efficiency and overall

biofumigation efficacy. Soil samples were initially screened to determine levels of myrosinase activity. Five soils with the lowest levels of myrosinase activity were dropped from the experiment (soil sample numbers 2, 5, 7, 12, and 14) (Table 2.1). The experimental design was split-plot with 4 replications. The whole plot factor was soil sample. Sub-plot factors were: added *R. solani* inoculum ((+) Rz); added *S. rolfsii* inoculum ((+) Sr); no-pathogen control (no added inoculum). Whole-plot factor experimental units were 72-cell flats. Sub-plot factor experimental units were 18 cells of a 72-cell flat. Autoclaved soil was included as a separate control to eliminate the effect of soil biological activity. Soil autoclaving was done based on the procedures by Bennett et al. (2003). Soil obtained from Clemson University CREC was placed in 4 separate aluminum trays to a depth of 2.5 cm and sealed with aluminum foil. Soil was then autoclaved for 30 min and left at ambient temperatures for 24 h. Soil was then autoclaved a second time for 30 min. On 31 January 1 kg soil from each of the 12 soil samples was placed in 1 of 3 labeled 9.5- liter Ziploc bags for replication 1. The procedure was repeated on 1 and 2 February for replications 2, 3, and 4. *S. rolfsii* and *R. solani* inoculum was added to each of the pathogen treatments separately, and the control treatment was left uninfested. *S. rolfsii* and *R. solani* were added at 0.069 g (~144 sclerotia) kg^{-1} soil and 0.212 g (~100 sclerotia) kg^{-1} soil, respectively. Before being added to soil, mustard was placed in a glass test tube and combined with DDI H_2O at a rate of 1 g dry mustard 18.32 mL^{-1} H_2O and vortexed for 30 s. Freeze-dried mustard leaf tissue was added to each of the soil bags at a rate of 0.89 g dry mustard 1 kg^{-1} dry soil. The mustard+H_2O solution was then added to each bag, immediately sealed, and mixed thoroughly by hand for 30 s. Bags were then left sealed at ambient temperature for 4 h, at which time each of the (+)

Sr and (+) Rz treatments from each soil were sampled for ITCs. Bags were immediately resealed and stored at ambient temperature for a total of 48 h.

Perlite was added to each soil sample at 25% v/v (250 ml perlite kg^{-1} soil) to reduce water retention. Soil from each bag was spread evenly in 18 cells of a 72 cell flat, with a row of cells left empty between each experimental unit. Beans were directly seeded to a depth of 1.5 cm, 2 seeds per cell. Plant counts were taken weekly for 3 weeks. Plants were considered emerged of two true leaves had emerged from the soil. Diseased plants were counted 2 weeks after seeding. Plants were considered diseased if any part of the shoot portion had a visible lesion. Stem portions were harvested and weighed after 3 weeks. Six root systems from both diseased and healthy plants were taken from non-infested control treatments (soil sample numbers 1, 6, 10, 15, and autoclaved), surface disinfested for 30 seconds in 5 % bleach and plated on WAS to identify the presence of *Pythium* spp. or *R. solani*. The same was done for stem lesions taken from 20 different plants representing all pathogen treatments and soil samples. Microbial growth was observed and recorded two days after plating.

Determination of ITC concentrations in soil samples

Prior to each soil sampling, one 50-ml plastic centrifuge tube was prepared for each (+) Rz and (+) Sr experimental unit. Each tube contained 17 μL cyclohexane as an internal standard, 12 ml dichloromethane, and 5 ml 0.2M $CaCl_2$. Four hours after incorporation of freeze-dried mustard tissue, 50 g soil samples were deposited into prepared centrifuge tubes and sealed. Samples were shaken for 15 minutes at 270 rpm, and then centrifuged for 10 minutes at 8000 x g. The dichloromethane fraction of each

sample was then removed using a disposable glass pipette, and placed in a glass test tube containing 0.4 g anhydrous sodium sulfate for desiccation. After 1 hour, 1.5 – 2.0 ml was removed from each tube and passed through a 0.45 μm filter into a GC vial and capped. Samples were then placed in a -80°C freezer pending analysis (Gardiner et al. 1999). On 7 March 2011, ITC samples were analyzed in the Clemson University multiuser analytical laboratory using a Hewlett Packard 5890 gas chromatograph with a 5971A mass selective detector (GC-MS) and a DB5-MS column (30 m x 0.25 mm, 0.5 μm film, Agilent Technologies). Three replicates were analyzed per treatment.The temperature program was at 40°C for 3 min, then increased 6°C min^{-1} to 240°C, followed by 30°C min^{-1} to 280°C which was held for 1 min. Injection volume was 3 μL per sample, with helium as the carrier gas. Allyl ITC peak areas were converted into allyl ITC concentrations using a standard curve (Figure 2.3). Allyl ITC concentrations used to generate the standard curve were $5.0x10^{-6}$ μg mL^{-1}, $9.0x10^{-6}$ μg mL^{-1}, $1.7x10^{-5}$ μg mL^{-1}, and $3.3x10^{-5}$ μg mL^{-1}. Each sample was also checked for the presence of benzyl ITC and 2-phenylethyl ITC, however neither were detected.

Determination of inoculum viability and effect of mustard amendment on pathogen populations

To determine whether or not the added inoculum was viable, as well as the effect of mustard on pathogen populations, a separate experiment was done with and without mustard added to two soils. Pathogen populations with and without added inoculum were compared directly by removing any pesticidal effect of the mustard amendment. Similarly, pathogen populations were compared from (+) mustard and (-) mustard

samples to quantify the effect of the mustard amendment. Soil samples 3 and 15 were chosen because of the variability of the sample characteristics such as soil type, previous plantings, and location (Table 2.1). Samples were prepared, three replications each, and beans were sown the same way as previously described except mustard was not added to half of the experimental units. Pathogens were quantified using the same techniques as previously described with the exception of *R. solani*. Due to the fact that perlite floats, it was not feasible to separate the soil organic matter from the perlite using the wet-sieving technique. As a result, the technique described by Papavizas et al. (1975) which uses beet seeds to quantify *R. solani* was used instead. First, 'Detroit Dark Red' beet seeds were weighed, one 1.1 g portion per soil sample. Seeds were then autoclaved for 10 min before being combined with 100 g of sample soil in a GA 7 tissue culture vessel. Vessels were then incubated at 25°C for 3 days. Following incubation, beet seeds were recovered in a sieve, rinsed with water, and dried with a paper towel. Seeds were then placed on plates of EPN2 with prochloraz, 7 seeds per plate and 4 plates per sample. Plates were then incubated at room temperature in the dark and incidence of *R. solani* infection was observed after 3 days. Also, the quantity of soil used in the *S. rolfsii* assay was adjusted from 300 g to 310 g to account for the added perlite.

Statistical analysis

All data were checked for normality using the Shapiro-Wilk test. Data from split-plot designs were analyzed using PROC MIXED in SAS (SAS, Inc., Cary, NC). Marginal means were used whenever treatment interactions were not detected ($P<0.05$), otherwise cell means were used. All correlation analyses were done using PROC CORR in SAS.

Results

Soil treatment and infestation rate

Table 2.2 shows data on rates of damping-off and emergence of beans associated with three different inoculum densities. Higher inoculum densities generally resulted in higher levels of damping-off and lower emergence, with the exception of pre-emergence damping-off from *S. rolfsii*. However, there were still high rates of damping-off in the non-infested control treatments. *Pythium* was detected in 6 out of the 8 diseased seeds plated (data not shown). Table 2.3 contains data on emergence and disease incidence from the emergence optimization experiment. There were no significant differences detected among any of the seeding treatments, Ridomil treatments, or soil physical treatments. Ridomil is a Group 4 fungicide and is susceptible to the development of insensitive fungal strains. My soil samples may have contained mefenoxam-insensitive strains of *Pythium* spp., resulting in Ridomil's ineffectiveness. Among stem lesions plated for pathogen identification, 6 out of 12 contained *R. solani* and 6 out of 12 contained *Pythium* spp. Among roots plated for pathogen identification, 1 out of 7 contained *R. solani* and 5 out of 8 contained *Pythium* spp. (data not shown).

Background microorganism levels

Table 2.4 contains the background population levels of *R. solani*, *Pythium* spp., *S. rolfsii*, and thermotolerant *Bacillus* spp. from each of the 17 soil samples. Population densities of *R. solani* ranged from 0 – 4.38 g *R. solani*-infested dry organic matter kg^{-1}

soil, with a mean of 1.79 g *R. solani*-infested dry organic matter kg^{-1} soil. *Pythium* spp. population densities ranged from 0 – 114 CFU g^{-1} soil, with a mean of 24 CFU g^{-1} soil. *S. rolfsii* population densities ranged from 0 – 7 CFU 300 g^{-1} soil, with a mean of 1.5 CFU 300 g^{-1} soil. *Bacillus* spp. population densities ranged from 23 – 77 CFU g^{-1} soil, with a mean of 41 CFU g^{-1} soil. *Bacillus* spp. were not quantified in samples 2, 5, 7, 12, and 14 because those samples had already been eliminated from the greenhouse experiment based on the results of the soil myrosinase assay.

Soil myrosinase activity and ITC concentration after mustard incorporation

Table 2.5 shows myrosinase activity prior to mustard incorporation and allyl ITC concentration after incorporation of freeze-dried mustard tissue for each soil sample. Myrosinase levels were used as one of the criteria for selecting samples to be included in the greenhouse study. Generally, samples with the highest levels of myrosinase activity were included in the greenhouse study, and those with the lowest levels were eliminated. However, a few samples with low myrosinase activity were included for comparison. Soil myrosinase activity was represented as glucose concentration (μg mL^{-1}) after samples incubated for 4 h at 37°C with sinigrin, followed by 30 min incubation with the assay reagent at 37°C. All samples were compared to their control, and the differences between controls and samples are listed in Table 2.5. Myrosinase activity in the 17 soil samples ranged from -0.71 μg glucose g^{-1} soil 4 h^{-1} to 5.87 μg glucose g^{-1} soil 4 h^{-1}. Allyl ITC values ranged from 1.8 to 13.4 μg g^{-1} soil. Myrosinase activity and allyl ITC values were converted to percentages relative to the sample with the highest value. This was done to equalize the scales used for myrosinase activity and allyl ITC concentrations, thus simplifying comparisons between them.

Inoculum viability

Samples 3 and 15 were used to determine if added *R. solani* and *S. rolfsii* inoculum was viable. Table 2.6 shows pathogen populations in soil samples with and without added inoculum. There was a significant sample by treatment interaction ($P<0.05$), so soil sample treatment means were not combined. Added *R. solani* inoculum significantly increased the percentage of beet seeds colonized by *R. solani* in both soil samples. Added *S. rolfsii* inoculum significantly increased the mean CFU 110 g^{-1} soil compared to the non-infested control in both soil samples.

Effect of mustard amendment on pathogen populations and bean emergence, disease incidence, and shoot weight

Soil samples 3 and 15 were used to determine if freeze-dried mustard added to soil had an effect on populations of *R. solani*, *S. rolfsii*, and *Pythium* spp. Table 2.7 shows pathogen populations in soil samples with and without mustard amendment. There were no differences found between soil samples 3 and 15 ($P<0.05$), nor were there significant soil sample by mustard treatment interactions, so treatment means were combined. Freeze-dried mustard amended into the soil did not significantly affect the levels of any of the three pathogens assayed. The largest difference was observed in the *Pythium* assay, in which the (+) mustard treatment mean was 40.5 CFU compared to 14.5 CFU in the (-) mustard control, which was not significant (LSD, $P=0.4081$).

Soil samples 3 and 15 were also used to determine if the mustard amendment affected bean emergence, disease incidence, or shoot weight. All three pathogen

treatments with mustard (+Rz, +Sr, and control) were compared to the same pathogen treatments without the mustard amendment. Mustard did not significantly affect emergence, disease incidence, or shoot weight in either sample 3 or sample 15 in the (+)Rz, (+)Sr, or control treatments. There was a significant soil-sample by pathogen-treatment interaction for both emergence and shoot weight, so data were analyzed separately by soil sample ($P<0.01$). Since there were no other significant treatment interactions marginal means were analyzed within soil samples. Mustard did not have a significant effect on emergence, disease incidence, or shoot weight means across all three pathogen treatments within soil sample ($P<0.05$) (Table 2.8).

Diseased bean pathogen identification

Bean roots taken from non-pathogen infested control samples and autoclaved control samples were observed 26 days after planting. All of the roots that were plated on WAS were positive for *Pythium* spp., including those taken from visibly healthy plants in the autoclaved control soils. *R. solani* was identified in 2 of the 6 root systems plated, both of which were taken from visibly diseased plants. Of the 20 stem lesions plated on WAS, 9 were found to contain *Pythium* and 10 contained *R. solani*. Three of the stems were neither infected with *Pythium* nor *R. solani*, and two were infected with both pathogens. Lesions were described as ‘dry and scaly’, ‘dark and sunken’, or ‘water-soaked’. *R. solani* was not identified in any of the lesions described as dry and scaly, the majority of which were positive for *Pythium*. Both *Pythium* and *R. solani* were indentified in the other two lesion types. The autoclaving procedure may have failed to

completely disinfest those samples, which could explain the *Pythium* recovered in beans grown in them.

Effect of soil sample and pathogen infestation treatment on bean emergence, disease incidence, and shoot weight

Soil sample and pathogen infestation treatment significantly affected bean emergence, disease incidence, and shoot weight overall ($P<0.01$) in treatments which received freeze-dried mustard. There was also a significant soil-sample*pathogen-treatment interaction for bean emergence, disease incidence, and shoot weight ($P<0.01$) (Table 2.9). Mean values of bean emergence for each pathogen infestation treatment were 58.3% for non-infested control, 56.2% for (+)Rz, and 47.6% for (+)Sr, and ranged from 28.0% to 75.9% among soil samples. Mean values of disease incidence for each pathogen infestation treatment were 29.1% for non-infested control, 43.9% for (+)Sr, and 73.3% for (+)Rz, and ranged from 8.3% to 79.7% among soil samples. Mean values of shoot weight for each pathogen infestation treatment were 53.8 g for non-infested control, 43.7 g for (+)Rz, and 41.5 g for (+)Sr, and ranged from 72.0 g to 24.4 g among soil samples. Means for each soil sample are also listed, however significant differences are not shown for the marginal means due to the significant sample*treatment interaction. Compared to the non-infested controls either (+)Rz or (+)Sr pathogen treatments significantly affected emergence in 5 soil samples (samples 3, 13, 15, 16, and 17), disease incidence in 10 soil samples (samples 3, 4, 6, 8, 10, 11, 13, 15, 16, and 17), and shoot weight in 6 soil samples (samples 3, 8, 13, 15, 16, and 17).

Correlations between ITC concentrations, soil myrosinase, and *Bacillus* spp. population densities

Correlation analyses were performed to determine if ITC concentrations correlated positively with soil myrosinase activity and negatively with soilborne *Bacillus* spp. population densities. Additionally, ITC concentrations were correlated separately with bean emergence, disease incidence, and shoot weight. None of the comparisons yielded a significant correlation coefficient ($P<0.05$) (Table 2.10).

Discussion

Myrosinase activity in the 17 soil samples ranged from -0.71 μg glucose g^{-1} soil 4 h^{-1} to 5.87 μg glucose g^{-1} soil 4 h^{-1}. Negative myrosinase values resulted from samples which had higher glucose concentrations in the controls than the samples after the 4-h incubation. This either meant that those samples had no myrosinase activity and the added sinigrin stimulated glucose consumption in the samples, or that those samples had higher background levels of glucose in the controls vs. the samples. In either case, negative myrosinase values were considered to be the equivalent of zero soil myrosinase activity for the purposes of this study. Al-Turki and Dick (2003) reported soil myrosinase activity between 71 μg glucose g^{-1} soil 4 h^{-1} and 323 μg glucose g^{-1} soil 4 h^{-1} among 5 different soil types tested. In that study pots containing each soil type were planted in 'Ida Gold' mustard (*Sinapsis alba*), and rhizosphere soil adhering to the roots of the plants was used for soil myrosinase analysis. Although their findings do accurately reflect

mustard-rhizosphere soil myrosinase activity, the levels that I detected are more indicative of soil myrosinase activity that would generally be observed in typical bulk field-soil samples. In unpublished work Warren Dick found large and rapid decreases in soil myrosinase activity as samples were taken farther from plants roots (W. Dick, *personal communication*). Despite the differences in myrosinase activity observed among my soil samples, soil myrosinase was not significantly positively correlated with ITC concentrations released after incorporation of freeze-dried mustard leaves. Soil myrosinase activity was not clearly associated with recent crops of brassicas planted in the fields from which the soil samples were collected, further supporting the hypothesis that there are myrosinase producers in the soil which are not dependent upon an association with brassicas (Gimsing et al. 2006, Sakorn et al. 2002, Rakariyatham et al. 2006). Although exogenous myrosinase was common among my soil samples, it was not present at high enough levels to have an identifiable effect on ITC release efficiency. Since I did not quantify known myrosinase producing *Aspergillus* spp. I cannot say whether or not samples which came from fields with repeated brassica plantings had higher levels of them. It is clear that even if *Aspergillus* spp. were being selected for in such fields, the levels of myrosinase being produced by them were negligible and did not affect the efficacy of biofumigation in the greenhouse.

Similar results were obtained for ITC-degrading *Bacillus* spp. Population densities of *Bacillus* spp. ranged from 23 CFU g^{-1} soil to 77 CFU g^{-1} soil, and were not significantly negatively correlated with ITC concentrations. The range in population densities across soil samples was probably too small to detect any effect they may have had on ITC biodegradation. Additionally, *Bacillus* spp. population densities were not

clearly associated with recent brassica plantings, suggesting that brassicas were not selecting for them. It is probable that the brassica crops that had recently been grown in the fields sampled (broccoli, turnip, kale, collards, radish) did not release sufficiently high levels of ITCs to select for ITC-degrading microorganisms. Brassica vegetable varieties are often selected for low levels of GSLs due to the bitter taste associated with the ITCs they release (D'Antuono et al. 2009). Warton et al. (2001) clearly demonstrated that repeated application of metam sodium resulted in the enhanced biodegradation of its active ingredient, MITC. There is no doubt that repeated incorporation of a specific crop residue into soil imposes a selection pressure on the soil microbial community. Although *Bacillus* has been implicated in ITC degradation (Warton et al. 2001), the biodegradation of a chemical in soil is affected by a number of factors including soil abiotic characteristics and the reaction of the entire soil biological community to the presence of the chemical (Aislabe & Lloyd-Jones, 1995). As a result, *Bacillus* spp. population densities may not have been an accurate biological parameter to associate with the biodegradation of allyl ITC, the primary ITC released from mustard leaf tissue. The difficulty in dealing with diverse field soil samples is that they have different biotic and abiotic makeups, thus making controlled comparisons across them challenging. Despite this, since neither soil myrosinase nor populations of soilborne *Bacillus* spp. had an effect on ITC concentrations detected in soil after incorporation of mustard leaves, it was not surprising that they also failed to correlate significantly with populations of soilborne pathogens and disease incidence in beans. Therefore, I conclude that soil myrosinase activity and soilborne *Bacillus* spp. populations are not useful in predicting the outcome of biofumigation with 'Pacific Gold' mustard.

Freeze-dried 'Pacific Gold' mustard leaves incorporated into soil samples 3 and 15 did not reduce populations of *R. solani, S. rolfsii,* or *Pythium* spp. The mustard amendment had no significant effect on any of the 3 pathogens assayed, although *Pythium* counts were more than 2.5 times higher in the (+) mustard treatments compared to the (-) mustard controls. The lack of statistical significance in the *Pythium* assays was attributed to the fact that only two replications were analyzed per treatment because there was no (+) *Pythium* treatment to analyze along with the control treatments. *Pythium* spp. populations have been reported to increase after incorporation of canola into field soil, and ITCs were inconsistently detected in soil samples following incorporation (Njoroge et al. 2008). In my study, allyl ITC was detected in all of the 72 soil samples analyzed. Allyl ITC concentrations of 5.5 μg g^{-1} soil and 2.3 μg g^{-1} soil were detected in samples 3 and 15, respectively, suggesting that concentrations higher than these are required to achieve pathogen suppression. Smith and Kirkegaard (2002) reported that concentrations between 496 μg and 85.5 mg (0.005 mM – 0.862 mM) of 2-phenylethyl ITC were required to reduce in vitro radial growth of various species of *Pythium* by 90%. It is likely that the added carbon from the mustard tissue had a stimulatory effect on the *Pythium*, which outweighed any fungicidal effect that the ITCs may have had (Omirou et al. 2011). In my controlled laboratory experiment I achieved total brassica tissue disruption by freeze-drying and blending mustard leaf tissues, added the mustard tissue to DDI water to ensure adequate moisture for GSL hydrolysis, and sealed the mixture in a Ziploc bag to limit volatile escape. My rate of mustard application, 0.89 g freeze-dried dry mustard 1 kg^{-1} dry soil, was chosen to simulate field-biomass potential of *B. juncea* as determined by Duke (1997) and Price et al. (2005). Although the glass jars used by

Price were probably less permeable to ITCs than the Ziploc bags I used, the bags were completely sealed and probably contained the ITCs as well or better than plastic mulch sealed with soil. Since I failed to identify a significant effect of the mustard soil amendment on populations of *R. solani, S. rolfsii,* or *Pythium* spp., or the diseases they cause in beans, I conclude that 'Pacific Gold' mustard is not a good biofumigant candidate for the control of these three pathogens in the field, where ITC release efficiency would be expected to be significantly lower due to the logistical limitations of implementing biofumigation in the field.

Literature Cited

Aislabie, J., & Lloyd-Jones, G. 1995. A review of bacterial degradation of pesticides. Australian Journal of Soil Research 33: 925–942.

Al-Turki, A.I., & Dick, W.A. 2003. Myrosinase activity in soil. Soil Science Society of America Journal 67: 139-145.

Bennett, A.J., Leifert, C., & Whipps, J.M. 2003. Survival of the biocontrol agents *Coniothyrium minitans* and *Bacillus subtilis* MBI 600 introduced into pasteurised, sterilised and non-sterile soils. Soil Biology & Biochemistry 35: 1565–1573.

Bangarwa, S.K., Norsworthy, J.K., Mattice, J.D., & Gbur, E.E. 2011. Glucosinolate and isothiocyanate production from Brassicaceae cover crops in a plasticulture production system. Weed Science 59: 247-254.

Collins, H.P., Alva, A., Boydston, R.A., Cochran, R.L., Hamm, P.B., McGuire, A., & Riga, E. 2006. Soil microbial, fungal, and nematode responses to soil fumigation and cover crops under potato production. Biology and Fertility of Soils 42: 247–257.

D'Antuono, L.F., Elementi, S., & Neri, R. 2009. Exploring new potential health-promoting vegetables: glucosinolates and sensory attributes of rocket salads and related *Diplotaxis* and *Eruca* species. Journal of the Science of Food and Agriculture 89: 713-722.

Dhingra, O.D., Costa, M.L.N., Silva Jr, G.J., & Mizubuti, E.S.G. 2004. Essential oil of mustard to control *Rhizoctonia solani* causing seedling damping off and seedling blight in nursery. Fitopatol. bras 29: 6.

Duke, J.A. 1997. Handbook of energy crops. http://www.hort.purdue.edu/newcrop/duke_energy/Brassica_juncea.html

Fenwick, G.R., Heaney, R.K., Mullin, W.J. 1983. Glucosinolates and their breakdown products in food and food plants. CRC Critical Reviews in Food Science and Nutrition 18:123–201.

Gamliel, A., & Stapleton, J. J. 1993. Characterization of antifungal volatile compounds evolved from solarized soil amended with cabbage residues. Phytopathology 83: 899-905.

Gardiner, J.B., Morra, M.J., Eberlein, C.V., Brown, P.D., Borek, V. 1999. Allelochemicals released in soil following incorporation of rapeseed (*Brassica napus*) green manures. Journal of Agricultural and Food Chemistry 47: 3837-3842.

Gimsing, A.L., & Kirkegaard, J.A. 2009. Glucosinolates and biofumigation: fate of glucosinolates and their hydrolysis products in soil. Phytochemistry Reviews 8: 299-310.

Gimsing, A.L., Sorensen, J.C., Tovgaard, L., Jorgensen, A.M.F., & Hansen, H.C.B. 2006. Degradation kinetics of glucosinolates in soil. Environmental Toxicology and Chemistry 25: 8, 2038–2044.

Jeffers, S.N., & Martin, S.B. 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Disease 70: 1038-1043.

Keinath, A.P. 1995. Reductions in inoculum density of *Rhizoctonia solani* and control of belly rot on pickling cucumber with soil solarization. Plant Disease 79: 1213-1219.

Keinath, A.P. 1996. Soil amendment with cabbage residue and crop rotation to reduce gummy stem blight and increase growth and yield of watermelon. Plant Disease 80: 564-570.

Larkin, R.P., & Griffin, T.S. 2006. Control of soilborne potato diseases using brassica green manures. Crop Protection 26: 1067-107.

Matthiessen, J.N., & Kirkegaard, J.A. 2006. Biofumigation and enhanced biodegradation: opportunity and challenge in soilborne pest and disease management. Critical Reviews in Plant Sciences 25: 235-265.

Morra, M.J., & J.A. Kirkegaard. 2002. Isothiocyanate release from soil-incorporated brassica tissues. Soil Biology and Biochemistry 34: 1683-1690.

Njoroge, S. M. C., Riley, M. B., & Keinath, A. P. 2008. Effect of incorporation of *Brassica* spp. residues on population densities of soilborne microorganisms and on damping-off and Fusarium wilt of watermelon. Plant Disease 92: 287-294.

Omirou, M., Rousidou, C., Bekris, F., Papadopoulou, K. K., Menkissoglou-Spiroudi, U., Ehaliotis, C., & Karpouzas, D. G. 2010. The impact of biofumigation and chemical fumigation methods on the structure and function of the soil microbial community. Microbial Ecology 61: 201-213.

Papavizas, G.C., Adams, P.B., Lumsden, R.D., Lewis, J.A., Dow, R.L., Ayers, W.A., & Kantzes, J.G. 1975. Ecology and epidemiology of *Rhizoctonia solani* in field soil. Phytopathology 65: 871-877.

Rakariyatham, N., Butr-Indr, B., Niamsup, H., & Shank, L. 2006. Improvement of myrosinase activity of *Aspergillus* sp. NR4617 by chemical mutagenesis. Electronic Journal of Biotechnology 9 (4).

Roberts, P.D. 2003. Damping-off and root rot. In Compendium of Pepper Diseases (pp. 12-13). The American Phytopathological Society.

Roberts, P.D. 2003. Southern blight. In Compendium of Pepper Diseases (pp. 20-21). The American Phytopathological Society.

Rodriguez-Kabana, R., Beute, M. K., & Backman, P. A. 1980. A method for estimating numbers of viable sclerotia of *Sclerotium rolfsii* in soil. Phytopathology 70: 917-919.

Sakorn, P., Rakariyatham, N., Niamsup, H., & Nongkunsarn, P. 2002. Rapid detection of myrosinase-producing fungi: a plate method based on opaque barium sulphate formation. World Journal of Microbiology & Biotechnology 18: 73-74.

Smith, B.J., & Kirkegaard, J.A. 2002. In vitro inhibition of soil microorganisms by 2-phenylethyl isothiocyanate. Plant Pathology 51: 585–593.

Sneh, B., Jabaji-Hare, S., Neate, S. & Dijst, G. 1996. *Rhizoctonia* species: taxonomy, molecular biology, ecology, pathology, and disease control. Dordrecht, The Netherlands: Kluwer Academic Publishers.

Stapleton, J.J., & Duncan, R.A. 1998. Soil disinfestation with cruciferous amendments and sublethal heating: effects on *Meloidogyne incognita*, *Sclerotium rolfsii* and *Pythium ultimum*. Plant Pathology 47: 737-742.

Trujillo, E.E., Cavin, C.A., Aragaki, M., & Yoshimura, M.A. 1987. Ethanol-potassium nitrate medium for enumerating *Rhizoctonia solani*-like fungi from soil. Plant Disease 71: 1098-1100.

van Bruggen, A. H. C., & Arneson, P. A. 1986. Quantitative recovery of *Rhizoctonia solani* from soil. Plant Disease 70: 320-323.

Vincelli, P.C., & Beaupre, C.M.S. 1989. Comparison of media for isolating *Rhizoctonia solani* from soil. Plant Disease 73: 1014-1017.

Warton, B. Matthiessen, J.N., & Roper, M.M. 2001. The soil organisms responsible for the enhanced biodegradation of metham sodium. Biology and Fertility of Soils 34:264–269

Table 2.1. Greenhouse soil sample characteristics and reference numbers.

Soil number	Farm name (field number), location	Conventional vs. organic	Recent planting history[y]	Soil type	Sample collection date
1	USDA (F7), Charleston, SC	Conventional	Broccoli, rye & clover	Yonges loamy fine sand	11/9/2010
3	Ambrose, Charleston, SC	Organic	Turnip greens	Edisto loamy fine sand	11/9/2010
4	Ambrose, Charleston, SC	Conventional	Corn	Edisto loamy fine sand	11/9/2010
6	Clemson CREC (B8), Charleston, SC	Organic	Non-brassica	Yonges loamy fine sand	11/9/2010
8	Clemson University student organic farm, Clemson, SC	Organic	Grass	Toccoa coarse loam	10/31/2010
9	Rawl's, Ridge Springs, SC	Conventional	Kale	Ruston sandy loam	11/2/2010
10	Clemson University heirloom garden, Clemson, SC	Organic	Clover	Cecil sandy loam	10/31/2010
11	Beechwood, Marietta, SC	Conventional	Collards	Congaree loam	11/1/2010
13	Titan, Ridge Springs, SC	Conventional	Old peach orchard, fallow	Ruston sandy loam	11/2/2010
15	Beechwood, Marietta, SC	Conventional	Corn	Congaree loam	11/1/2010

16	Field's, John's Island, SC	Organic	Collards	Loamy fine sand[z]	1/25/2011
17	Thornhill, McClellanville, SC	Organic	Peas, kale	Chipley loamy fine sand	1/27/2011
2*	Ambrose, Charleston, SC	Conventional	Brassica	Edisto loamy fine sand	11/9/2010
5*	Ambrose, Charleston, SC	Organic	Corn	Edisto loamy fine sand	11/9/2010
7*	Clemson CREC (B11), Charleston, SC	Conventional	Non-brassica	Yonges loamy fine sand	11/9/2010
12*	Rawl's, Gilbert, SC	Conventional	Radish, collards	Troup B sand	11/2/2010
14*	Delano and Kneece, Pelion, SC	Conventional	Corn	Fuquay B loamy sand	11/2/2010

[y] Recent planting history listed oldest to most recent left to right.
[z] Mixture of Wando loamy fine sand, Kiawah loamy fine sand, and Seabrook loamy fine sand.
*Samples dropped from greenhouse experiment due to low myrosinase activity.

Table 2.2. Preliminary emergence and damping-off data used to determine rate of soil infestation with *R. solani* and *S. rolfsii* for greenhouse experiments.

Pathogen Inoculum Density (sclerotia kg^{-1} soil)	**Emergence**[a]	**Pre-emergence damping off**	**Post-emergence damping off**
Rhizoctonia solani			
Non-infested control	14[b]	3	1
25	10	7	1
50	13	2	3
100	7	8	3
Sclerotium rolfsii			
Non-infested control	6	12	0
36	4	14	0
72	6	12	0
144	8	10	0

[a] A total of 18 seeds were planted per treatment.
[b] Treatments were not replicated to collect this preliminary data.

Table 2.3. Rates of bean emergence and disease incidence to determine optimal soil treatment for bean emergence and reduced disease incidence.

[w]Seed Treatment [x]Ridomil Treatment [y]Soil Treatment	% Emerged	% Diseased
Direct Seeded	67a[z *]	13a
(+) Ridomil	62a	12a
Mixed	61a	17a
Layered	58a	11a
Control	67a	8a
Direct Seeded		
(-) Ridomil	71a	14a
Mixed	67a	22a
Layered	72a	8a
Control	75a	11a
Pre-Germinated	68a	21a
(+) Ridomil	68a	25a
Mixed	81a	25a
Layered	69a	19a
Control	53a	31a
Pre-Germinated		
(-) Ridomil	69a	18a
Mixed	83a	19a
Layered	56a	14a
Control	67a	19a

[w] Whole-plot factor 1: pre-germinated seeds were held on a temperature gradient table at 26.7°C for one week prior to seeding.

[x] Whole-plot factor 2: Ridomil was applied at 1.054 µL 50 mL^{-1} H_20 (approximately 1 pt $acre^{-1}$) to each treatment prior to seeding.

[y] Sub-plot factors: mixed treatment = 75% field soil mixed with 25% perlite; layered treatment = 50% field soil above 50% potting mix; control = 100% field soil

[z] Means followed by the same letter within columns by factor levels are not significantly different ($P<0.05$).

* Values are the mean of two replicates.

Table 2.4. Population levels of selected microorganisms in greenhouse soil samples.

Soil sample number	***R. solani*-infested dry organic matter per kg soil (g)**	***Pythium* spp. CFU g^{-1} oven-dry soil**	***S. rolfsii* CFU 300 g^{-1} soil**	***Bacillus* spp. CFU g^{-1} oven-dry soil**
1	0.18[y]	28[y]	2[y]	40[y]
3	0.28	13	2	55
4	2.05	0	1	26
6	3.53	17	7	26
8	2.08	0	1	77
9	0.85	23	0	33
10	4.35	114	0	47
11	0.93	26	1	50
13	1.03	33	1	23
15	1.43	38	0	49
16	ND[z]	ND	ND	43
17	ND	ND	ND	26
2*	3.55	0	1	ND
5*	4.38	11	2	ND
7*	1.70	6	3	ND
12*	0.00	0	0	ND
14*	0.50	49	2	ND

[y] Soil microorganism assays were not replicated.
[z] Not done.
*Samples excluded from greenhouse experiment.

Table 2.5. Myrosinase activity of each soil sample prior to greenhouse experiment and allyl ITC concentration 4 h after incorporation of freeze-dried mustard.

Soil #	**Myrosinase activity (µg glucose g^{-1} soil 4 h^{-1}) [t]**	**Relative myrosinase activity (%)[u]**	**Allyl ITC concentration (µg g^{-1} soil)**	**Relative allyl ITC concentration (%)**
1	0.71[v]	12[v]	2.9[w]	21.6[w]
3	0.61	10	5.5	41.0
4	0.2	3	5.8	43.2
6	5.87	100	3.3	24.6
8	1.02	17	2.3	17.1
9	1.32	22	2.8	20.9
10	1.42	24	2.2	16.4
11	1.93	33	1.8	13.4
13	2.75	47	2.6	19.4
15	1.23	21	2.3	17.1
16	0.82	14	13.4	100
17	0.41	7	11.8	88.1
Autoclaved	ND[x]	NA[y]	7.0	52.2
2*	-0.71[z]	-12	ND	NA
5*	0.61	10	ND	NA
7*	1.88	32	ND	NA
12*	-0.56	-10	ND	NA
14*	0	0	ND	NA

[t] Glucose concentrations represent the mean difference between the sample glucose concentration and control glucose concentration across four experimental repetitions after 4 h incubation. High glucose values correspond to high levels of myrosinase activity.
[u] Values relative to each soil sample, with the highest values listed as 100%.
[v] Myrosinase values are the mean of four replicates.
[w] ITC values are the mean of three replicates.
[x] Not done.
[y] Not applicable.
*Samples dropped from greenhouse experiment.
[z] Negative values indicate that samples had glucose concentrations lower than their controls i.e. no myrosinase activity.

Table 2.6. Determination of inoculum viability in infested and non-infested soil.

		R. solani	***S. rolfsii***
Soil sample number	Inoculum added to soil	Beet seeds colonized (%)	Mean CFU 110 g^{-1} soil
3	Yes	97.3[x] a[y]	16.5 a
3	No	8.9 b	0.8 b
15	Yes	84.0 A	21.3 A
15	No	25.9 B	0.3 B

[x] Values are the mean of two replicates.
[y] Means followed by the same letter within column by soil sample number are not significantly different (P<0.05).

Table 2.7. Effect of freeze-dried 'Pacific Gold' mustard on pathogen populations in soil samples 3 and 15.[w]

	R. solani	***S. rolfsii***	***Pythium* spp.**
Freeze-dried mustard added to soil	% beet seeds colonized	Mean CFU 110 g^{-1} soil	Mean CFU g^{-1} soil
Yes	51.3[x] (5.9)[y] a[z]	8.1 (3.8) a	40.5 (19.0) a
No	56.7 (5.9) a	11.3 (3.8) a	14.5 (19.0) a

[w] There was not a significant difference found between samples 3 and 15, nor was there a significant sample by mustard interaction (P<0.05), so treatment means were combined.
[x] Values are the mean of four replicates.
[y] Value in parentheses is one standard error of the mean.
[z] Means followed by the same letter within a column are not significantly different (P<0.05).

Table 2.8. Effect of freeze-dried 'Pacific Gold' mustard leaves on bean emergence, disease incidence, and shoot weight in samples 3 and 15.

Freeze-dried mustard added to soil	(+) *R. solani*	(+) *S. rolfsii*	No added inoculum control	Mean
	Soil sample 3			
	Emergence (%)			
Yes	46.3[x] a[y]	25.9 a	46.3 a	39.5 a
No	68.5 a	25.9 a	47.2 a	47.2 a
	Disease incidence (%)[z]			
Yes	92.1 a	56.3 a	38.4 a	62.3 a
No	80.0 a	63.3 a	44.3 a	62.5 a
	Shoot weight (g)			
Yes	34.6 a	24.2 a	43.7 a	34.2 a
No	47.1 a	20.2 a	36.4 a	34.6 a
	Soil sample 15			
	Emergence (%)			
Yes	51.9 a	55.6 a	74.1 a	60.5 a
No	62.0 a	68.5 a	62.0 a	69.1 a
	Disease incidence (%)			
Yes	71.5 a	33.4 a	4.4 a	36.5 a
No	64.3 a	14.3 a	3.5 a	27.4 a
	Shoot weight (g)			
Yes	42.3 a	41.6 a	68.1 a	50.7 a
No	51.7 a	67.0 a	74.7 a	64.5 a

[x] Values are the mean of four replicates.
[y] Means followed by the same letter within a column under a given measurement parameter are not significantly different, LSD ($P<0.05$).
[z] Disease incidence represents the percentage of emerged seedlings with disease.

Table 2.9. Effect of soil sample and pathogen infestation on bean emergence, disease incidence, and shoot weight following freeze-dried mustard incorporation into soil.[w]

Soil Sample Pathogen treatment	Emergence (%)	Disease incidence (%)	Shoot weight (g)
1	28.0[x]	79.7	24.4
(+) Rz	32.6 a[y]	85.0 a	22.6 a
(+) Sr	24.3 a	85.7 a	24.8 a
(-) Pathogen control	27.1 a	68.5 a	26.0 a
3	41.3	63.3	33.6
(+) Rz	55.5 a	87.0 a	40.1 a
(+) Sr	24.1 b	58.2 b	22.0 b
(-) Pathogen control	44.4 a	44.8 b	38.8 a
4	47.7	63.0	37.6
(+) Rz	57.7 a c	93.2 a	38.0 a
(+) Sr	35.4 b	52.4 b	30.3 a
(-) Pathogen control	50.0 b c	43.3 b	44.4 a
6	57.9	52.7	46.0
(+) Rz	70.2 a c	81.0 a	47.5 a
(+) Sr	47.9 b	44.4 b	39.3 a
(-) Pathogen control	55.6 b c	32.8 b	51.1 a
8	62.3	29.7	57.6
(+) Rz	63.2 a	48.1 a	57.7 a b
(+) Sr	55.6 a	24.8 b	49.3 b
(-) Pathogen control	68.1 a	16.2 b	65.9 a
9	75.9	8.3	72.0
(+) Rz	81.3 a	5.1 a	79.0 a
(+) Sr	71.5 a	11.3 a	67.4 a
(-) Pathogen control	75.0 a	8.5 a	69.5 a
10	51.6	46.6	50.5
(+) Rz	56.3 a	78.6 a	51.0 a
(+) Sr	45.8 a	38.4 b	43.8 a
(-) Pathogen control	52.8 a	22.8 b	56.8 a
11	51.4	47.0	49.5
(+) Rz	54.9 a	69.3 a	51.1 a
(+) Sr	47.9 a	35.7 b	44.5 a
(-) Pathogen control	51.4 a	36.0 b	53.0 a

13	62.5	47.6	44.6
(+) Rz	55.6 b	86.2 a	35.2 b
(+) Sr	60.4 a b	46.5 b	44.4 a b
(-) Pathogen control	71.6 a	10.0 c	54.3 a
15	62.1	32.1	55.3
(+) Rz	52.7 b	61.8 a	43.4 b
(+) Sr	60.6 a b	29.1 b	53.5 b
(-) Pathogen control	73.1 a	5.3 c	68.9 a
16	41.7	57.0	35.2
(+) Rz	36.1 b	95.4 a	23.8 b
(+) Sr	34.0 b	47.3 b	31.3 b
(-) Pathogen control	54.9 a	28.2 b	50.6 a
17	66.0	58.4	49.8
(+) Rz	58.3 b	88.9 a	35.5 b
(+) Sr	63.2 a b	53.5 b	47.1 b
(-) Pathogen control	76.4 a	32.9 c	66.8 a
Autoclaved control	70.2	2.0	48.3
(+) Rz	ND[z]	ND	ND
(+) Sr	ND	ND	ND
(-) Pathogen control	70.2	2.0	48.3

[w] All treatments received freeze-dried mustard tissue.
[x] All values are the mean of four replicates.
[y] Means followed by the same letter in a column within soil sample*pathogen treatment are not significantly different, LSD ($P<0.05$).
[z] Not done because autoclaved controls were only included for (-) pathogen control soils in order to verify that autoclaving effectively killed soilborne pathogens.

Table 2.10. Correlation coefficients and *P*-values for relevant greenhouse comparisons.

Comparison	**Correlation coefficient**	**p-value**
ITC concentrations and soil myrosinase activity	-0.334	0.288
ITC concentrations and *Bacillus* spp. populations densities	-0.244	0.444
ITC concentrations and bean disease incidence	0.352	0.262
ITC concentrations and bean emergence	-0.147	0.647
ITC concentrations and bean shoot weight	-0.305	0.334

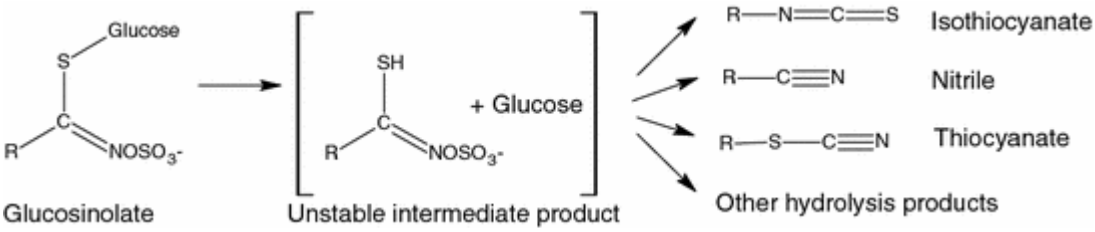

Figure 2.1. Hydrolysis of glucosinolates by the enzyme myrosinase. A number of hydrolysis products can be formed depending on the specific glucosinolate, presence of other enzymes and environmental factors, but isothiocyanate, nitrile and thiocyanate are the ones most often encountered in soil. Reprinted from "Glucosinolates and biofumigation: fate of glucosinolates and their hydrolysis products in soil," by A.L. Gimsing and J.A. Kirkegaard, 2009, *Phytochemistry Reviews*, *8*, p. 300. Copyright 2008 by Springer Science+Business Media B.V.

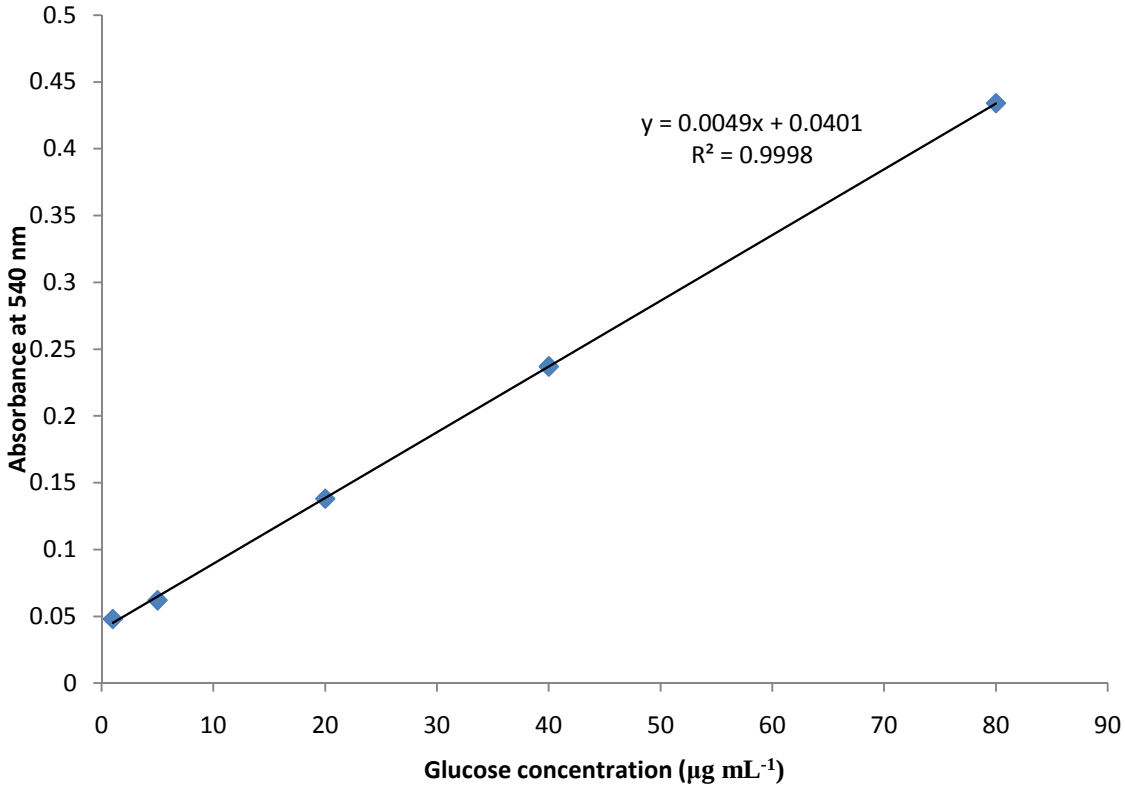

Figure 2.2. Standard curve used to quantify myrosinase activity in soil. Absorbance values at 540 nm were converted into glucose concentrations expressed in µg mL^{-1}.

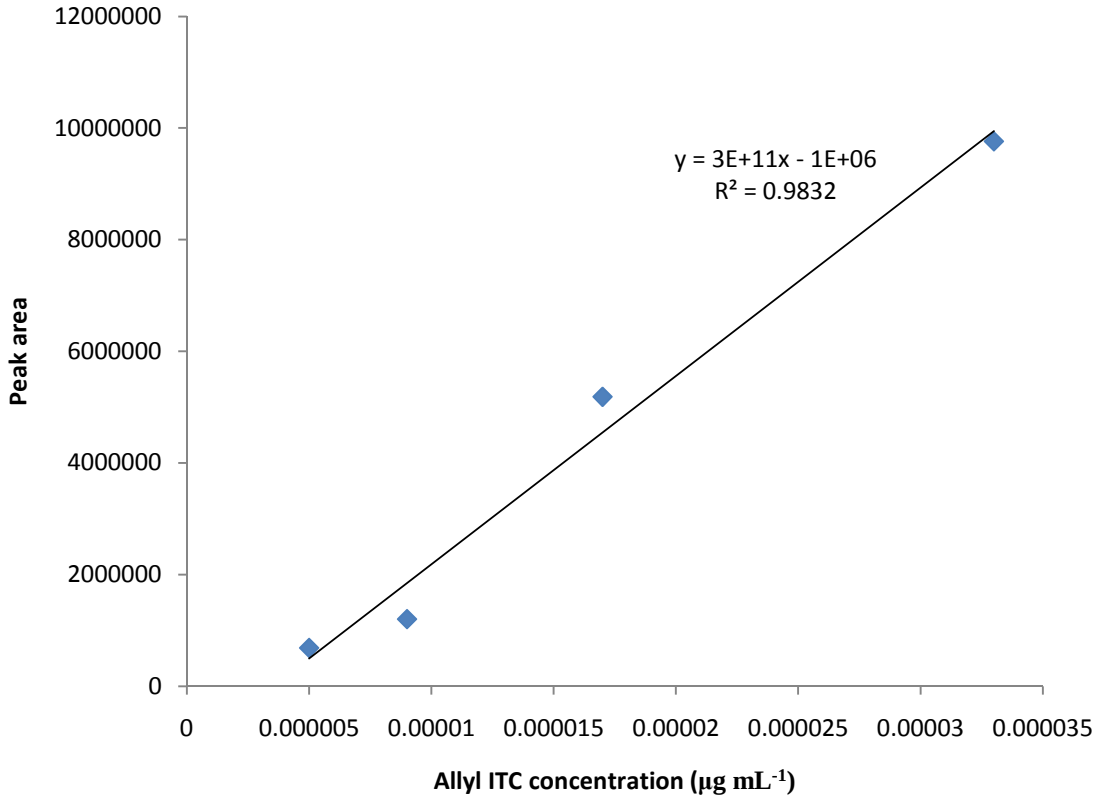

Figure 2.3. Standard curve used to quantify allyl ITC concentrations in soil samples following incorporation of freeze-dried 'Pacific Gold' mustard leaves.

CHAPTER THREE

FIELD STUDY

Introduction

Biofumigation is the use of *Brassica* species and their natural chemicals to control soilborne pathogens, nematodes, or weeds. This is accomplished by a reaction between two isolated compounds that occur naturally within the plant: the enzyme myrosinase (thioglucoside glucohydrolase), located in myrosin cells, and the secondary metabolites glucosinolates (GSLs), which are contained in cell vacuoles. Upon tissue disruption the myrosinase catalyzes a hydrolysis reaction which converts GSLs into isothiocyanates (ITCs), the compounds primarily responsible for the plant's pesticidal properties, as well as a variety of other breakdown products such as nitriles, thiocyanates, oxazolidine-2-thiones, hydroxynitriles, and epithionitriles (Fenwick et al. 1983, Morra et al. 2002,). Biofumigation has been the subject of research since the early 1990's with varying levels of success, and remains an active area of research.

Management of soilborne pathogens can be particularly challenging as a result of both their biology and physical location. Many soilborne pathogens, such as fungi, bacteria, and nematodes, are microscopic. As a result, scouting for and monitoring their populations is often not feasible. Furthermore, once crops have been planted there are no control agents available to manage soilborne pathogens because of the physical disruption caused by fumigant application and the potential damage caused by phytotoxic fumigants (Matthiessen et al. 2006). For this reason there is a need for an effective pesticide which can be applied to the soil prior to planting in areas where soilborne

pathogen populations are consistently at economic threshold levels. Methyl bromide successfully filled this niche until 2005, by which time the EPA had phased out the chemical except for critical use exemptions due to its role in ozone depletion (USEPA, 2011). This phase out of methyl bromide has brought biofumigation back into the spotlight as one potential replacement.

There is a great deal of variation among different species and cultivars of brassicas with regard to their biofumigation potential. *Brassicaceae* species should be selected based on the amount of GSL that they contain, the type of resulting ITC that will be produced, as well as the amount of biomass they are capable of producing. Antonious et al. (2009) reported *Brassica juncea* cv. Pacific Gold ('Pacific Gold' mustard) to have the highest above-ground biomass (5.7± 1.7 kg m^{-2}) and GSL content of seven *B. juncea* species tested. The allyl ITC released from macerated *B. juncea* tissue has been shown to be suppressive to in vitro growth of several plant pathogens including *Pythium ultimum* and *Rhizoctonia solani*, with suppressive AITC concentrations ranging from 0.18-0.36 mg g^{-1} leaf tissue (Mayton et al. 1996). *Brassica napus* PI 169083 (winter rapeseed) had the highest above ground biomass (7.7±3.4kg m^{-2}) of ten *Brassica* species studied (Antonious et al. 2009). Although its GSL content was found to be relatively low, its high biomass gave it high GSL yields per unit area relative to the other ten species tested. Furthermore, winter rapeseed requires a vernalization period to flower. As a result it will continue to produce vegetative growth and fail to seed when sown in the spring (Sarwar et al. 1998). Winter rapeseed overcomes the limitation due to early flowering, which affects many other brassica species, making it an excellent candidate for a biofumigant

crop. *Raphanus sativus* (oilseed radish), according to Ohio State University Extension (Sundermeier 2008), has the potential to produce approximately 10 kg m^{-2} biomass.

Different brassicas produce different GSLs, and they are produced at different concentrations. As a result the type and concentration of ITC released upon tissue disruption varies among brassica species and cultivars, which can in turn affect their biofumigation potential. Thirty-five genotypes of *B. juncea* and 28 genotypes of *B. nigra* were screened for ITC production and suppression of the plant pathogens *Helminthosporium solani* and *Verticillium dahliae* (Olivier et al. 1999). They found a correlation between the concentration of allyl ITC (AITC) and pathogen suppression. Although genotypes that released low concentrations of AITC were generally less suppressive, a few still resulted in moderate to high suppression. Such genotypes released other ITCs, such as 2-phenylethyl and benzyl, at sufficient concentrations to suppress the pathogens. Allyl ITC has been shown to be suppressive to *R. solani* and *Pythium ultimum* in a controlled laboratory study (Charron et al. 1999). *S. rolfsii* has been shown to be susceptible to suppression with brassica soil amendments, although specific information relating ITC type to suppression is lacking (Gamliel et al. 1993).

In addition to brassica species and cultivar, many factors relating to incorporation can affect the success of biofumigation. Two such factors are the level of tissue disruption prior to incorporation and the soil moisture at incorporation. Morra et al. (2002) showed that ITC release efficiency could be increased from 1% to 26.4% by switching from whole plant incorporation to freezing and thawing plants prior to incorporation. They also found that extractable ITC concentrations were higher in waterlogged vs. non-waterlogged soil. Thus the level of tissue disruption and soil

moisture play key roles in the percentage of GSLs that will be converted to ITC. Time of planting and growth stage at incorporation are also important to consider. GSL concentration was highest at the bud-raised growth stage, prior to flowering, and higher in spring-seeded vs. fall-seeded brassicas (Sarwar et al. 1998). Although spring-seeded brassicas were shown to have higher concentrations of GSLs, it is important to note that a key to the success of biofumigation is the total amount of GSL present in the field at the time of incorporation. As a result, unless spring-seeded brassicas can accumulate comparable biomass to fall-seeded brassicas by the time of incorporation, it may still be beneficial to seed in the fall. The type of plastic used to cover the fumigated area has also been an important consideration. Virtually impenetrable film (VIF) greatly reduces the quantity of volatile chemicals allowed to escape from the soil into the atmosphere compared to conventional low density polyethylene mulch (LDPE), which will be referred to as conventional plastic (CP) (Noling et al. 2010, Hanson et al. 2010). This both reduces the rate at which volatile chemicals must be applied to the soil to obtain adequate fumigation results and the harmful effects that may result from releasing toxic volatile chemicals into the atmosphere.

Although ITCs are generally considered to be the primary factor behind pathogen suppression from biofumigation, other factors may be involved. The application of plastic mulch reduces the loss of volatile chemicals and creates an anaerobic environment. Soil anaerobiosis has been reported to suppress a number of soilborne plant pathogens including *R. solani* (Blok et al. 2000). Other plant breakdown products, such as acetic acid and butyric acid, have been shown to suppress several plant pathogens (Momma et

al. 2006). This may help to explain why Kasuya et al. (2006) found that dried plant residues of *B. rapa*, peanut, and clover were all suppressive to *R. solani* in vitro.

R. solani, a soilborne fungal plant pathogen within the phylum Basidiomycota with a wide host range, causes both pre-emergence and post-emergence damping-off. It can also lead to reduced vigor and yield of mature pepper plants (Roberts, 2003). Control of *R. solani* through biofumigation has shown varying success, most likely due to the conditions discussed above. Njoroge et al. (2008) found no significant difference in *R. solani* population densities between brassica-amended and non-amended treatments in a field experiment. However, Larkin et al. (2006) found significant *R. solani* population reductions in greenhouse assays and disease reductions in field tests following incorporation of brassica plant tissue, including *B. juncea*, *B. napus*, and *Raphanus* spp.. Dhingra et al. (2004) also found that essential oil extracted from mustard seeds (*B. rapa*) significantly reduced the saprophytic growth of *R. solani* both in vitro and in field soils, although significantly higher mustard oil concentrations were required for field soil.

S. rolfsii is another soilborne fungal plant pathogen within the phylum Basidiomycota with a wide host range that can cause the economically important disease Southern blight in coastal South Carolina. Infection by *S. rolfsii* occurs at the soil line, and symptoms on peppers include yellowing, wilting, and eventual plant death (Roberts, 2003). Stapleton and Duncan (1998) demonstrated that germination of *S. rolfsii* sclerotia was inhibited by brassica amendments in conjunction with a diurnal heat treatment with a maximum and minimum of 38°C and 27°C. The heat treatment alone was not suppressive.

Pythium spp. are commonly occurring fungus-like soilborne plant pathogens that are classified in the kingdom Straminopila and the phylum Oomycota. They can cause damping-off and root rot in a variety of vegetable crops. Many species of *Pythium*, with varying degrees of pathogenicity, are capable of colonizing pepper roots. The diversity in *Pythium* spp. results in differential sensitivity to control strategies. Smith and Kirkegaard (2002) reported significant variations in growth rate response to 2-phenylethyl ITC among 13 different isolates across 3 species of *Pythium* (*P. sulcatum*, *P. ultimatum*, and *P. violae*). The ITC concentration required to reduce in vitro radial growth by 90% ranged from 0.005 mM to 0.862 mM for the different isolates. The least sensitive isolate was also the only isolate of *P. ultimatum*. There was little intraspecific variation in sensitivity among isolates. These results indicate that species of *Pythium* may vary significantly in sensitivity to toxins. Njoroge et al. (2008) and Collins et al. (2006) both found that the incorporation of brassica tissue did not significantly reduce, and in some cases increased, the population densities of *Pythium* spp. In contrast, Stapleton and Duncan (1998) reported significant reductions in survival of *P. ultimum* following the incorporation of 2% brassica tissue into soil in a controlled experiment. This suggests that under field conditions there is more involved in *Pythium* suppression than simply ITC exposure. To better understand the variation in pathogenicity among species of *Pythium*, Chellemi et al. (2000) conducted a study analyzing ten different species of *Pythium* collected from fresh market pepper plants in Florida. A large range in pathogenicity was found among the species. *P. aphanidermatum* and *P. myriotylum* were the most aggressive pathogens on pepper, leading to approximately 50% mortality in inoculated

pepper plants. *P. periplocum* and *P. spinosum*, although capable of colonizing pepper roots, resulted in no adverse affects on root system weight or plant growth.

Peppers (*Capsicum annuum*), members of the plant family Solanaceae, are good candidate crops for the incorporation of biofumigation into the production system. Peppers are susceptible to a variety of bacterial, viral, and fungal diseases. Genetic resistance and tolerance is available in some cultivars; however, all are susceptible to the pathogens *R. solani*, *S. rolfsii*, and *Pythium* spp. Methyl bromide is still allowed in pepper production in South Carolina where there is a moderate to severe nut sedge, nematode, or *Pythium* infestation under an EPA critical use exemption. This highlights the need for the development of alternative disease management strategies in pepper so that methyl bromide can be entirely phased out of pepper production. Many growers may also be attracted to the relatively low cost of implementing biofumigation compared to conventional management strategies like methyl bromide or metam sodium.

Peppers are a warm-season crop, and grow best at temperatures between 21°C to 24°C. Rows should be spaced 4 to 5 feet apart, with plants in double rows spaced 12 to 18 inches apart (Kemble et al. 2011). *Capsicum annuum* 'Hunter', the green bell pepper variety chosen for this study, has resistance to tobacco etch virus, tomato mosaic virus, and bacterial spot. It produces large to extra large, four-lobed, blocky fruit. It is sold by Rogers Seed Company, Boise, ID. Bell pepper production in the Southeastern US was valued at over half of the country's total production value, $239 million, making bell peppers an important crop for Southeastern vegetable growers (USDA ERS, 2011).

The first objective of this study was to determine if biofumigation with oilseed radish, 'Pacific Gold' mustard, or winter rapeseed would reduce populations of the

soilborne plant pathogens *R. solani*, *S. rolfsii*, and *Pythium* spp. in the field. The second objective was to determine the overall effect of biofumigation on peppers including plant vigor, incidence of damping-off, root rot, and Southern blight, and yield. The third objective was to determine the relative pathogenicity of distinct *Pythium* spp. isolates on pepper in order to better understand which were responsible for the majority of damping-off and root rot observed in the field.

Materials and Methods

Field plot establishment

The experiment was conducted at the Clemson University Coastal Research and Education Center, Charleston, SC. The soil in the fields used is sandy loam. Prior to planting the experimental cover crops, the fields were planted in a mixture of rye and clover during the winter each year. The experimental design was a randomized complete block design with four replications. In 2011 the spring pepper experiment was reduced to three replications due to poor brassica emergence in one of the blocks. Prior to planting brassica cover crops, each experimental field was sectioned into 16 equally-sized plots. Each plot measured 9.1 m by 2.4 m, with a 1.5 m fallow space between each plot. On 4 April and 18 October 2010, prior to planting brassica cover crops, field plots were infested with *S. rolfsii* at a rate of approximately 21,800 sclerotia per plot (16.8 g and 13.0 g sclerotia, respectively) by spreading sclerotia over the surface of each plot. Sclerotia were grown using green beans. First, 100 g of frozen cut green beans were placed into GA-7 vessels, capped, and autoclaved for one hour on three consecutive days.

Once cooled, each vessel was inoculated with 3 5-mm disks of three-week-old *S. rolfsii* growing on quarter strength potato dextrose agar. Vessels were incubated for three weeks at room temperature. Excess liquid was then drained off and beans were spread onto trays covered with paper towels and allowed to dry for three to four days. Dried beans were then blended in a blender and sclerotia were separated from larger bean pieces with a sieve with 2-mm openings. Sclerotia were stored in a refrigerator until use. The three experimental brassica treatments were oilseed radish (*Raphanus sativus)*, Pacific Gold mustard (*B. juncea* 'Pacific Gold'), and winter rapeseed (*B. napus* 'Dwarf Essex') seeded at 28.0 kg hectare^{-1} for radish and 11.2 kg hectare^{-1} for mustard and rapeseed. Seeds were obtained from Johnny's Selected Seeds. Seeding rates were based on the highest rates recommended by Johnny's Selected Seeds. All brassica treatments received VIF after incorporation. Controls included weedy fallow plots with and without VIF, the latter being bare-soil plots. The final experimental repetition included an additional control of fallow with conventional plastic (CP). Brassicas were direct seeded on 6 April 2010 (repetition 1), 18 October 2010 (repetition 2), and 23 February 2011 (repetition 3) and irrigated as needed throughout the growing season with overhead irrigation.

Brassica incorporation and pepper planting

Brassica biomass measurements were taken 0 to 9 days prior to incorporation. Plants were dug from one 0.25-m^2 area from each brassica treatment plot to a depth of 30 cm to ensure that all root material was removed. Plants were washed and counted prior to roots and shoots being separated. Roots and shoots were weighed separately to measure fresh weights. The roots and shoots were placed in separate paper bags and dried in an

oven at 50°C for one week. Plants were then weighed again and dry weights were recorded.

On 28 May 2010, 16 February 2011, and 21 April 2011 plants were pulverized using a flail mower that was run twice over each plot to ensure thorough disruption of plant tissue. In May 2010 and April 2011 'Pacific Gold' mustard was 100% flowering, winter rapeseed was approximately 50% flowering and 50% bud raised, and oilseed radish had no flowers or buds at incorporation. None of the plants had buds raised at incorporation in February 2011. The mower was run over the fallow plots first, followed by each separate brassica treatment; the mower was pressure washed between treatments to avoid contamination. Within three hours of mowing all treatments were incorporated into soil with a disk in the same order in which they were mowed. The disk was washed between treatments. Prior to laying plastic in repetitions 1 and 2 15-0-15 fertilizer was applied at 673 kg ha^{-1}, and in repetition 3 10-10-10 was applied at 448 kg ha^{-1}. Raised beds were shaped and covered with a continuous strip of black VIF with drip irrigation tape underneath. The VIF was then cut away from the fallow-without VIF plots and the ends were sealed with soil. After all VIF had been laid, irrigation was run for at least one hour to ensure high soil moisture for GSL hydrolysis.

On 22 July 2010, 14 April 2011, and 12 July 2011 5-week-old pepper seedlings, which had been growing in the CREC greenhouse, were transplanted in the field in double rows at 0.3 m spacing. The first 2 repetitions had 60 plants per plot, and the final repetition had 50 plants per plot. Plots were irrigated immediately after planting. After the first day of growth, irrigation was run daily at 10:00 am and 10:00pm, applying 800 gallons to the entire field at each watering. Irrigation was later switched to once daily.

The fallow (-) VIF plots were hand weeded throughout the growing season to reduce the effect of weeds on pepper growth and yield. In repetition 1 insects were managed by applications of the insecticides Radiant SC (spinetoram) at 0.44 L hectare^{-1} on 13 August 2010; Venom (dinotefuran) at 0.29 L hectare^{-1} on 26 August 2010; Sniper (bifenthrin) at 0.47 L hectare^{-1} on 2 September 2010; Radiant (spinetoram) at 0.73 L hectare^{-1} plus Venom (dinotefuran) at 0.29 L hectare^{-1} on 16 September 2010; and Coragen (rynaxypyr) at 0.55 L hectare^{-1} plus Brigade (bifenthrin) at 0.47 L hectare^{-1} on 22 September 2010. Powdery mildew was managed with the fungicides Switch (cyprodinil and fludioxonil) at 1.02 L hectare^{-1} on 16 September 2010 and Bravo (sulfur and chlorothalonil) at 3.51 L hectare^{-1} on 22 September 2010. In repetition 2 insects were managed by application of the insecticide Lannate (methomyl) at 0.56 kg hectare^{-1} on 29 April 2011. In repetition 3 insects were managed by applications of the insecticides Coragen (rynaxypyr) at 0.37 L hectare^{-1} on 19 July 2011; Intrepid (methoxyfenozide) at 0.73 L hectare^{-1} on 29 July 2011; Coragen (rynaxypyr) at 0.37 L hectare^{-1} on 9 August 2011; and Oberon (spiromesifen) at 0.51 L hectare^{-1} on 31 August 2011. Powdery mildew was managed with the fungicides Organocide (sesame oil) at 9.19 L hectare^{-1} on 9 August 2011. Weeds were managed prior to planting by application of the herbicides Poast (sethoxydim) at 1.75 L hectare^{-1} on 7 April 2011 and Honcho (glyphosate) at 32.15 L hectare^{-1} on 17 June 2011 and 8 July 2011.

Plant mortality was recorded weekly, separated into Southern blight and root rot. Plants were considered infected with Southern blight if they showed symptoms and signs commonly associated with the disease, such as rapid wilting accompanied by white mycelia and dark brown sclerotia at the plant base (Roberts, 2003). Dead plants which

demonstrated gradual wilting, chlorosis, and overall decline were considered to have died from root rot associated with *Pythium* and *R. solani*. In repetitions 1 and 2 plants were removed from the field 26 days after transplanting to test for *Pythium* spp. and *R. solani* infection. In repetition 1 twelve plants showing decline and five healthy plants were removed for testing. All treatments were represented in the sampling. Roots were cut into small pieces approximately 1 mm long, surface disinfested for 30 seconds in 5% bleach (0.3% sodium hypochlorite) and placed on pimaricin-ampicilin-rifampicin-pentachloronitrobenzene medium prepared with pimaricin at 5 mg L^{-1} (P_5ARP), 10 small heaps per plate (Jeffers and Martin, 1986). In repetition 2 five plants showing decline were removed for testing. Roots were surface disinfested as previously described for *Pythium* spp., and disinfested for 30 seconds in 10% bleach (0.6% sodium hypochlorite) for *R. solani*, and placed on water agar amended with 100 μg L^{-1} streptomycin for *Pythium* spp. and ethanol-potassium nitrate (EPN_2) medium with prochloraz (Trujillo et al. 1987) prepared with 2% ethanol (Vincelli et al. 1989) for *R. solani*. Two to three heaps of roots were plated per sample and three to six pieces cut from the lower stem were plated per sample for each pathogen. In repetition 3, 101 days after transplanting, three plants which were stunted for the entire duration of the experiment and one healthy plant were removed from each treatment in one block, surface disinfested, and cultured on P_5ARP as previously described to test for *Pythium* spp.

Plant stunting was recorded five weeks after transplanting in all 3 repetitions. Plant height was measured from the soil line to the highest point on the plant. Plants were considered stunted if they measured less than 2/3 the height of the most vigorous plant in the field. Peppers were harvested weekly between 15 September and 20 October 2010, 6

June and 13 July 2011, and 6 September and 18 October 2011. In summer and fall 2011 peppers were separated by healthy fruit and fruit with blossom end rot (BER) or sun scald because of high BER incidence observed in summer 2011. Any fruit with a visible dark-brown or tan lesion was considered to have BER or sun scald.

Spinach planting

On October 19 2010 the unplanted ends of the existing pepper plots were planted in 'Melody' spinach in a split-plot design. Each plot measured 46 cm by 0.6 m. Untreated spinach seeds were obtained from SeedWay. Ridomil Gold EC (mefenoxam) was randomly assigned to half of the sub-plots and applied at 2.3 L ha^{-1} to manage *Pythium*, with the whole plot factor remaining cover crop treatment. Each plot was planted in 3 rows of 9 seeds. On 28 October 2010 spinach emergence was recorded. On 17 November 2010 final plant counts and stunting were recorded. To quantify stunting, plants were measured from the farthest edges. Plants were considered stunted if they measured less than 50% the width of the largest plant in the field. On 1 December 2010, 43 days after planting, forty plants were removed from the field, half healthy and half stunted. Roots were rinsed thoroughly in tap water and whole root systems were placed on P_5ARP without surface disinfestation. Plates were incubated at room temperature and presence of *Pythium* spp. was observed two days after plating.

Determination of ITC concentrations in soil following brassica incorporation

Soil samples were taken after brassica incorporation to determine ITC levels at the following time intervals: 4 hours, 2 days, and 4 days (only done in April 2011). Prior

to each sampling, one 50-ml plastic centrifuge tube was prepared for each plot excluding the fallow (-) VIF plots. Each tube contained 17 μL cyclohexane as an internal standard, 12 ml dichloromethane, and 5 ml 0.2 M $CaCl_2$ (Gardiner et al. 1999). The weight of a prepared test tube was recorded prior to sampling to determine the amount of soil used in each sample. Soil samples were taken with a stainless steel soil probe to a depth of 15 cm, one per plot, in the same order in which the treatments were incorporated. Samples were immediately deposited into a prepared and labeled centrifuge tube and sealed. Samples were weighed, shaken for 15 minutes at 270 rpm on a New Brunswick Scientific Innova 2300 platform shaker, and then centrifuged for 10 minutes at 8000 x g. Soil samples weighed approximately 90 g. The dichloromethane fraction of each sample was then removed using a disposable glass pipette, and placed in a glass test tube containing 0.4 g anhydrous sodium sulfate for desiccation. After one hour, 1.5 – 2.0 ml were transferred from each tube and passed through a 0.45-μm filter into a GC vial and sealed. Samples were placed in a -80°C freezer pending analysis (Gardiner et al. 1999). On 7 March and 28 June 2011, ITC samples were analyzed in the Clemson University multiuser analytical laboratory using a Hewlett Packard 5890 gas chromatograph with a 5971A mass selective detector (GC-MS) and a DB5-MS column (30 m x 0.25 mm, 0.5 μm film, Agilent Technologies). The temperature program began at 40°C for 3 min, and was then increased 6°C min^{-1} to 240°C, then 30°C min^{-1} to 280°C which was held for 1 min. Injection volume was 3 μL per sample, with helium as the carrier gas. ITC peak areas were converted into ITC concentrations using external standards (Figure 3.1).

Estimating soilborne pathogen populations

The effect of biofumigation treatments on soilborne pathogen populations was determined by estimating soilborne populations before and after incorporation of brassicas. Percent change in pathogen population was then determined using the following formula:

$$((\text{pre-incorporation count} - \text{post-incorporation count})(\text{pre-incorporation count})^{-1})*100$$

Population estimates were converted into percent change to account for variability in initial population densities among plots. This calculation was done for estimates of *R. solani* and *Pythium* spp. but not *S. rolfsii* because sclerotia were scattered on the soil surface prior to sampling in repetitions 1 and 2. *S. rolfsii* inoculum was not added to soil in repetition 3 due to low recovery among fallow plots in the first 2 repetitions. Soilborne pathogen population densities of *R. solani* and *Pythium* spp. were estimated before and after incorporation of brassica cover crops in each of the three experimental repetitions. Population density of *S. rolfsii* was determined before incorporation only in repetition 1. Pre-incorporation soil samples were collected on May 11 2010, 17 days prior to incorporation of Spring 2010 planted brassicas, February 15 2011, 1 day prior to incorporation of Fall 2010 planted brassicas, and 21 April 2011, same day as incorporation of Spring 2011 planted brassicas. *S. rolfsii* pre-incorporation population densities were only estimated in repetition 1 because direct comparisons between pre-incorporation estimates and post-incorporation estimates could not be made because soil

cores taken pre-incorporation did not adequately sample the sclerotia that were scattered on the soil surface but not incorporated in the field in repetitions 1 and 2. Post-incorporation soil samples used to quantify all three pathogens were collected on June 28 2010, 31 days after incorporation of brassicas. Soil samples for *R. solani* estimation were collected on 2 March 2011, 14 days after incorporation of brassicas planted in Fall 2010, and 4 May 2011, 13 days after incorporation of brassicas planted in Spring 2011. Soil samples for *Pythium* spp. estimation were collected on 2 March 2011, 14 days after incorporation of Fall 2010 brassicas, and 15 June 2011, 55 days after incorporation of Spring 2011 brassicas. Soil samples for *S. rolfsii* estimation were collected on 5 July 2011, 139 days after incorporation of brassicas in repetition two, and 5 September 2011, 137 days after incorporation of brassicas in repetition three.

To estimate the inoculum density of *R. solani* in the soil, 400 g of soil from each plot in 2010 and 200 g soil from each plot in 2011 was placed in a #18 mesh sieve (1-mm openings) and rinsed thoroughly with tap water (Keinath, 1995). The organic matter trapped by the sieve was then air-dried on paper towels at ambient temperature overnight. The organic matter was then weighed and placed, ten small heaps of equal size per plate (van Bruggen et al. 1986), on EPN_2 medium with prochloraz (Trujillo et al. 1987), prepared with 2% ethanol (Vincelli et al. 1989). Plates were held in the dark at 23°C-25°C. After three days the numbers of heaps with and without colonies of *R. solani* growing from them was recorded. The percentage of organic matter by weight that contained *R. solani* was calculated by multiplying the percentage of heaps infested with *R. solani* with the weight of organic matter recovered.

To estimate the inoculum density of *Pythium* spp. in soil, 10 g of soil from each plot was added to 200 ml of 0.3% water agar and vortexed for 30 seconds. Five aliquots from each sample, 0.5 ml each, were then spread on plates of P_5ARP (Jeffers and Martin, 1986) using a wide-mouth glass pipette. The plates were incubated at 20°C for 20 h and then the adhering soil was washed away using DDI water. Plates then incubated at 20°C, and colonies of *Pythium* > 0.5 cm in diameter were counted 48 hours after plating. Colony counts were expressed as colony-forming units (CFU) per gram of dry soil.

To estimate the inoculum density of *S. rolfsii* in the soil, 300 g of soil from each plot were air dried over night in aluminum pans (30 x 24 x 4 cm) at ambient temperatures. Soil was then crushed in the aluminum pans to break apart soil aggregates. The dried soil was spread evenly in the pans and moistened with 75 ml 1.33% methanol (Rodriguez-Kabana et al., 1980). Pans were placed in large Ziploc® plastic bags and incubated at 30°C for three days. Colonies of *S. rolfsii* were counted by visually examining the soil surface.

Determination of *Pythium* spp. pathogenicity on peppers in the greenhouse

Morphological variations existed among *Pythium* spp. isolates collected from field-soil samples, so an experiment was set up to determine if morphologically distinct isolates demonstrated varying pathogenicity on peppers. The experimental design was a randomized complete block with six replications. The experiment was done twice. The seven experimental treatments included five distinct *Pythium* spp. isolates tested separately, a treatment with all five isolates mixed together in equal proportions, and a non-infested control. There were three distinct morphological types observed in field-soil

Pythium isolates growing on P5ARP. The slow-growing and fast-growing types were extremely common in field soil samples and comprised the majority of the CFUs counted during field soil assays. The type showing a moderate growth rate was extremely uncommon and was only detected sporadically during field soil assays. Two isolates each of slow-growing and fast-growing *Pythium* were used in the experiment, and one isolate of the relatively uncommon type showing a moderate growth rate (Table 3.21). Flasks (250 ml) were prepared with 20 g wheat seeds and 25 ml distilled de-ionized H_2O, two flasks per treatment (Chellemi et al. 2000). Wheat seeds were allowed to soak at room temperature for 24 hours. Flasks were then autoclaved twice daily on two consecutive days. Each flask was inoculated with five 5-mm disks of two-day-old *Pythium* cultures growing on V8 juice agar. Each control flask received five 5 mm disks of sterile V8 juice agar. Flasks were then incubated in the dark at 25°C for eight days and were shaken by hand periodically to ensure uniform *Pythium* growth. On 26 July 2011 and again on 12 September 2011, 2.5 g infested wheat seeds were added to 150 g potting mix and shaken thoroughly in a Ziploc bag. Control flasks received 2.5 g uninoculated wheat seeds. In repetition 1 Fafard 3B potting mix was used, and in repetition 2 Metro-Mix 350 potting mix was used. Six-week-old 'Hunter' bell pepper seedlings grown in 72-cell flats were then replanted in 10-cm pots containing 150 g infested soil. Pepper seeds were obtained from Rogers Seed Company. Plants were maintained at 27.8°C during the day and 25.6-°C at night and watered daily to ensure high soil moisture. After 29 days fresh root and shoot weights were recorded. Roots were rinsed thoroughly and excess moisture was drained prior to weighing. Root disease was then rated based on the following index: 0 = no root symptoms; 1 = 1 to 25% of roots necrotic; 2 = 25 to 50% of roots necrotic; 3 = 50

to 95% of roots necrotic and localized necrotic lesions on crown; 4 = extensive root rot with few white roots and extensive crown rot; 5 = root system completely necrotic and plant dead or moribund (Chellemi et al. 2000). In repetition 2 *Pythium* was recovered from diseased roots taken from control plants. Hyphal tips were then transferred to cornmeal agar (CMA) for morphological identification of *Pythium* at the genus level.

Pathogenic *Pythium* spp. identification

On 5 October 2011 the *Pythium* spp. isolate found to be pathogenic on pepper was identified using a grass-leaf blade culturing technique (Van der Plaats-Niterink 1981). Non-pathogenic isolates were not identified at the species level. Hyphal tips growing on V8 agar were transferred to CMA and allowed to grow for three days at 21.1°C. St. Augustine grass blades were then cut into 1-cm sections and boiled in distilled H_20 for 10 minutes. A sterile petri dish was then filled with 0.5 cm of sterile DDI H_20. Six 1-cm plugs of 3-day-old *Pythium* culture growing on CMA were then transferred to the petri dish containing 0.5cm H_20 along with six sterile grass blades. After three to five days grass blades were placed on slides, stained with aniline blue, and identified morphologically (Van der Plaats-Niterink 1981).

Statistical analysis

All data were checked for normality using the Shapiro-Wilk test. Data were analyzed using proc GLM in SAS, except for data from split-plot designs which were analyzed using proc MIXED (SAS, Inc., Cary, NC). For split-plot analyses marginal means were used whenever treatment interactions were not detected, otherwise cell

means were used. All correlation analyses were done using proc CORR in SAS. Treatment differences were considered significant at $P<0.05$ unless otherwise noted.

Results

Effect of biofumigation on soilborne pathogen populations

R. solani data from the three experimental repetitions were not combined due to a significant repetition-by-treatment interaction. *R. solani* population reductions were observed in all treatments which received VIF during all 3 experimental repetitions (Table 3.1). Reductions ranged from 1.4% in fallow (+) VIF in repetition 3 to 98.3% in 'Pacific Gold' mustard in repetition 2. Reductions observed in brassica treatments were not significantly different from those observed in fallow (+) VIF treatments in any of the 3 repetitions. Minor population reductions were also observed in the fallow (+) CP treatment included in repetition 3, although the observed reduction did not significantly differ from any of the treatments receiving VIF. Significant increases in *R. solani* populations were observed in the fallow (-) VIF treatments in all 3 experimental repetitions. Increases ranged from 110.1% in repetition 1 to 211.1% in repetition 2.

Pythium spp. data from the three experimental repetitions were not combined due to a significant repetition-by-treatment interaction. Treatment effects on populations of *Pythium* spp. were generally inconsistent across experimental repetitions (Table 3.2). In repetition 1 reductions in *Pythium* population densities were observed in all treatments except oilseed radish, which had an increase in *Pythium*. The only significant difference in percent population change observed among (+) VIF treatments was that between

oilseed radish (+11.77%) and ‘Pacific Gold’ mustard (-85.68%) in repetition 1. In repetition 2 fallow (-) VIF had a significant increase in *Pythium* (+1133.8%) compared to the other treatments. There were no significant differences among any of the other treatments in repetition 2, which had changes in *Pythium* populations ranging from -54.5% in winter rapeseed to +118.3 in oilseed radish. In repetition 3 a reduction in *Pythium* was observed in all treatments after cover crop incorporation, with no significant differences detected among them. Changes in populations ranged from -30.3% in ‘Pacific Gold’ mustard to -47.9% in fallow (+) VIF.

S. rolfsii population densities were estimated before and after incorporation of brassicas in repetition 1, and only after incorporation in repetitions 2 and 3. Prior to incorporating brassicas in repetitions 1 and 2, soil was infested with *S. rolfsii* sclerotia, which were still present on the soil surface when pre-incorporation soil samples were collected. Since the sclerotia had been disked into the soil at the time of post-incorporation soil sampling, direct comparisons between the two could not be made. As a result the pre-incorporation sampling was not done in repetitions 2 and 3, and post-incorporation population densities from each treatment were compared directly. In repetition 1 pre-incorporation counts did not differ significantly among treatments. After brassica incorporation, winter rapeseed and fallow (+) VIF had significantly less *S. rolfsii* than oilseed radish and fallow (-) VIF (Table 3.3). In repetition 2 oilseed radish had significantly less *S. rolfsii* than fallow (-) VIF. In repetition 3 winter rapeseed had significantly higher *S. rolfsii* counts than fallow (+) VIF. When repetition 3 was analyzed with fallow (+) CP, fallow (+) CP had significantly higher *S. rolfsii* counts than OR, fallow (+) VIF and fallow (-) VIF. Those statistical differences are not shown in Table

3.3 because fallow (+) CP was not included in repetitions 1 and 2. None of the significant differences among treatments were observed in all 3 repetitions.

Brassica biomass

Biomass in each plot was measured prior to incorporation of brassicas. Brassicas were spring-planted in repetitions 1 and 3, and fall-planted in repetition 2. Repetition 1 brassica biomass was an order of magnitude lower than the biomass accumulated in repetitions 2 and 3 (Tables 3.4, 3.5, and 3.6). Repetition 2 had the highest overall biomass which was the expected outcome for fall-planted brassicas. Oilseed radish had significantly higher fresh-weight biomass (shoots and roots) than winter rapeseed and 'Pacific Gold' mustard in all 3 repetitions. Oilseed radish fresh root weight was higher than the fresh root weight of the other two brassicas in all 3 repetitions, and fresh shoot weight was higher in the first and third repetitions. Fresh weight totals did not differ significantly between winter rapeseed and 'Pacific Gold' mustard in any of the repetitions. Dry weight totals from repetitions 1 and 3 did not significantly differ among the 3 brassicas. However, in repetition 2 oilseed radish had significantly lower total dry weight compared to winter rapeseed and 'Pacific Gold' mustard. 'Pacific Gold' mustard had the highest stand count in all three repetitions, followed by winter rapeseed and oilseed radish (Table 3.7). Repetition 2 had the highest mean brassica stand count, and was significantly higher than repetition 3.

Soil isothiocyanate concentrations

ITCs were quantified in soil samples 4 hours and 2 days after incorporation of brassicas in repetition 2, and 4 hours, 2 days, and 4 days in repetition 3 (Tables 3.8 and 3.9). ITCs were not quantified in repetition one because of low biomass. Because ITC detection was inconsistent within treatments, statistical comparisons were not made. ITCs were detected at every sampling of the 'Pacific Gold' mustard treatment, which also had the highest mean amount of ITC per treatment in repetitions 2 and 3 (5.91 and 1.58 $\mu g\ g^{-1}$ dry soil respectively). ITCs were detected in only one oilseed radish plot in repetition 2 (allyl-ITC, 1.67 $\mu g\ g^{-1}$ dry soil), and were not detected in any of the oilseed radish plots in repetition 3. ITCs were also only detected in 1 winter rapeseed plot in repetition 2 (allyl-ITC, 14.96 $\mu g\ g^{-1}$ dry soil). Although it was only detected once, it was detected at the highest concentration of all 3 treatments in either repetition. This single observation was enough to give winter rapeseed the highest ITC mean total at the two-day sampling of any treatment in both repetitions. ITCs were detected more consistently in the winter rapeseed treatment during repetition 3. Interestingly, only benzyl-ITC and 2-phenylethyl ITC were detected in winter rapeseed soil in repetition 3, whereas only allyl-ITC was detected in winter rapeseed soil in repetition 2. ITC concentrations generally decreased with increased time from incorporation, although allyl-ITC was only detected at the 2 day sampling of oilseed radish and winter rapeseed in repetition 2. In general, allyl-ITC was more commonly detected than benzyl-ITC and 2-phenylethyl ITC, and was generally detected in higher quantities.

Benzyl-ITC was detected in the weedy fallow (+) VIF treatments in repetitions 2 and 3, and allyl-ITC was detected in repetition 3. In repetition 2, benzyl-ITC was detected in only 1 weedy fallow (+) VIF plot. In repetition 3, allyl-ITC was detected in only one weedy fallow (+) VIF plot, while benzyl-ITC was detected in 2/3 of the plots. The most common weeds observed in the weedy fallow plots were nutsedge (*Cyperus rotundus*), corn spurry (*Spergula arvensis*), purslane (*Portulaca oleracea*), Carolina geranium (*Geranium carolinianum*), and wild radish (*Raphanus raphanistrum*), although wild radish was pulled from fallow plots whenever it was observed to limit the introduction of GSLs into fallow treatments. Of the weeds commonly observed, only wild radish is a member of the order Capparales, which is known to contain nearly all of the GSL-producing plants (Matthiessen and Kirkegaard, 2006). Despite the relatively consistent detection of benzyl-ITC in the fallow (+) VIF treatment during repetition 3, the mean ITC concentrations were lower than those of 'Pacific Gold' mustard and winter rapeseed. With the exception of the 2-day sampling during repetition 2, the weedy fallow (+) VIF treatment consistently had higher mean ITC amounts than the oilseed radish treatment.

Pepper disease and yield

In repetition 1, five weeks after peppers were transplanted, diseased plants were noted and symptoms were divided into four categories: chlorotic, >50% defoliated, wilted, and stunted (Table 3.10). Fallow (-) VIF had a significantly higher incidence of chlorosis, defoliation, and stunting compared to the other treatments. There were no significant differences among any of the other treatments for any of the disease

symptoms. ‘Pacific Gold’ mustard and winter rapeseed had 11% and 10% greater percentage healthy plants, respectively, compared to fallow (+) VIF, although the differences were not significant. As a result, only stunting data was collected in subsequent repetitions because it was the most objective of the four symptoms initially measured. Five weeks after peppers were transplanted pepper stunting was recorded in all three repetitions (Table 3.11). Fallow (-) VIF had the highest percentage of stunted plants. Both oilseed radish and winter rapeseed had significantly less stunting than fallow (-) VIF in all three repetitions. Significant differences were not detected among brassica treatments during any of the experimental repetitions. ‘Pacific Gold’ mustard had less stunting than fallow (-) VIF in repetitions 1 and 3. Stunting was not significantly different in brassica treatments compared to fallow (+) VIF in the first two repetitions. Oilseed radish had significantly less stunting than fallow (+) VIF in the third repetition. Fallow (+) VIF had significantly less stunting than fallow (-) VIF in repetitions 1 and 2, but not repetition 3. Stunting was not significantly different among fallow (+) VIF, fallow (-) VIF, or fallow (+) CP treatments in repetition 3.

Pepper mortality was recorded weekly during all 3 repetitions, and categorized by root rot, Southern blight, and total plant death (Table 3.12). Brassica treatments had significantly more plant death than the fallow (-) VIF treatment during the first repetition. The same result was generally observed for deaths from root rot, although ‘Pacific Gold’ mustard was not significantly different from fallow (-) VIF. Incidence of Southern blight was not significantly different among any treatments during repetition 1. During repetition 2 winter rapeseed had significantly more plant deaths than all of the other treatments. Winter rapeseed also had a significantly higher incidence of Southern blight

compared to all other treatments. Oilseed radish also had significantly more Southern blight than fallow (-) VIF. Incidence of mortality due to root rot was not different among treatments during repetition 2. The fewest pepper deaths overall were recorded during repetition 3, with no significant differences among treatments in incidence of plant death from root rot, Southern blight, or total plant death. Incidence of plant death from root rot, Southern blight, or total plant death was not lower in the brassica treatments compared to the fallow (+) VIF treatment in any of the three repetitions.

In all three repetitions pepper plants were removed from each treatment to identify pathogens associated with root rot symptoms. In repetition 1 96% of pepper roots cultured from plants showing root rot symptoms were positive for *Pythium* spp., while 1% of roots from healthy plants were positive for *Pythium* spp. (data not shown). In repetition 2 100% of roots and 33% of shoots cultured from symptomatic plants were positive for *Pythium* spp. and 53% of roots and 0% of shoots were positive for *R. solani*. In repetition 3 91% of pepper roots cultured from symptomatic plants were positive for *Pythium* spp. and 46% of roots taken from healthy plants were positive for *Pythium* spp. Roots were not tested for *R. solani* infection in repetition 1 or 3.

Once plants matured, peppers were harvested weekly (Table 3.13). There was a high incidence of BER observed during repetition 2 (Table 3.14). In repetitions 2 and 3 yield was categorized by total yield, healthy fruit yield, and blossom end rot (BER). Only total yield was recorded in repetition 1. When all three repetitions were combined, the year-by-treatment interaction for total yield was not significant. Oilseed radish and winter rapeseed both had significantly higher mean total yields than fallow (+) VIF. Mean total yield for ‘Pacific Gold’ mustard was not significantly different than fallow (+) VIF. Mean

total yield for fallow (+) VIF was significantly higher than fallow (-) VIF. Based on orthogonal contrasts, mean yields (+) VIF were significantly higher than yields (-) VIF, and mean yields with brassica cover crops were significantly higher than yield with fallow (+) VIF. In all three repetitions, all four (+) VIF treatments had significantly higher yields than fallow (-) VIF with the exceptions of oilseed radish in repetition 1 and fallow (+) VIF in repetition 2. In repetition 1, mean yields with the two *Brassica* cover crops were significantly higher than yield with a *Raphanus* cover crop, based on an orthogonal contrast. In repetitions 2 and 3, there were no significant differences in total yield among brassica treatments. During repetition 1 the highest total yields were observed in 'Pacific Gold' mustard and winter rapeseed, although they were not significantly different from oilseed radish and fallow (+) VIF. In repetition 2 winter rapeseed had a significantly higher yield than fallow (+) VIF. 'Pacific Gold' mustard and oilseed radish had numerically higher yields than fallow (+) VIF, and based on an orthogonal contrast brassica cover crops had significantly higher yields than fallow (+) VIF. In repetition 3 oilseed radish had a significantly higher yield than fallow (+) VIF. Based on an orthogonal contrast, brassica cover crops had a significantly higher yield than fallow (+) VIF, and the *Raphanus* cover crop had a significantly higher yield than the two *Brassica* cover crops ($P<0.10$). Fallow (+) CP was not significantly different from fallow (+) VIF or fallow (-) VIF, and total yield was significantly lower than the brassica cover crop treatments.

Yield data from repetitions 2 and 3 were not combined due to a significant year-by-treatment interaction. In repetition 2, fallow (-) VIF had a significantly lower incidence of BER than all (+) VIF treatments (from 10.03% to 17.83% less) (Table 3.14).

As a result, the mean total healthy yield in fallow (-) VIF was not significantly different from any of the other treatments. Brassica cover crops had significantly higher healthy fruit yields than fallow (+) VIF, based on an orthogonal contrast (P=0.0558). Healthy fruit yield with winter rapeseed was significantly greater than yield with fallow (+) VIF. In repetition 3, incidence of BER was relatively low. As in repetition 2, fallow (-) VIF had a significantly lower incidence of BER than the mean of (+) VIF treatments. In addition, BER cull weight was higher with brassica cover crops than with fallow (+) VIF. Although all three brassica cover crop treatments had slightly higher healthy fruit yields than fallow (+) VIF, only oilseed radish was significantly higher. The trend was significant at 10% when analyzed in an orthogonal contrast (P=0.0997). Fallow (-) VIF had a significantly lower healthy fruit yield than all other treatments in repetition 3.

2010 spinach emergence and disease

On 28 October 2010, 9 days after spinach was planted, emergence was recorded. 'Pacific Gold' mustard (+) Ridomil and winter rapeseed (-) Ridomil had significantly lower emergence than winter rapeseed (+) Ridomil, fallow (+) VIF (+) Ridomil, and fallow (-) VIF (+) Ridomil (Table 3.15). Since there was no significant main effect of Ridomil and no significant Ridomil-by-brassica interaction, Ridomil treatment data were combined (Table 3.16). With the Ridomil effect removed, there were no significant differences in percent emergence detected among any of the cover crop treatments.

On 17 November 2011, 29 days after spinach was planted, stunting was recorded (Table 3.17). Fallow (-) VIF had the highest percentage of stunted plants in both (+) Ridomil and (-) Ridomil treatments (95.8% stunted and 100.0% stunted, respectively).

There were no significant differences detected among brassica treatments or between brassica treatments and fallow (+) VIF with the exception of oilseed radish (-) Ridomil, which had significantly less stunting than fallow (+) VIF (+) Ridomil. As with emergence there was no significant effect of Ridomil and no significant Ridomil*brassica interaction, therefore Ridomil treatment data were combined (Table 3.18). With the Ridomil effect removed fallow (-) VIF had significantly more spinach stunting compared to all other treatments. There were no significant differences detected among brassica treatments or between those and fallow (+) VIF.

On 17 November 2011, 29 days after spinach was planted, plant counts were taken to determine total plant death. Winter rapeseed (+) Ridomil had significantly less plant death than 'Pacific Gold' mustard (+) Ridomil and winter rapeseed (-) Ridomil (Table 3.19). None of the brassica treatments differed significantly in percent plant death compared to fallow (+) VIF or fallow (-) VIF, either (+) or (-) Ridomil. Again, there was no significant effect of Ridomil and no significant Ridomil*brassica interaction, so Ridomil treatment data were combined (Table 3.20). With the Ridomil effect removed there were no significant differences in percent plant death detected among any of the treatment means. On 1 December 2010 healthy and stunted plants were sampled and roots were cultured for *Pythium* spp. infection. *Pythium* spp. were recovered from 80% of the roots taken from stunted plants and from 60% of the roots taken from healthy plants (data not shown).

Greenhouse *Pythium* spp. pathogenicity on peppers

The only *Pythium* isolate found to be pathogenic on pepper was isolate 3. It was the only isolate which alone caused a significant degree of root rot in peppers: it caused an average root disease rating of 3.8 and 2.3 in repetitions 1 and 2, respectively (Table 3.22). A root disease rating of 3.8 meant that root rot was extensive with few white roots and extensive crown rot, and a rating of 2.3 meant that root necrosis ranged from 25-50%. Peppers inoculated with the mixed treatment in which equal parts of each isolate were added to soil also had root rot, with an average disease rating of 2.0 and 2.2 in repetitions 1 and 2, respectively. Those ratings meant 25-50% of roots were necrotic. The mixed treatment had significantly less disease than the isolate 3 treatment and significantly more disease than the control in repetition 1. There was no disease observed in any of the treatments besides isolate 3 during repetition 1. At least minor root discoloration was observed in all treatments during repetition 2. Isolate 3 was again the only treatment to have a significantly higher root disease rating than the control. In repetition 1 pepper shoot and root fresh weights were significantly lower in the *P. aphanidermatum* treatment compared to all other treatments. Shoot and root fresh weights in treatments infested with isolates 1, 2, and 4 were significantly higher than the control. In repetition 2 there was a trend of higher shoot and root fresh weights in treatments infested with isolates 1, 2, 4, and 5, although the values were not significantly different from the control. The inverse was observed in isolate 3 compared to the control and was also not significant. In repetition 2, after root fresh weights were taken, discolored roots from each treatment were cultured on WAS to test for the presence of

Pythium spp. *Pythium* spp. were present in all root samples, including those taken from the control treatments. Two morphologically distinct cultures recovered from control treatment roots were transferred to cornmeal agar (CMA) and identified morphologically as *Pythium* spp. and *Fusarium* spp.

Pathogenic *Pythium* spp. identification

Pythium isolate 3, the only isolate found to be pathogenic on peppers during the first repetition of the greenhouse experiment, was identified as *P. aphanidermatum*. The pool of potential *Pythium* species was narrowed down to ten species already known to be pathogenic on pepper (Chellemi et al. 2000). The following characteristics were used for species identification: smooth-walled oogonia; filamentous inflated sporangia; oogonia average 23 μm diameter; one antheridia per oogonium. The last characteristic was used to distinguish *P. aphanidermatum* from the morphologically similar *P. myriotylum*, which has three to six antheridia per oogonium (Van der Plaats-Niterink 1981).

Discussion

Treatment effects on *R. solani* populations were relatively consistent across experimental repetitions. Population reductions were observed in every treatment which received plastic mulch (either VIF or CP). Population increases were observed in every fallow (-) VIF treatment. Fallow (+) VIF treatments generally had less of a percent decrease in *R. solani* compared to brassica treatments. However, since none of the (+) VIF treatment means differed significantly, which includes fallow (+) VIF and all three

brassica treatments, it cannot be concluded that the observed decreases in *R. solani* populations were due to any effect of the brassica cover crops. Reductions in *R. solani* populations appear to have been induced by an effect associated with the application of plastic mulch, like anaerobiosis, rather than biofumigation. Blok et al. (2000) reported significant reductions in survival of *R. solani* 15 weeks after incorporation of grass or broccoli residue followed by the application of plastic mulch compared to a non-covered control. They also found that pathogen suppression was not observed in a covered treatment that did not receive a soil amendment. Their results indicate that soil amended with a non-brassica, followed by tarping, is sufficient to suppress *R. solani* compared to a non-covered control. During one of two repetitions they reported significantly greater *R. solani* suppression in broccoli-amended soil with plastic mulch compared to grass-amended soil with plastic mulch. Such a result may be related to the effect of ITCs in the soil, which may explain the small differences observed in my brassica treatments compared to fallow (+) VIF. However, it is difficult to say whether or not ITCs played a role in *R. solani* suppression because those treatments also had a larger amount of organic matter incorporated into the soil compared to fallow (+) VIF. It is more likely that another factor was responsible for the slightly higher suppression observed in brassica treatments, such as increased oxygen consumption resulting from organic decomposition, or proliferation of competitive saprophytes or antagonistic anaerobic microorganisms. Kasuya et al. (2006) demonstrated that *B. rapa* subsp. *rapifera* 'Saori', clover, and peanut were all suppressive to damping off in sugar beet caused by *R. solani*. They also demonstrated that the application of antibiotics to the test soil eliminated the suppressive effect, indicating that bacteria played a role in the suppressive effect. In my study, higher

concentrations of ITCs were detected in 'Pacific Gold' mustard and winter rapeseed, and to a lesser extend fallow (+) VIF compared to oilseed radish. If ITCs were contributing to *R. solani* suppression then suppression would have been greater in the mustard and rapeseed treatments compared to radish, but that result was not observed. The observed increases in *R. solani* in fallow (-) VIF treatments was probably a result of the incorporation of organic matter (weeds) into the soil which the pathogen could then colonize. Papavizas et al. (1975) studied the field ecology and epidemiology of *R. solani* and reported that field populations and saprophytic activity both peaked after the incorporation of a bean crop into the soil. Blok et al. (2000) also reported a slight increase in *R. solani* populations following incorporation of broccoli and grass residues without tarping.

Treatment effects on *Pythium* spp. populations were inconsistent across experimental repetitions. *Pythium* decreased after 'Pacific Gold' mustard and winter rapeseed were incorporated into soil in all 3 repetitions, although those decreases were not significantly different from population changes observed in fallow (+) VIF. The fact that *Pythium* increased by 118.3% following incorporation of oilseed radish in repetition 2, but that increase was not significantly different from the decrease of 54.5% observed in winter rapeseed during the same repetition, is indicative of the high variability of population estimates within treatments. Nevertheless, the lack of statistical significance detected among seemingly large differences in *Pythium* populations across treatments probably accurately reflects the ineffectiveness of biofumigation at reducing populations of soilborne *Pythium* spp. Njoroge et al. (2008) reported similar results in 2004 and 2005 in a field study which was also conducted at the Clemson University CREC in

Charleston, SC. They found that canola (*B. napus*) and mustard (*B. juncea*) were both ineffective at reducing *Pythium* spp. compared to a non-amended control. They also found that in some cases *Pythium* spp. populations increased following the incorporation of brassica amendments when plastic mulch was not applied compared to control plots with plastic. These results resemble my findings with *Pythium* spp. and *R. solani* and those reported by Blok et al. (2000), and provide further evidence supporting the stimulatory effect of organic amendments on pathogen populations in the absence of an anaerobic environment. *Pythium* spp. have shown sensitivity to high soil temperatures, such as those achieved during soil solarization. Deadman et al. (2006) demonstrated that a soil temperature of 46.6°C was sufficient to significantly reduce populations of *P. aphanidermatum*. Stapleton and Duncan (1998) found that an increased diurnal temperature treatment of 27°C and 38°C enhanced the suppressive effects of various brassica amendments on suppression of *P. ultimum*. In my experiments average soil temperatures were relatively consistent across seasons. Mean temperatures under VIF during the fall 2010, summer 2011 and fall 2011 pepper seasons were 27.7°C, 27.7°C, and 28.4°C, respectively. Mean temperatures in bare soil plots during the same seasons were 26.7°C, 27.1°C, and 25.9°C, respectively. Maximum temperatures under VIF reached 41.6°C, 38.4°C, and 41.4°C, while maximum temperatures in bare soil plots reached 38.1°C, 38.3°C, and 39.7°C, respectively. It is unlikely that soil heating significantly affected pathogen populations during my experiment because differences between (+) VIF and (-) VIF temperature averages and maxima were small and temperatures were never sustained at levels comparable to those achieved during soil solarization (Deadman et al. 2006, Klein et al. 2007).

Njoroge et al. (2008) also detected 2-phenylethyl ITC and benzyl ITC in soil following brassica incorporation. Allyl-ITC detection was not reported, nor did they report the concentrations of the ITCs detected. They did report that ITC detection was sporadic among soil samples. Biomass measurements (kg ha^{-1}) of 3984 and 8489 were observed in 2004 and 2005 mustard treatments, and 4754 and 9659 in 2004 and 2005 canola treatments, respectively, with fall-seeded brassicas (Njoroge et al., 2008). My biomass measurements (kg ha^{-1}) were 7400 and 4280 for mustard, and 7480 and 4360 for winter rapeseed in repetitions 2 and 3, respectively. Since the biomass accumulated in the Njoroge experiment was similar to mine, certain important comparisons between the two studies can be made. Since ITC detection was sporadic in both studies, the use of VIF and application of irrigation immediately following incorporation was not adequate to increase detection of ITCs in the soil, nor was spring-seeding.Since biomass in the two experiments was comparable, it is likely that ITC concentrations in my experiment were comparable, or higher, than those in the Njoroge experiment because of the measures taken to increase biofumigation efficiency. Whether or not my ITC concentrations were higher, it can be concluded that my attempts to increase the efficiency of biofumigation did not result in increased pathogen suppression compared to the Njoroge experiment. Although spring-seeded brassicas have been reported to contain higher concentrations of GSLs (Sarwar et al. 1998), I detected higher concentrations of ITCs after incorporation of fall-2010 planted brassicas compared to spring-2011 planted. This probably resulted from the higher biomass accumulated in the fall vs. spring planted brassicas.

Treatment effects on *S. rolfsii* populations were also inconsistent across experimental repetitions. None of the brassica treatments had significantly lower post-

incorporation population densities compared to fallow (+) VIF in any of the 3 repetitions. In a controlled laboratory experiment Stapleton and Duncan (1998) reduced germination of *S. rolfsii* by 38% to 55% through the incorporation of cabbage residue into soil at a rate of 2%. They also found that increasing the temperature from 23°C to a 27°C-38°C diurnal heat treatment during the brassica exposure period enhanced the suppressive effect. Since none of the differences in post-incorporation *S. rolfsii* populations among treatments were observed consistently across repetitions, it is likely that these results are also explained by environmental conditions and naturally inconsistent soil populations. The low number of germinated sclerotia counted in each individual plot may have also contributed to the lack of statistical significance detected among treatments. In repetition 3 the fallow (+) CP treatment had significantly higher population densities than fallow (+) VIF treatment. This may be explained by the greater restriction of gas exchange following the application of VIF compared to CP. If *S. rolfsii* is inhibited by anaerobiosis then this could explain the observed difference between the two treatments. Further research is needed to understand how VIF effects the soil aerobic environment and how that influences soil microbial populations.

Brassica biomass was highest in repetition 2, which also had the highest concentrations of soil ITCs after brassica incorporation. Since ITCs are known to be suppressive to the pathogens examined in this study, the effects of brassica incorporation on pathogen populations were expected to be more pronounced in repetition 2 than repetitions 1 and 3; however this result was not observed (Charron et al. 1999, Dhingra et al. 2004, Olivier et al. 1999, Smith and Kirkegaard et al. 2002). In fact biofumigation treatments in repetition 2 and 3 did not outperform the same treatments in repetition 1

even though brassica biomass was nearly an order of magnitude higher in repetitions 2 and 3 compared to repetition 1. These results indicate that *R. solani*, *Pythium* spp., and *S. rolfsii* are not susceptible to population reductions caused by exposure to ITCs at the highest concentrations achieved in my field trials. This is further supported by the lack of consistent differences in pathogen populations between the high-ITC producing 'Pacific Gold' mustard and winter rapeseed treatments and the low to non-ITC producing oilseed radish treatment. The weedy fallow (+) VIF treatment also had low concentrations of ITCs detected. Certain GSL-producing weeds, such as wild radish (*Raphanus raphanistrum*), may have been the source of ITCs in the weedy fallow treatments. Wild radish, a common weed at the Clemson CREC, is known to produce several GSLs and ITCs (Malik et al. 2010).

Pepper yields were generally highest following the brassica treatments, although the differences between those and the fallow (+) VIF treatment were not always significant. When experimental repetitions were combined, yields were significantly higher following brassica incorporation compared to fallow (+) VIF, based on an orthogonal contrast. Higher yields following brassica incorporation could have significant implications for South Carolina pepper growers, especially those interested in reducing their reliance on synthetic pesticides. The yield differences observed were probably due to the general benefits associated with cover cropping rather than disease reductions resulting from biofumigation. For example, Weil et al. (2009) at the University of Maryland's Cooperative Extension found that oilseed radish alleviated soil compaction and the need for deep tillage through the growth of a long taproot, which can penetrate six feet or more into the soil. In addition to improving soil structure, cover crops also

improve nutrient and moisture availability, increase soil organic matter, suppress weeds, and reduce soil erosion (Clark, 2007). Brassicas in particular are known to be efficient nutrient scavengers. Cover crops can also contribute to pathogen and disease suppression through non-host and trap-crop effects, proliferation of antagonistic microorganisms, and the release of non-ITC decomposition products (Matthiessen and Kirkegaard, 2006). Such effects may partially explain the trend of pathogen suppression observed in some of the brassica treatments. Pepper yield was consistently lowest in the fallow (-) VIF treatment, which also had the greatest incidence of stunting. The same stunting result was observed in spinach in 2010. *R. solani* population increases following brassica incorporation in fallow (-) VIF treatments seem to indicate that *R. solani* likely played a role in the stunting observed in those treatments. Despite the similarity in results between pepper stunting and yield and *R. solani* soil assays, only half of the roots from stunted pepper plants tested positive for *R. solani* during repetition 2, indicating that pathogenic *Pythium* spp. most likely also contributed to stunting. Roots from symptomatic plants tested for *Pythium* during repetitions 1 and 2 were 96% and 100% positive for *Pythium* spp., respectively. Healthy plant roots were only 1% positive for *Pythium* spp. All pepper roots tested for *Pythium* spp. were surface sterilized prior to plating. Spinach roots tested for *Pythium* were not surface sterilized prior to plating and only rinsed to remove soil. In that case 80% of roots from stunted spinach plants tested positive for *Pythium* spp. and 60% of roots from healthy plants tested positive for *Pythium* spp. These results appear to indicate that non-pathogenic *Pythium* spp. are capable of forming a symbiotic relationship with host plant roots without directly penetrating or parasitizing them. The results from the greenhouse *Pythium* pathogenicity experiment support this theory,

because a relatively small percentage of the morphological types counted as *Pythium* spp. in the field soil assays were actually pathogenic on pepper. The majority of colonies were either non-pathogenic or growth-promoting. The inconsistencies observed between repetitions 1 and 2 of the greenhouse pathogenicity experiment were most likely the result of a change in the potting mix used. In repetition 1 Fafard 3B potting mix was used but was switched to Metro-Mix 350 potting mix for repetition 2. Since there was no *Pythium* added to the control treatments, the root discoloration observed in repetition 2 was a surprising result. The apparent presence of *Pythium* spp. in Metro-Mix 350 potting mix most likely explains the root disease observed in *Pythium* treatments that were non-pathogenic and growth-promoting during repetition 1. Preliminary results from an additional experiment further support the hypothesis that *Pythium* isolates 1, 2, 4, and 5 are non-pathogenic on pepper. In that experiment 18 cells per isolate were filled with potting mix and infested with one of the four non-pathogenic *Pythium* isolates, followed by the direct seeding of peppers. After five weeks there were no disease symptoms observed on any of the 72 seedlings. The results do not mean that *Pythium* spp. estimates obtained from field soil using P_5ARP are inaccurate. However, such estimates may be less useful in predicting disease associated with *Pythium* spp. in the field than previously thought. *Pythium* spp. isolated from field soil should not be assumed to be pathogenic on any given crop. Connecting estimates of *R. solani* to plant disease may be more accurate because morphological identification of *R. solani* is significantly easier than morphological *Pythium* species identification. *Pythium* species identification may be a necessary component of soil population estimates if the intention is to connect the estimates with observed plant disease. Additionally, the role played by non-pathogenic

Pythium spp. in the rhizosphere needs to be investigated in more detail. Non-pathogenic *Pythium* spp., once established on a host's root system, may serve to outcompete or even parasitize pathogenic *Pythium* spp. Al-Rawahi and Hancock (1998) found *P. oligandrum* to be suppressive to *Verticillium dahliae.* They also found that *P. oligandrum* increased fresh weight of pepper plants in the greenhouse compared to controls both in the presence and absence of *V. dahliae*. Takenaka et al. (2007) also found that *P. oligandrum* was capable of reducing bacterial wilt in tomato caused by *Ralstonia solanacearum* in a greenhouse study. Such results warrant further investigation of the role played by non-pathogenic *Pythium* spp. in the field.

The results indicate that the use of brassica cover crops as a means of soil disinfestation is ineffective against *Pythium* spp., *R. solani*, and *S. rolfsii* in Charleston, SC. These results reflect the specific environmental conditions, brassica species selected and biomass achieved during my field experiments. Considering the complexity of the biofumigation system, it is not possible to infer that the results of one field study are indicative of the efficacy of biofumigation in general. However, the data suggest that there is an overall benefit to cover cropping in terms of total pepper yield. The application of plastic mulch, specifically VIF, had the most consistent and pronounced effect on pepper yield and stunting. This suggests that the changes to the soil ecosystem that occur after plastic mulch application, such as anaerobiosis or proliferation of anaerobic bacteria, have a beneficial effect on plant health, which may be amplified following cover cropping (Blok et al. 2000, Chung et al. 2008). Further research on the mechanisms of plant-health promotion following the application of plastic mulch could

lead to a targeted increase of the beneficial effects and another tool to manage soilborne pathogens for growers interested in sustainable agriculture.

Literature Cited

Al-Rawahi, A.K., and Hancock, J.G. 1998. Parasitism and biological control of *Verticillium dahliae* by *Pythium oligandrum*. Plant Disease 82: 1100-1106.

Antonious, G F., Bomford, M., & Vincelli, P. 2009. Screening brassica species for glucosinolate content. Journal of Environmental Science and Health Part B 44: 3, 311-316.

Blok, W. J., Lamers, J. G., Termorshuizen, A. J., & Bollen, G. J. 2000. Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. Phytopathology 90:253-259.

Charron, C. S., & Sams, C. E. 1999. Inhibition of *Pythium ultimum* and *Rhizoctonia solani* by shredded leaves of brassica species. Journal of the American Society of Horticultural Science 124(5): 462–467.

Chellemi, D. O., Mitchell, D. J., Kannwischer-Mitchell, M. E., Rayside, P. A., & Rosskopf, E. N. 2000. *Pythium* spp. associated with bell pepper production in Florida. Plant Disease 84: 1271-1274.

Chung, S., Kong, H., Buyer, J.S., Lakshman, D.K., Lydon, J., Kim, S., Roberts, D.P. 2008. Isolation and partial characterization of Bacillus subtilis ME488 for suppression of soilborne pathogens of cucumber and pepper. Applied Microbiology and Biotechnology 80: 115-123.

Clark, A. 2007. Managing cover crops profitably. Third edition. Sustainable Agriculture Network, Beltsville, MD.

Collins, H.P., Alva, A., Boydston, R.A., Cochran, R.L., Hamm, P.B., McGuire, A., & Riga, E. 2006. Soil microbial, fungal, and nematode responses to soil fumigation and cover crops under potato production. Biology and Fertility of Soils 42: 247–257.

Deadman, M., Al Hasani, H., & Al Sa'di, A. 2006. Solarization and biofumigation reduce *Pythium aphanidermatum* induced damping-off and enhance vegetative growth of greenhouse cucumber in Oman. Journal of Plant Pathology 88: 3, 335-337.

Dhingra, O.D., Costa, M.L.N., Silva Jr, G.J., & Mizubuti, E.S.G. 2004. Essential oil of mustard to control *Rhizoctonia solani* causing seedling damping off and seedling blight in nursery. Fitopatol. bras 29: 6.

Fenwick, G.R., Heaney, R.K., Mullin, W.J. 1983. Glucosinolates and their breakdown products in food and food plants. CRC Critical Reviews in Food Science and Nutrition 18:123–201.

Gardiner, J.B., Morra, M.J., Eberlein, C.V., Brown, P.D., & Borek, V. 1999. Allelochemicals released in soil following incorporation of rapeseed (*Brassica napus*) green manures. Journal of Agricultural and Food Chemistry 47: 3837-3842.

Hanson, B.D., J.S. Gerik, & S.M. Schneider. 2010. Effects of reduced rate methyl bromide applications under conventional and virtually impermeable plastic film in perennial crop field nurseries. Pest Management Science 66: 892-899.

Jeffers, S.N., & Martin, S.B. 1986. Comparison of two media selective for *Phytophthora* and *Pythium* species. Plant Disease 70: 1038-1043.

Kasuya, M., Olivier, A. R., Ota, Y., Tojo, M., Honjo, H., & Fukui, R. 2006. Induction of soil suppressiveness against *Rhizoctonia solani* by incorporation of dried plant residues into soil. Phytopathology 96: 1372-1379.

Keinath, A.P. 1995. Reductions in inoculum density of *Rhizoctonia solani* and control of belly rot on pickling cucumber with soil solarization. Plant Disease 79: 1213-1219.

Kemble, J.M., Louws, F.J., Jennings, K.M., & Walgenbach, J.F. 2011. Southeastern U.S. 2011 vegetable crop handbook: 74-75.

Klein, E., Katan, J., Austerweil, M., and Gamliel, A. 2007. Controlled laboratory system to study soil solarization and organic amendmenteffects on plant pathogens. Phytopathology 97:1476-1483.

Larkin, R.P., & Griffin, T.S. 2006. Control of soilborne potato diseases using brassica green manures. Crop Protection 26: 1067-107.

Malik, M.S., Riley, M.B., Norsworthy, J.K., and Bridges, W. 2010. Variation of glucosinolates in wild radish (*Raphanus raphanistrum*) accessions. Journal of Agricultural and Food Chemistry 58: 11626–11632.

Matthiessen, J.N., & Kirkegaard, J.A. 2006. Biofumigation and enhanced biodegradation: opportunity and challenge in soilborne pest and disease management. Critical Reviews in Plant Sciences 25: 235-265.

Mayton, H. S., Olivier, C., Vaughn, S. F., & Loria, R. 1996. Correlation of fungicidal activity of brassica species with allyl isothiocyanate production in macerated leaf tissue. Phytopathology 86: 267-271.

Momma, N., Yamamoto, K., Simandi, P., & Shishido, M. 2006. Role of organic acids in the mechanisms of biological soil disinfestation (BSD). Journal of General Plant Pathology 72: 247–252.

Morra, M.J., & J.A. Kirkegaard. 2002. Isothiocyanate release from soil-incorporated brassica tissues. Soil Biology and Biochemistry 34: 1683-1690.

Njoroge, S. M. C., Riley, M. B., & Keinath, A. P. 2008. Effect of incorporation of *Brassica* spp. residues on population densities of soilborne microorganisms and on damping-off and Fusarium wilt of watermelon. Plant Disease 92: 287-294.

Noling, J.W., Botts, D.A., & MacRae, A.W. 2010. Alternatives to methyl bromide soil fumigation for Florida vegetable production. University of Florida IFAS extension vegetable production handbook: 47-54.

Olivier, C., Vaughn, S.V., Mizubuti, E.S.G., & Loria, R. 1999. Variation in allyl isothiocyanate production within brassica species and correlation with fungicidal activity. Journal of Chemical Ecology 25: 2687-2701.

Papavizas, G.C., Adams, P.B., Lumsden, R.D., Lewis, J.A., Dow, R.L., Ayers, W.A., & Kantzes, J.G. 1975. Ecology and epidemiology of *Rhizoctonia solani* in field soil. Phytopathology 65: 871-877.

Roberts, P.D. 2003. Damping-off and root rot. In Compendium of Pepper Diseases (pp. 12-13). The American Phytopathological Society.

Roberts, P.D. 2003. Southern blight. In Compendium of Pepper Diseases (pp. 20-21). The American Phytopathological Society.

Rodriguez-Kabana, R., Beute, M. K., & Backman, P. A. 1980. A method for estimating numbers of viable sclerotia of *Sclerotium rolfsii* in soil. Phytopathology 70: 917-919.

Sarwar, M., & Kirkegaard, J.A. 1998. Biofumigation potential of brassicas. II. Effect of environment and ontogeny on glucosinolate production and implications for screening. Plant and Soil 201: 91-101.

Smith, B.J., & Kirkegaard, J.A. 2002. In vitro inhibition of soil microorganisms by 2-phenylethyl isothiocyanate. Plant Pathology 51: 585–593.

Stapleton, J.J., & Duncan, R.A. 1998. Soil disinfestation with cruciferous amendments and sublethal heating: effects on *Meloidogyne incognita*, *Sclerotium rolfsii* and *Pythium ultimum*. Plant Pathology 47: 737-742.

Sundermeier, A. 2008. Oilseed radish cover crop. Ohio State University Extension SAG-5-08.

Takenaka, S., Sekiguchi, H., Nakaho, K., Tojo, M., Masunaka, A., and Takahashi, H. 2008. Colonization of *Pythium oligandrum* in the tomato rhizosphere for biological control of bacterial wilt disease analyzed by real-time PCR and confocal laser-scanning microscopy. Phytopathology 98:187-195.

Trujillo, E.E., Cavin, C.A., Aragaki, M., & Yoshimura, M.A. 1987. Ethanol-potassium nitrate medium for enumerating *Rhizoctonia solani*-like fungi from soil. Plant Disease 71: 1098-1100.

[USDA ERS] U.S. Department of Agriculture Economic Research Service. 2011. US bell & chili pepper statistics. http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1659. Accessed: June 20, 2011.

[USEPA] U.S. Environmental Protection Agency. 2011. Ozone layer depletion-regulatory programs: the phaseout of methyl bromide. http://www.epa.gov/ozone/mbr/index.html. Accessed: June 10, 2011.

van Bruggen, A. H. C., & Arneson, P. A. 1986. Quantitative recovery of *Rhizoctonia solani* from soil. Plant Disease 70: 320-323.

Van der Plaats-Niterink, A.J. 1981. Monograph of the Genus *Pythium*. Studies in Mycology No. 21. Centraalbureau Voor Schimmelcultures, Baarn, The Netherlands.

Vincelli, P.C., & Beaupre, C.M.S. 1989. Comparison of media for isolating *Rhizoctonia solani* from soil. Plant Disease 73: 1014-1017.

Weil, R., White, C., & Lawley, Y. Forage radish: new multi-purpose cover crop for the Mid-Atlantic. University of Maryland Cooperative Extension Fact Sheet 824.

Table 3.1. *R. solani* pre-incorporation and post-incorporation field soil mean population densities for each of three field experimental repetitions.

Repetition 1 - 2010 Spring-planted brassicas[v]			
Treatment	Pre-incorporation count (%)[w]	Post-incorporation count (%)	Difference (%)[x]
'Pacific Gold' mustard	70.8	31.7	-52.1 a[y]
Oilseed radish	54.5	29.5	-17.2 a
Winter rapeseed	62.1	22.5	-67.7 a
Fallow (+) VIF	43.0	30.0	-35.2 a
Fallow (-) VIF	26.6	56.1	+110.1 b

Repetition 2 - 2010 Fall-planted brassicas[z]			
Treatment	Pre-incorporation count (g infested organic matter kg^{-1} soil)	Post-incorporation count (g infested organic matter kg^{-1} soil)	Difference (%)
'Pacific Gold' mustard	0.755	0.017	-98.3 a
Oilseed radish	0.838	0.052	-94.6 a
Winter rapeseed	0.538	0.123	-69.4 a
Fallow (+) VIF	0.365	0.202	-51.6 a
Fallow (-) VIF	0.365	0.972	+211.1 b

Repetition 3 - 2011 Spring-planted brassicas[v]			
Treatment	Pre-incorporation count (g infested organic matter kg^{-1} soil)	Post-incorporation count (g infested organic matter kg^{-1} soil)	Difference (%)
'Pacific Gold' mustard	1.221	0.543	-32.4 a
Oilseed radish	1.200	0.596	-45.7 a
Winter rapeseed	0.719	0.336	-51.2 a
Fallow (+) VIF	0.716	0.573	-1.4 a
Fallow (-) VIF	0.433	0.838	+148.6 b
Fallow (+) CP	0.605	0.571	-6.3 a

[v] All values in repetitions 1 and 3 are the mean of four replicates.
[w] Counts represent the percentage of organic matter heaps infested with *R. solani.*
[x] Difference between pre-incorporation counts and post-incorporation counts. Calculated as ((pre-incorporation count – post-incorporation count) (pre-incorporation count)$^{-1}$)*100.
[y] Means followed by the same letter within column*brassica planting date are not significantly different, LSD (P<0.05).
[z] All values in repetition 2 are the mean of three replicates.

Table 3.2. *Pythium* spp. pre-incorporation and post-incorporation field soil mean population densities for each of three field experimental repetitions.

Repetition 1 - 2010 Spring-planted brassicas[t]			
Treatment	Pre-incorporation count (CFU g^{-1} dry soil)	Post-incorporation count (CFU g^{-1} dry soil)	Difference (%)[u]
'Pacific Gold' mustard	19.00	2.75	-85.68 a[v]
Oilseed radish	15.00	13.00	+11.77 b[w]
Winter rapeseed	18.50	9.00	-39.20 a b
Fallow (+) VIF	12.00	5.00	-60.38 a b
Fallow (-) VIF	11.25	6.00	-54.48 a b

Repetition 2 - 2010 Fall-planted brassicas[x]			
Treatment	Pre-incorporation count (CFU g^{-1} dry soil)	Post-incorporation count (CFU g^{-1} dry soil)	Difference (%)
'Pacific Gold' mustard	16.67	10.33	-46.7 a
Oilseed radish	31.33	58.67	+118.3 a
Winter rapeseed	38.00	14.00	-54.5 a
Fallow (+) VIF	14.67	18.33	+29.1 a
Fallow (-) VIF	14.67	179.67	+1133.8 b

Repetition 3 - 2011 Spring-planted brassicas[t]			
Treatment	Pre-incorporation count (CFU g^{-1} dry soil)	Post-incorporation count (CFU g^{-1} dry soil)	Difference (%)
'Pacific Gold' mustard	149.3	97.3	-30.3 a
Oilseed radish	172.5	108.0	-37.5 a
Winter rapeseed	161.5	107.5	-33.3 a
Fallow (+) VIF	171.5	88.0	-47.9 a
Fallow (-) VIF	166.0	101.0	-38.7 a
Fallow (+) CP	ND[y]	47.5	NA[z]

[t] All values are the mean of four replicates.

[u] Difference between pre-incorporation counts and post-incorporation counts. Calculated as ((pre-incorporation count – post-incorporation count) (pre-incorporation count)$^{-1}$)*100.

[v] Means followed by the same letter within column*brassica planting date are not significantly different, LSD (P<0.05).

[w] Although the pre-incorporation mean was higher than the post-incorporation mean, the mean % difference was positive because a large increase in *Pythium* was observed in one of the replications which influenced the overall mean % difference.

[x] All values in repetition 2 are the mean of three replicates.

[y] Not done.

[z] Not applicable.

Table 3.3. *S. rolfsii* field soil population densities.

	Repetition 1 - 2010 Spring-planted brassica field[v]		**Repetition 2 - 2010 Fall-planted brassica field[w]**	**Repetition 3 - 2011 Spring-planted brassica field[v]**
Treatment	Pre-incorporation count (CFU 300 g^{-1} field weight soil)	Post-incorporation count (CFU 300 g^{-1} field weight soil)	Post-incorporation count (CFU 300 g^{-1} dry soil)	Post-incorporation count (CFU 300 g^{-1} dry soil)
'Pacific Gold' mustard	5.75 a[x]	0.50 a b	2.77 a b	2.03 a b
Oilseed radish	6.25 a	1.50 a	0.77 b	1.15 a b
Winter rapeseed	6.75 a	0.25 b	2.03 a b	2.28 a
Fallow (+) VIF	6.75 a	0.25 b	3.53 a b	0.58 b
Fallow (-) VIF	2.75 a	1.50 a	7.60 a	0.85 a b
Fallow (+) CP	ND[y]	ND	ND	3.58[z]

[v] All values in repetitions 1 and 3 are the mean of four replicates.
[w] All values in repetition 2 are the mean of three replicates.
[x] Means followed by the same letter are not significantly different, LSD (P<0.05).
[y] Not done.
[z] Fallow (+) CP was not included in the statistical analysis because it was not included in repetitions 1 and 2.

Table 3.4. Repetition 1 - 2010 Spring-planted brassica biomass.[y]

	'Pacific Gold' mustard	**Oilseed radish**	**Winter rapeseed**
Dry shoot weight (g m^{-2})	64 a[z]	84 a	44 b
Dry root weight (g m^{-2})	16 a	30 a	28 a
Total dry weight (g m^{-2})	80 a	114 a	72 a
Fresh shoot weight (kg m^{-2})	0.312 b	0.572 a	0.216 b
Fresh root weight (kg m^{-2})	0.076 b	0.228 a	0.148 b
Total fresh weight (kg m^{-2})	0.388 b	0.800 a	0.364 b

[y] All values are the mean of four replicates.
[z] Means within a row followed by the same letter are not significantly different, LSD (P<0.05).

Table 3.5. Repetition 2 - 2010 Fall-planted brassica biomass.[y]

	'Pacific Gold' mustard	**Oilseed radish**	**Winter rapeseed**
Dry shoot weight (g m^{-2})	584 a[z]	300 b	596 a
Dry root weight (g m^{-2})	156 b	324 a	152 b
Total dry weight (g m^{-2})	740 b	624 a	748 b
Fresh shoot weight (kg m^{-2})	3.76 a	3.96 a	4.08 a
Fresh root weight (kg m^{-2})	1.40 b	4.16 a	0.84 b
Total fresh weight (kg m^{-2})	5.16 b	8.12 a	4.92 b

[y] All values are the mean of three replicates.
[z] Means within a row followed by the same letter are not significantly different, LSD (P<0.05).

Table 3.6. Repetition 3 - 2011 Spring-planted brassica biomass.[y]

	'Pacific Gold' mustard	**Oilseed radish**	**Winter rapeseed**
Dry shoot weight (g m^{-2})	308 a	352 a	304 a
Dry root weight (g m^{-2})	120 b	200 a	132 b
Total dry weight (g m^{2-})	428 a	552 a	436 a
Fresh shoot weight (kg m^{-2})	1.92 b	3.84 a	2.20 b
Fresh root weight (kg m^{-2})	0.60 b	2.04 a	0.56 b
Total fresh weight (kg m^{-2})	2.52 b	5.88 a	2.76 b

[y] All values are the mean of four replicates.
[z] Means within a row followed by the same letter are not significantly different, LSD (P<0.05).

Table 3.7. Brassica stand counts from all 3 experimental repetitions.

Repetition 1 - 2010 Spring-planted brassicas[u]	
Treatment[v]	Mean stand count (number of plants 0.25 m^{-2})
'Pacific Gold' mustard	81 a[w]
Oilseed radish	31 c
Winter rapeseed	53 b
Total	55 A[x y] B
Repetition 2 - 2010 Fall-planted brassicas[z]	
Treatment	Mean stand count (number of plants 0.25 m^{-2})
'Pacific Gold' mustard	75 a
Oilseed radish	43 b
Winter rapeseed	67 a
Total	61 A
Repetition 3 - 2011 Spring-planted brassicas[v]	
Treatment	Mean stand count (number of plants 0.25 m^{-2})
'Pacific Gold' mustard	75 a
Oilseed radish	26 c
Winter rapeseed	42 b
Total	48 B

[u] All values in repetitions 1 and 3 are the mean of four replicates.
[v] 'Pacific Gold' mustard and winter rapeseed were seeded at 11.2 kg ha^{-1}. Oilseed radish was seeded at 28.0 kg ha^{-1}.
[w] Means followed by the same lower-case letter within experimental repetitions are not significantly different (P<0.05).
[x] Total brassica stand counts were compared across years because there was not a significant year*treatment interaction.
[y] Means followed by the same upper-case letter across experimental repetitions are not significantly different (P<0.05).
[z] All values in repetition 2 are the mean of three replicates.

Table 3.8. Mean amount of isothiocyanate detected in soil samples after incorporation of repetition 2 - 2010 Fall-planted brassicas.[y]

Sampling Time[z] Isothiocyanate	**Pacific Gold Mustard ITCs (µg g^{-1} dry soil) (*data range*)**	**Oilseed Radish ITCs (µg g^{-1} dry soil) (*data range*)**	**Winter Rapeseed ITCs (µg g^{-1} dry soil) (*data range*)**	**Weedy Fallow ITCs (µg g^{-1} dry soil) (*data range*)**
4 hours				
Allyl	5.91 *(4.36-8.58)*	nd	nd	nd
Benzyl	nd*	nd	nd	0.14 *(nd-0.43)*
2-phenylethyl	nd	nd	nd	nd
Subtotal	**5.91**	**nd**	**nd**	**0.14**
2 Days				
Allyl	3.01 *(0.65-5.89)*	0.56 *(0-1.67)*	4.99 *(0-14.96)*	nd
Benzyl	nd	nd	nd	nd
2-phenylethyl	0.13 *(nd-0.39)*	nd	nd	nd
Subtotal	**3.14**	**0.56**	**4.99**	**nd**

[y] All values are the mean of three replicates.

[z] Sampling times correspond to time elapsed after brassica incorporation.

* nd = none detected above the GC-MS limit of detection (limit of detection = 0.005 picogram ITC mL^{-1}.

Table 3.9. Mean amount of isothiocyanate detected following incorporation of repetition 3 - 2011 Spring-planted brassicas.[y]

Sampling Time[z] Isothiocyanate	**Pacific Gold Mustard ITCs (µg g^{-1} dry soil) (*data range*)**	**Oilseed Radish ITCs (µg g^{-1} dry soil) (*data range*)**	**Winter Rapeseed ITCs (µg g^{-1} dry soil) (*data range*)**	**Weedy Fallow ITCs (µg g^{-1} dry soil) (*data range*)**
4 hours				
Allyl	1.58 (*nd-4.86*)	nd	nd	0.13 (*nd-0.53*)
Benzyl	nd*	nd	0.16 (*nd-0.63*)	0.17 (*nd-0.36*)
2-phenylethyl	nd	nd	0.28 (*nd-1.12*)	nd
Subtotal	**1.58**	**nd**	**0.44**	**0.3**
2 Days				
Allyl	0.57 (*0.5-0.62*)	nd	nd	nd
Benzyl	0.06 (*nd-0.23*)	nd	nd	0.11 (*nd-0.43*)
2-phenylethyl	0.15 (*nd-0.32*)	nd	0.44 (*nd-0.88*)	nd
Subtotal	**0.78**	**nd**	**0.44**	**0.11**
4 Days				
Allyl	0.22 (*nd-0.89*)	nd	nd	nd
Benzyl	nd	nd	nd	0.07 (*nd-0.27*)
2-phenylethyl	nd	nd	0.14 (*nd-0.54*)	nd
Subtotal	**0.22**	**nd**	**0.14**	**0.07**

[y] All values are the mean of four replicates.
[z] Sampling times correspond to time elapsed after brassica incorporation.
* nd = none detected above the GC-MS limit of detection (limit of detection = 0.005 picograms ITC mL^{-1}.

Table 3.10. Effect of biofumigation treatments on various pepper disease symptoms from repetition 1 - Fall 2010.[y]

Treatment	**Chlorotic (%)**	**>50% defoliated (%)**	**Wilted (%)**	**Stunted (%)**	**Healthy (%)**
'Pacific Gold' mustard (+) VIF	5.75 a[z]	9.50 a	11.50 a	26.25 a	66.00 a
Oilseed radish (+) VIF	6.50 a	10.75 a	21.00 a	33.75 a	58.50 a
Winter rapeseed (+) VIF	6.50 a	6.75 a	15.75 a	21.25 a	65.75 a
Fallow (+) VIF	7.75 a	6.50 a	24.50 a	32.75 a	55.25 a
Fallow (-) VIF	18.75 b	37.25 b	3.50 a	75.75 b	14.50 b

[y] All values are the mean of four replicates. Values across rows do not add to 100% because individual plants may have possessed multiple disease symptoms.
[z] Means within a column followed by the same letter are not significantly different, LSD ($P<0.05$).

Table 3.11. Effect of biofumigation treatments on pepper stunting in all three repetitions.[v]

Treatment	**Repetition 1 - 2010 Fall peppers stunted plants[w] (%)**	**Repetition 2 - 2011 Spring Peppers stunted plants[x] (%)**	**Repetition 3 - 2011 Fall peppers stunted plants[w] (%)**
'Pacific Gold' mustard (+) VIF	26.25 a[y]	18.33 a b	43.25 a b
Oilseed radish (+) VIF	33.75 a	6.33 a	24.00 a
Winter rapeseed (+) VIF	21.25 a	4.33 a	44.00 a b
Fallow (+) VIF	32.75 a	7.00 a	67.00 b c
Fallow (-) VIF	75.75 b	38.67 b	86.00 c
Fallow (+) CP	ND[z]	ND	83.25 c

[v] Plants were considered stunted if they measured <2/3 the height of the most vigorous plant in the field.
[w] Values in repetitions 1 and 3 are the mean of four replicates.
[x] Values in repetition 2 are the mean of three replicates.
[y] Means within a column followed by the same letter are not significantly different, LSD (P<0.05).
[z] Not done.

Table 3.12. Pepper mortality in all three experimental repetitions separated by cause of death and total plant death.

Repetition 1 - 2010 Fall peppers[v w]			
Treatment	Number of plants that died from root rot	Number of plants that died from Southern blight	Total plant death (%)
'Pacific Gold' mustard	16.5 a[x] b	3.8 a	33.8 b[z]
Oilseed radish	18.3 b	2.5 a	34.6 b
Winter rapeseed	18.8 b	2.5 a	35.4 b
Fallow (+) VIF	14.8 a b	1.8 a	27.5 a b
Fallow (-) VIF	9.3 a	0.3 a	15.9 a

Repetition 2 - 2011 Spring peppers[y w]			
Treatment	Number of plants that died from root rot	Number of plants that died from Southern blight	Total plant death (%)
'Pacific Gold' mustard	3.0 a	2.0 a b	7.8 a
Oilseed radish	3.0 a	2.7 b	8.9 a
Winter rapeseed	5.3 a	6.3 c	19.4 b
Fallow (+) VIF	4.7 a	1.7 a b	10.6 a
Fallow (-) VIF	3.3 a	0.3 a	6.1 a

Repetition 3 - 2011 Fall peppers[v z]			
Treatment	Number of plants that died from root rot	Number of plants that died from Southern blight	Total plant death (%)
'Pacific Gold' mustard	1.0 a	1.3 a	4.5 a
Oilseed radish	0.8 a	1.3 a	4.0 a
Winter rapeseed	1.5 a	0.8 a	4.5 a
Fallow (+) VIF	3.0 a	0.3 a	6.0 a
Fallow (-) VIF	3.3 a	0.3 a	7.0 a
Fallow (+) CP	2.0 a	0.3 a	4.5 a

[v] All values in repetitions 1 and 3 are the mean of four replicates.
[w] Each plot was planted in 60 pepper seedlings.
[x] Within experimental repetitions, means within a column followed by the same letter are not significantly different, LSD ($P<0.05$).
[y] All values in repetition 2 are the mean of three replicates.
[z] Each plot was planted in 50 pepper seedlings.

Table 3.13. Total pepper yields from all three experimental repetitions.

Treatment	**Repetition 1 - 2010 Fall yield (kg)[s t]**	**Repetition 2 - 2011 Spring yield (kg)[t u]**	**Repetition 3 - 2011 Fall yield (kg)[s v]**	**Mean yield (kg)[w]**
'Pacific Gold' mustard	54.98 a[x]	51.38 a b	31.79 a b	45.92 a b
Oilseed radish	47.17 a b	52.21 a b	41.38 a	46.79 a
Winter rapeseed	55.36 a	54.15 a	34.88 a b	48.00 a
Fallow (+) VIF	48.46 a	47.26 b c	28.13 b c	41.16 b
Fallow (-) VIF	38.94 b	41.05 c	13.35 d	30.98 c
Fallow (+) CP	ND[y]	ND	19.83 c d	NA[z]

Contrasts	***P*-value**	***P*-value**	***P*-value**	***P*-value**
(+) VIF vs. (-) VIF	0.0025	0.0018	0.0002	<0.0001
Brassica vs. Fallow (+)VIF	0.2590	0.0494	0.0754	0.0089
Brassica spp. vs. *Raphanus sativus*	0.0471	0.8271	0.0860	0.9383
Winter rapeseed vs. 'Pacific Gold' mustard	0.9304	0.3533	0.5462	0.4177

[s] Values in repetitions 1 and 3 are the mean of four replicates.
[t] Each plot was planted in 60 pepper seedlings. Plots measured 9.1 m long with peppers planted in double rows.
[u] Values in repetition 2 are the mean of three replicates.
[v] Each plot was planted in 50 pepper seedlings. Plots measured 9.1 m long with peppers planted in double rows.
[w] There was not a significant year*treatment interaction so data were combined ($P<0.05$).
[x] Within experimental repetitions, means within a column followed by the same letter are not significantly different, LSD ($P<0.05$)
[y] Not done.
[z] Not applicable.

Table 3.14. 2011 Spring and fall pepper yields separated by healthy fruit and fruit with blossom end rot.

Treatment	Repetition 2 - 2011 Spring[w x]				Repetition 3 - 2011 Fall[y]			
	Healthy weight (kg)	**Blossom end rot weight (kg)**	**Healthy yield (% of total yield)**	**Blossom end rot yield (% of total yield)**	**Healthy weight (kg)**	**Blossom end rot weight (kg)**	**Healthy yield (% of total yield)**	**Blossom end rot yield (% of total yield)**
'Pacific Gold' mustard	30.79 a[z] b	12.97 a	75.50 a	24.50 a	29.24 a b	2.55 a	92.13 a	7.88 a
Oilseed radish	29.62 a b	14.15 a	67.70 a	32.30 a	38.77 a	2.61 a	93.65 a	6.35 a
Winter rapeseed	31.71 a	11.63 a	73.30 a	26.70 a	32.75 a b	2.13 a b	93.98 a	6.03 a
Fallow (+) VIF	27.32 b	10.39 a	72.80 a	27.20 a	26.68 b	1.45 b c	95.38 a	4.63 a
Fallow (-) VIF	30.69 a b	5.21 b	85.53 b	14.47 b	12.46 c	0.89 c	93.23 a	6.78 a
Contrasts	***P*-value**	***P*-value**			***P*-value**	***P*-value**		
(+) VIF vs. (-) VIF	0.5870	0.0008			0.0002	0.0070		
Brassica vs. Fallow	0.0558	0.1119			0.0997	0.0351		
(+)VIF *Brassica* spp. vs. *Raphanus sativus*	0.3401	0.2502			0.0828	0.5534		

Winter rapeseed vs. 'Pacific Gold' mustard	0.6358	0.4607		0.4736	0.4131

[w]All repetition 2 yield values exclude the first of 6 harvests because yield was not separated into two categories during the first harvest.
[x] Values in repetition 2 are the mean of three replicates.
[y] Values in repetition 3 are the mean of four replicates.
[z] Within experimental repetitions, means within a column followed by the same letter are not significantly different, LSD ($P<0.05$).

Table 3.15. 2010 Fall spinach emergence.[w]

Ridomil treatment[x] / Brassica treatment[y]	Spinach emergence (%)
(+) Ridomil	76.2 a[z]
'Pacific Gold' mustard	53.8 a b
Oilseed radish	74.8 b c
Winter rapeseed	84.3 c d
Fallow (+) VIF	82.3 c d
Fallow (-) VIF	86.0 c d
(-) Ridomil	70.9 a
'Pacific Gold' mustard	75.0 b c
Oilseed radish	72.3 b c
Winter rapeseed	62.8 a b
Fallow (+) VIF	74.3 b c
Fallow (-) VIF	70.3 b c

[w] All values are the mean of four replicates.
[x] Sub-plot factor.
[y] Whole-plot factor.
[z] Means within a column followed by the same letter are not significantly different, LSD (P<0.05).

Table 3.16. 2010 Fall spinach emergence with Ridomil treatment data combined.[x y]

Treatment	Spinach emergence (%)
'Pacific Gold' mustard	64.4 a[z]
Oilseed radish	73.5 a
Winter rapeseed	73.5 a
Fallow (+) VIF	78.3 a
Fallow (-) VIF	78.1 a

[x] All values are the mean of four replicates.
[y] Ridomil data were combined because the Ridomil treatment did not significantly affect emergence overall and there was not a significant Ridomil*brassica interaction, LSD (P<0.05).
[z] Means followed by the same letter are not significantly different, LSD (P<0.05).

Table 3.17. 2010 Fall spinach stunting.[w]

Ridomil treatment[x] / Brassica treatment[y]	Stunted plants (%)
(+) Ridomil	68.8 a[z]
'Pacific Gold' mustard	48.7 a b
Oilseed radish	65.3 a b c
Winter rapeseed	60.5 a b
Fallow (+) VIF	73.6 b c d
Fallow (-) VIF	95.8 c d
(-) Ridomil	63.7 a
'Pacific Gold' mustard	56.9 a b
Oilseed radish	38.3 a
Winter rapeseed	67.4 a b c
Fallow (+) VIF	56.0 a b
Fallow (-) VIF	100.0 d

[w] All values are the mean of four replicates.
[x] Sub-plot factor.
[y] Whole-plot factor.
[z] Means within a column followed by the same letter are not significantly different, LSD (P<0.05).

Table 3.18. 2010 Fall spinach stunting with Ridomil treatment data combined.[x y]

Treatment	Stunted plants (%)
'Pacific Gold' mustard	52.8 a[z]
Oilseed radish	51.8 a
Winter rapeseed	63.9 a
Fallow (+) VIF	64.8 a
Fallow (-) VIF	97.9 b

[x] All values are the mean of four replicates.
[y] Ridomil data were combined because the Ridomil treatment did not significantly affect stunting overall and there was not a significant Ridomil*brassica interaction, LSD (P<0.05).
[z] Means followed by the same letter are not significantly different, LSD (P<0.05).

Table 3.19. 2010 Fall spinach deaths 29 days after seeding.[w]

Ridomil treatment[x] **Brassica treatment[y]**	**Total plant death (%)**
(+) Ridomil	28.9 a[z]
'Pacific Gold' mustard	44.4 b
Oilseed radish	33.3 a b
Winter rapeseed	18.5 a
Fallow (+) VIF	23.1 a b
Fallow (-) VIF	25.0 a b
(-) Ridomil	35.3 a
'Pacific Gold' mustard	33.3 a b
Oilseed radish	29.6 a b
Winter rapeseed	45.4 b
Fallow (+) VIF	35.2 a b
Fallow (-) VIF	33.3 a b

[w] All values are the mean of four replicates.
[x] Sub-plot factor.
[y] Whole-plot factor.
[z] Means within a column followed by the same letter are not significantly different, LSD (P<0.05).

Table 3.20. 2010 Fall spinach deaths 29 days after seeding with Ridomil treatment data combined.[x y]

Treatment	**Total plant death (%)**
'Pacific Gold' mustard	38.9 a[z]
Oilseed radish	31.5 a
Winter rapeseed	31.9 a
Fallow (+) VIF	29.1 a
Fallow (-) VIF	29.1 a

[x] All values are the mean of four replicates.
[y] Ridomil data were combined because the Ridomil treatment did not significantly affect stunting overall and there was not a significant Ridomil*brassica interaction, LSD (P<0.05).
[z] Means followed by the same letter are not significantly different, LSD (P<0.05).

Table 3.21. Description of *Pythium* spp. isolates used in the greenhouse *Pythium* pathogenicity experiment.

Isolate number	**Relative growth rate on P5ARP**	**Relative abundance in field-soil samples**
1	slow	high
2	slow	high
3 (*P. aphanidermatum*)	moderate	low
4	fast	high
5	fast	high

Table 3.22. Pathogenicity of five isolates of *Pythium* spp. collected from field-soil samples in 2011 on 'Hunter' peppers and their effect on pepper shoot and root fresh weight.[w]

***Pythium* spp. isolate number**	**Pepper shoot weight (g)**	**Pepper root weight (g)**	**Root disease rating[x]**
	Repetition 1		
1	25.1 a[y]	51.8 a	0.0 c
2	24.2 a	50.9 a	0.0 c
3 (*P. aphanidermatum*)	4.8 d	5.7 e	3.8 a
4	23.7 a	47.0 a b	0.0 c
5	16.9 b	40.4 b c	0.0 c
Mix (all five isolates combined)	13.0 c	19.9 d	2.0 b
Control (non-infested)	15.4 b c	35.8 c	0.0 c
	Repetition 2		
1	11.0 a b	13.4 a b	1.3 a b c
2	15.9 a	16.5 a	0.5 c
3 (*P. aphanidermatum*)	9.0 b	8.6 c	2.3 a
4	13.7 a b	16.3 a	1.2 a b c
5	13.6 a b	12.8 a b c	1.3 a b c
Mix (all five isolates combined)	10.8 a b	10.1 b c	2.2 a b
Control (non-infested)	11.0 a b	13.0 a b c	1.0 b c

[w] All values are the mean of six replicates.
[x]Root disease ratings based on the following scale: 0 = no root symptoms; 1 = 1 to 25% of roots are necrotic; 2 = 25 to 50% of roots are necrotic; 3 = 50 to 100% of roots are necrotic with localized stem lesions; 4 = extensive root rot with few white roots and extensive crown rot; 5 = root system completely necrotic and plant is dead or moribund.
[y]Means in a column within a repetition followed by the same letter are not significantly different, LSD ($P<0.05$).

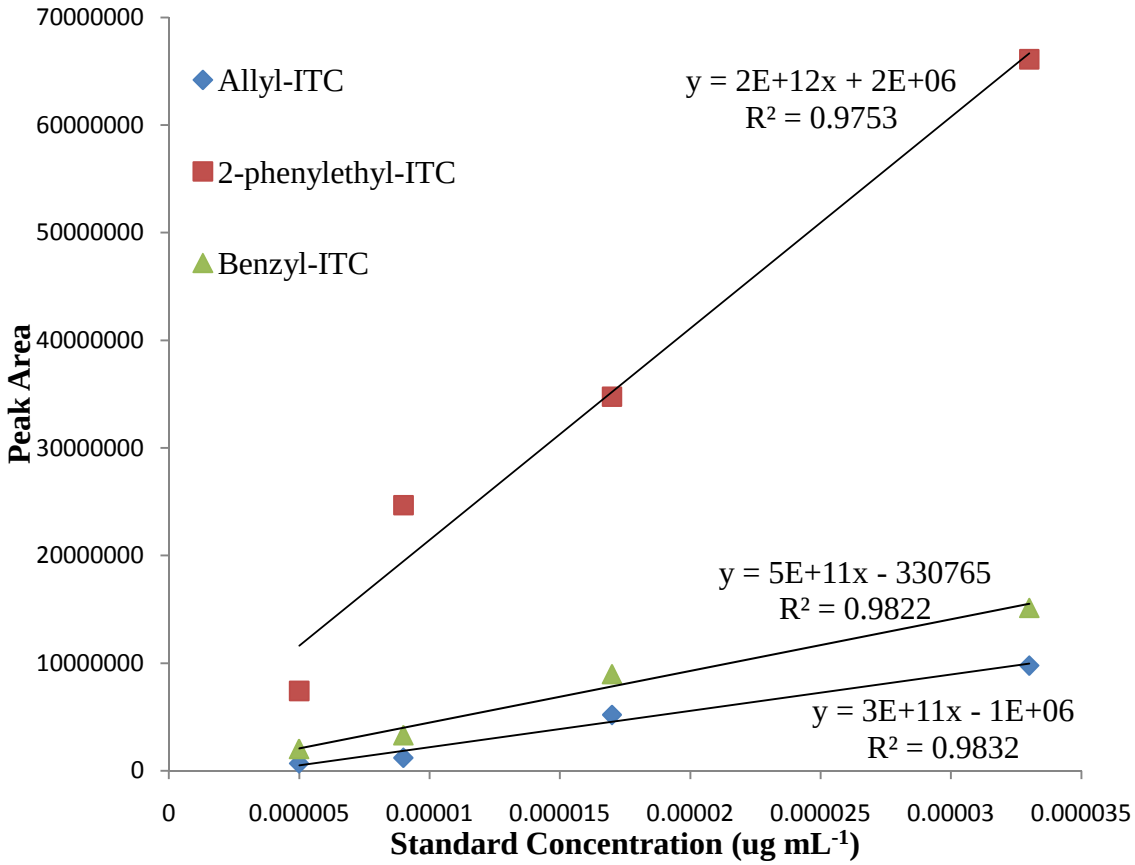

Figure 3.1. Standard curves used to quantify ITC concentrations in field soil samples following incorporation of brassica cover crops.

CHAPTER FOUR

CONCLUSION

Greenhouse Study

The purpose of the greenhouse study was to identify causes of variability in the results of field biofumigation studies (Collins et al. 2006, Larkin et al. 2006, Njoroge et al. 2008, Stapleton and Duncan 1998). It was hypothesized that naturally high levels of soil myrosinase would correlate positively with ITC concentrations following mustard incorporation into soil. ITC release efficiency is known to be low following brassica tissue disruption, and maximizing ITC release could enhance the effect of biofumigation (Morra et al. 2002). It was also hypothesized that *Bacillus* spp. populations would correlate negatively with ITC concentrations following mustard incorporation into soil because *Bacillus* spp. are known to be capable of degrading ITCs (Warton et al. 2001). Significant correlations between soil myrosinase, soil *Bacillus* spp. populations and ITCs would have implicated the value of using myrosinase and *Bacillus* spp. measurements as screening tools for a given soil's receptiveness to biofumigation. This idea was based on the premise that, following mustard soil incorporation, soils with relatively high ITC concentrations would have lower pathogen levels and greater overall bean health than those with relatively low ITC concentrations.

Soil myrosinase and *Bacillus* spp. populations were not significantly correlated with ITC concentrations and are probably not useful screening tools for biofumigation site selection. The soil myrosinase assay used in this study was developed to quantify myrosinase activity in rhizosphere soil (Al-Turki and Dick 2003). The myrosinase

activity detected in my bulk field-soil samples was extremely low, and probably below the assay's limit of detection in at least some samples. The soil used for this assay should be restricted to rhizosphere soil in future work. Furthermore, since ITC concentrations were not significantly correlated with soilborne pathogen populations or overall bean health, there is probably more involved in the inconsistency of biofumigation results than ITC release efficiency and degradation alone. Future research should focus on the variables that separate field biofumigation experiments which yielded positive results, like the Larkin et al. (2006) experiment, from those which yielded negative results, like the Njoroge et al. (2008) experiment. Geographic location and environment are two such variables that may significantly affect the outcome of biofumigation studies. Additionally, research should be conducted to determine the cause of variability in ITC concentrations following mustard incorporation into diverse soil samples, such as those used in this study. Results from such a study could then be applied to field work to determine if selected soil variables have a measurable effect on the efficacy of biofumigation.

Field Study

The field experiment was designed to determine if biofumigation could control soilborne pathogens and the diseases they cause in peppers. This was done by implementing the best practices available in the current literature known to maximize the beneficial effect of biofumigation. Biofumigation with 'Pacific Gold' mustard, winter rapeseed, and oilseed radish was not effective at reducing populations of soilborne

pathogens or the diseases they cause in peppers. The application of VIF decreased populations of *R. solani*, incidence of pepper stunting, and increased pepper yield compared to a bare soil treatment. There was a trend of higher yield in the biofumigant treatments, although it was not apparently related to disease reductions induced by the introduction of ITCs into the soil. This result is probably related to the enhanced anaerobic soil environment created following the incorporation of organic matter into the soil compared to the fallow (+) VIF treatment (Blok et al. 2000). The overall benefits associated with cover cropping, such as improved nutrient and moisture availability, and changes to the soil microbial community may have also contributed to the higher yields observed in those treatments (Clark, 2007). Future research in this area should focus on identifying how plastic mulch affects the soil aerobic environment and the specific causes of plant-health promotion following organic matter incorporation into soil and plastic mulch application. Such research could lead to a targeted increase of the beneficial effects and another tool to manage soilborne pathogens for growers interested in sustainable agriculture.

Biofumigation is a highly complex system with many variables that must be considered prior to its implementation. In the Southeastern United States brassicas should be seeded in the fall to maximize biomass and ITC release into soil following incorporation. Future research should focus on the development of high GSL-producing brassica cultivars, like ‘Ida Gold’ and ‘Pacific Gold’ mustard, which grow especially well in the southeastern United States. Additionally, a screening of existing brassica varieties here at the Clemson Coastal Research and Education Center for relative biomass and ITC production following soil incorporation could help to identify which are best suited for

biofumigation in the southeastern United States. Such a screening could also be used to identify species and cultivars which demonstrate resistance to *R. solani* and especially *Pythium* spp., so disease problems are not exacerbated by growing a host crop. It is unclear whether oilseed radish, winter rapeseed, and 'Pacific Gold' mustard were hosts of *Pythium* spp. or if the *Pythium* only proliferated saprophytically following incorporation. This question could have important implications for controlling *Pythium* with biofumigation and warrants further investigation.

During the field experiment *P. aphanidermatum* was identified in field-soil samples and demonstrated to be pathogenic on pepper in the greenhouse. Four other isolates, which comprised the majority of *Pythium* spp. isolated from field-soil samples, were found to be non-pathogenic or growth-promoting on pepper. Non-pathogenic *Pythium* spp. may increase overall plant health by competing with or parasitizing pathogenic *Pythium* spp. (Al-Rawahi and Hancock 1998, Takenaka et al. 2007). Further research investigating the role played by non-pathogenic *Pythium* spp. could lead to the development of biological control agents. This topic, as it relates to biofumigation, may be particularly relevant. It is hypothesized that brassicas produce glucosinolates and myrosinase, and subsequent ITCs, as a natural defense against pathogens and pests. If certain brassica varieties are hosts of pathogenic *Pythium* spp., then those pathogens probably have some tolerance of the ITCs produced by that plant. The application of broad-spectrum ITCs could reduce populations of beneficial *Pythium* while leaving populations of ITC-tolerant *Pythium* intact. The host-pathogen relationship between *Pythium* spp. and brassicas showing high biofumigation potential needs to be investigated

in order to understand how biofumigation affects populations of both pathogenic and non-pathogenic *Pythium* spp.

Finally, the vast quantity of literature available on biofumigation must be carefully condensed into practical guidelines for use by growers or extension agents. A thorough review of the literature exists (Matthiessen and Kirkegaard, 2006), although it is unlikely to be widely adopted as a field-guide to biofumigation due to its length and esoteric language. This highlights the need for a concise set of guidelines which growers can follow to maximize the likelihood of successful biofumigation.

Literature Cited

Al-Rawahi, A.K., and Hancock, J.G. 1998. Parasitism and biological control of *Verticillium dahliae* by *Pythium oligandrum*. Plant Disease 82: 1100-1106.

Al-Turki, A.I., & Dick, W.A. 2003. Myrosinase activity in soil. Soil Science Society of America Journal 67: 139-145.

Blok, W. J., Lamers, J. G., Termorshuizen, A. J., & Bollen, G. J. 2000. Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. Phytopathology 90:253-259.

Clark, A. 2007. Managing cover crops profitably. Third edition. Sustainable Agriculture Network, Beltsville, MD.

Collins, H.P., Alva, A., Boydston, R.A., Cochran, R.L., Hamm, P.B., McGuire, A., & Riga, E. 2006. Soil microbial, fungal, and nematode responses to soil fumigation and cover crops under potato production. Biology and Fertility of Soils 42: 247–257.

Larkin, R.P., & Griffin, T.S. 2006. Control of soilborne potato diseases using brassica green manures. Crop Protection 26: 1067-107.

Matthiessen, J.N., & Kirkegaard, J.A. 2006. Biofumigation and enhanced biodegradation: opportunity and challenge in soilborne pest and disease management. Critical Reviews in Plant Sciences 25: 235-265.

Morra, M.J., & J.A. Kirkegaard. 2002. Isothiocyanate release from soil-incorporated brassica tissues. Soil Biology and Biochemistry 34: 1683-1690.

Njoroge, S. M. C., Riley, M. B., & Keinath, A. P. 2008. Effect of incorporation of *Brassica* spp. residues on population densities of soilborne microorganisms and on damping-off and Fusarium wilt of watermelon. Plant Disease 92: 287-294.

Stapleton, J.J., & Duncan, R.A. 1998. Soil disinfestation with cruciferous amendments and sublethal heating: effects on *Meloidogyne incognita*, *Sclerotium rolfsii* and *Pythium ultimum*. Plant Pathology 47: 737-742.

Takenaka, S., Sekiguchi, H., Nakaho, K., Tojo, M., Masunaka, A., and Takahashi, H. 2008. Colonization of *Pythium oligandrum* in the tomato rhizosphere for biological control of bacterial wilt disease analyzed by real-time PCR and confocal laser-scanning microscopy. Phytopathology 98:187-195.

Warton, B. Matthiessen, J.N., & Roper, M.M. 2001. The soil organisms responsible for the enhanced biodegradation of metham sodium. Biology and Fertility of Soils 34:264–26.