EFFECTS OF OILSEED MEALS AND ISOTHIOCYANATES (ITCS) ON *PHYMATOTRICHOPSIS OMNIVORA* (COTTON ROOT ROT) AND SOIL MICROBIAL COMMUNITIES

A Dissertation

by

PING HU

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Soil Science

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ABSTRACT

Effects of Oilseed Meals and Isothiocyanates (ITCS) on *Phymatotrichopsis omnivora* (Cotton Root Rot) and Soil Microbial Communities. (May 2012)

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The meals from many oilseed crops contain biocidal chemicals that are known to inhibit the growth and activity of several soil pathogens, though little is known concerning impacts on whole soil microbial communities. We investigated the effect of oilseed meals (SMs) from both brassicaceous plants, including mustard and camelina, as well as non-brassicaceous plants, including jatropha and flax, on *P. omnivora* (the casual agent of cotton root rot) in Branyon clay soil (at 1 and 5% application rates). We also investigated the effect of SMs from camelina, jatropha, flax, and wheat straw on microbial communities in Weswood loam soil. We also used four types of isothiocyanates (ITCs) including allyl, butyl, phenyl, and benzyl ITC to test their effects on *P. omnivora* growth on potato dextrose agar (PDA), as well as on soil microbial communities in a microcosm study. Community qPCR assays were used to evaluate relative abundances of soil microbial populations. Soil microbial community composition was determined through tag-pyrosequencing using 454 GS FLX titanium technology, targeting ITS and 16S rRNA gene regions for fungal and bacterial

communities, respectively.

The results showed that all tested brassicaceous and jatropha SMs were able to inhibit *P. omnivora* sclerotial germination and hyphal growth, with mustard SM being the most effective. Flax didn't show any inhibitory effects on sclerotial germination. All tested ITCs inhibited *P. omnivora* OKAlf8 hyphal growth, and the level of inhibition varied with concentration and ITC type. Total soil fungal populations were reduced by ITC addition, and microbial community compositions were changed following SM and ITC application. These changes varied according to the type of SM or ITC added. Our results indicated that SMs of several brassicaceous species as well as jatropha may have potential for reducing cotton root rot as well as some other pathogens. Different SMs releasing varied ITCs may result in differential impacts on soil microorganisms including some pathogens.

.

DEDICATION

To my parents

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NOMENCLATURE

ITC	Isothiocyanate
GLS	Glucosinolates
NMDS	Non-metric multidimensional scaling
PCA	Principal component analysis
SM	Oilseed meal
CLPP	Community level physiological profiling

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CHAPTER I

INTRODUCTION AND

LITERATURE REVIEW

1.1. Introduction

Interest in bioenergy has recently intensified due to increasing fuel prices and concerns regarding the worldwide supply of fossil fuels. Another potential benefit of bioenergy is a possible reduction in greenhouse gas emissions relative to traditional fuels. The Energy Independence and Security Act (December 2007) required changes to the previous Renewable Fuel Standard (RFS) Program, and resulted in the new RFS2 Program (effective in July 2010) that mandated significantly increased volumes of renewable fuel to 36 billion gallons by 2022.

Using oilseed crops to extract oils and subsequently transform them into bioethanol or biodiesel is one of several major means of bioenergy production. The processing of higher amounts of oilseed to meets these demands will result in large quantities of by-products known as oilseed meals (SMs) - the residual remaining after the oil extraction process. Many of these SMs including those from crops such as corn (*Zea mays* L.), cottonseed (*Gossypium* spp.), canola (*Brassica* spp.), olive (*Olea*

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europaea L.), peanut (*Arachis hypogaea* L.), safflower (*Carthamus tinctorius* L.), sesame (*Sesamum indicum* L.), soybean (*Glycine max* L.), and sunflower (*Helianthus annuus* L.) have markets as food or animal feed due to their high energy and nutrient content. However, SMs from some type of plants are not suitable for food or animal feed due to the presence of toxic compounds such as those produced by some members of the *Brassicaceae* family (Mithen et al., 2000), *Jatropha curcas* (Makkar et al., 1997), castor bean (*Ricinus communis*) (Nicolson et al., 1974), Indian beech (*Pongamia pinnata*) (Bhatia et al., 2008), and neem (*Azadirachta indica*) (Paul et al., 1996). Thus, land application as an organic fertilizer may be the most promising option for use of these SMs. Additionally, numerous studies have demonstrated that some of these oilseed plants, such as many *Brassica* spp., can inhibit numerous different plant pathogens due to the release of biocidal compounds such as ITCs (Angus et al., 1994; Sarwar et al., 1998). However, relatively little is known regarding the potential impacts of these SMs on major microbial processes in soil (e.g., C and N cycling) and non-target soil microbial populations.

1.2. Background

1.2.1. Biofuel oilseed crops

Members of the *Brassicaceae* family such as canola and rapeseed, are typically grown for their seeds, which are harvested for oil production. Mustard has also been grown for spicy condiment production. The flour of *Sinapis alba* (yellow or white

mustard) is used for mustard paste with mild hotness, while *Brassica juncea* (brown or oriental mustard) is used to produce a sharp hot flavor paste (Yu et al., 2007). From the 1980's, *Brassica* species have also been used increasingly as cover crops in temperate regions of North America due to the presence of biocidal compounds produced by these plants leading to their utility in biofumigation (Haramoto and Gallandt, 2004; Yu et al., 2007).

Jatropha curcas tree is a member of the *Euphorbiaceae* family which grows in most of the tropics including Africa, Central and South America, India and South East Asia (Cano-Asseleih et al., 1989). It is resistant to drought and disease and can survive in very poor soil conditions, and is thus suitable to grow on marginal lands. It is also well-known for uses in medicines and as a green manure (Jones and Miller, 1991) due to its medicinal and nutritional contents of plant tissues (Kumar and Sharma, 2008). Its oil has been used for centuries in lamps in homes and for producing soap, candles, and varnish. *Jatropha curcas* has a high seed production (up to 5 tons/ hectare) and high oil content in the seeds (more than 60% in the kernel) with a high proportion of unsaturated fatty acids (78.6%), which has made *J. curcas* a desirable feedstock for non-edible oil and biodiesel production (Kumar and Sharma, 2008; Akbar et al., 2009). *Jatropha curcas* SM contains a high concentration of proteins and lipids but its utility in food and feed is limited by the presence of toxic chemicals such as phorbol esters, trypsin inhibitor, lectin and phytate (Makkar et al., 2008).

Castor bean (*Ricinus communis*) is another crop in the *Euphorbiaceae* family that is very important in producing non-edible oil and biodiesel. India is currently the leading

producer of castor and a major exporter to the US (Sujatha et al., 2008). The world demand for castor oil has been increasing steadily due to its multiple uses in industries, such as the production of paint and varnish, nylon fiber production, jet engine lubricants, hydraulic fluids, plastics, manufacture of fiber optics, and as antifreeze for fuels and lubricants in aircraft and spacecraft (Scarpa and Guerci, 1982; Ogunniyi, 2006; Sujatha et al., 2008). Moreover, castor is also important as a source of vegetable and medicinal oil and has numerous benefits to human. Castor also contains toxic compounds such as lectins (ricin, *Ricinus communis* agglutinin) and alkaloid ricinine (Rich et al., 1989), which have been found able to reduce plant parasitic nematodes (Marban-Mendoza et al., 1987; Mashela and Nthangeni, 2002).

Indian beech (*Pongamia pinnata*) is a member of the subfamily *Papilionoideae*, a fast-growing leguminous tree that originated from India and south-east Asia and has been introduced to the world including the USA (Scott et al., 2008). It is cultivated as an ornamental plant in gardens in India and Polynesia and can be grown on marginal lands (Bhatia et al., 2008). The plant tissues have been used in medicine for treating bronchitis, whooping cough, rheumatic joint scabies, and herpes (Kirtikar and Basu 1995). The seed contains pongam oil, and its fruits and sprouts are used to treat abdominal tumors in India (Buccolo and David, 1973). Recently, *Pongamia pinnata* has been recognized as an important resource for the biofuel industry due to its high oil seed production (Scott et al., 2008). The de-oiled SM contains up to 30% protein that has the potential for animal feed, although the effects of potentially toxic compounds in the meal have not been fully elucidated (Scott et al., 2008).

1.2.2. SMs as organic fertilizers

Oilseed meals, especially those not suitable for animal feed due to the presence of inherent toxic compounds, have been used as organic fertilizers for plants. Land application becomes an even more important if not the only method to reuse the nutrients in these types of SMs, may also increase levels of soil carbon (C), and contribute positively to the net C effect of biofuels. Oilseed meals contain substantial amounts of C, N, P and varying levels of other nutrients needed for plant growth (Wang et al., 2012). A significant forms of C in SMs are protein and fatty acids, thus these materials are often labile and easily decomposable within days of incubation (Berglund, 2002; Wang et al., 2012). Some have reported a priming effect after several SM application to soil (Snyder et al., 2010), while others have reported that nearly 50% of C in SM was respired into the atmosphere within 51 days (Wang et al., 2012). Both these possibilities should be considered in C sequestration calculations. Besides C, SMs also contain high level of organic N, which give them great potential as a slow-release N fertilizer when applied to soil (Moore et al., 2010; Wang et al., 2012).

In addition to supporting plant growth, previous studies have also reported a successful role of SMs in pathogen and weed control as a result of systematic response, which will be discussed in detail in the following sections. Nevertheless, relatively limited research has been focused on dedicated bioenergy oilseed crops such as jatropha (Mazzola et al., 2007; Moore et al., 2010; Snyder et al., 2010; Wang et al., 2012), or on the effects of bioenergy oilseed crops on soil ecology.

1.2.3. Chemicals in various SMs

1.2.3.1. Brassicaceae

It is known that *Brassicaceae* and their SMs can release biocidal compounds as glucosinolates (GLS) and their hydrolysis products to soil (Mazzola et al., 2001; Cohen et al., 2007). GLS are organic chemicals with a β-D-thioglucose moiety, a sulfonated oxime, and aliphatic or aromatic R groups (Borek and Morra, 2005). Although GLS stored in the cell vacuole possess limited biological activity, enzymatic degradation by thioglucoside glucohydrolase or myrosinase in the cell wall or cytoplasm (Poulton and Moller, 1993) can result in the formation of a number of toxic hydrolysis products, including ITCs, nitriles, organic thiocyanates, SCN^-, oxazolidinethione, and epthionitriles (Cole, 1976; Borek and Morra, 2005). The ITCs are commonly among the major products from these plants. Higher concentrations of ITCs are typically produced from the seed than other parts of the plant (Woods et al., 1991). For example, Tsao et al. (2000) reported that mustard seeds released 1% allyl ITC compared with 0.5% from mustard bran or husk.

Because of the biological toxicity of ITCs, they have been used as insecticides, fungicides, and nematicides. There have also been studies showing their inhibiting effects on soil nitrifying bacteria (Bending and Lincoln, 2000).

1.2.3.2. Jatropha curcas

The toxic chemicals contained in *J. curcas* have been examined in numerous studies. The lectin (curcin) from *J. curcas* seeds has been reported to be toxic to organisms, but the toxicity level was much less than other lectins and ricin (Oslnes and Phil, 1973). Trypsin inhibitor activity and phytate levels in *J. curcas* meals have been reported to be very high, possibly resulting in decreased mineral bioavailability (especially Ca^{2+} and Fe^{2+}) (Makkar et al., 1997) and decreased protein digestibility through interaction with enzymes such as trypsin and pepsin (Reddy and Pierson, 1994). Phorbol esters from *J. curcas* have also been found to be toxic to some fungal pathogens. They have also been found to be a skin-irritant, have purgative effects, and promote tumors (Hirota et al., 1988; Makkar et al., 1997; Saetae and Suntornsuk; 2010). Among all of the tested toxic compounds derived from *J. curcas*, the phorbol esters have been considered to be the primary contributor to toxicity, while lectins and trypsin inhibitors could be more easily mitigated by heat due to their being more labile (Makkar et al., 1997).

1.2.3.3. Other oilseed plants

Numerous other SMs are known to produce biocidal compounds. Perhaps one of the best known is the castor bean (*Ricinus communis*). Castor contains toxic and/or medicinal compounds such as lectins (ricin, *Ricinus communis* agglutinin), alkaloid ricinine, and an allergenic protein polysaccharide CB-1A (Rich et al., 1989; Audi et al., 2005). Marban-Mendoza et al. (1987) reported that application of the lectin limax flavus

agglutinin from castor significantly reduced tomato root knot caused by nematodes. The toxicity and medicinal applications of ricin and *Ricinus communis* agglutinin have been examined (Hegde and Podder, 1998) and may act as a principal compound in castor SM that adversely affect plant parasitic nematodes (Marban-Mendoza et al., 1987; Rich et al., 1989; Mashela and Nthangeni, 2002).

Various parts of *Pongamia pinnata* have been reported to be of medicinal value (Buccolo and David, 1973; Kirtikar and Basu, 1995; Bhatia et al., 2008), but there has been no detailed characterization of the active constituents in these extracts. The reported chemical composition of *P. pinnata* includes the alkaloids demethoxy-kanugin, gamatay, glabrin, glabrosaponin, kaempferol, kanjone, kanugin, karangin, neoglabrin, pinnatin, pongamol, pongapin, quercitin, saponin, β-sitosterol, and tannin (Chander et al. 1992, 2003). Some of these chemicals have been demonstrated to control a wide range of animal and human pathogens (Alam et al., 2004; Simin et al., 2002).

1.2.4. Environmental fate and persistence of allelochemicals from SMs

Glucosinolates themselves do not have obvious biocidal effects unless hydrolyzed to ITCs etc. by the enzyme myrosinase. Oilseed meal of rapeseed containing GLS in the presence of myrosinase were found suppressive to *Aphanomyces* root rot of pea, while no obvious disease reduction was observed by this SM after the myrosinase was inactivated (Smolinska et al., 1997).

Individual SMs in combination with environmental conditions such as soil type and depth, pH, and microbial activities determines which reaction products exist and

how quickly they will degrade within the soil. Generally speaking, the time for complete degradation of oilseed-derived allelochemicals is rapid, ranging from minutes to weeks depending on individual chemical characteristics and environmental conditions. In a study conducted by Hansson et al. (2008), the mobility of ionic thiocyanate (SCN^-) was evaluated and was found to occur predominately between 0 and 10 cm of depth and to be nearly completely degraded within 44 d of application. Gimsing et al. (2009) evaluated the sorption and degradation of 2-propenyl isothiocyanate (PrITC) and benzyl isothiocyanate (BeITC), in which the half-lives of the two ITCs were determined to range from about 1 to 4 h depending on soil type and depth. In another study on PrITC and BeITC soil degradation by Warton et al. (2003), the half-lives of the chemicals were measured to be 5 h for PrITC and 10 h for BeITC in a MITC-degrading soil (a soil that has been found to quickly degrade MITC), compared with 1.5 d for PrITC and 6.5 d for BeITC in a non-degrading soil (a soil that did not rapidly degrade MITC). Borek and Morra (2005) conducted a pH stability study by incubating SM extract containing 4-hydroxybenzyl isothiocyanate dissolved in buffers ranging from pH 3.0 to 6.5. The shortest half-life (6 min) of 4-hydroxybenzyl isothiocyanate occurred at the highest pH of 6.5, and the longest half-life (321 min) occurred when the pH was decreased to 3.0.

The importance of microbial activities in allelochemical degradation has been indirectly confirmed by Warton et al. (2003), who investigated to investigate the rate of degradation of PrITC and BeITC in sterilized and non-sterilized soil. The half-lives of the chemicals were measured to be 5 h for PrITC and 10 h for BeITC in the non-sterilized soil compared with 3.1 d for PrITC and 9 d for BeITC in the sterilized soil.

Similar results were observed by Gimsing et al. (2008) who found that ITC concentration in sterilized soil was not degraded in comparison with fast first-order degradation in non-sterilized soil.

1.3. Effects of SMs on soil microorganisms

1.3.1. Soil fungal pathogens

Numerous studies have demonstrated the potential of various *Brassicaceae* SMs on soil borne plant pests. Murray and Brennan (1998) reported suppression of take-all (a serious soilborne wheat disease) by *Brassica spp*. Other studies on SMs derived from *Brassica juncea*, *B. napus*, or *Sinapis alba* (Cohen et al., 2005; Mazzola et al., 2007) demonstrated that the SMs could suppress root infection by soilborne pathogens including *Rhizoctonia* spp.

Despite their reported fungicidal, nematicidal, and insecticidal effects, to-date there has been no report of *Jatropha curcas*, castor, or *Pongamia pinnata* SMs being used to supress fungal pathogens *in-situ*. Extracts from *J. curcas* SM has been recently found to be toxic to several fungal pathogens including *Fusarium* and *Colletotrichum* spp. (Saetae and Suntornsuk, 2010). Moreover, *J. curcas* was found to reduce viral and fungal infection in transgenic tobacco due to an induced ribosome-inactivating protein (curcin 2) (Huang et al., 2008). Castor (*Ricinus communis*) SM has been reported to suppress plant parasitic nematodes, indicating that it may have possible application for the control of other organisms as well. Moreover, *Pongamia pinnata* SM amendment to

soil has been found to significantly reduce root rot disease incidence of sage caused by the plant pathogenic fungi *Fusarium solani* and *Rhizoctonia solani* (Mallesh et al., 2008).

The mechanism involved in fungal inhibition has been partially explained by the effect of allelochemicals induced from the SMs or tissues after soil amendments. Previous studies used either compounds volatilized from SMs or pure chemicals such as ITCs and phorbol esters that were believed major toxic products from SMs to investigate their influence on various plant pathogenic fungi. Smolinska et al. (1997) found that volatile compounds (enzymatic hydrolysis products of GLS) from *B. napus* SM inhibited *Aphanomyces euteiches* mycelia growth and germination of encysted zoospores and reduced root rot of pea. Kirkegaard et al. (1996) tested volatile compounds produced from *B. napus* and *B. juncea* tissues on several plant pathogenic fungi and found that they effectively suppressed *G. graminis var. tritici* and *Rhizoctonia solani*, and the level of inhibition was different in various types of tissue with different ITC concentrations.

Other studies have tested the potential for a variety of ITCs as fungicides. For instance, Sarwar et al. (1998) examined the effect of 6 common ITCs on numerous plant pathogenic fungi including *Gaeumannomyces graminis* var. *tritici*, *R. solani*, *Fusarium graminearum*, *Bipolaris sorokiniana*, and *Pythium irregular*. A general suppression effect of these ITCs on tested fungi was discovered, although the relative sensitivity depended upon the fungal group and ITC types. Additionally, more recent studies have found similar results including Manici et al. (2000) who found thiofunctionalised GLS degradation products effectively inhibited *P. irregulare oospore* germination and *R. solani* soil colonization.

Allelochemicals from SMs other than the *Brassicaceae* family have not been reported to control plant pathogenic fungi, although there have been reports on using ricin to control nematodes (Rich et al., 1989). A summarized list of studies testing the effects of various allelochemicals on selected microorganisms is presented in Table 1.1, and a summarized table of studies on effects of various SMs on selected organisms is presented in Table 1.2.

1.3.2. Other soil microorganisms

1.3.2.1. Brassicaceae SMs effects on soil microbial communities

Relatively little literature exists on how soil microbial ecosystems respond to SM applications. Cohen et al. (2005) analyzed the effect of adding *Brassica napus* SM on soil microbial community structure (Table 1.2). They found an increase in total culturable bacteria, *Streptomyces* spp., and *Pythium* spp., with a decrease in fluorescent *Pseudomonas* spp. Since biological activity is vital for soil quality and is the driving force for most nutrient cycling, it is critically important to determine the effects of land application of various SMs on soil microbial ecosystems before this can be recommended as a management practice.

Table 1.1. Effects of selected oilseed-associated allelochemicals on soil organisms.

	Tested organisms	Allelochemicals	Major findings	Reference
Bacteria	NH_4^+-oxidizing and NO_2^--oxidizing bacteria	ITCs (Methyl, 2-Propenyl, Butyl, Butyl, Phenyl, Benzyl, Phenethyl), 3-Butene-nitrile, 3-Phenyl-propionitrile.	1) ITCs inhibited NH_4^+-oxidizing bacteria in soil; 2) ITCs inhibited NO_2^--oxidizing bacteria in clay-loam soil; 3) ITCs were more effective inhibitors of nitrification than intact GLS or nitriles; 4) Phenyl-ITC was found the most toxic of the ITCs tested.	Bending and Lincoln, 2000.
Fungi	*Gaeumannomyces graminis* var. *tritici*, *R.solani*, *Fusarium graminearum*, *Bipolaris sorokiniana*, *Pythium irregulare*	methyl-ITC, propenyl-ITC, butenyl-ITC, pentenyl-ITC, benzyl-ITC and 2-phenylethyl-ITC	Tested soil pathogens were generally suppressed by ITCs, though the sensitivity depended on fungal groups and ITC types.	Sarwar et al., 1998
	G. graminis var. *tritici*, *R. solani*, *F. graminearum*. *P. irregulare*,and *B. sorokiniana*.	Volatiles from *Brassica napus*, *Brassica juncea* tissues of different plant parts	1) *Gaeumannomyces* and *Rluzoctonia* were generally the most sensitive to the volatiles released, while *Pythium* and *Bipolaris* the least. 2) *Brassica* tissue age and type released different the type and concentration of ITCs, thus difference in effectiveness of fungal suppression.	Kirkegaard et al., 1996
	Aphanomyces euteiches f. sp. *pisi* (root rot of pea)	Volatile compounds produced from rapeseed (*B. napus*) meal	1) Volatile compounds produced from rapeseed suppressed the fungal growth and germination, and reduced root rot of pea; 2) Autoclaved rapeseed meal produced mainly nitriles, while intact rapeseed meal produced mainly ITCs.	Smolinska et al., 1997
	Aspergillus niger, *Penicillium cyclopium*, *Rhizopus oryzae*, and 13 additional fungi	11 natural ITCs	A remarkable antifungal activity was observed in some analogues of benzyl and B-phenylethyl isothiocyanate.	Drobnica et al., 1967
	P. irregulare and *R. solani*	Thiofunctionalised, hydroxy-alkenyl, and alkenyl GLS degradation products	Thiofunctionalised GLS degradation products were most effective inhibiting of *P. irregulare* oospore germination and *R. solani* soil colonization.	Manici et al., 2000
	Wood-colonizing fungi	methyl isothiocyanate (MITC)	MITC (up to 0.018 μg kg^{-1}) was uniformly toxic to most of the tested fungi.	Canessa and Morell, 1995

Table 1.2. Effects of selected SMs on soil microorganisms.

Organisms		SMs	Major findings	References
Fungi	*Rhizoctonia* spp., *Pythium* spp., *Rhizoctonia solani* AG-5, *Cylindrocarpon* spp.	*B. juncea, B. napus, S. alba*	1) All SMs reduced infection by native *R.* spp. and introduced *R. solani* AG-5; 2) Only *B. juncea* suppressed *Pratylenchus* spp. while didn't stimulate *Pythium* spp.; 3) *B. juncea* and *B. napus* SM mixture can better control apple replant disease.	Mazzola et al., 2007; Cohen et al., 2005; Mazzola and Mullinix, 2005
	Fusarium solani, Rhizoctonia solani	*Pongamia pinnata*	*Pongamia pinnata* SM amendment to soil significantly reduced root rot disease incidence of sage caused by *Fusarium solani* and *Rhizoctonia solani.*	Karnataka, 2008
Chromalveolata	*Phytophthora* spp.	*B. juncea, B. napus, S. alba*	Seed meals used in this study didn't suppress *Phytophthora* spp.	Cohen et al., 2005; Mazzola and Mullinix, 2005;
	Aphanomyces euteiches f. sp. *pisi*	*B. napus*	1) *B. napus* SM severely inhibited infection by oospores and mycelial growth; 2) Autoclaved *B. napus* SM produced little disease reduction due to denatured myrosinase and low GLS.	Smolinska et al., 1997
Bacteria	*Streptomyces* spp., *Pseudomonas* spp.	*B. juncea, B. napus, S. alba*	*1) B. napus* SM altered communities of both pathogenic and saprophytic soil microorganisms, elevated *Streptomyces* spp., but suppressed fluorescent *Pseudomonas*. 2) Suppression of *Rhizoctonia* root rot by *B. napus* SM amendment requires resident soil microbiota (*Streptomyces* spp.) and generation of nitric oxide through nitrification.	Cohen and Mazzola, 2006

1.3.2.2. ITCs effects on soil microbial communities

Although studies on the impact of SM application on general soil microbial communities are few in number, more (although still limited) studies have investigated what, if any, effects various allelochemicals from SMs have on non-target soil microorganisms. Several studies have investigated the effects of pure ITCs on a wide range of soil fungi and bacteria (primarily plant pathogens), in which a general suppression effect was discovered, and the sensitivity varied among different microbial groups and ITC types (Kirkegaard et al., 1996; Smolinska et al., 1997; Manici et al., 2000; Bending and Lincoln, 2000; Smith and Kirkegaard, 2002; Hu et al., 2011). In the study of Haramoto and Gallandt (2004), daily applications of low concentrations of 2-phenylethyl ITC were found to change the active portion of the soil microbial community composed of bacteria and eukaryotes. The effects of fumigants like methyl ITC on microbial biomass may be short-term with biomass recovery after a few weeks or they may be long-term. In a 12-week study by Ibekwe et al. (2001), the structural diversity of the dominant microbial community in general decreased with increasing methyl ITC concentration, but the effect was different among various groups of microorganisms. The effect of methyl ITC on soil microbial biomass was found to be dramatic during the first week of application. After this period when methyl ITC has been decomposed (Ibekwe, 2004), microbial composition in methyl ITC treated soil were quite similar to an unamended control regardless of the doses of methyl ITC used. Another study found long-term (a full year) changes in soil microbial composition and

activities, as indicated by soil respiration, after repeated exposure to methyl ITC (Taylor et al., 1996).

Moreover, Rumberger and Marschner (2003) demonstrated that phenyl-ethyl-isothiocyanate (PEITC) released by microbial degradation of canola crop residue affected both bacterial and eukaryotic community structure as determined by PCR-DGGE. Results from Bending and Lincoln (2000) suggested that ITCs inhibited soil nitrifying bacteria communities through direct reduction of the size of nitrifying community bacteria and their activities, and the magnitude of the negative effects were related to various soil properties (Table 1.1). These results indicate that *Brassicaceae* SM could potentially affect important microbial processes, such as nitrification, nitrogen fixation, and mycorrhizal symbiosis.

1.3.2.3. Effects of other oilseed plants on selected organisms

Extracts from various parts of *J. curcas*, such as seeds and leaves, have been shown to have fungicidal, molluscicidal, and insecticidal properties (Nwosu and Okafor, 1995; Meshram et al., 1996; Liu et al., 1997; Rug and Ruppel, 2000; Saetae and Suntornsuk, 2010). Emeasor et al. (2005) reported insecticidal effects by *J. curcas* seed powder application to soil comparable to the synthetic insecticide pirimiphos-methyl. Castor plant tissues, fruit, and seed extracts also been found to have antimicrobial effects (Jombo and Enenebeaku, 2007). For example, Kabir et al. (2001) reported and tested acute toxicity of *P. pinnata* leaf extract on American cockroach (*Periplaneta*

Americana). However, there is little-to-no information on the effects of these other SMs and associated allelochemicals on soil microorganisms.

1.3.2.4. Summary

The effect of SM application on soil microbial communities, such as nitrifying bacteria, general bacteria and fungi, and specifically selected microbial groups, still needs to be explored, though existing studies have given some guiding information, such as potential suppression of nitrifying bacteria and *Pseudomonas* spp., and promotion of total bacteria, *Streptomyces* spp., and *Pythium* spp.

1.4. Conclusions

With the increasing demand for energy and concerns about environmental sustainability, biofuel and biodiesel provides a promising alternative energy source. This could be potentially both more economical and environmentally friendly with appropriate use of the SM by-products. In parts of the world, these SMs have been used as organic fertilizers for years; however, the effects of these SMs, especially many of the dedicated biofuel SMs, on many plant pathogens and general soil microbial communities and soil processes has not been adequately investigated. Many of these dedicated biofuel SMs contain toxic chemicals that are potentially useful in controlling a variety of pathogens including several fungi, bacteria, and some insects. There is a limited amount of information on the use of *Brassicaceae* SMs to control selected pathogens. Even less literature exists on the use of jatropha, castor, and other SMs, although these plants are

very important in biodiesel production and possibly are very promising for pathogen control. In addition, almost no literature exists documenting the impacts of applying these SMs on soil microbial community composition and ecology. Before land application of these SMs can be recommended as an agricultural management practice, additional studies need to be conducted in order to increase our understanding of the impact these SMs will have on the microbial communities and processes in soil ecosystems.

CHAPTER II

PHYMATOTRICHOPSIS OMNIVORA INHIBITION BY OILSEED MEALS AND ISOTHIOCYANATES*

2.1. Introduction

The fungus *Phymatotrichopsis omnivora* (Duggar) Hennebert, a serious and recalcitrant soilborne pathogen, can infect the roots of over 2,000 different species of plants and often results in rapid plant wilting and death. As the causal agent of cotton root rot, the fungus has greatly hindered the production of cotton [*Gossypium hirsutum* (L.)] and alfalfa [*Medicago sativa* (L.)] in alkaline (pH from 7.0 to 8.5), low-organic-matter soils of Texas and the Southwestern USA (Walla and Janne, 1982; Whitson and Hine, 1986; Kenerley et al., 1998). The average statewide yield loss of cotton due to this disease has been estimated to be 3.5% in Texas, but it may range from 8-13% in severely infested areas (Kirkpatrick and Rothrock, 2001). This yield loss corresponds to approximately $100 million in annual losses to the US cotton industry (Marek et al., 2009). One aspect that makes the disease particularly damaging to agriculture is that the pathogen can infect such a wide variety of plants, including cotton, alfalfa, vegetable crops, fruits, and nut orchards in a large region ranging from eastern Texas and southern

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*Reprinted with permission from "Inhibition of the Germination and Growth of *Phymatotrichopsis omnivora* (Cotton Root Rot) by Oilseed Meals and Isothiocyanates" by Hu P, Wang AS, Engledow AS, Hollister EB, Rothlisberger KL, Matocha JE, Zuberer DA, Provin TL, Hons FM & Gentry TJ, 2011, *Applied Soil Ecology*, 49, 68-75, Copyright [2011] by Elsevier Ltd.

Oklahoma westward through Arizona and south into Mexico (Streets and Bloss, 1973; Walla and Janne, 1982; Marek et al., 2009).

The taxonomy of *P. omnivora* has varied over the past several decades. Recent research, through phylogenetic analysis, indicates that *P. omnivora* is a member of the family *Rhizinaceae*, *Pezizales* (*Ascomycota*: *Pezizomycetes*) related to *Psilopezia* and *Rhizina* (Marek et al., 2009). The primary inocula of *P. omnivora* are strands in soil or on host plant root systems and sclerotia that can survive as deep as 2.6 m in soil (although most occur at 0.5-0.9 m) (CABI/EPPO, 2004).

Over the past several decades, numerous approaches have been investigated for the potential to control *P. omnivora* and cotton root rot. Management practices such as applying chemical and organic fertilizers and fungicides have been studied for disease control. Some research has suggested that cotton root rot may be reduced through application of fertilizers high in certain forms of nitrogen, such as ammonia (Walla and Janne, 1982). Organic amendments such as green manures have also been reported to suppress cotton root rot, though the mechanism causing this is still not clear. Possible mechanisms include an unfavorable increase in acidity after incorporation of organic matter, increases in populations of antagonist microorganisms, and release of toxic compounds during decomposition of the added material (Streets and Bloss, 1973). Different types of fungicides, such as telone and propiconazole, have also been demonstrated to have some potential efficacy in controlling cotton root rot (Hine and Whitson, 1982; Matocha, 2008). Other sterol-inhibiting fungicides have also been reported to have potential for cotton root rot control and continue to be investigated

(Whitson and Hine, 1986). In a recent study by Isakeit et al. (2010), the fungicide flutriafol showed promise for economical control of cotton root rot. However, to-date the use of chemical amendments and fungicides to control cotton root rot has been limited by the high cost of the chemicals (especially at effective rates) and unpredictable behavior of the fungus in different crops and soils with varied activity in even the same field from year to year (Walla and Janne, 1982).

Another possible alternative for the control of *P. omnivora* may be the use of biofumigation. Compounds such as GLS in some plants, including many *Brassica* spp., are partially degraded by plant and/ or microbial enzymes upon incorporation into soil. Resultant biocidal products of hydrolysis include isothiocyanates (ITCs), nitriles, organic thiocyanates, SCN^-, oxazolidinethione, and epthionitriles (Cole, 1976; Borek and Morra, 2005) which can inhibit pathogens. Numerous studies have demonstrated the potential for using green tissues of brassicaceous crops as biofumigants to control soilborne plant pathogens, such as *Rhizoctonia* spp. and take-all disease of wheat (Murray and Brennan, 1998; Charron and Sams, 1999). Additionally, recent field studies showed that using a brassicaceous winter cover crop in rotation with cotton resulted in 50% reduction in plant mortality from *P. omnivora* compared with the untreated control (Matocha, 2008).

A limited number of studies have also investigated the use of brassicaceous oilseed meals (SMs) (the by-product remaining after extraction of oil) as biofumigants to control root rot caused by soil fungal pathogens such as *Aphanomyces euteiches* f. sp. *pisi* (Smolinska et al., 1997) and *Rhizoctonia* spp. (Cohen et al., 2005; Mazzola et al.,

2007). The use of SMs, instead of green tissue, may be particularly attractive since large quantities of SMs may be produced from *Brassica* spp., and other crops such as *Jatropha curcas* L. (jatropha), as a by-product of the biofuel industry. Like *Brassica* spp., jatropha has also been studied for its biocidal chemical contents, such as phenols, tannins, saponin, amylase inhibitors, cyanogenic glucosides, phorbol esters, phytate, a trypsin inhibitor, and lectins (curcin) (Makkar et al., 1997; Wink et al., 1997; Aregheore et al.,1998) which have been reported to inhibit nematodes, insects, fungi, and viruses (Huang et al., 2008).

To date, no research has been published on the effects of brassicaceous and jatropha SMs, and their specific biocidal compounds, on *P. omnivora* survival and growth. The objectives of our study were: 1) to evaluate the impact of various SMs on *P. omnivora* sclerotial germination and active hyphal growth in soil, and 2) to determine the effect of selected isothiocynates (allyl, benzyl, phenyl, and butyl ITC) on *P. omnivora* hyphal growth in pure culture.

2.2. Materials and Methods

2.2.1. Soil collection and characterization

The soil used in this experiment is mapped as Branyon clay (Fine, smectitic, thermic Udic Haplustert) and has a history of supporting *P. omnivora* growth. The soil was sampled near Snook, Texas (30.5 N, 96.5W) from 0-15 cm depth and passed through a 4-mm sieve to remove plant residues or rocks. The soil water content was

determined by oven-drying a subsample of 30 g of field moist soil for 24h at 105 ℃ and calculated to be 24.3% (w/w). Soils were analyzed for organic C and total N by a combustion method using an Elementar Vario Max CN analyzer (Storer, 1984; McGeehan and Naylor, 1988; Schulte and Hopkins, 1996). Organic C was determined at 650 ℃. Soil phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), and sodium (Na) were extracted with Mehlich III solution and analyzed by inductively coupled plasma (ICP) spectrometry (Mehlich, 1978; 1984). Soil micronutrients including copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn) were extracted using a 0.005 M DTPA, 0.01 M $CaCl_2$ and 0.1 M triethanolamine solution mixture and determined by ICP (Lindsay and Norvell, 1978). Soil particle size distribution was determined by the hydrometer method (Day, 1965).

The soil was mapped as Branyon clay, but soil analysis showed a pH of 7.0 (Table 2.1), which is slightly lower than a typical Branyon clay soil of around 8.0. Soil organic C was 11.8g kg^{-1}. The soil also contained various concentrations of extractable macro and micronutrients. Extractable P, Fe, and Zn were in the moderate category, while K, S, Mn, and Cu were rated as high in availability according to Texas AgriLife Extension Guidelines. Sodium was rated as very low, while and Ca and Mg were rated as very high.

2.2.2. Phymatotrichopsis omnivora *cultures and sclerotia*

Phymatotrichopsis omnivora (San Angelo isolate) was provided by Dr. Charles Rush, Texas AgriLife Research, Amarillo, TX. The fungus was isolated from diseased

cotton stalks collected from a field near San Angelo, Texas (C. Rush, personal communication). *P. omnivora* cultures were maintained on Difco potato dextrose agar (PDA) (Becton, Dickinson and Company, Sparks, MD) at 25 ℃. Sclerotia were generated by adding the *P. omnivora* culture from PDA into Houston Black clay (Fine, smectitic, thermic Udic Haplustert) amended with sterilized sorghum [*Sorghum bicolor* (L.) Moench] seeds. The soil was previously autoclaved and mixed with sterilized water to approximately 50% (w/w) water content. After 3-4 weeks of incubation at 28 ℃, sclerotia were recovered from the soil and stored at room temperature. *P. omnivora* OKAlf8 was obtained from the American Type Culture Collection (ATCC MYA-4551) (Marek et al., 2009) and was used to test the impact of selected ITCs in pure culture (PDA).

Table 2.1. Selected characteristics of Branyon clay soil.

Total N	**Organic C**	**P**	**K**	**Ca**	**Mg**	**S**	**Na**	**Fe**	**Zn**	**Mn**	**Cu**	**Sand**	**Silt**	**Clay**	**pH**
-------g kg^{-1}------		---------- mg kg^{-1} ----------										---------- % ---------			
1.06	11.8	24	297	6422	753	18	104	3.87	0.44	2.24	0.36	33	27	40	7.0

2.2.3. SM analysis

Mustard (*Brassica juncea* L.) SM was obtained from the *Brassica* Breeding and Research group at the University of Idaho. SMs of flax (*Linum usitatissimum* L.), *Camelina sativa* (L.) Crantz (camelina), *Jatropha curcas* L. (jatropha), and *Triadica sebifera* (L.). Small (Chinese tallow) were obtained by processing seeds with a Komet

Oil Press (Model CA59, IBG Monforts Oekotec, Germany). Seed meals were ground with a mortar and pestle and passed through a 1-mm sieve. The water content of SMs was determined by drying sub-samples at 60 ℃ for 3 days. Organic C and total C and N in the SMs were determined by a high-temperature combustion process using an Elementar Vario Max CN analyzer (Nelson and Sommers, 1973; Sheldrick, 1986; McGeehan and Naylor, 1988; Sweeney, 1989). Organic C was determined at 650 ℃ while total C was determined at 950 ℃. Plant SM B, Ca, Cu, Fe, K, Mg, Na, P, S, and Zn concentrations were determined using a nitric acid digestion and ICP analysis (Isaac and Johnson, 1975; Havlin and Soltanpour, 1989).

Glucosinolate concentrations of selected SMs were determined using methods similar to those found in the International Organization of Standardization (IOS, 1992), but with a few additions. Mustard, flax, and camelina SMs were first defatted with one extraction and two rinses of petroleum ether by vacuum filtration using a Büchner funnel. Defatted SMs were weighed (300 mg) into 50 ml centrifuge tubes to which 500 mg of 5-mm glass beads (Borek and Morra, 2005) were added and then immediately vortexed. A hot (70 ℃) 70% methanol: H_2O solution (10 ml) was added to the samples that were then placed in a hot water bath at 65 ℃ for 20 minutes and vortexed intermittently. The samples were then centrifuged at 2500 g for 5 min and the supernatant was collected. An additional extraction was performed similar to above, but with 5 ml of hot methanol rather than 10 ml. The extracts were combined and 2 ml were added to a 0.6 ml plug of DEAE Sephadex A-25 anion exchanger and allowed to drain freely. The poly-prep chromatography columns (BioRad, Hercules, CA) were then rinsed

with 1 ml deionized water and finally with two aliquots of 1 ml 0.02M sodium acetate buffer (pH 4.5). Sulfatase solution (100 μl) was added to the columns and allowed to sit overnight (16 hrs). Desulfo GLS were eluted with 3 consecutive 1 ml volumes of deionized water. Samples were immediately separated and quantified using HPLC with a Waters 600s System Controller, 717 autosampler and 996 photodiode array detector. The system was equipped with a Waters 3.5 μm Symmetry Shied RP8 column (2.1 x 150 mm), in which mobile phases flowed at 0.3 ml min^{-1} and compounds were separated using an acetonitrile gradient starting at 2.0% and increasing to 95.0%. Expected retention behavior, such as time and sequence, and UV spectra were used to identify GLS peaks. A calibration curve was constructed using sinigrin monohydrate (Science Lab, Houston, TX) as an external standard.

2.2.4. Experimental plan

2.2.4.1. Inhibition of sclerotial germination by SMs

A preliminary experiment was conducted to test the effects of various SMs on *P. omnivora* sclerotial germination. Different rates (1 and 5% [w/w]; dry weight basis) of brassicaceous SMs from mustard and camelina, as well as non-brassicaceous SMs from jatropha, Chinese tallow, and flax were added to the Branyon soil with 3 replicates for each treatment. Another 3 microcosms receiving no SM were used as the control. A total of 27.5 g (22.1 g dry weight equivalent) of moist soil for each microcosm was mixed thoroughly with SM and placed into a 30- ml sterile polypropylene centrifuge tube along

with two *P. omnivora* sclerotia on top of the soil surface. The tubes were partially opened every 2-3 days to allow for gas exchange.

Based upon the initial results from the above experiment, a second experiment of completely randomized design (CRD) and two factorial treatments were used. The two treatment factors were application rate and type of SM. Application rates were 0.5, 1 and 5% (w/w), while added SMs included mustard and camelina, as well as non-brassicaceous plants including jatropha and flax. Chinese tallow SM was not included because no effect was observed in the initial experiment. Each treatment had 3 replications. Another 3 microcosms receiving no SM were used as the control. A total of 30 g of moist soil (24.1 g dry weight equivalent) for each microcosm was mixed thoroughly with SM and then placed into a Petri dish (100×15 mm) to provide the growth medium for sclerotia. Twenty *P. omnivora* sclerotia were added to each Petri dish, and the dishes were sealed with parafilm and incubated at 28 ℃. At the end of each week (for a total of 4 weeks), the dishes were opened to read sclerotial germination rates and then sealed with parafilm again. At the end of the 4^{th} week, all of the remaining, non-germinated sclerotia in the Petri dishes were isolated from soil, placed onto wet filter paper, and incubated at 28 ℃ for 1 week to determine viability. The sclerotia that germinated in soil were identified as "germinated" sclerotia, while those that failed to germinate in soil but did germinate on filter paper were identified as "inhibited" sclerotia. The remaining ungerminated sclerotia were considered to be "inactivated" sclerotia. Survival rates [(number of germinated sclerotia + number of inhibited sclerotia)/ 20] for each treatment were recorded.

2.2.4.2. Inhibition of hyphal growth by SMs

To test the effect of SMs on *P. omnivora* (San Angelo isolate) hyphal growth, different rates [1 and 5% (w/w)] of mustard, camelina, and jatropha SMs were added to soil with 3 replicates for each treatment. Another 3 microcosms receiving no SM were used as the control. The soil water content was adjusted to 24.3% (w/w) for consistency with sclerotial experiments described above. A total of 65 g (52.2 g dry weight equivalent) of moist soil for each microcosm was mixed thoroughly with SM and then packed into a glass tube (25 cm long × 2.2 cm diameter). Soils were compressed to a similar bulk density of ~0.75 g cm^{-3}. Ten sorghum seeds that had been soaked in sterilized water overnight were added on top of the soil surface to provide a C source along with a 1 × 1 cm agar plug of a one-week-old *P. omnivora* culture on PDA. The glass tubes were sealed with a rubber stopper at the bottom and a cotton plug at the top. The top of the tubes were elevated slightly (~10 °) during incubation at 28 ℃. The length of hyphal growth was recorded every day, or as needed, for 4 weeks. No data were recorded for the control after one week since the hyphae had reached the bottom of the tube, while hyphal growth was monitored for 4 weeks for the other treatments.

2.2.4.3. Inhibition of P. omnivora *OKAlf8 growth by ITCs*

A laboratory study with completely randomized design was conducted to evaluate the effects of selected ITCs applied at different rates on *P. omnivora* OKAlf8 growth. A plug of PDA-grown *P. omnivora* OKAlf8 (1.5 cm in diameter) was transferred to the center of a Petri dish (8.5 × 1.3 cm) with fresh PDA (20 ml) using a 15-ml sterile

polypropylene centrifuge tube. The ITC dilutions used ranged from 10^{-1} to 10^{-5} from pure allyl ITC (Acros Organics, New Jersey, USA), benzyl ITC (Acros Organics), phenyl ITC (MP Biomedicals, Ohio, USA), and butyl ITC (Alfa Aesar, MA, USA). All ITC dilutions were made by mixing with 1,4-dioxane (Acros Organics) as the solvent to ensure a complete vaporized form of ITCs. A 100-µl aliquot of each solution was added onto the center of the lid of a Petri dish and then the Petri dish was sealed with parafilm, resulting in initial ITC concentrations in the head space of the Petri dishes from approximately 0.02-200 µg cm^{-3}. The Petri dishes were then incubated at 25 ℃ for 5 days, and *P. omnivora* hyphal growth in length was recorded every 12 hours or as needed. Control plates received 100-µl of 1,4-dioxane (used in data analysis) or sterilized water (used as a comparison for discussion only). Each treatment had 3 replicates. The experiment was conducted twice using the same method and under the same conditions, except for additional ITC concentrations being used in the second experiment in order to provide better resolution between treatments.

At the end of the ITC experiment, *P. omnivora* hyphae were tested to confirm that they were *P. omnivora*. Hyphae were collected from selected plates (1,4-dioxane treated control, ITC dilution treated with 10^{-5} for allyl, 10^{-4} for benzyl, 10^{-4} for phenyl, and 10^{-3} for butyl). Fungal DNA was extracted with a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA). The primers ITS1-F and TW13 (Taylor et al., 2008), which amplify an ~1200 bp region spanning both internal transcribed spacer regions (ITS1, ITS2) and a portion of the large ribosomal subunit, were used to generate PCR products for sequencing. Each 25 µl PCR reaction contained ~8 ng DNA template,

FailSafe buffer E (Epicentre Biotechnologies, Madison, WI, USA), 0.25 µl Taq polymerase, and forward and reverse primers at a final concentration of 0.5 µM each. Thermocycling was conducted in a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 96 ℃ for 2 min; 28 cycles of denaturation at 94 ℃ for 30 sec, annealing at 55 ℃ for 40 sec, and extension at 72 ℃ for 3 min, and a final extension at 72 ℃ for 10 min. Three replicate amplifications were performed for each sample, and their products were combined for downstream use.

The PCR-amplified DNA products were then purified using 1% (w/v) agarose gel. DNA bands at ~1200bp were excised from the gel and then extracted using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Purified PCR products were sent to Beckman-Coulter Genomics (Danvers, MA) for single-pass Sanger sequencing with the primer ITS1F. BLAST searches of the GenBank-nr database were used to assign identities to the post-ITC-inhibition cultures.

2.2.5. Statistical analysis

Statistical analyses were conducted using SAS version 9.2 (SAS Institute Inc., 2003). A generalized linear model (Proc GLM) was used for two-way ANOVA on treatment factors. The pair-wise treatment mean comparisons were made using Least Significance Difference (LSD) when these were shown to be significant. Unless otherwise indicated, all statistical significance levels were set at $P \leq 0.05$. Data on sclerotial germination and survival rates were arcsin square root transformed before

statistical analysis. The IC_{50} values for the various ITCs were calculated with BioDataFit 1.02 (Chang Bioscience, Castro Valley, CA) using the Sigmoidal (LogEC50) model.

2.3. Results

2.3.1. SM chemical composition

All SMs generally had very high concentrations of C, N, P, and K (Table 2.2). The brassicaceous SMs, mustard and camelina, had higher concentrations of P, S and Zn than the SMs of other families, namely jatropha, flax, and Chinese tallow. The Chinese tallow SM contained the lowest concentrations of N, P, K, S, and Mg compared to other meals. Greater Ca, however, was detected in jatropha SM. Other elemental concentrations such as Na, Mg, Cu, Fe, Mn, and B were comparable among different SMs. The C:N ratios of SMs ranged from 7.4 for camelina to 21.4 for Chinese tallow.

Brassicaceous SMs varied in GLS profile and content. Mustard contained the highest level of GLS with 99.6% being allyl GLS at a concentration of 157.0 ± 15.6 μmol g^{-1} of defatted SM. Camelina possessed a lower level of GLS with 51.9% being 10-methyl-sulfinyl-decyl-GLS (glucocamelinin), followed by 30.2% being 11-methyl-sulfinyl-decyl and 17.9% being 9-methyl-sulfinyl-decyl GLS at concentrations of 12.2 ± 7.5, 7.1 ± 3.5, and 4.2 ± 2.6 μmol g^{-1} of defatted SM, respectively. No GLS compounds were detected in flax SMs.

Table 2.2. Selected elemental concentrations of mustard, camelina, jatropha, flax, and Chinese tallow SMs.

Oilseed Meals	C	N	P	K	Ca	S	Mg	Na	Zn	Fe	Cu	Mn	B
	---------- g kg^{-1} ----------								--------- mg kg^{-1} -----				
Mustard	472	56	11.1	10.3	3.9	18.2	5.3	0.3	58	71	10	35	11
Camelina	439	59	10.1	14.5	5.3	12.3	4.2	0.4	71	53	12	42	24
Jatropha	477	35	6.2	12.9	9.6	2.3	5.2	1.1	31	42	21	39	30
Flax	491	51	6.8	10.3	3.0	3.3	5.1	0.7	38	33	13	34	27
Chinese tallow	492	23	3.7	6.0	3.2	1.4	1.9	0.3	30	19	14	107	41

2.3.2. Sclerotial germination and survival rate

The preliminary experiment showed that germination of *P. omnivora* sclerotia was inhibited in mustard, camelina, and jatropha SM treated soils (Table A-2.1 in Apprndix B). After 4 weeks of incubation, none of the 6 sclerotia (2 per tube × 3 reps) germinated in the 1% mustard, 5% mustard, and 5% jatropha SM treatments, and only 2 of 6 sclerotia for the 1% jatropha treatments had germinated. Only 1 of 6 sclerotia for the 5% camelina treatment had germinated at the end of the incubation. The 1% application rate of camelina SM did not suppress sclerotial germination. No inhibitory results were noted with flax and Chinese tallow SMs at either application rate.

In the subsequent experiment, a significant two-way interaction was detected with respect to the effects of SM type and application rate on *P. omnivora* sclerotial germination rate at the end of the 1st, 2nd, 3rd, and 4th weeks of incubation at 28 ℃ (Table A-2.2). The higher (5% w/w) application rate of mustard, camelina, and jatropha SM

addition significantly inhibited *P. omnivora* sclerotial germination (Table 2.3). Mustard treated soil, however, most effectively suppressed *P. omnivora* sclerotial germination compared with all other SM treatments except for the 5% camelina treatment during the first 3 weeks of incubation. Both the 1% and 5% mustard SM treated soils resulted in no sclerotial germination. Jatropha SM showed less inhibition of sclerotial germination than the brassicaceous SMs. Flax SM had no effect on sclerotial germination. Camelina and jatropha SMs applied at the higher rate suppressed sclerotial germination throughout the 4-week incubation, but suppression decreased over time. The 0.5% application rate for all SM treatments did not inhibit sclerotial germination (data not included in figures).

A significant two-way interaction was detected with respect to the effects of SM type and application rate on *P. omnivora* sclerotial survival rate (Table A-2.2) and the survival rates were significantly different among treatments (Fig. 2.1). The 5% application rate of brassicaceous and jatropha SMs resulted in significantly lower *P. omnivora* sclerotial survival rates, while only mustard SM significantly decreased survival rate at the 1% application rate. Camelina SM at the higher application rate resulted in the greatest number of inhibited sclerotia (those that did not germinate in treated soil but did when removed and placed on filter paper).

2.3.3. P. omnivora *hyphal growth in soil*

All SMs at both application rates significantly reduced *P. omnivora* hyphal growth in soil compared with the control (Fig. 2.2). After one week of incubation, hyphal growth was reduced by 92% for 1% camelina, 95% for 1% jatropha, and 100% for all other treatments including 1% mustard, 5% mustard, 5% camelina, and 5% jatropha. No further growth was found in the following 3 weeks of incubation.

Table 2.3. Effects of SMs on *Phymatotrichopsis omnivora* sclerotial germination rates (arcsin square root transformed) in SM-amended Branyon clay soil at the end of the 1st, 2nd, 3rd, and 4th week of incubation at 28 ℃.

Treatments	Week 1	Week 2	Week 3	Week 4
	---------- Germination rates (%) ----------			
Control	100a†	100a	100a	100a
Mustard 1%	0d	0d	0d	0d
Mustard 5%	0d	0d	0d	0d
Camelina 1%	100a	100a	100a	100a
Camelina 5%	3d	7d	7d	40c
Jatropha 1%	100a	100a	100a	100a
Jatropha 5%	38b	57b	65b	70b
Flax 1%	100a	100a	100a	100a
Flax 5%	100a	100a	100a	100a

†Means within columns followed by the same letter are not significantly different at $P<0.05$.

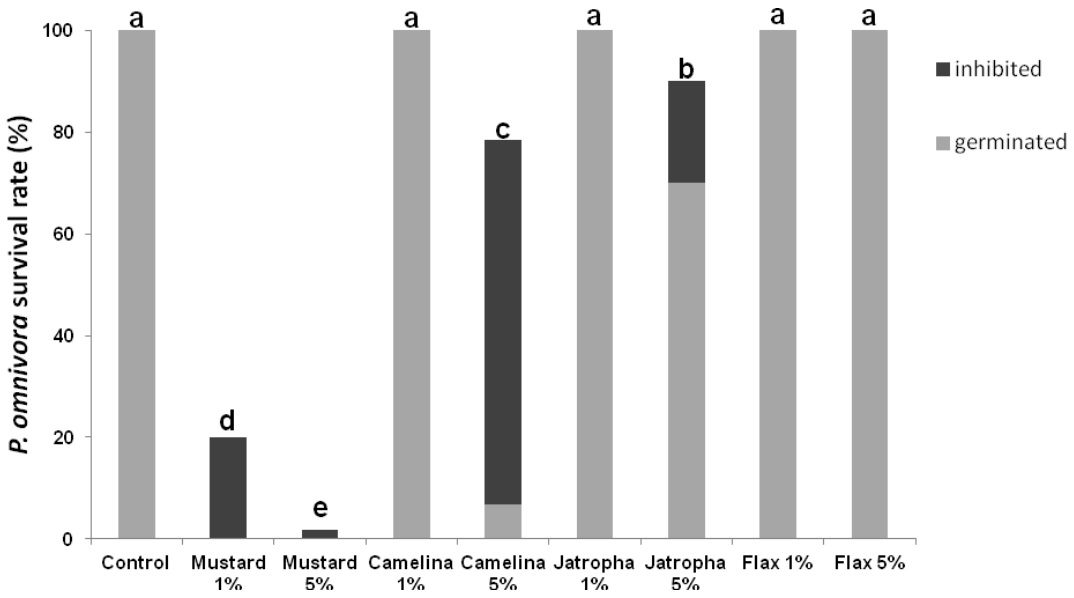

Fig. 2.1. Effects of SMs on *Phymatotrichopsis omnivora* sclerotial survival rates in SM-amended Branyon clay soil following 4 weeks of incubation at 28 ℃. Lighter bars represent "germinated" sclerotia that successfully germinated in soil, while those that did not germinate in soil but did germinate after being removed from soil and incubated on filter paper for 1 week were identified as "inhibited" sclerotia and are represented by the darker bars. Survival rates were calculated as [(number of germinated sclerotia+ number of inhibited sclerotia)/ 20, expressed as percentages]. Means with the same letter are not significantly different at $P<0.05$.

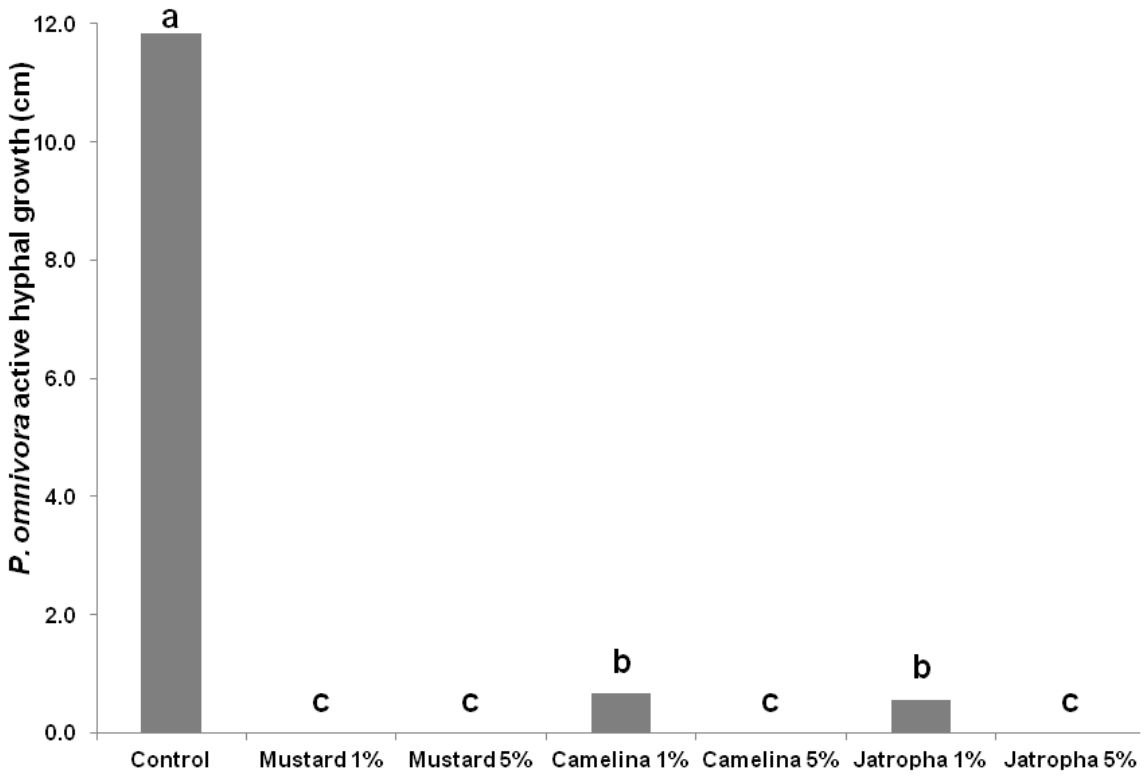

Fig. 2.2. Effects of SMs on *Phymatotrichopsis omnivora* hyphal growth in SM-amended Branyon clay soil after 1 week of incubation at 28 ℃. Means with the same letter are not significantly different at $P<0.05$.

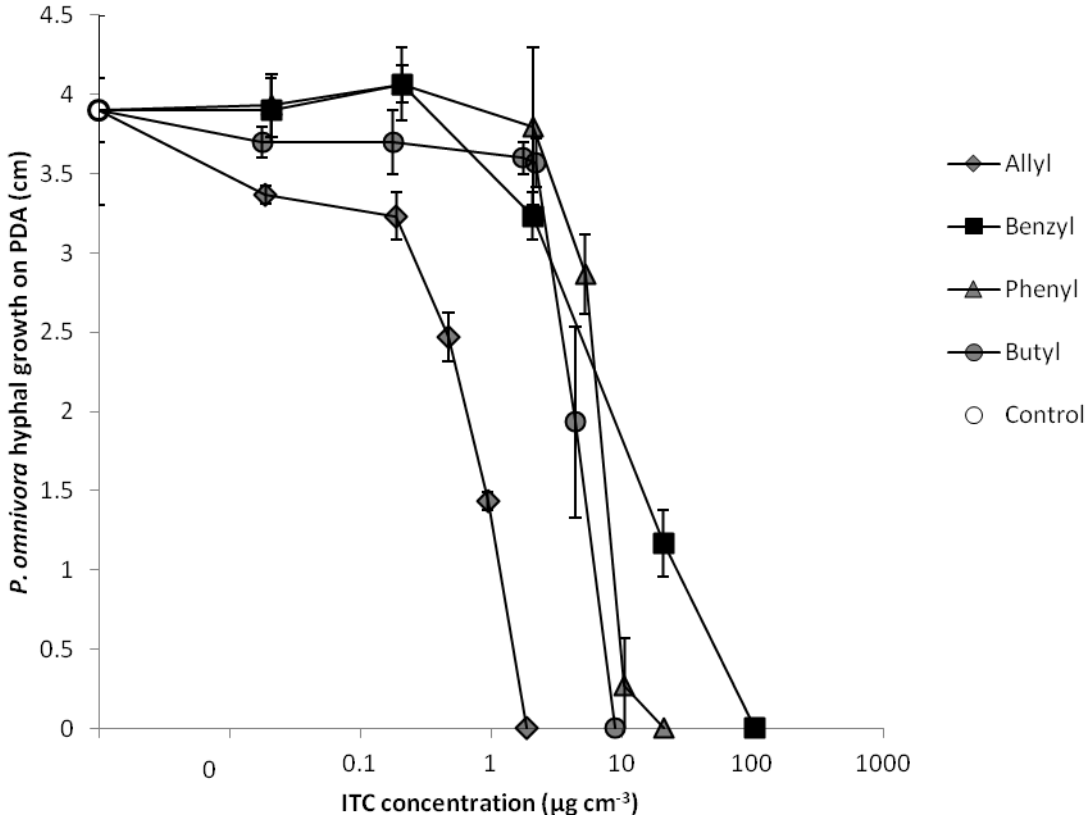

Fig. 2.3. Effects of selected ITCs on *Phymatotrichopsis omnivora* OKAlf8 hyphal growth on potato dextrose agar (PDA) after 84 h of incubation at 25 ℃. (♦: Allyl ITC; ■: Benzyl ITC; ▲: Phenyl ITC; ●: Butyl ITC; ○: 1,4-dioxane Control). Means are based on 3 replicates. Bars represent ± standard deviation of the mean. Error bars are hidden when smaller than the symbols.

2.*3.4.* P. omnivora *OKAlf8 hyphal growth on PDA*

Results from the first experiment, presented as supplementary data (Fig. A-2.1) were similar to those of the following more extensive experiment (Fig. 2.3), in which all tested ITCs inhibited *P. omnivora OKAlf8* hyphal growth, although the level of inhibition varied with concentration. The IC_{50} values were 0.62 ± 0.19, 4.47 ± 0.08, 5.67 ± 0.10, and 20.48 ± 0.30 μg cm^{-3} for allyl, butyl, phenyl, and benzyl ITC, respectively. Allyl ITC showed the highest level of inhibition of hyphal growth, with no growth observed at a concentration of 1.89 μg cm^{-3}, followed by butyl and phenyl ITC with no growth at 8.87 μg cm^{-3} and 21.0 μg cm^{-3} respectively. Benzyl ITC was the least inhibitory, with no hyphal growth occurring at a concentration of 104.2 μg cm^{-3}.

The DNA sequencing confirmed that the fungal cultures on the post-ITC inhibition experiment plates were *P. omnivora* as indicated by ≥ 99% identity with *P. omnivora* OKAlf8.

2.4. Discussion

2.4.1. GLS in SMs and ITC concentrations in the soil

In our study, the dominant GLS compound in the mustard SM was found to be allyl GLS at a concentration of 157.0 ± 15.6 μmol g^{-1}defatted SM, which is similar to the results reported by Rice et al. (2007) and Hansson et al. (2008) who found the dominant compound contained in *B. juncea* meal to be allyl GLS at concentrations of 123.8 ± 15.3μmol g^{-1} and 152.0 ± 12.3 μmol g^{-1}, respectively. A higher allyl GLS concentration

of up to 303 μmol g^{-1} in mustard SM was reported by Mazzola et al. (2007). The major GLS compound detected in camelina SM was 10-methyl-sulfinyl-decyl (glucocamelinin) at 12.2 ± 7.5 μmol g^{-1}, which is comparable with a glucocamelinin concentration of 15.5 μmol g^{-1} reported for camelinain in the study of Schuster and Friedt (1998).

Assuming the ratio of GLS to hydrolysis product (ITC) is 1:1 (Hansson et al., 2008), the 1% (w/w) mustard SM treatment would have produced approximately 170 μg allyl ITC cm^{-3} soil. The camelina SM in our study contained much lower concentrations (and different forms) of GLS compounds compared with mustard and thus is likely to have produced less ITC in the soil.

2.4.2. P. omnivora *inhibition by SMs: sclerotial germination*

Mustard was the most effective SM at reducing germination of sclerotia in our study. Addition of mustard SM completely inhibited *P. omnivora* sclerotial germination after 4 weeks of incubation, and a large proportion (80% -100%) of these inhibited sclerotia appeared to be completely inactivated. Camelina SM showed less effective control of sclerotial germination than mustard. For camelina, only the 5% application rate resulted in significant inhibition of sclerotial germination. Further, inhibition by camelina and jatropha SMs seemed to decrease over time, possibly due to the volatilization of allelochemicals that may have occurred when opening the sealed Petri dishes at the end of each week for observation and/ or degradation of the compounds in soil (Warton et al., 2003; Gimsing et al., 2006; Poulsen et al., 2008).

Our results indicated that jatropha SM also inhibited *P. omnivora*, though to a

lesser extent than the brassicaceous SMs. Although a relatively limited volume of literature exists regarding the chemical composition of jatropha SMs, a number of biocidal proteins and compounds, such as lectin (curcin), trypsin, and phorbol esters have been found in various parts of jatropha plants (Makkar et al., 1997; Wink et al., 1997; Rakshit et al., 2008).

Based on our results and publications by other researchers, we believe that the inhibition of *P. omnivora* was likely due to allelochemicals produced from the added SMs. The flax SM had similar elemental composition to the brassicaceous SMs, except for the S concentration which was higher (~2- to 6-fold) in the brassicaceous meals. It is likely that S-containing compounds such as carbon disulfide, dimethyl disulfide, dimethyl sulfide and methanethiol were produced during degradation of the SMs in soil and may have played an important role in suppressing *P. omnivora* (Bending and Lincoln, 1999; Vargas-Arispuro et al., 2005). Further, the higher elemental S concentrations likely correspond to higher levels of initial sulfur-containing biocidal chemicals (e.g., GLS) in *Brassica* spp. as we discussed before. Other researchers have demonstrated that several species of *Brassicaceae* release biocidal products of GLS hydrolysis including ITCs, nitriles, organic thiocyanates, SCN^-, oxazolidinethione, and epthionitriles (Cole, 1976; Brown et al., 1991; Borek and Morra, 2005) to soil. It is also possible that high N concentrations in selected SMs may also have inhibited *P. omnivora* development. However, this did not appear to be the major mechanism of inhibition in our experiments as the flax meal, with N content similar to the brassicaceous species but no GLS content, did not show any inhibitory effect on *P. omnivora*.

2.4.3. P. omnivora *inhibition by SMs: hyphal growth*

Active hyphal growth of *P. omnivora* in soil was significantly inhibited by mustard, camelina, and jatropha SMs at both 1% and 5% application rates. The SMs were generally more effective in suppressing active hyphal growth than in inhibiting sclerotial germination. Although, the mechanism of hyphal inhibition may be similar to that for inhibition of sclerotial germination, the morphological and structural differences between sclerotia and hyphae may cause a greater sensitivity of hyphae to biocidal chemicals in soil (Gunasekaran et al., 1974; Whitson and Hine, 1986). The effective control of hyphal growth indicated that even if sclerotia germinate in soil, the presence of appropriate SMs may inhibit the growth of hyphae and thus may reduce the virulence of *P. omnivora*. We did not test the impact of flax SM addition on hyphal growth since the flax did not affect sclerotial germination; however, inclusion of flax as a non-GLS-containing SM control may have provided additional evidence that the observed reductions in hyphal growth were due to biocidal compounds and not just the addition of decomposable biomass. It is also possible that the mechanisms involved were not just a simple biofumigation effect by the allelochemicals induced, but also may have included plant systemic resistance conferred by changes in the soil faunal and floral communities (Cohen and Mazzola, 2006).

2.4.4. P. omnivora *inhibition by ITCs*

Isothiocyanates are considered to be the main allelochemicals produced from brassicaceous seeds (Borek and Morra, 2005). Here, we have shown that 4 types of pure

ITCs, including allyl, phenyl, butyl, and benzyl ITC significantly suppressed *P. omnivora* hyphal growth in pure culture. In addition to our SM results, these ITC results further suggest that suppression of *P. omnivora* by brassicaceous SMs may be largely due to the production of allelochemicals (e.g., allyl ITC) following application. Although there is no other published literature documenting the effects of ITCs on *P. omnivora*, ITCs have been found to inhibit many other soilborne pathogens including *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia solani*, *Fusarium graminearum*, *Pythium irregular*, *Bipolaris sorokiniana*, and *Aphanomyces euteiches* f. sp. *pisi*, though the effectiveness of suppression depends on the pathogen species and ITC tested (Kirkegaard et al., 1996; Smolinska et al., 1997; Sarwar et al., 1998).

Allyl ITC, which was the dominant form of ITC in the mustard used in this experiment, was found to be highly suppressive to the growth of *P. omnivora*. If the IC_{50} value (0.62 μg cm^{-3}) for ally ITC is converted to a soil basis (using a soil bulk density of 1.1 g cm^{-3}), this would correspond to approximately 0.36 μg of allyl ITC cm^{-3} soil. This value is much lower than the estimated amount of allyl ITC produced by addition of 1% mustard SM (~170 μg cm^{-3} soil) where significant effects on sclerotia were detected, but it is also much lower than the 0.5% mustard amendment (~85 μg cm^{-3} soil) where no inhibition of sclerotial germination was detected. This large difference may be due to methodological differences because the ITC experiment was conducted on pure cultures in the parafilm-sealed PDA Petri plates. In a soil environment, the ITCs may volatilize, be degraded within hours, or be bound by soil thus requiring a higher concentration of ITCs for the same level of effectiveness (Gimsing et al., 2009). We should also note that

the ITC experiment was conducted using actively growing *P. omivora* cultures and that we did not test the effects of mustard SM against hyphal growth at rates lower than 1% (which would have been closer to ITC levels used in the pure culture experiment). Even though rates of mustard SM less than 1% were not effective against *P. omnivora* sclerotia, they may have been effective against hyphal growth. This is important since application of larger amounts of SM materials (e.g. 5%) as a biofumigant would likely not be cost-effective, at this time, for crops such as cotton.

Consistent with our results describing differing rates of effectiveness among the four types of ITC tested, other studies have found that aromatic ITCs, such as benzyl ITC, were generally less effective than alkenyl aliphatic ITCs, such as allyl and butyl ITC, when using them in vaporized form, due to the lower volatility of aromatic ITCs (Sarwar et al., 1998). Within alkenyl aliphatic ITCs, butyl ITCs have been found to be less effective than allyl ITC (Angus et al., 1994). Additional research on the field-effectiveness of these SMs and the development of plants with greater concentrations of specific GLS may further improve the prospects for land-application of SMs as a cotton root rot control strategy.

2.5. Conclusions

Application of 1 to 5 % brassicaceous and jatropha SMs inhibited *P. omnivora* sclerotial germination and active hyphal growth in soil, suggesting that field application of select SMs, especially mustard which showed the highest toxicity to *P. omnivora*, may potentially reduce cotton root rot. Although the specific mechanisms responsible for the

inhibition of *P. omnivora* by SMs were not elucidated, our results, along with previous studies, suggest that biocidal chemicals released from the SMs played a major role. Further, our results demonstrate that different ITCs have varying levels of effectiveness in controlling the growth of *P. omnivora*.

CHAPTER III

SOIL MICROBIAL COMMUNITY CHANGES DUE TO OILSEED MEAL APPLICATION

3.1. Introduction

Extraction of oil from oilseed crops and subsequently transforming the oil into biodiesel is one of the major pathways for the production of biofuels. With increased interest in the use of biofuels to supplement fossil fuel supplies, increasing amounts of oilseed meals (SMs), which are the by-products (residual) remaining after the oil extraction process, will be produced. One of the possible uses for these SMs includes land application as soil organic amendments, due to their high nutrient contents (C, N, P etc.). Another related, potential application for some SMs (i.e., those containing allelochemicals) is in biofumigation strategies to control pathogens, insects, and/or weeds (Hu et al., 2011). Although there has been a relatively large amount of research on using SMs as organic fertilizers and for controlling pathogens and weeds, relatively limited research has been focused on dedicated bioenergy oilseed crops such as jatropha (Mazzola et al., 2007; Moore et al., 2010; Snyder et al., 2010; Wang et al., 2012). In addition, very limited information is available about the impact of these SMs on the soil microbial community, despite the critical importance of the soil microorganisms to many soil processes, plant health, and soil quality.

Many SMs have a potential role in biofumigation and have been demonstrated to suppress several plant pathogens including fungi and oomycetes such as

Phymatotrichopsis omnivora (Duggar) Hennebert (Hu et al., 2011), *Aphanomyces euteiches* f. sp. *pisi* (Smolinska et al., 1997), and *Rhizoctonia* spp. (Cohen et al., 2005). Such studies using SMs are still relatively limited although meals from the oilseed crop seeds (where the allelochemicals tend to be more concentrated) have been demonstrated to be more efficient and effective as a biofumigant than other plant tissues (Mazzola et al. 2001; Mazzola and Zhao, 2010). The mechanisms for pathogen control by SMs have been attributed to either the allelochemicals released or/ and plant systemic resistance conferred by changes in the soil microbial community (Cohen and Mazzola, 2006; Hu et al., 2011). Other possible nutritional consequences included an unfavorable increase in acidity and increases in populations of antagonistic microorganisms (Streets and Bloss, 1973). However, the relative importance of these mechanisms is still not clear. Thus, it would be beneficial to further explore soil microbial community changes as a result of SM application in order to gain understanding of its potential role in soil pathogen control.

In addition to a potential impact on plant pathogens, changes in soil microbial communities may impact other ecosystem processes. Soil microorganisms play key roles in ecosystem processes such as nutrient cycling and organic matter degradation. Numerous studies have demonstrated that the addition of organic amendments can alter, at least transiently, soil microbial communities with these impacts possibly being either beneficial or detrimental to soil quality and pathogen control (Hamel et al., 2005; Yao et al., 2006; Lejon et al., 2007; Omirou et al., 2011). In addition, studies have shown that isothiocyanates (ITCs), such as those produced by many *Brassica* spp., can negatively

impact soil microbial communities and specifically nitrifying bacteria (Bending and Lincoln, 2000; Rumberger and Marschner, 2003).

In the past, most of microbial community studies have been focused upon the soil bacterial community (Lauber et al., 2009), even though the soil fungal community is likely to be of as much, or even greater, importance than bacteria to many processes such as organic matter formation and decomposition (De Boer et al., 2005; Baldrian et al., 2011). For example, previous studies have shown that soil fungi often quantitatively dominate the soil microbial community over soil bacteria in litter decomposition, while soil bacteria in many cases were little impacted or undisturbed by amendment of fresh organic matter (Baath and Anderson, 2003; Lindahl et al., 2010; Baldrian et al., 2011). Nevertheless, such studies on soil fungal communities have been quite limited primarily because it has been difficult to describe most fungal species, estimate their diversity, distinguish individual taxa, and understand the ecological roles that fungi played (Hawksworth, 2001; Bailey et al., 2002; McGuire and Treseder, 2010). Moreover, the handful of studies that have investigated the impacts of SMs on soil fungal composition have used low resolution techniques such as fatty acid methyl ester analysis which provided information regarding community shifts but little-to-no information regarding which specific organisms were being impacted (Wang et al., 2012). To our knowledge, no study has been published that details the effects of SMs on soil fungal community structure on a taxonomic level.

The objectives of this study were: 1) to track changes in soil fungal and bacterial abundance due to SMs through time; 2) to elucidate soil fungal community composition

changes after SM addition; and 3) to investigate functional changes in soil microbial communities [community level physiological profiles (CLPPs)] due to various SM treatments.

3.2. Materials and Methods

3.2.1. Soil collection and characterization

We used Weswood loam (fine-silty, mixed, superactive, thermic, Udifluventic Haplustept) in this study. Selected characteristics can be found in Wang et al. (2012). This type of soil is well drained and has been used as irrigated cropland (USDA NRCS, 2008). Soil samples were collected from 0-15 cm depth, homogenized and air-dried. Water was added to field moisture level at the beginning of incubation.

3.2.2. SM analysis

Oilseed meals of *Jatropha curcas* L. (jatropha), *Camelina sativa* (L.) Crantz (camelina) and *Linum usitatissimum* L. (flax) were obtained by processing seeds with a Komet Oil Press (Model CA59, IBG Monforts Oekotec, Germany). *Triticum aestivum* L.(wheat) straw was from the Texas AgriLife Research Farm near College Station, TX. Seed meal pellets were ground with a mortar and pestle and passed through a 1-mm sieve. The water content of SMs was determined by drying at 60 ℃ for 3 days. Total C, total N and organic C of the SMs were determined by a high-temperature combustion process using Elementar Vario Max CN analyzer (Nelson and Sommers, 1973;

Sheldrick, 1986; McGeehan and Naylor, 1988; Sweeney, 1989). Organic C was determined at 650 ℃, and total C was determined at 950 ℃. Biomass elemental contents including B, Ca, Cu, Fe, K, Mg, Na, P, S, and Zn were determined by nitric acid digestion and ICP analysis (Isaac and Johnson, 1975; Havlin and Soltanpour, 1989).

3.2.3. Experimental plan

Each microcosm contained 400 g dry soil in a 1-L glass jar, and organic amendments of SMs and wheat straw were incorporated into soils at an application rate of 1.0 (w/w), or a field-equivalent of approximately 18 Mg ha^{-1}. The microcosms were maintained at 13% (w/w) water content (approximately 40% field capacity) and incubated at 25 ℃ under aerobic conditions. Each treatment had 3 replicates, with a series of unamended controls receiving no organic addition. To track potential changes in community level physiological profiling with Biolog Ecoplates, 1 g of soil from each microcosm was subsampled and processed at days 3, 7, 14, 28, 77, and 133. Another 5 g of sub samples were collected at days 3, 7, 14, 21, 28, and 77 to investigate temporal changes in soil bacterial and fungal abundance using quantitative real time polymerase chain reaction (qPCR). These soil sub-samples at days 3, 21, and 77 were used in soil fungal community analysis with pyrosequencing. Soil sub-samples for qPCR and pyrosequencing were stored at -80 ℃ until DNA extraction.

3.2.4. Functional diversity using BIOLOG analysis

Soil CLPP based on carbon (C) source utilization patterns was obtained using Biolog EcoPlates (Hayward, CA, USA) containing 31 different C sources. To carry out the procedure, 1 g of wet soil was collected from each sample and suspended in 9 mL of 0.87% saline solution (8.7 g NaCl/L w/v), and then diluted 100 times further. Aliquots of 150 μL of the resulting soil solution were injected in EcoPlate wells with a multichannel pipette and incubated at 25 ℃ in the dark for at least 96 hours, during which absorbance was measured at 590 nm every 24 hours using an ELx808 Microplate Reader (Biolog, Inc., Hayward, CA, USA). The average well-color development (AWCD) was calculated from each plate at each time point. For each plate, those time points of readings that had an AWCD closest to 0.75 were selected for data analysis (Garland, 1996) and normalized dividing by the AWCD to reduce biases due to different inoculum densities (Garland, 1997). For data analysis, the 31 C sources were grouped into 6 categories including carboxylic acids, carbohydrates, complex C sources, phosphate-associated C, amino acids, and amines (Table 3.1).

3.2.5. DNA extraction and quantification

Community DNA was extracted from 0.5 g soil samples using a PowerSoil DNA extraction kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). Extracted DNA was purified with illustra MicroSpin S-400 HR columns (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA), and quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Quant-iT PicoGreen dsDNA

assay kit (Invitrogen Corp, Carlsbad, CA, USA). Data generated from the latter was used in the analysis.

Table 3.1. Carbon (C) groupings of 31 C sources used in Biolog EcoPlate™.

C group*	**C source**	**Well location**
Carboxylic acids	Pyruvic acid methyl ester	B1
	D-glucosamic acid γ-lactone	F2
	D-galactonic acid	A3
	D-galacturonic acid	B3
	2-hydroxy benzoic acid	C3
	4-hydroxy benzoic acid	D3
	γ-hydroxy butyric acid	E3
	Itaconic acid	F3
	α-ketobutyric acid	G3
	D-malic acid	H3
Complex C sources	Tween 40	C1
	Tween 80	D1
	α-cyclodextrin	E1
	Glycogen	F1
Carbohydrates	D-cellobiose	G1
	α-D-lactose	H1
	β-methyl-D-glucoside	A2
	D-xylose	B2
	i-erythritol	C2
	D-mannitol	D2
	N-acetyl-D-glucosamine	E2
Phosphate C	Glucose-1-phosphate	G2
	D,1-α-glycerol phosphate	H2
Amino Acids	L-arginine	A4
	L-asparagine	B4
	1-phenylalanine	C4
	L-serine	D4
	L-threonine	E4
	Glycyl-L-glutamic acid	F4
Amines	Phenylethyl-amine	G4
	Putrescine	H4

*Adapted from Chazarenc et al., 2010.

3.2.6. qPCR on general bacterial and fungal abundance

Community qPCR assays based on Fierer et al. (2005) and Boyle et al. (2008) were used to evaluate relative abundances of soil general bacterial and fungal populations in each sample. Assays were performed in triplicate using a Rotor-Gene 6000 series thermal cycler (Qiagen, Valencia, CA, USA). Each 10 μL reaction for qPCR contained: 4.5 μL 2.5x RealMasterMix with 20x SYBR solution (5Prime, Inc., Gaithersburg, MD, USA), 1.0 μL BSA (10 mg mL^{-1}), 0.5 μL of each primer (10 μM,), 2.5 μL molecular-grade water, and 1.0 μL template DNA (2.5 ng μL^{-1}). Thermocycling consisted of an initial denaturation at 95 ℃ for 15 min, followed by 40 cycles of 95 ℃ for 1 min and annealing temperature at 53 ℃ for 30 s, and 72 ℃ for 1 min. Primer sets of Eub338/518 (Fierer et al., 2005) and 5.8S/ ITS1F (Boyle et al., 2008) were used for bacteria and fungi, respectively. Plasmid standards for the bacterial and fungal relative abundance by qPCR were generated as described by Somenahally et al. (2011). Values representing the mean of 3 biological replicates for each treatment were used to create the graphs on soil fungal and bacterial abundance.

3.2.7. Fungal tag-encoded amplicon pyrosequencing and analysis

Purified community DNA samples from all treatments including 3 biological replicates each were submitted to the Research and Testing Laboratory (Lubbock, TX, USA) for tag-pyrosequencing using 454 GS FLX titanium technology (454 Life Sciences, Branford, CT, USA). The fungal ITS region was amplified using primers

ITS1F and ITS4 for the initial generation of the amplicons (Amend et al., 2010), and fungal amplicons were sequenced in the forward direction, generating reads from ITS1F.

All sequences were preprocessed in MOTHUR v.1.20.0 (Schloss et al., 2009) to remove primers and barcodes, check quality (Q25), discard sequences that contained ambiguous base calls, cap the homopolymer length at 8, remove sequences that were shorter than 300 bp in length, and trim all sequences to the same length of 300 bp. Chimeric sequences were then identified from the ITS sequence libraries using the Fungal Metagenomics Pipeline chimera tool (http://www.borealfungi.uaf.edu) provided by the University of Alaska Fairbanks. All potentially chimeric reads were flagged and excluded from downstream analysis. Sequences from all samples were combined in one single file and clustered into operational taxonomic units (OTUs) at 97% similarity using CD-HIT-EST (Li and Godzik 2006). Identities were assigned to the OTUs using the UNITE database's 454 pipeline (Tedersoo et al 2010) by submitting representative sequences for BLAST. Hits with BLAST scores ≤ 200 or query percentage of alignment $\leq 60\%$ were considered to represent unknown or unclassified fungi. The three biological replicates for each treatment were grouped for calculations on Theta-YC (Yue and Clayton, 2005) similarity metrics, and neighbor-joining tree based on Theta-YC values in MOTHUR v.1.20.0 (Schloss et al., 2009). The most abundant OTUs (top 200), that represented the majority (97%) of all the sequences produced, were selected for community taxonomic composition descriptions at genus level.

Since a number of biological diversity and richness estimators tended to suffer from sample size bias (Magurran, 2004), we "re-sampled" our fungal sequence libraries

by using sub.sample function in MOTHUR resulting in randomly selected sequences from each library with equally sized sequence numbers. Only the community diversity indices and richness estimators were calculated based on these reduced sized libraries. Shannon and inverse Simpson diversity indeces and ChaoI richness used in our study were previously described by Schloss et al. (2009). Sequence coverage refers to how well the sub-sampled libraries represent the original whole data set for each sample.

3.2.8. Statistical analysis

Variation in community qPCR values and fungal community composition at the genus level among amendment types and over time were assessed using SAS version 9.2 (SAS Institute Inc., 2003). Proc GLM was used to test individual treatment significance. Pair-wise treatment mean comparisons were made using Least Significance Difference (LSD) when treatment was shown to be significant. Unless otherwise indicated, all statistical significance levels were set as $P \leq 0.05$. Values of qPCR were log-transformed, and fungal genus composition values were arcsin transformed prior to analysis.

Nonmetric multidimensional scaling (NMDS) of the fungal communities based upon OTU composition was carried out using the Bray-Curtis similarity metric in the PAST (Paleontological Statistics, University of Oslo) software package, version 2.08 (Hammer et al, 2001). Data are presented using the means of biological replicates for each treatment with error bars represented for standard deviation among the three replications. Data from Biolog EcoPlate™ analysis were subject to principal component

analysis (PCA). Ordinations of replicate-level CLPP values for each treatment at different time points were performed for the first two principal components which loaded most of the variance of original data. A PCA biplot was created to show how each category of C source contributed to the separation among treatments.

Heatmaps were used to show the relative abundances of fungal genera for each amendment type and time point. To create the graph, values of the mean across three biological replicates for each treatment were used with heatmap function included in the gplots package for R version 2.13.0. The colored rectangles for each taxonomic group represented sequence abundances relative to the mean of all samples. All treatments were clustered with Euclidian distance-based hierarchical agglomerative clustering.

3.3. Results

3.3.1. SM and wheat straw chemical composition

The chemical compositions of the SMs, as previously determined (Hu et al., 2011) and wheat straw used in this experiment are summarized in Table 3.2. All SMs generally had very high concentrations of C, N, P, and K. The C:N ratios of SMs ranged from 7.4 for camelina to 13.6 for jatropha. The brassicaceous SM (camelina) had higher concentrations of P, S and Zn than did the other SMs. Greater Ca, however, was detected in jatropha SM. Other elemental concentrations such as Na, Mg, Cu, Fe, Mn, and B were comparable among different SMs. Wheat straw had a different chemical composition profile than the SMs. It had similar levels of C and K, but much lower amounts of N and

P than the SMs did. The C:N ratio of the wheat straw was 32, which was much higher than that of the SMs. Other elemental contents such as Ca, S, Mg, Zn, Cu, and B in wheat straw were also lower, but Fe concentration was much higher (3-6 times more) than the selected SMs.

Table 3.2. Selected elemental concentrations of SMs of jatropha, camelina, flax, and wheat straw.*

Biomass	C	N	P	K	Ca	S	Mg	Na	Zn	Fe	Cu	Mn	B
Type	---------- g kg^{-1} ----------								---------- mg kg^{-1} -------				
Jatropha	477	35	6.2	12.9	9.6	2.3	5.2	1.1	31	42	21	39	30
Camelina	439	59	10.1	14.5	5.3	12.3	4.2	0.4	71	53	12	42	24
Flax	491	51	6.8	10.3	3.0	3.3	5.1	0.7	38	33	13	34	27
Wheat	416	13	1.1	16.0	2.6	1.1	1.0	1.3	15	194	7	53	<9

(* Modified from Hu et al., 2011)

3.3.2. Abundance of soil bacterial and fungal populations

Within 7 days, SM application enhanced soil fungal abundance dramatically (~40-fold) compared with the unamended control (Fig. 3.1-A). Wheat straw addition also increased the soil fungal population though to a smaller extent (~10-fold at day 7). Soil fungal abundance dropped significantly at day 14 and then stabilized maintaining a relatively lower level after day 21 for all treatments, though flax and camelina SM amended treatments still contained significantly higher fungal populations compared with the control.

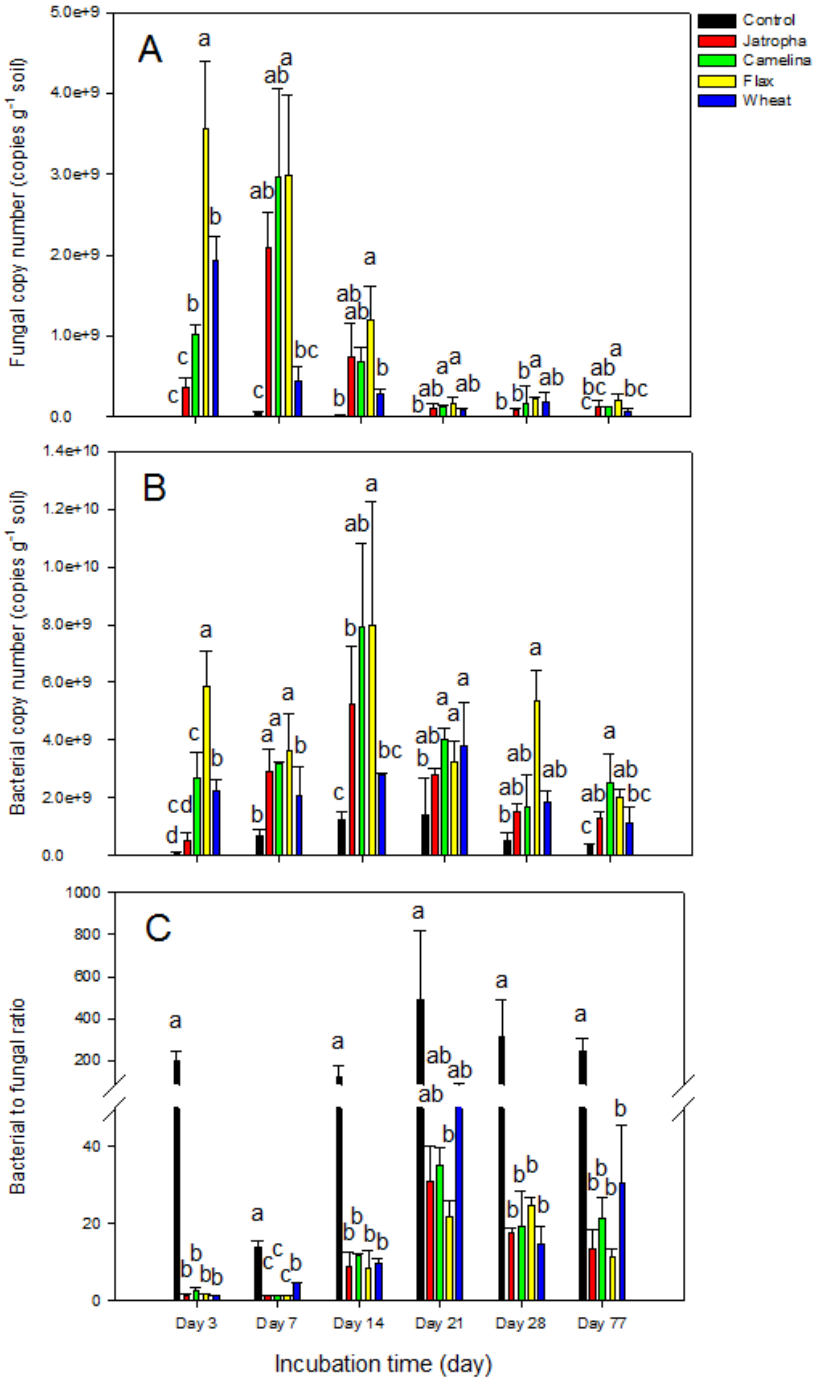

Fig. 3.1. Microbial abundance by qPCR in 1% (w/w) organic material (SMs of jatropha, camelina, flax, and wheat straw) treated Weswood loam soil after 3, 7, 14, 21, 28, and 77 days of incubation at 25 ℃. (A) Soil fungal copy number. (B) Soil bacterial copy number. (C) The ratio of soil bacterial to fungal copy number. Different letters indicate significant difference at $P<0.05$ within each day. Bars represent the means of three biological replicates for each treatment, and error bars represent the standard deviation among biological replicates.

Soil bacterial abundances were also increased following SM amendments compared with the control though to a smaller extent (4- to 7-fold at day 14) than for fungi (Fig. 3.1-B). There was not a clear temporal trend for soil bacterial responses in abundance during the 77 days of incubation. Nevertheless, bacterial populations seemed to peak in most treatments at day 14, which was 1 week later than the peak for fungal populations. At day 77, soil bacterial abundance was still significantly higher in all three SM-amended soils than the unamended control. Across time, wheat straw amended soil yielded bacterial abundances that were higher than the control but lower than the SM treatments.

Oilseed meal application resulted in significantly decreased soil bacterial to fungal ratios (75- to 150-fold at day 3) compared with the unamended treatment throughout the entire experiment (Fig. 3.1-C). The ratio tended to increase over time and then stabilized after day 21. At day 77, the soil bacterial to fungal ratio was still significantly lower in SM amended soils than unamended control. Similar to SM treatments, wheat straw amendment also resulted in a decreased bacterial to fungal ratio compared to the unamended control.

3.3.3. Soil fungal community composition

The NMDS analysis indicated that amendment of soil with the SMs of jatropha, camelina, and flax altered the soil fungal community composition (Fig. 3.2). At day 3 when soil fungal abundances had been promoted by SMs, fungal community composition in all three SM-amended treatments were significantly different from the

unamended control (Fig. 3.2-A). However, by day 21 when both the soil fungal and bacterial populations stabilized, the soil fungal communities were very similar in all three SM treatments and the control (Fig. 3.2-B). In contrast, the wheat straw amendment also altered soil fungal community composition compared with the unamended control, but the shift was significantly different from that for the SMs and persisted throughout the 77 days of the experiment.

The soil fungal community composition as described by Theta-YC similarity metrics also showed that regardless of time of incubation, all three SMs altered the fungal community composition (Fig. 3.3). All fungal communities grouped by amendment type with camelina being most similar to flax, then the control, jatropha, and wheat amendments (in order of decreasing similarity). Within each treatment, the fungal communities from day 21 and day 77 were generally more similar to each other than the communities at day 3. One exception was the jatropha SM amended soils, where more similarity was shared between day 3 and day 77 samples, though their differences from day 21 samples were relatively small.

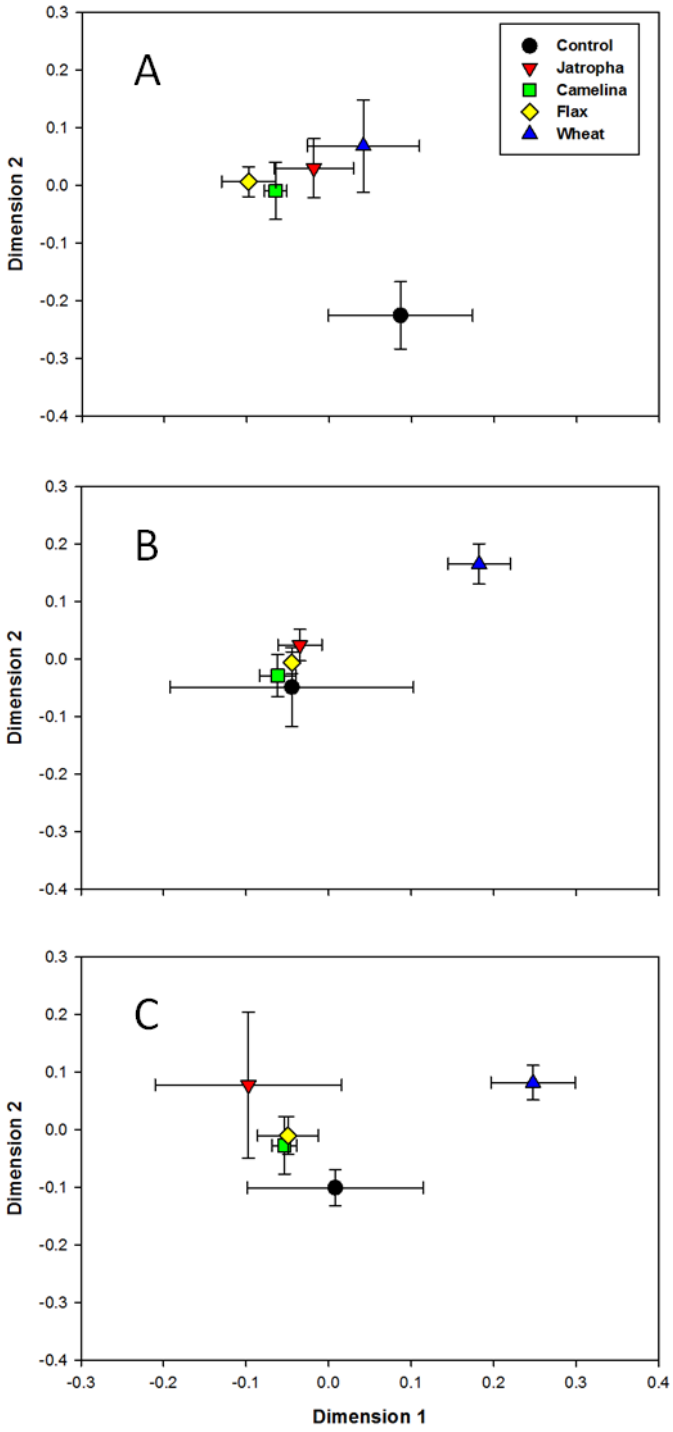

Fig. 3.2. NMDS ordination based on 1741 OTUs in 1% (w/w) organic material (SMs of jatropha, camelina, flax, and wheat straw) treated Weswood loam soil after 3 (A), 21 (B), and 77 (C) days of incubation at 25 ℃. Symbols represent the mean ordination of 3 biological replicates in each treatment, and the error bars represent the standard deviation among biological replicates.

As suggested by results from NMDS and Theta-YC, soil fungal taxonomic distribution patterns were also altered by SM applications, and these changes varied over time (Table 3.3, Fig. 3.4, and Fig. A-3.1). All three SMs decreased soil fungal diversity compared with the unamended soil at all 3 time points (Table A-3.1). *Ascomycota* (>90%) was the dominant phylum of classified fungal groups in all treatments and the fungal genera shown in Table 3.3 all belonged to this phylum. *Fusarium* was the dominant genus detected in all SM treatments as well as the unamended control across time (Fig. 3.4). Oilseed meals of camelina and flax, which greatly enhanced soil fungal abundance at the early stages of incubation, showed very similar proportions of *Fusarium* that was significantly higher than the unamended control.

The *Fusarium* was mainly (~90%) composed of three species that were most closely related to *F. equiseti*, *F. brachygibbosum*, and *F. oxysporum* in our microcosms (Table A-3.2 and Fig. A-3.2). Camelina and flax SM amendments resulted in greater levels of all 3 *Fusarium* species, indicating a general enhancement of *Fusarium* without species selectivity. In contrast, the *Fusarium* composition in jatropha SM treatments was very similar to the control at the genus level. However, at the species level, the relative level of *F. brachygibbosum* was increased by jatropha SM.

Another interesting result from the jatropha SM amendment was the relatively large proportion (40-50%) of unclassified fungi (most closely related to *Chaetomium*). The fungal taxonomic distribution for wheat straw on the other hand, was significantly different from both the unamended control and all the other three SM amended soils (Fig. 3.4). The dominant fungal genera in wheat straw treatments were *Schizothecium*

and *Humicola* in addition to *Fusarium*, with the *Fusarium* composition being much lower than in the control and SM-amended microcosms. The relative abundance of unclassified fungi was relatively high (40-60%) in some of the time points, but a large proportion of these unclassified fungi were found to be most closely related to either *Schizothecium* or *Humicola* albeit with a low percentage of the query sequence being covered by the BLAST alignment.

3.3.4. Soil community level physiological profiles (CLPP)

Microbial communities in SM and wheat straw treated soils were generally able to utilize all the 31 types of C sources. The highest average C utilization sampled from day 3 to 28 was observed in C sources of galacturonic acid, acetyl glucosamine, mannitol, L-asparagine, cellobiose, methyl glucoside, and glucose-phosphate sources. After 77 days of incubation, the most utilized C sources were similar to those at the earlier days with more complex C sources including malic acid, pyruvic acid methyl ester, and Tween 80 being more utilized by soil microorganisms (data not shown).

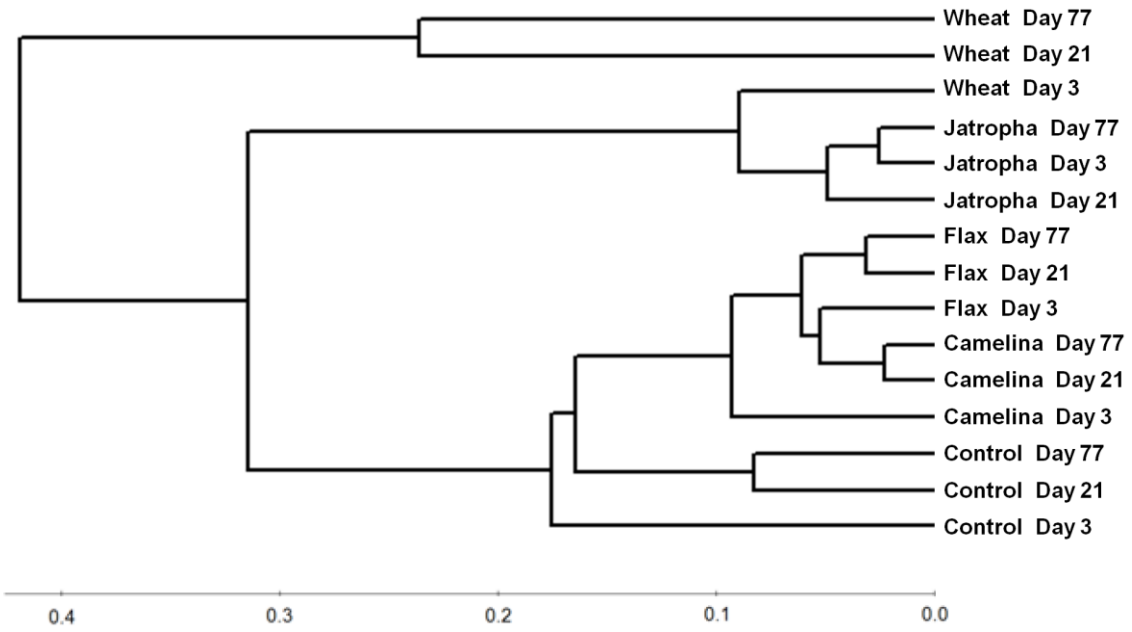

Fig. 3.3. Neighbor joining tree based on Theta-YC similarity metrics for a Weswood loam soil amended with different organic amendments including SMs of jatropha, camelina, flax, and wheat straw as well as unamended control at sampling time points of 3, 21, and 77 days of incubation at 25 ℃. Biological replicates for each treatment were treated as one group to calculate the Theta-YC similarity metrics. Analysis was based on 1741 operational taxonomic units (OTUs) clustered at 97% sequence identities.

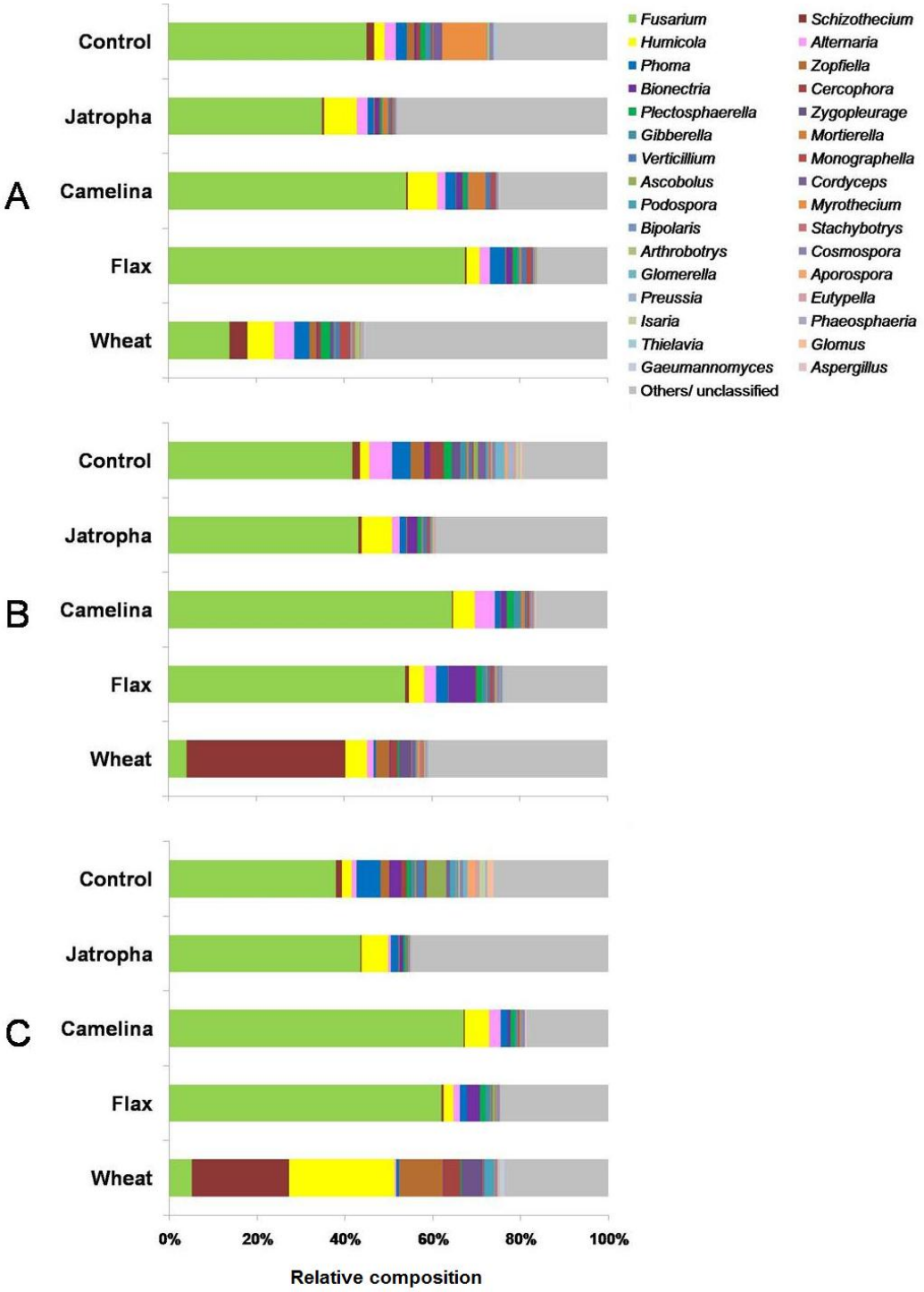

Fig. 3.4. Fungal OTU distribution patterns summarized at the genus level in Weswood loam soils treated with SMs of jatropha, camelina, and flax, as well as wheat straw and unamended control after 3 (A), 21 (B), and 77 (C) days of incubation at 25 ℃. Bars represent the means of relative abundance of different OTUs of 3 biological replicates in each treatment at each time point.

Table 3.3. Fungal OTU composition at the genus level in Weswood loam soils treated with SMs of jatropha, camelina, and flax, as well as wheat straw and unamended control after 3, 21, and 77 days of incubation at 25 ℃. Values represent the means of 3 biological replicates in each treatment.

Genus	Control	Jatropha	Camelina	Flax	Wheat	Control	Jatropha	Camelina	Flax	Wheat	Control	Jatropha	Camelina	Flax	Wheat
	Fungal OTU Composition %														
	Day 3					Day 21					Day 77				
Fusarium	45.07b*	34.94b	54.19ab	67.53a	13.82c	41.85c	43.24c	64.59a	53.85b	4.07d	37.95b	43.53b	67.03a	61.95a	5.07c
Schizothecium	1.81b	0.44b	0.32b	0.36b	4.21a	1.82b	0.76b	0.22b	0.96b	36.20a	1.31b	0.17c	0.21c	0.53bc	22.15a
Humicola	2.34b	7.47a	6.75a	2.98ab	6.05a	2.00b	6.94a	4.92ab	3.39ab	4.89ab	2.26c	6.06b	5.64b	2.20c	24.12a
Alternaria	2.59ab	2.51ab	1.81b	2.32ab	4.53a	5.28a	1.77bc	4.68ab	2.79abc	1.53c	1.11bc	0.65cd	2.55a	1.48ab	0.35d
Phoma	2.44a	1.32a	2.42a	3.46a	3.53a	4.25a	1.48bc	1.19cd	2.69ab	0.51d	5.43a	1.75b	1.60b	1.57b	0.64b
Zopfiella	1.79a	0.28b	0.12b	0.25b	1.56a	3.01a	0.25b	0.07b	0.16b	3.11a	2.05b	0.28bc	0.03c	0.03c	9.99a
Bionectria	0.41ab	1.01ab	1.35a	1.39a	0.20b	1.36b	2.21b	1.30b	6.10a	0.19c	2.70ab	0.88bc	0.59bc	2.99a	0.09c
Cercophora	0.96a	0.27a	0.13a	0.15a	0.80a	3.15a	0.12b	0.07b	0.17b	1.70a	1.27b	0.01c	0.10bc	0.05bc	3.85a
Plectosphaerella	1.10a	0.31a	0.87a	0.99a	2.07a	1.88a	0.72ab	1.70ab	1.20ab	0.35b	0.84a	0.43a	0.91a	1.17a	0.32a
Zygopleurage	0.00c	0.01bc	0.05b	0.05bc	0.74a	1.83a	0.04b	0.00b	0.01b	2.67a	0.13b	0.00b	0.04b	0.00b	4.78a
Ascobolus	0.17a	0.01b	0.01b	0.01b	0.01b	1.03a	0.00a	0.05a	0.01a	0.02a	4.50a	0.10a	0.04a	0.66a	0.01a
Cordyceps	1.95a	0.22a	0.18a	0.27a	0.31a	1.69a	0.28b	0.16b	0.30b	0.42b	0.87a	0.03b	0.12b	0.05b	0.06b
Cosmospora	0.78a	0.11b	0.11b	0.17b	0.12b	0.85a	0.08bc	0.27b	0.26b	0.05c	0.80a	0.01b	0.09b	0.07b	0.02b
Glomerella	0.05a	0.00a	0.01a	0.01a	0.01a	2.09a	0.01b	0.01b	0.00b	0.02b	0.94a	0.00b	0.00b	0.01b	0.00b
Aporospora	0.00c	0.05b	0.10ab	0.05b	0.16a	0.77a	0.18ab	0.04b	0.06b	0.05b	1.80a	0.03b	0.07b	0.01b	0.08b
Isaria	0.00c	0.00b	0.00bc	0.00c	0.02a	0.66a	0.02ab	0.02b	0.01b	0.06b	1.33a	0.02b	0.00b	0.02b	0.31b
Others /unclassified	38.54a	51.04a	31.59a	20.01a	61.87a	26.49a	41.91b	20.70b	28.03b	44.15b	34.70a	46.05c	20.98c	27.22c	28.15b

*Different letters indicate significant difference within each row at a specific time point.

Soil microbial functional patterns of CLPPs based upon C source utilization indicated by principal component analysis (PCA) were significantly affected by SM additions (Fig. 3.5). All three SMs (jatropha, camelina, and flax) resulted in significantly different CLPPs from the control from day 3 through day 77. However, by day 133, the three SM treatments had CLPPs similar to the unamended soil. Wheat straw on the other hand, produced a CLPP similar to the three SMs at an early stage of the experiment (up to 7 days) that slowly diverged (14 to 77 days) and then became similar to the unamended control and SM-treatments at the end of the experiment (133 days). A PCA biplot was created to show how each of the six categories of C sources (Table 3.1) contributed to the separation among treatments. The cumulative percentage of variation explained by principal components 1 and 2 ranged from 29.5% to 50.7%. At day 3, we found complex C and phosphate-associated C sources were the main contributors for the separation of organic amendments from the unamended control (Fig. 3.5-A). At day 7, the primary C sources contributing to CLPP separation were slightly different from day 3, with greater utilization of carboxylic acids and carbohydrates differentiating the organic amendments away from the unamended control (Fig. 3.5-B). By day 14, there was a differentiation between the SMs and wheat straw with the SMs observing greater utilization of phosphate-associated C and amines and the wheat straw having greater utilization of carbohydrates. This pattern continued with the wheat straw producing greater levels of utilization of carbohydrates and also phosphate-associated C at days 28 and 77. Likewise, the pattern continued for the SMs at days 28 and 77, with utilization of amines, amino acids, and then carboxylic acids being most positively impacted. At the

end of the experiment (day 133), there was no substantial separation in CLPP among treatments (Fig. 3.5-F).

3.4. Discussion

3.4.1. Soil fungal and bacterial abundance enhanced by SMs

The incorporation of jatropha, camelina, and flax SMs increased soil microbial abundance, especially fungi, which is not surprising given the high nutrient content (C, N, P etc.) present in the SMs. Previous studies found similar positive responses in soil microbial abundance due to SM applications although the researchers used different measuring techniques. For example, Cohen et al. (2005) reported increased soil fungal and bacterial populations caused by soybean and rapeseed meals through use of culture-based methods. Moreover, Wang et al. (2012) found that soil microbial abundance was increased by mustard and flax SMs revealed from total lipid fatty acid methyl esters (TL-FAME) analysis. Likewise, the addition of the SMs enhanced soil microbial activities with C mineralization rates increasing rapidly after biomass application to soil (Wang et al., unpublished). A similar increase in soil respiration after SM amendments was reported by Wang et al. (2012). As compared with the soil respiration data which indicated only a transient elevated soil C mineralization rate due to addition of SMs (Wang et al., unpublished), the increase in soil microbial abundance was more persistent and significant over time.

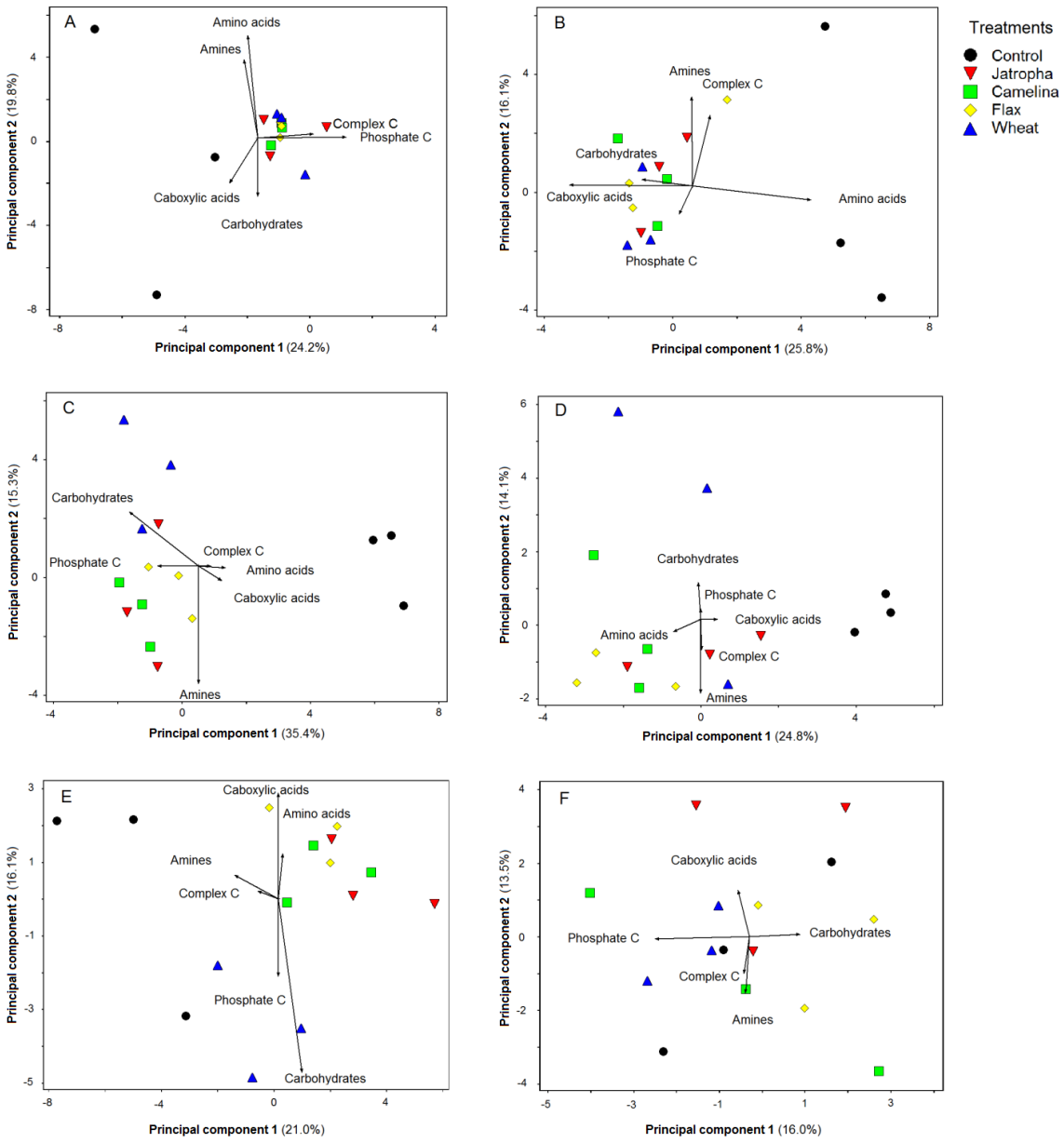

Fig. 3.5. Principal component analysis (PCA) on Weswood loam soil microbial CLPP indicated by Biolog EcoPlate™ with C sources grouped into carboxylic acids, complex C, carbohydrates, phosphate-containing C, amino acids, and amines after 3 (A), 7 (B), 14 (C), 28 (D), 77 (E), and 133 (F) days of incubation at 25 ℃. Treatments included unamended control, 1.0% (w/w) SMs of jatropha, camelina, and flax, and wheat straw. Symbols represent mean values from each replicate-level sample.

Comparing the roles of soil bacteria and fungi in soil nutrient cycling and organic matter degradation, the soil fungal communities appeared to have been more greatly impacted by the SMs and were likely more critical to SM degradation (De Boer et al., 2005). Soil fungal abundances increased (~40 fold) much more than bacterial abundances (4-7 fold) by addition of the SMs. This resulted in the ratio between soil bacterial and fungal abundances to be decreased suggesting that soil fungi were more responsible for the decomposition of added SMs than were soil bacteria. Wheat straw, which was used as a non-SM organic amendment in our study, also increased soil fungal abundances temporarily but to a much lesser extent. This is likely due to the wheat straw's significantly higher C:N ratio compared with SMs. Soil bacterial abundances in the oilseed amended treatments peaked at day 14, after soil fungal abundances had decreased. This delayed response may have resulted from bacteria feeding on living or dead fungal hyphae (Boer et al., 2001), or because of less competition for resources from the largely decreased number of fungi.

Besides their roles as energy and nutrient resources for soil microorganisms, selected SMs could also serve as biofumigants since they can inhibit specific groups of soil microorganisms due to production of biocidal compounds (Mazzola et al., 2007; Hu et al., 2011). Our fungal abundance data showed some evidence for a biofumigation effect by the camelina SM, which contained a moderate level of GLS (Hu et al., 2011). The fungal abundances peaked at day 3 in the flax treatment (no GLS) but did not peak until day 7 in the camelina treatment. This delayed peak in fungal abundances in the camelina SM treatment may have resulted from the release of fungicidal chemicals such

as ITCs from the GLS. However, other studies have reported that such chemicals do not always produce negative impacts on soil total fungal abundance. For example, Wang et al. (2012) reported that soil amended with mustard SM that contained high levels of allyl GLS (Hu et al., 2011) resulted in a significantly higher fungal abundance than did flax at the early stages of incubation. However, this appeared to be due to selection and proliferation of a small number of specific fungal groups that were apparently more resistant to ITCs.

3.4.2. Soil fungal community structural shifts by SMs

Soil fungal community composition was altered by addition of SMs, and these shifts were relatively persistent over time. One explanation for these changes could be attributed to the nutritional impacts of the SMs. Numerous studies have reported microbial community structural changes, usually transient, following the input of various organic compounds (Aneja et al., 2006; Liu et al., 2007; Schlatter et al., 2009; Pascault et al., 2010). However, the impacts appeared to be somewhat persistent when using SMs as found in our study as well as by others (Wang et al., 2012). Although the differences in the composition of the soil fungal communities among the three SM amendments was not apparent as indicated by NMDS, we still found a trend that various SM amendments, especially jatropha versus camelina and flax, selected their unique fungal communities through time as indicated by Theta-YC similarities. Wheat straw treatment, on the other hand, also changed the soil fungal community composition but selected for a significantly different community than did the SMs. This is likely due to the much

higher C:N ratio and different elemental composition of the wheat straw. It is also interesting that wheat straw had a large impact on soil fungal community composition even though it did not alter fungal abundance much over time.

Another explanation for changes in soil fungal community composition by SM application could be attributed to biofumigation effects by allelochemicals released from selected SMs. A previous study by Wang et al. (2012) found significantly different microbial communities in soil amended with SMs of mustard (containing high concentration of allyl GLS) and flax (containing no GLS) that had similar nutrient compositions. However, there were generally no significant differences in soil fungal community structure between soils amended with SMs of camelina and flax throughout time in our study, although moderate levels of several types of GLS had been previously detected in the camelina SM (Hu et al., 2011). The only result that may suggest allelochemical impacts on soil fungal composition in camelina SM amendment would be its relatively higher dissimilarity at day 3 compared with flax SM treatment. Taken along with the previously discussed fungal abundance responses to camelina and flax SMs, these results suggest that relatively low concentrations of GLS-induced allelochemicals could reduce the overall soil fungal abundance temporarily but without having a large impact on fungal community composition.

One more explanation for shifts in the soil fungal community composition due to SM applications could be attributed to soil bacterial community responses and the interactions between the fungal and bacterial abundances. Previous studies showed that the incorporation of green manures and specific SMs could increase fungal antagonists

such as fluorescent *Pseudomonas* spp., and actinomycetes such as *Streptomyces* spp. (Mazzola et al., 2001; Cohen et al., 2005). Although we did not directly analyze soil bacterial community compositions, we did find significantly increased total bacterial abundances, which would result in direct competition with fungal groups for space and food, and possibly also include members that would be directly antagonistic toward soil fungi.

In terms of taxonomic identification of the soil fungal communities, ascomycetes were the dominant fungi detected. This is not surprising since they are the largest group of the true fungi (Larena et al., 1999) with their members consisting of both noxious plant pathogens and non-pathogenic saprotrophic fungi (Osono et al., 2003). Although biofumigation using plant residues has been reported to be a successful strategy for controlling many soil pathogens, it generally fails to suppress common plant pathogenic ascomycetes, although ascomycete abundances have been temporarily changed (Omirou et al., 2011). Similarly, in our fungal community study using SMs, we found that the ascomycete taxonomic diversity decreased as indicated by the Shannon and Inverse Simpson diversity indices (Table A-3.1). However, these changes were maintained until the late stages of incubation (day 77), suggesting a possible greater potential of SMs than green manures that contains high N to alter the composition of these fungal groups.

Among all of the ascomycete groups found in our soils, SMs of camelina and flax that are rich in N content mainly promoted *Fusarium* spp., which was also the dominant genus in SM amended and unamended soil. Such increase of *Fusarium* in our study may not be detrimental to the ecosystem since most *Fusarium* species in soil are

non-pathogenic and harmless to the environment. In addition, the soil in our study did not have a history of *Fusarium*-caused disease in the past. On the contrary, such increased *Fusarium* could be beneficial for agriculture since non-pathogenic *Fusarium* has been found economically and ecologically important and positively correlated to pathogenic *Fusarium* suppression in support of the competition theory for nutrients between non-pathogenic and pathogenic *Fusarium* (Scher and Baker, 1982; Alabouvette et al., 1985). Increasing soil N input by cultivation of cover plants has also resulted in promoting abundances of soil non-pathogenic *Fusarium* that contributed to soil suppressiveness to pathogenic *Fusarium*-caused disease (Alabouvette et al., 1996; Abadie et al., 1998). However, there were also documented members of noxious pathogenic *Fusarium* that could be increased by SMs such as some *F. oxysporum* that causes plant wilts (St-Arnaud et al., 1997; Abadie et al., 1998; Steinberg et al. 2007), *F. graminearum* that induces wheat head blight (Luz et al., 2003; Perez et al., 2008), and *F. proliferatum* that leads to asparagus crown and root rot (Seefelder et al., 2002; Hamel et al., 2005).

We found an overall increase in fungi similar to three dominant *Fusarium* species (*F. equiseti*, *F. brachygibbosum*, and *F. oxysporum*) following the addition of camelina and flax SMs. Jatropha SM, on the other hand, increased *F. brachygibbosum* but decreased or did not change the other two dominant *Fusarium* species (*F. equiseti* and *F. oxysporum*), thus resulting in an overall *Fusarium* relative abundance similar to the unamended soil. Previous research found similar results as ours using jatropha SM extracts and found it suppressive to some *Fusarium* spp. (Saetae and Suntornsuk, 2010).

Different results were reported in biofumigation studies using *Brassicaceae* plant materials with no change in *Ascomycota* structure including *Fusarium* being detected after soil amendment (Omirou et al., 2011).

Differences in the fungal composition in the jatropha amended soil were mostly due to the high percentages of unclassified fungi. Many of these fungi were most closely related to *Chaetomium*, some members of which have been used in biotechnological industry due to their high selectivity for assimilating polysaccharides, especially hemicelluloses such as xylan, as well as their ability to produce enzymes such as cellulase and laccase (Ankudimova et al. 1999; Mimura et al. 1999; Suyanto et al., 2003). Research by Suyanto et al. (2003) indicates the potential of particular *Chaetomium* in decomposition of palm-oil mill fiber, which was somewhat similar to our kernel-containing jatropha SM (Suyanto et al., 2003). Other *Chaetomium* have been demonstrated to assist biological control of particular pathogens through production of toxic metabolites and/ or competition for living space and nutrient resources (Aggarwall et al., 2004; Zhang and Yang, 2007; Syed et al., 2009; Kharwar et al., 2010). Similar results in enhanced *Chaetomium* were reported previously by Lang et al. (2011), where they amended soil with amino acid and manure composts that contained high N and found suppressed disease of Cotton *Verticillium* wilt with co-occurrence of several fungal groups including *Chaetomium*. The differential impact on *Fusarium* compared with camelina and flax SMs may come from phorbol esters contained in jatropha SMs, which have recently been demonstrated to be toxic to several notable fungal pathogens including *Fusarium* members (Saetae amd Suntornsuk, 2010).

Wheat straw amended soil had a unique profile with dominant fungal taxonomic groups of *Schizothecium*, similar to those that have been found in opossum and rabbit dung (Cai et al., 2005; Kwaśna et al., 2008), and *Humicola*, which are considered to be beneficial soil fungi with some representatives having been used to produce important enzymes for hydrolyzing lignocellulosic materials in the renewable energy industry (Lang et al., 2011). Although there were relatively high percentages of unclassified fungi in wheat straw amended soils at some time points, a great proportion was found to be most closely related to either *Schizothecium* or *Humicola*. Some of the similarity between the fungal communities selected for by the jatropha SM and the wheat straw may be due to the fact that the jatropha seeds were not de-hulled prior to processing. They therefore had a higher C:N ratio and greater content of recalcitrant C compounds than the other SMs. Overall, all of the SM amendments had less of an impact on soil fungal community composition than did wheat straw. Although the certainty of species identification from partial ITS sequencing should be viewed cautiously, these results do reveal differential impacts of the biomass treatments on various *Fusarium* spp. and other fungal abundances.

3.4.3. Soil microbial functional changes due to SM applications

Similar to the fungal community composition results, soil microbial function (CLPP patterns) was also changed by SM application, as indicated by PCA. These changes were relatively persistent, lasting through day 77. However, similar to the soil fungal community composition results, there were not major differences between the

three types of SMs. Wheat straw also changed microbial CLPP compared with the control, but in a substantially different way from the SMs, again also agreeing with the soil fungal community composition results. Although utilization of C sources in the Biolog EcoPlates do not necessarily represent *in situ* degradation of the C substrates in the microcosms, changes in the utilization patterns over time do indicate shifts in the capacity of the microbial communities to metabolize different C sources (Gomez et al., 2006). The most utilized C sources (galacturonic acid, acetyl glucosamine, mannitol, L-asparagine, cellobiose, methyl glucoside, and glucose-phosphate) shown in our results were comparable to those reported in other studies (Gomez et al., 2006; Weber and Legge, 2009; Chazarenc et al., 2010). It was expected that the C sources supporting higher microbial growth would be those easier to be utilized by microorganisms, such as elemental sugars or very simple amino acids rather than relatively complex C sources such as Tween 80.

The PCA biplots of the CLPP results indicated a temporal trend of shifts in microbial degradation of various types of organic materials (Fig. 3.5). At day 3, soil microorganisms in SM-applied microcosms tended to utilize more complex C and phosphate-associated C than the control. This trend shifted to carbohydrates and carboxylic acids by day 7 and then amines and amino acids at days 14-77. The apparent sequential hierarchy for C metabolism in the oilseed-amended microcosms was generally complex C > phosphate-associated C > carboxylic acids > carbohydrates > amines > amino acids. This suggests that the residual oils were degraded first followed by P-containing compounds, then the other C-containing compounds, and lastly the N-

containing compounds. The control at day 7 had been separated from all the organic amendments primarily by amino acids, which could be related to dry soil re-wetting consequences that may have been masked by excess available nutrient sources in organic treatments (Sorensen, 1974; Kieft et al., 1987; Miller et al., 2005; Cosentino et al., 2006). The wheat straw treatment was not significantly different from the SM amendments at early stages of the experiment, but showed increased utilization of carbohydrates and phosphate-associated C sources from day 14 to day 77. This is likely due to its different decomposition product profile (resulting from higher cellulose and hemicelluloses content) and lower N and P concentrations as compared with SMs.

3.4.4. Differences in soil microbial communities among different types of SM treatments

Previous studies have shown that biocidal chemicals such as isothiocyanates (ITCs) produced by many *Brassica* spp. can negatively impact soil microbial communities (Rumberger and Marschner, 2003). The SM of camelina used in our study added total GLS compounds at a concentration of approximately 23.5 nmol g^{-1} soil [23.5 nmol total GLS g^{-1} SM (Hu et al., 2011) applied at 1%], which could then hydrolyzed to several ITCs. Jatropha has also been reported to contain biocidal compounds such as lectins, ricin, trypsin inhibitor, and phorbol esters (Reddy and Pierson, 1994; Makkar et al., 1997) that have been demonstrated to be toxic to several important soil pathogenic fungi (Saetae and Suntornsuk, 2010). In our study, we did observe some inhibitory impacts on soil fungal population sizes and some influences on community composition in camelina and jatropha SM treatments early in the experiment (day 3). Similar impacts

have been discovered previously following ITC- producing SM application. Using the same soil as in our study, Hollister et al. (2011) found that *B. juncea* SM (157 nmol allyl GLS g^{-1} defatted meal) applied at 2.5% (w/w) that contributed 390 nmol g^{-1} allyl GLS in soil (>16 times higher than in our study) also delayed soil fungal proliferation compared with flax SM that contains no GLS. However, the composition of fungal community in their study was quite different from ours, with 87% to 98% of the community accounted for by a single genus of *Retroconis*, and this effect seemed to be persistent over time. The camelina SM used in our study was less selective on soil fungal members, which could be related to lower biological toxicity of released ITCs compared with the well-known highly toxic allyl ITC (Matthiessen and Shackleton, 2005) when such SMs were applied to soil.

Nevertheless, we did not find any large impacts of biocidal chemicals on microbial CLPP or mineralization rates (Wang et al., unpublished). Similar to our findings, Snyder et al. (2010) did not detect any inhibitory effect on soil CO_2 efflux after applying SM of *B. napus* that was estimated to add GLS at a concentration of 29.5 nmol g^{-1} soil, which is comparable to the GLS application rate for the camelina SM in our experiment (23.5 nmol g^{-1} soil) (Hu et al., 2011). Nevertheless, similar to our soil fungal community composition responses, higher biocidal chemical concentration could lead to significant changes in soil microbial activity. Previous studies showed that SMs such as *B. juncea* and *Sinapis alba* that added high levels of GLS (150-340 nmol g^{-1} soil) have been reported to temporarily inhibit soil microbial activity (Snyder et al., 2010; Wang et al., 2012; Hu et al., 2011). Despite the impacts on specific microbial abundances and

fungal community composition, it appears that the microbial communities in our study adapted and were functionally redundant resulting in little differential impacts of the biocidal SMs (camelina and jatropha) on overall microbial activity and function as compared to the non-biocidal SM (flax).

The larger and more long-lasting impacts on soil microorganisms appeared to be due to other characteristics of the added biomass such as nutritional composition (e.g., C:N ratio). For example, the jatropha SM that had a higher C:N ratio than camelina and flax due to seeds not being de-hulled prior to processing resulted in microbial impacts that, in many instances, shared more similarity with the wheat straw treatment than the other SMs (camelina and flax). The other nutritional impacting factor could be SM application rate, which resulted in varied amounts of total available nutrients. A similar study conducted by Hollister et al. (2011) using the exact same soil and flax SM under the same growing conditions but at a higher application rate of 2.5% found a largely differentiated soil fungal community structure compared with ours. Based on their results, *Microdochium* and *Bionectria* dominated their flax SM treatment in addition to *Fusarium*. However, these two genera were absent from all of our amendments.

3.5. Conclusions

Application of SMs to soil rapidly increased microbial, especially fungal, abundances. The composition of the fungal community was also impacted by SM additions with the resulting shifts being relatively persistent over time. Each of the SMs selected for specific fungal groups such as *Fusarium* in camelina and flax treatments and

Chaetomium in the jatropha treatment. In addition to changes in the soil fungal community composition, the SMs altered microbial CLPP patterns, suggesting that changes in microbial population size and composition also impacted the functionality of the soil microbial community.

The different SM amendments seemed to result in slightly varied soil microbial community responses depending on either the allelochemicals involved, nutrient composition (e.g., C:N ratio), or both. Compared with flax SM that contained no biocidal chemicals, the camelina and jatropha SMs produced a smaller increase in soil fungal abundances in the first few days of the experiment. In addition, the composition of the soil fungal community was also differentially impacted by the camelina and jatropha SMs at early stages of the experiment. Nevertheless, these transient impacts on population size and community composition did not result in discernible impacts on microbial community functionality – likely due to functional redundancy of the microbial community and/ or relatively low concentrations of biocidal chemicals in the SMs used in this experiment. The more persistent differential impacts of the various SMs appeared to be due to variable chemical composition (e.g., C:N ratio) of the amendments. For example, the camelina and flax SMs selected for a fungal community more similar to each other over time than to the jatropha SM which had a higher C:N ratio and several other properties that were in many ways intermediate between the other SMs and the wheat straw (lignocellulosic comparison). However, even this impact on soil functionality was diminished by the end of the study with the amended microcosms not being different than the unamended control.

These findings elucidate the impacts that various SMs have on soil microbial abundance, community composition, and functionality and also highlight the critical role that the soil fungal community plays in the decomposition of organic amendments.

CHAPTER IV

SOIL MICROBIAL COMMUNITY CHANGES DUE TO THE APPLICATION OF ISOTHIOCYANATES

4.1. Introduction

With increased production of biodiesel to supplement traditional fossil fuels, greater amounts of SMs, which are the by-products (residual) remaining after oil extraction from oilseeds, will be produced. Many of these SMs contain biocidal compounds. For example, many *Brassicaceae* SMs contain GLS, which can be degraded by plant and/ or microbial enzymes upon incorporation into soil. Resultant biocidal products of hydrolysis of GLS include isothiocyanates (ITCs), nitriles, organic thiocyanates, SCN^-, oxazolidinethione, and epthionitriles (Cole, 1976; Borek and Morra, 2005), which have been reported to contribute to control of soil pathogens such as *Phymatotrichopsis omnivora* (Duggar) Hennebert (Hu et al., 2011), *Aphanomyces euteiches* f. sp. *pisi* (Smolinska et al., 1997), *Rhizoctonia* spp. (Cohen et al., 2005), and other pathogens.

Among all of the allelochemicals produced from *Brassicaceae*, the ITCs have received particular attention due to their high biological toxicity. Several studies have investigated the effects of pure ITCs on a wide range of soil fungi and bacteria (primarily plant pathogens), in which a general suppression effect was discovered, and the sensitivity varied among different microbial groups and ITC types (Kirkegaard et al., 1996; Smolinska et al., 1997; Manici et al., 2000; Bending and Lincoln, 2000; Smith and

Kirkegaard, 2002; Hu et al., 2011). These studies provided very important information for determining relative effects according to the specific organism and ITC type; however, most of these studies were conducted on pure cultures of organisms instead of organisms within their natural environment, e.g., soil. Impacts of the various ITCs may be very different within the soil environment due to complex interactions with soil and phase-partitioning of ITCs (Borek et al., 1998; Matthiessen and Shackleton, 2005). Moreover, studies adding only pure ITCs would not resemble real-world biofumigation strategies where the ITCs would be added in the form of plant biomass (e.g., SMs). Studies adding only pure ITCs would miss the impacts from SMs, which alone could change soil microbial abundances and community structure during decomposition (Hollister et al., 2011; Baldrian et al., 2011).

Most studies investigating the impacts of ITCs on soil microbial communities have been focused upon bacteria (Lauber et al., 2009), even though soil fungi may be more sensitive to ITCs and organic amendments, and are likely to be of as much, or even greater, importance than bacteria to many soil processes (Smith and Kirkegaard, 2002; De Boer et al., 2005; Baldrian et al., 2011). This situation may be at least partly due to the lower historical emphasis placed upon the taxonomy of fungal communities and resulting lower number of taxonomic tools available for describing fungal species, estimating their diversity, distinguishing individual taxa, and understanding the ecological roles that various fungi play (Hawksworth, 2001; Bailey et al., 2002). Futhermore, the handful of studies that have investigated the impacts of ITCs on soil fungal composition have used low-resolution techniques such as fatty acid methyl ester

analysis which provided information regarding community shifts but little-to-no information regarding which specific organisms were being impacted (Wang et al., 2012). To our knowledge, no study has been published that details the impacts of various ITCs on soil fungal community composition at a taxonomic-level.

In order to build upon these previous studies that used pure ITCs only and/ or pure cultures, we applied various pure ITCs in the presence of flax SM to soil and focused on both soil fungal and bacterial community changes following amendment. The specific objectives of this study were to determine the impacts of different ITCs, in the presence of flax SM, on soil fungal and bacterial abundance and community composition through time.

4.2. Materials and Methods

4.2.1 Soil and SM of flax

Weswood loam (fine-silty, mixed, superactive, thermic, Udifluventic Haplustept) was used in this study (low nutrients and organic matter). It is an alluvial soil in the flood plain of the Brazos River in south central Texas. Weswood soils are well drained loamy soils and are used as irrigated cropland (USDA NRCS, 2008). Bulk soil samples were collected from 0-15 cm depth and then homogenized and passed through a 2-mm sieve. The soil water content was then determined by oven-drying a subsample of 20 g of field moist soil for 24h at 105 ℃ and calculated to be 14.4% (w/w). Soil samples were incubated at room temperature (~24 ℃) for 24 h before use. Soils were tested for

total C, organic C, and total N by a combustion method using an Elementar Vario Max CN analyzer (Elementar Analysensysteme, Hanau, Germany) (Storer, 1984; McGeehan and Naylor, 1988; Schulte and Hopkins, 1996). Organic C was determined at 650 ℃ while Total C was determined at 950 ℃. Soil phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), and sodium (Na) were extracted with Mehlich III solution and analyzed by inductively coupled plasma (ICP) spectrometry (Mehlich, 1978; 1984). Soil micronutrients including copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn) were extracted using a 0.005 M DTPA, 0.01 M $CaCl_2$ and 0.1 M triethanolamine solution mixture and determined by ICP (Lindsay and Norvell, 1978). Soil particle size distribution was determined using the hydrometer method (Day, 1965).

Oilseed meal of flax (*Linum usitatissimum* L.) was obtained by processing seeds with a Komet Oil Press (Model CA59, IBG Monforts Oekotec, Germany). The resulting flax SM was ground with a mortar and pestle and passed through a 1-mm sieve. The water content of SMs was determined by drying sub-samples at 60 ℃ for three days. Organic C and total C and N in the SMs were determined by a high-temperature combustion process using an Elementar Vario Max CN analyzer (Nelson and Sommers, 1973; Sheldrick, 1986; McGeehan and Naylor, 1988; Sweeney, 1989). Organic C was determined at 650 ℃ while total C was determined at 950 ℃. Plant B, Ca, Cu, Fe, K, Mg, Na, P, S, and Zn were determined using a nitric acid digestion and ICP analysis (Isaac and Johnson, 1975; Havlin and Soltanpour, 1989).

4.2.2 Experimental Plan

This was a laboratory microcosm study investigating soils treated with different types of ITCs, including allyl ITC (Acros Organics, New Jersey, USA), butyl ITC (Alfa Aesar, MA, USA), phenyl ITC (MP Biomedicals, Ohio, USA), and benzyl ITC (Acros Organics) at a concentration of 50 μg ITC g^{-1} soil. Each treatment had three replications, and there were three controls receiving no ITC but sterile water. The microcosms were set up in 127.6 cm^3 sterile specimen containers (VWR International, LLC., Sugar Land, TX, USA) filled with 57.2 g (50 g dry soil equivalent) fresh soil. A total of 0.52 g (0.5 g dry SM equivalent) flax SM was then added to each of the microcosm including the three controls. ITC stock solutions were prepared by adding 10 mg pure ITCs into 1.0 ml sterilized water and vortexing for 1 min to homogenize before adding to the microcosms to generate an initial ITC concentration of 50 μg g^{-1} soil. The lids on the microcosms were loose to assure aerobic condition, and the microcosms were incubated at 25 ℃ for 28 days. A subsample of 2 g soil were collected at days 2, 7, 14, 21, and 28 and stored at -80 ℃ until DNA extraction. Soil moisture was adjusted to 14.4% every 24 hours by addition of sterilized water.

4.2.3 DNA extraction and quantification

Community DNA was extracted from 0.5 g aliquots of each soil sample using a PowerSoil DNA extraction kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). Extracted DNA was purified with illustra MicroSpin S-400 HR columns (GE Healthcare Bio-Sciences Corp, Piscataway, NJ, USA), and quantified using both a NanoDrop ND-

1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and a Quant-iT PicoGreen dsDNA assay kit (Invitrogen Corp, Carlsbad, CA, USA). Data generated from the latter was used in the analysis.

4.2.4 qPCR on general bacteria and fungi

Community qPCR assays, based upon Fierer et al. (2005) and Boyle et al. (2008) were used to evaluate the relative abundances of general bacteria and fungi in the microcosm communities. Assays were performed in triplicate, using a Rotor-Gene 6000 series thermal cycler (Qiagen, Valencia, CA, USA). For general bacterial and fungal qPCR, each 15 μL reaction contained: 6.75 μL 2.5x RealMasterMix with 20x SYBR solution (5Prime, Inc., Gaithersburg, MD, USA), 1.5 μL BSA (10 mg mL^{-1}), 0.75 μL of each primer (10 μM,), 0.25 μL molecular-grade water, and 5.0 μL template DNA (1.0 ng μL^{-1}). Thermocycling consisted of an initial denaturation at 95 ℃ for 15 min, followed by 40 cycles of 95 ℃ for 1 min and annealing temperature at 53 ℃ for 30 s, and 72 ℃ for 1 min. Primer sets of Eub338/518 (Fierer et al., 2005) and 5.8S/ ITS1F (Boyle et al., 2008) were used for bacteria and fungi respectively. Plasmid standards for the bacterial and fungal relative abundance by qPCR were generated as described by Somenahally et al. (2011).

4.2.5 Fungal and bacterial tag-encoded amplicon pyrosequencing and analysis

Purified community DNA samples were submitted to the Research and Testing Laboratory (Lubbock, TX, USA) for tag-pyrosequencing using 454 GS FLX titanium

technology (454 Life Sciences, Branford, CT, USA). The fungal ITS region was amplified using primers ITS1F and ITS4 for the initial generation of the amplicons (Amend et al., 2010), and fungal amplicons were sequenced in the forward direction, generating reads from ITS1F. Bacterial 16S rRNA genes were sequenced in a similar manner as the fungal sequences substituting primers 530F and 1100R as described by Acosta-Martínez et al. (2008) to generate initial amplicons. Bacterial amplicons were also sequenced in the forward direction.

Fungal sequences were preprocessed in MOTHUR v.1.20.0 (Schloss et al., 2009) to remove primers and barcodes, check quality (Q25), discard sequences that contain ambiguous base calls, cap the homopolymer length at 8, and remove sequences that were shorter than 300 bp in length. Chimeric sequences were then identified from the ITS sequence libraries using the Fungal Metagenomics Pipeline chimera tool (http://www.borealfungi.uaf.edu) provided by the University of Alaska Fairbanks. All potentially chimeric reads were flagged and excluded from downstream analysis. Sequences from all samples were combined in one single file and clustered into OTUs (97% similarity) using CD-HIT-EST (Li and Godzik 2006). Identities were assigned to the OTUs using the UNITE database's 454 pipeline (Tedersoo et al 2010) by submitting representative sequences for BLAST. Hits with BLAST scores ≤ 200 or query percentage of alignment $\leq 60\%$ were considered to represent unknown or unclassified fungi. Theta-YC (Yue and Clayton, 2005) similarity metrics, neighbor-joining tree based on Theta-YC values, and rarefaction curves based upon the OTU data were calculated in MOTHUR v.1.20.0 (Schloss et al., 2009).

Bacterial sequence processing was carried out as described by Schloss et al. (2011). Initial sequences were all preprocessed in MOTHUR v.1.22.0 (Schloss et al., 2009) to remove primers and barcodes, check quality (Q25), discard sequences that contained ambiguous base calls, cap the homopolymer length at 8, remove sequences that were shorter than 250 bp in length. Resulting sequence data were then aligned, and chimera checked with the chimera.uchime function. All sequences that were flagged as potential chimeras were excluded from downstream analysis. Following chimera detection, we calculated the distance matrix for all bacterial samples, assigned sequences to OTUs (97% similarity) using cluster function, and determined Theta-YC similarity (Yue and Clayton, 2005) treating three biological replicates as one group.

Since a number of biological diversity and richness estimators tend to suffer from sample size bias (Magurran, 2004), we "re-sampled" our fungal and bacterial sequence libraries by using sub.sample function in MOTHUR resulting in randomly selected sequences from each library with equally sized sequence numbers. Only fungal and bacterial diversity indices and richness estimators were calculated based on these reduced sized libraries.

4.2.6. Statistical analysis

Variation in community qPCR values among amendment types and over time were assessed using SAS version 9.2 (SAS Institute Inc., 2003). Proc GLM was used to test individual treatment significance. Pair-wise treatment mean comparisons were made using Least Significance Difference (LSD) when treatment was shown to be significant.

Unless otherwise indicated, all statistical significance levels were set as $P \leq 0.05$. Values were log-transformed prior to analysis.

Nonmetric multidimensional scaling of the bacterial and fungal communities based upon OTU composition was carried out using the Bray-Curtis similarity metric in the PAST software package, version 2.03 (Hammer et al., 2001). Heatmaps were used to show the relative abundances of fungal genera and bacterial phyla for each amendment type and time point. To create the graph, values of the mean across three biological replicates for each treatment were used with heatmap function included in the gplots package for R version 2.13.0. The colored rectangles for each taxonomic group represented sequence abundances relative to the mean of all samples. All treatments were clustered with Euclidian distance-based hierarchical agglomerative clustering.

4.3. Results

4.3.1. Abundance of soil fungal and bacterial populations

Within 2 d of incubation, allyl ITC significantly reduced fungal abundance by 80%, while the other three ITCs (benzyl, phenyl, and butyl) resulted in no significant difference from the control (Fig. 4.1-A). Fungal abundance peaked after 2 d of incubation in the control and benzyl and phenyl ITC-amended microcosms but not until 7 d in the allyl and butyl ITC-amended microcosms. Soil fungal abundances decreased substantially in all treatments after 7 d and then stabilized after 14 d of incubation. By 28

d, fungal population levels in all treatments were not significantly different than the control.

Soil bacterial abundances were generally not impacted by the ITCs as much as the fungal abundances were. The butyl ITC appeared to slightly inhibit bacterial abundances at 2 d; however, the bacterial levels were not statistically different from the control (Fig. 4.1-B). Similar to the soil fungal responses, soil bacterial abundance peaked either at day 2 or 7 and then stabilized after 14 days of incubation, with the exception of the butyl ITC treatment which still contained bacterial abundances significantly higher than the control and other treatments at 28 d.

Due to greatly suppressed fungal abundances, the soil bacterial to fungal ratio in the allyl ITC-amended soil was significantly higher than the control (~6-fold) and for the other three types of ITCs after 2 d (Fig. 4.1-C). At the early stages of incubation (2-7 d), the butyl ITC-amended soil generated a significantly lower bacterial to fungal ratio than the allyl and benzyl ITC treatments did. However, this was reversed at 14 d when the butyl ITC-amended soil had a significantly higher bacterial to fungal ratio than all of the other treatments as a result of a significantly higher soil bacterial population. By 28 d, the ratio among all treatments was not significantly different.

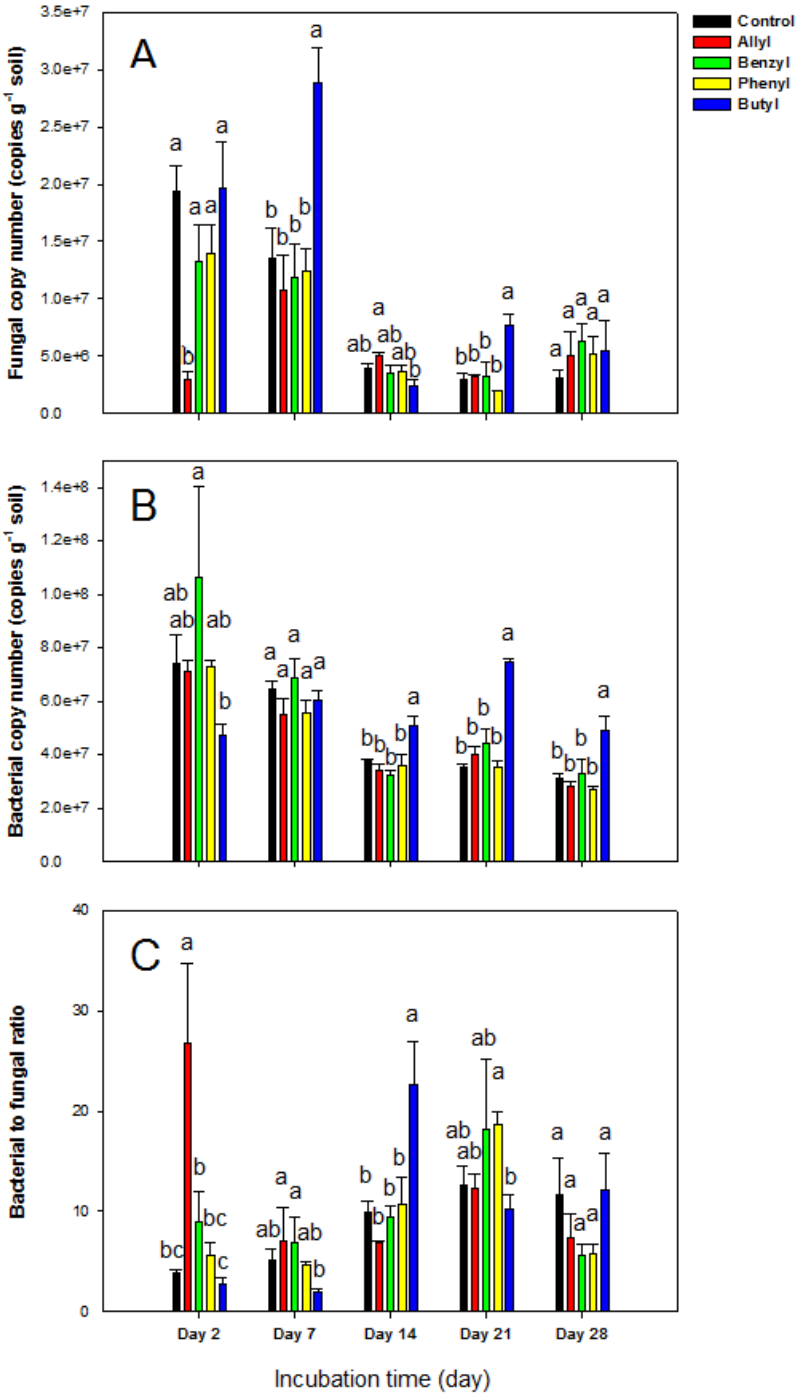

Fig. 4.1. Microbial abundance by qPCR in Weswood loam soil 2, 7, 14, 21, and 28 days after amendment with 1% flax SM and 50 μg g^{-1} allyl, benzyl, butyl or phenyl isothiocyanate (ITC). The controls received 1% flax SM but no ITC. Bars represent the mean of 3 biological replicates for each treatment, and error bars represent standard deviation. (A) Soil fungal copy number. (B) Soil bacterial copy number. (C) The ratio of soil bacterial to fungal copy number. Different letters indicate significant difference at $P<0.05$ within each day.

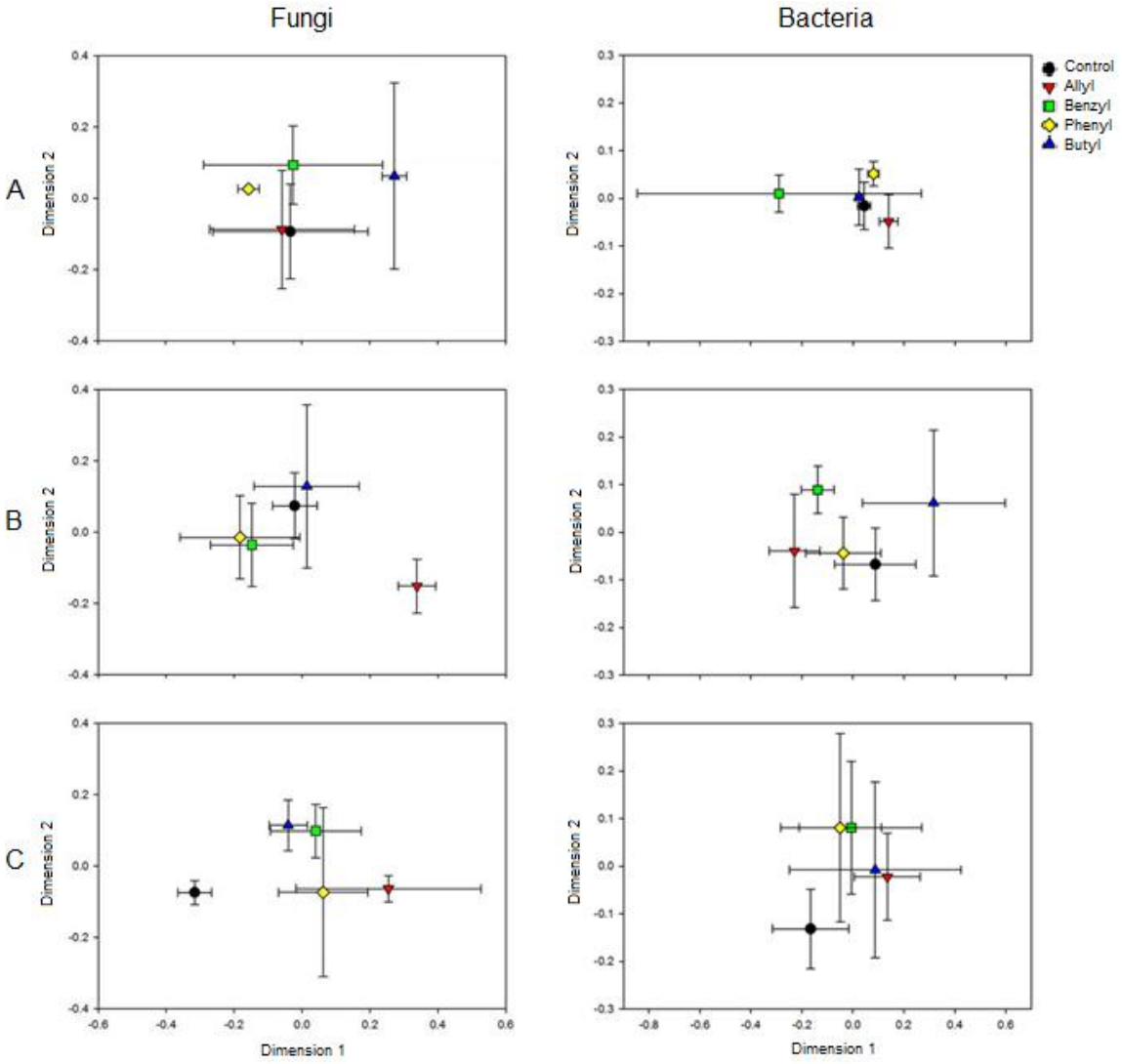

Fig. 4.2. NMDS graphs of fungal and bacterial communities in Weswood loam soil at 2 (A), 7 (B), and 28 (C) days after amendment with 1% flax SM and 50 μg g^{-1} allyl, benzyl, butyl or phenyl isothiocyanate (ITC). Analysis based on operational taxonomic units (OTUs) in all samples (800 for fungi and 18,392 for bacteria) clustered at 97% sequence identities The controls received 1% flax SM but no ITC. Symbols represent the mean of 3 biological replicates for each treatment, and error bars represent standard deviation.

4.3.2. Soil fungal community composition

The NMDS analysis indicated that amendment of soil with various ITCs altered the soil fungal community composition (Fig. 4.2). At day 2 when the soil fungal population was greatly inhibited by allyl ITC, the composition of fungal community in that treatment was surprisingly similar to the control. Likewise, there were no significant differences in the benzyl and phenyl ITC treatments compared with the control. On the contrary, the butyl ITC treatment, which did not suppress soil fungal abundance, did change the composition of the soil fungal community. By 7 d, the allyl ITC had resulted in a dramatic shift in the soil fungal community composition. These differences in the allyl ITC treatments persisted through 28 d. After 28 d, the soil fungal community compositions in all of the ITC amendments were still different from the control with the phenyl ITC becoming more similar to the ally ITC treatment and the benzyl and butyl treatments being similar to each other.

According to the Theta-YC similarity metrics, there was not a consistent trend regarding the relative impacts of ITC type and time on the soil fungal community composition (Fig. 4.3). The unamended soil, with no flax or ITC addition, was the outgroup, as expected. In general, the benzyl ITC treatment produced fungal communities that were more similar to those in the butyl ITC treatment and the allyl ITC-produced communities were more similar to those in the phenyl ITC treatment.

Soil fungal taxonomic distribution patterns were also shifted by ITC applications (Fig. 4.4). Allyl ITC addition increased fungal diversity through time, while the other three types of ITCs decreased soil fungal diversity temporarily at 2 d and later were not

different from the control (Table 4.1). *Ascomycota* and *Zygomycota* were the dominant phyla of classified fungi in all treatments (89%-97%). *Fusarium*, *Chaetomium*, *Humicola*, *Mortierella*, and *Ascobolus* were the dominant genera detected in all treatments as well as the control through time (Table 4.2). Among those, the fungal genera that responded the most to ITC amendments were *Chaetomium*, *Humicola*, and *Mortierella*. There was no significant difference in the relative abundance of *Fusarium* among all treatments through time. Similar to the NMDS and Theta-YC results, allyl, benzyl, and phenyl ITC amendments had similar fungal taxonomic distributions to the control at 2 d. The butyl ITC treatment on the other hand, had significant lower compositions of *Chaetomium* and *Humicola*, and significantly higher composition of *Mortierella*, both of which contributed to its unique fungal taxonomic distribution from all the other treatments. Later at 7 d, allyl ITC application significantly suppressed *Chaetomium* but enhanced *Humicola* compared with the control and the other ITC treatments (Fig. 4.4). After 28 d, *Chaetomium* were the dominant fungi (28-62%) and *Mortierella* had decreased to a minor component (< 2%) of fungal communities in all treatments. However, even after 28 d, the allyl ITC treatment contained a significantly lower proportion of *Chaetomium* and more *Humicola* than most of the other treatments did.

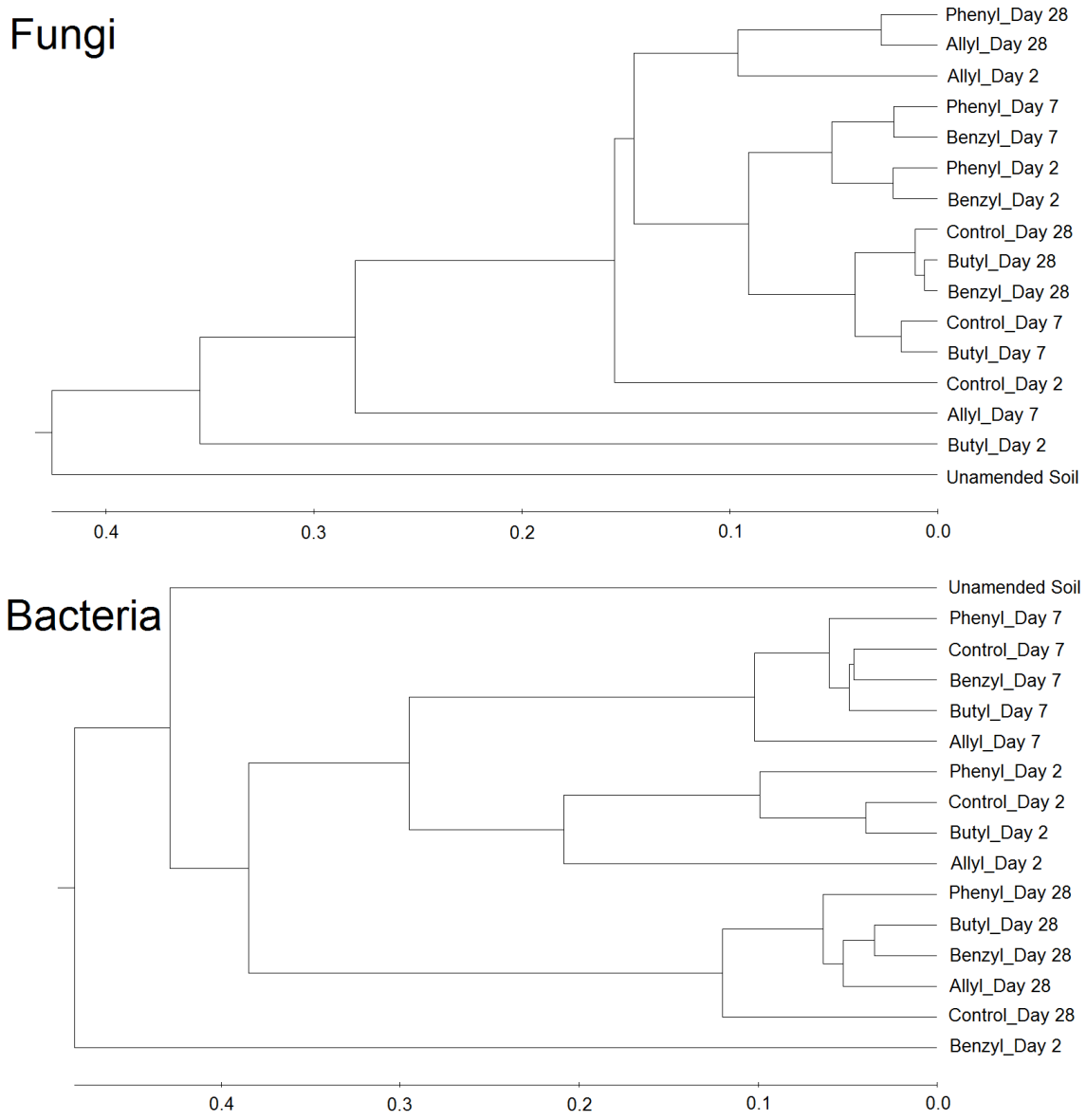

Fig. 4.3. Neighbor joining trees based on Theta-YC values of fungal and bacterial communities in Weswood loam soil at 2, 7, and 28 days after amendment with 1% flax SM and 50 µg g^{-1} allyl, benzyl, butyl or phenyl isothiocyanate (ITC). The 3 biological replicates for each treatment was grouped for calculations on Theta-YC similarity metrics. Biological replicates for each treatment were treated as one group to calculate the Theta-YC similarity metrics. Analysis was based on operational taxonomic units (OTUs) (800 for fungi and 18,392 for bacteria) clustered at 97% sequence identities. The controls received 1% flax SM but no ITC. The unamended soil was sampled at 0 d and had not received any amendment (neither flax nor ITC).

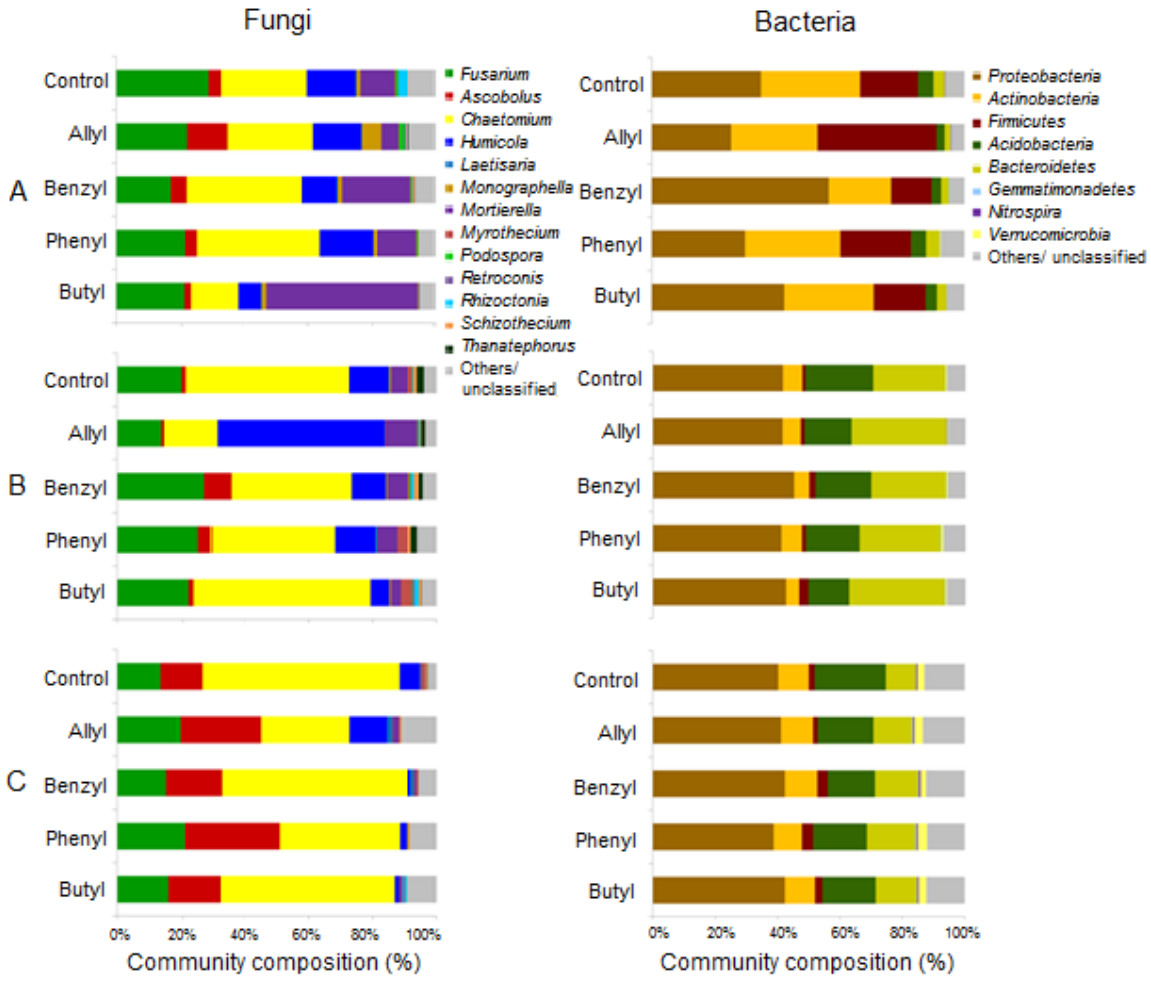

Fig. 4.4. Soil microbial operational taxonomic unit (OTU) distribution patterns in a Weswood loam at 2 (A), 7 (B), and 28 (C) days after amendment with 1.0% flax SM and 50 μg g^{-1} allyl, benzyl, phenyl, or butyl isothiocyanate (ITC). The controls received 1% flax SM but no ITC. Bars represent the mean of 3 biological replicates for each treatment.

Table 4.1. Fungal and bacterial community diversity indexes based upon operational taxonomic units (OTUs; 97% similarity) and their relative abundances in a Weswood loam soil mixed with 1% flax SM and treated with 50 μg g^{-1} allyl, benzyl, phenyl, or butyl isothiocyanate (ITC) and the control receiving no ITC after 2, 7, and 28 days of incubation at 25°C. Diversity and richness estimates were based on reduced sized sequence libraries (1036 sequences for fungal and 1558 sequences for bacterial community respectively). Values displayed represent the mean of 3 biological replicates for each treatment.

Sample		Community characteristics (mean ± std)									
		Fungal communities					Bacterial communities				
Treatment	Day	Sequence Coverage (%)	Observed OTUs	Chao I Richness	Shannon (H')	Inverse Simpson	Sequence Coverage (%)	Observed OTUs	Chao I Richness	Shannon (H')	Inverse Simpson
Control	2	98 ±1	52 ± 9	69 ±15	2.40 ±0.11	6.5 ±0.5	88 ±3	366 ± 39	658 ±202	4.81 ±0.03	47 ± 9
Allyl	2	98 ±0	61 ± 5	86 ± 9	2.55 ±0.12	7.4 ±1.7	89 ±4	334 ±141	575 ±185	4.28 ±1.40	46 ±35
Benzyl	2	98 ±0	48 ± 6	75 ±15	2.09 ±0.09	4.4 ±0.2	84 ±5	452 ± 68	837 ±360	5.21 ±0.11	78 ± 3
Phenyl	2	98 ±0	55 ± 2	76 ±10	2.15 ±0.14	4.8 ±1.5	87 ±3	392 ± 51	645 ±114	4.99 ±0.18	61 ± 9
Butyl	2	99 ±0	46 ±14	57 ±13	2.12 ±0.65	5.5 ±3.0	83 ±2	455 ± 29	901 ±159	5.20 ±0.12	77 ±19
Control	7	98 ±1	53 ± 6	79 ±25	1.91 ±0.15	3.3 ±0.7	82 ±1	469 ± 13	992 ± 45	5.23 ±0.02	76 ± 2
Allyl	7	98 ±0	51 ± 6	82 ± 7	2.08 ±0.04	5.4 ±0.7	80 ±2	494 ± 44	1091 ±120	5.29 ±0.20	82 ±26
Benzyl	7	98 ±0	52 ± 9	73 ±10	2.05 ±0.21	4.1 ±0.5	81 ±0	504 ± 27	1055 ± 97	5.40 ±0.21	106 ±37
Phenyl	7	98 ±0	56 ±10	77 ± 7	2.02 ±0.41	3.8 ±1.5	84 ±1	459 ± 19	785 ± 10	5.24 ±0.17	76 ±21
Butyl	7	98 ±0	44 ± 6	58 ± 3	1.59 ±0.45	2.9 ±1.4	80 ±1	554 ± 8	1005 ± 96	5.65 ±0.11	133 ±36
Control	28	97 ±0	49 ± 7	90 ±12	1.56 ±0.09	2.5 ±0.1	75 ±0	607 ± 14	1401 ± 9	5.79 ±0.08	183 ±28
Allyl	28	98 ±0	42 ±10	61 ±18	2.06 ±0.24	5.1 ±1.0	77 ±2	627 ± 12	1090 ±112	5.91 ±0.09	214 ±44
Benzyl	28	99 ±0	38 ± 2	48 ± 5	1.56 ±0.14	2.7 ±0.4	78 ±4	585 ± 60	1171 ±186	5.76 ±0.16	171 ±43
Phenyl	28	99 ±0	40 ± 2	56 ± 8	1.70 ±0.10	3.1 ±0.2	78 ±2	593 ± 18	1208 ±232	5.78 ±0.09	173 ±33
Butyl	28	98 ±1	44 ± 7	64 ±20	1.65 ±0.15	2.8 ±0.4	77 ±2	670 ± 29	1072 ± 89	5.99 ±0.06	195 ±33

Table 4.2. Fungal operational taxonomic unit (OTU) composition summarized at the genus level in a Weswood loam soil mixed with 1% flax SM and treated with 50 μg g^{-1} allyl, benzyl, phenyl, or butyl isothiocyanate (ITC) and the control receiving no ITC after 2, 7, and 28 days of incubation at 25°C. Values displayed represent the mean of 3 biological replicates for each treatment.

Treatment	Day	Fungal OTU Composition (%)													
		Fusarium	*Ascobolus*	*Chaetomium*	*Humicola*	*Laetisaria*	*Monographella*	*Mortierella*	*Myrothecium*	*Podospora*	*Retroconis*	*Rhizoctonia*	*Schizothecium*	*Thanatephorus*	Other/ unclassified
Control	**2**	29.00a*	3.51ab	27.16ab	15.89a	0.16a	1.13b	10.56b	0.42a	0.64ab	0.06b	2.89a	0.18b	0.02b	8.38a
Allyl	**2**	22.00a	12.93a	26.54ab	15.57a	0.22a	6.00a	5.32b	0.13a	1.09a	0.33a	0.14a	0.70a	0.82a	8.21a
Benzyl	**2**	17.44a	4.46ab	35.94a	10.75ab	0.73a	1.27b	21.65ab	0.21a	0.28b	0.08b	0.13a	0.52a	0.18ab	6.34ab
Phenyl	**2**	21.38a	4.37ab	38.58a	16.05a	0.16a	1.32b	12.10b	0.28a	0.35b	0.01b	0.05a	0.25b	0.08ab	5.02ab
Butyl	**2**	20.71a	2.36b	15.41b	6.84b	0.22a	1.21b	47.65a	0.32a	0.17b	0.06b	0.04a	0.19b	0.22ab	4.60b
Control	**7**	19.61a	1.71a	52.00a	12.14b	0.24a	0.41a	4.79a	1.66a	0.12a	0.08c	0.28a	0.91ab	2.55a	3.51a
Allyl	**7**	13.43a	1.36a	15.99b	53.11a	0.22a	0.24a	9.60a	0.15a	0.13a	0.82a	0.34a	0.31b	1.03a	3.25a
Benzyl	**7**	27.51a	8.20a	38.43ab	10.62b	0.11a	0.31a	5.91a	0.73a	0.26a	0.23bc	0.81a	1.46a	1.55a	3.87a
Phenyl	**7**	25.79a	3.98a	38.22ab	12.78b	0.62a	0.18a	6.46a	2.53a	0.30a	0.13c	0.17a	1.07ab	1.72a	6.06a
Butyl	**7**	22.13a	1.33a	55.41a	6.82b	0.01a	0.44a	2.87a	3.57a	0.25a	0.51ab	1.54a	0.87ab	0.07a	4.19a
Control	**28**	13.17a	13.94a	61.51a	6.65a	0.25a	0.06a	0.37b	0.85a	0.03a	0.14a	0.00b	0.42a	0.09a	2.51b
Allyl	**28**	19.35a	25.70a	28.41c	11.57a	1.43a	0.11a	1.70a	0.35ab	0.04a	0.07a	0.01b	0.29a	0.04a	10.92a
Benzyl	**28**	15.76a	16.87a	58.26ab	1.10b	0.98a	0.15a	0.57b	0.44ab	0.02a	0.18a	0.01b	0.40a	0.00a	5.25ab
Phenyl	**28**	20.87a	30.44a	37.39bc	1.39b	0.05a	0.12a	0.60b	0.55ab	0.04a	0.03a	0.01b	0.51a	0.01a	7.99ab
Butyl	**28**	16.47a	15.23a	55.49ab	1.33b	0.00a	0.14a	0.60b	0.14b	0.05a	0.12a	0.61a	0.52a	0.01a	9.30a

*Different letters indicate significant difference at $P<0.05$ within each time point for each genus.

4.3.3. Soil bacterial community composition

The NMDS analysis indicated that ITC applications to soil altered the bacterial community compositions, although these effects seemed to be somewhat less-pronounced than for the fungi (Fig. 4.2). Allyl ITC shifted the soil bacterial composition compared with the control through time, while butyl ITC behaved in the opposite way leading to no difference from the control through time. Benzyl and phenyl ITC had a transient effect and altered soil bacterial community composition after 2 or 7 days of incubation, but these differences were diminished by 28 d.

The soil bacterial community similarities described by Theta-YC similarity metrics clustered treatments primarily by time instead of ITC type (Fig. 4.3). The exception was the benzyl ITC at 2 d, which was an outgroup from all of the other treatments [this resulted from large variation among the replicate samples due to a spike in *Proteobacteria* (primarily *Pseudomonas* spp.; 88% of the bacterial community in one sample) in one replicate of the phenyl ITC treatment]. Bacterial community structures at the earlier stages of the experiment (2 and 7 d) were more similar to each other than those at the end of the experiment (28 d).

Soil bacterial taxonomic distribution patterns were also transiently shifted by the ITC applications, with no apparent differences being detected after 7 d at the phylum level (Fig. 4.4). Soil bacterial diversity was generally increased at 2 and 7 d by benzyl, phenyl, and butyl ITC addition, but was not significantly changed by the allyl ITC (Table 4.1). The diversity metrics were similar for all treatments by 28 d. The dominant bacterial phyla detected were *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Acidobacteria*, and *Bacteroidetes* (Fig. 4.4). The only bacterial phylum that differentially responded to ITC addition (compared to the control) was *Firmicutes* at 2 d, which consisted of 4 dominant genera detected in our microcosms including *Bacillus, Brevibacillus*, *Lysinibacillus*, and *Paenibacillus* (Table 4.3). Allyl ITC significantly increased the proportion of *Firmicutes*, mainly *Brevibacillus* and *Paenibacillus*, compared to the control and all the other ITC treatments. However, after 7 d, *Firmicutes* was a less dominant component (< 4%) of bacterial communities in all treatments being replaced largely by *Bacteroidetes* and *Acidobacteria*. The benzyl ITC treatment appeared to increase the relative level of *Proteobacteria* at 2 d, but the variation among the three biological replicates were quite large.

Table 4.3. Bacterial operational taxonomic unit (OTU) composition summarized at the genus level for *Firmicutes* in a Weswood loam soil mixed with 1% flax SM and treated with 50 μg g^{-1} allyl, benzyl, phenyl, or butyl isothiocyanate (ITC) and the control receiving no ITC after 2, 7, and 28 days of incubation at 25 ℃. Values displayed represent the mean of 3 biological replicates for each treatment.

Treatment	**Day**	**Total *Firmicutes***	***Firmicutes* OTU Distribution (% of Bacterial Community)**				
			Bacillus	***Brevibacillus***	***Lysinibacillus***	***Paenibacillus***	**Others/ Unclassified**
Control	2	18.46b*	7.28a	0.74ab	0.01b	2.99b	7.44a
Allyl	2	38.00a	8.92a	6.48a	0.67a	14.15a	7.77a
Benzyl	2	12.80b	5.03a	0.29b	0.00b	1.97b	5.51a
Phenyl	2	22.58b	8.65a	1.92ab	0.00b	4.05b	7.95a
Butyl	2	16.61b	5.89a	0.52b	0.00b	4.85b	5.35a
Control	7	0.96a	0.41a	0.00a	0.00a	0.05b	0.50a
Allyl	7	1.32a	0.70a	0.00a	0.00a	0.14ab	0.48a
Benzyl	7	1.93a	0.87a	0.00a	0.00a	0.08b	0.98a
Phenyl	7	1.19a	0.42a	0.00a	0.00a	0.08b	0.69a
Butyl	7	3.02a	1.37a	0.01a	0.00a	0.22a	1.42a
Control	28	1.86a	0.90a	0.03a	0.00a	0.08a	0.85a
Allyl	28	1.48a	0.78a	0.00a	0.00a	0.06a	0.64a
Benzyl	28	3.39a	1.70a	0.00a	0.00a	0.12a	1.57a
Phenyl	28	3.54a	1.78a	0.02a	0.00a	0.19a	1.55a
Butyl	28	2.42a	1.25a	0.01a	0.00a	0.09a	1.06a

*Different letters indicate significant difference at $P<0.05$ within each time point for each phylum or genus.

4.4. Discussion

4.4.1. Soil fungal and bacterial abundance inhibited by ITCs

The incorporation of different types of ITCs with the presence of flax SM only temporarily inhibited soil fungal abundance, and the level of suppression varied according to the ITC type, similar to results of previous studies on ITC fungicidal toxicity (Yulianti et al., 2007; Hu et al., 2011). Allyl ITC had a greater inhibitory effect on soil fungal population size than did the aromatic ITCs (benzyl and phenyl). Similarly, other researchers have reported that aliphatic ITCs such as allyl ITC are more biologically toxic to targeted microorganisms than aromatic ITCs are (Angus et al., 1994; Matthiessen and Shackleton, 2005; Troncoso-Rojas et al., 2009; Hu et al., 2011). The inhibiting effect of allyl ITC released from SM on soil fungal abundances has been demonstrated by Hollister et al. (2011), who compared application of *Brassica juncea* (containing compounds that produce allyl ITC) and flax (induce no ITC) SMs and found that amendment of soil with 2.5% of *B. juncea* (estimated to release approximately 390 $\mu g\ g^{-1}$ allyl ITC in soil) delayed soil fungal proliferation. Such inhibition however, did not occur when a lower application rate of 0.5% *B. juncea* SM (estimated to release ~80 μg allyl ITC g^{-1} soil; higher than the 50 $\mu g\ g^{-1}$ level used in this study) was added (Wang et al., 2012). Butyl ITC, although also an aliphatic ITC, was not initially as inhibitory to soil fungal abundances. However, the presence of a delayed peak in fungal abundance in the butyl ITC-treatment suggested that soil fungal abundances may have been inhibited by butyl ITC addition initially, though other mechanisms (e.g., reduced bacterial

abundances) may be responsible for its higher fungal abundance level. The toxicity of various ITCs has been linked to specific chemical and physical properties such as water solubility and bioavailability; however, it can be much more difficult to predict their toxicity in the soil environment due to ITC phase (solid, liquid, and gas) partitioning and complex interactions with the soil (Borek et al., 1998; Matthiessen and Shackleton, 2005). The observed higher inhibiting level of aliphatic than aromatic ITCs in our study may come from the higher chemical volatility and/ or higher biological activity of aliphatic ITCs (Matthiessen and Shackleton, 2005; Hu et al., 2011).

Soil bacterial abundance was less affected than fungi by ITC addition, regardless of the ITC type. Similar findings of lower susceptibility of soil bacteria than fungi to various ITCs were reported by Matthiessen and Shackleton (2005). Nevertheless, we did observe a slight inhibiting effect in the butyl ITC treatment, indicating that butyl ITC could be generally more suppressive to soil bacterial abundances than the other ITCs. This was concurrent with higher fungal abundance and a lag in fungal growth peak due to butyl ITC addition, which suggested that butyl ITC did suppress soil fungal abundances initially but this may have been masked by lower level of competition from the reduced bacterial community. Moreover, although allyl ITC seemed not to suppress soil bacterial abundance, this may have been masked by the reduction in fungal abundances and thus less competition from soil fungi. Previous studies agreed with our results that allyl ITC, such as that released from some SMs, can suppress soil bacterial abundances. For example, Hollister et al. (2011) found that soil bacterial abundance was significantly reduced (compared to a flax control) by applying *B. juncea* SM (2.5%) that

was estimated to release approximately 390 μg g^{-1} allyl ITC in soil. Moreover, Wang et al. (2012) observed a slightly inhibited bacterial population within only a few days after *B. juncea* SM addition (5%) resulting in a total of 780 μg g^{-1} allyl ITC in soil. However, they found that a lower level of *B. juncea* SM (0.5%; ~ 80 μg allyl ITC g^{-1} soil), which was comparable to ITC level used in our experiment (50 μg g^{-1}), produced no significant impact on soil bacterial population size (Wang et al., 2012), although the way the ITC was incorporated into soil differed from ours (SM v. pure chemical). At later time points (≥14 d), there were significantly higher bacterial abundances in the butyl ITC amendments than the other treatments, which could be related to its higher fungal population early in the experiment later serving as additional nutrient resources for the bacterial community (Bernard et al., 2011). The differential effect of the two aliphatic ITCs (allyl and butyl) on the soil microbial community was further revealed by the bacterial to fungal ratios, where we found that allyl ITC led to a transient increase in the bacterial to fungal ratio due to inhibition of soil fungal abundances. In contrast, the butyl ITC treatment led to a reduction in the bacterial to fungal ratio as a result of this ITC initially inhibiting bacterial abundances.

4.4.2. Soil fungal community structural shifts by ITCs

Numerous studies have demonstrated the ability of pure ITCs to inhibit a wide range of soil fungi (usually focused on specific plant pathogens), with the level of inhibition varying depending upon the ITC and organism (Kirkegaard et al., 1996; Sarwar et al., 1998; Smith and Kirkegaard, 2002). Far fewer studies have investigated

ITC impacts on the soil fungal community. Of those, even fewer studies have investigated the impacts of ITCs produced from added biomass (e.g., *Brassicaceae* SMs, plant residues, or root exudes etc.) as would be applied for biofumigation (Hollister et al., 2011; Baldrian et al., 2011). Our study extended the studies investigating pure ITC influences on soil organisms by adding the effect of SM and found that soil fungal community composition was also altered due to ITCs, and these changes were different according to ITC type. Generally speaking, aliphatic ITCs (allyl and butyl) seemed to impact the fungal community more than aromatic ITCs (benzyl and phenyl) did. To be specific, butyl ITC had the largest initial impact on soil fungal community composition. Since butyl ITC did not initially alter total fungal abundance, this indicated that various fungal groups responded to butyl ITC differently with some groups being inhibited while others were promoted. Allyl ITC on the other hand, although it greatly reduced total fungal abundance, did not change the composition of the fungal community compared with the control. This indicated that the allyl ITC had a wide-spectrum effect on the fungal community and impacted all of the dominant fungi similarly. These results are the first to detail the impacts of various ITCs on soil fungal community composition, so there is no direct point-of-comparison in the published literature. A related study by Hollister et al. (2011) using a relatively higher amendment rate of *B. juncea* SM (2.5%) producing allyl ITC reported that the amendment did alter soil fungal community structure within 3 days of incubation. Our results agreed with their study given a longer time of incubation (1 week), when we detected a recovery growth of fungal population in allyl ITC treatment that had a significantly different community structure from all the

other treatments. This suggested that fungal re-colonization in the soil may result in differentiation due to the varied initial ITC impacts, especially in the case of allyl ITC. Another possible explanation is that the ITCs were added in pure chemical form at day 0 in our experiment, while in the oilseed experiments, the GLS would have to first be transformed into ITCs before they would be effective. This might at least partially explain the seemingly conflicting results between this experiment and those using SMs (Hollister et al., 2011; Wang et al., 2012). The effects of the aromatic ITCs (benzyl and phenyl) on soil fungal community composition were less apparent early in the experiment, but given a longer time of up to 4 weeks, they also shifted soil fungal structure. This was likely due to systematic impacts instead of pure chemical effects, considering that ITCs typically degrade rapidly (within hours to days) when incorporated into soil (Warton et al., 2003; Gimsing et al., 2008).

In terms of taxonomic classification of the soil fungal communities, *Ascomycota* and *Zygomycota* were the dominant fungal phyla detected. This is not surprising since *Ascomycota* are the largest group of the true fungi (Larena et al., 1999) with their members consisting of both noxious plant pathogens and non-pathogenic saprotrophic fungi (Osono et al., 2003). *Zygomycota* comprise only 1% of the described species of true fungi, they often live in soil close to plants, are able to degrade plant materials, and are ecologically diverse (Kirk et al. 2001; James and O'Donnell, 2007). *Zygomycota* in soil include fast-growing members that utilize substrates that are high in sugar (James and O'Donnell, 2007).

The only genus belonging to *Zygomycota* that dominated our soil fungal community was most similar to *Mortierella*, and its relative abundance varied among treatments. This genus is considered to be sugar fungi, which means that they mainly utilize simple nutrient sources such as sugars, amino acids, and organic acids (Kirk et al., 2001; Weber and Tribe, 2003). Interestingly, they are typically unable to utilize polymeric C sources such as lignins, celluloses, and hemicelluloses, which comprise the major components of many plant residues (Weber and Tribe, 2003). The proliferation of *Mortierella* early in our study may have resulted from their degradation of oils, organic acids, and proteins in the SMs (Wang et al., 2012), which have been reported to be good nutrient sources for supporting *Mortierella* growth (Weber and Tribe, 2003). Residual oils in the flax SM were likely degraded early in the incubation (Chapter 3), which could also explain why *Mortierella* became a minor proportion of the fungal community over time.

In addition, the relative proportion of *Mortierella* was impacted by the type of ITC added. *Mortierella* has been reported to be tolerant of several fungicides and can often survive soil fumigation and then rapidly re-colonize the soil (Warcup, 1976; Kuthubutheen and Pugh, 1979). Based upon our results, it is possible that the various ITCs had differing levels of toxicity to *Mortierella*. We observed a dramatic proliferation of *Mortierella* composition in the butyl ITC treatment, which could result from a higher tolerance of this genus to butyl relative to the other ITCs. Increased abundances of *Mortierella* seems to be the main reason for the lack of suppression on soil total fungal abundance in the butyl ITC treatment as indicated by qPCR.

Among all the *Ascomycota* groups found in our soils, *Humicola* and *Chaetomium* were the ones that responded to ITC additions and differed according to ITC type, while *Fusarium* and *Ascobolus* compositions were generally not influenced by ITC amendment or type. After the large, initial suppression of soil fungal abundances, the allyl ITC-treated soil was rapidly re-colonized, primarily by *Humicola*, which are considered to be beneficial soil fungi with some representatives having been used to produce important enzymes for hydrolyzing lignocellulosic materials in the renewable energy industry (Lang et al., 2011). The ability of *Humicola* to re-colonize soil rapidly after application of fungicides has also been reported in previous research (Kuthubutheen and Pugh, 1979). It could partially explain our finding that *Humicola* out-competed other genera such as *Chaetomium*, and this phenomenon may be related to the initial fungal inhibition by allyl ITC. As some *Chaetomium* such as *C. globosum* are notable infectious human pathogens (Guarro et al., 1995), it seems that allyl ITC-producing SMs could be a good candidate for controlling *Chaetomium* pathogens.

4.4.3. Soil bacterial community structural shifts by ITCs

In our experiment, soil bacterial community composition was temporarily altered by the addition of ITCs, and these changes were different according to ITC type. Unlike the soil fungal community responses to the ITCs, there was not a clear distinction between the aliphatic and aromatic ITCs. The allyl ITC seemed to have a greater impact on the soil bacterial community throughout the experiment. At the same time, various bacterial groups responded to allyl ITC differently, indicating a selectivity of this

chemical on the soil bacterial community. Interestingly, the butyl ITC behaved in almost the opposite way by reducing soil bacterial abundance after only a few days of incubation but not impacting the composition of bacterial community. Benzyl and phenyl ITCs also influenced the bacterial community composition as indicated by NMDS that separating one another at the early days in the incubation. The later changes in bacterial community structure were likely due to whole systematic effects that related to changes in the soil fungal community and nutrient status instead of pure chemical influence (Bressan et al., 2009).

In terms of taxonomic composition of the soil bacterial communities, the largest difference detected, at the phylum level, was in the proportion of *Firmicutes* at the very early stages of the experiment. *Firmicutes* includes fast-growing bacteria that produce endospores and thus can survive in extreme environments (Clark and Hirsch, 2008; Teixeira et al., 2010; Hollister et al., 2010). The dominance of *Firmicutes* only a few days after treatments were imposed in our study is likely due to the energy- and N-compounds (Ibekwe et al., 2007; Teixeira et al., 2010; Collignon et al., 2011; Bernard et al., 2011) contained in the flax SM (Hu et al., 2011). Allyl ITC in our experiment significantly increased the composition of *Firmicutes* compared with the other three types of ITCs. It was likely that this group of bacteria was more resistant to allyl ITC toxicity than the other bacterial members were. Previous results from Hollister et al. (2011) also found the proportion of *Firmicutes* increased in response to addition of allyl ITC (in the form of *B. juncea* SM). When our results were examined at the genus level, we found that the most dominant *Firmicutes* were similar to *Paenibacillus*, which

includes members tolerant to pesticides (Singh et al., 2009) and suppressive to soil-borne fungal pathogens (notably *Fusarium* and *Chaetomium* app.) through various mechanisms such as chitinase production (Guemouri-Athmani et al., 2000; Budi et al., 2000; Da Mota et al., 2005; Singh et al., 2009; Kyselkova et al., 2009). Although there was no significant difference in soil bacterial taxonomic composition at the phylum-level after 7 d, the communities in the allyl and benzyl ITC-amended treatments remained different than the control after 28 d, likely due to differences at lower taxonomic levels. Also at 7 d, *Bacteroidetes* largely replaced *Firmicutes* and became one of the dominant genera in all treatments. This may be partially due to the large amounts of N from the flax SM, since *Bacteroidetes* are fast-growing r-strategy bacteria that have been reported to respond to N-rich amendments (Blagodatskaya and Kuzyakov, 2008; Bernard et al., 2011). A similar trend was found in the study by Hollister et al. (2011) that used flax SM at a higher application rate (2.5%).

4.4.4. Soil microbial responses due to ITC additions- an overall perspective

The soil fungal community was more greatly impacted than bacteria by addition of the various ITCs in the presence of flax SM. Although many of these influences were relatively short-lived, some of the changes persisted until the end of the experiment. The impacts of the ITCs at the early stages of the experiment appeared to be directly due to reactions of the microbial populations to the ITCs themselves and then later shifted to systematic responses due to the previously altered microbial communities. The most interesting results came from the aliphatic ITCs (allyl and butyl). Allyl ITC had a broad

and intensive toxicity on the soil fungal community, while butyl ITC selectively reduced *Chaetomium* and increased *Mortierella*. The increase in *Mortierella* may have resulted from its higher tolerance of or ability to degrade butyl ITC. Along with the large suppression of fungal abundance in the allyl ITC treatment, the concurrent proliferation of *Paenibacillus*, which includes bacteria reported to be antagonist to numerous fungal pathogens, could therefore also have contributed to overall fungal inhibition. *Humicola* appeared to selectively re-colonize the allyl ITC-amended soil, as compared with other genera such as *Chaetomium* and *Fusarium*, after the initial fungal suppression. This may have been due to *Humicola*'s reported ability to rapidly re-establish its populations after fumigation and/ or alterations in the bacterial community such as the proliferation of *Paenibacillus* that subsequently inhibited *Chaetomium* and *Fusarium*. These results suggest that allyl ITC-producing SMs could be a good candidate for controlling *Chaetomium*-related pathogens through direct fungal toxicity and/ or increased numbers of antagonists.

When comparing our results with other studies, especially allyl ITC-related research, we found that the soil fungal community structure was very sensitive to differences in amendments among similar studies. Using the same type of soil and incubation conditions, a previous study by Hollister et al. (2011) who applied SM of *B. juncea* (estimated to release 390 μg g^{-1} allyl ITC in soil), found that the soil fungal community was dominated (87-98%) by a single genus of fungi (*Retroconis*). However, this fungus was just a minor component of the fungal community in our study. The observed large difference in fungal taxonomic distribution could be related to multiple

impacting factors such as ITC level, nutrient amount, and the way the ITC was incorporated, each of which alone has been reported to greatly affect microbial responses to amendments in previous studies (Borek et al., 1998; Sarwar et al., 1998; Smith and Kirkegaard, 2002; Matthiessen and Shackleton, 2005; Wang et al., 2012; Hu et al., 2011). To be specific, the total allyl ITC estimated to be released in the soil environment in the Hollister et al. (2011) study was ~8 times more than in ours, and essential nutrient application (C, N, and P) was also estimated to be 2 to 4 times more than in our study. Furthermore looking at the way the ITCs were applied, in the Hollister et al. (2011) experiment, allyl ITC was gradually released to soil from hydrolyzing allyl GLS contained in *B. juncea* SM during incubation. This represented a more chronic effect of allyl ITC on the soil fungal community instead of a one-time acute dosing as in our experiment. All of the described differences in amendments between Hollister et al. (2011) and our study could contribute to the largely varied results in soil fungal taxonomic structural responses.

For soil bacterial community responses to allyl ITC, our overall results were very similar to those reported previously by Hollister et al. (2011), who found that the soil bacterial population was inhibited by allyl ITC-inducing *B. juncea* SM, and the community composition was also temporarily differentiated between SM application of *B. juncea* and no-ITC-inducing flax. *Firmicutes* (particularly *Bacillus* spp.) proliferated within days of incubation in their study, likely due to being more resistant to allyl ITC. However, impacts of ITCs (SMs) seemed to be greater in their study compared with our results. To be specific, bacterial population size in our study was likely to be reduced,

but seemed to be more transient and less apparent than reported by Hollister et al. (2011). Furthermore, *Firmicutes* at an early stage comprised a much higher proportion of the total bacterial community (~85%) in their study than in ours (~38%). All of the above differences could have resulted from either varied ITC and nutrient levels, and/ or systematic influence related to soil fungal community responses (Smith and Kirkegaard, 2002; Baldrian et al., 2011; Wang et al., 2012).

4.5. Conclusions

The application of ITCs in the presence of flax SM temporarily reduced soil fungal and bacterial population though to differing extents depending upon the ITC type. Both the soil fungal and bacterial communities seemed to be more sensitive to aliphatic (allyl and butyl) than to aromatic (benzyl and phenyl) ITCs. Allyl and butyl ITCs had a wide-spectrum initial inhibiting effect on soil fungal and bacterial abundances in soil. On the other hand, selectivity of these ITCs was also apparent among several fungal and bacterial genera with ITC amendment leading to microbial community composition changes. *Humicola* re-colonized allyl ITC-treated soil rapidly, indicating its higher tolerance to allyl ITC and/ or its greater ability for survival and proliferation when provided adequate nutrients. Allyl ITC suppressed *Chaetomium* possibly indicating its utility for control of this pathogen. *Mortierella*, as a sugar fungus, was enhanced by flax SM application, and its proportion was particularly higher in the butyl ITC treatment, indicating that this genus may be more resistant to butyl than to the other 3 ITCs. *Firmicutes* as a bacterial phylum, which included members that are fast-growing and

resistant to environmental stress, was found to be most responsive to both ITC type and flax SM addition compared with other bacterial phyla; however, this effect was only transient. Several genera within the phylum *Firmicutes* dominated bacterial community composition. This included *Paenibacillus* spp. which appeared to be temporarily enhanced by allyl ITC concurrent with the initial suppression of fungal abundances in this treatment. Since some *Paenibacillus* spp. is known to produce antifungal compounds, this suggests that increases in abundances of these bacteria may have also (in addition to direct ITC effects on fungi) contributed to decreased fungal abundances.

Our experiment was designed to determine how different ITCs released from SMs would impact soil microbial communities. By using pure ITCs and single type of SM, this enabled us to focus specifically upon differential effects of the ITCs and eliminate other variables such as varying chemical composition (C, N, S, other biocidal chemicals, etc.) inherent in comparisons of ITC-producing (e.g. mustard) and non-ITC-producing (e.g., flax) SMs directly. Our findings are the first to detail the impacts of various ITCs, as part of SM application, on soil fungal and bacterial community composition. This information will be very useful for producers designing biofumigation strategies for pathogen control, for plant breeders selecting plants for controlling specific pathogens, and for ecologists attempting to determine the effects of land-applied ITC-containing SMs on soil quality.

CHAPTER V

CONCLUSIONS

Application of 1 to 5 % brassicaceous and jatropha oilseed meals (SMs) inhibited *P. omnivora* sclerotial germination and active hyphal growth in a Branyon clay soil, suggesting that field application of select SMs, especially mustard which showed the highest toxicity to *P. omnivora*, may potentially reduce cotton root rot. Although the specific mechanisms responsible for the inhibition of *P. omnivora* by SMs were not elucidated, our results, along with previous studies, suggest that the biocidal chemicals released from SMs played a major role. Further, our results demonstrated that different ITCs have differing levels of effectiveness in controlling the growth of *P. omnivora*.

Application of 1% SMs to Weswood loam soil rapidly increased microbial, especially fungal, abundances. The composition of the fungal community was also impacted by SM additions with the resulting shifts being relatively persistent over time. Each of the SMs selected for specific fungal groups such as *Fusarium* in camelina and flax treatments and *Chaetomium* in jatropha treatment. In addition to changes in the soil fungal community composition, the SMs altered microbial CLPP patterns, suggesting that changes in microbial population size and composition also impacted the functionality of the soil microbial community.

The different SM amendments seemed to result in slightly varied soil microbial community responses depending on either the allelochemicals involved, nutrient composition (e.g., C:N ratio), or both. Compared with flax SM that contained no

biocidal chemicals, camelina and jatropha SMs produced a smaller increase in soil fungal abundances in the first few days of the experiment. In addition, the composition of the soil fungal community was also differentially impacted by camelina and jatropha SMs at early stages of the experiment. Nevertheless, these transient impacts on population size and community composition did not result in discernible impacts on microbial community functionality – likely due to functional redundancy of the microbial community and/ or relatively low concentrations of biocidal chemicals in the SMs used in this experiment. The more persistent differential impacts of the various SMs appeared to be due to variable chemical composition (e.g., C:N ratio) of the amendments. For example, camelina and flax SMs selected for fungal communities more similar to each other over time than to jatropha SM which had a higher C:N ratio and several other properties that were in many ways intermediate between the other SMs and the wheat straw (lignocellulosic comparison). However, even this impact on soil functionality was diminished by the end of the study with the amended microcosms not being different than the unamended control. These findings elucidate the impacts that various SMs have on soil microbial abundance, community composition, and functionality and also highlight the critical role that the soil fungal community plays in the decomposition of organic amendments.

The application of ITCs in the presence of flax SM temporarily reduced soil fungal and bacterial abundances though to differing extents depending upon the ITC type. Both the soil fungal and bacterial communities seemed to be more sensitive to aliphatic (allyl and butyl) than to aromatic (benzyl and phenyl) ITCs. Allyl and butyl

ITCs had a wide-spectrum initial inhibiting effect on soil fungal and bacterial abundances in soil. On the other hand, selectivity of these ITCs was also apparent among several fungal and bacterial genera with ITC amendment leading to microbial community composition changes. *Humicola* re-colonized allyl ITC-treated soil rapidly, indicating its higher tolerance to allyl ITC and/ or its greater ability of survival and proliferation when provided adequate nutrients. Ally ITC suppressed *Chaetomium* possibly indicating its utility for control of this pathogen. *Mortierella*, as a sugar fungus, was enhanced by flax SM application, and its proportion was particularly higher in the butyl ITC treatment, indicating that this genus may be more resistant to butyl than to the other 3 ITCs. *Firmicutes* as a bacterial phylum, which included members that are fast-growing and resistant to environmental stress, was found to be most responsive to both ITC type and flax SM addition compared with other bacterial phyla; however, this effect was only transient. Several genera within the phylum *Firmicutes* dominated bacterial community composition. This included *Paenibacillus* spp. which appeared to be temporarily enhanced by allyl ITC concurrent with the initial suppression of fungal abundances in this treatment. Since some *Paenibacillus* spp. are known to produce antifungal compounds, this suggests that increases in abundances of these bacteria may have also (in addition to direct ITC effects on fungi) contributed to decreased fungal abundances.

Our experiment focusing on ITC-related microbial community responses was designed to determine how different ITCs released from SMs would impact soil microbial communities. By using pure ITCs and single type of SM, this enabled us to

focus specifically upon differential effects of the ITCs and eliminate other variables such as varying chemical composition (C, N, S, other biocidal chemicals, etc.) inherent in comparisons of ITC-producing (e.g. mustard) and non-ITC-producing (e.g., flax) SMs directly. Our findings are the first to detail the impacts of various ITCs, as part of SM application, on soil fungal and bacterial community composition. This information will be very useful for producers designing biofumigation strategies for pathogen control, for plant breeders selecting plants for controlling specific pathogens, and for ecologists attempting to determine the effects of land-apply ITC-containing SMs on soil quality.

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APPENDIX A

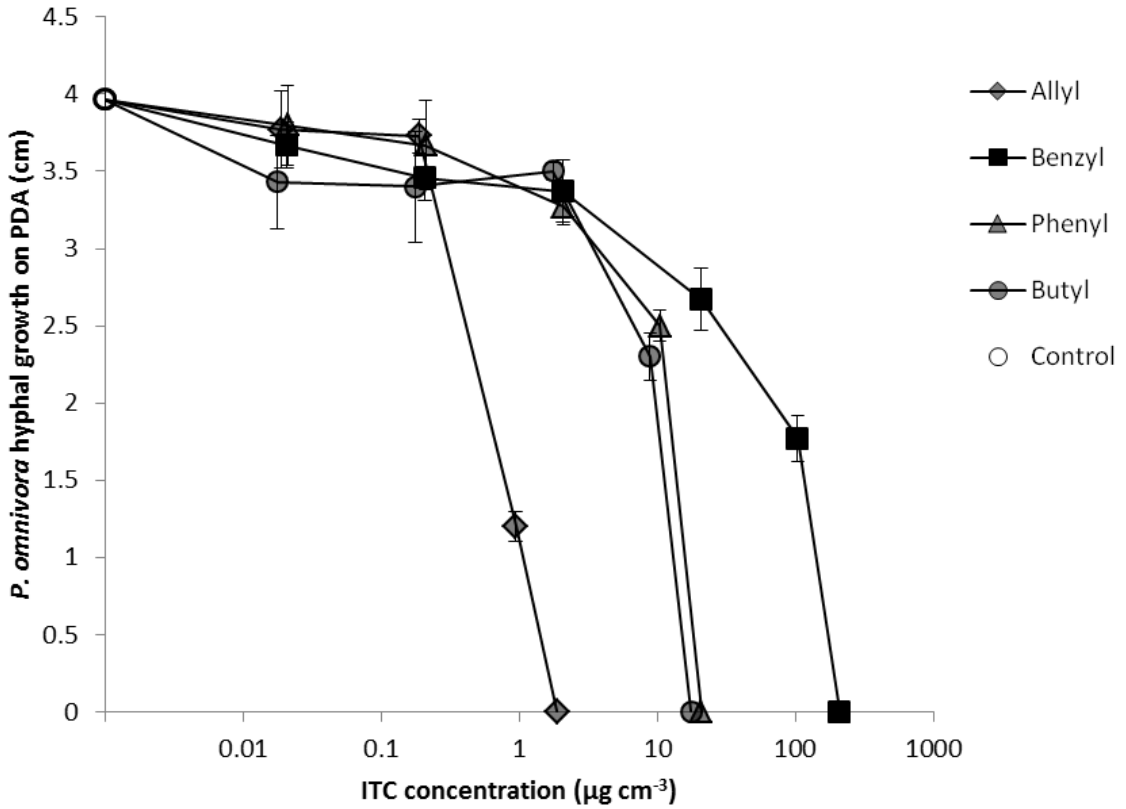

Fig.A-2.1. Effects of selected isothiocyanates on *Phymatotrichopsis omnivora* OKAlf8 hyphal growth on potato dextrose agar (PDA) after 84 h of incubation at 25 ℃. (◊: Allyl ITC; □: Benzyl ITC; Δ: Phenyl ITC; ×: Butyl ITC; ○: 1,4-dioxane Control). Means are based on 3 replicates. Bars represent ± standard deviation of the mean. Error bars are hidden when smaller than the symbols.

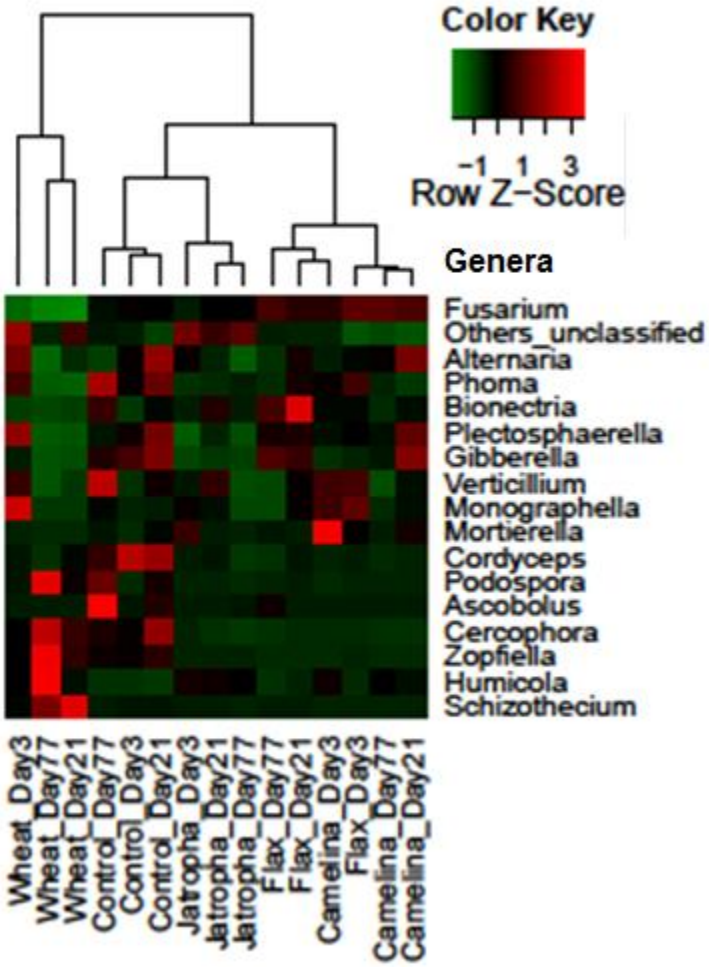

Fig. A-3.1. Heatmap on soil fungal communities genus level in Weswood loam soils treated with oilseed meals of jatropha, camelina, and flax, as well as wheat straw and unamended control after 3, 21, and 77 days of incubation at 25 ℃. This heatmap was created based on the fungal relative abundances of the mean of the 3 biological replicates. Abundances for each taxonomic group were scaled relative to the mean across all samples. The dendrogram depicts Euclidian distance-based hierarchical agglomerative clustering of samples with one another.

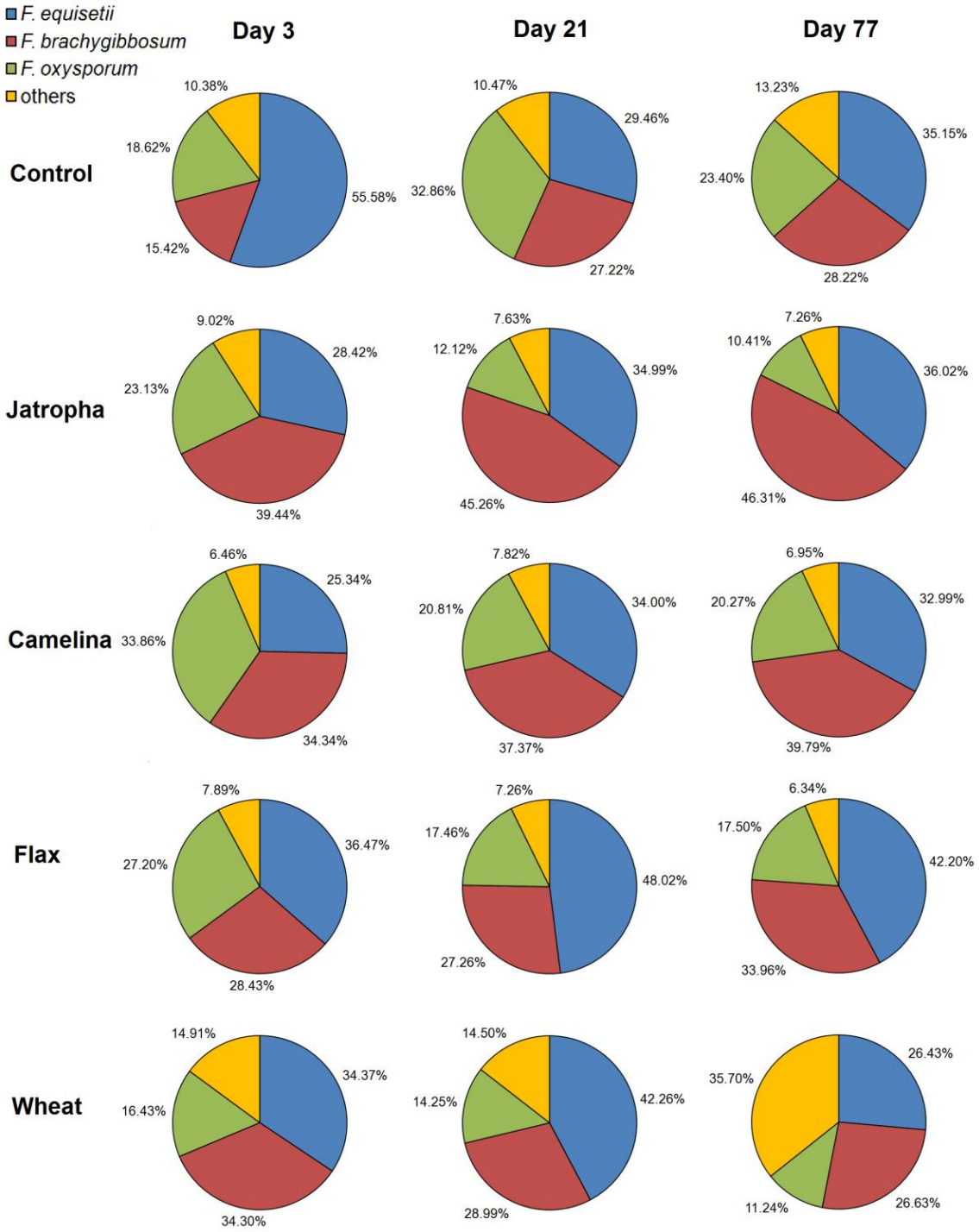

Fig. A-3.2. *Fusarium* OTU distribution patterns at the species level in Weswood loam soils treated with oilseed meals of jatropha, camelina, and flax, as well as wheat straw and unamended control after 3, 21, and 77 days of incubation at 25 ℃. The means of each *Fusarium* species abundance relative to total *Fusarium* genus abundance for the 3 biological replicates in each treatment are represented by different color at each time point.

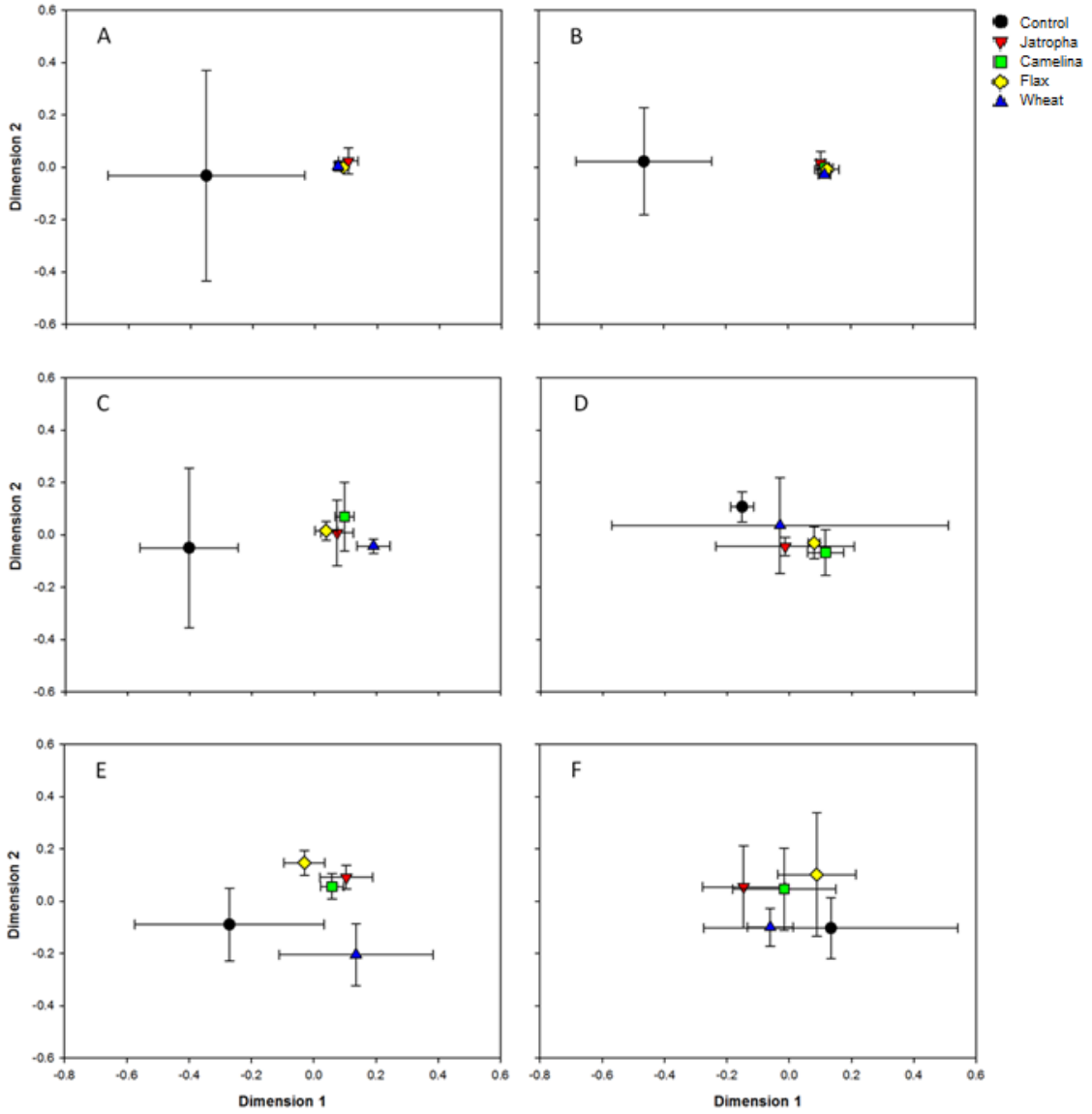

Fig. A-3.3. NMDS ordination of microbial community-level physiological profiling based on Biolog EcoPlate™ readings in 1% (w/w) organic material (oilseed meals of jatropha, camelina, flax, and wheat straw) treated Weswood loam soil after 3 (A), 7 (B), 14 (C), 28 (D), 77 (E), and 133 (F) days of incubation at 25 ℃. Symbols represent the means of 3 biological replicates in each treatment, and the error bars represent the standard deviation among biological replicates.

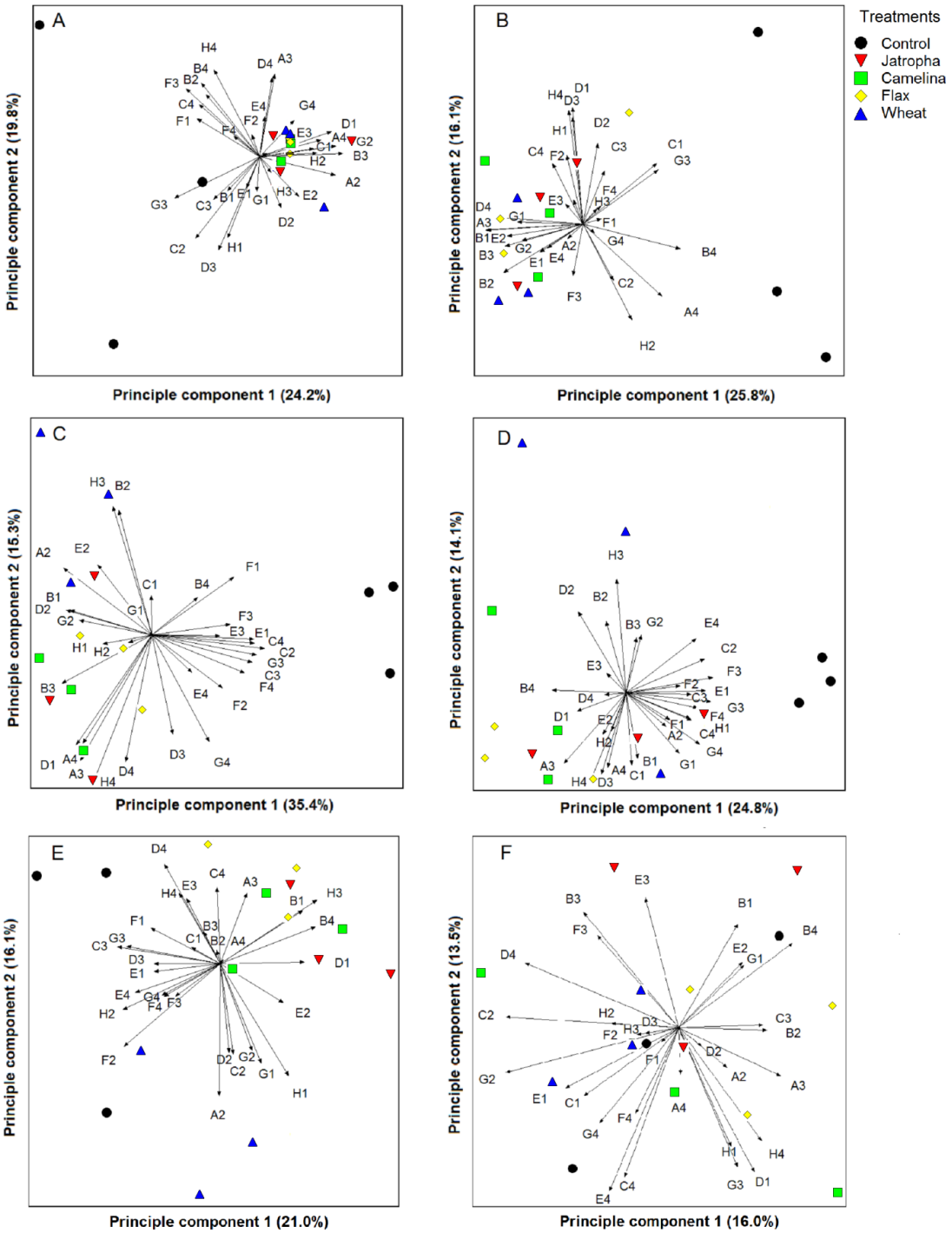

Fig. A-3.4. Principal component analysis (PCA) on Weswood loam soil microbial CLPP indicated by Biolog EcoPlate™ after 3 (A), 7 (B), 14 (C), 28 (D), 77 (E), and 133 (F) days of incubation at 25°C. Treatments included unamended control, 1.0% (w/w) oilseed meals of jatropha, camelina, and flax, and wheat straw. Symbols represent CLPP values from all the replicate-level samples. Codes for the 31 carbon sources are explained in Table 3.1.

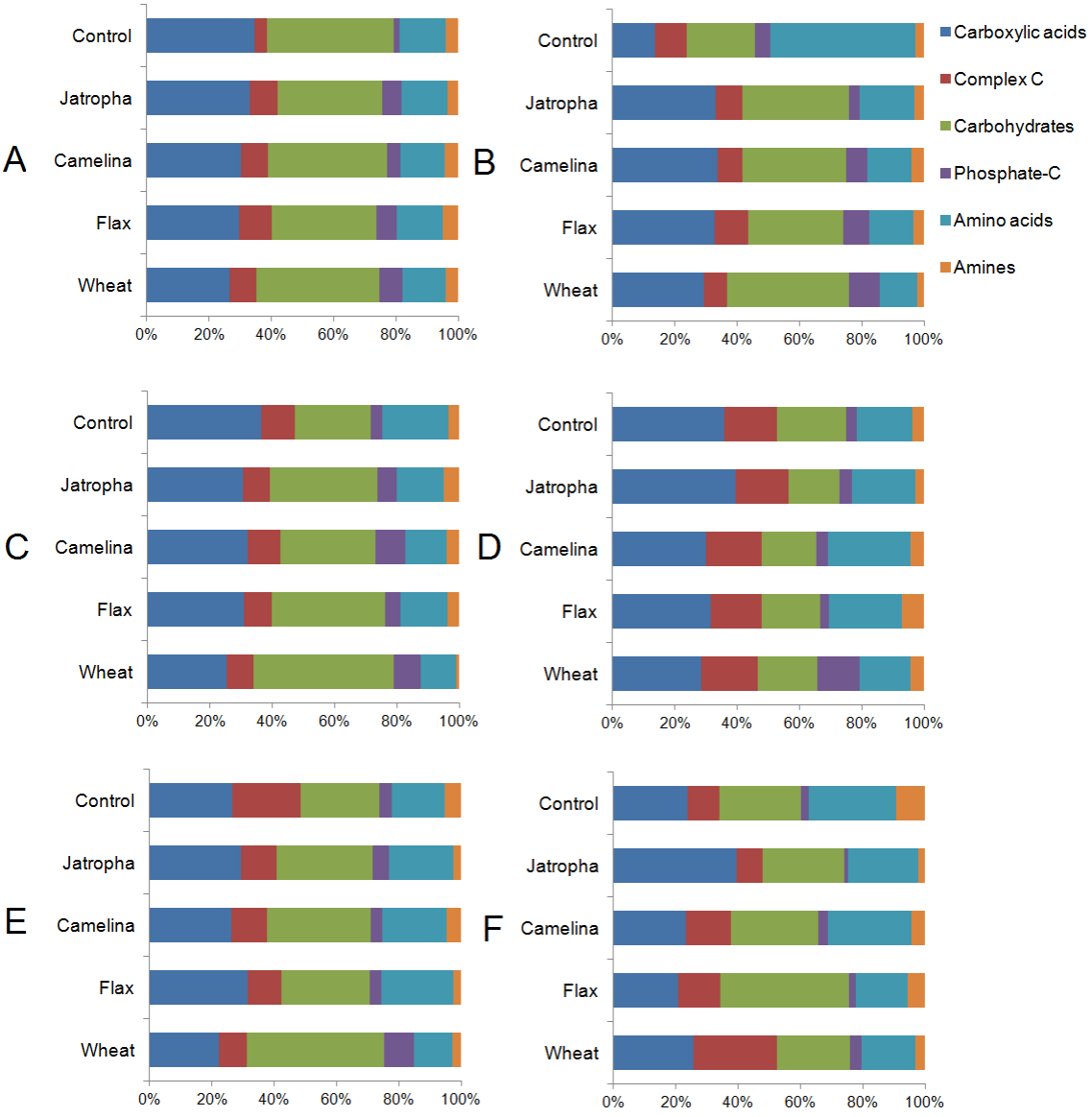

Fig. A-3.5. Percent of CLPP response grouped by C source after 3 (A), 7 (B), 14 (C), 28 (D), 77 (E), and 133 (F) days of incubation at 25 ℃ in a Weswood loam soil. The 31 C sources in Biolog EcoPlate had been grouped into 6 types including carboxylic acids, complex C, carbohydrates, phosphate-containing C, amino acids, and amines explained in Table 3.1. Bars represent the mean of 3 biological replicates on CLPP values for each group of C source in each treatment at each time point.

APPENDIX B

Table A-2.1. Number of germinated *Phymatotrichopsis omnivora* sclerotia in seed meal-amended Branyon clay soil at the end of the 1st, 2nd, 3rd, and 4th week of incubation at 28 ℃ from preliminary studies.

Treatments	**Week 1**	**Week 2**	**Week 3**	**Week 4**
	----------Number of sclerotia germinated---------			
Control	6	6	6	6
Mustard 1%	0	0	0	0
Mustard 5%	0	0	0	0
Camelina 1%	6	6	6	6
Camelina 5%	0	0	1	1
Jatropha 1%	0	0	2	2
Jatropha 5%	0	0	0	0
Flax 1%	6	6	6	6
Flax 5%	6	6	6	6
Chinese tallow 1%	6	6	6	6
Chinese tallow 5%	6	6	6	6

Table A-2.2. Results of analysis of variance of *Phymatotrichopsis omnivora* sclerotial germination rate (arcsin square root transformed) in seed meal-amended Branyon clay soil at the end of the 1st, 2nd, 3rd, and 4th week of incubation, and survival rate (arcsin square root transformed) at the end of at 4th week of incubation at 28 ℃.

Effects	Germination Rate								Survival Rate	
	Week 1		Week 2		Week 3		Week 4		Week 4	
	F	*P* value	*F*	*P* value	*F*	*P* value	*F*	*P* value	*F*	*P* value
Type	350.0	<0.0001	290.2	<0.0001	251.7	<0.0001	330.4	<0.0001	326.6	<0.0001
Rate	412.5	<0.0001	311.7	<0.0001	322.9	<0.0001	406.0	<0.0001	226.0	<0.0001
Type*Rate	122.5	<0.0001	90.8	<0.0001	105.4	<0.0001	137.2	<0.0001	89.0	<0.0001

Type=oilseed meal type, Rate=application rate.
Survival rates were calculated as [(number of germinated sclerotia+ number of inhibited sclerotia)/ 20 expressed as percentages]

Table A-3.1. Summary of fungal community characteristics based on OTUs (97% similarity) and their relative abundances in a Weswood loam soil amended with 1.0% (w/w) oilseed meals of jatropha, camelina, flax, and wheat straw after 3, 21, and 77 days of incubation at 25 °C. Diversity and richness estimates are based upon normalized (reduced-sized) sequence libraries, each of which contained 730 sequences. Values represent the means of 3 biological replicates with standard deviations.

Sample		Community characteristics (mean ± std)				
Amendment	Day	Sequence Coverage (%)	Observed OTUs	Chao I richness	Shannon (H')	Inverse Simpson
Control	3	97 ±2	67 ±18	82 ±31	2.90 ±0.65	12.0 ±9.3
Jatropha	3	97 ±1	51 ±3	83 ±15	2.12 ±0.13	4.3 ±0.8
Camelina	3	97 ±0	55 ±6	88 ±15	2.55 ±0.17	7.6 ±1.0
Flax	3	96 ±1	59 ±8	94 ±8	2.47 ±0.12	6.7 ±1.0
Wheat	3	95 ±2	73 ±7	147 ±69	2.55 ±0.46	6.3 ±4.9
Control	21	93 ±2	103 ±10	176 ±54	3.41 ±0.15	14.5 ±2.4
Jatropha	21	95 ±2	64 ±12	107 ±42	2.33 ±0.07	5.4 ±0.6
Camelina	21	96 ±1	62 ±14	94 ±29	2.48 ±0.17	6.7 ±0.5
Flax	21	95 ±1	66 ±8	123 ±17	2.57 ±0.11	7.3 ±0.4
Wheat	21	95 ±2	75 ±24	157 ±33	2.46 ±0.34	5.1 ±0.6
Control	77	95 ±1	92 ±13	138 ±34	3.38 ±0.36	17.0 ±6.2
Jatropha	77	96 ±2	48 ±10	123 ±66	1.93 ±0.28	4.1 ±1.1
Camelina	77	95 ±1	60 ±9	134 ±55	2.32 ±0.13	5.9 ±0.4
Flax	77	96 ±1	56 ±15	103 ±23	2.32 ±0.25	6.2 ±1.1
Wheat	77	97 ±0	61 ±2	87 ±7	2.68 ±0.09	7.8 ±0.2

Table A-3.2. *Fusarium* composition at the species level in Weswood loam soils treated with oilseed meals of jatropha, camelina, and flax, as well as wheat straw and unamended control after 3, 21, and 77 days of incubation at 25 ℃. Values represent the means of 3 biological replicates in each treatment.

***Fusarium* Species**	**Control**	**Jatropha**	**Camelina**	**Flax**	**Wheat**	**Control**	**Jatropha**	**Camelina**	**Flax**	**Wheat**	**Control**	**Jatropha**	**Camelina**	**Flax**	**Wheat**
	***Fusarium* Species Composition %**														
	Day 3					**Day 21**					**Day 77**				
F. equiseti	25.05a	9.93ab	13.73ab	24.63a	4.75b	12.33c	15.13bc	21.96ab	25.86a	1.72d	13.34c	15.68bc	22.11ab	26.14a	1.34d
F. brachygibbosum	6.95b	13.78a	18.61a	19.20a	4.74b	11.39c	19.57ab	24.14a	14.68bc	1.18d	10.71b	20.16a	26.67a	21.04a	1.35c
F. oxysporum	8.39b	8.08b	18.35a	18.37a	2.27c	13.75a	5.24b	13.44a	9.40a	0.58c	8.88ab	4.53b	13.59a	10.84a	0.57c

*Different letters indicated significant difference within each row at a specific time point.

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