Production and Roles of Volatile Secondary Metabolites in Interactions of the Host Plant Tomato (*Solanum lycopersicum* L.) with Other Organisms at Multi-Trophic Levels.

A Dissertation

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Dedication

I dedicate my dissertation to my Gurudev, and my parents, Vijay Shrivastava and Usha Srivastava.

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Abstract

Tomato (*Solanum lycopersicum* L.) produces an array of volatile secondary metabolites that act as constitutive and induced defenses against a variety of insect pests and diseases. We studied the effect of beneficial microorganisms, an arbuscular mycorrhiza fungus (AM), *Glomus intraradices*, an entomopathogenic fungus (Bb), *Beauveria bassiana* and a combination of both (AM+Bb), as well as, pests, such as rootknot nematode (RKN) (*Meloidogyne incognita)* and beet armyworm (BAW) (*Spodoptera exiguae* H.) on the production of volatile compounds in the leaves and roots of tomato. Benzyl alcohol, 3-hexenoic acid, total other compounds and β-myrcene were significantly increased ($P<0.05$) in the leaves by AM and Bb colonization. Upon herbivory, control plants showed a trend for high volatile contents among all four treatment groups contrary to the trend observed without herbivory, when control showed low volatile contents. Herbivory significantly decreased all the volatiles in the leaves compared to those without herbivory. Pest bioassay revealed that these two fungi together can significantly induce resistance against BAW. In the experiment with RKN, a trend was observed with low production of most volatile compounds in the leaves from the RKN plants. Varying durations of herbivory exposure had significant effects on many leaf volatile compounds compared to those without herbivory. Effect of RKN was significant ($P<0.05$) on the production of methyl salicylate, and (*Z*)-geraniol in the roots. Interaction effect of RKN with 18 hours of herbivory was significant for (*Z*)-geraniol, and with 42 hours of herbivory, it was significant for (*Z*)-geraniol, benzyl alcohol, and total volatiles in the roots. Beet armyworm preferred RKN plants and caused greater damage to them compared to the control plants. Semi-quantitative RT-PCR showed higher expression of the *SlSAMT* gene in the roots colonized with RKN, compared to the control roots and those from a resistant line. The experiments demonstrated that interaction with these organisms can change the volatile compounds in the leaves and roots of tomato plant, can alter herbivore preference, and can upregulate defense genes such as *SlSAMT.*

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Chapter I. Introduction and Literature Review: Production and Roles of Volatile Secondary Metabolites in Interactions of the Host Plant Tomato (*Solanum lycopersicum* L.) with Other Organisms at Multi-Trophic Levels.

Plant volatile secondary metabolites are a wide spectrum of compounds that are produced and emitted from different plant parts. They are involved in an array of complex physiological processes, as well as interactions with the surrounding environment. Chemically complex, these compounds have multiple ecological roles in plant-insect interactions including attracting pollinators (Reinhard et al., 2004), acting as cues for herbivores, as well as functioning as direct defenses, indirect defenses (Turlings et al., 1990), plant–pathogen interactions (Arimura et al., 2000) and/or interplant priming (Ton et al., 2007). Various biotic and abiotic factors have been found to alter the volatile profile of the plants at multitrophic levels (Holopainen and Gershenzon, 2010) and therefore, altering the plant interaction with other organisms. Floral volatiles often serve as attractants for species-specific pollinators, whereas, the volatiles emitted from vegetative parts, appear to mediate interactions with organisms having different functions, detrimental or beneficial (Pichersky and Gershenzon, 2002). The ecological functions of the volatiles are often species specific and have great potential for their utilization in agriculture. In this chapter, I will provide a short review about my dissertation research dealing with production and roles of the volatile secondary metabolites in the interaction of tomato plant with two beneficial organisms and two pests at above and below ground levels and will further discuss the research goal with the specific objectives of my research.

Introduction to plant volatile secondary metabolism

Plant volatile compounds are a class of lipophilic secondary metabolites with low molecular weights and high vapor pressure. So far, approximately 1700 volatile compounds have been identified from more than 90 plant families, and this number is very likely to increase with studies on more plants and the development and application of new detection methods (Knudsen et al., 2006). These compounds come from three major groups: terpenoids, fatty acid derivatives, and phenylpropanoids. Of these, terpenoids make up the largest group (Dudareva et al., 2006). Terpenoids are made from a basic five carbon unit called isoprene (Figure 1.1) and are further classified on the basis of the number of isoprene units in the compound, such as, hemiterpenes have one; monoterpenes, two; sesquiterpenes,three; and homoterpenes, four isoprene units. For terpenoid synthesis, plants use two distinct pathways, the 2-C-methyl-d -erythritol 4-phosphate (MEP) and the mevalonate (MVA) pathways, for Isopentanyl diphosphate (IPP) and

Dimethylallyl pyrophosphate (DMAPP) synthesis which are universal precursors for various isoprenoides. In general, the MVA pathway produces IPP for production of sesquiterpenes in the cytosol. Whereas, the MEP pathway produces precursors for monoterpenes, as well as sesquiterpenes in the plastid (Ganjewala et al., 2009; Paetzold et al., 2010; Strack et al., 2003) and although they are localized in different compartments there is cross talk of IPP, the five carbon molecules universal precursors to all isoprenoids, between the cytosol and plastid (Liao et al., 2006). Some examples of terpenoids are: α-pinene, β-myrcene, α-terpinene, 1-phellandrene, and β-phellandrene, which are monoterpenes; δ-elemene, β-elemene, (*Z*)-caryophyllene, and α-humelene, which are sesuiterepenes. Many terpenoids functions a phytoalexins in plants direct defense and/or as cues for indirect defense, for example, gossypol and related sesquiterpene aldehydes, provide both constitutive and inducible protection against pests and diseases in cotton (*Gossypium* spp.) (Cheng et al., 2007). Volatile fatty acid (VFA) derivatives are another large class, originating from an eighteen carbon unsaturated fatty acid. These VFAs are often associated with flavors described as “tomato,” “green,” or “grassy.” Examples of major compounds in this class are, linoleic acid derived, hexanal and linolenic acid derived , trans-2-hexenal (Figure 1.2) via lipoxygenase activity . The six-carbon aldehydes and alcohols derived from omega-3-linolenic acid are also important constituents of the flavors of apple, sweet cherry, olive, bay leaf, and tea (Goff and Klee, 2006). Phenylpropanoids, the last group, contain structurally diverse compounds and are made from one or more hydroxyl groups attached to an aromatic hydrocarbon ring such as methyl salicylate (Figure 1.3) and eugenol. These compounds play an important role in plant defense. Other relatively less common amino acid derived volatiles use alanine, valine, leucine, isoleucine, and methionine to produce aldehydes, alcohols, esters, nitrogen- and sulfur-containing volatile compounds (Dudareva et al., 2006).

Production and role in plant defense

During the evolutionary timeline, plants have developed various strategies for defending themselves against, or at least tolerating insect herbivore attack. Plant defense strategies can be broadly categorized into either structural or chemical defenses, though these tactics can work in concert. Chemical defenses are characterized by the constitutive or induced production of myriad secondary metabolites. A large portion of these defense compounds are volatile compounds that are involved in different modes of defense: direct defense, indirect defense and inter-plant priming. Direct defense involves the production of toxic compounds or volatiles that directly kill or repel herbivores. Plant compounds that are antifeedant, antinutritive, repellent, and/ or toxic to the herbivore are called direct defenses. Direct defenses also include physical barriers, such as leaf thickness and presence of foliar and stem trichomes. Some direct defense traits are constitutive and have well developed biosynthesis programs independent of herbivory. However, in addition to these constitutive levels, pest invasion activates the induced defense response in the damaged tissues that may be further transmitted systemically to undamaged tissues (Howe and Jander, 2008).

Indirect defenses involve the production of compounds that minimize infestations of herbivores by attracting natural enemies of the herbivore (Guerrieri et al., 2004). Plants defend themselves indirectly by attracting natural enemies of herbivores through release of volatiles, by providing food, such as floral nectar, and/ or by providing shelter, such as domatia (Agrawal, 2000). Isothiocyanate, a volatile compound produced from glucosinolates after tissue damage in brassicas, has been found to attract aphid parasitoids *Diaeretiella rapae* (Bradburne and Mithen, 2000). Rice has been found to emit a group of volatile compounds that attracts females of the parasitoid wasp *Cotesia marginiventris* when it was fed on by fall armyworm caterpillar (Yuan et al., 2008). Another aphid parasitic wasp, *Aphidius ervi* is able to discern between volatiles emitted by broad bean plants attacked by host and non- host species (Guerrieri et al., 2004).

In addition to mediating the interaction of a plant with both its pest arthropod species and their natural enemies, volatile compounds induced by herbivory may also signal adjacent and downwind plants of a possible herbivore threat, which is called inter-plant priming (Howe and Jander, 2008). In plant defense, priming is a physiological process through which a plant prepares to more quickly or aggressively respond to future biotic or abiotic stress (Frost et al., 2008). The condition of readiness achieved by priming is termed the "primed state" (Conrath et al., 2006). Corn seedlings previously exposed to green leaf volatiles (GLVs) from neighboring plants produced significantly more JA and sesquiterpenes when mechanically damaged and induced with caterpillar regurgitant, than the seedlings not exposed to GLVs. Therefore, GLVs specifically primed neighboring plants against herbivory by enhancing induced defense response

(Engelberth et al., 2004). Fitness parameters of native tobacco was improved through interplant signaling via above ground volatile, when they were transplanted adjacent to clipped sagebrush. Transcriptional responses were found in tobacco growing adjacent to clipped sagebrush foliage (Kessler et al., 2006). Further investigations about the interplant priming in different plant species can reveal interesting and potentially useful information for agriculture.

Many volatiles are produced in plant tissues at specific developmental stages such as flowering, ripening, or maturation and serve various functions (Goff and Klee, 2006). In addition to biotic agents, like fungi, bacteria, and herbivores, abiotic factors, like nutrient stress, drought, UV radiation, and temperature have been found to up regulate or induce certain volatiles. A large number of secondary metabolites is produced mainly through three major biochemical pathways. Carbohydrate, fatty acid, and nitrogen metabolism precede photosynthesis. Each leads to one or more major metabolites that produce several derivatives through various enzymatic transformations (Hartmann, 1996) such as hydroxylation, acetylation, and methylation (Dudareva et al., 2006). In many species, herbivory activates the octadecanoid pathway, which regulates the production of certain volatiles, that attract host-seeking parasitic wasps (Thaler, 1999). The lipoxygenase, the shikimic acid, and the terpenoid pathways are the major biosynthetic pathways producing the most common volatiles that mediate plant defense (Pichersky and Gershenzon, 2002). The jasmonate pathway is also involved in increasing resistance to a wide range of insects in different kinds of tissue (Howe and Jander, 2008). The chemical diversity of plant volatiles is related to the unique biology and ecology of the host plants that produce them.

Species and cultivars with different volatile profiles

The natural host preferences of some insects for a specific plant species or variety were identified long ago (Ellis et al., 1979; Guerin and Stadler, 1984; Guerin and Ryan, 1984). Along with other factors, such as color, different volatiles emitted by specific genotypes play a critical role in host recognition. Volatile profiles in different domesticated genotypes and their wild relatives confer different levels of compounds related to direct and indirect defense against herbivores. Undamaged leaves from one naturalized and five commercial cotton varieties showed no significant difference in their volatile profiles. However, average volatile emissions from the beet army worm damaged leaves of the naturalized variety were found to be seven times higher than those from the leaves of commercial varieties even though larvae preferred commercial cultivars to feed on. These volatiles were monoterpenes, sesquiterpenes, and lipoxygenase products (Loughrin et al., 1995).

Volatile compounds released by leaves of 10 willow varieties that vary in their susceptibility to herbivory by blue (*Phratora vulgatissima*), brassy (*P. vitellinae*), and brown (*Galerucella lineola*) willow beetles. The concentration of cis-3-hexenyl acetate and its relative proportion to cis-3-hexenol were significantly different among the varieties after physical wounding. Some of these varieties showed a significantly negative correlation between the resistance of each variety to blue and brown willow beetles and the yield of cis-3-hexenyl-acetate from wounded plants. The green leaf volatile ratio of damaged plants was also negatively correlated with the relative resistance of the willow variety to these two beetle species. Blue and brown willow beetles preferred *Salix dasyclados* whereas, brassy beetle preferred two species *S. burjatica* and *S. purpurea* (Peacock et al., 2001). Leaves from seven tomato lines with different levels of resistance to early blight and late blight were analyzed, and quantitative and qualitative difference in volatile compounds were observed (Table 1.1). This information about volatile profiles can be utilized to breed cultivars with enhanced resistance for various insect pests and diseases.

Model organisms

In this dissertation tomato (*Solanum lycopersicum* L.) was chosen as the host plant. Beet armyworm (*Spodoptera exiguae* H.) was chosen as the above ground insect pest. Two microorganisms, *Glomus intraradices*, a symbiotic arbuscular mycorrhiza fungus, and *Beauveria bassiana*, an entomopathogenic endophyte were chosen as beneficial organisms. Tomato root knot nematode (RKN): *Meloidogyne incognita* was selected as the below ground pest.

Tomato (Solanum lycopersicum L.) Tomato was chosen as the plant model for this study as it is one of the most important vegetable crops in the world. It is grown worldwide for its edible fruits, with thousands of cultivars selected based on fruit size, shape, growing conditions, disease and insect pest resistance, and several other factors. The US is one of the biggest producers in the world, and fresh and processed tomatoes account for more than $2 billion in annual farm cash receipts in the country (Lucier and Glaser, 2009). Wherever they are grown, tomatoes host a wide range of insect pests and diseases, and all parts of the plant provide food and shelter to pests. The range of tomato insect and arthropod pests can range roughly between 100 to 200 species. In some situations secondary pests can become primary pests. Therefore, constant efforts are required to keep the pest below economic threshold (Lange and Bronson, 1981). This becomes even more important in organic production system due to limitations of pest protection strategies. During the past 25 years, considerable research has been directed toward identifying and developing a mechanistic understanding of pest resistance in tomato. That body of research reveals a level of detail and complexity in plant-symbiont-herbivore-natural enemy interactions at the tri-trophic level. These unique interactions give insight into the plant's mechanism to survive and thrive in the natural ecosystem, as well as give direction towards developing more eco-friendly strategies to manage pests in the agricultural crop production system (Kennedy, 2003).

Due to the commercial importance of tomatoes and wide range of pests pest management is one of the most critical aspects of tomato production. Much research has been done on pest management in tomato and arrays of commercial pest control methods are available in the market. However, recently, as consumer demand is increasing for organic and/or low pesticide sprayed tomatoes and due to growing environmental concerns, researchers and growers alike are looking for alternate strategies.

Like every plant, tomato has its own constitutive and induced defense system, which helps the plant protect itself from various kinds of disease and insect pests (e.g. glandular trichomes, α- tomatine in tomato, some terpenoids, etc.) (Kennedy, 2003). However, these mechanisms are not sufficient to impart complete protection to the plant in today's intensive agricultural system. However, tomato's trait of emitting volatile compounds especially when induced by various biotic factors like herbivory or physical damage, and microorganisms can be utilized in different ways to minimize insect pests and pesticide use in tomato production. The reasons why tomato was chosen for this study are: importance as a vegetable crop, cultivation techniques are very well standardized for the field, as well as greenhouse conditions, ability to emit myriad of secondary metabolites including volatile compounds. Tomato and many other members of the solanaceae family serve as model plants to study biosynthesis of particular secondary metabolites as well as plant defense (Mueller et al., 2009). In addition, many genomic studies have been done on tomato, and so we have good information about tomato at the molecular level (Mueller et al., 2009).

*Beet armyworm (Spodoptera exigua H. Insecta: Lepidoptera: Noctuidae)*Beet armyworm was selected as the above ground pest model as it is one of the most important generalist pests of fresh- market and processed tomato (Brewer et al., 1990) and is known to induce defense responses in many crops, such as cotton (*Gossypium hirsutum*) (Loughrin et al., 1994), (Rose et al., 1996), maize (Schmelz et al., 2001), tropical soda apple (*Solanum viarum*), a relative of tomato (Hix et al., 2008). Moreover, they are commercially available, which makes it easier to have a supply constant throughout the course of study, and are easy to rear.

*Mycorrhizaea: Glomus intraradices (Glomerales: Glomeraceae)*Arbuscular mycorrhizal fungi (AMF, phylum *Glomeromycota*) are one of the most common fungi in the rhizosphere, having symbiotic associations with plant roots. Mycorrhizae are being utilized in crop production for several purposes such as enhanced phosphorus and other nutrient supply, better drought resistance, and improved soil structure. They are found in an extremely broad range of habitats, indicating a high tolerance for environmental factors and a generalist life history strategy (Gianinazzi et al., 2010). Recently, AMF have also been studied for their potential utilization for pest control, as they have been found to have enhanced protection against nematodes, fungi, and insect pests. In a study with Potato aphid (*Macrosiphum euphorbiae*) and its parasitoid, *Aphidius ervi*, on tomato, with and without mycorrhizae, it was found that both plants inoculated with mycorrhizae and plants infested with aphids were twice as attractive to parasitoids as the control (without aphids) plants. This indicates that volatiles produced from mycorrhizal plants and aphid infested plants were similar. Significantly, lower reproduction and growth of aphids was found in plants associated with mycorrhizae compared to the control (Guerrieri et al., 2004).

These defense responses have been found to be systemic in nature (Guerrieri and Digilio, 2008). Enhanced pest protection might be due to increased availability of soil nutrients through AMF, thus improving overall plant health (Hodge et al., 2001). Apart from that, mycorrhizae can induce reorganization of host cell structure, and secrete chitin elicitors, which induce defense responses. Many reports indicate that they have been most effective against chewing type herbivores. Performance of polyphagous chewers and sucking type mesophyll feeders was reduced on mycorrhizal plant whereas, sucking type phloem feeders are reported to be benefited from mycorrhizal infection (Koricheva et al., 2009).

Entomopathogenic endophyte: Beauveria bassiana (Hypocreales: cordycipitaceae) An endophyte is a bacterium or fungus, which lives within a plant without causing apparent disease. *Beauveria bassiana* is an entomopathogenic fungi that can exist inside the plants (Ownley et al., 2008). They have been found to attack a wide range of arthropod hosts. They grow systemically in intercellular spaces in different plant tissues in all plant parts (Rudgers and Clay, 2008). A study with *Beauveria bassiana* in banana reported reduced crop damage by banana weevil (up to 42-86.7% less) (Akello et al., 2008). Endophytes have been found to alter the volatile profile by altering the physiology of the plant. This altered physiology also affects herbivore food utilization. In addition, endophytes also induce production of feeding–deterrent alkaloids. These alkaloids make the host plants toxic or distasteful to herbivores. It has been reported in several field and laboratory studies that herbivores avoid feeding on endophyte infected plants and increased mortality and decreased growth has been found on infected grasses (Clay, 1996).

Tomato root knot nematode: Meloidogyne incognita (Tylenchida: Heteroderidae) Root-knot nematodes (RKN) are minute, thread like worms that are very common in soil. They are sedentary endoparasites and have a very wide host range. RKNs pose the greatest overall nematode threat to tomatoes worldwide (Duncan and Noling, 1998). *Meloidogyne incognita* spp. is the most widespread and damaging RKN in tropical and subtropical regions and in Tennessee (E. Bernard, personal communication). They are obligate parasites that require living plant tissue and their life cycle includes the egg, four juvenile stages and the adult.

RKNs do not produce specific symptoms above ground during early plant growth. However, in later stages plants show an unhealthy appearance characterized by stunting, wilting or chlorosis. Symptoms are severe when plants are infected soon after planting. Affected plants begin to wilt, die back with flowering, fruit set and fruit development (Duncan and Noling, 1998). As RKNs feed on a wide range of hosts and symptoms are visible in the later stage of growth, it is difficult to control this pest on time. The two most popular controls are applying toxic nematicides for prevention and growing RKN resistant cultivars. Nematicide application has limitations as it is not ecofriendly and it is expensive. As for resistant cultivars, some cultivars might not be suitable for all growing conditions and might not be best agronomically (Molinari, 2005). Therefore, there is an emphasis on studying and finding alternative methods for nematode control.

Methyl Salicylate (MeSA), and Salicylic acid methyl transferase (*SAMT*)

A vast array of genes work in coordination to provide basal and systemic acquired resistance (SAR) and salicylic acid (SA) is one of the three most important biochemical compounds, along with jasmonic acid (JA), and ethylene (ET), known to regulate the defense response (Glazebrook, 2005). The importance of SA in plant defense has been demonstrated in a large number of experiments. Several Arabidopsis mutants that express systemic acquired resistance (SAR) show a high level of SA accumulation (Bowling et al., 1997). In tomato *Mi-1* gene confers resistance against three species of root-knot nematode and SA is an important part of *Mi* gene mediated resistance. Transformation of tomato carrying *Mi-1* with a construct expressing *NahG,* which encodes salicylate hydroxylase, a bacterial enzyme that degrades salicylic acid (SA) to catechol, has been found to result in partial loss of resistance to root-knot nematode *M. javanica* (Branch et al., 2004). It was recognized that SAR is characterized by the accumulation of SA, which act as a mediator for SAR (Durner et al., 1997).

For a long time, scientists worked to identify the mobile signal that alarms the whole plant about potential danger and SA had been considered as the endogenous long distance signal molecule that accumulates in the non-infected region to induce plant defense response, which is supported by the detection of SA in the phloem of pathogen-infected tobacco and cucumber (Métraux et al., 1990; Rasmussen et al., 1991). One lipid derived signal JA was already suggested to be active in SAR, especially in the case of wounding and herbivory. However, the role of SA as the long distance signal was challenged through many experiments. Grafting experiments suggested that SA may not be a primary mobile signal in SAR but it is necessary for the induction of SAR (Vernooij et al., 1994).

Methyl Salicylate (MeSA) esterase activity of the salicylic acid–binding protein 2

(*SABP2*), which converts MeSA into salicylic acid (SA), is required for SAR signal perception in distal tissue that does not receive the primary (initial) infection. In plants with mutant *SABP2*, with unregulated MeSA esterase activity in the SAR signal–generation, SAR is shown to be suppressed and MeSA levels are decreased in the infected areas. Therefore, MeSA was confirmed as mobile SAR signal in tobacco (Park et al., 2007). They indicated that MeSA was needed to be made at the site of infection and SAR can be induced in the graft even if the enzyme that makes MeSA is missing there, but not if this enzyme was absent in the rootstock. The tissue first produces MeSA, which is distributed throughout the plant by phloem. *SABP2* then converts MeSA in to SA, which triggers SAR in the target tissue (Park et al., 2007).

In addition to its role as mobile defense signal, MeSA is also an important contributor to the taste and scent of many fruits and flowers (Tieman et al., 2010a). MeSA is found to have an important role in multiple trophic interactions, acting as an attractant of insect pollinators and seed dispersers, and inducing defenses against microbial pathogens. Several reports have shown that MeSA is the constituent of floral flavor, which can attract the insect pollinators (Knudsen et al., 2006). MeSA is an organic ester produced by plants attacked by pathogens or herbivores. Most insect infested plants release methyl salicylate, which aids in the recruitment of beneficial insects to kill the herbivorous insects and also possess anti-microbial properties. Aside from its toxicity, methyl salicylate may also be used by plants as a pheromone to warn other plants of pathogens. MeSA is synthesized from salicylic acid by salicylic acid methyl transferase enzyme, a member of a family of O-methyltransferases. MeSA is produced by a reaction where the methyl group is transferred from donor S-adenosine-L-methionine to carboxyl group of SA catalyzed by salicylic acid methyl transferase (*SAMT*), which is regulated by the *SAMT* gene (Tieman et al., 2010b).

The *SAMT* gene, has been isolated and characterized from a variety of plant species. The first SAMT genes are identified from the flower of the annual plant *Clarkia breweri* (Ross et al., 1999). However, there is no induced expression of *SAMT* during various treatments reported in these papers. Later, a new *SAMT* identified from Arabidopsis, was named *AtBSMT1*, which showed *SAMT* activity. *AtBSMT* was the first one of *SAMT* genes to be shown to have a defense role (Chen et al., 2003). Recently, tomato *SAMT* has been identified, characterized, and validated by analyzing over and under expressed lines. Analysis of MeSA emissions from an introgression population derived from a cross with *Solanum pennellii* showed higher MeSA emissions associate with significantly higher *SpSAMT* expression (Tieman et al., 2010b)

Goal and major objectives

The goal of my dissertation is to enhance the understanding of production and roles of volatile secondary metabolites in interactions of the host plant tomato (*Solanum lycopersicum* L.) with other organisms above and below ground.

In this dissertation research I propose to answer three central questions:

1. Does plant interaction with one organism lead to change in the secondary metabolism eventually leading to alterations in the volatile profile?

2. Does this alteration, due to one organism, lead to changes in the plant's interaction with another organism?

3. How are below ground and above ground interactions of the plant linked together?

This dissertation contains three major objectives **1. Discuss plant volatile compound based insect pest management in organic farming; 2. Investigate the effect of two fungi: *Glomus intraradices*, mycorrhizae, and *Beauveria bassiana*, an entomopathogenic endophyte, on the volatile secondary metabolite profile of tomato plant and on induced defense against Beet Armyworm; 3. Investigate the effect of RKN *Meloidogyne incognita* on volatile secondary metabolites of the plant, it's induced resistance against Beet armyworm and Salicylic acid methyl transferase *SlSAMT* gene expression in resistant and susceptible tomato lines.**

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Appendix

Tables.

Table 1.1. Volatile compounds [means ng g (fresh wt)$^{-1}$±SE] from the leaves of tomato lines with different levels of resistance against late blight and early blight. Seeds were generously provided by Dr. Dilip Panthee (NC State University, Raleigh, NC). Seven lines were grown in the green house in potting mix (Conrad Fafard Inc. Agawam, MA). Second leaf from the top from four-week old randomly selected plants of same size from each line was ground with liquid nitrogen and extracted with ethyl acetate containing Octanol (0.003%) as internal standard was used for extraction. Extracts were injected into GC-MS (Shimadzu Corp, Columbia, MD) for separation and identification of compounds. Identification of compounds was based on NIST database[#]. N=5

Compounds	NC6113	Culbpt04	860A	NC946	Celbr1	Ebr1	Ncics
α-Pinene	1.2±0.4	3.1±1.4	2.7±1.0	2.0±0.5	3.7±2.1	1.7±1.0	2.8±1.4
β-Myrcene	0.3±0.0	0.6±0.2	0.6±0	0.4±0.1	2.5±2.1	0.3±0.1	1.6±0.6
2-Carene	2.3±0.6	6.2±0.9	4.3±1.5	3.5±1.2	7.1±1.5	3.9±1.9	4.5±0.5
α-Phellandrene	0.7±0.1	0.9±0.3	1.6±	1.1±0.6	1.7±0.4	1.2±1.0	1.4±0.7
Limonene	1.5±0.4	3.8±1.0	2.5±0.8	3.6±1.2	4.4±0.6	2.4±1.2	3.1±0.4
β-Phellandrene	7.4±2.2	23.5±3.8	12.1±3.9	16.4±4.5	22.1±2.9	13.2±6.3	14.8±1.6
β-(*E*)-Ocimene	ND[*]	0.3±0.1	0.3±0.1	ND	0.3±0.1	ND	ND
<u>Total Monoterpenes</u>	12.6±3.7	36.2±3.8	21.6±6.9	26.7±7.6	39.1±5.2	22.0±10.2	26.4±3.9
δ-Elemene	0.4±0.1	2.1±0.5	0.5±0.1	1.3±0.3	1.0±0.1	0.7±0.3	1.6±0.1
β-elemene	ND	0.2±0.1	ND	ND	ND	ND	0.1±0.0
(*Z*)-Caryophyllene	0.7±0.2	3.8±0.6	1.3±0.4	2.8±0.6	1.0±0.2	1.1±0.5	2.6±0.2
α-Humulene	0.2±0.0	0.8±0.1	0.4±0.2	0.6±0.1	0.2±0.1	0.3±0.1	0.5±0.1
<u>Total Sesquiterpenes</u>	1.2±0.4	6.9±1.1	1.9±0.6	4.6±1.0	2.1±0.4	2.0±0.9	4.7±0.4
2-Hexenal	6.1±2.2	1.4±	18.6±7.5	7.4±0.7	2.5±0.2	6.1±1.1	2.0±1.8
1,3,5-Cycloheptatriene, 3,7,7-trimethyl-	0.8±0.1	0.7±0.2	1.0±0	1.0±0.4	ND	0.8±0.2	1.3±0.4
Benzyl Alcohol	0.5±0.1	0.2±0.1	0.7±0.1	0.9±0.1	1.3±0.1	0.4±0.1	0.3±0.1
Eugenol	ND	ND	ND	0.1	ND	ND	ND
<u>Total others</u>	4.4±1.9	0.8±0.6	19.5±7.7	5.4±2.4	1.5±0.5	5.8±1.8	2.0±1.4
Total volatiles	18.1±6.0	43.8±5.6	43.1±15.1	36.7±9.7	42.7±5.5	29.7±11.6	33.0±4.8

[*] Not detected

[#] Detailed description about method of volatile extraction and analysis in chapter 3.

Figures

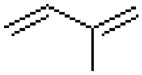

Figure 1.1: Isoprene (C_5H_8), the basic five carbon unit of terpenoids.

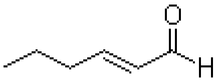

Figure 1.2: Trans-2-Hexenal ($C_6H_{10}O$), an example of volatile fatty acid derivative.

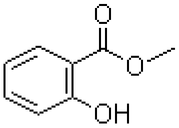

Figure 1.3: Methyl salicylate ($C_8H_8O_3$), a phenylpropanoid.

Chapter II. Plant Volatiles-based Insect Pest Management in Organic Farming

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Abstract

Organic agriculture is increasing in popularity worldwide due to the rapidly growing market for organic products. In organic production, insects present a major pest challenge that negatively impacts crop health and yield. To successfully manage an organic farmland, an effective insect pest management program is key. In this review, we first describe the approaches currently used for pest management in organic farming. Next, we review natural plant defense mechanisms, especially those based on plant volatile organic compounds. Chemically complex, plant volatiles have multiple ecological roles in plant-insect interactions including attracting pollinators, acting as cues for foraging herbivores as well as functioning as direct defense, indirect defense, or interplant priming. Based on the ecological roles of plant volatiles, we then discuss in-depth how pest management may be improved through a variety of strategies including using resistant cultivars, polyculture, using beneficial microorganisms such as mycorrhizal fungi and endophytes, and using plant-derived pesticides, all of which are reviewed in the context of plant volatiles. Lastly, integration of these different strategies based on the trait of plant volatiles for a successful and sustainable pest management program in organic farming is discussed.

Keywords organic farming, insect pests, plant volatiles, plant defenses

Introduction

Promoted early in the 1940s by J. I. Rodale, and spurred by writings of British agriculturalist Sir Albert Howard, organic farming in the U.S. has been politicized and polarizing, despite growing popularity and consumer demand. Organically grown produce is an alternative to conventionally grown produce. Although sometimes difficult to verify scientifically (Dangour et al., 2009; Woese et al., 1999), organic products are often perceived by consumers as healthier and more environmentally friendly (Yue and Tong, 2009; Williams and Hammitt, 2001; Thompson and Kidwell, 1998). The movement of organic farming in the U.S. was directly related to the indiscriminate use of chemical pesticides, herbicides, and fertilizers. Heavy reliance on synthetic agrochemicals, although undeniably an important factor contributing to increased food production and subsequent food security, led to significant health and environmental concerns. Despite reluctance of certain agricultural communities, organic agriculture gradually began to gain a foothold in U.S. culture. In 1979, the California Organic Food Act was signed into law, which established standardized production practices for organic food products.

In 1990, the Federal Organic Foods Production Act was passed, which established national standards for organic products, thus allowed for consistency in interstate trade. It took twelve years from that date to finalize a set of standards administered by the USDA's National Organic Program. Since 2002, foods that are certified organic are accompanied by the USDA organic label. The USDA currently defines organic agriculture as "an ecological production management system that promotes and enhances biodiversity, biological cycles and soil biological activity. It is based on minimal use of off-farm inputs and on management practices that restore, maintain and enhance ecological harmony." Since the adoption of national standards, organic sales in the U.S. have increased by 20% or more per year, representing one of the fastest growing sectors in agriculture (Oberholtzer et al., 2007).

In a survey of organic farmers administered by the Organic Farming Research Foundation, farmers identified several major challenges faced by organic producers. Among these were issues related to practical weed management, followed by management of insect pests, vertebrate pests, and diseases (Walz,1999). In this review, we restrict our discussion to management of insect pests. Among the key insect pests of concern, the most significant challenges come from cucumber beetles, flea beetles, Colorado potato beetles, aphids, codling moth, leafhoppers, and grasshoppers (Walz, 1999). These insect pests can cause significant losses in crop yield and produce aesthetics, especially in organic production systems. For example, despite implementation of various organic management efforts, codling moth larvae caused damage levels exceeding 25% in organic apple orchards in British Columbia (Judd et al., 1997). It is important to recognize that insects can cause differing levels of crop damage depending upon both the life stage of the insect pest and the developmental stage of the crop. In addition to direct feeding damage, insects can vector many different plant viruses. Wounds caused by insect feeding also become potential entry points for pathogenic fungi and bacteria. Therefore, adequate management of key insect pests remains critical to the health of organically grown crops and longevity of perennial plants within an organic production system.

Undoubtedly, insect pests are an important factor in yield and commercial salability of conventional agricultural commodities. Various management strategies have been developed to control insects, including botanical, biorational and synthetic insecticides as well as creation of transgenic pest- and disease-resistant crops, such as Bt corn and cotton. Genetically modified crops are not permitted for use in organic farming systems, and options of chemical insecticide are limited. In light of these constraints, it can be significantly more challenging to sufficiently manage insect pests using approved organic options. In this review, we first discuss approaches currently used to manage insect pest populations in organic production systems. We then review and discuss the potential for volatile compounds produced by plants to optimize or improve insect pest management in organic production.

Current approaches to insect pest management in organic farming

A major difference between conventional and organic pest management is the limited choices of pesticides for organic production. Consequently, the focus in organics has been placed on preventative measures, which emphasize an ecosystem approach in pest management. This “agroecosystems” approach drives systems-based research, where relationships between complex elements such as plants and insects are explored. A systems approach to pest management is knowledge-based rather than product-based, which takes the emphasis off therapeutic management, or reactive strategies, and focuses on prevention and proactive strategies for management (Lewis et al., 1997). Additionally, the advance of Integrated Pest Management (IPM) practices focuses on using a variety of strategies to keep pest populations below pre-determined economic or aesthetic threshold levels, rather than targeting pest eradication. In this section we discuss current pest management strategies in organic production, which include prevention, biological control and chemical control.

A. Prevention

Due to limitations of allowable and available insecticides and/or interest in conservation and reducing synthetic inputs, organic farmers must focus on pest prevention by stopping arthropod outbreaks before they occur. This is most commonly attempted through use of crop rotation programs, increasing overall crop biodiversity and potential habitat for beneficial organisms (Lotter, 2003). Besides increasing yields, rotating crops improves soil conditions and fertility, and reduces insect and disease pressure. Crop rotations normally follow a three year schedule where genetically diverse crops are alternated. Cover crops are ideal for in-between years where vegetable crops are not grown, and can be cultivated to address specific soil needs. For example, oats provide biomass that builds soil organic matter, while legumes such as clovers and vetch provide nitrogen for the following crop. In addition, cover crops increase the biodiversity of the farm. In particular, by providing a constant supply of nectar, cover cops may help sustain beneficial insect populations. For example, cover crops such as phacelia (*Phacelia tanacteflolia*) and buckwheat (*Fagopyrum esculentum*) provide nectar for hoverflies and bees, which are beneficial insects (Pontin et al., 2006). As a consequence, pest cycles can be broken and pest outbreaks reduced on such farmland.

Timing to avoid pest outbreaks and using resistant varieties can be successful cultural management strategies. The female Hessian fly, *Mayetiola destructor*, has a small window of time after emergence in which to lay eggs and is primarily associated with wheat. However, if this preferred host is not available, eggs may be deposited on other grasses such as oats that do not support larval development (Harris and Rose, 1989). In order to avoid damage by Hessian fly, growers can delay planting wheat to avoid adult emergence. In addition to timing, breeding wheat varieties for resistance to Hessian fly has also been successful (Williams et al., 2003). Other integrated management practices can involve use of row covers, colored plastic and conventional mulches, physical barriers, sticky traps and barriers, scent lures, and pheromone lures and traps that prevent target insect pests from finding host plants and reproducing. Row covers can be put up and removed fairly easily to provide protection from insect pests. Some research has shown that mulches can reduce insect pest pressure. Whitefly (*Bemisia argentifolii*) colonization was also hindered in zucchini crops when wheat straw and reflective mulches were used (Summers et al., 2004). Sticky traps and pheromone traps are often used for insect monitoring, but can be used in organic systems to attract pests away from crops and disrupt mating. Yellow sticky traps visually stimulate insect pests such as greenhouse whiteflies (*Trialeurodes vaporariorum*) and tarnished plant bugs (*Lygus lineolaris*). When paired with traps, pheromone and scent lures utilize both chemical and visual attractants (Foster and Harris, 1997). These can be sex or aggregation pheromones, or kairomones, which manipulate insect behavior by mimicking chemical cues that elicit a response (Foster and Harris, 1997). Squash vine borer (*Melittia cucurbitae*) is a destructive pest that is difficult to manage in organic systems. Mass trapping using pheromones can lure males away from females that would otherwise mate and lay eggs (Jackson et al., 2005).

B. Biological Control

Biological control is the use of natural enemies to manage pests. There are three different types of biological control recognized: conservation, augmentation and importation. Conservation biological control involves habitat manipulation to increase populations of predators and parasitoids, that can help keep pest populations from building up to injurious levels. An example of conservation biological control is planting strips of nectar-producing flowers that provide energy-rich sugars to beneficial insects. Dill (*Anethum graveolens*), coriander (*Coriandrum sativum*), and phacelia (*Phacelia tanacetifolia*) produce nectar and pollen, and have adequate flower morphology to support predatory lacewings (*Chrysoperla* sp.), lady beetles (*Coleomegilla maculata*), and syrphid flies (family *Syrphidae*) in agricultural areas (Landis et al., 2000). These generalist predators eat insect eggs and soft bodied insects, such as aphids. Parasitoid insects lay their eggs in or on a host, and developing larvae exploit host resources eventually causing death. Parasitoid insects are often host-specific, and are adept at finding their hosts. Adult wasps that have access to food sources will live longer and lay more eggs. Adult encyrtid wasps (*Copidosoma koehleri*) lived twice as long as controls when given nectar from buckwheat, faba beans, phacelia, and nasturtium (Baggen et al., 1999). Additional habitat management strategies to conserve beneficial insects are adjusting mowing, height, providing overwintering habitat, and providing alternate hosts (Landis et al., 2000). Augmentative biological control involves the deliberate release of beneficial species, such as predatory bugs, mites, beetles and midges, parasitic wasps, entomopathogenic nematodes, fungi, bacteria and viruses, to manage pests in a controlled environment. This type of biological control is best applied before pest outbreaks occur. Over 100 different species are commercially available for augmentative biological control, mostly used in greenhouse situations (van Lenteren, 2000). Importation biological control refers to use of non-native natural enemies that are sought out, imported, reared, tested and introduced from their indigenous habitat to manage an exotic invasive pest. One of the oft-cited successes of this type of biological control is management by the vedalia beetle (*Rodolia cardinalis*) of cottony cushion scale (*Icerya purchasi*), a serious citrus pest. The beetle was imported to the U.S. in 1888 to help control the scale insect and remains effective at managing populations of the pest (Greathead, 1995).

C. Chemical Control

In the past 50 years, insects have mainly been controlled by synthetic insecticides (Kabaru and Gichia, 2001). While many insecticides are not allowed in organic production, there are some organic insecticide options that are approved by the Organic Materials Review Institute for use in organic cropping systems. The Organic Materials Review Institute is a nonprofit organization that independently reviews products for compliance with the USDA National Organic Program, and publishes this information for organic certifiers, producers, and manufacturers (www.omri.org). The permitted list of insecticidal products includes plant-based extracts, such as neem oils, pyrethrums, ryania, rotenone, and sabadilla, and microbe-derived chemicals, such as spinosad, which is derived from a naturally occurring soil bacterium called *Saccharopolyspora spinosa*. Soaps, mineral oils, and potassium salts of fatty acids may also be used to help manage small, soft-bodied insects. Mineral products such as kaolin clay and diatomaceous earth are also allowed. Most organic pesticides have low residual activity, but some are rather stable. For example, veratridine and cevidine, two alkaloid components of sabadilla, an organic pesticide, degrade slowly with 50% and 10% of degradation, respectively, when exposed to 22 days of sunlight (Rosen and Zang, 2007). There is much contention and controversies surrounding the safety, risks, and environmental impacts of organic versus conventional pesticides, with many consumers believing that organic foods produced using organic pesticides are safer than conventionally grown foods using synthetic pesticides (Williams and Hammitt, 2001). Scientific data supporting or rejecting this perception are still needed (Dangour et al., 2009).

Biology of plant volatiles

Despite availability of a variety of pest control options, efficient and effective insect pest management in organic farming remains challenging. When developing new pest management methods, it is important to fully consider the plant's natural defense mechanisms as well as mechanisms that direct interactions of the host plant with its environment. During a long co-evolutionary timeline, plants have evolved various strategies for defending against or tolerating insect herbivore attack. In the past few decades, there has been tremendous academic and commercial interest in elucidating plant natural defense mechanisms related to enhanced insect resistance with a long-term goal of genetic crop improvement. Some approaches presently used to assist insect management in organic farming described in the previous section are in essence attempts to emulate plant natural defense mechanisms. Plant defense strategies can be broadly categorized into either structural or chemical defense, though these tactics can work in concert. Chemical defenses are characterized by the constitutive or induced production of a myriad of secondary metabolites (Chen et al., 2009b). Some of these secondary metabolites are small molecular weight lipophilic compounds with high vapor pressure and therefore easily volatilize at normal atmosphere. Plant volatiles play important roles for the host plant to interact with its environment, especially plant insect interactions. In order to effectively utilize plant volatiles for insect management in organic farming, it is necessary to understand the biology of plant volatiles: what they are, how they are synthesized, and what biological/ecological functions they have.

A. Plant Volatiles: Chemistry and Biosynthesis

Approximately 1,700 volatile secondary metabolites have been identified from more than 90 plant families and this number is very likely going to increase with studies on more plants and development and application of new analytical methods (Knudsen and Gershenzon, 2006). Although very complex, the majority of plant volatiles belong to three groups: terpenoids, fatty acid derivatives, and phenolics (Dudareva et al., 2006). Volatile terpenoids, including monoterpenes and sesquiterpenes, are synthesized through the non mevalonate and the mevalonate pathways, respectively (Eisenreich et al., 2001). Terpene synthases are the key enzymes for terpene production. They catalyze the formation of monoterpenes, sesquiterpenes and diterpenes using geranyl diphosphate, farnesyl diphosphate, and geranylgeranyl diphosphate as substrate, respectively. Phenolic volatiles are produced from the shikimic acid pathway (Herrmann and Weaver, 1999). Methyl salicylate is the most important representative in this group. Methyl salicylate is formed from salicylic acid, which is a defense signal, through the action of salicylic acid methyltransferase (Chen et al., 2003). Salicylic acid methyltransferase belongs to a plant-specific protein family called “SABATH” (Zhao et al., 2007, 2008, 2009). Fatty acid-derived volatiles are synthesized through the lipoxygenase pathway. Lipoxygenase catalyzes the dioxygenation of polyunsaturated fatty acids such, as linoleate and linolenate to yield hydroperoxides (Porta and Rocha-Sosa, 2002). Hydroperoxides are further metabolized via the hydroperoxide lyase pathway to produce volatile aldehydes and alcohols, which are also known as green leaf volatiles.

Many plant volatiles are produced in specific tissues at specific developmental stages, such as flowering, ripening, or maturation and serve various functions (Goff and Klee, 2006). Some plant volatiles are constitutively produced. Some are induced by abiotic stresses such as nutrient stress, drought, UV radiation, and temperature or biotic agents such as fungi, bacteria, and herbivores. Stress factors can activate various biochemical pathways that lead to the production of a single or mixture of different volatile compounds through the regulation of a number of plant signaling molecules, such as jasmonic acid, ethylene (Schmelz et al., 2003), and salicylic acid (Ozawa et al., 2000).

B. Ecological Functions of Plant Volatiles in Plant-Insect Interactions

Flowers of many plants release a complex mixture of volatile compounds and are therefore strongly scented. Floral volatiles are used by many pollinators as an olfactory cue for visitation (Zhuang et al., 2008). Therefore, floral volatiles are important for the reproductive success of many cross-pollinated plants. In addition to attracting pollinators, floral volatiles may function in deterring unwanted floral visitors (Omura et al., 2000). For example, as a floral scent compound of *Nicotiana attenuata*, nicotine has a defense role against herbivores (Euler and Baldwin, 1996). The functions of vegetative volatiles are much more complex. Specialist arthropod herbivores may use vegetative volatiles as the major cue for host finding. Different insect species have preference for different volatile compounds.

Plant volatiles may have multiple functions in defenses against insects: direct defense, indirect defense, and interplant priming. When plant volatiles function as toxins that kill herbivores or reduce plant digestibility, they are called direct defense. Volatiles derived from glucosinolate degradation are a good example of direct defense against insects (Halkier and Gershenzon, 2006). Several herbivory-induced plant volatiles, such as linalool, were shown to function as a direct defense in nature (Kessler and Baldwin, 2001). The production of plant volatiles for direct defense may be constitutive independent of herbivory. Young and reproductive tissues generally contain large amounts of these defense compounds. In addition to constitutive production, some toxic plant volatiles are induced by herbivory, in which the jasmonic acid signaling pathway plays a key role (Howe and Jander, 2008).

Plants may also defend themselves indirectly. This defense strategy is based on tri-trophic interactions involving plants, herbivores and carnivores (Kessler and Baldwin 2002). When damaged by herbivores, plants emit a diverse array of volatiles, which attract natural enemies, either carnivorous predators or parasitoids, of the herbivores to the herbivore-damaged plants (Takabayashi and Dicke, 1996). These carnivores prey upon the herbivores or lay eggs in them. Further damage to the plant by the herbivores may thus be prevented or reduced. This phenomenon is therefore called “indirect defense.” Since the first time it was demonstrated about two decades ago, plant volatile-mediated indirect defense has been documented in many systems. Many species of plants, arthropod herbivores and carnivores have been used in different combinations in the studies of herbivore induced indirect defense (Takabayashi and Dicke 1996). Some investigations were carried out in natural ecosystems (Kessler and Baldwin 2001), and most have been performed in a lab setting (Yuan et al., 2008).

In addition to mediating the interaction of a plant with both its pest arthropod species and their natural enemies, volatile compounds induced by herbivory may also signal adjacent and down-wind plants about a possible herbivore threat. Such signaling may activate the neighboring plant's defense system. This interplant signaling from herbivore-damaged plants to undamaged neighboring plants is called inter-plant priming (Howe and Jander, 2008). Priming often results in even stronger resistance among undamaged neighboring plants when subsequent herbivory occurs. In addition, undamaged cotton plants become more attractive to predatory mites and less susceptible to spider mites when exposed to volatile compounds emitted by cotton plants damaged by spider mites (Bruin et al., 1992). Most of these studies have been conducted in laboratory conditions and whether these results can be reproduced in field conditions has not been thoroughly studied. A study combining both laboratory and field tests reported that volatiles produced from clipped sagebrush (*Artemesia tridentata*) increased the fitness of native tobacco when they were planted adjacent to each other. Clipped sagebrush emits many volatiles, including methyl jasmonate, methacrolein, terpenoids, and green leaf volatiles. When infested by *Manduca sexta* caterpillars, plants that were already exposed to clipped sagebrush showed an enhanced production of trypsin proteinase inhibitors. This activation of a defense response led to reduced total insect damage on plants activated by clipped sagebrush and also to a higher mortality rate of young Manduca caterpillars (Kessler et al., 2006).

Insect Pest management in organic farming based on plant volatiles

When underlying mechanisms for insect management approaches currently employed in organic farming are examined, it becomes evident that several emulate either natural defense mechanisms of plants or natural interactions between organisms in agroecosystems. Some of them are fully or partly based upon volatile chemistry of plants, which has many important ecological roles in plant-insect interactions. When we develop new practices to improve the effectiveness of insect management approaches in organic farming, it is important that we fully appreciate the natural defenses that plants employ and implement them in practice to their full potential. In this section, we will discuss various types of existing and prospective approaches for insect control in organic farming taking into the full consideration of the trait of plant volatiles.

A. Cultivars with Enhanced Indirect and/or Direct Defense

Natural preferences of insects for a specific plant species or variety were identified long ago (Guerin and Stadler, 1984; Guerin and Ryan, 1984), which were due to the different volatiles emitted by a specific genotype. However, limited progress has been made in insect resistance breeding (Miklas et al., 2006). With the development of new technologies, it is possible to obtain the entire profile of the volatiles emitted by the plants (Chen et al., 2009; Laothawornkitkul et al., 2008) at particular growth stage due to internal factors or in response to external factors such as wounding. With the determination of a specific volatile profile that is beneficial for self-defense, it can be manipulated through breeding or genetic transformation (Ng et al., 2008). As an example, responses of wheat varieties Betta (susceptible) and Elands (resistant) to aphids (*Diuraphis noxia*) were evaluated with respect to the application of volatiles from plant essential oils, methyl salicylate, 1,8-cineole and menthol (Prinsloo et al., 2007). *D. noxia* settled less on Elands plants that were exposed to the volatiles, whereas the effect of the volatiles on Betta was not noticeable. The impact of the three chemicals on aphid numbers varied according to the wheat variety, indicating that there was an interaction between those chemicals and wheat genotype. Volatiles of different genotypes and wild species confer different levels of direct and indirect defense against herbivores. One naturalized and five commercial cotton varieties were studied for their volatile profiles. Undamaged leaves from all the varieties showed no significant difference in their volatile profiles. However, average volatile emissions from the leaves of naturalized variety damaged by beet armyworm were found to be seven times higher than those from the leaves of commercial varieties even though larvae preferred commercial cultivars to feed on. These volatiles include monoterpenes, sesquiterpenes, and lipoxygenase products (Loughrin et al., 1995).

These differences in the volatile profile can be utilized by breeders to develop new cultivars with volatile profiles that are repulsive for herbivores or attractive to their natural enemies or a combination of both. Breeding of new crop varieties with increased direct and/or indirect defense based on plants volatiles may be based on existing varieties. The western corn rootworm (*Diabrotica virgifera*) is the most devastating pest of maize in the U.S. Recent study showed that (E)-β-caryophyllene, a sesquiterpene, is emitted by the roots of maize in response to feeding by larvae of western corn rootworm (Rasmann et al., 2005). (E)-β-caryophyllene is highly attractive to entomopathogenic nematodes, which parasitize and kill western corn rootworm larvae effectively (Rasmann et al., 2005). Most North American maize lines lack the ability of emitting (E)-β-caryophyllene from roots (Rasmann et al., 2005). This provides a knowledge base for creating novel maize varieties with improved resistance against western corn rootworm based on the restoration of (E)-β-caryophyllene emission in the roots. Breeding of new crop varieties for organic agriculture should also take advantage of existing genetic diversity in the wild. For example, medium length methylketones are one class of compounds highly effective in protecting plants against pests (Williams et al., 1980). These compounds are not found in cultivated tomatoes. However, they are produced in greater quantities by some wild tomato species (Williams et al., 1980). Transferring the trait of methylketone production from wild tomato species to cultivated elite tomato varieties through breeding may lead to novel tomato varieties with enhanced pest resistance better suited for organic production.

B. Polyculture

When managing insect pests in organic production, it is important to consider natural interactions of a host plant with its environment. The interactions of a host plant with insects as well as the interactions of insects with their natural enemies can be influenced by the diversity of plants in an agroecosystem. Polyculture is an agricultural practice in which multiple plants are grown in the same space. It aims at increasing crop yield by making full use of resources of a given piece of land. In contrast to monoculture, polyculture imitates the diversity of natural ecosystems, which has an important impact on the insect populations. The population density of arthropod herbivores in polyculture is found to be lower than that in monoculture. In contrast, the population density of natural enemies, especially parasitoids, are found to be lower in monoculture (Andow, 1991). Growing plants of different species in close physical proximity may aid insect control in several different ways depending on the volatile traits of various plants (Perrin and Phillips, 1978; Uvah and Coaker, 1984).

Intercropping is one form of polyculture, in which two or more types of crops are cultivated in the same space at the same time. Examples of intercropping strategies include planting a deep-rooted crop with a shallow-rooted crop, or planting a tall crop with a shorter crop that requires partial shade. The presence of multiple crops in a same agroecosystem provides a habitat for a variety of insects and therefore increases the local biodiversity, especially the beneficial insects, such as parasitic wasps.This can lead to the reduction of outbreaks of crop pests. For example, Colorado potato beetles (*Leptinotarsa decemlineata*) are attracted to volatiles from potato (*Solanum tuberosum*), but are repelled or not attracted by mixtures of potato and tomato (*Lycopersicon esculentum*) (Thiery andVisser, 1986, 1987). Another study was conducted to investigate the effectiveness of combined cropping of cultivated and wild varieties to manage stem borer. Intercropping with nonhost molasses grass (*Melinis minutiflora*) was found to significantly decrease stem borer infestation in the main crop as well as increase larval parasitism by parasitoid *Cotesia sesamiae*. Volatile compounds emitted by *M. minutiflora* were found to repel female stem borers and to attract females of *C. sesamiae* (Khan et al., 2007).

Companion planting refers to the planting of noncommercial plants along with the main crop for the purpose of facilitating nutrient uptake and pollination of the main crop as well as impacting other factors that affect crop productivity. Companion plants may affect crop-insect interactions. For example, when clover was grown as a companion plant for Brussels sprouts (*Brassica oleracea gemmifera*), the infestation of Brussels sprouts by diamondback moth was significantly reduced (Dover, 1986). For many of these studies, the associated resistance has been suggested to be provided by the olfactory effect of volatiles emitted from the non host. Some of the companion crops have also been found to increase the number of pests on themain crop. For example, when rue (*Ruta graveolens*), garlic chives (*Allium scheonparum*), and zonal geranium (*Pelargonium X hortorum* Bailey) were grown as companion plants for roses, the first two companion plants had no effect on Japanese beetles in the main crop. Geranium attracted more Japanese beetle and significantly increased the number of beetles on rose instead of reducing it (Held et al., 2002). Therefore, careful selection of companion plants for specific main crops is important for this strategy to work.

Trap cropping is a specific form of companion planting. Being planted around the field to be protected or interspersed among them, trap crops help reduce pest pressure on the main crop by being more attractive to insects than nearby crops. For example, perimeter rows of zucchini (*Cucurbita pepo* ‘Elite’), buttercup (*Cucurbita maxima* ⍰’Burgess’), and blue hubbard squash (C. maxima) can attract striped cucumber (*A. vittatum*) away from the butternut (*Cucurbita moschata*) main crop (Adler and Hazzard, 2009).Different species of plants may vary in their ability in serving as a trap crop. For example, to control the diamondback moth (*Plutella xylostella*) on cabbage, several plants including glossy and waxy collards, Indian mustard, and yellow rocket, were evaluated as trap crops. Yellow rocket was found to be the best candidate for use as a trap crop for *P. xylostella* (Fransisco et al., 2004). In addition to the species to grow, other factors that may affect the effectiveness of trap cropping include the proper timing of planting and adequate spacing and size of the trap crop (Hokkanen, 1991).

Weedy species, which are usually undesired, may have some positive impacts on pest control in organic production under certain circumstances. Choice experiments in the laboratory demonstrated that volatiles emitted by ragweed (*Ambrosia artemisiifolia*) interfered with the host finding and feeding behavior of *P. cruciferae* on tomato (Tahvanainen and Root, 1972). Therefore, leaving the ragweed to grow in the tomato field can help control this insect. In addition to growing multiple plants at the same place simultaneously, decomposed plants may also benefit pest control in organic production. This is called biofumigation, which is an agricultural practice for control of soil-born pests through the toxic substances released from the decomposed plants. Brassica plants are commonly used as biofumigant crops due to the production of secondary compounds especially glucosinolates. When glucosinolates are hydrolyzed, a variety of volatile compounds, including isothiocyanates, nitriles, epithionitriles and thiocyanates, are being produced, many of which are toxic to insect pests (Halkier and Gershenzon, 2006).

Interactions of insect herbivores and their natural enemies mediated by plant volatiles are complex processes. Some of the underlying mechanisms of such interactions are still poorly understood. In addition, these interactions may vary depending on the interacting species. Therefore, fundamental characterization of these interactions, especially in the field conditions, is greatly needed. When choosing crops or companion plants for polyculture, in addition to considering their volatile chemical traits, it is also important to bear in mind that these crops should not compete for physical space, nutrients, water, or sunlight when growing together, the factors that are equally important for crop yield.

C. Association with Beneficial Microorganisms

There is a rich diversity of soil organisms that can provide benefits to host plants through association. Growing evidence supports that some of these plant-microorganism associations may impact plant defenses against insect pests. Mycorrhizal fungi are specialized soil fungi that colonize plant roots to form a symbiotic association. Mycorrhizal associations with plant roots are widespread in nature. For example, arbuscular mycorrhizae are found in association with almost 85% of all plant families (Wang and Qiu, 2006). Mycorrhizal fungi gain constant and direct access to carbohydrates from the plant roots. In return, mycorrhizal fungi assist plant in uptake of water and mineral nutrients such as phosphorus. In addition, there is evidence that mycorrhizal association play an important role in the suppression of pests and diseases of associated plants, including herbivores (Gosling et al., 2006). For example, mycorrhizal soybean was found to be more resistant to corn earworm (*Heliothis zea*) and fall armyworm (*Spodoptera frugiperda*) than non-mycorrhizal soybean (Rabin and Pacovsky, 1985). And mycorrhizal ribwort plantain (*Plantago lanceolata)* is more resistant to attacks by the larvae of Arctia caja (Gange and West, 1994). Enhanced pest resistance of mycorrhizal plants might be due to increased availability of soil nutrients through mycorrhizal association, thus improving overall plant health (Gosling et al., 2006). Some studies showed that mycorrhizal plants produce more toxic compounds. For example, significant increase in accumulation of terpenpoids was found in cucumber (*Cucumis sativus*) roots upon mycorrhizae inoculation (Akiyama and Hayashi, 2001).

How mycorrhizal association affects the production and emission of volatiles from host plants is, however, little understood. Some studies showed that mycorrhizae colonization can alter a plant’s volatile profile. For example, mycorrhizal inoculation led to altered accumulation of certain sesquiterpenes and enhanced emission of limonene and artemisia ketone in *Artemisia annua* plants (Rapparini et al., 2008). In another study, mycorrhizal ribwort plantain (*Plantago lanceolata*) was shown to release one green leaf volatile (Z)-3-hexenyl acetate at a rate that was much higher than that of non-mycorrhizal plants and similar to that of insect-infested plants (Fontana et al., 2009), suggesting this compound may affect direct and indirect defenses. In the same study, it was found that mycorrhizal plants emitted significantly lower quantities of sesquiterpenes than non-mycorrhizal plants when fed by insects, suggesting that indirect defense may be weakened (Fontana et al., 2009). These contradictory implications from the same study highlight the complexity of multitrophical interactions. In a recent meta-analysis of more than 30 independent studies, it was found that in general the density of chewing insects on mycorrhizal plants were higher than that on non-mycorrhizal plants. However, because herbivore survival on mycorrhizal plants tends to be lower, more consumption did not lead to greater plant damage. Also from this analysis, it was found that while mycorrhizal colonization provides benefits to mono- and oligophagous chewing insects, it negatively affects the performance of polyphagous chewing insects (Koricheva et al., 2009). A number of studies showed that mycorrhizal association negatively affects parasitism (Gange et al., 2003). Whether this is due to altered production of plant volatiles is not yet clear. While mycorrhizal association may benefit crop production in organic farming in many different ways (Gosling et al., 2006), its impact on plant-insect interactions needs to be further investigated.

Endophytes are another type of microorganisms that can affect host plant defense via the alteration of volatile production. An endophyte is a bacterium or fungus that grows systemically in intercellular spaces throughout the plant without causing apparent disease (Clay, 1996). Some endophytes produce mycotoxins. Therefore, plants infested by endophytes are usually more resistant to insects. A study with *Beauveria bassiana* in banana reported reduced crop damage by banana weevil (up to 42.86.7%) (Akello et al., 2008). Some endophytes have been found to alter the volatile profile of host plants. For example, the presence of endophyte resulted in qualitative and quantitative differences in the production of volatile compounds from tall fescue (*Festuca arundinacea*). Treatment of endophyte-infected and endophyte-free plants with jasmonic acid dramatically altered the volatile compound profile (Yue et al., 2001). The altered production of plant volatiles may be responsible, at least partly, for the increased resistance of endophyte-infected plants against insects. Under certain conditions, the enhanced insect resistance of endophyte-infected plants may be due to the volatiles produced by endophytes. For example, the endophytic fungus *Muscodor vitigenus*, produces naphthalene under certain conditions. This endophyte-derived naphthalene has been found to effectively repel adult wheat stem sawfly (*Cephus cinctus*) in a Y-tube olfactory bioassay (Daisy et al., 2002). *Muscodor albus* is another endophyte that has been known to produce certain antimicrobial volatile compounds. This fungus was tested for its insecticidal activity against Potato tuber moth (*Phthorimaea operculella*). Adults and neonate larvae exposed to the volatile produced by *M. albus* showed significant higher percent mortalities than control (Lacey and Neven, 2006).

Despite many encouraging results, some plant-endophyte association was found to increase the pest infestation of the host plant. A study examining the interaction between an unspecialized root fungal endophyte *Acremonium strictum* and polyphagous moth *Helicoverpa armigera* provided such evidence. In a bioassay under greenhouse and laboratory conditions *H. armigera* was found to be more attracted to endophyte *A. strictum* inoculated tomato plants as compared to the endophyte-freeplants. Endophyte-inoculated plants released significantly lower levels of terpenes as compared to endophyte free plants. This study demonstrated that *A. strictum* was able to systemically influence the host selection of *H. armigera* moths for oviposition probably mediated by the induced changes in volatile emissions (Jallow et al., 2008). Therefore, endophyte selection is very critical and the impact can be specific to crops and pests.

D. Extracted Plant Volatiles for Pest Control

Based on their biological activities, plant volatiles may be developed into various products for insect control in organic production. They can be developed into organic pesticides. For example, a number of plant-originated monoterpenoids have been found to be more toxic to nematode *Caenorbditis elegans* than a commercial nematicide. Compounds like thymol and carvacrol have been found to be most effective with 100% mortality and eugenol and geraniol moderately effective (Tsai and Yu, 2000). In addition, many plant essential oils have been found to have nematicidal activity. Essential oil of ajowan (*Trachyspermum ammi*), allspice (*Pimenta dioica*) and litsea (*Litsea cubeba*) have been tested against pinewood nematodes and compounds like geranial, isoeugenol, and methyl isoeugenol have been identified to have good nematicidal activity (Park et al., 2006). Similarly volatile compounds from clove oil have been found to reduce egg hatch and the viability of hatched juveniles of tomato root knot nematodes (*Meloidogyne incognita*) (Meyer et al., 2008). Altieri et al. (1981) studied the application of the extract of the preferred host plant on the less preferred host plant in order to augment parasitoid preference and reported increases in parasitization by Trichogramma when Amaranthus extract was applied on soybean plants. Aqueous extracts of marigold were also found to trigger oviposition of Tricogramma (Ravi et al., 2006). Plant volatiles may also be developed into products for luring insects. For example, traps baited with compounds obtained from Canada thistle (*Cirsium arvense*) floral volatiles have been found to exhibit strong oviposition response from many lepidopteran insect pests. Soybean looper has been found to be attracted significantly to the Canada thistle floral volatiles and more females were trapped than males. Further study showed that phenylacetaldehyde plays the major role in attracting soybean looper, particularly the females (Stringer et al., 2008). Much research is still needed to further develop plant volatiles-based agents for insect control, especially from those plants that are high producer of volatiles, such as Copaifera trees (Chen et al., 2009a). Essential oils are often a mixture of many volatiles compounds. The identification of the most active compound will help develop highly effective products. The advance of metabolomics techniques combined with novel approaches for screening of effective volatile compounds (Enan, 2005) will play important roles in such new endeavors.

Conclusion and perspective

Pest management is a key component in organic crop production systems. Because synthetic pesticides and genetically modified crops are prohibited in this system, pest management presents a challenging task for the success of organic production. One key strategy currently used for pest management in organic farming involves preventive measures through selection of insect-resistant varieties, planting trap crops, following crop rotation, and nutrient management. Other strategies involve control measures through releasing or attracting natural enemies of herbivores and employing organic pesticides. Facing the challenging task of effective pest management, in this article, we have extensively reviewed and discussed how we may improve current strategies and design new strategies based on the production of volatile organic compounds from various plants.

Plant volatile compounds play important roles in mediating complex interactions between plants and organisms in the same ecosystem (Dudareva et al., 2006). These compounds are of great importance to plant defense as well as reproduction. Volatile production is genetically controlled and can be affected by different biotic (herbivores, microorganisms like mycorrhizae, and endophytes) and abiotic (light, temperature, nutrients) factors. To successfully control pests in an organic system, it is important to understand the interactions of different components in a specific ecosystem. For instance, restriction of use of pesticides may result in increase in the pest pressure in some crop species or varieties. However, it may positively impact the biodiversity in the crop ecosystem and increase natural enemies' populations. Therefore, it is important to emphasize the integration of different approaches that work synergistically and be in harmony with the nature. In addition to their roles in plant herbivore interactions, plant volatiles are involved in many other aspects of plant biology and ecology, such as defense against pathogens (Pichersky and Gershenzon, 2002). Volatiles released from below-ground tissues may have synergistic or allelopathic activity on the germination and growth of competitive neighboring plants. When leveraging the benefits of plant volatiles for enhanced defense against insects in organic farming, their potential negative impact also need to be considered. More basic research regarding the functions of plant volatiles, especially in an organic agroecosystem, is still needed. While much research in this area is still in its infancy, a multidisciplinary approach involving molecular biology, biochemistry, plant breeding, entomology, crop management and economics can facilitate the investigation and subsequently provide real world solutions for pest control in organic agriculture.

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Chapter III. Effect of colonization with arbuscular mycorrhizae, *Glomus intraradices,* and the fungal endophyte *Beauveria bassiana* on the volatile profile of tomato (*Solanum lycopersicum* L.), and on induced response against beet armyworm (*Spodoptera exigua* H).

Potential journals: Journal of Chemical Ecology

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Abstract:

A greenhouse study was conducted to study the impact of two important beneficial microorganisms, arbuscular mycorrhizae (*Glomus intraradices)* (AM) and an entomopathogenic fungal endophyte *(Beauveria bassiana)* (Bb), on the production of volatile compounds in the leaves of tomato (*Solanum lycopersicum* L) cv Castlemart, and on the induced direct resistance against beet armyworm (*Spodoptera exigua*. Hübner) (BAW). Four treatments, AM, Bb, AM+Bb, and control (C) were compared in terms of volatile compound produced in the leaves (n=6), and the response of BAW to these treatments. Mycorrhizal and non-mycorrhizal plants were of similar size as the phosphorus levels were adjusted, given that mycorrhizal plants require less phosphorus than nonmicorrhizal plants. Plants colonized with AM alone, and in combination with Bb, had significant effect ($P<0.05$) on some compounds, whereas showed a trend (different means but $P>0.05$) towards high production of total volatiles, total monoterpenes, and total sesquiterpenes, but were not significantly different. The effect of AM and Bb was significant for β-myrcene, benzyl alcohol, 3-hexenoic acid and overall total other compounds ($P<0.05$). Interaction between AM and Bb was not significant for any compound. Herbivory treatment for 18 hours significantly decreased the production of volatile contents in the leaves ($P<0.001$).The fungi colonized plants showed a trend towards high volatile production than the control plants without herbivory whereas, the trend was in opposite direction after herbivory when the control showed a trend towards high production of volatiles among all four treatments. However, the differences were not significant ($P>0.05$) among the four treatment groups after herbivory. Pest bioassay showed a trend for larvae choosing the control (n=12).The larval performance test showed significant ($P<0.05$) weight gain by the larvae fed on the control leaves compared to the other three treatments.

Key words: arbuscular mycorrhizae, *Beauveria bassiana*, beet armyworm, endophyte, entomopathogen, *Glomus intraradices,*induced resistance, volatile compounds.

Introduction

Herbivorous insects and diseases are major problems in agriculture leading to reduced productivity and economic losses. Chemical pesticides have undeniably contributed to minimize pest pressure, reduce losses due to pest damage, and increase food production and security. However, their indiscriminate use has created major environmental and health concerns. Alternative pest management strategies are gaining recognition and popularity among farmers, and research is being done to explore alternative and effective methods for insect pest management.

Plant defense systems have captured much interest for their potential role in defense against insect pests (Shrivastava et al., 2010). More specifically, physiological and chemical changes induced in plants after interaction with biotic factors have attracted researchers in plant pathology, entomology, and physiology (Cardoza et al., 2003). Various pathogenic, as well as nonpathogenic fungi can induce biochemical changes in plants (Cardoza et al., 2003). One of the major focuses of such studies is to explore and develop methods to enhance the plant's own defense/ immune system to prevent and/or resist pest attack (Lucas, 1999) through direct (Stout et al., 2009) and indirect mechanisms (Yuan et al., 2008).

Arbuscular mycorrhizal fungi (AM, phylum *Glomeromycota*) are among the most common fungi in the rhizosphere, having symbiotic associations with roots of almost 85% of the plant families (Barea et al., 2005). They constitute an important functional group of soil biota that positively impacts plant performance and soil health (Gianinazzi et al., 2010). They provide plants with mineral nutrients (mainly phosphorus and nitrogen) and water through their extensive hyphal network, and in return, the fungi receive carbohydrates from the host plant. They are found in every terrestrial ecosystem and are important in many, ecological and agricultural processes, such as nutrient cycling, and plant growth, as well as crop yield, land revegetation, and plant community structure (Koricheva et al., 2009).

In addition to their well known function in enhancing nutrient availability and uptake, they are also gaining widespread recognition for their bioprotective functions (Pozo et al., 2002). Currently, AM are being studied for their substantial impacts on secondary metabolism and defense traits induced in colonized plants. They can induce many morphological, physiological, and biochemical changes in the plants, which in turn can change the plant’s traits as a host for insect herbivores (Koricheva et al., 2009). A number of effects on plant physiology and ecology have been reported (Leitner et al., 2010). For example, changes in the patterns of flavonoid production and their correlating biosynthetic enzymes have been reported in *Medicago truncatula* Gaertn. and *Medicago sativa* L. during mycorrhizal colonization (Harrison and Dixon, 1993). Production of triterpenoids (Akiyama and Hayashi, 2002), apocarotenoids in colonized roots (Fester et al., 2002), and jasmonate (Hause et al., 2007) have been reported in various plant species.

Endophytes are the microorganisms that form unapparent infections within healthy plants. They are closely related to plant pathogens but do not cause disease in plants. For the most part, endophytes protect host plants from their enemies (Carroll, 1988) with additional chemical defenses (Hartley and Gange, 2009). *Beauveria bassiana* (Ascomycota: Hypocreales) is an entomopathogenic endophytic fungus that can exist inside plants (Ownley et al., 2008) and has been found to provide dual biological control for both insect pests and plant pathogens (Ownley et al., 2010). *Beauveria bassiana* has been studied mainly for pathogenicity against insects, and more recently for protection against plant pathogens; and the potential of inducing direct and indirect defenses against herbivores (Leckie, et al., 2008; Hare and Andreadis, 1983; Migiro et al., 2010; Quesada-Moraga et al., 2009).

Beet armyworm, (*Spodoptera exigua* (Hübner), Lepidoptera: Noctuidae), caterpillars are destructive insect pests responsible for enormous economic losses annually in various crops. It is challenging to manage these generalist herbivores due to a diversity of dietary choices that can impact their survival, development and fecundity (Merkx Jacques et al., 2008). It is one of the most important pests of fresh-market and processed tomato (Brewer et al., 1990), and has been found to induce defenses in tomato (Hix et al., 2008).

Microorganisms affecting different types and amounts of volatile emissions in different plant species, and their impact on herbivores and their natural enemies make these volatile-based herbivore-plant interactions very complex. For example, mycorrhizal colonization influences insect herbivore performance, but the magnitude and direction of impact depends upon the mode of feeding, generalist and specialist nature of the herbivore, as well as the fungal species (Koricheva et al., 2009; Leitner et al., 2010). Much research has been done in the recent past

(Dudareva et al., 2006; Jallow et al., 2004; Leitner et al., 2010; Powell et al., 2009; Rabin and Pacovsky, 1985; Shrivastava et al., 2010), yet more is needed to determine the link between single or combination of microbial colonization, plant volatile profiles and herbivory. Not many studies involving the impact of these two individual organisms, alone, or in combination, on the volatile profile of tomato, and on the response of the above ground pest beet armyworm have been reported. Although tomato plants have high levels of constitutive defenses, such as glandular trichomes, and α-tomatine, induced defenses are also of great importance and have been well reviewed (Guerrieri et al., 2004).

The present study elucidates the production of monoterpenes and sesquiterpenes and some other volatile compounds in the interactions of the host plant tomato with *Beauveria bassiana* (Bb) and *Glomus intraradices* (AM) and their role in defense against beet armyworm *Spodoptera exigua* (BAW) above ground. Our hypothesis is that colonization with these two fungi can lead to alteration in secondary metabolite production in the tomato plant. This alteration could potentially lead to induced direct resistance in the plant against beet armyworm.

Materials and Methods

Treatments and experimental design The experiment consisted of four treatments, control (C), arbuscular mycorrhizal fungi (*Glomus intraradices*) (AM), *Beauveria bassiana* (Bb), and *Glomus intraradices* + *Beauveria bassiana* (AM+Bb) with a randomized complete block design (RCBD). Two separate benches were assigned as two blocks. Twenty replicate plants per treatment were grown on one bench. Therefore both benches contained a total of 160 plants, with 80 plants on each bench/block.

Host plant, microorganisms, and insect culture Tomato (*Solanum lycopersicum*, Mill cv Castlemart; Family: Solanaceae) was selected for the experiment and seeds were generously provided by Dr. G. A. Howe, Michigan State University, East Lansing.*Glomus intraradices* cultures were originally obtained from the International Culture Collection of Vesicular Arbuscular Mycorrhizae Fungi (INVAM). The cultures were provided by Dr. Robert Augé (University of Tennessee, Knoxville). Pot cultures were established on *Sorghum bicolor* DK39Y roots in pure calcined montmorillonite clay (Turface, Profile products LLC, Buffalo Grove, IL).

After eight weeks of growing sorghum with mycorrhizal culture, sorghum roots were harvested just below the crown. Planting medium (Turface) with sorghum roots was evenly mixed and placed in pots. To control for effects of soil microflora other than AM, a filtrate solution from both AM and non-mycorrhizal roots (NM) was prepared that contained other microflora, but excluded fungal propagules. Culture media (AM and NM) along with roots were mixed with deionized water, each culture separately at the rate of one pot culture per 750 ml water. The suspension was then filtered through a 25-micron filter twice, giving the filtrate. Enough filtrate was prepared to inoculate all the experimental pots.

Two types of pots were prepared, one group contained calcined montmorillonite clay with chopped sorghum roots colonized by AM, and another group contained pure calcined montmorillonite clay (control). For the control and Bb treatmentst, untreated and Bb-coated seeds were sown in pure calcined montmorillonite clay growing media. Whereas, for AM and AM+Bb treatments, untreated and Bb coated seeds were sown in the pots with mycorrhiza culture. Filtrate from both AM and control (no mycorrhizae) cultures was prepared and applied in the same way as mentioned earlier. *Beauveria bassiana* strain 11-98 cultures are maintained as a part of Dr. Ownley's research program. Seeds of tomato cv Castlemart were coated with 1.15×10^5 CFU/seed of *B. bassiana* 11-98 and provided by Dr. Bonnie Ownley (University of Tennessee, Knoxville).

Mycorrhizal cultures were inoculated with non-mycorrhizal filtrate and non mycorrhizal plants were inoculated with AM filtrate (100 ml each). Plants were grown in the greenhouse with a 16/8 light/dark photoperiod, 75^o F/70^o F day and night temperatures, and 60% relative humidity. Mycorrhizal cultures were fertigated with 0.8 mM potassium phosphate and the non-mycorrhizal control received 1.6 mM potassium phosphate weekly. Peter's professional fertilizer (15-0-15) was applied to both treatments weekly at the rate of 150 ppm.

Beet armyworm (BAW) larvae were used for the insect (herbivory) treatment. Eggs (Benzon Research Inc, Carlisle, PA) were kept in 37.5-ml cups in darkness at 28^oC to hatch. The cups contained approximately 15 ml of pinto bean based artificial diet (Benzon Research Inc., Carlisle, PA) as a food source for the larvae.

Herbivory treatment Twenty, second instar BAW larvae were placed on tomato leaves with the help of a brush and allowed to feed for 18 hours. Larvae were removed and the leaves were processed for extraction of volatiles.

Test of colonization for mycorrhizae and Beauveria bassiana Mycorrhizal colonization was determined from the pot cultures before sowing the seeds and on experimental plants after the experiment, using histology techniques and light microscopy based on methods described by Phillips and Hayman (1970). In short, fresh lateral root samples (100mg) were collected from each pot; placed the samples into plastic histology cassettes for staining; cassettes were submerged in a 10% KOH solution (Fisher Scientific, Waltham, MA) in a beaker and brought to a simmer for 5 minutes; the KOH was removed, and a 2% hydrochloric acid solution (HCL) (Fisher Scientific, Waltham, MA) was added; samples were kept at room temperature for 1.5 hours; HCL solution was removed and samples were stained with 0.05% Trypan Blue solution (Mallininckrudt, inc., Hazelwood, MO) solution for 1 hour; destained the samples in a lactoglycerol solution (Glycerol-Acrose, Geel, Belgium; Lactic acid-Fisher Scientific, Waltham, MA) for a minimum of 48 hours; roots from each sample were mounted with lactoglycerol, covered with a cover slide and viewed with a light microscope (Fisher Scientific, Waltham, MA) at 20X power.

Percent colonization values for each sample was determined using method described by McGonicle et al.(1990). In short, counts were made by moving the slide in a grid-like pattern across the microscope field of view; each visual intersection with a root is scored based on visual identification of AM fungal organs, hyphae, arbuscules and vesicles in the root cortex; a standard percent colonization value for each sample was determined based on the total score, out of 100.

Detection of *B. bassiana* was done with PCR amplification of ITS sequences of plant genomic DNA with *B. bassiana*-specific primers. Genomic DNA was isolated using the CTAB (N-acetyl-N, N, N-trimethylammonium bromide) method (Murray and Thompson, 1980). PCR was conducted with primers cbITSf and cbITSr, which are specific for the ITS regions of *B. bassiana* (Griffin, 2007). The reaction mixture contained 25 µl Takara PerfectShot ExTaq (1.25 units Takara *Ex Taq*, final concentration 0.2 mM dNTPs and 2 mM $MgCl^{2+}$, Takara Biotechnology, Otsu, Shiga, Japan), 2 µM each primer (5 µl of 20 µM), and 60-300 ng template DNA (2 µl) and sterile DNA grade water to a final reaction volume of 50 µl. Reaction conditions were 95°C for 2 min, 40 cycles of 94°C for 1 min, 59°C for 1 min, 72°C, with a final extension of 72°C for 3 min, conducted with a Mastercycler gradient thermal cycler (Eppendorf AF Hamburg, Germany). PCR products were visualized on a 1.5% agarose gel stained with SybrSafe (Invitrogen, Eugene, OR).

Volatile extraction Six plants of similar size per treatment were used for volatile extractions according to a standard protocol (Chen et al., 2009). In short, the second leaf from the top was detached from 8-week-old plants and immediately ground to powder with liquid nitrogen. A one ml aliquot of ethyl acetate, which contained 0.003% w/v 1-octanol (internal standard), was added to 200 mg of powdered leaf tissue. Extraction was done by continuous shaking on an orbit shaker (Lab-line instruments Inc, Melrose Park, IL) at room temperature for 2.5 hours. A 5-µl aliquot of extract was injected into a GC-MS for separation and identification of the compounds. Two technical replicates were run to reduce variability.

Volatile analysis Compounds were analyzed by Shimadzu GC (GC-17A) (Shimadzu Corp, Columbia, MD). Separation was performed on a Restek SHR5XLB column (30 m × 0.25 mm internal diameter × 0.25 µm thickness). Helium was used as the carrier gas (flow rate of 1 ml min^{-1}), a splitless injection (injection injector temperature 250°C) was used, and a temperature gradient of 5°C min^{-1} from 40°C (3 min hold) to 240°C was applied. The coupled mass spectrometer was a Hewlett-Packard model 5973 with a quadrupole mass selective detector. Products were identified using the National Institute of Standards and Technology mass spectra database. Quantification was performed as reported previously by comparing samples with the peak area of the internal standard (Chen et al., 2009).

BAW performance bioassay For leaf disc choice test and leaf area consumption, leaf discs of 1.54-cm^2 were cut by a corer from 10-week-old tomato plants of each treatment. Discs were arranged on the periphery of a clean 150mmX15mm Petri dish, containing a moist qualitative filter paper of the same size (Ahlstrom, Helsinki, Finland). Eight starved second instar larvae were placed in the center of the dish; larvae were allowed to choose any disc to feed on for 15 min. The number of larvae showing any preference for a particular treatment was recorded. The assay was replicated 12 times with new plants and new larvae for each replicate. After 2.5 hours, larvae were removed and the leaf area consumed by the larvae was measured with a LI-3100C Area Meter (LI-COR Biosciences, Lincoln, NE). Leaf area consumed by BAW larvae was calculated by subtracting the area of BAW damaged leaf from the area of an intact leaf. The assay was performed at room temperature. For Beet army worm performance test, one second instar larva per cup was fed with a leaf detached from ten-week-old tomato plants from different treatments. Plants and cups were assigned numbers and new leaves were added to the respective cups every day to ensure that larva received leaves from the same plant each time. Larvae were weighed when they reached the wandering stage.

Statistical analysis Analysis of variance was carried out with AM, Bb, and herbivory treatment and their three-way interaction as fixed effects. Block, and block with AM, Bb, and herbivory were random effects in the mixed models SAS 9.2 (SAS 2008) for RCBD factorial. Analyses were performed using program code contained in the "DANDA" macro for SAS designed by Dr. Arnold Saxton (http://dawg.utk.edu/). Data were checked for normality and equality of variance and those, not meeting the criteria were log (0.05 log value) or rank transformed. Pair wise means were compared with LSD mean separation test at *P*=0.05 level of significance. Leaf disc choice test data are presented as percent larvae attracted (±SE). Leaf area consumption and performance tests were analyzed with AM, and Bb and their two-way interaction as fixed effects. Block, and block with AM, Bb were set as the random effect using mixed models ANOVA SAS 9.2 (SAS 2008) for RCBD factorial. Pair wise means were compared with LSD mean separation test at *P*=0.05 level of significance.

Results

Test for colonization of tomato with AM and Bb More than 70% colonization was confirmed in all the mycorrhizal plants before and after the experiment. The non-mycorrhizal plants were also tested to confirm no colonization. Two plants contaminated with mycorrhizae were discarded from the analysis. For Bb detection, DNA was isolated from leaf and root tissues from twelve randomly selected Bb and AM+Bb plants. ITS sequences of Bb were amplified with primers designed specifically for *B. bassiana*. Out of twelve plants eleven (91.7 percent) were found to be positive for Bb, indicating that *B. bassiana* can successfully colonize the Castlemart line.

Volatile compounds analysis Mycorrhizal and non-mycorrhizal plants were of similar size at the time of analysis as the phosphorus levels were doubled for nonmycorrhizal plants, given that nonmycorrhizal plants require more phosphorus than the mycorrhizal plants. The monoterpenes that were detected and analyzed from the control plants were comprised of α-pinene, α-terpinene, and β-phellandrene. Total sesquiterpenes consisted of δ-elemene, β-elemene, (*Z*)-caryophyllene, and α-humelene. 'Other compounds' were comprised of benzyl alcohol, 3-hexenoic acid, and eugenol. All of these compounds were also detected in AM plants in addition to two other monoterpenes, β-myrcene, and 1-phellandrene. Bb treated plants produced all the compounds that were detected in AM except, 1-phellandrene, and AM+Bb plants also produced same compounds as Bb plants. Most individual compounds as well as groups of compounds had a pattern of low production in the control plants, whereas, AM and Bb treated plants showed a trend towards high production of total volatiles including total monoterpenes, sesquiterpenes, and total 'Other compounds' (Tables 3.1). The monoterpene β-myrcene was significantly higher in plants colonized with fungi AM (P=0.021) and Bb (P=0.002) as compared to the control which did not produce this compound. Benzyl alcohol was significantly increased by Bb (P=0.007) and AM (P=0.029) colonization, and 3-hexenoic acid was also significantly increased by AM (P=0.005) and Bb (P=0.018). There was a trend of increased production of total other volatiles in all three treatments containing AM, Bb, or both with the effect of AM (P=0.06) and Bb (P=0.006). Sesquiterpenes, α-Humulene was significantly influence by Bb (P=0.032). The presence of Bb showed a trend for slightly enhanced production of total sesquiterpenes (P=0.07) as evident by (*Z*)-caryophyllene (*P*=0.054), and α-humelene (*P*=0.032) contents (Tables 3.1). The effect of the two way interaction between AM and Bb (AM*Bb) was not significant for any of the volatile compounds.

The effect of herbivory was significant on all the volatile compounds (*P*<0.01) compared to those without herbivory. Effect of interaction between AM and herbivory was significant on the total others (*P*=0.043), α-humulene (*P*=0.022), 1-phellandrene (*P*=0.035) and β-myrcene (*P*=0.021). Effect of interaction between herbivory and Bb was significant for the total others (*P*=0.003), total sesquiterpenes (*P*=0.037), total monoterpenes (*P*=0.031), total volatiles (*P*=0.023), α-humulene (*P*=0.017), (*Z*)-caryophyllene (*P*=0.04), δ-elemene (*P*=0.03), 3-hexenoic acid (0.002), benzyl alcohol (*P*=0.002), β-phellandrene (*P*=0.04), α-terpinene (*P*=0.026), and β-myrcene (*P*=0.002). After herbivory, a trend for high total volatiles, monoterpenes, and sesquiterpenes production was observed in control plants (Table 3.2). Although, the differences for any compound among the four treatments were not significant after herbivory, a pattern towards high volatile production in the control plants was observed. Effect of three way interaction between AM, Bb and herbivory (AM*Bb*H) was not significant for any compound.

BAW Performance Bioassay In the leaf disc choice test, a pattern was seen with the control treatment attracting the most (28%) larvae followed by AM+Bb (22%), AM (20%) and Bb (20%) treated plants (Figure 3.1), that led to high consumption of leaf disc from control plants (0.64 cm^2) (Figure 3.2). The remaining larvae did not make any choice. Despite the pattern of larvae choosing the control plants over other treatments, the difference we not significant. However, AM (*P*=0.022) and Bb (*P*=0.024) had significant effect on the larval weight, which higher for those fed with the control leaves (0.084 g) compared to those fed with the other three treatments, with the lowest weights for AM+ Bb (0.063 g) (Figure 3.3). Effect of interaction between AM and Bb was not significant.

Discussion

Volatile compounds analysis Higher production of β-myrcene, a monoterpene, and marginally enhanced production of sesquiterpenes in the fungi-treated plants may be due to the activation of the methylerythritol phosphate (MEP) pathway, the principal route to monoterpene production, and the mevalonate (MVA) pathway, responsible for sesquiterpenes (Fontana et al., 2009).A common sesquiterpene synthesis intermediary molecule known as E,E-Farnesyl diphosphate (*E,E*-FPP, C_{15}) and a monoterpene synthesis molecule, geranyl diphosphate (GPP, C_{10}) may be influenced (Chappell, 1995) by fungal colonization in the ubiquitous terpenoid pathway. The MEP pathway also leads to production of carotenoids and apocarotenoids, which are responsible for the yellow colored roots after mycorrhizal colonization (Strack et al., 2003), that may explain AM activating MEP pathway. Mycorrhizae colonization has been found to enhance the transcription of genes encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS), an enzyme that catalyzes the initial step of the MEP pathway in several species, including tomato. Accumulation of transcripts for 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), an enzyme that is immediately downstream from DXS in the MEP pathway, also has been reported in wheat roots after *G. intraradices* colonization (Walter et al., 2000). Therefore, activation of the MEP pathway due to fungal colonization may be responsible for high production of monoterpenes in the colonized plants. Sesquiterpenes are produced via the MAV pathway, which is not very well explained in terms of the effect of mycorrhizal colonization (Fontana et al., 2009).

However, several terpene synthase (TPSs) enzymes, controlled by multiple TPS genes, act upon the intermediary molecules farnysl diphosphate (FPP) and geranyl diphosphate (GPP), to produce different sesquiterpenes and monoterpenes. A single TPS enzyme acts to produce trans-caryophyllene and α-humulene (Chen et al., 2003; Yuan et al., 2008), which is marginally high in the plants infected with the Bb. This suggests, that the pattern is the result of altered activity of multiple enzyme systems, and more likely that the common intermediary molecules (*E,E*)-FPP and GPP are influenced by fungal colonization. The mechanism behind increased production of benzyl alcohol, and 3-hexenoic acid is not yet clear. However, 3-hexenoic acid has been reported to be used by lepidopteron insects for communication (www.pherobase.com). Its increased production by the fugal colonized plants may indicate that the AM and Bb colonization can alter the interaction of plants with BAW by producing this compound in larger quantities. The direction of the effect on the communication is not very well studied for this compound. Benzyl alcohol dehydrogenase (BAD) generated in *Arabidopsis* plants during certain fungal infections, is reported to be involved in the conversion of benzaldehyde derivatives into benzyl alcohol and incorporation of important phenolic defense molecules into the cell wall. It is also involved in the metabolism of soluble compounds in signaling for plant defense using phenolics (Somssich et al., 1996). Benzyl alcohol also has structural similarity with salicylic acid, an established signal molecule in plants. Therefore, increased benzyl alcohol may be expected to fulfill different physiological and defense roles in the plant after fungal colonization. Direct effect of benzyl alcohol is not yet clear, but it may have a role in structural defense and signaling.

An induced systemic response seems a likely source of some of the observed changes in the volatile compounds. Some endophytes have been reported to induce the production of phytohormones and other signaling molecules, which in turn can influence host gene expression and metabolism resulting in enhanced resistance (Smith and Gianinazzi-Pearson, 1988). These endophytes have also been found to alter the volatile profile by altering the physiology of the plant. This altered physiology also affects herbivore food utilization. It has been reported in several field and laboratory studies that herbivores avoid feeding on endophyte infected plants and increased mortality and decreased growth and development of herbivores has been found on infected grasses (Clay, 1996). This might explain lesser larval weight gain on the fungal colonized plant. This is in the agreement with the reports of *Beauveria bassiana* pathogenicity against insects and its potential of inducing direct and indirect defenses against herbivores (Leckie et al., 2008; Ownley et al., 2010).

Significant reduction in the leaf volatiles after herbivory compared to those without herbivory, might be due to the release or loss of the volatiles through rupture of glandular trichomes and leaf tissue upon herbivore damage. In addition, short duration of insect feeding may not be sufficient to upregulate production of the volatiles. This is known as lag-time, which is the time required for the induction of defense after the first contact with the herbivore (Heil and Baldwin, 2002; Radhika et al., 2008).

Trend of low production of volatiles in mycorrhizal plants after herbivory is in agreement with the report, where herbivory decreased the sesquiterpenes in the mycorrhizal *Plantago lanceolata* L. than in the herbivore-damaged non-mycorrhizal/control one (Fontana et al., 2009). Another report of no impact of herbivory on the volatiles of mycorrhiza colonized *M. truncatula* plants also reinforces this finding (Leitner et al., 2010) which may have important implications for the indirect defense.

Defense responses are vital but metabolically costly for the plant (Pozo and Azcon-Aguilar, 2007). That might be the reason for trend towards high production of volatiles in the control plants in response to herbivore attack. AM, Bb, AM+Bb treatment provided induced defense to some extent, and did not require the plant to produce as great of quantity of defense compounds as the control plants after herbivory. Control plants produced higher amounts of volatiles in response to herbivory, possibly as cues for the herbivore's natural enemies (Agrawal, 2000). A trend towards high production of phenolic compounds in control+herbivory plants suggests activation of JA regulated defense responses after herbivory (Pozo and Azcon-Aguilar, 2007). Jasmonate has been found to enhance phenolics (Kim et al., 2006), as well as sesquiterpene production (Schmelz et al., 2001), which might explain the pattern for high sesquiterpenes, and other volatiles in the control + herbivory. AM + herbivory plants were closer to control + herbivory in terms of these compounds as compared to other treatment combinations. The reason might be that the SA dependent pathway is activated during plant-fungal interactions, with increased SA levels during establishment of colonization as a defense response. However, in the case of symbiotic colonization, SA levels decrease upon perceiving certain symbiont specific receptors to let the fungus reach and colonize the cortex. As the SA levels decrease, JA levels increase in the later stages of colonization (Pozo and Azcon-Aguilar, 2007). Mutually inhibitory crosstalk exist between JA and SA signaling in many plant species (Gutjahr and Paszkowski, 2009), and this mutually inhibitory cross talk may be responsible for low sesquiterpenes contents in the fungal colonized plants with herbivory when compared to control + herbivory.

BAW Performance Bioassay The results indicate that AM, as well as Bb, induced resistance in tomato to beet armyworm to some extent through systemic mechanisms, and through local as well as systemic mechanisms with Bb. Higher β-myrcene produced from AM+Bb plants may have played a role in the plant's induced resistance against herbivory as this compound has been reported to be an effective semiochemicals utilized by insects for different purposes such as repellent to some insects like thrips, as well as, attractants to aphidophagous hoverflies in the terrestrial orchid *Epipactis veratrifolia* (Stökl et al., 2011). It has also been previously reported to be produced in grape roots in response to herbivory (Lawo et al., 2011) and from the herbivore damaged *M. truncatula* along with (*E*)-β-ocimene (Navia-Giné et al., 2009). This indicates its important implications regarding direct and indirect defense after herbivory. Higher benzyl alcohol and 3-hexenoic acid may also be responsible for poor performance of BAW on fungus-colonized plants. Although the role of benzyl alcohol is not clear, it is likely that it has role in structural and chemical defense. Its structural similarity with salicylic acid indicates its potential role as defense signal. The compound 3-hexenoic acid has also been reported to have a role in communication for lepidopteron species, and is likely to have influenced the performance of BAW. AM has also been known to enhance resistance against many soil-borne pathogens, nematodes, and root insects through local as well as systemic mechanisms (Pozo and Azcon-Aguilar, 2007). These systemic changes may also be effective against BAW.

The result is also in agreement with the reports of AM induced resistance against generalist chewing herbivores (Gange, 2006; Pozo and Azcon-Aguilar, 2007). One of the explanations for this might be easy availability of a broader range of alternative hosts for the generalists compared to the specialists, in addition to the transcriptional regulation of mycorrhiza-regulated defense genes (Liu et al., 2007), and accumulation of insect antifeedents (Gange, 2006). Mycorrhizal formation has also been found to prime tissue for quicker and greater activation of JA dependent defense responses upon herbivore attack, resulting in enhanced resistance (Pozo and Azcon-Aguilar, 2007). In addition, terpenoids are also structural components of the cell and organelle membranes (Hunter, 2007), which might also induce structural direct defenses when these compounds are influenced in AM plants. AM has also been reported to decrease aphid population growth by 47% in the grass *Phleum pratens* (Hempel et al., 2009).

The results suggest that colonization with symbiotic AM fungi, such as G*lomus intraradices* and the entomopathogenic endophyte *Beauveria bassiana*, may induce some level of resistance against beet armyworm by reducing their growth. They also seem to influence the volatile secondary metabolites. Therefore, the experiment was successful at addressing the original question of interest. However, some aspects could be improved in future work. The fact that volatile secondary metabolites have low molecular weight, and have high vapor pressure, very sensitive to the method of extraction, time of storage and analysis. Differences in time of analysis after extraction can add variability to the results. Therefore, these are important factors to consider for extraction and analysis of samples. Although the sample size was acceptable for such experiments conducted in the greenhouse, a larger sample size might result in less variability in the data and clearer treatment effects on more compounds.

The effects of *G. intraradices* and *B. bassiana* can have important ecological and economic implications. The degree of potential nutritional benefits related to AM suggest that mycorrhizal species distribution may not only enhance plant nutrition, but also can act as an important bioprotectant against certain species of insect pests through direct or indirect defense (Hempel et al., 2009). With the vast number of such fungal species, morphology and distribution, these effects can be widely adopted and can have an important ecological impact. These relationships could present a unique tool to enhance plant bioactive compounds, and pest resistance in crops. Optimal benefits can be obtained by introducing these fungi in the agricultural system, where they are depleted or absent, as well as introducing responsive plant species into the system. For example, *Brassica* spp are not responsive to mycorrhiza, and given the obligate symbiotic nature of AM fungi, if the proper host is not available, the fungi population can drastically diminish in that system. Transition to organic systems from conventional systems could benefit from such fungi in enhancing soil quality and biodiversity more quickly. In addition, they can be a useful tool in greenhouse or nursery production.

AM and Bb have been studied in many crop species yet many more crops need to be explored. Herbivore damage to the plant has been reported to be reduced or unaltered depending on the fungal and plant species (Gange et al., 2003). Therefore, the effect on more insect pests needs to be studied in different crop species. This work demonstrates that application of these fungi have potential benefits by enhancing pest protection and altering the plant bioactive compounds. These tools can be incorporated into an integrated pest management program and could play an important role in the future of sustainable agriculture.

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Appendix

Tables

Table 3.1: Volatile compounds [means ng g (fresh wt)$^{-1}$±SE] from the leaves of control, AM, Bb, and AM+Bb plants. Eight-week old plants (three from each block) were randomly selected. Leaves were ground with liquid nitrogen and extracted with ethyl acetate containing Octanol (0.003%) as internal standard was used for extraction. Extracts were injected into GC-MS for separation and identification of compounds. N=6, except control with N=4.

Compounds	Control	AM	Bb	AM+Bb
β-Myrcene	ND[*] **cd**[#]	0.1 ±0.1**bc**	0.5 ±0.2**b**	1.2 ±0.2**a**
α-Terpinene	2.1 ±0.7	4.9 ±1.2	6.4 ±0.8	7.6 ±1.5
l-Phellandrene	ND	0.7 ±0.3	ND	ND
β-Phellandrene	16.2 ±5.2	27.6 ±6.4	35.4 ±4.3	36.8 ±5.3
<u>Total Monoterpenes</u>	18.3 ±5.9	33.4 ±7.9	42.2 ±5.1	45.6 ±6.7
Benzyl alcohol	0.9 ±0.2**c**	1.5 ±0.1**b**	1.8 ±0.2a**b**	1.9 ±0.1**a**
3-Hexenoic acid	0.1 ±0.1**e**	0.5 ±0.1**bc**	0.8 ±0.1**ab**	1.8 ±0.6**a**
Eugenol	1.8 ±0.2	2.1 ±0.1	2.4 ±0.2	2.1 ±0.1
<u>Total Others</u>	2.8 ±0.4**c**	4.1 ±0.3**b**	4.9 ±0.5**ab**	5.8 ±0.5**a**
δ-Elemene	1.0 ±0.2	1.9 ±0.4	2.5 ±0.4	2.2 ±0.1
β-Elemene	0.2 ±0.1	0.2 ±0.1	0.2 ±0.1	0.5 ±0.2
(*Z*)-Caryophyllene	1.4 ±0.4	3.1 ±0.6	4.5 ±0.8	4.2 ±0.4
α-Humulene	0.3 ±0.1**bc**	0.6 ±0.1**ab**	0.8 ±0.2**a**	1.0 ±0.1**a**
<u>Total Sesquiterpenes</u>	2.8 ±0.8	5.7 ±1.3	8.2 ±1.5	7.8 ±0.4
<u>Total Volatiles</u>	24.0 ±7.0	43.2 ±9.1	55.3 ±6.9	59.4 ±7.4

[*] Not detected.

[#] Different letters a,b,c,d denote significant differences among treatments (P<0.05).

Table 3.2: Volatile compounds [mean ng g (fresh wt)$^{-1}$±SE] from the leaves of control, AM, Bb, and AM+Bb plants after herbivory. Eight-week old plants (three from each block) were randomly selected. Twenty, two-instar BAW larvae were placed on the leaves at 5 PM with a brush and were left to feed on the leaves overnight. Larvae were removed at 11 AM the next morning after 18 hours of herbivory and were ground with liquid nitrogen and extracted with ethyl acetate containing octanol (0.003%) as an internal standard. Extracts were injected into GC-MS for separation and identification of compounds. Herbivory significantly decreased all the volatiles compared to non-herbivory (P<0.001). N=6.

Compounds	Control+H[#]	AM+H	Bb+H	AM+Bb+H
β-Myrcene	ND[*]	ND	ND	ND
α-Terpinene	1.05 ±0.24**cd**[$]	1.10 ±0.40**cd**	0.86 ±0.48**d**	0.56 ±0.11**cd**
l-Phellandrene	0.13 ±0.09	ND	0.18 ±0.15	ND
β-Phellandrene	5.18 ±1.46	4.13 ±1.20	4.25 ±1.79	2.63 ±0.11
<u>Total Monoterpenes</u>	6.35 ±1.66	5.23 ±1.51	5.29 ±2.42	3.18 ±0.17
Benzyl alcohol	0.35 ±0.07	0.43 ±0.13	0.32 ±0.04	0.30 ±0.04
3-Hexenoic acid	0.25 ±0.03**cde**	0.37 ±0.06**bcd**	0.23 ±0.09**de**	0.26 ±0.06**cde**
Eugenol	0.59 ±0.06	0.39 ±0.01	0.58 ±0.07	0.41 ±0.07
<u>Total Others</u>	1.18 ±0.10	1.19 ±0.19	1.13 ±0.16	0.97 ±0.11
δ-Elemene	0.31 ±0.09	0.24 ±0.11	0.23 ±0.08	0.15 ±0.01
β.-Elemene	0.01 ±0.01	0.01 ±0.01	0.01 ±0.01	ND
(*z*)-Caryophyllene	0.52 ±0.14**c**	0.45 ±0.14**c**	0.54 ±0.13**bc**	0.29 ±0.02**c**
α-Humulene	0.09 ±0.02**cd**	0.05 ±0.03**d**	0.10 ±0.03**cd**	0.03 ±0.01**d**
<u>Total Sesquiterpenes</u>	0.91 ±0.26	0.74 ±0.26	0.88 ±0.25	0.47 ±0.04
<u>Total Volatiles</u>	8.45 ±1.97	7.16 ±1.81	7.29 ±2.76	4.63 ±0.17

[#] Herbivory;

[*] Not detected;

[$] Different letters a,b,c,d denote significant difference (P<0.05) in continuation with Table 3.1.

Figures

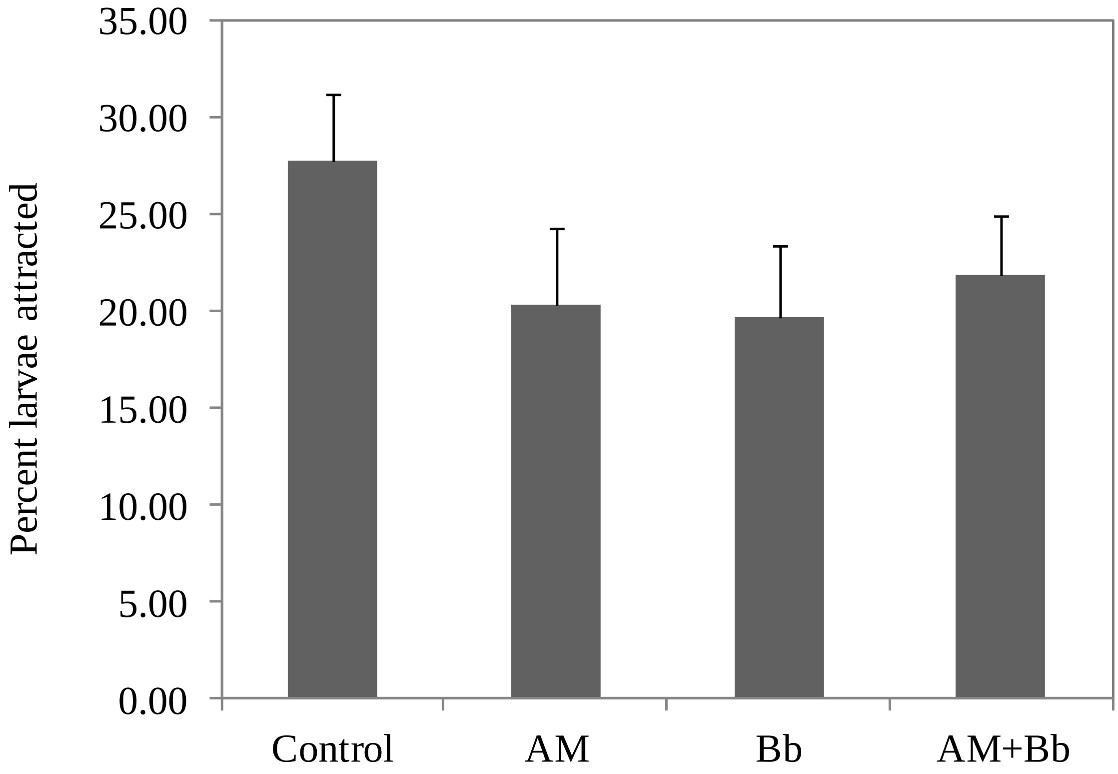

Figure 3.1: Larvae [mean percent ±SE] attracted to leaf disc from the control (untreated plants) and those treated with AM, Bb or AM+Bb. Leaf discs of 1.54 cm^2 were cut out from 10-week-old plants of each treatment and arranged on the periphery of clean Petri dish containing a moist qualitative filter paper disc. Eight starved second instar larvae were put in the center of the dish and allowed to choose any disc to feed on for 15 minutes. N=12.

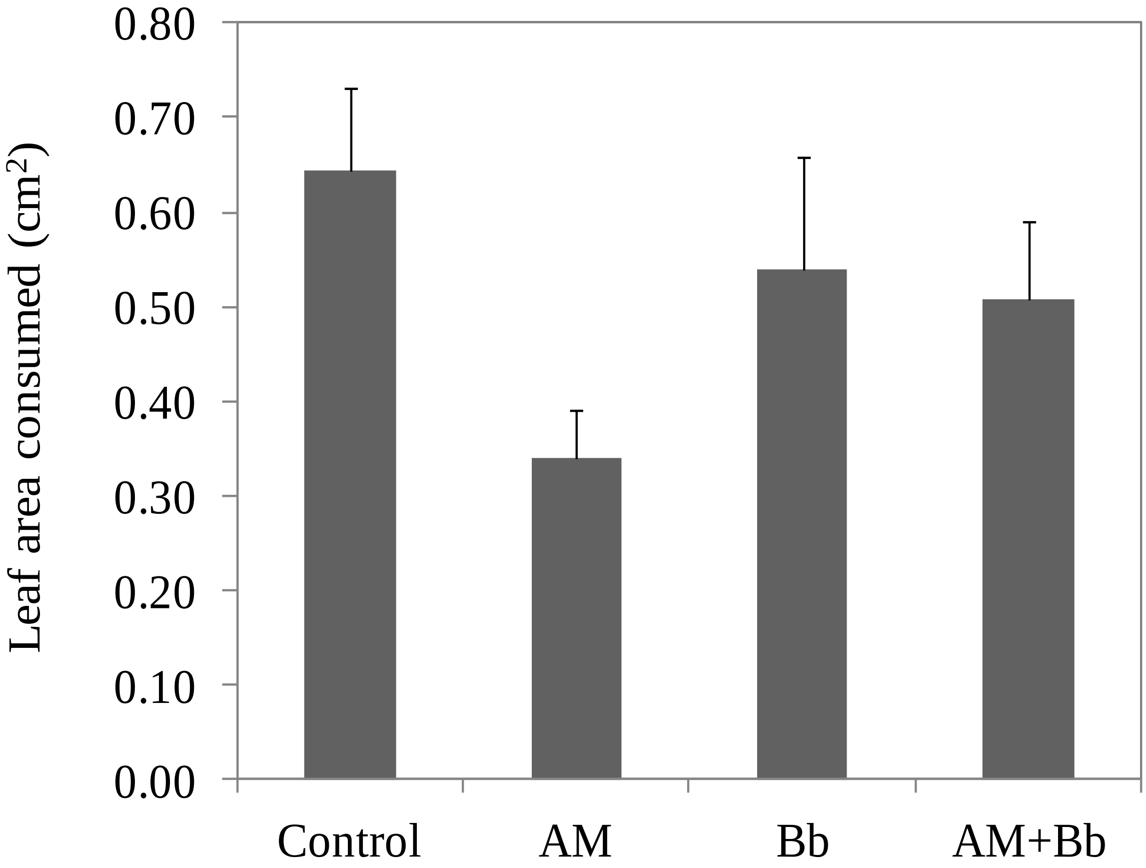

Figure 3.2: Leaf area consumed [mean cm^2 ±SE] by beet armyworm larvae from the leaf discs from tomato plants treated with AM, Bb, or AM+Bb and untreated control. Leaf discs of 1.54 cm^2 were cut out from about 10 week old plants of each treatment and were arranged on the periphery of a clean Petri dish with a wet qualitative Filter Paper. Eight starved second instar larvae were put in the center of the dish and were allowed to feed for 2 ½ hours. After larvae were removed, the leaf area consumed by the larvae was measured with a LI-3100C Area Meter. Leaf area consumed by BAW larvae was calculated by subtracting area of BAW damaged leaf from area of intact leaf. n=12.

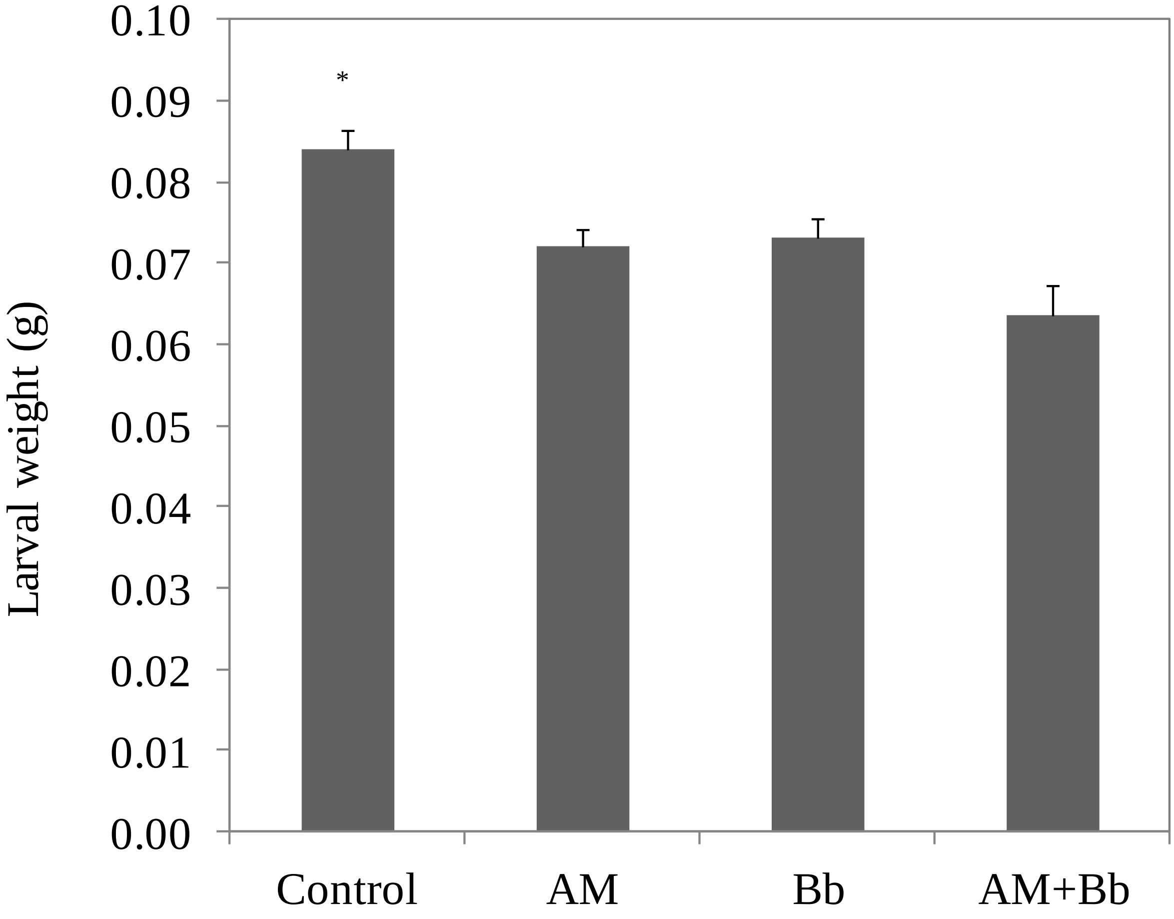

Figure 3.3: Larval weight [mean g ±SE]. One second instar larva per cup was fed with a leaf detached from ten-week-old plants from different treatments.Larvae were weighed when they reached wandering stage. Control (n=14), arbuscular mycorrhizae (n=11), *Beauveria* (10), AM+*Beauveria* (n=10).

* Indicates statistically significant difference ($P<0.05$).

Chapter IV. Effect of root knot nematode on the volatile secondary metabolites from the above and below ground tissues of tomato (*Solanum lycopersicum* L.) plant and on the induced defense against Beet armyworm (*Spodoptera exiguae* Ḧ.).

Target journal: Journal of Chemical Ecology**.**

Authors: Gitika Shrivastava, EC Bernard, Chen Hao, Feng Chen*.

Abstract

A green house study was conducted to determine the effect of double parasitism by beet armyworm (BAW) and rootknot nematode (RKN) on the production of volatile in the leaves and roots of tomato (*Solanum lycopersicum*; line Castlemart).Two sets of plants were studied. The second set was grown and analyzed the same way as the first set after the analysis for the first set was completed. Herbivory exposure times for the first and second sets were 18 and 42 hours respectively. Methyl salicylate (MeSA), an important airborne defense molecule, was also studied with respect to tomato *SlSAMT* gene expression in the RKN resistant and susceptible lines after inoculation with RKN. RKN had a significant effect (P<0.05) on benzyl alcohol, eugenol and increased their production in the RKN leaves (n=6). On the contrary, terpenes, such as α-pinene, 2-carene, β-phellandrene, δ-elemene, and β-elemene and overall total volatiles showed a trend for high production in the control leaves. Herbivory for18 hours significantly (P<0.05) decreased many compounds compared to no herbivory. The effect of interaction between 18 hours of herbivory and RKN was significant for eugenol and total other compounds in the leaves. A different pattern however, was seen with 42 hours of herbivory (n=3) when a trend was seen for high volatile production in the leaves of RKN plants, a trend contrary to that observed with 18 hours of herbivory. RKN infestation had a significant effect (P<0.05) on methyl salicylate, benzyl alcohol, and (*Z*)-geraniol production in the roots. Herbivory for 18 hours, as well as, for 42 hours had a significant effect (P<0.05) on methyl salicylate and (*Z*)-geraniol production in the roots. Interaction effects of RKN with 18 hours of herbivory was significant (P<0.05) for (*Z*)-geraniol, whereas, with 42 hours of herbivory, the interaction effect was significant for benzyl alcohol, (*Z*)-geraniol and overall total volatiles. The BAW damage to the leaves was greater in the RKN infested plants (P<0.05) (n=6), possibly due to compromised above ground defense. *SlSAMT* was highly expressed in roots of susceptible lines, whereas no or less expression was seen in susceptible lines without RKN and resistant lines, with and without RKN inoculation. The tissue that showed higher expression also produced higher levels of MeSA.

Key words: Root Knot nematode, volatile compounds, double parasitism, above ground, below ground, beet armyworm, root volatiles, *SlSAMT*

Introduction

Plant defense against pathogens and insects is mediated in part by an array of constitutive and inducible chemical resistance factors (Agrawal et al., 1999; Van Wees et al., 2000). Since constitutive defenses effective against all challengers are absent, plants must coordinate multiple defense strategies that may have different effects on different pests. There has been considerable interest in developing chemicals to induce the biochemical pathways in economically important plants that provide resistance to either pathogens or insects (Thaler et al., 1999). An understanding of how pathways controlling SAR and IR interact will be critical for using chemical inducers to provide effective pest management (Thaler, 1999)

Root-knot nematodes (*Meloidogyne spp.* Chitwood) (RKN) are minute, thread-like obligate plant parasites which are very common in the rhizosphere and have a very wide host range. Tomato RKN species *Meloidogyne incognita* is the most widespread and damaging RKN in tropical and subtropical regions and in Tennessee (E. Bernard, personal communication). They have been found to generate initial responses similar to any biotrophs when perceived by plants (De Vos et al., 2005). The two most popular RKN control methods are application of toxic nematicides and growing RKN resistant cultivars. Nematicide application has limitations as it is not ecofriendly and is expensive, and as for resistant cultivars, some cultivars might not be suitable for all growing conditions and might not be agronomically advantageous (Molinari, 2005). The *Mi-1* gene confers resistance against three species of root-knot nematode in tomato. Salicylic acid (SA) is an important molecule in *Mi*-mediated resistance and its degradation has been found to results in partial loss of resistance to some species of root-knot nematodes (Branch et al., 2004). Temperature has also been found to adversely affect Mi-gene mediated defense (Bhattarai et al., 2008; Cooper et al., 2005b). Therefore, studies to find alternative methods for nematode control are important. Apart from exogenous methods, and *Mi-gene* mediated defense, the plant’s own defense system has received a lot of attention and various studies are underway to understand the defense mechanisms. A comprehensive understanding of the plant’s defense mechanisms under various abiotic and biotic conditions can lead to finding potential strategies to utilize the plant’s defense system for agricultural pest management.

Three pathways, salicylic acid (SA), jasmonic (JA), and ethylene (ET) have been found to be most important in plant defense signaling (Glazebrook, 2005). In general, JA and ET signaling pathways seem to work synergistically, whereas SA and JA/ET signaling pathways are antagonistic and communicate mainly by negative cross talk, indicating a tradeoff between these pathways (Thaler et al., 1999). However, cooperation and sequential positive interactions have also been reported between SA and JA/ET pathways (Bostock, 2005). The interaction and crosstalk between these pathways regulate both above and below-ground defense responses, which are characterized by production of defense compounds (Ament et al., 2004; Heil and Baldwin, 2002; Kunkel and Brooks, 2002; Owen et al., 2007; Thaler et al., 1999). Plants utilize two types of defense strategies, constitutive and inducible (Kaplan et al., 2008; Van Loon et al., 2006). Inducible defenses, however, have captured wider attention as a strategy for defense at specific spatial and temporal levels in a cost effective manner (Heil and Baldwin, 2002). Still, studies on induced defenses have predominantly focused on the induction by above-ground herbivores and pathogens despite the fact that below ground parts are attacked by as many, if not more, pests than the above ground parts (Bezemer et al., 2004).

This study elucidates the effect of tomato RKN (*Meloidogyne incognita*:Tylenchida: Heteroderidae) infections on the production of volatile secondary metabolites in tomato leaves and roots. The specific objectives of this study were: to determine the effect of RKN infection on the production of volatile secondary metabolites of tomato leaves and roots; to test the induced effect against beet armyworm (BAW); and to test the effect of duel pest pressure, RKN below ground and BAW above ground, on the volatile profile of the leaves and roots. Methyl salicylate is an important molecule produced by methylation of salicylic acid (SA), and regulated by *SAMT* gene in defense response to pest attack. We also studied the *SlSAMT* gene expression in the RKN infested and non-infested roots.

Materials and methods

Treatments and experimental design This experiment consisted of two RKN levels: 1. RKN infested; and 2. Control (untreated), combined with herbivory producing four treatments, 1. RKN, 2. RKN+ herbivory, 3. control, 4. control+herbivory. The experiment was conducted in completely randomized design (CRD) in the greenhouse. Two sets of plants were grown and analyzed separately. The second set of plants were grown in the same way as the first set, after the analysis for the first set of plants was completed.

Host plant, Root Knot nematode and insect culture Tomato (*Solanum lycopersicum*, Mill cv Castlemart; Family: Solanaceae) was selected as the host plant for the experiment and seeds were generously provided by Dr. G. A. Howe, Michigan State University, East Lansing. Castlemart is susceptible to RKN (*M. incognita*). Tomato seeds were sown in autoclaved growing media containing 4:1 mixture of sand (Quikrete International inc. Atlanta, GA) and Sequatchie loam soil obtained from ETREC – Plant Sciences Unit, Knoxville, in 4.25" terracotta pots (New England Pottery, Foxboro, MA). Plants were grown in the greenhouse with day/night temperatures of 75^{o} F/70^{o} F, 60% relative humidity and 16/8-h light/dark condition. Four week old plants were used for inoculation with RKN eggs.

M. incognita cultures were originally obtained from Plant Sciences Research unit, University of Tennessee, Knoxville by Dr. EC Bernard (Department of Plant Pathology and Entomology, UT, Knoxville). These cultures were initially maintained on tomato plant cultivar 'Rutgers' in similar soil/sand mixture in similar pots. Maximum egg yield is obtained after eight weeks of inoculation. Therefore, eggs were harvested from the roots of the tomato in the eighth week to get the maximum number of eggs for further inoculation.

Seeds of Castlemart for the experiment were sown at the time so that they were ready to be inoculated (four weeks old) by the time the nematode eggs were ready to harvest. Eggs were harvested as per standard protocol (EC Bernard, personal communication). In short, bleach solution is prepared by adding 40 ml chlorox (6% hypochlorite) (The Chlorox company, Oakland, CA) and 160 ml tap water; colonized roots were separated from the plants; roots were cut into pieces and covered with the bleach solution and blended with blender at low for 3 minutes; let the blended roots sit for 1 minute with occasional stirring; placed 200 mesh sieves on 500 mesh sieves and poured the blended roots on it and washed thoroughly with warm tap water for 30 seconds; eggs were passed through 200 mesh and were accumulated over 500 mesh, which were then poured in a flask by adding little water; made the volume up to 100 ml by adding more water. One ml egg solution was put on nematode counting slide (Chalex Corporation, Wallowa, OR) and number of eggs/ml solution was counted under a light microscope. For inoculation, roots were slightly exposed by removing the soil around the roots with fingers. Growing media was first soaked with water so that soil became easy to remove around the roots. This also was done to avoid any possible root injury. After the roots were exposed, 5 ml solution containing~5000 eggs was poured per plant with a pipette. Egg solution was thoroughly shaken each time before pipetting to maintain uniform suspension of eggs and keep the concentration even among all the plants.

Beet armyworm (BAW) (*Spodoptera exiguae* Hübner) (Insecta: Lepidoptera: Noctuidae) larvae were used for herbivory treatment. Eggs (Benzon Research Inc., Carlisle, PA.) were kept in 37.5 ml cups in the dark at 28^{o} C to hatch. The cups contained approximately 1 tea spoon of pinto bean based artificial diet (Benzon Research Inc, Carlisle, PA.) as a food source for larvae.

Herbivory treatment The effect of herbivory was studied for two different durations with two different sets of plants. For the first set of plants (n=6), twenty, two-instar BAW larvae were placed on the leaves at 5 PM with a brush and were left to feed on the leaves overnight. Larvae were removed at 11 AM the next morning after 18 hours of herbivory and leaves and roots were used for extraction. For the second set (n=3), herbivory was applied the same way as the first set, except the larvae were removed at 11 AM following the second night after 42 hours of feeding.

Volatile extraction Plants of similar size was selected for the volatile extractions. Leaves and roots with and without herbivory treatment, were used for the extraction and analysis was done according to the standard protocol (Chen et al., 2009). In short, the second leaf from the top was detached from around eight weeks old plant and immediately ground to powder with liquid nitrogen. One ml Ethyl acetate which contained 0.003% w/v 1-octanol (internal standard) was added to 200 m g powdered leaf tissues. Extraction was done by continuous shaking on an orbit shaker (Lab-line Instruments Inc., Melrose Park, IL) at room temperature for two and half hours. Roots were washed with tap water and were extracted the same way as leaves. Five micro liters of extract was injected into GC-MS for separation and identification of the compounds. Two replicates were run to decrease variability due to any technical factor in both experiments.

Volatile analysis Compounds were analyzed by Shimadzu GC (GC-17A) (Shimadzu Corp, Columbia, MD). Separation was performed on a Restek SHR5XLB column (30 m × 0.25 mm internal diameter × 0.25 μm thickness). Helium was used as the carrier gas (flow rate of 1 ml min^{-1}), a splitless injection (injector temperature 250°C) was used, and a temperature gradient of 5°C min^{-1} from 40°C (3 min hold) to 240°C was applied. The coupled mass spectrometer was a Hewlett-Packard model 5973 with a quadrupole mass selective detector. Products were identified by using the National Institute of Standards and Technology (NIST) mass spectra data base. Quantification was performed as reported previously by comparing with the peak area of internal standard (Chen et al., 2009). Extracts from the first set were stored at -20^{o}C for around three weeks before the analysis was done, whereas extracts from the second set of plants were analyzed immediately after extraction.

Beet army worm performance assay Four independent observers were chosen and asked to observe and note percent damage on the plants after the 18 hour herbivory treatment was given and larvae were removed. No leaf damage was considered as 0%, and all leaves eaten was considered as 100% damage. For leaf area consumption, leaf discs of 1.54 cm^2 were cut with a corer from around eight week old RKN infested and control plants. Four plants per treatment were chosen and four leaves per plant were taken for leaf discs. One disc was placed on a wet qualitative Filter Paper (Ahlstrom, PA, USA) in one 37.5 ml cup ((Benzon Research Inc., Carlisle, PA.). One, second-instar starved larvae was placed in each cup containing one disc and was allowed to feed. After 2 ½ hours of feeding, larvae were removed and leaf area consumed by larvae was measured by using a LI-3100C Area Meter (LI-COR Biosciences, Lincoln, NE). Leaf area consumed by BAW larvae was calculated by subtracting area of BAW damaged leaf from area of intact leaf. The assay was performed at room temperature.

Semi-quantitative RT-PCR analysis of SlSAMT Two RKN susceptible lines: Castlemart, and NC-946 and two RKN resistant lines: CULBPT-4 and NC-06113 were grown and infested with RKN as mentioned in previous sections. Roots from eight week old plants were cut, cleaned with distilled water and ground with liquid nitrogen. Total RNA extraction and subsequent first-strand cDNA synthesis were performed as previously described (Chen et al., 2003b). For PCR analysis primer was designed to amplify *SlSAMT* fragment of 1.1kbp as follows: forward primer 5'-ATGAAGGTTGTTGAAGTTCTTCACATGAA-3' and reverse primer

5'-TTATTTTTTCTTGGTCAAGGAGACAGTAA-3'. Two primers used for PCR amplification of *SlActine* were designed as previously described (Hu et al., 2011): forward primer 5'-AGGACGTGACCTCACTGATAG -3' and reverse primer 5'-CACTTCCTGTGGACAATAGAG -3'. Initially, *SlActin*- was normalized for all the lines before amplifying *SlSAMT*. The program used to amplify *SlActin* was as follows: 94^{o}C for 2 min followed by 34 cycles at 94 ^{o}C for 30 sec, 58^{o}C for 30 sec and 72 ^{o}C for 30 sec, with a final extension at 72 ^{o}C for 5 min. Amplified products were separated on 1.0% agarose gel. Gels were stained with ethidium bromide, visualized under UV-light, using the Bio-Rad Quantity One software (Bio-Rad, Hercules, CA). The program used to amplify the *SlSAMT* fragment was as follows: 95°C for 2 min followed by 34 cycles of 94°C for 30 sec, 56 °C for 30 sec and 72 °C for 1:10 min, and then followed by an extension step of 72 °C for 7 min. All PCRs were replicated twice using first strand cDNA made from two independent RNA preparations.

Statistical analysis Volatile compounds were compared by analysis of variance (*ANOVA*) with mixed models SAS 9.2 (SAS 2008) as CRD factorial. *ANOVA* included RKN, herbivory and interaction between RKN and herbivory as fixed effects for both, leaf and root volatile analysis. Analyses were performed using program code contained in the “DANDA” macro for SAS designed by Dr. Arnold Saxton (http://dawg.utk.edu/). Data were checked for normality and equality of variance and those not meeting the criteria were log transformed (log value=0.05). Rank transformation was done if any of these criteria could not be met even by log transformation. Pair wise means were compared with LSD mean separation at $P<0.05$. BAW response to test the induced defense was analyzed by *ANOVA* with mixed models SAS 9.2 (SAS 2008) for the fixed effect of RKN with CRD. Observations from four observers were averaged to find percent damage for each replicate and those data were used for analysis. For leaf area consumption assay, results were calculated and reported as mean (±SE) leaf area consumed by BAW larvae.

Results

Volatile compounds analyses The leaf volatile compounds detected in first set of plants (n=6) included three monoterpenes: α-pinene, 2-carene, and β-phellandrene; five sesquiterpenes:

δ-elemene, β-elemene, (*Z*)-caryophyllene, α-humulene, and germacrene-D. Total other compounds that were not terpenes, included benzyl alcohol, methyl salicylate and eugenol. All of the above compounds were detected from the control plants except methyl salicylate. From RKN plants, all the compounds except α-pinene and methyl salicylate were detected. RKN had significant effect on the total other compounds (P <0.001) comprised of benzyl alcohol (P=0.001) and eugenol (P=0.007) that were significantly higher in the leaves of RKN plants compared to the control (Table 4.1). However, for the total volatile compounds, total monoterpenes, and total sesquiterpenes, the trend was contrary to that for total others. Compounds such as α-pinene (P=0.13), 2-carene (P=0.072), β-phellandrene (P=0.18), total monoterpenes (P=0.095), δ-elemene (P=0.18), β-elemene (P=0.062), germacrene-D (P=0.088), total sesquiterpenes (P=0.18), and overall total volatiles (P=0.187) showed a pattern for low production in the leaves of RKN plants, but the effect was not significant (Table 4.1).

Herbivory for 18 hours significantly affected some compounds and lowered the volatile contents in the leaf tissues compared to those without herbivory. All the compounds except α-pinene were detected from the control+herbivory. From RKN+herbivory, all the compounds were detected except α-pinene and β-elemene. Methyl salicylate was detected from both, RKN and control plants only after herbivory. Total monoterpenes (P=0.002), β-phellandrene (P=0.002), δ-elemene (P=0.029), β-elemene (P=0.008), eugenol (P <0.001), total other compounds (P <0.001) and overall total volatiles (P<0.001) were significantly decreased after 18 hours of herbivory. Some other compounds such as α-pinene (P=0.1), (*Z*)-caryophyllene (P=0.06), α-humulene (P=0.05), benzyl alcohol (P=0.19), and methyl salicylate (P=0.07) were influenced to some extent but not significantly. The effect of interaction between RKN and herbivory was significant for eugenol (P=0.003) and total other compounds (P<0.001). Some compounds such as α-pinene (P=0.13), and germacrene-D (P=0.061) showed similar trend but the effect was not significant. The pattern for group of compounds above ground after herbivory was same as that without herbivory, except that the quantities decreased after herbivory (Table 4.1).

Leaf volatiles detected in all the treatments from the second set of plants included three groups of compounds. The monoterpenes included α-pinene, β-myrcene, 2-carene, α-phellandrene, β-phellandrene, β-(*E*)-ocimene, and γ-terpinene; sesquiterpenes included δ-elemene, β-elemene, (*Z*)-caryophyllene, aristolene, α-humulene, germacrene-D, and α-selinene;

and total other compounds that were not terpenes, included benzyl alcohol, methyl salicylate, and eugenol. From the control plants, all but α-pinene and γ-terpinene; from RKN, all but α-pinene, β-(*E*)-ocimene, γ-terpinene, aristolene, and α-selinene; from control+herbivory, all, and from RKN+ herbivory, all but aristolene were detected. Total volatile compounds, total monoterpenes, and total sesquiterpenes from the leaf tissue showed a pattern for low production in the RKN infested plants, same as the first set of plants. The RKN infestation showed a trend, with γ-terpinene (*P*=0.09), β-elemene (*P*=0.145), α-selinene (*P*=0.093), the effect was nonsignificant for any compound (Table 4.2).

After herbivory treatment for 42 hours, the pattern was different than that in the first set of plants with herbivory for 18 hours. An increase in volatile compounds was seen in the RKN plants contrary to those in first set of the plants, where, decreased volatile content was observed in RKN plants after herbivory. Herbivory for 42 hours, increased α-pinene (*P*<0.001), 2-carene (*P*=0.011), β-phellandrene (*P*=0.022), total monoterpenes (*P*=0.011), and overall total volatiles (*P*=0.04) compared to those without herbivory. Some other compounds such as β-myrcene (*P*=0.18), α- phellandrene (*P*=0.075), β-phellandrene (*P*=0.055), β-(*E*)-ocimene (*P*=0.154), benzyl alcohol (*P*=0.192), (*Z*)-caryophyllene (*P*=0.189), and germacrene-D (*P*=0.155) showed similar trends towards increased production after herbivory but the effect was not significant. The effect for interaction between RKN and 48 hours herbivory was not significant for any compound, however, showed a trend towards high production of compounds such as β-phellandrene (*P*=0.19), δ-elemene (*P*=0.19), (*Z*)-caryophyllene (*P*=0.15), total monoterpenes (*P*=0.198), and total sesquiterpenes (*P*=0.177) (Table 4.2).

Roots from the first set of the plants produced only three compounds, (*Z*)-geraniol, methyl salicylate, and benzyl alcohol unlike leaves, which produce a large number of volatiles. RKN had significant effect on the production of all the three compounds, and produced higher methyl salicylate (*P*=0.01), benzyl alcohol (*P*=0.006), and overall total volatile (*P*=0.004) compared to the control. (*Z*)-geraniol (*P*<0.001) was produced only by RKN roots. Above ground herbivory for 18 hours significantly decreased the production of (*Z*)-geraniol (*P*=0.032), and methyl salicylate (*P*=0.038) in the roots. The same trend was observed for benzyl alcohol (*P*=0.12), and overall total volatiles (*P*=0.052) but the effect was not significant. The effect of interaction between RKN and 18 hours of herbivory was only significant for (*Z*)-geraniol (*P*=0.032) (Table

4.3).

Roots from the second set of the plants produced the same three volatiles as from the first set. The monoterpene (*Z*)-geraniol was again produced only from RKN infested roots and was not detected from the control roots. The effect of RKN was significant for methyl salicylate ($P=0.022$), (*Z*)-geraniol ($P<0.001$) and overall total volatiles ($P=0.021$). The same trend was observed for benzyl alcohol ($P=0.062$) but the effect was nonsignificant. Herbivory for 42 hours had a significant effect on methyl salicylate ($P=0.048$), (*Z*)-geraniol ($P<0.001$), and overall total volatiles ($P=0.034$). Benzyl alcohol ($P=0.093$) had a similar trend but the effect was nonsignificant. The effect of the interaction between RKN and 42 hours of herbivory was significant for benzyl alcohol ($P=0.003$), (*Z*)-geraniol ($P<0.001$), and total volatiles ($P=0.005$). (*Z*)-geraniol was not detected from RKN roots after longer exposure to herbivory. Methyl salicylate ($P=0.057$) showed a similar trend but the effect was not significant (Table 4.4).

An interesting pattern was observed for the volatiles from roots from the first and second set of plants after two different durations of herbivory exposure. The first set of plants with shorter exposure to herbivory showed the same pattern as those without herbivory for both, RKN and the control. However, the second set of plants with longer exposure to herbivory showed a trend that was opposite to that in the first set.

Beet army worm performance assay A significantly higher percent leaf damage was observed in RKN infested plants than in control plants ($P<0.001$) with the first set of plants (Figure 4.1). In the second set, the mean leaf area consumed by beet armyworm larvae was not significantly different between both treatments. However, the trend was still towards high consumption of leaves from the RKN plants (Figure 4.2).

Semi-quantitative RT-PCR analysis Higher expression of *SlSAMT* gene was seen in the root tissue from the RKN infested Castlemart plant than the control plants of the same line. Another susceptible line NC-946 also showed high expression of the gene than the control. Resistant lines NC-06113 did not show any sign of RKN infestation and plants showed equal but very low expression of the *SlSAMT* gene, despite inoculation and no-inoculation. Another resistant line Culbpt-4 showed no expression of the gene (Figure 4.4). The two susceptible lines were also found to produce MeSA from the RKN infested roots (Figure 4.5.A). However, non-infested roots of these lines as well as those of resistant lines did not produce MeSA (Figure 4.5.B).

Discussion

Volatile compound Upon perception of any 'foreign' organism, different signaling pathways operate within the plant to produce the appropriate response. These pathways are co-ordinated by 'alarm signals' that regulate differential sets of defense-related genes (De Vos et al., 2005). Higher MeSA in roots indicates upregulation of alarm signals in response to RKN infestation. SA is one of the major alarm signals that coordinate defense mechanisms that are generally effective against biotrophic pathogens. RKNs are obligate biotrophs and generate an initial response similar to any biotroph when perceived by plants (De Vos et al., 2005). SA is converted to MeSA, a critical mobile defense signal to the distant tissue, through activity of *SAMT* (Zhao et al., 2010). Secondary metabolite compounds play a pivotal role in plant's defense and survival but are metabolically costly for the plant (Pozo and Azcon-Aguilar, 2007) that might be the reason why RKN infested plants produced higher quantities of volatiles in the roots but lower quantities in the leaves to compensate for the metabolic cost.

Production of (*Z*)-geraniol, a terpene by the RKN infested roots and relatively low production of terpenes in the leaves, is likely due to economic utilization of common substrates such as monoterpene synthesis molecule which generates geranyl diphosphate (GPP, C_{10}) (Chappell, 1995) in the ubiquitous terpenoid pathway. Several terpene synthase (TPSs) enzymes, controlled by multiple TPS genes, act upon intermediatery molecules farnysl diphosphate (FPP) and geranyl diphosphate (GPP), to produce different sesquiterpenes and monoterpenes (Chen et al., 2003a; Yuan et al., 2008). Also, the volatiles are carbon-based defense compounds and thus, might compete for a common pool of substrates for their synthesis (Radhika et al., 2008). This may explain higher volatile production in the roots but less in the leaves in the RKN plants.

Plants from the first set were subjected to herbivory for a shorter time (42 hours) and those from second set, for a longer time (42 hours). RKN infested plants responded by producing higher quantities of volatiles above ground (in leaves) after a longer time of herbivory than the control contrary to the trend seen in those with shorter exposure to herbivory. The possible reason might be that its defense system in the RKN plants was already primed (Hulten et al., 2010) and systemic acquired resistance was induced due to RKN infestation. Since defense genes are not activated directly in primed plants or plant parts, it is hypothesized that the primed state is based on accumulation or posttranslational modification of signal molecules that remain inactive until a subsequent and enough of a stress stimulus is perceived (Conrath et al., 2006). Due to the enhanced level of signaling components, subsequent pest attack leads to an increased activation of the appropriate defense pathway and thus to a potential activation of defense-related genes (Hulten et al., 2010). Therefore, after being subjected to above ground pest pressure for longer time, RKN infested plants that may have accumulated or modified the posttranslational signal molecule through priming, produced higher volatiles in leaves by allocating its resources to above ground defense resulting in decrease in the below ground volatiles.

Decrease in root volatiles upon herbivory in this case may be to maintain balance of plants metabolic cost, which is in agreement with growth-differentiation balance hypothesis (GDBH). This hypothesis assumes that a balance must be maintained between resources used for growth and defense (Barto and Cipollini, 2005). This was not the case with the plants subjected to shorter period of herbivory. Shorter exposure to herbivory might not be sufficient for the RKN plants to up regulate their above ground defenses to a level that could enhance the above ground volatile production. This is known as lag-time, which is the time required for the induction of the defense after the first contact with the herbivore (Heil and Baldwin, 2002; Radhika et al., 2008) The overall decrease in root volatiles in RKN infested and control plants upon herbivory as compared to those without herbivory is in agreement with optimal defense hypothesis (ODH) which assumes that organisms allocate their defenses in a way that maximize fitness (Radhika et al., 2008). Higher production of (*Z*)-geraniol in RKN infested roots and subsequent decrease or no detection after herbivory, from the plants of both sets is in agreement with the report of significantly higher levels of terpenoid aldehyde production in root tissue after root herbivory, and no such effect following foliar herbivory (Bezemer et al., 2004).

Increase in leaf volatiles and decrease in root volatiles from RKN plants after 42 hours of herbivory may also be due to higher activation of jasmonic acid (JA) regulated defense response above ground (Pozo and Azcon-Aguilar, 2007). Volatile compounds are inducible and their production rate increases in response to herbivory or mechanical damage and this response is regulated by the octadecanoid pathway, in which JA plays a key role (Heil and Baldwin, 2002). Putative terpene synthase transcripts has been found to be induced upon herbivory (Ament et al., 2004) which might explain higher terpenoid content upon longer herbivory exposure. Relatively high levels of terpenoids, as well as methyl salicylate were also observed after longer time of herbivory, which is also in agreement with earlier reports of increased proportion of MeSA and terpenoids upon herbivory through induction of the octadecanoid pathway (Thaler, 1999). Relatively low MeSA in the leaves of RKN plant, but high MeSA in roots of RKN plants may be an indication of competition for substrate utilization for above and below ground defense, and that RKN infestation has stronger impact on induced MeSA production below ground as compared to induced MeSA production by herbivory above ground.

The JA and SA pathways are known to conflict, and therefore, induction of one signaling compound can in some cases, inhibit induction of the other (Pena-Cortés et al., 1993; Thaler et al., 1999). The JA pathway has been found to enhance phenolics (Kim et al., 2006) as well as sesquiterpenes production (Schmelz et al., 2001), which might explain increase in sesquiterpenes and other volatiles in leaves but decrease in volatiles in roots. *Mi-1* is the only known source of heritable RKN resistance in cultivated tomato. Given that SA is involved in *Mi*-mediated resistance, induction of JA could have a negative impact on the function of *Mi-1* by affecting the SA pathway (Cooper et al., 2005b). This may explain the relatively low MeSA in roots after herbivory, as herbivory is known to induce the JA pathway (Demkura et al., 2010).

One significant compound produce by RKN roots was benzyl alcohol. The role and mechanism of the production of this compound is not well known. However, significantly larger quantities in the RKN roots may indicate its possible role in direct and indirect defense below ground. Benzyl alcohol dehydrogenase (BAD) generated in *Arabidopsis* plants during certain fungal infections, is reported to be involved in the conversion of benzaldehyde derivatives into benzyl alcohol and incorporation of important phenolic defense molecules into the cell wall. It is also involved in the metabolism of soluble compounds in signaling for plant defense using phenolics (Somssich et al., 1996). Benzyl alcohol also has structural similarity with SA, an established signal molecule in plants. Therefore, increased benzyl alcohol may have different physiological and defense roles in the plant after a pathogen invasion. Direct effect of benzyl alcohol is not yet clear, but it may have a role in structural defense and signaling.

Differences in the number and overall quantities of the volatiles detected from the first set and the second set of plants may be due to different time of growing the two set of plants and different durations of the storage of the extracts before analysis. Extracts from the first set were stored at -20 °C for around three weeks before the analysis was done, whereas, extracts from the second set of plants were analyzed within the first week after extraction.

Beet army worm performance assay Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), are known to regulate both basal and resistance (*R*) gene-mediated defense responses (Glazebrook, 2005). In general, SA and JA/ET signaling pathways are antagonistic and communicate mainly by negative cross talk, indicating a tradeoff between these pathways (Hulten et al., 2010). SA mimic, benzothiadiazole, has been found to attenuate the jasmonate-induced expression of the antiherbivore defense-related enzyme polyphenol oxidase, leading to compromise in host-plant resistance to BAW larvae (Thaler et al., 1999). RKN related defense system in the plant involves SA that generally affects the JA dependent pathway, important for herbivore defense, in a negative way. This may result in compromised herbivore resistance in the plant, which might explain higher damage in root knot nematode infested plants compared to the control. Although in some cases, cooperation and sequential positive interactions have also been reported between SA and JA pathways (Bostock, 2005), conflict of these pathways seems more plausible in this case.

Semi-quantitative RT-PCR analysis Both resistant lines contain the *Mi*-gene for RKN resistance and, therefore, were not successfully colonized by RKNs (Dilip Panthee, personal communication). Based on the nucleotide sequence, *Mi* gene is grouped in a family of plant resistance genes (R-genes) characterized by leucine-rich repeats and nucleotide-binding sites (Milligan et al., 1998). R-gene-mediated resistance depends on rapid, local defense responses triggered by direct or indirect interactions between the R-gene product and highly specific elicitors from the pest (Takken and Joosten, 2000).

Mi gene confers resistance against three species of root-knot nematode in tomato and SA is part of the *Mi* mediated defense response (Branch et al., 2004). MeSA is an organic ester and a critical mobile defense signal produced by reaction where the methyl group is transferred from a donor S-adenosine-L-methionine to carboxyl group of SA catalyzed by methyl transferase, which is regulated by *SAMT* gene (Chen et al., 2003a). *SAMT* and putative terpene synthase transcripts have been found to be induced upon herbivory in some cases (Ament et al., 2004) and plants produce or increase MeSA production as defense signal. This might explain the higher expression of *SlSAMT* in RKN infested lines. As discussed earlier, plant defense compounds are vital but metabolically costly and plants produce them mostly when they are required to defend themselves. This explains higher MeSA and higher *SlSAMT* expression in susceptible lines as induced defense upon RKN infestation. No or little production of MeSA with lower or no expression of the *SlSAMT* in susceptible lines without RKN, and resistant lines underscore this theory. Since they were not infested, they did not require producing higher levels of MeSA to defend them.

Production of (*Z*)-geraniol in susceptible roots with RKN infestation and not in any other roots also agrees with same theory of need based volatile production. The (*Z*)-geraniol, in combination with (E)-and (Z)-(±)-nerolidol has been demonstrated an additive interaction against *Bacillus cereus* (Seatlholo, 2008), which is an endemic, soil dwelling bacterium. Geraniol has also been reported to play role in microbial interactions below ground. The microbial communities in the rhizosphere are usually limited by carbon availability. The carbon-containing root volatiles, especially monoterpenes such as geraniol, have been reported to contribute significantly to the belowground carbon cycle (Owen et al., 2007). Therefore, production of geraniol by the RKN infested roots might have a role in direct and/or indirect defense below ground.

The results suggest an important link between above and below ground plant defense mechanisms. It has been suggested that defense against biotrophic pathogens is largely associated with activation of defense responses regulated by the SA–dependent pathway (Glazebrook, 2005) and response to herbivory is largely associated with the JA dependent pathway. However, varied level of crosstalk between these pathway have been reported in various cases (Spoel et al., 2003), such as enhancement of induced disease resistance by simultaneous activation of SA and JA dependent pathways in *A. thaliana* (Van Wees et al., 2000).

The present study shows how above ground and below ground pest attacks influence production of volatile secondary metabolites in a cost effective manner. Temporal and spatial allocation of resources and sharing of common substrates for optimum defense has been observed in this study. Higher volatiles released by RKN infested roots here, are in agreement with various reports of increased volatile production as a defense response and may have important implication for direct and indirect defense against RKNs. The results could be used to narrow down specific compounds to study, such as (*Z*)-geraniol and benzyl alcohol, or a group of compounds such as monoterpenes and sesquiterpenes, to determine more specific and comprehensive roles and regulatory mechanisms of these compounds. Although a link between above and below ground defense responses is observed in this case, the responses could vary according to host and species, temporally and spatially. The gene expression study showing increased *SlSAMT* expression in RKN infested roots, further underscores the need based production of compounds, and activation of genes for metabolically cost effective defense. Future studies are required to look into specific compounds and mechanisms, which could be important for pest management in agricultural crops. The findings could be utilized through breeding crops containing higher levels of defense compounds, as well as through exogenous application of the compounds to alter the host preference or for indirect defense.

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Appendix

Tables

Table 4.1: Volatile compounds [means ng g (fresh wt)$^{-1}$±SE] from the leaves of RKN infested and control plants without herbivory and with 18 hours of herbivory. Twelve eight-week old RKN infested and control plants each were randomly selected. Herbivory treatment was given by placing twenty, second instar BAW larvae on the leaves of six plants from each treatment group at 5 PM. Larvae were removed at 11 AM the next morning after 18 hours of herbivory. Leaves from both, with and without herbivory treatments were ground with liquid nitrogen and extracted with ethyl acetate containing Octanol (0.003%) as internal standard. Extracts were injected into GC-MS for separation and identification of compounds. N=6.

Compounds	Control	RKN	Control+Herbivory	RKN+Herbivory
α-Pinene	2.4 ± 1.6	ND[*]	ND	ND
2-Carene	6.3 ± 1.8	3.1 ± 1.4	4.0 ± 1.1	1.6 ± 0.8
β -Phellandrene	34.4 ± 5.9**a**[#]	25.7 ± 5.6**ab**	14.6 ± 4.5**bc**	9.4 ± 2.4**c**
Total Monoterpenes	43.1 ± 7.9**a**	28.8 ± 6.4**ab**	18.6 ± 4.9**bc**	11.0 ± 3.1**c**
δ-Elemene	3.6 ± 0.5**a**	3.1 ± 0.7**ab**	2.6 ± 0.6**ab**	1.5 ± 0.4**b**
β-Elemene	0.5 ± 0.1**a**	0.2 ± 0.1**ab**	0.1 ± 0.1**b**	ND **b**
(*Z*)-Caryophyllene	6.1 ± 1.0	4.9 ± 1.5	4.0 ± 0.9	2.6 ± 0.9
α -Humulene	1.4 ± 0.2	1.2 ± 0.4	0.9 ± 0.2	0.7 ± 0.2
Germacrene D	0.1 ± 0.0	0.0 ± 0.0	0.8 ± 0.0	0.1 ± 0.0
Total Sesquiterpenes	11.7 ± 1.9	9.5 ± 2.6	7.7 ± 1.9	4.8 ± 1.5
Benzyl Alcohol	1.3 ± 0.4**bc**	2.4 ± 0.4**b**	0.8 ± 0.2**c**	1.8 ± 0.4**ab**
Methyl salicylate	ND	ND	0.2 ± 0.2	0.1 ± 0.0
Eugenol	3.9 ± 0.5**b**	6.9 ± 0.6**a**	1.8 ± 0.4**c**	1.6 ± 0.4**c**
Total Others	5.2 ± 0.8**b**	9.3 ± 0.5**a**	2.8 ± 0.4**c**	3.5 ± 0.6**bc**
Total Volatile	60.0 ± 9.1**a**	47.6 ± 9.4**ab**	29.1 ± 6.1**bc**	19.4 ± 4.8**c**

[*] Not detected.

[#] Different letters a, b, c denote significant difference among treatments (P<0.05).

Table 4.2: Volatile compounds [mean ng g (fresh wt)$^{-1}$±SE] from the leaves of RKN infested and control plants without herbivory and with 42 hours of herbivory. Six eight-week old RKN infested and control plants each were randomly selected. Three plants from each treatment were given herbivory and three from each treatment were kept without herbivory. For herbivory, twenty, second instar BAW larvae were placed on the leaves at 5 PM and were left to feed for 42 hours. Leaves from both, with and without herbivory treatments were ground with liquid nitrogen and extracted with ethyl acetate containing Octanol (0.003%) as internal standard. Extracts were injected into GC-MS for separation and identification of compounds. N=3.

Compounds	Control	RKN	Control+Herbivory	RKN+Herbivory
α-Pinene	ND[*] **b**[#]	ND **b**	5.8 ±1.1 **a**	6.3 ±1.9 **a**
β-Myrcene	1.7 ±1.7	0.4 ±0.4	3.5 ±0.8	2.2 ±0.4
2-Carene	8.7 ±3.6 **ab**	3.3 ±2.7 **b**	22.5 ±5.9 **a**	22.3 ±3.7 **a**
α-Phellandrene	1.0 ±0.9	0.5 ±0.5	1.9 ±0.2	3.6 ±1.2
Limonene	1.8 ±1.8	1.5 ±1.5	4.0 ±2.2	4.1 ±3.6
β-Phellandrene	40.7 ±9.2**ab**	23.0 ±8.3**b**	78.6 ±29.8**a**	93.6 ±27.2 **a**
β-(*E*)-Ocimene	1.0 ±0.9	ND	1.5 ±0.5	1.6 ±0.2
γ-Terpinene	ND	ND	0.1 ±0.1	0.3 ±0.1
Total Monoterpenes	54.8 ±9.7**ab**	28.7 ±12.9**b**	117.9 ±35.6**a**	133.8 ±32.1**a**
δ-Elemene	7.1 ±2.2	4.7 ±1.5	5.4 ±3.5	9.6 ±1.6
β -Elemene	0.7 ±0.2	0.4 ±0.2	0.6 ±0.4	1.0 ±0.1
(*Z*)-Caryophyllene	17.9 ±3.7	15.9 ±5.6	23.4 ±15.9	21.8 ±3.9
Aristolene	0.2 ±0.1	ND	0.3 ±0.2	ND
α -humulene	3.9 ±0.8	3.5 ±1.3	5.3 ±3.3	5.9 ±1.8
Germacrene D	0.3 ±0.1	0.1 ±0.1	0.4 ±0.3	0.5 ±0.1
α -Selinene	ND	ND	0.3 ±0.3	0.5 ±0.0
Total Sesquiterpenes	30.1 ±7.0	24.6 ±8.6	35.8 ±24.1	39.1 ±7.5

Compounds	Control	RKN	Control+Herbivory	RKN+Herbivory
Benzyl Alcohol	3.7 ±1.0	3.7 ±0.4	4.5 ±0.3	3.6 ±0.2
Methyl salicylate	0.9 ±0.1	0.1 ±0.0	1.6 ±0.9	1.4 ±0.2
Eugenol	2.5 ±0.9	1.4 ±0.5	1.8 ±0.4	1.1 ±0.5
<u>Total Others</u>	7.1 ±1.5	5.2 ±0.8	7.8 ±1.5	6.1 ±0.5
<u>Total Volatiles</u>	92.1 ±17.0	58.5 ±18.5	161.5 ±61.1	179.1 ±28.0

[*] Not detected.

[#] Different letters a, b, c denote significant difference among treatments (P<0.05).

Table 4.3: Volatile compounds [means ng g (fresh wt)$^{-1}$±SE] from the roots of RKN infested and control plants without herbivory and with 18 hours of herbivory. Twelve eight-week old RKN infested and control plants each were randomly selected. Six from each treatment were given herbivory and six from each treatment were kept without herbivory. For herbivory, twenty, two-instar BAW larvae were placed on the leaves at 5 PM with a brush and were left to feed on the leaves overnight. Larvae were removed at 11 AM the next morning after 18 hours of herbivory. Roots from both, with and without herbivory treatments were cleansed with tap water, ground with liquid nitrogen and extracted with ethyl acetate containing Octanol (0.003%) as internal standard. Extracts were injected into GC-MS for separation and identification of compounds. N=6.

Compounds	Control	RKN	Control+Herbivory	RKN+Herbivory
Benzyl alcohol	2.6 ±0.9**b**[#]	6.8 ±1.4**a**	1.6 ±0.9**b**	4.2 ±1.2**ab**
Methyl salicylate	1.5 ±0.5**ab**	4.7 ±1.4**a**	0.6 ±0.3**b**	2.0 ±0.5**a**
(*Z*)-Geraniol	ND [*] **c**	0.6 ±0.0**a**	ND **c**	0.2 ±0.1**b**
Total volatiles	4.1 ±1.3**b**	11.6 ±2.6**a**	2.2 ±1.1**b**	6.2 ±1.6**b**

[#] Different letters a, b, c denote significant difference among treatments (P<0.05).

[*] Not detected.

Table 4.4: Volatile compounds [means ng g (fresh wt)$^{-1}$±SE] from the leaves of RKN infested and control plants without and with 42 hours of herbivory. Eight-week old RKN infested and control plants each were randomly selected. Three plants from each treatment were given herbivory and three from each treatment were kept without herbivory. For herbivory, twenty, two-instar BAW larvae were placed on the leaves at 5 PM and were left to feed for 42 hours. Roots from both, with and without herbivory treatments were cleansed with tap water, ground with liquid nitrogen and extracted with ethyl acetate containing Octanol (0.003%) as internal standard. Extracts were injected into GC-MS for separation and identification of compounds. N=3.

Compounds	Control	RKN	Control+Herbivory	RKN+Herbivory
Benzyl alcohol	1.0 ±0.8**b**[#]	8.5 ±1.4**a**	8.2 ±1.0**a**	5.7 ±1.3**a**
Methyl salicylate	3.7 ±0.5**b**	8.1 ±1.1**a**	3.6 ±0.4**b**	4.2 ±1.2**b**
(*Z*)-Geraniol	ND [*]**b**	1.5 ±0.1**a**	ND **b**	ND **b**
Total volatiles	4.7 ±0.6**c**	17.6 ±1.2**a**	11.8 ±1.4**ab**	9.9 ±2.4**bc**

[#] Different letters a, b, c denote significant difference among treatments (P<0.05).

[*] Not detected.

Figures

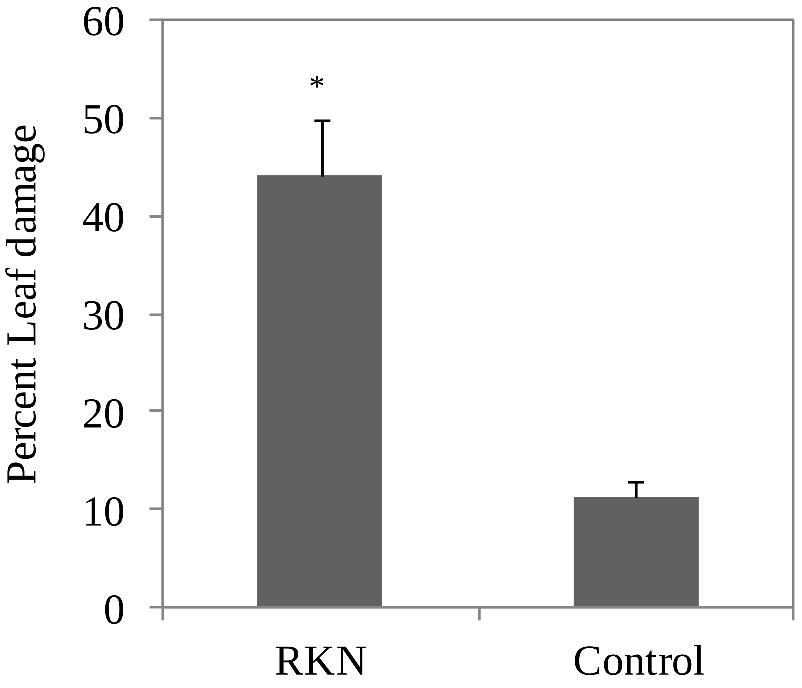

Figure 4.1: Leaf damage [mean percent ±SE] by beet armyworm larvae through visual observation. Six, eight-week old RKN infested and control plants each of similar size were randomly selected for the herbivory treatment. Twenty, two-instar BAW larvae were placed on the leaves at 5 PM and were left to feed on the leaves overnight. Larvae were removed at 11 AM the next morning. Four independent observers were chosen and asked to observe and note percent damage on the plants after the larvae were removed from the plants. N=6.

* Indicates statistically significant difference.

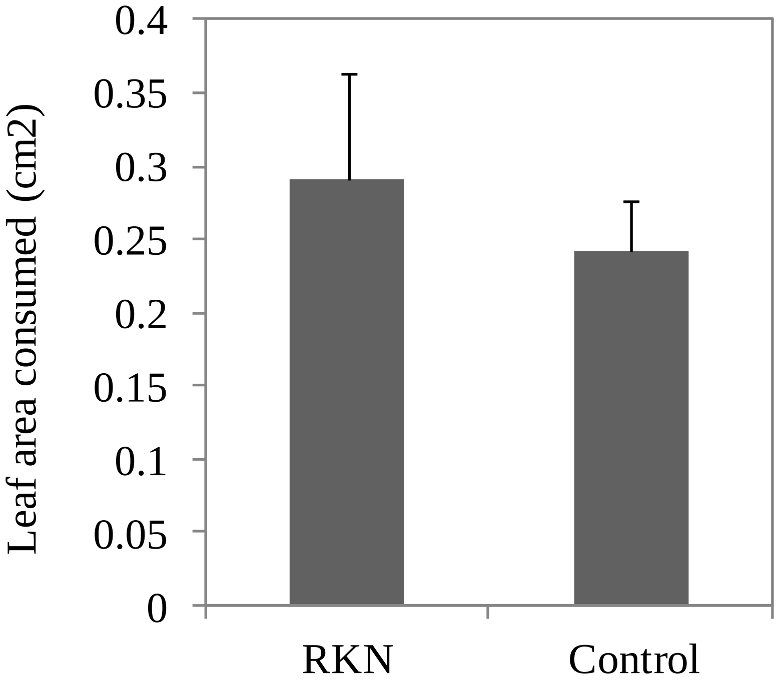

Figure 4.2: Leaf disc area consumed [cm^2 ±SE] per beet armyworm larvae. Leaf discs of 1.54 cm^2 were cut from around eight week old RKN infested plants and control plants. Four plants per treatment were chosen and four leaves per plant were taken for the leaf discs. One disc was placed on a wet qualitative Filter Paper in one 37.5 ml cup. Starved two-instar larvae were placed one in each cup containing one disc and were allowed to feed. After 2 ½ hours larvae were removed and leaf area consumed by larvae was measured by using a LI-3100C Area Meter. Leaf area consumed by BAW larvae was calculated by subtracting area of BAW damaged leaf from area of the intact leaf. The assay was performed at room temperature. N=4.

Figure 4.3:A. Beet army worm damage (18 hours) to the control plants.

Figure 4.3:B.Beet army worm damage (18 hours) to the root knot nematode infested plants.

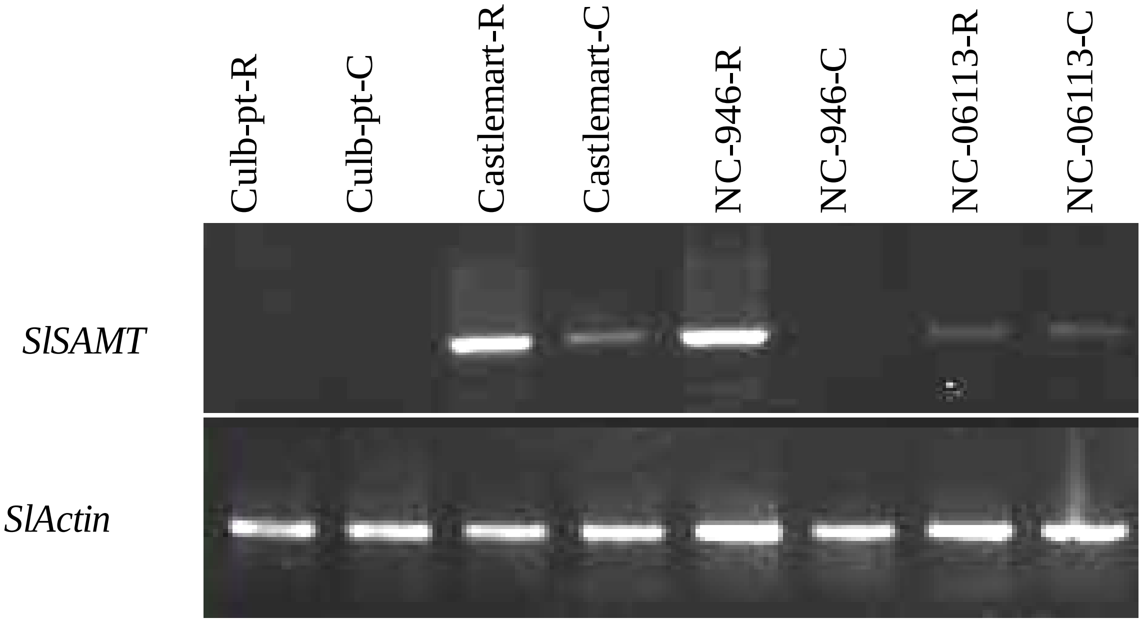

Figure 4.4: Semi-quantitative RT-PCR analysis of *SlSAMT* expression in two tomato breeding lines resistant to RKN and two susceptible breeding lines. Roots were collected from eight weeks old tomato plants grown in a greenhouse. Total RNA was extracted and used for RT-PCR analysis. PCR with primers for *SlActin* was normalized prior to *SlSAMT* amplification of different samples. RKN resistant lines are Culbpt-4, and NC-06113; RKN susceptible lines are Castlemart, and NC-946. Abbreviations: C-Control; R-RKN infested.

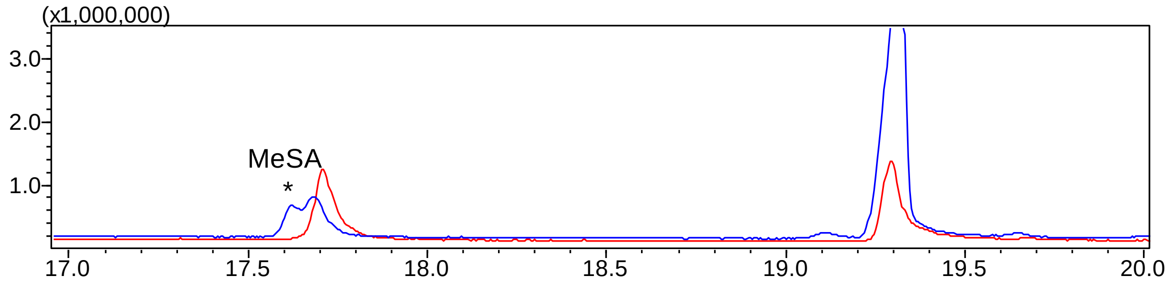

Figure 4.5:A. GC-Chromatogram of root Volatile compound from susceptible line NC-946. RKN infestation induced MeSA production (blue) and root without RKN infestation did not show any MeSA (Red).

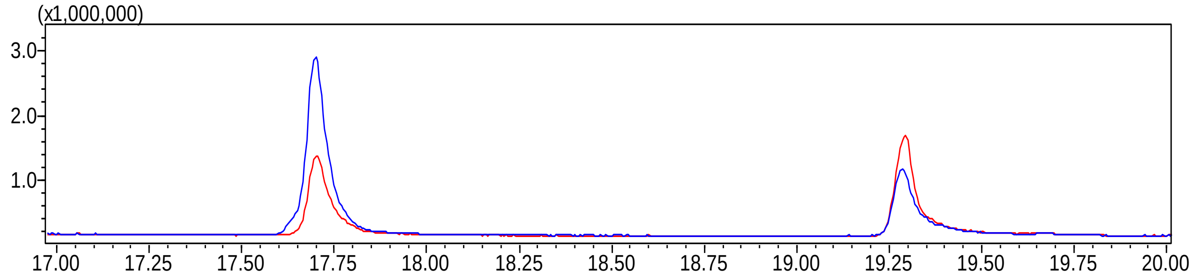

Figure 4.5:B. GC- Chromatogram of root volatile compound from resistant line Culbpt4. RKN (blue) and without RKN (Red) inoculum did not show any MeSA. The roots from resistant line could not be infested with RKN despite inoculation.

Chapter V. Conclusion and Perspective

Conclusion

In the previous chapters, metabolomics approaches were applied to study the production of volatile secondary metabolites from above and belowground parts of the tomato plant (*Solanum lycopersicum* L.). Influence of the plant's interaction with an aboveground pest, beet armyworm (BAW) (*Spodoptera exiguae* Hübner), belowground pest tomato rootknot nematode (RKN) (*Meloidogyne incognita*) and two beneficial organisms, a symbiotic arbuscular mycorrhizal fungi (AM) (*Glomus intraradices*) and entomopathogenic endophyte (Bb) (*Beauveria bassiana*) on the volatile profiles were studied. Effects of the beneficial organisms and RKN on the performance of BAW, as well as the influence of dual pest attack (above and belowground) on the volatile profile were also studied.

First, previous work involving volatile compounds were reviewed in terms of their potential utilization for insect pest management in organic crop production systems. The Integrated pest management (IPM) approach is a major component of organic agricultural system which are characterized by selection and integration of biological, cultural, and chemical practices to manage pests that will ensure favorable economic and ecological consequences (Sandler, 2010). This system is based on dynamic principles and biological knowledge which can vary according to types of crops and cultivars, season, climatic and growing conditions. Some plant volatile based pest management strategies are suggested for their potential integration into existing strategies as they have been found to play important role in mediating interactions between plant and other organisms in the surrounding environment (Dudareva et al., 2006).

Host preference of the insects is mediated through color and smell of the plant species and cultivars (Weaver et al., 2009). Knowledge about the plant scent, specifically volatile compounds influencing the natural preference of insects can be utilized by choosing appropriate cultivars and crop species to reduce pest pressure. These differences in volatile profiles leading to direct and indirect defense can also be used by plant breeders to develop novel cultivars with enhanced direct and indirect defense. Polyculture, intercropping, companion planting, trap cropping and other types of cropping systems can be studied and utilized to better understand the influence of crop volatiles on the pests of other crops grown in the vicinity. Use of beneficial organisms like mycorrhizae and endophytes require further study to determine their role in altering volatiles of colonized plants and in turn altering the pest performance in many cases. Study and utilization of natural plant volatile extracts, as well as their synthetic analogues can be used as pesticides, as well as repellent, attractants, confusants to deter, repel, and reduce viability of the pest and to lure their natural enemies (Altieri et al., 1981; Stringer et al., 2008; Tsao and Yu, 2000).

Second, the study on the influence of beneficial microorganisms on the volatile profile of the plant revealed interesting information, which underscores their role in altering the compounds, as well as pest performance. The volatile profiles from the plants from different treatments showed that colonization with either of the fungi *G. intraradices* and *B. bassiana* alone or in combination has potential to alter the volatiles emitted from the plant. The different and very contrary pattern in the volatile profiles before (Table 3.1) and after herbivory (Table 3.2) show that in addition to these organisms, herbivory for overnight significantly decreased the volatile content of the leaves for all the four treatments. Herbivory had significant interaction with AM and Bb for certain compounds, however, there were no three-way interactions for any compound. The alterations in the volatile content among the four treatments without herbivory was significant for β-myrcene, benzyl alcohol, 3-hexenoic acid, and overall total others. The pest bioassays revealed that these fungi can significantly influence the performance of beet armyworm and might be an effective control against them and provide some level of protection (Figure 3.3). The significant difference for the bioassay among the treatments is likely due to several other secondary compounds that were not quantified but might be influenced by these fungi, in addition to the volatile compounds. The volatile compounds, however, seemed to play some role in host preference by the BAW (Figure 3.1).

Third, the study involving root knot nematodes provides information that can be useful in understanding plant response to below ground pests. We found that the plant roots responded significantly to the pest invasion in the roots, which was characterized by significantly higher amount of volatiles from the infested roots (Table 4.3; Table 4.4). Root infestation also altered the leaf volatiles. Above ground herbivory and below ground pest invasion together, as well as individually showed an effect on the root and leaf volatiles (Table 4.1; Table 4.3). Root infestation also induced production of (*Z*)-geraniol, a monoterpene generally not produced otherwise. RKN infestation was also found to adversely affect plant resistance against BAW.

MeSA, a mobile defense molecule was also produced in higher quantities from RKN infested roots. The salicylic acid methyl transferase (*SlSAMT*) gene that regulates the production of MeSA was also upregulated and showed higher gene expression in the roots of susceptible lines infested with RKN.

Overall, the results address the major objectives and the goal of this dissertation research and are able to answer the central question. Production of plant volatile secondary metabolites may be altered by their interaction with above and below ground organisms and the interaction with one organism may also influence plant interaction with other organisms as a result of those altered metabolites. Although, statistically non-significant differences in some cases make some of these findings inconclusive, specific trends provide certain idea about direction of change in the volatile profiles that could be useful.

Perspectives

Despite the progress made in addressing the research questions raised, many new questions arose. The experimental results provide some useful information, however, some aspects of the study could be improved. In terms of analysis, considering the sensitiveness of volatile compounds more could be done to reduce the variability in data. Maintaining similarity in the growing conditions, handling the plant material, extraction and storage of the samples before analysis is important to minimize variability.

The magnitude of the effect in nature is yet to be discussed and more studies need to be conducted to find the applicability and suitability of the beneficial organisms in field conditions for pest management. The factors influenced by colonization by *B. bassiana* and *G. intraradices* other than volatile compounds, and the mechanism involved also needs further study of direct and indirect defense against insect pests.

The effects on volatile compounds can vary with plant and organism type and species and they can act differently in various plant interactions. In most cases, these beneficial organisms are found to induce resistance, there are some cases where they have also increased plant susceptibility, and therefore, the effects cannot be generalized. In addition, results from lab studies are not applicable to field conditions but can certainly provide clues about positive mechanisms in the field situations.

Compounds released from RKN infested roots should be further studied to determine their role in plant direct and indirect defense as some monoterpenoids, including geraniol, have been found to control some economically important nematodes (Rong and Qing, 2000). It would also be interesting to further study the influence of degree of infestation both above- and belowground. Plant allocation of metabolites for their defense below- and above ground also would be interesting to study in depth.

On one hand, the yet unclear mechanisms involved in plant interactions with beneficial organisms and pests need to be studied and be fully understood, on the other hand, potential utilization of volatile compounds also need to be explored. These beneficial organisms can be utilized in transitioning from a conventional to an organic crop production system by introducing inoculum into the transitioning field. These organisms can especially be useful in semi-controlled conditions, such as a greenhouse, where pest pressure is generally less as compared to field conditions.

While research in the area of plant volatile compounds is still in its infancy, an approach involving different disciplines of applied agricultural sciences, such as plant breeding, entomology, crop management and economics and basic molecular biology, biochemistry, and chemistry can facilitate the investigation and subsequently contribute to environmentally friendly agriculture. Where studies at molecular and biochemical levels will help understand the mechanisms involved in plant interactions, studies that combine lab and field conditions will lead us towards the applicability of this knowledge in applied agricultural situations.

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Vita

Gitika Shrivastava was born in Rudrapur, Uttarakhand, India, on November 18th. After completing her early education in Rudrapur, India, she entered the Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India in 1995, where she obtained her B.Sc. Ag and AH degree in 1999 and her M.Sc. Ag degree in 2002 with horticulture major. After graduation, she assisted in managing her parental farm for one and half year, before joining Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow as a Council of Scientific and Industrial research intern in spring of 2004 and worked on *in-vitro* propagation and biochemical characterization of medicinal plants. She joined Dev Sanskrity University as a lecturer, in the department of rural management, in fall, 2004 and taught sustainable agriculture. In June, 2007, she became Ph.D. student at the University of Tennessee, Knoxville, in the department of Plant Science, where she is conducted dissertation research focused on the production and roles of volatile secondary metabolites in tomato plant in the interaction with beneficial and detrimental organisms. She received her PhD in 2011 with major, Plants, Soils, and Insects and concentrations, horticulture.