Soil bacterium *Bacillus subtilis* (GB03) augments plant growth and volatile emissions in *Eruca sativa* (Arugula)

By

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A Thesis

In

Chemistry

Submitted to the Graduate Faculty
of Texas Tech University in
Partial Fulfillment of
The Requirements for
the Degree of

MASTER OF SCIENCE

Approved

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December, 2013

ACKNOWLEDGEMENTS

Firstly, I would like to thank my advisor Dr. Paul W. Paré for his support, advice and patience throughout my time at Texas Tech University. He spent a great deal of time discussing data with me, helping me with problem solving, and teaching me useful techniques in the lab; techniques and knowledge that will provide me with a strong foundation for my future endeavors after graduate school.

I would also like to thank my graduate committee member Dr. David B. Knaff for his support and time here at Texas Tech. I will always appreciate the discussions and stories from him, and also for questions during seminar presentations that challenged me and made me think.

To my lab mates, thank you Mina Aziz, Kalpana Sandun, Ranjith Nadipalli, and Wael Elmasri for the many birthdays celebrated in the lab to countless discussions over research, teaching, and life in general; and for all of the support and help in the lab. I want to specifically thank Mina, who taught me the basic fundamentals of the lab and who has always taken the time to give me advice.

Finally, I would like to thank my family and friends for their love and support. My parents have been so supportive and encouraging; they are always cheering me on and have always wanted me to do my best and achieve my goals. Thank you to my friends (you know who you are) for always being there for me. I would not have been able to do any of this without everyone's support during my graduate studies.

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil microorganisms that colonize roots to stimulate plant growth and development by increasing harvest yields, plant weight, seed germination, and resistance to abiotic stress. A commercially available bacterial strain *Bacillus subtilis* GB03 emits a complex blend of bacterial volatile organic compounds (VOCs) that promote plant growth in Arabidopsis. Here we address the question whether GB03 VOCs induce growth in the agricultural salad crop *Eruca sativa* (Arugula) and how flavor components are influenced by GB03 exposure. *In vitro* plant exposure to GB03 increased fresh and dry plant tissue weight compared to water treated controls. To analyze for flavor components, crushed Arugula plant tissue was analyzed for volatile emissions. Headspace odors were collected onto solid phase micro extraction (SPME) fibers and subsequently analyzed by gas chromatography (GC) flame ionization detection (FID) and/or mass spectroscopy (MS). Total plant volatile emissions increased in Arugula by *ca*. 3 fold with GB03 exposure for 21 days compared with the water treated controls. The 6 peaks with the largest areas as observed by GC–FID were identified as hexanal, nonanal, 4-pentenyl isothiocyanate, cis-3-hexenyl butanoate, cumin aldehyde, and 4-methylthiobutyl isothiocyanate. Since such sulfur components are associated with Arugula flavor, GB03–induction of these volatiles may well maintain flavor at the same time that GB03 induces plant growth.

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CHAPTER I

INTRODUCTION

1.1 *Brassicaceae* family

Brassicaceae is a family classified in the plant order Brassicales according to the Angiosperm Phylogeny Group system (APC system) which contains an assortment of cruciferous plants and crops that are grown worldwide for oil, food and feed (Ahuja, 2010). *Brassicaceae* family comprises of vegetables ranging from cabbage, broccoli, Brussels sprouts, cauliflower, collard greens, kale, mustard, turnip, radish, bok choy to herbaceous vegetables such as watercress and *Eruca sativa* (Arugula). *Brassicaceae* ranks second behind the plant family *Solanaceae* in vegetable production and consumption worldwide (Bennett, 2007). *Arabidopsis thaliana*, a model plant system with a complete sequenced genome, is a member of the *Brassicaceae* family.

1.1.1 Defense mechanisms of Brassicaceae

Defense mechanisms, such as induced chemical and physical defenses are shared between many types of plants, activated in the presence of microbes and pests. In response to microbial infection in plant immune systems, biosynthesis of antimicrobial secondary metabolites can occur (Bednarek, 2012); herbivore damage causes an induction of defensive proteins, production of volatiles to attract predators against herbivores, and toxic secondary metabolites (Mello, 2002). Secondary metabolites which

distinguish *Brassicaceae* from other plant families contain sulfur compounds such as sulfides, glucosinolates and *Brassicaceae* derivatives isothiocyanates, thiocyanates, indole derivatives and nitriles that aid in plant defense (Ahuja, 2010). Insect herbivores reduce crop yields in *Brassicaceae* crop production in agriculture every year. Pests such as the diamondback moth, cabbage looper and moth, armyworm, pollen beetles, aphids attack *Brassicaceae* crops and vary by geographical location (Ahuja, 2010). Glucosinolate concentrations are augmented due to herbivore feeding and damage (Bodnaryk, 1992), and are a recognized class of natural pesticides because of their toxic and repellent effects (Mithen, 1992).

1.1.2 Health benefits of Brassicaceae

Brassicaceae plants have medicinal properties and have been used as an aphrodisiac, for eye infections, for digestion issues, for kidney problems, as a deodorant, as an anti-inflammatory, for blood circulation, and an acne treatment (Yaniv, 1998) because of the phytochemicals produced by plant. *Brassicaceae* phytochemicals produced are carotenoids, flavonoids, vitamin C, and glucosinolates (Barillari, 2005).

Sulfur-containing compounds, such as glucosinolates, indole derivatives, thiocyanates and isothiocyanates, are linked to anticarcinogenic mechanisms (Higdon, 2007). Epidemiological evidence has provided evidence that the consumption of vegetables in the *Brassicaceae* family is associated with the reduced incidence of cancer. It has been proposed that anticancer phytochemicals, such as glucosinolates,

isothiocyanoates and indole derivatives are responsible for this effect (Higdon, 2007, Jin, 2009).

1.1.3 Sulfur in Brassicaceae and flavor

Brassicaceae plants are rich in sulfur and sulfur containing compounds which are used for plant development and defense. The flavor profile of *Brassicaceae* vegetables and onion and garlic are due in part to sulfur compounds (Chin, 1994 & Carson, 1961). Sulfur derivatives give a distinct aroma specifically seen in the *Brassicacaea* family. Cysteine, a product of sulfur assimilation, is in sulfur-rich proteins and glutathione. The synthesized sulfur-containing secondary metabolites produced by *Brassicacaea* include glucosinolates, thiocyanates and indole derivatives (Bednarek, 2012).

1.2 Sulfur containing compounds in *Brassicaceae*

1.2.1 Glucosinolates

Glucosinolates are a diverse group of non-volatile, amino acid-derived sulfur-containing secondary plant metabolites and glycosides that occur naturally in the *Brassicaceae* family (Blažević, 2008 & Bennett, 2002). Glucosinolates are β-thioglucoside *N*-hydroxysulphates containing at least 2 sulfur atoms, one from cysteine and the other from phosphoadenosine phosphosulfate, having a β-D-glucopyranose moiety and a side chain synthesized from methionine, tryptophan or phenylalanine amino acids (Fig. 1.1) (Wittstock, 2002 & Rausch, 2005).

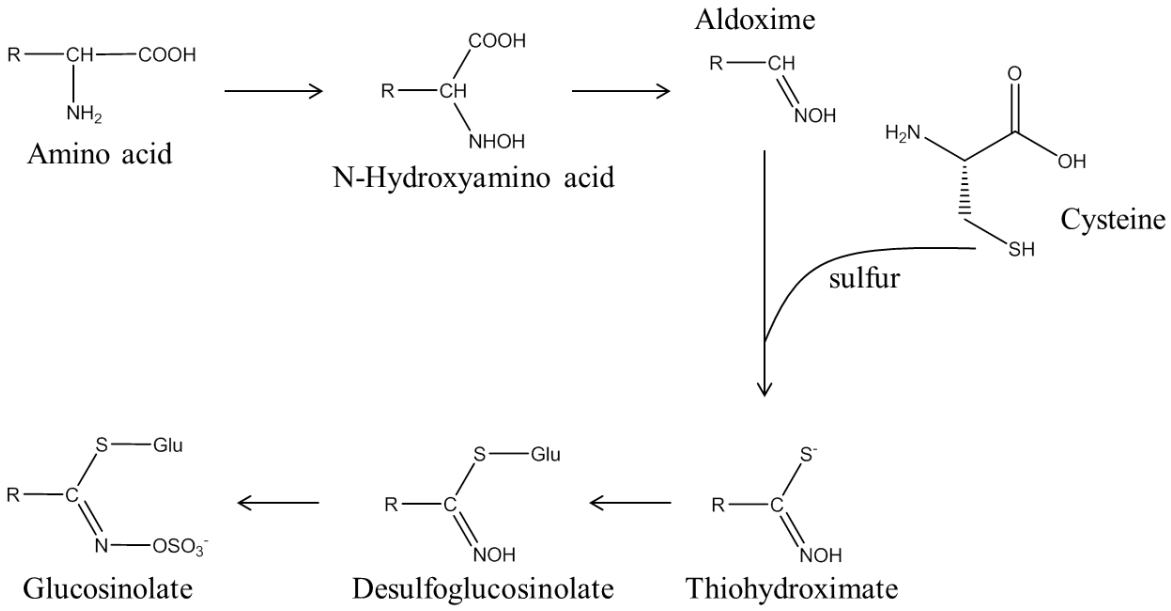


Figure 1.1 Biosynthesis of glucosinolates from an amino acid.

Glucosinolates are precursors to isothiocyanates (Barillari, 2005) and indolylglucosinolates (Bones, 2006). When the plant tissue is disrupted, glucosinolates are hydrolysed by the myrosinase enzyme, which cleaves thio-linked glucose, to form hydrolysis products (Fig. 1.2) such as 4-methylsulfinylbutylisothiocyanate and indole-3-carbinol (Bennett, 2002).

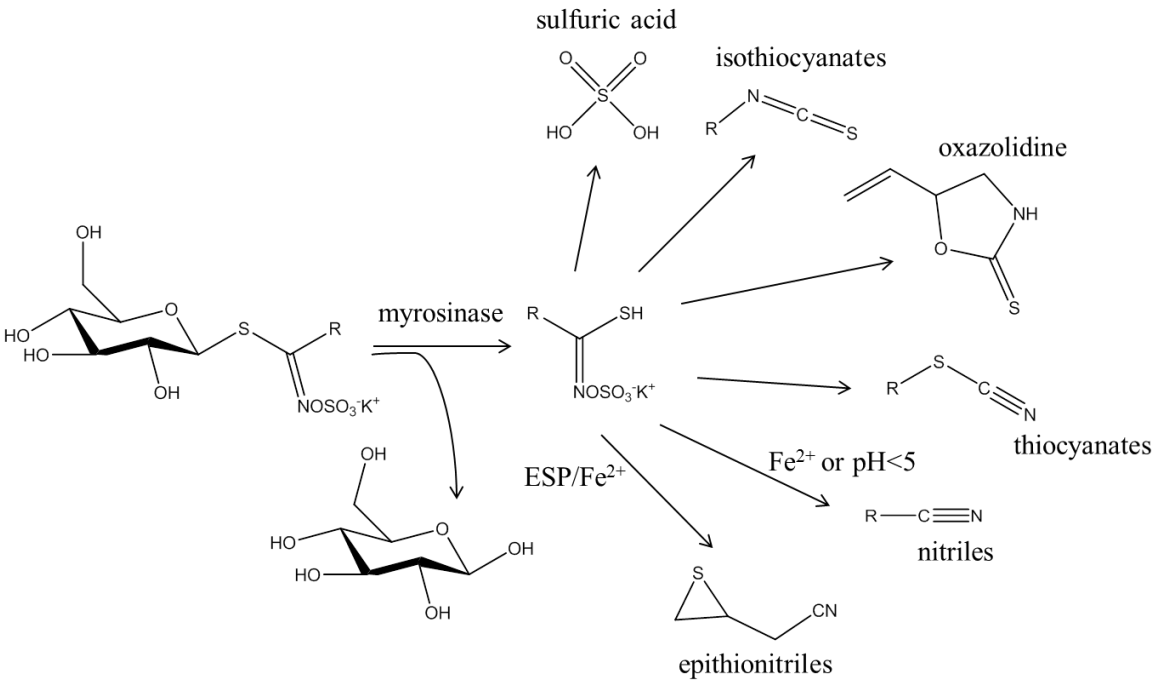


Figure 1.2 Products of myronsinase enzyme hydrolysis (glucosinolate degradation).

1.2.2 Thiocyanates, isothiocyanates and indole derivatives

Thiocyanates, isothiocyanates and indole glucosinolate derivatives (Fig. 1.3) have been isolated and identified in Arugula (Cerny, 1996).

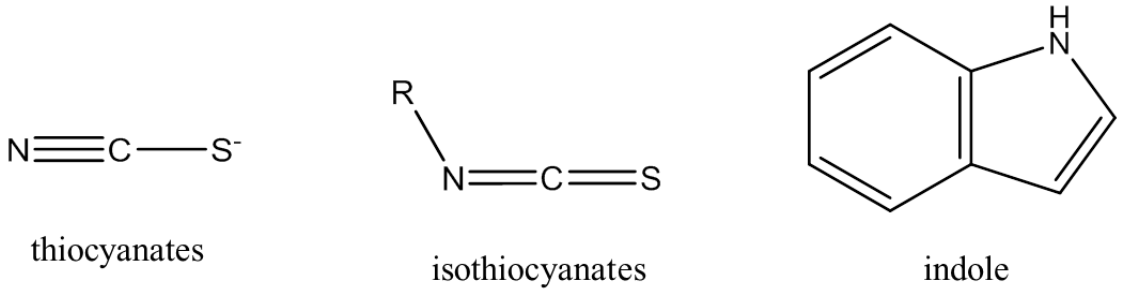


Figure 1.3 Structures of thiocyanate, isothiocyanate and indole glucosinolate derivative.

Thiocyanate formation requires myronsinase enzyme and a thiocyanate forming factor, while hydrogen sulfide suppresses the formation of isothiocyanates (Saarivirti, 1973). The most common compound formed from glucosinolate hydrolysis are isothiocyanates (Bones, 2006), then thiocyanates and nitriles (Fig. 1.4) (Bones, 1996). Isothiocyanates have anti-feedant activity and have been shown to protect *Brassicaceae* species from herbivore damage (Barillari, 2005). Glucosinolate hydrolysis also produces indole derivatives known as indolylglucosinolates (Fig. 1.4) (Bones, 2006).

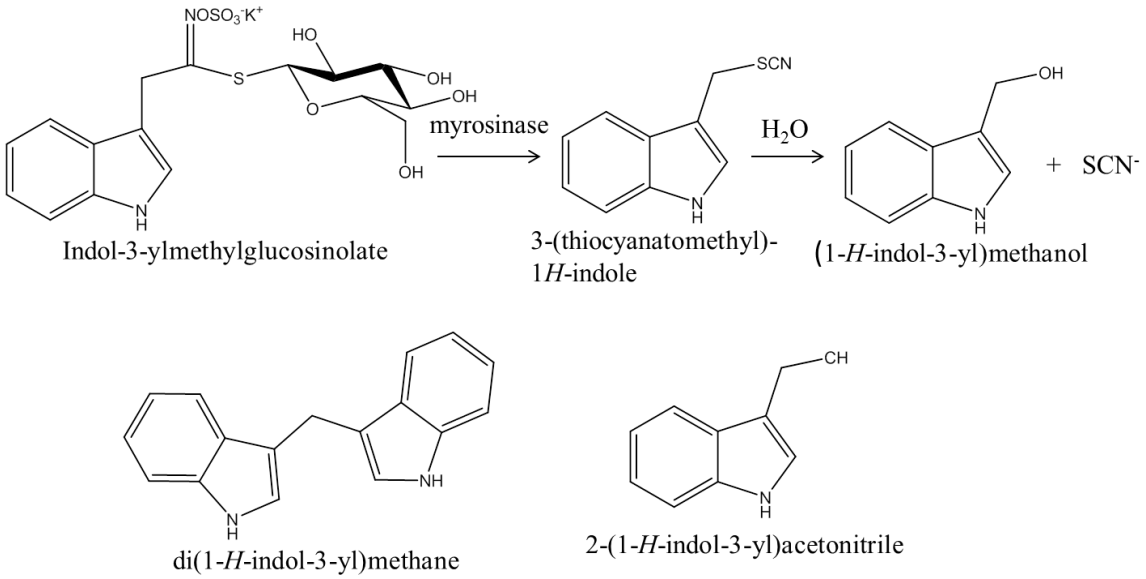


Figure 1.4 Structures of thiocyanates, isothiocyanates and indolylglucostinolate produced in *Brassicaceae*.

1.3 Plant growth promoting rhizobacteria (PGPR)

Plant growth promoting rhizobacteria (PGPR) are naturally occurring soil microorganisms that colonize in the plant-soil interphase to stimulate plant growth and are beneficial for plant development. Reported growth parameters include increased harvest yield, tissue weights, and seed germination (Kloepper, 1991 and 1980). Certain PGPR strains can mimic the synthesis of plant hormones and increase mineral and nitrogen availability; all processes that lead to increased plant growth (Ryu, 2003).

PGPR were first named by Kloepper in 1978 (Kloepper, 1978) and characterized in 1980 (Kloepper, 1980). PGPR have been applied to a diverse selection of agricultural crops to study and observe growth augmentation (Kloepper, 1991). Examples include an 8 to 40% increase in cotton plant weight, a 10 to 25% increase in peanut and rice plants, a 5 to 20% increase in barley plant, a 10 to 50% increase of seed emergence in canola (Kloepper, 1991) and a 80% increase in tuber-treated potato weight by midseason (Kloepper, 1981). Other vegetables crops that have yet to be tested may also benefit from plant-PGPR interactions.

Rhizobacteria are root-colonizing bacteria that form a symbiotic relationship with plants where their metabolites contain beneficial compounds. The colonization of PGPR on roots has been proposed to activate growth by bacterial synthesis of the plant hormones such as indole-3-acetic acid, cytokinin, and gibberellins (Xie, 2009). In the absence of physical contact of PGPR with the plant roots, blends of bacterial volatiles lacking traditional hormones such as auxin and GA can also augment growth promotion

(Xie, 2009). How PGPR influences the production of secondary metabolites and VOCs has not been well characterized yet.

1.3.1 Bacillus subtilis GB03 bacterial VOCs

Bacillus subtilis GB03 is a commercially available bacterial strain (PGPR) that emits a complex blend of bacterial VOCs that augments plant growth (Ryu, 2003 & Farag, 2006). A bouquet of over 25 volatiles has been identified from GB03 that can activate growth mechanisms in plants. In addition, bacterial VOCs have been shown to aid in plant tolerance to abiotic stress such as drought and elevated salt levels in the soil.

1.3.2 GB03 exposed Arabidopsis

Arabidopsis has been used widely in plant research as a model system because of its small genome, short life cycle, availability of large number of mutants, ease of gene transformations, and their translucent roots and young seedlings are suited for light microscopy analysis.

Arabidopsis plants exposed to GB03 bacterial VOCs are known to have increased growth promotion and development. Exposed GB03 Arabidopsis plants also elicit differential expression of roughly 600 transcripts associated with primary and secondary metabolism, stress responses, hormone regulation (Zhang, 2007), and cell wall modifications (Ryu, 2003). Genetic and physiological studies have shown that the GB03 VOCs control auxin signaling to allow for growth promotion (Zhang, 2008). Growth-associated GB03 responses down-stream of transcriptional regulation include tissue

specific redistribution of endogenous auxin (Zhang, 2007), leaf-cell expansion (Zhang, 2007), augmented photosynthetic activity, increased chlorophyll content (Zhang, 2008), and increased iron (the third most limiting nutrient in plants) acquisition (Zhang, 2009). Long-term GB03 exposure with Arabidopsis can result in higher seed counts and can sustain growth promotion and development (Ryu, 2003 & Xie, 2009).

1.4 Plant volatile emissions

Bacteria are not the only emitters of VOCs. Plants release odors which include acids, alcohols, alkanes, alkenes, carbonyls, esters, ethers, isoprenes, terpenes; where isoprenes and monoterpenes are the most prominent compounds, followed by alcohols and carbonyls (Kesselmeier, 1999). Plant emissions are thought to serve many functions. Emitted plant volatiles can aid in defense, communication, and/or protection against stressful conditions (Peñuelas, 2004). Chemical signals via airborne transmissions from infested plants can warn neighboring undamaged plants (Farag, 2006).

Plant emissions can also be regulated by a variety of factors including the developmental stage of the plant and environmental factors such as light, temperature, and air quality (Kesselmeier, 1999). Examples of volatile compounds that are regulated by environmental factors are isoprenes, monoterpenes, and terpenoids. Isoprenoids depend on temperature and light. Temperature changes regulate isoprene and monoterpene synthase activities which in turn regulates the synthesis of isoprenoids (Niinemets, 2004). Isoprene emissions are beneficial because photosynthesis can recover

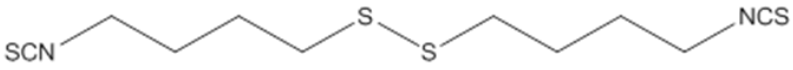
from high-temperature episodes, and are able to survive rapid temperature changes in the environment (Sharkey, 2001). Terpenoids are directly used as defense against herbivores (Gershenzon, 2007). Little is known about how PGPR may regulate plant volatiles. In this study the role of GB03 in *Eruca sativa* (Arugula) volatile emissions will be examined.

1.4.1 VOC Analysis

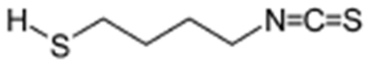
Solvent extractions of plant tissue for collecting VOCs can introduce artifacts into the results. Organic solvents can pull out much more than just volatile material. Headspace analysis provides a sampling method for characterizing airborne volatile components from biological systems (Farag, 2006). Two techniques for the collection of headspace volatiles for plant VOC emission studies are the use of absorbent filters and solid phase micro extraction (SPME). Absorbent filters require a continuous air flow that concentrates volatile metabolites on the filter; the filter can then be washed with organic solvent, spiked with an internal standard and injected onto a GC for chemical analysis. with SPME fibers, headspace volatiles are collected in the absence of air flow. SPME also concentrates the VOC sample over time, however, the sensitivity of SPME is dependent on the coating, compatibility of the specific analyte with the polarity and pore size of the coating (Farag, 2006).

1.5 *Eruca sativa* (Arugula)

Arugula, a member of the *Brassicaceae* family is primarily eaten as a salad or used as a spice because of its aromatic and spicy flavor. It is a perennial herb native to the Mediterranean coast (Stuart, 1988). The plant's brown seeds measure between 1.5 – 2.0 mm in length, basal leaves are 10 – 25 cm long, and the plant can be up to 80 centimeters in height (Miyazawa, 2002). Because of Arugula's uniquely pungent flavor, cultivation has spread around the globe (Jin, 2009). For example, in Italy, where it is known as "rucola," is used with garlic and oil to season spaghetti because of its sesame seed scent (Miyazawa, 2002). In Asia, Arugula is an important source of oil seeds in addition to being a vegetable. Specifically in Japan, Arugula is known as "kibanasuzushiro," which has been increasing in popularity and economic potential since the 1990s because of its distinct flavor in spiciness, and nutritional value (Miyazawa, 2002). Arugula is also a popular salad crop for farmers because it has a short growing season of 45 – 60 days (Kim, 2004). Arugula's horseradish scent and sharp peppery taste is associated with glucosinolates and isothiocyanates (Bennett, 2002). 2 of the most produced compounds in Arugula are 1,2-bis(4-isothiocyanatobutyl)disulfane and 4-mercaptobutyl isothiocyanate (Fig 1.5), both responsible as flavor determinants in Arugula (Bennett, 2002 & Bones, 2006).



1,2-bis(4-isothiocyanatobutyl)disulfane



4-mercaptobutyl isothiocyanate

Figure 1.5 Flavor compounds in Arugula.

1.6 Objective of the project

In previous studies, *Bacillus subtilis* GB03 promotes plant growth in Arabidopsis (Ryu, 2003), but GB03 exposure has yet to be tested on Arugula. In this project, Arugula will be exposed to *Bacillus subtilis* GB03 to investigate plant growth. Also, the plant volatile emissions will be analyzed to determine whether GB03 has an effect on Arugula emissions. Studies will be analyzing GB03 effects on Arugula, and if there is a correlation between growth and plant emissions.

CHAPTER II

MATERIALS AND METHODS

2.1 Plant Materials and Treatments

2.1.1 Eruca sativa (Arugula)

Eruca sativa (Arugula) seeds were surface sterilized with 70% (v/v) ethanol for 1 minute, then with 1% (v/v) sodium hypochlorite soaking for 20 minutes and rinsed (4-6x) with sterile double-distilled water (DDW) in a 1.5 mL microcentrifuge tube. The seeds were suspended in sterile DDW and were vernalized for 2 days at 4ºC in the absence of light before planting.

2.1.2 Growth Chambers

Eruca sativa (Brassicaceae) seeds were planted 1 per closed sterilized chamber in Magenta boxes (75 mm x 75 mm x 97 mm; size GA-7, Sigma-Aldrich), where the two chamber halves were coupled together as pairs by a plastic collar (75 mm x 75 mm x 20 mm); each contained *ca.* 100 mL of half-strength Murashige and Skoog (MS) media containing 0.8% (w/v) agar and 1.5% (w/v) sucrose. A glass vial (4 dr.) also containing MS media was placed in the chamber to provide a restricted yet open to the magenta box headspace area within the chamber for the bacterial treatment. This provided an area for the bacterial volatiles to interact with the plant without physically touching the plant. Magenta boxes were all grown in a growth room with metal halide and high-pressure

sodium lamps with a total light intensity of 200 µmol photons m^{-2} s^{-1}, set to a 14-/10-h light/dark cycle respectively and with a temperature of 21 ± 4ºC and relative humidity 40 ± 10%.

2.1.3 Bacterial Cultures

B. subtilis GB03 was streaked onto LB agar plates and incubated in the absence of light at 30ºC for 24 – 48 hours. Bacteria cells were harvested from the plates into LB broth to yield 10^9 CFU mL^{-1} as determined by serial dilutions and optical density measurements using a UV-VIS spectrophometer (OD_{600}). Bacteria stocks were stored with 50% sterile glycerol (v/v) at -80ºC.

2.1.4 Bacterial Treatments

Two days after seed germination, treatments were added to the plants. A bacterial suspension culture of the GB03 and sterile DDW (50 µL) were added into the glass vials containing MS media inside the Magenta boxes for bacterial treated and control conditions, respectively. Plants were grown for 21 days total after treatment was added for gas chromatography analysis, and 2, 6, 11, 15, 18, 21 days for fresh and dry weight determinations.

2.2 Fresh and Dry Weight Analysis

Seven, fourteen, twenty-one, and twenty-eight day old plants were used for fresh and dry weight analysis. After fresh weight measurements, these samples were placed in an oven

at 70 ºC for them to dry completely. After two days, samples were measured for their dry weights.

2.3 Gas Chromatography Analysis

2.3.1 Solid-Phase Micro Extraction (SPME) Analysis

Plant volatiles were collected and measured using stable flex divnylbenzene/carboxen/PDMS (DCP, 2 cm – 50/30 µm, Supelco) commercially available SPME fibers. Sealed glass vials (8 dr.) contained 1 g of crushed plant material and an internal standard (5 µL of 800 ng μL^{-1} of octanal) on a 1 cm^2 filter paper disk were placed in a water bath heated at 30 ºC ± 2 ºC to keep the temperature constant. The SPME fiber was inserted above the plant material and the headspace volatiles were collected for 1 hour.

2.3.2 GC–FID Parameters and Kovats Index

SPME fibers were desorbed and pre-conditioned at 250 ºC for 5 minutes in the injection port of an HP 5890A GC–FID (Hewlett-Packard, Palo Alto, CA) before collecting plant volatiles. After the headspace volatiles were collected for 1 hour, the SPME fiber was inserted for 1 minute into the GC and the compounds were separated on a DB5 column (J&W Scientific, Folson, CA) column (60 m, 0.25 mm i.d., 0.25 µm film thickness). The GC–FID method was 25 minutes, and the injection port was in splitless mode with a constant He flow of 1.0 ml/min. The initial oven temperature began at 40 ºC, held for 3

minutes, ramped at 12 ºC/min to 230 ºC and held for 3 minutes, then ramped at 20 ºC/min to 250 ºC and remained for 3 minutes. The injector temperature was 220 ºC, and the detector (FID) temperature was 250 ºC.

Kovats retention indices of major compounds were calculated using the equation

$$I = 100 \times \left[n + (N - n) \frac{t_{r(unknown)} - t_{r(n)}}{t_{r(N)} - t_{r(n)}} \right]$$

where I = Kovats retention index, n = number of carbon atoms in the smaller n-alkane, N = the number of carbon atoms in the larger n-alkane, t_r = the retention time; an alkane mixture of C_8 – C_{20} (commercially available through Sigma) was used to determine these values for the calculation of the Kovats indices. Kovats indices were compared with literature values (Jirovetz, 2002).

2.3.3 GC–MS Parameters and Analysis

SPME fibers were desorbed and pre-conditioned at 250 ºC for 5 minutes in the injection port of an HP 5890A GC–FID (Hewlett-Packard, Palo Alto, CA). A Trace GC Ultru GC with (Triplus HS Autosampler) and ISQ quadrupole MS was used for the GC–MS studies. The GC–MS method was the same as the GC–FID method stated previously, but the column was a TR-FAME (30 m 0.25 mm id. 0.25 um film thickness). The injection port was in splitless mode with a constant He flow of 1.0 ml/min. The mass spectrometer had an electron ionization mode at 30 eV, and both the ionization temperature and transfer line temperature were at 220 ºC.

Identification of each chemical constituent was concluded by comparison of its Kovats indices which were calculated using retentions times (GC–FID) and mass spectra with an internal standard from GC–MS.

2.4 Salt Experiment

For the salt tolerance experiment (future studies), all materials and methods were the same except for the additional 0.58% (w/v) sodium chloride that was added to the media in the growth chambers before the inoculation of the seeds and GB03 and water treatments.

CHAPTER III

RESULTS

3.1 PGPR augments growth in Arugula

Growth promotion in *E. sativa* associated with GB03 bacterial volatiles were tested in the laboratory. To expose plants only to GB03 VOCs, the bacteria were cultured in glass vials within Magenta boxes in which the Arugula was grown. GB03 exposed plants exhibited a significant increase in areal and root growth compared to the water controls (Fig. 3.1, A). Fresh and dry weight measures were performed at 2, 6, 11, 15, 18, and 21 days after bacterial exposure (Fig. 3.1, B & C). At 11 days, GB03 exposed plants were statistically significantly larger with respect to both fresh and dry weight and GB03 exposed plants were more than doubled at 15 days.

3.2 PGPR increases volatile emissions in Arugula

Volatile emissions of *E. sativa* were determined after 21 days of bacterial treatment of the plant. GB03 augmented the volatile organic compounds emitted by the plant compared to the water treated controlas (Fig. 3.2). Quantification of amounts of compounds emitted was determined using octanal as an internal standard.

Compounds [1] hexanal, [2] nonanal, [3] 4-pentenyl isothiocyanate, and [5] cumin aldehyde, and the combined total of the 6 most prominent compounds showed significant differences between the GB03 treated plants and the water treated controls.

GB03 more than doubled the volatiles emitted from the bacterial treated plants in compounds [1] hexanal, [2] nonanal, [3] 4-pentenyl isothiocyanate, and [5] cumin aldehyde; and more than tripled the total amount of the 6 most prominent compounds emitted from the plant volatile emissions.

3.3 Volatile organic compounds emitted by Arugula

The volatile organic compounds emitted by *E. sativa* were determined using retention times (Kovats index) from GC–FID with correlations of mass spectra using GC–MS. The 6 most prominent peaks were determined as [1] hexanal, [2] nonanal, [3] 4-pentenyl isothiocyanate, [4] cis-3-hexenyl butanoate, [5] cumin aldehyde, [6] 4-methylthiobutyl isothiocyanate, respectively in the order of lower to higher retention times (Fig. 3.3 & Fig. 3.4).

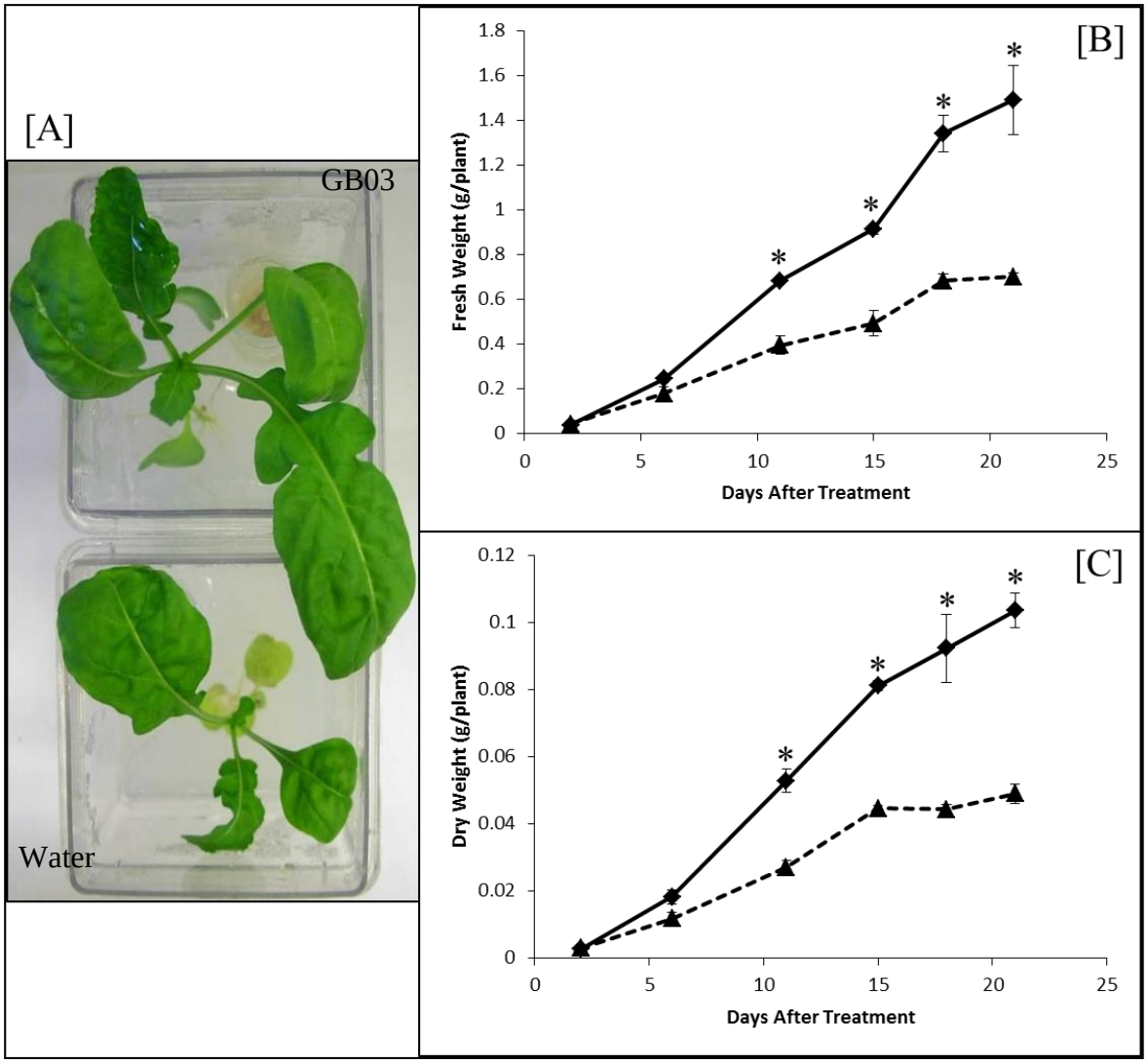


Figure 3.1 *Eruca sativa* growth promotion was augmented by GB03 exposure. *E. sativa* weights were taken after 21 days after exposure [A]. Quantification of growth was determined at 2, 6, 11, 15, 18, and 21 days after treatment for fresh weights [B] and dry

weights [C]. An asterisk (*) represents a statistical difference between GB03 and water using t-test analysis with a p-value ≤ 0.05, mean $\pm$ SD, n = 3.

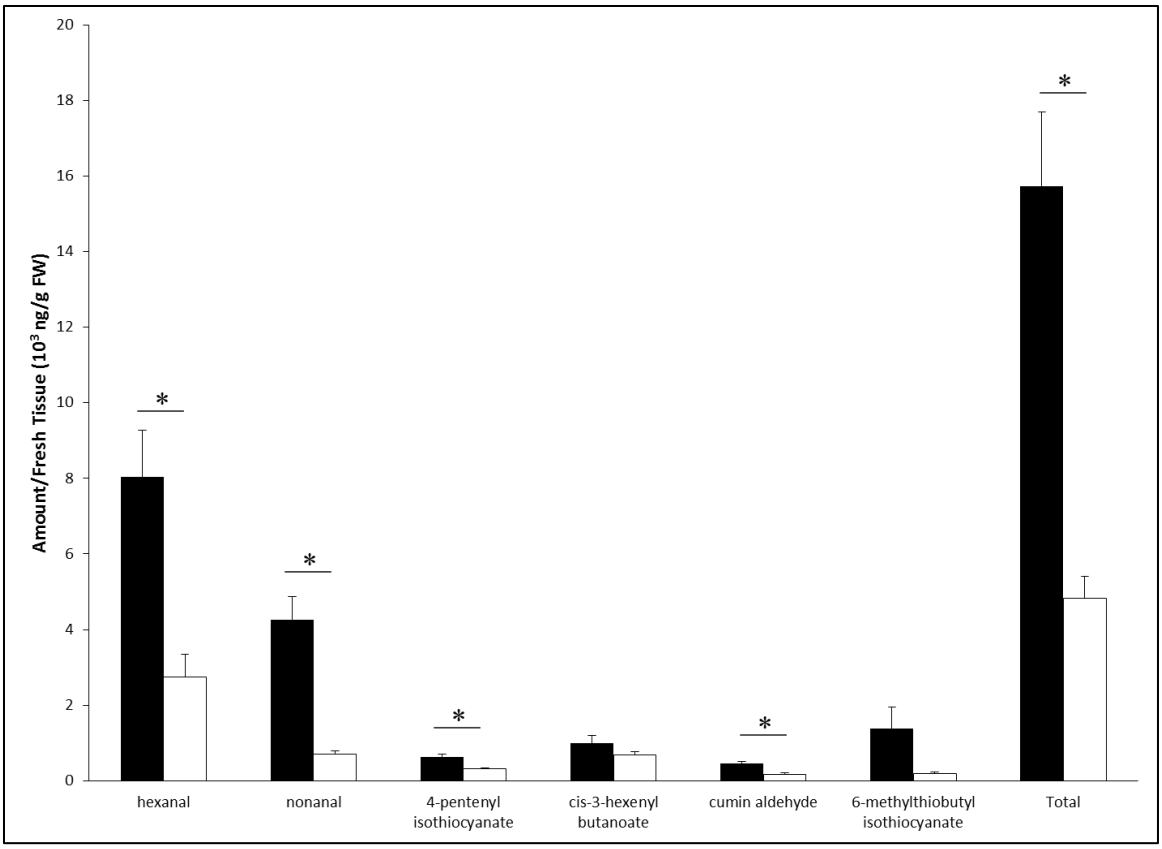


Figure 3.2 The plant volatile emissions of *E. sativa* were significantly increased by GB03 with 21 days of exposure. An asterisk (*) represents a statistical difference between GB03 and water using t-test analysis with a p-value ≤ 0.05, mean $\pm$ SD, n = 4.

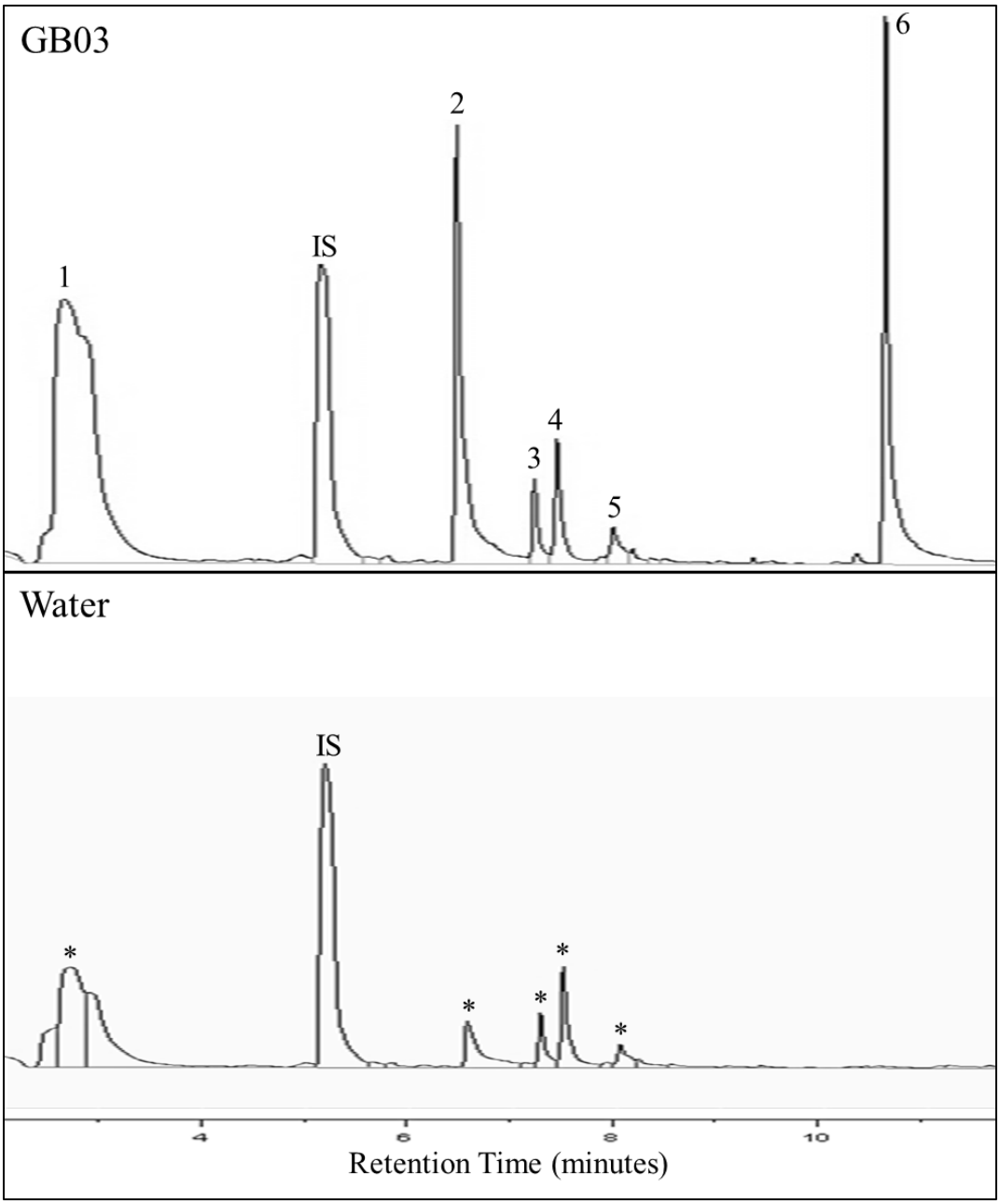


Figure 3.3 Chromatographic profiles of volatile emissions from bacterial treated *E. sativa* were augmented compared to the water treated plants. Compounds positively

identified include [1] hexanal, [2] nonanal, [3] 4-pentenyl isothiocyanate, [4] cis-3-hexenyl butanoate, [5] cumin aldehyde, [6] 4-methylthiobutyl isothiocyanate; octanal was added as an internal standard (IS). Asterisks in the water chromatogram designate compounds that align with numbered peaks in the GB03 chromatogram.

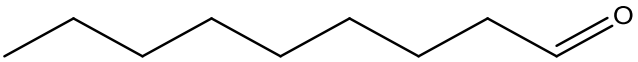
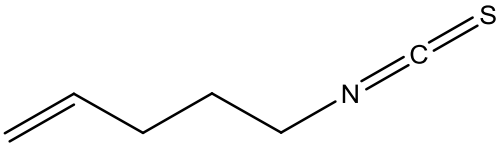
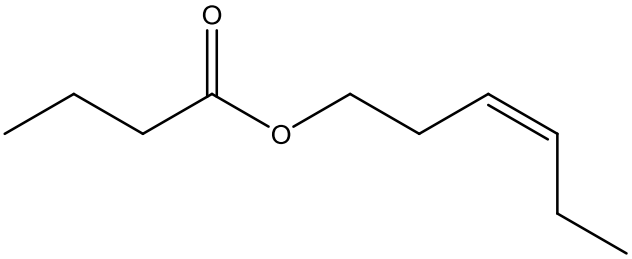
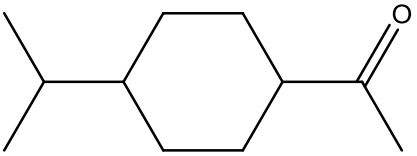
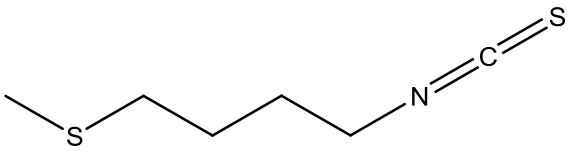
Compound	Structure	Kovats Indices
hexanal[a]		746
nonanal[a]		1087
4-pentenyl isothiocyanate[a]		1155
cis-3-hexenyl butanoate[a]		1174
cumin aldehyde[a]		1223
4-methylthiobutyl isothiocyanate[a,b]		1459

Figure 3.4 The 6 compounds of the volatile emissions of *Eruca sativa* were identified using [a]retention time (Kovats index) from GC–FID, in correlation with [b]mass spectra from GC–MS.

CHAPTER IV

DISCUSSION

PGPR are naturally occurring soil microorganisms that colonize roots to stimulate plant growth and development by increasing harvest yields, plant weight, seed germination, and resistance to abiotic stress. *Bacillus subtilis* GB03 emits a complex blend of bacterial VOCs that promote plant growth in Arabidopsis and sweet basil.

In the laboratory, agricultural salad crop Arugula was exposed to GB03 to determine if GB03 would have similar effects on arugula as it did with Arabidopsis. Plant VOCs were also studied to determine if GB03 influenced plant emission amounts because of their important with the flavor profile of Arugula. GB03 increased both growth promotion and plant volatile emissions in Arugula. Plant exposure to GB03 increased both fresh and dry plant tissue weight compared to water treated controls. Headspace odors were also analyzed, and the total plant volatile emissions increased in arugula by *ca*. 3 fold with GB03 exposure for 21 days compared with the water treated controls.

Arugula had an increase in leaf surface area and in areal and root growth with GB03 compared to water controls (Fig. 3.1, A). Fresh and dry weights of GB03 exposed plants were increased by *ca*. 2 fold after 11 days, and increased by *ca*. 2 to 3 fold after 15 days (Fig. 3.1 B & C). Arabidopsis plants exposed to GB03 are reported to have an increase in growth (Ryu, 2003). GB03 exposed Arabidopsis had an increase of *ca*. 3 fold

in the leaf surface area (Ryu, 2003). PGPR growth has also been reported in agricultural crops such as canola, cotton, rice, and potatoes (Kloepper, 1981 & 1991). This increase in growth development of Arugula can provide a natural way to potentially deliver larger production yields of Arugula crops to consumers.

GB03 increases volatile emissions with 21 days of GB03 exposure. Arugula emissions increased by *ca*. 3 fold. In a previous study, *Ocimum basilicum* L. (sweet basil) was exposed to GB03 and plant growth was augmented *ca*. 2 fold after 14 days, and sweet basil volatiles increased *ca.* 2 fold (Banchio, 2009). Arugula is consumed as a vegetable and spice because of its unique flavor and aroma profile caused by glucosinolate and isothiocyanate compounds (Bennett, 2002). The value of understanding the volatile profile of Arugula and its production can be beneficial because of its connection to its flavor.

The total volatile emissions identified of the 6 compounds with the largest areas ([1] hexanal, [2] nonanal, [3] 4-pentenyl isothiocyanate, [4] cis-3-hexenyl butanoate, [5] cumin aldehyde, [6] 4-methylthiobutyl isothiocyanate) in Arugula (Fig. 3.4) increased *ca*. 3 fold in GB03 plants compared with water controls (Fig. 3.2). Sweet basil volatile emissions increased by *ca.* 2 fold, and are utilized for flavor enhancement in foods, as well as a natural defense mechanism against herbivore pests (Banchio, 2009). In Arugula, the unique flavor is thought to be related to the production of glucosinolate and isothiocyanate compounds (Bennett, 2002).

Two of the compounds identified in Arugula were isothiocyanates, specifically [3] 4-pentenyl isothiocyanate and [6] 4-methylthiobutyl isothiocyanate (Fig. 3.4). 4-pentenyl isothiocyanate and 4-methylthiobutyl isothiocyanate compounds increased in amounts per fresh tissue in GB03 exposed Arugula; and there was a *ca.* 2 fold increase in 4-pentenyl isothiocyanate (Fig. 3.2). Isothiocyanates are the products of glucosinolate hydrolysis, formed by chewing, cutting, and/or processing of the plant (Barillari, 2005). Isothiocyanates and glucosinolate derviatives are responsible for the protection of the plant when it is being threatened (Higdon, 2007). Since glucosinolates and isothiocyanates are linked to the unique aroma and flavor profile of Arugula, the increase in Arugula emissions, and specifically the increase in isothiocyanate compounds provides evidence that the flavor of Arugula is not compromised when growth promotion is augmented. GB03 enhances the flavors and possibly also the defense mechanism of the plant as well.

In this project, GB03 is shown to increase growth and volatile emissions in Arugula. The volatile emission is coupled with the enhancement of sulfur metabolites associated with the distinct flavors of Arugula. Increasing plant growth without compromising flavor should be of interest to the production of other fruits and vegetables within the *Brassicaceae* family and beyond.

CHAPTER V

CONCLUSIONS & FUTURE STUDIES

Soil salinity has been an agricultural issue because approximately 20% of irrigated land in the world is salt contaminated (Frommer, 1999). Elevated sodium content has decreased plant growth which affects agricultural productivity (Zhang, 2008). GB03 has been used to study the relationship between plants that are under salt stress to determine if GB03 can augment growth promotion in these plants as well.

In future laboratory experiments, salt tolerance studies will be conducted on Arugula to determine if GB03 will cause an increase in growth promotion in salt stressed plants compared to the water treated controls. These results will also be compared to the Arugula plants grown under no salt stress. Also, plant volatile emissions will be analyzed to determine if they are augmented due to the relationship between GB03 and salt stressed plants. Since salt tolerance is an issue in agricultural crops, these studies can help determine if Arugula crops can have increased yields due to GB03 treatments and better flavor components.

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