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Colonization by arbuscular mycorrhizal and endophytic fungi enhanced terpene production in tomato plants and their defense against a herbivorous insect

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Abstract Terpenoids serve as an important form of chemical defense for plants. A greenhouse study was conducted to investigate the effects of two types of beneficial fungi on the accumulation of terpenoids in tomato plants and on defense against herbivorous insects. Control tomato plants without any fungal inoculation constitutively made monoterpenes and sesquiterpenes. Inoculation by *Rhizophagus intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & A. Schüßler, an arbuscular mycorrhizal fungus, and *Beauveria bassiana* (Bals.-Criv.) Vuill., an endophytic entomopathogenic fungus, individually or in combination, led to enhanced levels of monoterpenes and sesquiterpenes, which included new monoterpenes not found in the control plants. Herbivore feeding assays using beet armyworm (*Spodoptera exigua* Hübner) were performed to compare the levels of defense in tomato plants with or without fungal inoculation. Beet armyworm larvae fed on tomato plants inoculated by either or both types of fungi were found to gain significantly less weight than those fed on control non-inoculated plants. This suggests that fungus-inoculated tomato plants had a stronger defense response against beet armyworm than control plants, which may be partly attributed to the difference in the levels of terpenoids.

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1 Introduction

Chemical pesticides have played critical roles in reducing crop losses from herbivorous insects, but environmental and health concerns surrounding their use has led to extensive exploration of alternative strategies for insect pest management (Onstad 2014; Wezel et al. 2014). One promising strategy relies on bolstering the innate defense system of plants to prevent and/or resist insect pest attack (Lucas 1999; Shrivastava et al. 2010). Plants produce an enormous variety of secondary metabolites (Zhao et al. 2013), many of which are toxic to insect pests and therefore can function as direct defense by affecting the growth and development of the pest (Chen et al. 2009b; Chen et al. 2012). Some secondary metabolites function in indirect plant defense by affecting the recruitment of natural enemies of insect pests (Yuan et al. 2008). The production of plant secondary metabolites can be modulated by a number of biotic and abiotic factors (Brunetti et al. 2013). Mycorrhizal and endophytic fungi, often demonstrated to benefit plant health and vigor (Smith and Read 2008), can modulate secondary metabolism in plants (Walker et al. 2012) and thus potentially fortify both direct and indirect plant defense systems (Jung et al. 2012; Borowicz 2013).

Arbuscular mycorrhizal fungi are among the most common fungi in the rhizosphere, developing symbiotic root associations with almost 85 % of plant families (Barea et al. 2005). Mycorrhizal symbioses are found in every terrestrial ecosystem and can exert controlling influences on ecological and agricultural processes, such as carbon and nutrient cycling

(Phillips et al. 2013) and water relations (Ruiz-Lozano et al. 2012), with substantial consequences for plant growth, crop yield, land revegetation and plant community structure (Koricheva et al. 2009; Dickie et al. 2014). In addition to promoting abiotic stress resistance in the host plant, e.g., drought (Augé 2001), salinity (Ruiz-Lozano et al. 2012), heavy metals (Forgy 2012), and temperature extremes (Zhu et al. 2010; Maya and Matsubara 2013), mycorrhizal fungi are also gaining recognition for their bioprotective functions against both fungi and insects (Pozo et al. 2002; Vannette and Hunter 2009). While the mechanisms underlying such enhanced defense are still being investigated, inoculation with mycorrhizal fungi is known to induce many morphological, physiological, and biochemical changes in plants, including the production of secondary metabolites. For example, mycorrhizal colonization has been shown to enhance the production of flavonoids (Harrison and Dixon 1993), triterpenoids (Akiyama and Hayashi 2002), apocarotenoids (Fester et al. 2002), and jasmonate (Hause et al. 2007) in various plants. Involvement of the jasmonic acid pathway has been implicated in the mycorrhiza-enhanced priming of systemic defense responses in tomato leaves upon attack by a chewing caterpillar, *Helicoverpa arimigera* (Song et al. 2013).

Endophytes are microorganisms that form inconspicuous infections within healthy plants (Ownley et al. 2010). In some cases, endophytes (e.g., *Fusarium* spp.) are closely related to plant pathogens but do not cause disease (Aimé et al. 2013). The majority of plants form relationships with endophytic fungi (Saikkonen et al. 1998). In mutualistic associations, while providing carbohydrate energy resources to the fungus, the plant harnesses a number of benefits from the fungus that include increased nutrient uptake (Faeth and Fagan 2002) and in some cases protection from insect pests (as reviewed in Ownley and Griffin 2012). It has been proposed that endophytic fungi protect host plants from their enemies (Carroll 1988) with additional chemical defenses based on secondary metabolites (Hartley and Gange 2009). Some defense secondary metabolites are produced by endophytic fungi (Vey et al. 2001). Other studies suggest that the presence of endophytic fungi affects the biosynthesis of secondary metabolites in the host plant (Sivasundaram et al. 2008; Gómez-Vidal et al. 2009).

Our objectives were to determine how inoculation by an arbuscular mycorrhizal symbiont *Rhizophagus intraradices*, formerly *Glomus intraradices* and recently reclassified (Redecker et al. 2013) and an endophytic fungus *Beauveria bassiana*, alone or in combination, affect the production of secondary metabolites in the host plant. We selected tomato (*Solanum lycopersicum*, Mill cv. Castlemart; Family: Solanaceae) for study because it is an important vegetable crop worldwide whose production is often hindered by insect pests (Bergougnoux 2014; Megido et al. 2014). Tomato tissues produce a variety of secondary metabolites (Spyropoulou et al. 2014), including terpenes (Kang et al. 2014) and a clear demonstration of the influence of beneficial fungi on these metabolites and on plant defense may add new tools for pest management in tomato production. *R. intraradices* is one of the most widely distributed and investigated arbuscular mycorrhizal fungi (Borowicz 2013; Augé et al. 2014). *B. bassiana* has been shown to have entomopathogenic properties. As an endophytic fungus, it provides protection to the host plant against plant diseases (Ownley et al. 2008). Each fungus shows promise for inducing direct and indirect defenses against herbivores (Hare and Andreadis 1983; Koricheva et al. 2009; Migiro et al. 2010; Quesada-Moraga, et al. 2009; Jung et al. 2012). We were particularly interested in the effect of fungal inoculation on the production of terpenoids, the largest class of secondary metabolites made by plants, which have various biological functions, including important functions in defense (Reid and Purcell 2011; Ahern and Whitney 2014). In addition to chemical profiling, we also examined the effect of fungal inoculation on the growth of beet armyworm (BAW) (*Spodoptera exigua*), which causes considerable economic losses annually in a number of important crops, including tomato (Brewer et al. 1990).

2 Materials and methods

2.1 Treatments and experimental design

The experimental design included a control (C) and three treatments: *Rhizophagus intraradices* (AM), *Beauveria bassiana* (Bb), and *R. intraradices* + *B. bassiana* (AM + Bb), in 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. We tested two hypotheses: (1) Inoculation with *R. intraradices* and *B. bassiana* would each increase the production of the chief terpenoids in tomato leaves, and dual inoculation would increase terpenoid production more than inoculation with either fungus alone; (2) Inoculation with *R. intraradices* and *B. bassiana* would each decrease the herbivory of beet armyworm on tomato leaves, and dual inoculation would decrease herbivory more than inoculation with either fungus alone.

2.2 Host plant, microorganisms and insect culture

Tomato was selected as the host plant and seeds were generously provided by Dr. G. A. Howe, Michigan State University, East Lansing. *Rhizophagus intraradices* cultures were originally obtained from the International Culture Collection of Vesicular Arbuscular Mycorrhizae Fungi (INVAM;

Morgantown, WV) and pot cultures were established on *Sorghum bicolor* cv. DK39Y roots in pure calcined montmorillonite clay (Turface, Profile Products LLC, Buffalo Grove, IL). After 8 weeks of growing sorghum with mycorrhizal culture, sorghum roots were harvested just below the crown. Planting medium (Turface) with sorghum roots was evenly mixed with additional Turface and placed in pots. To control for effects of soil microflora other than AM, filtrate solutions from both AM and non-mycorrhizal roots (NM) were prepared separately that contained other microflora, but excluded fungal propagules. Culture media (AM and NM) and roots were mixed with deionized water, each culture separately at the rate of one pot culture per 750 mL water. The suspensions were filtered through a 25-μm filter twice, and the resultant filtrates were used to inoculate all the experimental pots.

Two types of pots were prepared, one group contained calcined montmorillonite clay with chopped sorghum roots colonized by *R. intraradices*, and another group contained pure calcined montmorillonite clay (control). For the control and *B. bassiana* treatments, both untreated and Bb-coated seeds were sown in pure calcined montmorillonite clay planting medium. Whereas, for *R. intraradices* and *R. intraradices* + *B. bassiana* treatments, untreated and Bb-coated seeds were sown in pots with mycorrhizal culture. Filtrates from both AM and control (no mycorrhizae) cultures were prepared and applied in the same way as mentioned earlier. Tomato seeds were coated with 1.15×10^5 cfu/seed of *B. bassiana* strain 11–98 (Ownley et al. 2008).

Based on the preliminary study that non-mycorrhizal tomato plants grew less vigorously than mycorrhizal plants under the same phosphorus regime, phosphorus amendments for control tomato plants were doubled to avoid differences in plant size due to treatment. At the time of chemical profiling, when plants were 8-weeks-old, control plants were similar in size to mycorrhizal plants.

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, 24 °C day/21 °C night temperatures, and 60 % RH. 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) (R.J. Peters Inc., Allentown, PA) was applied to both treatments weekly at the rate of 150 ppm.

Beet armyworm larvae were used for the herbivory assays. Eggs (Benzon Research Inc, Carlisle, PA) were kept in 37.5-mL cups in darkness at 28 °C to hatch. The cups contained approximately 15 mL pinto bean based artificial diet (Benzon Research Inc., Carlisle, PA) as a food source for the larvae.

2.3 Test of colonization for mycorrhizae

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) and Gualandi et al. (2014). Briefly, fresh lateral root samples (100 mg) were taken from each pot, washed thoroughly to remove debris and placed into plastic histology cassettes. The cassettes were submerged in a 10 % KOH solution (Fisher Scientific, Waltham, MA) in a beaker and brought to simmer for 5 min. The KOH was drained, the roots rinsed three times with deionized water, and a 2 % hydrochloric acid solution added to the beaker; samples were kept at room temperature for 1.5 h. The HCl solution was drained and samples were stained with 0.05 % Trypan Blue solution (Mallinckrodt, Inc., Hazelwood, MO) for 1 h. Samples were destained in lactoglycerol solution (1:1:1 lactic acid:glycerol:water by volume) for at least 48 h. Roots from each sample were mounted individually with lactoglycerol, covered with a cover slide and viewed with a light microscope (Fisher Scientific) at 20× power. Percent colonization for each sample was determined using the gridline-intersect method described by McGonigle et al. (1990).

2.4 Test of colonization for *Beauveria bassina*

Detection of *B. bassiana* was done with polymerase chain reaction amplification of the nucleotide sequences of the nuclear ribosomal DNA internal transcribed spacer (ITS) regions of genomic DNA with *B. bassiana*-specific primers (Griffin 2007); genomic DNA was extracted from 12 randomly selected plants grown from seed treated with Bb (Bb and AM + Bb treatments). 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 of each primer (5 μl of 20 μM), 60–300 ng of 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, 94 °C for 1 min, 59 °C for 1 min, 40 cycles of 72 °C for 1 min, with a final extension of 72 °C for 3 min, conducted with a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany). PCR products were visualized on a 1.5 % agarose gel stained with SybrSafe (Invitrogen, Eugene, OR); the resultant single band from plants grown from seed treated with Bb was compared with the single band from purified DNA of *B. bassiana* 11–98. To further confirm identification, PCR products from selected treatments were sequenced, and

sequences had 100 % identity with the ITS sequence of *B. bassiana* 11–98.

2.5 Organic extraction and analysis

Six plants of similar size per treatment were used for volatile extractions according to a standard protocol (Chen et al. 2009a). In short, the second leaf from the top was detached from 8-week-old plants and immediately ground to powder with liquid nitrogen. A 1-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 with continuous shaking on an orbit shaker (Lab-line instruments Inc, Melrose Park, IL) at room temperature for 2.5 h.

Compounds were analyzed with a 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 $mL^{\bullet}min^{-1}$), a splitless injection (injection injector temperature 250 °C) was used, and a temperature gradient of 5 $°C^{\bullet}min^{-1}$ from 40 °C (3 min hold) to 240 °C was applied. The coupled mass spectrometer was a Shimadzu QP5050A quadrupole mass selective detector. Products were identified using the National Institute of Standards and Technology (NIST) mass spectra database and authentic standards. Quantification was performed as reported previously by comparing samples with the peak area of the internal standard (Chen et al. 2009a). Two technical replicates were run to reduce variability.

2.6 Beet armyworm feeding bioassays

For the beet armyworm performance test, one 2nd instar larva per cup was fed with a leaf detached from 10-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.

2.7 Statistical analysis

Analysis of variance was carried out with herbivory treatment (AM, Bb, AM + Bb, and control) as fixed effects. Block, and the interaction of block with herbivory treatment were random effects in the mixed models procedure of SAS 9.2 (SAS 2008). The test was arranged as a RCBD. Analyses were performed with the program codes using SAS macro "Danda" designed by Dr. Arnold Saxton (http://dawg.utk.edu/). Prior to statistical analysis, data for plant compounds were transformed with $\log_{10}(0.5+X)$ where X=value of the measured variable. The effect of block×treatment was not significant for any of the measured plant compounds based on a Tukey's single degree of freedom test (P=0.05), therefore replicate plants sampled from the two blocks for the tomato tissue analysis were pooled for a total of six replications. The pooled data were checked for normality (Shapiro-Wilk test at P=0.05), and equality of variance (Levene's test at P=0.05). Significant treatment effects were further analyzed with an F-protected LSD test at P=0.05.

Herbivory data were checked for normality (Shapiro-Wilk test at P=0.05), and equality of variance (Levene's test at P=0.05). Two outliers were identified with a Cook's D test in the leaf area consumption data and removed from the final analysis. Significant treatment effects were further analyzed with an F-protected LSD test at P=0.05.

3 Results

3.1 Terpenoid chemistry of control tomato plants

In order to determine the effects of fungal inoculation on the terpene chemistry of tomato plants, the terpene chemistry of tomato plants without any fungal inoculation were first determined.

For all plants analyzed, the second leaf from the top was detached and subjected to organic extraction. At this stage, a total of six terpenoids were identified. These included two monoterpenes: δ-2-carene and sabinene, and four sesquiterpenes: δ-elemeney, β-elemene, (*E*)-β-caryophyllene and α-humulene (Fig. 1). The most abundant monoterpene in control leaves was sabinene with a concentration of 16.16 ng/g fresh weight followed by δ-2-carene with a concentration of 2.1 ng/g fresh weight. Sabinene composed 88.5 % of total monoterpenes. The concentration of all monoterpenes in control plants was 18.3 ng/g fresh weight (Table 1). The most abundant sesquiterpene in the control leaf tissues was (*E*)-β-caryophyllene, which had a concentration of 1.4 ng/g fresh weight; and it accounted for 50 % of total sesquiterpenes. Other sesquiterpenes detected from the leaf tissue were δ-elemeney, β-elemene, and α-humulene. The concentration of all sesquiterpenes was 2.8 ng/g fresh weight (Table 2) and total terpenoids accumulated by the leaves from control plants was 21.1 ng/g fresh weight (Tables 1 and 2).

3.2 Colonization of tomato with *R. intraradices* (AM) and *B. bassiana* (Bb)

More than 70 % colonization in each plant was confirmed in selected plants inoculated with AM alone or in combination with Bb. The non-mycorrhizal plants were also tested to confirm the lack of colonization. For Bb treatment, 92 % of selected tomato plants inoculated with Bb alone or in combination with AM, tested positive for the presence of Bb.

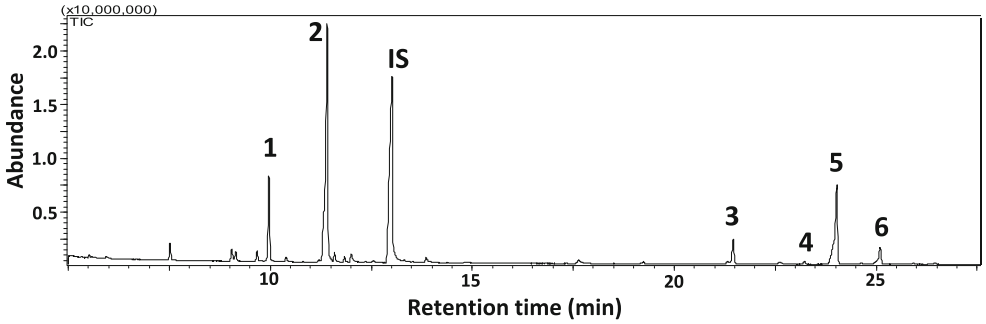

Fig. 1 Gas chromatography of terpenoids in leaves of 8-week old tomato plants without any fungal inoculation. These include two monoterpenes: δ-2-carene (peak 1) and sabinene (peak 2), and four sesquiterpenes: δ-elemene (peak 3), β-elemene (peak 4), (*E*)-β-caryophyllene (peak 5) and α-humulene (peak 6). *IS* internal standard

3.3 Effect of inoculation by *R. intraradices* (AM) on terpenoid chemistry of tomato plants

A total of eight terpenoids were detected from *R. intraradices*-inoculated tomato plants (Tables 1 and 2). These included the two monoterpenes and the four sesquiterpenes that were detected from control tomato plants. The concentrations of β-elemene in control and treated plants were almost the same (Table 2), whereas the concentrations of all of the other six terpenoids in *R. intraradices*-inoculated plants increased one fold compared to those in control plants. Of those, the concentrations of δ-2-carene and (*E*)-β-caryophyllene in *R. intraradices*-inoculated plants were significantly higher than those in control plants. In addition, two new monoterpenes, myrcene, and α-phellandrene, were detected from *R. intraradices*-inoculated plants. Their concentrations were 0.09 and 0.71 ng/g fresh weight, respectively (Table 1). The concentration of total monoterpenes in *R. intraradices*-inoculated plants was 33.4 ng/g fresh weight, higher than the concentration of 18.3. ng/g fresh weight in control plants (Table 1). However, this increase was not significantly different from the control. The concentration of total sesquiterpenes in *R. intraradices*-inoculated plants was 5.7 ng/g fresh weight, significantly higher than that in control plants (Table 2).

3.4 Effect of inoculation by *B. bassiana* (Bb) on terpenoid chemistry of tomato plants

A total of seven terpenoids were detected, which included all six terpenoids detected from control tomato plants. With the exception of β-elemene, the concentrations of which in *B. bassiana*-inoculated and control tomato were not significantly different, the concentrations of all of the other five terpenoids in *B. bassiana*-inoculated plants were significantly higher than those in control plants, showing 1–3 fold increases (Tables 1 and 2). The monoterpene myrcene was the only new terpene to be detected from *B. bassiana*-inoculated plants in comparison to control plants. The concentrations of total monoterpenes and total sesquiterpenes in *B. bassiana*-inoculated plants were 44.2 and 8.2 ng/g fresh weight, respectively. The concentration of total sesquiterpenes was

Table 1 Monoterpene content (ng/g fresh weight) in the leaves of control tomato plants and tomato plants inoculated with *Rhizophagus intraradices* (AM), *Beauveria bassiana* (Bb), or a combination of the two fungi (AM + Bb). Plants were not exposed to beet armyworm before terpenes were measured

Treatment[a]	Myrcene[b]	δ-2-Carene	α-Phellandrene[c]	Sabinene	Total monoterpenes
Control	0.00[d]±0.00 b	2.13±0.70 b	0.00[d]±0.00	16.16±5.23 b	18.29 b
AM	0.09±0.08 b	4.98±1.26 a	0.71±0.35	27.60±6.42 ab	33.38 b
Bb	0.47±0.22 b	6.36±0.84 a	0.00[c]±0.00	35.41±4.28 a	44.24 ab
AM + Bb	1.21±0.24 a	7.59±1.48 a	0.00[c]±0.00	36.85±5.35 a	45.65 a

[a] n=6, except for control and AM where n=4 and 5, respectively

[b] Within each column, different letters (a, b, c, d) denote significant differences between means based on an F-protected LSD (P<0.05). All values were transformed [$\log_{10}$ (0.5+X)], where X = measured variable, prior to analysis. Untransformed means ± SE are reported

[c] Data for α-phellandrene did not meet the criteria for ANOVA. The data were not normally distributed and variances were unequal

[d] Not detected, a value of zero was used for data analysis

Table 2 Sesquiterpene content (ng/g fresh weight) of leaves of control tomato plants and tomato plants inoculated with *Rhizophagus intraradices* (AM), *Beauveria bassiana* (Bb), or a combination of the two (AM + Bb). Plants were not exposed to beet armyworm before terpenes were measured

Treatment[a]	δ-Elemene[b]	β-Elemene[c]	(*E*)-β-Caryophyllene	α-Humulene	Total sesquiterpenes
Control	0.99±0.26 b	0.14±0.08	1.43±0.45 b	0.26±0.08 b	2.82 b
AM	1.89±0.43 ab	0.16±0.06	3.09±0.65 a	0.57±0.14 ab	5.71 a
Bb	2.56±0.44 a	0.25±0.04	4.48±0.86 a	0.87±0.18 a	8.16 a
AM + Bb	2.16±0.18 a	0.56±0.19	4.15±0.43 a	1.00±0.17 a	7.87 a

[a] n=6, except for control and AM where n=4 and 5, respectively

[b] Within each column, different letters (a, b, c, d) denote significant differences between means based on an F-protected LSD (P<0.05). All values were transformed [$\log_{10}$ (0.5+X)] prior to analysis. Untransformed means ± SE are reported

[c] Data for β-elemene did not meet the criteria for ANOVA. The data had unequal variances

significantly higher, while total monoterpenes were not significantly different than those in control plants (Tables 1 and 2).

3.5 Effect of Co-inoculation by AM and Bb on terpenoid profile of tomato plants

Since both the arbuscular mycorrhizae (*Rhizophagus intraradices*) (AM) and the entomopathogenic fungal endophyte (*Beauveria bassiana*) (Bb) affected the terpene chemistry of tomato plants, we further examined whether co-inoculation of these two fungal species had an additive or antagonistic effect on the terpene chemistry of tomato plants. The quality and quantity of terpene chemistry of tomato plants co-inoculated with *R. intraradices* and *B. bassiana* were highly similar to those of tomato plants inoculated with *B. bassiana* alone, with two exceptions (Tables 1 and 2). The first exception was for the monoterpene myrcene. The concentrations of this compound in control, AM-inoculated and Bb-inoculated plants were not significantly different. However, the concentration of this compound in tomato plants with AM and Bb co-inoculation was significantly higher than all other treatments (Table 1). The second exception was on the concentration of total monoterpenes. While the concentrations of total monoterpenes in control, AM-inoculated and Bb-inoculated plants were not significantly different, the concentration in tomato plants with AM and Bb co-inoculation was significantly higher than those in control and AM-alone plants (Table 1).

3.6 Performance of herbivorous insects on control and fungi-inoculated tomato plants

Plant treatment with AM, Bb, or AM + Bb had a significant effect on the weight of beet armyworm larvae (P<0.0001). Those fed with control leaves had significantly higher weight (0.084 g) compared to those fed with the leaves from AM, Bb, or AM + Bb treatments. Larvae fed leaves treated with AM + Bb had significantly lower weight than the larvae from all other treatments (Fig. 2).

4 Discussion

Arbuscular mycorrhizal symbioses have generally reduced attacks by root-feeding insects, while their effects on foliar-feeding insects have been more variable (Pozo and Azcón-Aguilar 2007; Gehring and Bennett 2009; Borowicz 2013). A number of studies have demonstrated a positive influence of mycorrhizal fungi on tolerance or resistance to above-ground herbivory, particularly with *R. intraradices*, and experiments using live insects have shown a large, positive overall effect of mycorrhizal fungi compared to simulated herbivory (Borowicz 2013). For example, colonization by *R. intraradices* reduced the

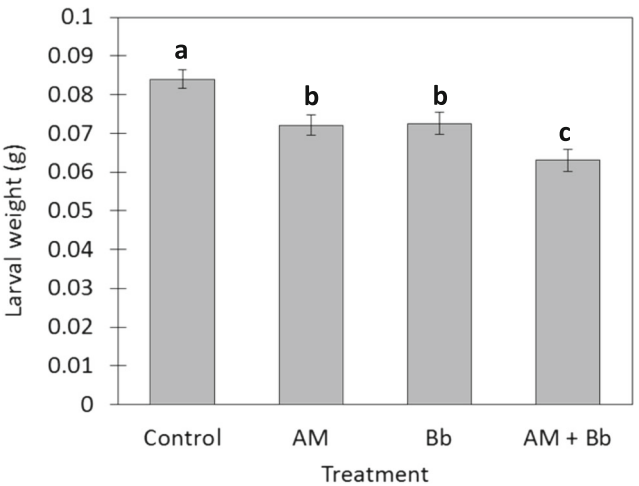

Fig. 2 Weight of beet armyworm larvae that were fed leaves of untreated control tomato plants (Control), or plants treated with *Rhizophagus intraradices* (AM), *Beauveria bassiana* (Bb) or both fungi (AM + Bb). One 2nd instar larva per cup was fed with leaves detached from 10-week-old plants from different treatments. Larvae were weighed when they reached wandering stage. Control (n=14), AM (n=11), Bb (n=10), AM + Bb (n=10). Bars with different letters indicate significant differences among treatments based on an F-protected LSD test (P<0.05)

level of leaf damage by *Arctia caja* in *Plantago lanceolata* (Gange et al. 2003), and *R. intraradices* symbiosis has been linked with the systemic induction of genes that play a regulatory role in the host defense response (Campos-Soriano et al. 2012). Mycorrhizal symbiosis has also reduced host tolerance or resistance to herbivory (Borowicz 2013). In general, mycorrhizal fungi have exerted a protective influence against chewing insects (Jung et al. 2012) and against generalist insects that feed on diverse plants (Fontana et al. 2009). Conversely, specialist insects usually perform better on mycorrhizal plants, perhaps due to better nutritional quality of the host (Gehring and Bennett 2009; Hartley and Gange 2009; Jung et al.. 2012). Mycorrhizal effects on herbivory have been highly dependent on the specific fungal and insect species investigated (Jung et al. 2012), and a meta-analysis revealed that *R. intraradices* has tended to diminish performance of chewing insects more than all other arbuscular mycorrhizal species examined (Koricheva et al. 2009). Degree of response can also vary with plant species. For example, the effects of mycorrhizal symbiosis on the expression of chemical resistance have been shown to vary among congeneric plant species (Vannette et al. 2013).

Mycorrhiza-induced increase in herbivory tolerance is likely often related to the better growth and nutrition that result from mycorrhizal colonization (Jung et al. 2012; Borowicz 2013). For example, mycorrhizal colonization benefitted plants in a tallgrass prairie subjected to grasshopper injury by stimulating compensatory growth (Kula et al. 2005). Others have demonstrated that the tradeoff between plant growth and defense observed in NM plants could be mitigated completely by mycorrhizal symbiosis (Vannette et al. 2013). Mycorrhizal effects on herbivory resistance, however, are not always merely a consequence of improved nutrition or compensatory growth (Liu et al. 2007; Pozo and Azcón-Aguilar 2007). Mycorrhization is thought to enhance resistance through a modulation of the plant defense responses that accompany the colonization process, priming the plant for subsequent attack by insect herbivores (Koricheva et al. 2009; Jung et al. 2012). This modulation pre-conditions shoot tissues, creating an "alert state" so that they respond more rapidly and more strongly when challenged by a pest. This mycorrhiza-induced resistance (MIR) has been linked to changes in the volatile profile of mycorrhizal plants, which may make them more attractive to natural insect enemies such as predators and parasitoids. For instance, tomato plants colonized by the arbuscular mycorrhizal fungus *Funnelformis mosseae* were more attractive to parasitoids of aphids than non-mycorrhizal plants (Guerrieri et al. 2004). In *Phaseolus* challenged by spider mites, mycorrhizal symbiosis with *F. mosseae* changed plant volatile composition, increasing the emission of β-ocimene and β-caryophyllene, with the accompanying effect that the mite predator preferred mycorrhizal plants (Schausberger et al. 2012). In *Medicago* colonized by *R. intraradices*, mycorrhization only slightly influenced herbivore-induced volatile emissions (Leitner et al. 2010).

Arbuscular mycorrhizal symbiosis can affect a number of volatile organic compounds (Jung et al. 2012), including terpenes (Rapparini et al. 2008; Toussaint et al. 2008). The stimulation of monoterpenes and sesquiterpenes by both *R. intraradices* and *B. bassiana* is significant because these terpenes generally function as a form of chemical defense for plants (Reid and Purcell 2011; Ahern and Whitney 2014). For example, myrcene, which was detected from fungus-inoculated tomato plants but not from control tomato plants, has been reported to be an effective semiochemical. Semiochemicals are utilized by insects for communication purposes, e.g., to repel other insects like thrips (Broughton and Harrison 2012), and to attract aphidophagous hoverflies in the terrestrial orchid *Epipactis veratrifolia* (Stökl et al. 2011). Myrcene has also been reported to be produced in grape roots in response to herbivory (Lawo et al. 2011) and, along with (*E*)-β-ocimene, in herbivore-damaged leaves of *Medicago truncatula* (Navia-Giné et al. 2009). These reports suggest important implications regarding direct and indirect defense after herbivory. Some plants constitutively produce terpenoids while others make terpenoids only under biotic or abiotic stress. Tomato is a species that synthesizes terpenoids both constitutively and by induction (Falara et al. 2011). By changing the concentrations of certain individual monoterpenes and sesquiterpenes as well as their total concentrations in the absence of herbivory, inoculation by *R. intraradices* may have induced resistance via priming in our study.

How might inoculation with *R. intraradices* modulate the biosynthesis of terpenoids in tomato? Monoterpenes are synthesized in plastids through the methylerythritol phosphate (MEP) pathway, whereas sesquiterpenes are synthesized in cytosol through the mevalonate (MVA) pathway (Fontana et al. 2009). These two pathways provide universal precursors for terpene biosynthesis. The conversions of geranyl diphosphate and farnesyl diphosphate to monoterpenes and sesquiterpenes, respectively, are catalyzed by terpene synthases, the pivotal enzymes of terpene biosynthesis. The induced production of monoterpenes and sesquiterpenes could happen at two levels: the elevation of substrate levels through the induction of the pathways or the elevated levels of terpene synthase enzymes. Mycorrhizal 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 (Walter et al. 2002). 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 *R. intraradices* colonization (Walter et al. 2000). Therefore, activation of the MEP pathway is partly responsible for the enhanced production of monoterpenes. 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).

With the considerable interest that has been generated regarding the potential for mycorrhizal symbiosis to enhance plant pest resistance via secondary metabolites and priming (Koricheva et al. 2009; Jung et al. 2012), it is interesting to find that *B. bassiana* tended to have even larger effects on host terpene production in tomato. Compared to *R. intraradices*, much less is known about the influence of *B. bassiana* on resistance to insect herbivory and host terpene biochemistry. It remains to be determined whether inoculation with *B. bassiana* affects the expression of the MEP and MVA pathway as well as the terpene synthase genes for the biosynthesis of monoterpenes and sesquiterpenes in tomato leaves.

The effects of co-inoculation by *R. intraradices* and *B. bassiana* were complex. The induction pattern of co-inoculation on individual terpenes was very similar to that of single inoculation by *B. bassiana*, except for myrcene, for which the co-inoculation showed an additive effect. At the levels of total monoterpenes and sesquiterpenes, the additive effect was insignificant. For the insect bioassays, co-inoculation has an additive effect on larval weight. As for inoculation with individual fungi, the molecular level analysis of the biochemical and physiological changes of tomato plants co-inoculated with *R. intraradices* and *B. bassiana* will help explain the mechanisms underlying the observed chemical and bioassay data.

Protective effects of beneficial endophytic fungi have been described for many host-pest interactions. These effects go beyond developing healthier host plants via improved nutrition, by strengthening plant immunity through defense priming (Jung et al. 2012). Understanding how beneficial fungi affect secondary metabolism and defense priming is of substantial agricultural interest. This is one more area in which mycorrhizal fungi and endophytic fungi are likely to make important contributions for biocontrol and integrated management of pests and diseases. To this end, it will be important to continue to investigate the occurrence and protective effects of different species of mycorrhizal and endophytic fungi on different crop plants.

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