Arbuscular mycorrhizal symbiosis alters plant gene expression and aphid weight in a tripartite interaction

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ABSTRACT

Mutualistic root associations with arbuscular mycorrhizal (AM) fungi are known to alter interactions with insects aboveground. In this study, we investigated whether root symbiosis with *Rhizophagus irregularis* modulates alterations in *Medicago truncatula* that affect pea aphid’s (*Acyrthosiphon pisum*) fitness. The results indicate that aphid colony weight increased after feeding on shoots of high *R. irregularis*-colonized plants relative to non-mycorrhizal controls. Moreover, when given a choice, aphid adults preferred high *R. irregularis*-colonized plants compared to non-mycorrhizal plants. Interestingly, we identified genes strongly regulated by aphid feeding in shoots (*β-1,3 glucanase*, *thaumatin*-like protein, *ethylene response factor*), by AM symbiosis in roots (*oxo-phytodienoic acid reductase*, *ERF1*, and *GA 2-oxidase*), and a gene (*GA 2-oxidase*) exclusively induced in roots during this tripartite interaction. Our data provide novel information about changes in plant gene expression during aphid-plant-AM fungus interactions, and serve as foundation for future transcriptome studies.

Introduction

Plants serve as intermediates of multiple interactions between belowground mutualists and aboveground antagonists like insects and pathogens (Pineda et al. 2010). The belowground mutualistic relationship between plants and arbuscular mycorrhizal (AM) fungi is widely studied. During mutualism with AM fungi, plants exchange carbon for nutrients (e.g. phosphate, P) that are supplied by the fungus, mainly in phosphorus-poor soils (Harrison 1999). AM fungi play an important role in plant nutrition (Smith 1988), chemical composition (Strack et al. 2003), and phytohormone signaling (Lopez-Raez et al. 2010), hence, the AM symbiosis affects plant interactions with organisms including pathogens (Dehne 1982; Pozo et al. 2002; Fritz et al. 2006; Song et al. 2015) and phytophagous insects (Bennett et al. 2006; Hartley and Gange 2009). Aphids are plant antagonists of agricultural importance that feed on a wide range of hosts, are fecund, transmit viruses, and can inject chemicals or protein effectors into their host plants (Kaloshian and Walling 2005; Walling 2008). In addition, there are reports of insecticide-resistant aphid populations (Sadeghi et al. 2009). Aphids are also known to manipulate their host plant by inducing defense responses through different signaling pathways (Moran and Thompson 2001; Thompson and Goggins 2006; Gao et al. 2007; Mai et al. 2014; Tzin et al. 2015).

AM fungi and aphids, although spatially separated, interact indirectly by altering their shared host plant’s chemistry. Despite progress on plant interactions with either AM fungi or insects and even tripartite interactions, limited data are available on changes in plant gene expression during AM fungus-plant-insect interactions. Previous reports showed variable effects of AM symbioses on aphids ranging from positive (Gange et al. 2002; Babikova et al. 2014a; Gilbert and Johnson 2015; Simon et al. 2017; Tomczak and Müller 2017) to negative (Guerrieri et al. 2004; Babikova et al. 2013; Gilbert and Johnson 2015; Tomczak and Müller 2017), or no effects (Gehring and Bennett 2009; Hartley and Gange 2009). A recent study explained that the variation in aphid outcome during a tripartite interaction could be attributed to the changes in plant quality that are driven by the level of AM fungus root colonization as well as plant age (Tomczak and Müller 2017). AM fungi can affect insects through multiple mechanisms by modulating changes in the plant’s nutritional quantity, nutritional quality, defensive strategies, and tolerance (Bennett et al. 2006). It was recently shown that milkweed (*Asclepias* spp.) tolerance and defense against insect herbivores could be predicted through mycorrhiza-induced changes in foliar P, and nitrogen levels, root biomass and plant growth rate (Tao et al. 2016).

Although previous studies have examined the role of plant age, mycorrhizal stage (Tomczak and Müller 2017), plant volatiles (Guerrieri et al. 2004; Babikova et al. 2014a), plant host quality and quantity in mediating aphid-plant-AM fungi interactions (Babikova et al. 2014a), to our knowledge, no study has yet examined the role of the AM symbiosis on plant signaling pathways during a tripartite interaction with aphids. To date, the transcript levels of four genes have been assessed in mycorrhizal tomato (*Solanum lycopersicum* L.) damaged by caterpillars (Song et al. 2013), but not much is known about aphid-induced changes in gene expression in mycorrhizal plants. Jasmonic acid (JA) has been proposed as a major player in mycorrhiza-induced resistance (Jung et al. 2012), but there are still limited data to support this hypothesis (Song et al. 2013). Aphids...
themselves are known to trigger an increase in salicylic acid (SA)-regulated transcripts, whereas, JA-regulated transcripts are reduced or slightly increased in aphid-infested leaves (Moran and Thompson 2001; Gao et al. 2007).

In the present study, we examined whether: (a) aphid fitness changes when aphids feed on mycorrhizal plants that exhibit different levels of AM fungus root colonization, (b) aphid feeding reduces plant growth and *R. irregularis* root colonization, and (c) pea aphids show a preference for non-mycorrhizal plants versus mycorrhizal plants. Our biological system consisted of barrel medic (*Medicago truncatula* Gaertn); the AM fungus, *Rhizophagus irregularis* N.C. Schenck & G.S. Sm.; and pea aphids (*Acyrthosiphon pisum* Harris). *M. truncatula* is a close relative of alfalfa (*Medicago sativa* L.) and is widely used as a model in legume biology. Pea aphids are found worldwide, they feed on legumes including *M. truncatula*, and they are closely related to other crop pests (The International Aphid Genomics Consortium 2010). Finally, the AM fungus *R. irregularis* is a generalist that forms symbioses with most plants, and is the most frequently used member of the Glomeromycota.

**Materials and methods**

**Experimental organisms and plant growth conditions**

The experimental system consisted of barrel medic plants (*M. truncatula* ‘Jemalong’, line A17), pea aphids (*A. pismum*), and the AM fungus *R. irregularis* (formerly known as *Glomus intraradices*). *M. truncatula* line A17 is a genotype known to be susceptible to pea aphids (Gao et al. 2008). Spores of *R. irregularis* were kindly provided by Dr. Maria Harrison (Boyce Thompson Institute for Plant Research, Ithaca, NY, USA), which was later propagated in *Daucus carota* L. root bi-plates (StArnaud et al. 1996) in our laboratory. Parthenogenetic, female pea aphids were provided by Dr. Kenneth Korth (University of Arkansas, Fayetteville, AR, USA) and were reared on fava bean (*Vicia faba* L.) plants, considered as universal hosts, in insect tents under laboratory conditions (16 h (29 ± 2°C): 8 h (24 ± 2°C), light:dark).

*M. truncatula* seeds were scarified in concentrated H₂SO₄ for 10 min, rinsed in sterile water three times, sterilized using 10% (v/v) bleach in 0.1% (v/v) Tween 20 solution for 10 min, and rinsed in sterile water five times. Seeds were spread on wet filter paper (sterile) in petri dishes and dishes were sealed with parafilm. Dishes were incubated at 4°C for three days (dark), at room temperature for one day (dark) and for three additional days under light (Liu et al. 2007). Once seedlings developed healthy roots and cotyledons, they were transplanted into azalea plastic pots (no-choice assay: 12 cm W × 8.5 cm H) or square pots (choice assay: 9 cm W × 5 cm H) filled with sterilized soil substrates low in mineral nutrients. One part of squeegee (Pioneer Sand Company, Windsor, CO, USA) mixed with one part of sand was used for experiments A and B (Table 1), and one part of topsoil mixed with eight parts of sand was used for experiments C-F (Table 1). Squeegee and Mason sand (Pioneer Sand Company) were washed 8–10 times prior to autoclaving (60 min, 121°C, at 15 psi). The topsoil (Pioneer Sand Company) was sieved (sieve no. 8) and autoclaved (60 min, 121°C, at 15 psi) three times prior to mixing it with sand. After transplanting, pots were placed on trays (54 × 28 × 6 cm) and were covered for seven days with clear plastic domes (55 × 31 × 17 cm). Plants were grown under laboratory conditions on wire shelves (152 × 60 cm) that were set up with a canopy of four fluorescent bulbs (mean: 131 µmol m⁻² s⁻¹) per shelf using a 16-h photoperiod.

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**Table 1.** Experimental set-up for pea aphid (*Acyrthosiphon pisum*) no-choice and choice assays.

| Experiment | Target AM fungus colonization | Soil substrate used | Days post inoculation with *R. irregularis* (%) | % RLC<sup>†</sup> when aphids were added | Aphid population type and days of feeding |
|------------|-------------------------------|---------------------|----------------------------------------------|------------------------------------------|-----------------------------------------|
| A Low      | squeegee: sand                | 37 (67)             | ND                                           | Adults for 1 day, nymphs for 6 extra days |
| B High     | squeegee: sand                | 53 (72)             | 42                                           | Adults for 1 day, nymphs for 6 extra days |
| C Low      | topsoil: sand                 | 27 (54)             | 29                                           | Adults + nymphs for 7 days                |
| D High     | topsoil: sand                 | 53 (85)             | 67                                           | Adults + nymphs for 7 days                |
| E Low      | topsoil: sand                 | 48 (69)             | 24                                           | Adults + nymphs for 7 days                |
| F High     | topsoil: sand                 | 55 (76)             | 46                                           | Adults + nymphs for 7 days                |

Pea aphids were added to experimental plants when the levels of *Rhizophagus irregularis* root colonization reached target levels in extra potted plants.

<sup>†</sup>RLC = root length colonized. ND = not determined because roots were accidentally damaged prior to quantification of colonization level. Nevertheless, visual observation of stained roots showed low level of colonization.

**Arbuscular mycorrhizal fungus inoculation**

Seedlings were either inoculated with *R. irregularis* spores (+AMF treatments) or ‘mock’ inoculated (-AMF treatments) when they had two to three trifoliate leaves. Prior to filling the pots, the soil substrate mixture was moistened with ½ strength modified Hoagland’s solution [100 µM P (reduced), 15 mM N (high)] pH 6.1 (Javot et al. 2011). Each pot consisted of three soil layers: (1) a bottom layer of sterile soil substrate mixture, (2) a 1.5–2 cm layer of Mason sand, and (3) a top layer of reused soil substrate mixture from seedlings’ pots. Roots from three seedlings per pot were inoculated with 300 or 500 *R. irregularis* spores per plant over the sand layer, and were covered with the reused soil mixture (with root exudates). Extra plants were inoculated to monitor the levels of *R. irregularis* colonization over time to add pea aphids to the experimental plants. Another group of seedlings were ‘mock’ inoculated with a filtered solution of the last rinse of the spore preparation (Gomez et al. 2009). After seven days, all plants were watered as normal and fertilized initially once, later twice a week with 45 ml of ½ strength modified Hoagland’s solution. To assess *R. irregularis* colonization levels, roots were cleared with 10% (w/v) KOH (85°C for 4 h), rinsed with deionized water, stained with 5% (v/v) Sheaffer black ink prepared in 5% (v/v) acetic acid (Vierheilig et al. 2000).
Assessing the effect of AM fungus colonization level on aphid fitness

To address whether AM fungus colonization level affects pea aphid’s fitness, we performed aphid no-choice biossays. Experiments A-D (Table 1) consisted of adding apterous pea aphids to either mycorrhizal or non-mycorrhizal plants without allowing insects to choose their host plant. Each experiment consisted of four treatments: (a) pea aphid-infested, mycorrhizal M. truncatula (+AMF +Aphid), (b) pea aphid-infested, non-mycorrhizal M. truncatula (-AMF +Aphid), (c) non-infested, mycorrhizal M. truncatula (+AMF), and (d) non-infested, non-mycorrhizal M. truncatula (-AMF). Each treatment had four to six biological replicates (three plants per pot; each pot was considered a replicate).

Nine-day old aphids that were reared on fava bean were transferred to experimental M. truncatula plants when the feeding period started. Three pea aphid adults of the same age were transferred, with the aid of a fine paintbrush, to the leaves of every plant that included an aphid treatment. Once the aphid transfer was completed, pots were placed inside bugdorm-1 insect cages (58.5 x 60 x 61 cm). The insect cages for the +AMF, and -AMF treatments were distantly separated from +AMF +Aphid, and -AMF +Aphid treatments. We used two types of aphid colonies to determine if the AM symbiosis affected insects differently. In experiments A and B, pea aphid adults were removed after one day, leaving only their offspring (referred as same age aphids in Results) to feed for six extra days. In experiments C-F, apterous pea aphid adults and their offspring (referred as mixed age aphids in Results) fed continuously on M. truncatula.

The indirect effect of R. irregularis on pea aphid-plant interactions was examined based on the aphids’ ability to survive and reproduce on mycorrhizal plants. The total number of pea aphids present on plants from each pot (referred as colony) was counted using a magnifying visor, aphids were removed and weighed. Before weighing, pea aphids were placed at -20°C to immobilize insects. All pea aphids present in a colony were weighed as a group using a microbalance (SE-2F Sartorius balance, Denver, CO, USA).

Total RNA isolation and cDNA synthesis

We chose to examine gene expression on experiments that included same age aphids after seven days of herbivory because plants were of similar age 67 and 72 days (experiments A and B, Table 1), and to detect a more consistent plant response induced by insects. Total RNA was extracted from shoot and root tissues using TRIzol reagent (Thermo-First, Scientific, Waltham, MA, USA) following the manufacturer’s protocol with minor modifications. Briefly, 33.3 µl of 10% PVP-40 (w/v) was added per ml of Trizol reagent in each sample. RNA samples were DNase-treated by adding 3 µl of Turbo DNAse (2 units µl⁻¹), 10 µl of buffer in a total volume of 87 µl and were incubated at 37°C for 40 min. The enzyme was inactivated with chloroform followed by sodium acetate-ethanol precipitation, and a total of three 75% ethanol washes were performed prior to air drying pellets. The integrity of RNA was verified using a native 1% (w/v) agarose-0.5x TAE gel. For cDNA synthesis, 1 µg of total RNA suspended in 11 µl autoclaved DEPC-treated water was mixed with 1 µl of dNTPs (10 mM) and 1 µl of anchored oligo dT₂₅ (500 ng µl⁻¹) in 0.2 ml PCR tubes. Each 13-µl reaction mixture was incubated at 37°C for 40 min. The reverse transcription reaction was performed using a previously validated M. truncatula reference gene, elongation factor 1-alpha (EF1-α) (Liu et al. 2007) to assess the quality
of the cDNAs. Each reaction consisted of 10 µl of GoTaq® colorless master mix (Promega Corporation, Madison, WI, USA), 0.4 µl each of 10 µM forward and reverse primers, 1 µl of cDNA (1:2) in a total volume of 20 µl. The thermal profile was as follows: 95°C for 2 min, 27 cycles at 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final incubation step at 72°C for 5 min, using a T100™ thermal cycler. EFl-α transcripts was visualized on a 2% (w/v) agarose-0.5x TAE gel. RT-qPCR consisted of 5 µl of SSO Advanced Universal SYBR® green Supermix (Bio-Rad), 1 µl each of 3 µM forward and reverse primers, 1 µl of cDNA (1:5 dilution) in a total volume of 10 µl. The thermal profile was as follows: 95°C for 30 s, 40 cycles at 95°C for 5 s and 57°C or 59°C for 30 s, followed by a melt curve cycle (65°C to 95°C: increment 0.5°C every 5 s) using a CFX384 Touch system™ (Bio-Rad). The reference gene EFl-α was included in every 384-well plate run, and each reaction consisted of four to six biological replicates and one technical replicate. Oligonucleotide sequences for target genes and reference genes are listed in Supp. Table S1. Genes of interest included those involved in oxylipin biosynthesis, the 9-lipoxygenase (LOX) branch (LOXA and divinyl ether synthase (DES)) and the 13-LOX branch (oxo-phytodienoic acid (OPR3)). JA signaling (transcription factor MYC2 and ethylene response factor 1 (ERF1)), SA-responsive defenses (β-1,3-glucanase (BGL) and pathogenesis-related protein 5 (PR5)), ethylene (ET) biosynthesis (1-aminocyclopropane-1-carboxylic acid (ACC0)), ABA biosynthesis (9-cis-epoxycarotenoid dioxygenase (NCED)), GA pathway (GA 20-oxidase (GA20ox) and GA 2-oxidase (GA2ox)), and phenylpropanoid biosynthesis (phenylalanine ammonia lyase (PAL)).

The relative expression of target genes was determined using the 2−ΔΔCq method (Livak and Schmittgen 2001). Each target gene was normalized to EFl-α. Genomic DNA contamination of plant RNA samples was tested by using primers designed on an intron region of the reference gene ubiquitin. Calculations were performed as follows: Fold Change = 2−ΔΔCq, where: ΔCq1 = Cq (Target gene in treated sample) − Cq (Reference gene in treated sample), ΔCq2 = Cq (Target gene in −AMF sample) − Cq (Reference gene in −AMF sample), and ΔΔCq = ΔCq1 − ΔCq2. Fold change reduction in gene expression shown in heatmaps was obtained by taking the negative inverse of 2−ΔΔCq (Schmittgen and Livak 2008). Heatmaps were generated using Heatmapper Plus developed by the University of Toronto (http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi).

**Determine whether pea aphids show a preference for non-mycorrhizal plants versus mycorrhizal plants**

We designed aphid choice bioassays (Experiments E and F, Table 1) to test the hypothesis that pea aphids would show a preference for non-mycorrhizal plants compared to mycorrhizal plants because of mycorrhiza-induced resistance. The same plant growth conditions and AM fungal inoculation procedure described previously were used for these assays. Each treatment consisted of nine biological replicates (one plant per pot, each choice arena was a replicate). Extra pots were prepared for +AMF to monitor the levels of AM fungus colonization over time. Once the desired colonization level was obtained, a mycorrhizal plant and a non-mycorrhizal plant were placed inside a bugdorm-1 insect cage using a double-blind experiment setup. A petri dish lid (6.5 cm) containing ten apterous pea aphids was placed in between two experimental potted plants, and insects were free to choose their preferred host. Apterous aphids were used previously to study antixenosis (deterrence) (Pegadaraju et al. 2007). The number of pea aphid adults and nymphs present on each plant was counted at 1, 3, 6, 12, 24, 48 and 72 h post aphid release.

**Statistical analyses**

Statistical analyses were performed using either SAS 9.4 software (SAS inst. Inc., Cary, NC, USA), Excel’s data analysis, or VassarStats (vassarstats.net). Raw data were checked for normality and homogeneity of variance (Quantile-quantile plot, Shapiro–Wilks, Anderson–Darling, Levene’s test and Cramer-von Mises test) before performing the parametric tests. Data that did not meet the criterion of normality were transformed using Box–Cox transformations. In the no-choice experiments, aphid number, aphid weight, and AM fungus colonization means were analyzed using Student’s t-tests, while plant root and shoot weights, and fold changes in gene expression were analyzed using one-way ANOVAs and post-hoc Tukey–Kramer test. For the choice experiments, proportions of adult aphids per plant, and number of aphid progeny per plant were square root-transformed, and analyzed with two-factor repeated measure ANOVA using type of inoculant and time point as the two factors (Gao et al. 2008; Barman et al. 2016). SAS output yields both, a multivariate repeated-measure ANOVA (MANOVA) and an univariate repeated-measure ANOVA for between-subjects effects (type of inoculant) and within-subjects effects (time point and time point*type of inoculant). If the sphericity assumption was violated, results obtained from the MANOVA were used, otherwise, results from the univariate analysis were used. Significance was declared at P < 0.05 for all the experiments.

**Results**

**Longer-term herbivory effects on plant growth and impacts of AM fungus root colonization on aphid performance**

The overall aim was to determine whether aphid fitness changes when aphids feed on mycorrhizal plants that exhibit different levels of AM fungus root colonization. We hypothesized that mycorrhizal plants would exhibit mycorrhiza-induced resistance against aphids, and root colonization in low AM fungus-colonized plants would be negatively affected by aphid feeding. Our data indicate that when a colony of pea aphids of the same age fed on mycorrhizal plants at low levels of AM fungus root colonization, neither insect number (t = 2.57; df = 5; P = 0.1227; Figure 1(a)) nor colony weight were affected (t = 1.50; df = 8; P = 0.1713; Figure 1(c)). When insects fed on plants at high levels of AM fungus colonization, aphid colony weight significantly increased (t = −3.33; df = 8; P = 0.0104; Figure 1(d)), but aphid number was not affected (t = −1.88; df = 8; P = 0.0963; Figure 1(b)). Contrastingly, when a colony of pea aphids of mixed age fed on mycorrhizal plants, aphids weighed less on plants that exhibited low levels of R. irregularis colonization (t = 2.33; df = 11;
In agreement with the first experiment, aphids from the mixed-age colony weighed more on plants at high levels of *R. irregularis* colonization (*t* = −4.33; *df* = 10; *P* = 0.0015; Figure 2(d)). The number of aphids that was present in a colony was unaffected by the mycorrhizal status, regardless if the level of colonization was low (*t* = 0.76; *df* = 11; *P* = 0.4651; Figure 2(a)) or high (*t* = −1.96; *df* = 10; *P* = 0.0781; Figure 2(b)). Our research asked whether plant growth is affected in a tripartite interaction, and whether aphid feeding aboveground indirectly affects *R. irregularis* root colonization. Herbivory by a colony of pea aphids of the same age did not alter shoot growth in the experiment at low level of AM fungus colonization. However, roots from non-mycorrhizal plants accumulated more fresh mass compared to roots from aphid-infested plants +/-AMF (Table 2). By contrast, mycorrhizal plants within the experiment at high levels of AM fungus colonization accumulated more shoot fresh mass compared to non-mycorrhizal plants +/-Aphids (Table 2). Herbivory by a colony of pea aphids of the same age or mixed age did not alter root colonization by *R. irregularis* (Tables 2 and 3). Pea aphid herbivory by a mixed age colony significantly decreased shoot fresh weight in both experiments (low and high levels of AM fungus colonization), however, root mass was unaffected (Table 3).

**Plant gene expression after longer-term aphid herbivory at two levels of AM fungus colonization**

We hypothesized that mycorrhizal plants would be 'primed' by root colonization leading to enhanced accumulation of transcripts involved in plant defenses, resulting in reduced aphid fitness. Our results showed several changes in gene expression (e.g. *LOXA*, *DES*, *ERF1*, *BGL*, *PR5*, *ACCO*, *GA20ox*, *GA2ox*, and *PAL*) in shoots caused by aphid herbivory and/or by AM symbiosis (Figure 3, Tables S2 and S3). In the low level of AM fungus colonization experiment, we found that *BGL* and *PR5* genes (SA-regulated defense genes) are strongly upregulated by aphid feeding in shoots, and aphid feeding alone triggered modest accumulation of *ACCO* transcripts in shoots (Figure 3, Table S2). In the high level of AM fungus colonization experiment, we found that aphid feeding triggered modest to strong accumulation of *DES*, *ERF1*, *BGL*, and *PR5* transcripts, while the AM symbiosis alone triggered modest induction or downregulation of these transcripts (Figure 3, Table S3). Aphid feeding triggered strong accumulation (ranging from 9.2 to 13.2 fold change) of *BGL* and *PR5* transcripts compared to AM symbiosis alone (Figure 3, Table 2).

Roots experienced fewer changes in gene expression (e.g. *OPR*, *MYC2*, *ERF1*, *ACCO*, *GA20ox*, and *GA2ox*) than shoots.

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**Figure 1.** Aphid number (a, b) and weight (c, d) after seven days of herbivory by pea aphids (*Acyrthosiphon pisum*) of the same age on non-mycorrhizal (-AMF +Aphids) and mycorrhizal (+AMF +Aphids) *Medicago truncatula* plants in two independent experiments (low and high level of *Rhizophagus irregularis* root colonization; experiments A and B in Table 1). Values represent the mean of five biological replicates ±SE. *P* < 0.05 indicates statistical significance based on Student’s *t* test.
In the low level of AM fungus colonization experiment, aphids alone triggered modest accumulation of OPR transcripts while AM symbiosis +/−Aphids caused downregulation of these transcripts (Figure 4, Table S4). AM symbiosis alone triggered the most significant increase (18.3 fold change) in GA20ox transcripts. In the high level of AM fungus colonization experiment, aphid feeding combined with AM symbiosis caused the highest accumulation (24.3 fold change) of GA20ox transcripts. GA2ox transcripts also accumulated during the tripartite interaction, but decreased during both two-way interactions (Figure 4, Table S5).

### Aphid preference for mycorrhizal plants with low or high levels of AM fungus root colonization

We hypothesized that pea aphids would show a preference for non-mycorrhizal plants compared to mycorrhizal plants because of mycorrhiza-induced resistance. In the aphid choice assay using plants with low level of AM fungus colonization experiment, aphids alone triggered modest accumulation of OPR transcripts while AM symbiosis +/−Aphids caused downregulation of these transcripts (Figure 4, Table S4). AM symbiosis alone triggered the most significant increase (18.3 fold change) in GA20ox transcripts. In the high level of AM fungus colonization experiment, aphid feeding combined with AM symbiosis caused the highest accumulation (24.3 fold change) of GA20ox transcripts. GA2ox transcripts also accumulated during the tripartite interaction, but decreased during both two-way interactions (Figure 4, Table S5).

### Table 2. Summary of results after seven days of herbivory by same age pea aphids (Acrithosiphon pisum) (Experiments A and B).

| Variable                      | -AMF | +AMF | -AMF +Aphid | +AMF +Aphid | F or t | DF | P value |
|-------------------------------|------|------|-------------|-------------|-------|----|---------|
| Shoot fresh weight (mg)       |      |      |             |             |       |    |         |
| Low level of Rhizophagus irregularis root colonization: |      |      |             |             |       |    |         |
| n = 5                         | n = 5| n = 5| n = 5       | n = 5       |       |    |         |
| Root length colonized (%)     |      |      |             |             |       |    |         |
| n = 4                         | n = 6| n = 5| n = 5       | n = 5       |       |    |         |
| High level of Rhizophagus irregularis root colonization: |      |      |             |             |       |    |         |
| n = 5                         | n = 5| n = 5| n = 5       | n = 5       |       |    |         |

*AMF = non-infested, non-mycorrhizal M. truncatula, +AMF = non-infested, mycorrhizal M. truncatula, +AMF +Aphid = pea aphid-infested, mycorrhizal M. truncatula, and -AMF +Aphid = pea aphid-infested, non-mycorrhizal M. truncatula. Herbivory by pea aphids (Acrithosiphon pisum) of the same age on non-mycorrhizal and mycorrhizal Medicago truncatula plants in two independent experiments (low and high level of Rhizophagus irregularis root colonization).

*Colonization level post aphid feeding. Student’s t tests were performed.

One-way ANOVA was followed by the Tukey-Kramer test when significant (α = 0.05; statistically significant P value is shown in bold). Different letters indicate significant difference among treatments. Values represent the mean ± SE.
colonization, more pea aphid adults and nymphs were present on non-mycorrhizal plants compared to mycorrhizal plants (Figure 5(a,c); Table S6), but there was only significant difference in the number of nymphs (Time*Treatment: *P* = 0.044; Figure 5(c); Table S6). The number of nymphs increased rapidly on non-mycorrhizal plants compared to mycorrhizal plants with a change in time (*P* < 0.0001; Figure 5(c); Table S6). In the choice assay with a high level of AM fungus colonization, adult aphids showed a distinctive preference for mycorrhizal plants compared to non-mycorrhizal plants (Time*Treatment: *P* < 0.0001, Figure 5(b); Table S6). Once again, the number of nymphs present per plant changed significantly over time (Time effect: *P* < 0.0001, Figure 5(d)).

An opposite trend was observed that showed more nymphs on mycorrhizal plants at high levels of AM fungus colonization compared to non-mycorrhizal plants, however this difference was not significant (Treatment effect: *P* = 0.1480; Figure 5(d)).

**Discussion**

In this study, we developed a system to examine aphid-plant-AM fungus interactions at the organismal and plant molecular levels. We found that the level of *R. irregularis* root colonization had an indirect impact on pea aphid colony weight (Figures 1 and 2). On one hand, mixed age aphids gained

**Table 3. Summary of results after seven days of herbivory by mixed age pea aphids (*Acyrthosiphon pisum*) (Experiments C and D).**

| Variable | -AMF | +AMF | +AMF +Aphid | +AMF +Aphid | F or t | DF | *P* value |
|----------|------|------|-------------|-------------|-------|----|-----------|
| Low level of *Rhizophagus irregularis* root colonization: | | | | | | | |
| Shoot fresh weight (mg) | 894 ± 98a | 732 ± 48a | 475 ± 16b | 913 ± 101a | 15.82 | 3, 21 | <0.0001 |
| Root fresh weight (mg) | 1574 ± 136 | 1124 ± 151 | 1038 ± 104 | 1161 ± 166 | 2.94 | 3, 21 | 0.0570 |
| Root length colonized (%) | 42 ± 2.3 | – | 38 ± 1.3 | 1.30 | 10 | 0.2220 |
| High level of *Rhizophagus irregularis* root colonization: | | | | | | | |
| Shoot fresh weight (mg) | 1816 ± 302bc | 2812 ± 111a | 1371 ± 97c | 1920 ± 94b | 11.98 | 3, 20 | 0.0001 |
| Root fresh weight (mg) | 2995 ± 497 | 2541 ± 143 | 2306 ± 209 | 1919 ± 165 | 2.94 | 3, 19 | 0.0594 |
| Root length colonized (%) | – | 74 ± 4.2 | – | 74 ± 2.4 | –0.04 | 10 | 0.9727 |

*-AMF = non-infested, non-mycorrhizal *M. truncatula* (used in calculations), +AMF = non-infested, mycorrhizal *M. truncatula*, +AMF +Aphid = pea aphid-infested, mycorrhizal *M. truncatula*, and -AMF +Aphid = pea aphid-infested, non-mycorrhizal *M. truncatula*. Herbivory by mixed age pea aphids (*Acyrthosiphon pisum*) on non-mycorrhizal and mycorrhizal *Medicago truncatula* plants in two independent experiments (low and high level of *Rhizophagus irregularis* root colonization). *Colonization level post aphid feeding. Student’s *t* tests were performed. One-way ANOVA was followed by the Tukey-Kramer test when significant (**α** = 0.05; statistically significant *P* value is shown in bold). Different letters indicate significant difference among treatments. Values represent the mean of six biological replicates ±SE.

**Figure 3. Heatmap of fold change in gene expression in shoots after seven days of herbivory by pea aphids (*Acyrthosiphon pisum*) of the same age on non-mycorrhizal and mycorrhizal *Medicago truncatula* plants in two independent experiments (low and high level of *Rhizophagus irregularis* root colonization; experiments A and B in Table 1). -AMF = non-infested, non-mycorrhizal *M. truncatula* (used in calculations), +AMF = non-infested, mycorrhizal *M. truncatula*, +AMF +Aphid = pea aphid-infested, mycorrhizal *M. truncatula*, and -AMF +Aphid = pea aphid-infested, non-mycorrhizal *M. truncatula*. The relative expression of each target gene was calibrated to the reference EF1α and calculated using the formula 2^ΔΔCq, where, ΔΔCq = ΔCq sample – ΔCq non-infested, non-mycorrhizal control (-AMF). Fold change reduction in gene expression was obtained by taking the negative inverse of 2^ΔΔCq. Values represent the mean of five and six biological replicates. *<0.05, **<0.01, and ***<0.001 denote significant differences among treatments at that level of *R. irregularis* colonization based on ANOVA.
less weight when they encountered mycorrhizal M. truncatula plants at low levels of R. irregularis root colonization when the feeding period started (Figure 2), while the weight of same age aphids was not affected by mycorrhizal status (Figure 1). It is possible that the aphid weight loss after feeding in low-AM fungus colonized plants was not consistent because of plant age differences in experiments A and C (Table 1) and/or aphid colony type (same age versus mixed age aphids). This is something that needs to be considered in future studies involving tripartite interactions. On the other hand, same age or mixed age aphids gained more weight when they fed on plants with high levels of R. irregularis root colonization (42%–67% RLC, Table 1, Figures 1 and 2). Previous research found a positive effect of the AM symbiosis on aphid adult weight (Gange and West 1994; Gange et al. 2002), percent growth (Gange et al. 2002), and aphid abundance (Babikova et al. 2014a), however, in most cases colonization levels were high. Few studies involving tripartite interactions specifically asked whether the extent of AM symbiosis had an effect on aphids. It was not until recently that it was demonstrated that green peach aphid’s (Myzus persicae Sulzer) relative growth rate was reduced when insects fed on mycorrhizal Plantago lanceolata L. plants that exhibited 10% RLC (Tomczak and Müller 2017). On the other hand, aphids were positively affected by feeding on mycorrhizal plants at 80% RLC (Tomczak and Müller 2017). It is worth noting that the results from our study and Tomczak and Müller’s are similar whether a generalist aphid M. persicae, or a legume specialist aphid, A. pisum, is used. It was shown recently that AM fungi alter plant tolerance to herbivores and chemical defenses simultaneously (Tao et al. 2016).

Plant tolerance increases with increased foliar P levels, whereas chemical defenses (in milkweed) increase with increased foliar N levels. Chemical defenses can increase or decrease based on plant growth, which can also be altered by AM symbiosis (Tao et al. 2016).

To our knowledge, this is the first study that investigates whether plants with varying levels of AM fungus colonization exhibit differential expression of defense-related genes against aphids. The only published work that measured gene expression in leaves (specifically) impacted by AM symbiosis, assessed transcript levels of four genes in tomato (Solanum lycopersicum L.) damaged by larvae of the chewing insect Heliocoverpa arimigera Hübner (Song et al. 2013). In this study, a single colonization level (51%–54% in wild-type tomato) was used and was recorded only after the three-day feeding period ended. It was found that leaves of insect-damaged mycorrhizal plants experienced stronger induction of defense-related genes (LOXD, AOC, PI-I, and PI-II) relative to non-mycorrhizal plants (Song et al. 2013). In the present study, transcript levels of ten to twelve genes reported as induced by insects, (Gao et al. 2007; Kusnierczyk et al. 2011; Verhage et al. 2011) or AM symbioses (Lopez-Raez et al. 2010; Floss et al. 2013) were measured in roots and shoots focusing on phytohormone biosynthesis and signaling, and defenses.

JA has been proposed as a key player in mycorrhiza-induced resistance; therefore, we focused our attention on the oxylipin pathway (Jung et al. 2012; Song et al. 2013). Plant defenses against insects and necrotrophic pathogens are known to be regulated by two distinct branches of the JA signaling pathway. The transcription factor MYC2 (MYC2-branch) and the APETALA2/ETHYLENE
RESPONSE FACTOR (AP2/ERF) domain transcription factors ORA59 (ERF-branch) regulate these two branches (De Vos et al. 2005; Verhage et al. 2011). Our data revealed a modest increase in shoot transcript levels of genes involved in oxylipin biosynthesis (LOXA and DES transcripts in +Aphid treatments, Figure 3), and in terms of JA signaling, the MYC2-branch did not seem to be involved, at least, after seven days of feeding (Figure 3).

We identified genes whose expression patterns are modulated by aphid feeding in shoots. Interestingly, we found that aphid feeding triggered downregulation of GA20ox and GA2ox transcripts in shoots in the high level of AM fungus colonization experiment (Figure 3). GA 20-oxidases (GA20ox) are involved in the synthesis of bioactive gibberellins (GA1, GA3, GA4, and GA7), while GA 2-oxidases (GA2ox) catalyze GA inactivation reactions. When GA is absent, DELLA proteins repress GA responses (e.g. germination and stem elongation), but when GA is present, it binds to the receptor GID1, which induces DELLA degradation, leading to GA responses (Schweighheimer 2012). DELLA proteins have been found to promote susceptibility to biotrophs and resistance to necrotrophs (dependent on JA) by altering the balance of SA/JA signaling (Navarro et al. 2008). Surprisingly, the role of GA in plant-aphid interactions is mostly unknown, and this is the reason we included this pathway in our study.

A proposed model of interactions between plant hormones in microbe-plant-insect interactions indicates that ‘plant growth promotion’ is regulated by GA, brassinosteroids, cytokinin, and auxin, whereas ‘induced systemic resistance’ is mediated by JA, SA, and ET (Pangesti et al. 2013). In the present study, aphid feeding might be reducing gibberellins in shoots leading to reduced growth. Alternatively, the plant might be reducing gibberellins as a defense mechanism to reduce its quantity of biomass available to aphids, or to shift the balance towards producing defenses. Nevertheless, the role of GA in aphid-plant-AM fungus interactions would be a promising area of further study.

We found that ERF1 transcript levels increased in shoots of aphid-infested plants compared to those of high AM fungus-colonized plants without insects (Figure 3). It is well documented that ERF1 is a key element in the integration of ET and JA signals for the regulation of defense genes (e.g. b-CHI and PDF1.2) against pathogens (Lorenzo et al. 2003), although, upregulation of ERF1 transcripts has also been reported in bluegreen-aphid-infested M. truncatula plants (Gao et al. 2007). Surprisingly, we found that transcript levels of SA-responsive defense genes (BGL and PR5) remained relatively high in shoots, even after seven days of aphid feeding, regardless of the level of AM fungus colonization (Figure 3). Our seven-day post-feeding results agreed with previous two-way interaction data showing that BGL and PR5 transcripts remained high after 72 h of bluegreen aphid infestation of M. truncatula plants compared to uninfested plants (Gao et al. 2007).
We also identified genes that are regulated by AM symbiosis alone, and aphid feeding combined with AM fungus colonization in roots (Figure 4). AM symbiosis triggered downregulation of OPR transcripts in roots of low AM fungus-colonized plants, perhaps as a way to reduce JA levels to promote fungal colonization (Jose Herrera-Medina et al. 2008). GA20ox transcript levels increased in roots from mycorrhizal plants regardless of the level of AM fungus colonization (Figure 4); although the highest accumulation of GA20ox transcripts occurred in roots from aphid-infested high AM fungus-colonized plants (Figure 4). Both of these genes have been shown to be induced in roots during plant-AM fungus interactions (Floss et al. 2013). It is possible that bioactive gibberellins are needed at different stages of development of AM symbiosis and/or as plants age. We found a unique expression pattern of the GA2ox gene in roots. Aphid feeding combined with AM fungus colonization triggered a modest accumulation of GA2ox transcripts, while high AM fungus-colonized plants and aphid-infested non-mycorrhizal plants showed downregulation of these transcripts (Figure 4). GA 2-oxidases are known to regulate plant growth by inactivating endogenous bioactive gibberellins (Figure 4). GA 2-oxidases are known to regulate plant growth by inactivating endogenous bioactive gibberellins (Figure 4). Given that we did not find differences in root weight, it is possible that the plant is trying to balance bioactive gibberellins in roots by inducing GA2ox transcripts.

We chose to use apterous aphids for the choice bioassay because there are no data available and apterous aphids have been used to determine deterrence (Pegadaraju et al. 2007). At least within a 72-h period, aphid adults preferred mycorrhizal plants at high levels of R. irregularis colonization compared to non-mycorrhizal plants (Figure 5(b)). More nymphs (progeny) were present on non-mycorrhizal plants compared to mycorrhizal plants at low levels of R. irregularis colonization (Figure 5(c)). It was shown previously that pea aphid abundance increased on mycorrhizal V. faba plants, and it was attributed to reduced release of defense-related sesquiterpene volatiles which made plants more attractive to aphids (Babikova et al. 2014a). A separate study indicated that increased Pi uptake by mycorrhizal plants was not the main mechanism causing attractiveness of pea aphids to plants (Babikova et al. 2014b). A recent study found that alate English grain aphids (Sitobion avenae Fabricius) were more attracted to mycorrhizal wheat (T. aestivum) variety Solstice (aphid-susceptible) compared to the control (Simon et al. 2017). It is tempting to speculate that the foliar anatomical changes (increases in the thickness of leaves, size of midrib vein, mesophyll cells, number of plastids, etc.) and increased insoluble polysaccharides and proteins in vascular regions of mycorrhizal plants (Krishna et al. 1981) could alter aphid feeding behavior. In fact, it was shown recently that AM fungi had a significant impact on feeding behavior...
as aphids fed on average longer, and spent a larger proportion of the overall time within the phloem (Simon et al. 2017). It was proposed that the changes in aphid feeding behavior are likely associated with increased vascular bundle size in mycorrhizal plants (Simon et al. 2017).

Conclusion
In conclusion, the data did not fully support the hypotheses that aphid-infested mycorrhizal plants would show significantly enhanced accumulation of target defense transcripts in shoots, and that prolonged feeding would affect R. irregularis root colonization in low AM fungus-colonized plants (Figures 6 and 7). Our future studies would focus on changes in gene expression occurring in less than seven days of feeding, now that we have a better understanding of how aphid fitness is affected by AM symbiosis. In addition, we identified two genes, GA20ox and GA2ox, which showed unique gene expression patterns in roots during this tripartite interaction (Figure 6). We found that low AM fungus-colonized plants are potentially ‘primed’ by fungus colonization leading to reduced weight in mixed age aphids (Figure 7(A)). In general, shoots of aphid-infested plants weighed less compared to shoots of mycorrhizal plants regardless of the level of AM fungus colonization (Table 3). Root weight was mostly not altered by aphid feeding and/or AM symbiosis. Additionally, the data partly support the hypothesis that pea aphids would show a preference for non-mycorrhizal plants compared to mycorrhizal plants. In this case, nymphs preferred to feed on non-mycorrhizal plants relative to low AM fungus-colonized plants, whereas adults showed a clear preference for high AM fungus-colonized plants relative to non-mycorrhizal plants.

We provided new insight in an interaction that has yielded conflicting reports on the effects of AM fungi on plant-aphid interactions. Further research is still needed to comprehend the underlying molecular mechanisms involved in mediating resistance or susceptibility to aphids. A recent study measured three phytohormones (JA, SA, and ABA) during M. truncatula-pea aphid interactions at 0, 12, 24, and 48 h post feeding (Stewart et al. 2016), but this type of information is lacking in tripartite interactions. Thus, our future studies would address early changes in plant transcriptome to have a better understanding of the underlying mechanisms involved in mycorrhiza-induced resistance and susceptibility against aphids. This information would help us understand how plants manage their resources to invest in growth and defense in the presence of both, insects and AM fungi.

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