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REGULAR PAPER

Effects of arbuscular mycorrhizal fungi on herbivory defense in two *Solanum* (Solanaceae) species

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Background and aims – Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil organisms that interact with plant roots and exchange soil nutrients for plant-derived carbohydrates, frequently resulting in growth and fitness benefits for plants. These benefits may be due partly to AMF effects on plants' resistance to insect herbivores, particularly through enhancement of induced defenses. Here we studied the impacts of AMF colonization on constitutive and induced resistance in two species of nightshades, *Solanum ptycanthum* and *S. dulcamara.*

Methods – We used a factorial design manipulating AMF presence and jasmonic acid application to determine if constitutive and induced resistance differ in the presence and absence of mycorrhizae. We measured three protein-based chemical defenses and performed a bioassay by feeding leaves of experimental plants to a specialist herbivore, *Manduca sexta.*

Key results – The presence of AMF influenced chemical defenses in *S. dulcamara,* including an interaction with jasmonic acid application for polyphenol oxidase activity. *Solanum ptycanthum* defenses were unaffected by AMF. Caterpillar growth was also unaffected by AMF but reduced by jasmonic acid treatments, indicating that, while AMF may influence certain chemical defenses in some plant species, this does not always translate to resistance against herbivores.

Conclusions – Our results emphasize the context dependency of fungi–plant–herbivore interactions and suggest that mycorrhizal effects on plant defense may vary with other plant traits or life history strategies.

Key words – Arbuscular mycorrhizal fungi, induced defense, herbivore, indirect effects, aboveground, belowground, *Solanum ptycanthum*, *Solanum dulcamara*.

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are abundant soil organisms that have wide-ranging impacts on plants and ecosystems. About 80% of land plants form symbiotic relationships with AMF (Schüßler et al. 2001), and these associations can affect plant growth, fitness, and community diversity (Klironomos et al. 2000, Smith & Read 2008, Jung et al. 2012). AMF hyphal networks reach parts of the surrounding soil that plant roots cannot and transfer nutrients, such as phosphate and nitrogen, to the plant in exchange for carbohydrates synthesized during photosynthesis (Smith & Smith 2011). The general increase in plant fitness due to AMF was historically attributed solely to the improved nutrition AMF provide (Pfleger & Linderman 1994), but more recent studies have found that plants colonized by AMF undergo physiological changes that alter their resistance to above- and belowground antagonists (Pozo et al. 2002, Koricheva et al. 2009, Song et al. 2013, Vannette et al. 2013, Cameron et al. 2013).

AMF may influence plant defenses by interacting with the jasmonic acid (JA) pathway (Pozo & Azcón-Aguilar 2007). Jasmonic acid is an essential hormone in the immune system of plants (Bari & Jones 2009, Pieterse et al. 2009), and when a plant is attacked by an insect herbivore, JA is produced, signaling cells to begin production of defensive compounds (Pozo et al. 2004). In many plants, this induced resistance includes the general defensive chemicals peroxidase (POD), polyphenol oxidase (PPO) and protease inhibitors (PI) (Farmer & Ryan 1992, Constabel & Ryan 1998, Moore et al. 2003), enzymes that have been shown to have inhibitory effects on insect herbivores. In *Populus* spp., POD inhibits herbivore growth by oxidizing phenols in the guts of caterpillars, damaging amino acids and denaturing proteins

(Barbehenn et al. 2010). The mechanism of PPO is not well studied, but it is thought to play a role in defense because of its induction by herbivores. Quinones produced from PPO may react with essential amino acids, causing a decrease in the nutritional value of the plant. Alternatively, PPO may oxidize phenols similarly to POD (Constabel & Barbehenn 2008). Application of JA to tomato (*Solanum lycopersicum* L.) increased PPO activity, and these plants also reduced growth of the caterpillar *Manduca sexta* (Linnaeus, 1763), a Solanaceae specialist and the bioassay herbivore used in this study (Cipollini & Redman 1999). Protease inhibitors interfere with the ability of caterpillars to digest protein by inhibiting protease enzymes in their gut (Green & Ryan 1972). Recently four serine PIs, isolated from *Solanum nigrum* L., were shown to decrease the growth of generalist caterpillars (Hartl et al. 2010), but effects on *M. sexta* are mixed: Hartl et al. (2010) concluded that proteinase inhibitors in *S. niger* do not affect growth, while Bosch et al. (2014) supported a role of induced PIs in conferring resistance against *M. sexta*. Taken together, the primary action of these defensive enzymes is to inhibit the ability of herbivores to obtain nutrients from their host plants, but their effects on herbivores, including *M. sexta*, in experiments seems variable.

Colonization of plant roots by AMF triggers a JA response which may cause the plants to become sensitized to attack by a pathogen or herbivore (Jung et al. 2012). This is not surprising given that the process of colonization results in a plant transcriptional response with extensive overlap to attack by various plant antagonists (Paszkowski 2006). In *Medicago truncatula* Gaertn. (Fabaceae), a build-up of transcription factors regulates the JA pathway upon colonization; however, the defenses are not actually activated until an attack (Van der Ent et al. 2009). This priming response has been shown in multiple species including carrot (*Daucus carota* L.), date palm (*Phoenix dactylifera* L.), and tomato (Pozo et al. 2010, Jung et al. 2012, Song et al. 2013). In priming the plant, AMF may help to amplify the immune response when a wound is detected and cause a greater amount of defensive chemicals to be synthesized.

Although mycorrhizal effects on induced resistance have been shown in several plant species, the outcomes of these interactions on both plants and herbivores are poorly understood and have produced contradictory results (Bennett et al. 2009, Kempel et al. 2010). The objective of this study was to investigate the effects of AMF on the induced defense response in two species of *Solanum* (Solanaceae). Although these species are evolutionarily related, they may not necessarily respond to AMF in the same way. In a study of mycorrhizal growth effects, congeneric host plants frequently showed strong differences in response to AMF colonization (Wilson & Hartnett 1998). We quantified induction of three defensive chemicals and growth of an insect herbivore in plants with and without AMF.

MATERIAL AND METHODS

Study species

Solanum ptycanthum Dunal (eastern black nightshade) is a widespread herbaceous native annual throughout North America. *Solanum dulcamara* L. (bittersweet nightshade) is a woody perennial vine, native to Eurasia and introduced across much of North America. In a comparative study of induced defenses, *S. dulcamara* displayed a moderately strong induced response to *M. sexta* herbivory compared to other Solanaceae (Campbell & Kessler 2013). Although *S. ptycanthum* was not included in this study, the closely related *S. nigrum*'s response was similar to *S. dulcamara*. Both *S. dulcamara* and *S. nigrum* are also colonized by AMF (Harley & Harley 1987), and previous trials had demonstrated that *S. ptycanthum* was readily colonized as well. Despite these similarities and their close relationship, the different growth forms of *S. ptycanthum* and *S. dulcamara* (herbaceous and woody vine, respectively) and the knowledge that congeners can differ in mycorrhizal response (Wilson & Hartnett 1998) suggested the possibility that AMF effects on induced resistance could differ, justifying a comparative study between the two species. Seeds of both species were obtained locally in DeKalb, IL, USA.

Defensive chemistry experiment

We used a factorial experimental design for each species, manipulating AMF presence and JA application. Seeds from each species were sterilized in 5% bleach solution and germinated on damp filter paper in a sterile petri dish in a growth chamber (14 h:10 h light:dark, 30°C day, 25°C night). Each germinated seedling was planted in a 473 mL pot of autoclaved soil obtained from a lawn outside the greenhouse where the study took place. Each pot then received either 50 mL of unsterilized soil containing root fragments from *S. ptycanthum* previously inoculated with *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C.Walker & A.Schüßler (formerly *Glomus intraradices* N.C.Schenck & G.S.Sm.) in gel suspension (Mycovitro, Grenada, Spain), or 50 mL of autoclaved soil from the same source. The previously-inoculated plants had been cultivated to produce sufficient inoculum for the experiment. We note that this soil also contained other microorganisms, but we expect these to be similar to what naturally colonized the experimental plants because we used the same soil source and the same greenhouse setting. The 50 mL soil was mixed into the top layer of the pot before transplanting took place. *Rhizophagus irregularis* is a generalist species that colonizes a wide variety of plant hosts (Öpik et al. 2006). Pots were placed in a greenhouse in blocks of four with each block containing two AMF+ and two AMF- plants. Plants received 14 h supplemental light in the greenhouse, and the greenhouse temperature was kept above 21°C. The blocks were rotated weekly and each pot was watered as needed with distilled (DI) water three times per week. Three weeks after the seedlings were planted, each AMF+ pot had an additional 1 mL of AMF inoculum (*R. irregularis* in gel suspension) injected into the soil at the base of the plant to ensure AMF presence. Each sterile pot received 1 mL of DI water. Although we did not collect roots from plants in this experiment, other trials have shown that both *Solanum* species are readily colonized by this *R. irregularis* inoculum and the inoculum used in the herbivore growth experiment below within four weeks (mean ± 1 s.e., 5.5 ± 2.6% colonization, vs. 0% in uninoculated plants, N. Barber, unpubl. data). Final sample sizes, following the death of several plants, were 41

Table 1 – Results of GLMM analyses of experimental treatments.
Models analysed activity of peroxidase (POD), polyphenol oxidases (PPO), and protease inhibitors (PI) in *Solanum ptycanthum* and *S. dulcamara,* and growth of *M. sexta* bioassay caterpillars. Fixed factors were evaluated using single degree of freedom likelihood ratio tests compared to a χ^2 distribution. Factors with $P < 0.05$ are shown in bold.

	Solanum ptycanthum							
	POD		PPO		PI		Herbivore mass	
	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
JA	**3.88**	**0.049**	–	–	1.56	0.211	**10.96**	**< 0.001**
AMF	0.18	0.672	–	–	0.11	0.738	1.95	0.163
AMF × JA	1.56	0.212	–	–	1.90	0.168	2.57	0.109
	Solanum dulcamara							
	POD		PPO		PI		Herbivore mass	
	χ^2	*P*	χ^2	*P*	χ^2	*P*	χ^2	*P*
JA	0.68	0.410	–	–	3.05	0.081	3.64	0.057
AMF	**7.56**	**0.006**	–	–	**4.83**	**0.028**	2.51	0.113
AMF × JA	2.98	0.085	**7.28**	**0.007**	1.96	0.161	2.54	0.111

S. ptycanthum (20 AMF+, 21 AMF-) and 20 *S. dulcamara* (11 AMF+, 9 AMF-).

Six (*S. ptycanthum*) or eight (*S. dulcamara*) weeks after the seedlings were planted, approximately half of the plants from each AMF treatment were treated with jasmonic acid (JA) to induce a defensive response (*S. ptycanthum*, n = 10 AMF+, 10 AMF-; *S. dulcamara,* n = 6 AMF+, 5 AMF-). Pure JA (Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in 250 µL of acetone and added to 119 mL of DI water to make a 10 mM solution that was diluted to 1 mM in a spray bottle (Thaler et al. 1996). A control spray bottle contained DI water and a trace amount of acetone. Plants were sprayed with either JA solution or DI water until all leaves were coated with spray, and treatments were kept separated during spraying and until plants dried to keep JA solution from drifting to control plants. Two days after the JA treatment, two 0.1 g fresh leaf samples were excised from each plant for chemical assays and stored at -80°C.

We measured activity of POD, PPO, and PI following methods of Thaler et al. (1996). POD and PPO activity was measured as change in absorbance over 60 s per g fresh leaf tissue (ΔOD/min/g), and PI was measured as percent inhibition of protease activity by the presence of leaf extract. PPO assays were not performed for *S. ptycanthum* because of limited leaf sample tissue. We used generalized linear mixed models (GLMMs) to analyze POD, PPO, and PI activity, treating JA treatment, AMF treatment, the JA × AMF interaction as fixed factors, and block as a random factor. Models assumed Gaussian error distribution and identity link function, and fixed factors were evaluated using likelihood ratio tests, starting with the interaction term. If the interaction term was significant, we did not test main effects. All analyses were carried out in R (R Development Core Team 2012) using the nlme() package.

Herbivore growth experiment

To determine how AMF induced response affected an insect herbivore, we repeated the previous experiment and fed leaves from experimental plants to *Manduca sexta* (Lepidoptera: Sphingidae), a Solanaceae specialist native to North America. Throughout its wide range, *M. sexta* feeds on a variety of plants in the genus *Solanum* including the two plants in this study (Madden & Chamberlin 1945, Yamamoto & Fraenkel 1960). Its growth can also be affected by the defensive proteins analyzed above (Cipollini & Redman 1999, Bosch et al. 2014), making it an appropriate bioassay organism.

The experiment was carried out identically as above, including the same soil source, except that there were 36 *S. ptycanthum* and 40 *S. dulcamara*, each with half of the plants inoculated with *R. irregularis* on a perlite carrier (Myke, PremierTech, Rivière-du-Loup, QC, Canada) because gel inoculum used above was unavailable. Two days after JA treatments were applied, the most recently grown leaf (or two if leaf was < 4 cm long) was excised through the petiole with a razor blade and placed in a petri dish containing moistened filter paper and resting on an inverted thumb tack to keep leaves elevated, so caterpillars could feed on either side of the leaf (Campbell & Kessler 2013). Eggs of *M. sexta* (Carolina Biological Supply Co., Burlington, NC, USA), were incubated in a growth chamber to hatch synchronously so that caterpillars were the same age and size at the start of bioassays. A single second-instar *M. sexta* was added to each petri dish and allowed to feed on the leaves for 48 hours (Campbell & Kessler 2013) in the growth chamber (14 h:10 h light:dark, 30°C day, 25°C night). The caterpillars were removed from the leaves for 15 hours to void their gut before each caterpillar's wet mass was recorded. We analyzed final caterpillar mass using GLMs as above.

RESULTS

Defensive chemistry experiment

Treatments affected defenses in both plant species. In *S. ptycanthum* JA induced POD activity (table 1, fig. 1A) but did not significantly change levels of PI activity (fig. 1B). AMF

did not affect defenses, and there was no interaction between the two treatments.

In *S. dulcamara*, there was a significant interaction between AMF and JA for PPO and a marginally significant interaction for POD (table 1, fig. 2), but the pattern of interaction was different for these responses. AMF increased POD response, and the response may be marginally stronger with JA application (fig. 2A). But JA application reduced PPO activity when AMF was absent and had no effect on AMF+ plants (fig. 2C). The pattern for PI activity was similar to POD, but the interaction was not significant; JA increased PI activity, while AMF had a marginal effect (table 1, fig. 2B).

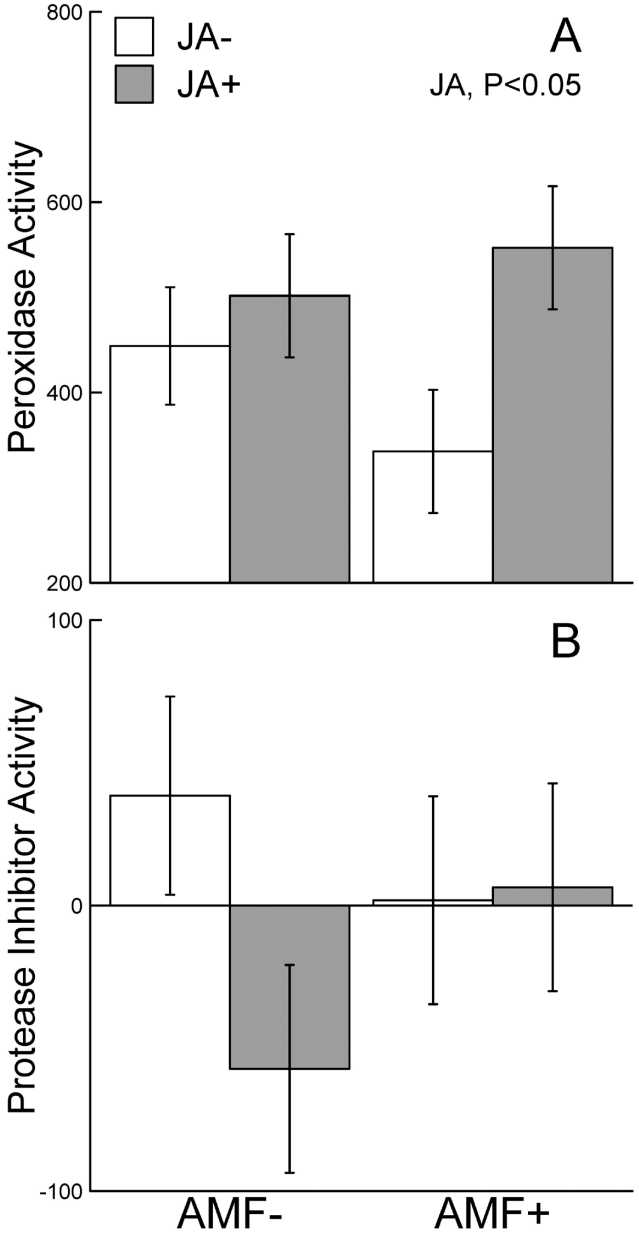


Figure 1 – Effects of AMF and jasmonic acid (JA) treatments on: A, peroxidase activity (POD); and B, percent protease inhibition (PI) in *Solanum ptycanthum*. Peroxidase activity is expressed in **Δ**OD/min/g leaf tissue, and values in both figures are based on estimated model coefficients ± 1 s.e.

Herbivore growth experiment

In *S. ptycanthum*, application of JA significantly reduced caterpillar mass, but AMF had no effect and there was no interaction (table 1, fig. 3A). Similarly, JA marginally reduced caterpillar mass when fed *S. dulcamara* but there were no other effects (fig. 3B).

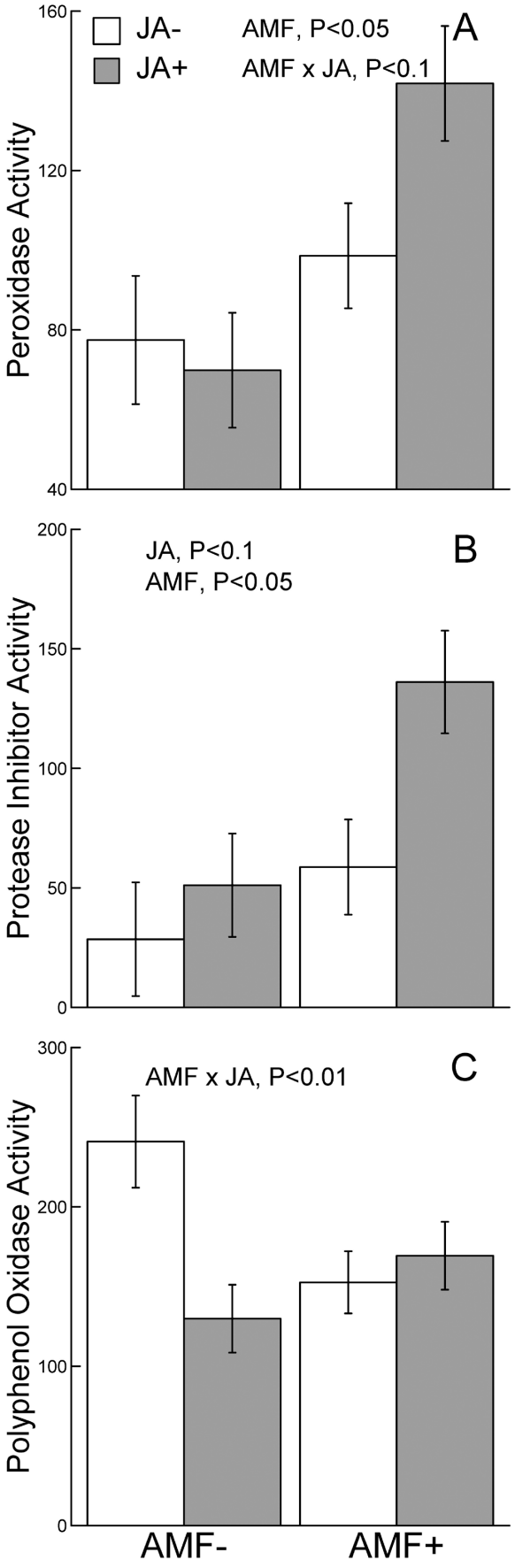
DISCUSSION

Colonization by AMF had variable effects on chemical defenses of *Solanum* spp., but colonization by AMF did not affect caterpillar growth. Application of JA to simulate herbivory and cause chemical defense induction affected chemical defense production and significantly reduced caterpillar growth in *S. ptycanthum*, with a trend in the same direction for *S. dulcamara*. Thus the effect of AMF on plant induced responses did not directly translate to increased resistance against herbivores, but AMF do have the potential to affect both constitutive and induced levels of defensive proteins, depending on the host plant species.

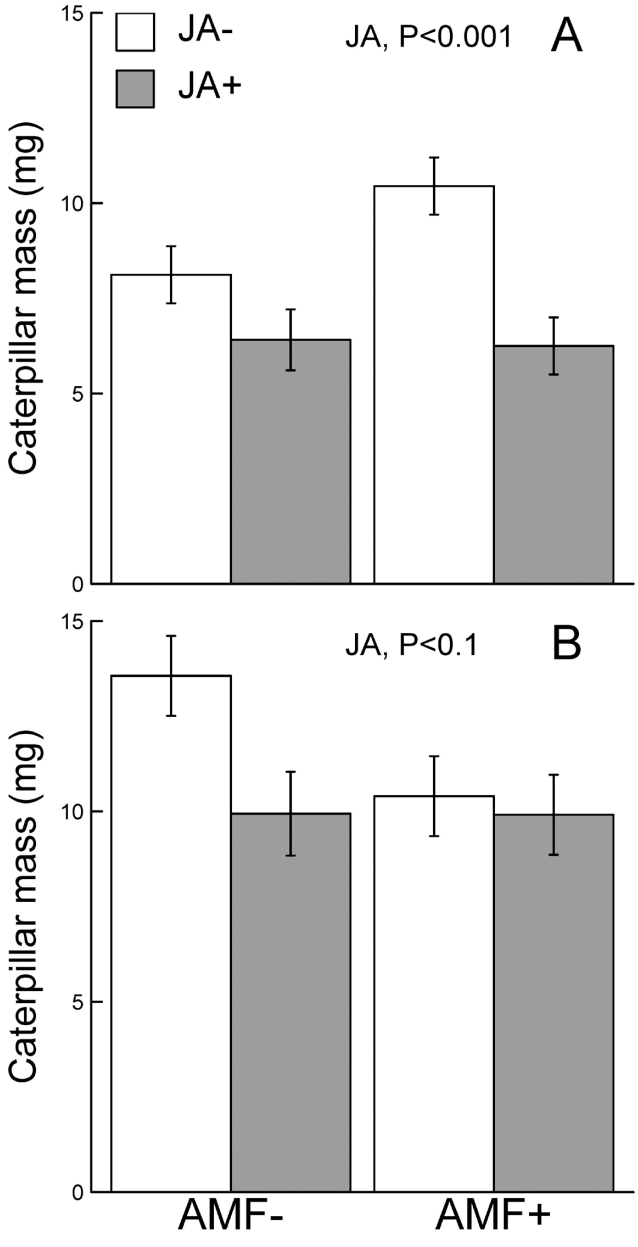
POD and PI responses in *S. dulcamara* to AMF were similar and followed predictions of AMF priming hypotheses, i.e., levels of both defenses were highest on induced plants with mycorrhizal colonization, although in neither case was the interactive effect significant. These results indicate that AMF can enhance plant defenses and, for POD, where there was a significant JA × AMF interaction, possibly enhance the induced response. This potential priming effect is consistent with studies in *S. lycopersicum* (tomato), in which transcription of PI genes following herbivory was greater in plants that had been inoculated with AMF than non-mycorrhizal tomato plants (Song et al. 2013).

JA application surprisingly reduced PPO in non-mycorrhizal *S. dulcamara* plants, and levels were similar for JA-treated and untreated mycorrhizal plants. In a previous study of tomato, PPO induction was strongest in response to feeding by the generalist herbivore *Spodoptera exigua* (Hübner, 1808), apparently due to a chemical elicitor in the insect's saliva (Bosch et al. 2014). The absence of this elicitor may partially explain the result of our study, but the elicitor was also absent in a tomato experiment that documented PPO induction by JA (Thaler et al. 1996). In two cases there was a main effect of JA on defenses with no mycorrhizal interaction: for POD in *S. ptycanthum* and PI in *S. dulcamara* the JA elicited an expected response with increased levels of defenses in JA-treated plants, which also does not support the AMF-priming hypothesis.

Despite AMF influences on defenses, caterpillar growth was unaffected by mycorrhizal inoculation. This contrasts a study of six different species representing several plant families, in which there was a consistent decrease in herbivore performance on plants inoculated with AMF after induction of defenses, with a significant interaction between AMF and induction similar to our results observed in several species (Kempel et al. 2010). In our study, JA treatments, however,



◄ **Figure 2** – Effects of AMF and jasmonic acid (JA) treatments on: A, peroxidase activity (POD); B, protease inhibitor activity (PI); and C, polyphenol oxidase activity (PPO) in *Solanum dulcamara*. Peroxidase and polyphenol oxidase activities are expressed in ΔOD/min/g leaf tissue. Values are based on estimated model coefficients ± 1 s.e.



▲ **Figure 3** – Effects of AMF and jasmonic acid (JA) treatments on growth of *Manducta sexta* bioassay caterpillars in: A, *Solanum ptycanthum*; and B, *S. dulcamara*. Values are based on estimated model coefficients ± 1 s.e.

did reduce caterpillar growth, and the effect was significant in *S. ptycanthum,* where JA application also triggered higher levels of POD activity. Herbivore growth was greater, and the JA effect on defenses was weaker, on *S. dulcamara*. Thus herbivore growth responses match treatment effects on POD in *S. ptycanthum* but do not correspond to defenses in *S. dulcamara.* The relative importance of these three chemical defenses for defense against herbivory, and their responses to other biotic interactions, appear to differ, even among the congeneric plant species in this study.

The fact that AMF inoculation affected the defenses measured here in *S. dulcamara* but did not influence herbivore growth suggests that additional resistance mechanisms may be important for *M. sexta* when feeding on *S. dulcamara*. Solanaceae produce secondary compounds such as alkaloids (Evans 1979), which we did not measure. However, *M.sexta* is a Solanaceae specialist and has high tolerance for alkaloids such as nicotine (Wink & Theile 2002). Although using an herbivore bioassay provides a more comprehensive assessment of resistance than measuring individual plant traits, growth effects alone may not reflect all resistance mechanisms. For example, protease inhibitors may also affect herbivores' abilities to avoid predation, enhancing indirect defenses (Schuman et al. 2012), which would not be apparent in our greenhouse study.

Mycorrhizal symbiosis influenced defenses of *S. dulcamara*, but we found no effect on the closely related *S. ptycanthum.* Clearly AMF effects on constitutive and induced plant defenses are species-specific, emphasizing the context dependency of direct and indirect interactions between soil fungi, plants, and insect herbivores and the necessity of further study with a variety of plant species, both among and within plant families (Gehring & Bennett 2009). Furthermore, these opposing effects may result from differences in functional traits, as variation in plant traits can be related to differential colonization by AMF in other species (Wilson & Hartnett 1998, Reinhart et al. 2012). While *S. dulcamara* and *S. ptycanthum* belong to closely related clades (Weese & Bohs 2007), they have different growth forms: *S. dulcamara* is a perennial semi-woody vine, whereas *S. ptycanthum* is an annual herbaceous forb. Perennial species often show stronger responses to AMF than annual plants, and mycorrhizal diversity may be greater in their roots (Gange et al. 1993, Alguacil et al. 2012). Plants with contrasting growth forms, such as herbaceous or woody, also have been shown to differ in their responsiveness to AMF, with woody and perennial species frequently exhibiting greater growth benefits from mycorrhizal association (Wilson & Hartnett 1998, Pérez & Urcelay 2009).

The divergent traits and life history strategies displayed by *S. dulcamara* and *S. ptycanthum* may be associated with dissimilar interactions with herbivores and different responsiveness to AMF, contributing to the differences that we observed. In the future, studying mycorrhizal effects on plant defense in multiple species, accounting for phylogeny (Pagel 1999), would make it possible to determine if particular plant life history traits are associated with increased chemical defenses or priming.

Members of Solanaceae represent important agricultural crops, such as tomato, potato, eggplant, and peppers. These and other solanaceous crops occupy tens of millions of hectares of cropland (Samuels 2015). Yields in these crops are frequently reduced by insect pests, and effective pest control can require large inputs of insecticides (Alyokhin et al. 2008). Continuing studies to understanding how AMF affect resistance against antagonists in both wild and domesticated Solanaceae species may help in the development of novel integrated pest management strategies that incorporate microbial symbionts as a way to minimize the use and negative side effects of chemical pesticides (Burketová et al. 2015).

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