100 YEARS Journal of Ecology

British Ecological Society

Journal of Ecology 2013, **101**, 1019–1029 doi: 10.1111/1365-2745.12111

Mycorrhizal abundance affects the expression of plant resistance traits and herbivore performance

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Summary

1. The effects of mutualistic interactions on partner phenotype and fitness can vary with many factors, including the abundance of interacting partners. Partner abundance may determine the relative costs and benefits associated with the interaction. Although arbuscular mycorrhizal fungi (AMF) can strongly influence plant phenotype and community interactions, the effects of AMF abundance on plant resistance traits and multitrophic interactions are not well understood. We tested the hypothesis that increasing AMF abundance in soil will increase mycorrhizal colonization and affect plant biomass, foliar phosphorus concentration, the expression of plant resistance and herbivore performance.

2. We inoculated *Asclepias syriaca* seedlings with *Glomus etunicatum*, *Scutellospora fulgida* and a mix of the two species in 11 AMF abundance treatments. We quantified plant phosphorus (P), growth and resistance phenotype and the performance of a specialist herbivore, *Danaus plexippus* on plants associated with varying amounts of fungi.

3. Increasing abundance of *S. fulgida* or *G. etunicatum* in soil increased the proportion of plant root colonized by AMF, but root colonization by a mix of the fungi was not related to inoculum density. The abundance of *S. fulgida,* but not *G. etunicatum*, increased per cent foliar P and trichome density, but decreased latex exudation. Abundance of all AMF treatments tended to decrease specific leaf mass (SLM), and the two single-species treatments unimodally affected the expression of total foliar cardenolides. Increasing abundance of the mix of AMF species also increased above-ground biomass, foliar P and trichome density, but had little effect on other traits. The presence of AMF, species identity and the AMF abundance all explained significant variation in the expression of plant traits, although their relative contribution varied depending on the trait examined. Mycorrhizal abundance strongly increased caterpillar growth rate, which was associated with a decline in SLM.

4. Synthesis. Variation in mycorrhizal abundance can profoundly influence the expression of plant resistance and subsequent herbivore performance. AMF abundance may be a key, but overlooked factor in determining the outcome of mycorrhizal mutualisms.

Key-words: above-below-ground interactions, cost/benefit, milkweed, multitrophic interactions, mutualism, plant–herbivore interactions, resource exchange model of plant defence

Introduction

Mutualisms are considered key interactions in structuring ecological communities (Bronstein 1994a; Stachowicz 2001). Despite their potential importance, the outcome of such interactions can range from mutualism to parasitism, depending on many factors including partner presence and abundance, partner identity and environmental context (Bronstein 1994b). Determining the relative importance of these factors may allow us to better predict the effects of mutualisms for interacting partners and ecological communities.

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More than 90% of plant species associate with mycorrhizal fungi in putatively mutualistic interactions that can profoundly affect fungal and plant nutrition, growth and subsequent plant–plant interactions, community composition (van der Heijden *et al.* 1998; Hartnett & Wilson 1999) and plant–consumer interactions (Gehring & Whitham 1994; Gange 2007). We focus here on arbuscular mycorrhizal fungi (AMF, Phylum Glomeromycota), common below-ground symbionts that associate with a majority of plant species examined to date. Plant hosts supply photosynthate to fungi and, in return, are provided with resources gathered from the soil, most often phosphorus (P), micronutrients and water (Smith & Read 2008).The majority of research on AMF associations has been

gathered from experiments that manipulate the presence or absence of symbiotic microbes. However, plants in natural systems are rarely, if ever, free of mycorrhizal symbionts: only nonmycotrophic plants and mycorrhizal plants growing in severely disturbed areas are ever found without mycorrhizal associates (Miller 1979). Instead, most plants vary in the degree of association with and the identity of below-ground symbionts (Gange & Ayres 1999).

Plant benefits gained from mycorrhizal partners vary widely due to a range of factors, including fungal community composition, soil nutrient levels, plant competition, etc. (Johnson, Graham & Smith 1997; Hoeksema *et al.* 2010 and references therein). Certainly, the presence or absence of fungal taxa can dramatically affect plant performance (Klironomos 2003), and nutrient availability can similarly modulate the outcome of AMF interactions (Treseder & Allen 2002). However, variation in mycorrhizal abundance is considered far less frequently than these variables, despite theoretical and empirical work which suggests that partner density may be a key factor in mutualisms more generally (Anderson & Midgley 2007; Chamberlain & Holland 2009; Morris, Vazquez & Chacoff 2010).

Indeed, mycorrhizal inoculum potential and root colonization by AMF vary on multiple scales, from within single habitats (Koide & Mooney 1987) to broad spatial scales (Lekberg & Koide 2005). For example, AMF spore abundance can vary on metre scales (Carvalho *et al.* 2003) and landscape scales, with variation in land use history (Boerner, DeMars & Leicht 1996). Mycorrhizal inoculation potential and subsequent colonization of indicator plants can also vary on very small (cm) scales (Wolfe *et al.* 2007). While not many studies have directly generated a range of mycorrhizal abundance to address its functional consequences (but see Carling, Brown & Brown 1979; Khan 1988; Garrido *et al.* 2010), mutualism theory provides a cost/ benefit framework for understanding how variation in mycorrhizal abundance may affect plants and their interactions with other organisms (Koide & Elliott 1989; Bronstein 1994b). Additionally, the effects of mycorrhizal abundance on species interactions are complicated by the fact that some plants exert significant control over the fungal colonization that results from a given level of inoculum (Vierheilig 2004; Grman 2012). It is therefore important to explore the relationship between inoculum availability and subsequent levels of colonization to understand the effects of mycorrhizal abundance on plant traits and species interactions.

Although previous research suggests that the abundance of mycorrhizal fungi alters plant performance (Clapperton & Reid 1992; Gange & Ayres 1999), the direct and indirect effects of mycorrhizal abundance on plant interactions with other organisms are poorly understood in large part because most recent studies of the ecological effects of AMF continue to rely on presence/absence manipulations of these fungi. Plant–consumer interactions may be strongly affected by variation in mycorrhizal abundance because mycorrhizal fungi influence a suite of traits important to herbivore performance (Vannette & Hunter 2011a). These include plant nutrient content (Borowicz 1997; Goverde *et al.* 2000), tolerance (Bennett & Bever 2007) and resistance to herbivores (Gange & West 1994; Thamer *et al.* 2011). In particular, recent work has emphasized that plant P content can be limiting for herbivores (Elser *et al.* 2000, Huberty & Denno 2006) and that herbivores may respond to microbially induced changes in chemical or physical defence, depending on their feeding mode and degree of specialization (Hartley & Gange 2009). However, the relative roles of changing nutrition and resistance in plant protection and herbivore performance remain elusive, as changes in plant resistance and nutrition in response to symbionts are often confounded or not quantified simultaneously.

Here, we address five hypotheses. First, increasing AMF abundance in the soil will increase mycorrhizal colonization of plant roots. Second, higher mycorrhizal abundance will also improve plant phosphorus nutrition and affect nonlinearly the expression of plant resistance. The expectation of nonlinear responses emerges from the assumption of saturating benefits with increasing partner density as costs continue to rise (Bronstein 1994b; Vannette & Hunter 2011a). Third, changes in plant phosphorus and resistance mediated by increasing AMF abundance will influence the performance of a specialist insect herbivore. Fourth, fungal species will differentially affect plant and herbivore responses to varying AMF abundance. Finally, we hypothesize that mycorrhizal abundance will explain a similar proportion of variation in plant traits and herbivore performance as does AMF species identity or the presence of AMF.

Materials and methods

STUDY SYSTEM

To test these hypotheses, we inoculated *Asclepias syriaca* L. (common milkweed) seedlings with a series of AMF soil treatments. *Asclepias syriaca* is a perennial herb that grows throughout eastern North America and associates with AMF throughout its range (Landis, Gargas & Givnish 2004). *Asclepias syriaca* is attacked by a variety of specialist insect herbivores including *Danaus plexippus* larvae. Milkweeds express several traits that can deter damage by herbivores or reduce the growth and reproduction of even specialists (Dussourd & Hoyle 2000; Zalucki *et al.* 2001; Agrawal 2005). Resistance traits include cardenolides – toxic, bitter-tasting steroids; latex – a sticky polyisoprene polymer that fills pressurized laticifers; trichomes; and leaf toughness, which is tightly correlated with specific leaf mass (SLM) (Frost & Hunter 2008). While all these resistance traits are primarily composed of carbon, their synthesis requires nutrient-rich enzymes (Gershenzon 1994). These same traits have been linked to decreased herbivore performance by *D. plexippus* or other milkweed specialists (Zalucki *et al.* 2001; de Roode *et al.* 2011).

Asclepias syriaca associates with AMF at colonization levels ranging from 10 to 80% of root length colonized at our field site in northern Michigan, USA, and spore density and abundance also vary within this site (authors' unpublished data and observations). In addition, genetic families of *A. syriaca* vary substantially in defence expression (Vannette & Hunter 2011b) and may respond differentially to mycorrhizal colonization or AMF identity.

We delineated five genets of *A. syriaca* growing in a natural population in northern Michigan (University of Michigan Biological Station, Pellston, MI) based on morphological, phenological, chemical and genetic similarity (Kabat, Dick & Hunter 2010). Follicles containing full-sibling seeds were collected from five different genets

at our field site, cold moist stratified for at least three months and germinated. Hereafter, we refer to the groups of seeds germinated from a single genet as genotypes. Pure cultures of *Glomus etunicatum* and *Scutellospora fulgida*, AMF species that associate with *A. syriaca* at our field site (R. Vannette, pers. obs.), were obtained from INVAM (http://invam.caf.wvu.edu/) and cultured on Sorghum roots to obtain sufficient inoculum for experiments.

Our experiments were designed to examine the evidence for the following predicted sequence: first, that AMF abundance (= inoculum availability) would influence subsequent AMF colonization of plants; second, that AMF abundance would influence plant traits important to herbivores (nutrition and resistance); and third, that herbivore performance would be altered accordingly. We therefore performed two simultaneous experiments. The 'plant phenotype' experiment was used to explore relationships among inoculum availability, AMF colonization of roots and plant traits (growth, nutrition and resistance). The 'herbivore performance' experiment was used to explore effects of AMF abundance on herbivore performance. We separated experiments for two reasons. First, monarch caterpillars consume considerable amounts of foliage and we could not measure all plant traits from the same plants on which caterpillars were feeding while maximizing the period over which caterpillars were being exposed to experimental plants. Second, herbivore damage itself can influence AMF colonization of roots (Kula, Hartnett & Wilson 2005; Wearn & Gange 2007), and we needed to establish the effects of inoculum availability on subsequent AMF colonization without the confounding effects of caterpillar damage.

For the plant phenotype experiment, we varied the availability of fungal inoculum to plants (Vannette & Hunter 2011a). Seedlings were planted in conical Deepots™ (Steuwe and Sons Inc., Corvallis, OR, U.S.A.), with a diameter of 6.4 cm and depth of 25 cm, filled with 600 mL 1:1 autoclaved Sunshine Metromix/sand containing mycorrhizal fungal inoculum. Inoculation volumes were determined from an initial trial with *A. syriaca* in which we had generated a wide range of colonization intensities. Based on the results of this trial, we added 150 mL (1/4 pot volume) of fungal inoculum consisting of spores, hyphae and colonized sorghum root pieces ranging across 11 AMF inoculation ratios from 100% autoclaved inoculum to 100% live inoculum (Table S1 in Supporting Information). We did not include a microbial wash treatment. The top and bottom of each pot contained 225 mL of autoclaved soil mixture to prevent contamination. AMF abundance treatments were established separately for *Glomus etunicatum*, *Scutellospora fulgida* and a mix of the two species. (N = 5 plant genotypes × 3 AMF species treatments × 10 AMF abundances × 2 replicates = 300 plants plus 10 non-AMF plants (5 plant genotypes × 2 replicates) = 310 total plants for the phenotype assay experiment (Table S1 in Supporting Information)).

For the herbivore performance experiment, we used the same soil inoculum described above, but used only six levels of AMF inoculum abundance (N = 3 AMF species treatments × 5 AMF abundances × 5 plant genotypes × 4 replicates, plus 20 non-AMF plants = 320 plants for herbivore assays) (Table S1 in Supporting Information). Due to constraints in the number of herbivores, we could rear and measure at one time; we could not fully replicate all 11 abundance treatments for the herbivore performance experiment, so we chose to maximize the variation in AMF abundance.

PLANT PHENOTYPE EXPERIMENT: HARVEST AND ANALYSIS OF PLANT TRAITS

At the end of three months of growth, plants were harvested destructively. We then measured levels of AMF colonization, above-ground biomass, phosphorus content and resistance traits from all plants. To quantify levels of AMF colonization that resulted from abundance treatments, a subset (*c.* 0.1 g) of dried fine root tissue was sampled from all plants. Roots were rehydrated for 24 h in water, cleared with 10% KOH for 10 min, acidified using 2% HCl and stained in 0.05% trypan blue in 1:1:1 water: glycerine/lactic acid. Stained roots were mounted on slides and scored using the magnified gridline intersect method (McGonigle *et al.* 1990) using a Nikon compound microscope (Melville, NY, USA). A site was considered colonized if AM hyphae, arbuscules or vesicles were present. Non-AMF hyphae were also detected at low levels (< 0.05%).

To measure foliar traits, six hole punches (424 mm^2 total) of fresh leaf tissue were taken from one half of the two largest leaf pairs on each plant, placed immediately into 1 mL of methanol and stored at −10 °C for cardenolide analysis. Six identical leaf discs were taken from the opposite half of the leaf pairs and stored in glassine envelopes to provide estimates of the dry mass of plant material used in chemical analysis and for measures of other leaf traits. Latex that flowed from the first six holes punched was collected on a preweighed cellulose disc (1 cm diameter), dried and weighed. Trichome density was determined by counting the number of trichomes on the lower surface of the leaf discs using a dissecting microscope at 4× magnification. Leaf discs were dried, weighed, and this mass was divided by disc area to provide an estimate of specific leaf mass (SLM). Plant chemical resistance traits were assessed following established protocols (Zehnder & Hunter 2007). Briefly, cardenolides were separated and quantified by extracting plant material in methanol. Samples were separated using UPLC (Waters Inc, Milford, MA, USA) using an Acquity BEH C18 column (1.7 μm, 2.1 × 50 mm, Waters, Milford, MA, USA). Each 2 μL injection was eluted at a constant flow of 0.7 mL min^{-1} with a gradient of acetonitrile (ACN) and water, maintained at 20% ACN for 3 min, increasing to 45% ACN through the 9 min run. Peaks were detected by a diode array detector at 218 nm, and absorbance spectra were recorded from 200 to 400 nm. Peaks with symmetrical absorbance between 218–222 nm were quantified as cardenolides (Malcolm & Zalucki 1996). Cardenolide concentrations were calculated using a digitoxin internal standard and total cardenolides were calculated as the sum of individual peaks.

To measure foliar phosphorus concentration, we dried and ground the remaining foliar tissue from all plants of the plant phenotype experiment. Foliar phosphorus in ground samples was converted to soluble P using acid reflux and quantified using the molybdenum method with ascorbic acid reduction (Allen 1989; American Public Health Association 1998).

HERBIVORE PERFORMANCE EXPERIMENT

To assess the effects of any AMF-induced changes in plant nutrition and resistance on herbivore performance, eggs of monarch butterflies, *D. plexippus,* were obtained from Michigan Monarchs (http://www.mi-monarchs.com) and attached to the leaves of the 'herbivore assay' group of plants. Eggs were applied to four replicates of each plant genotype × AMF treatment to expose caterpillars to a range of fungal abundance treatments (Table in Supporting Information, n = 4 replicates/treatment = 320 caterpillars). A single egg was applied to one leaf of the fourth expanded leaf pair from the apical meristem and held to the leaf using a single drop of water. Eggs hatched within 2 days, and the date of eclosion was recorded. Monarch caterpillars grow rapidly and consume large quantities of leaf tissue; individual plants could support larval growth until larvae reached the third instar. Therefore, all caterpillars were collected 5 days following

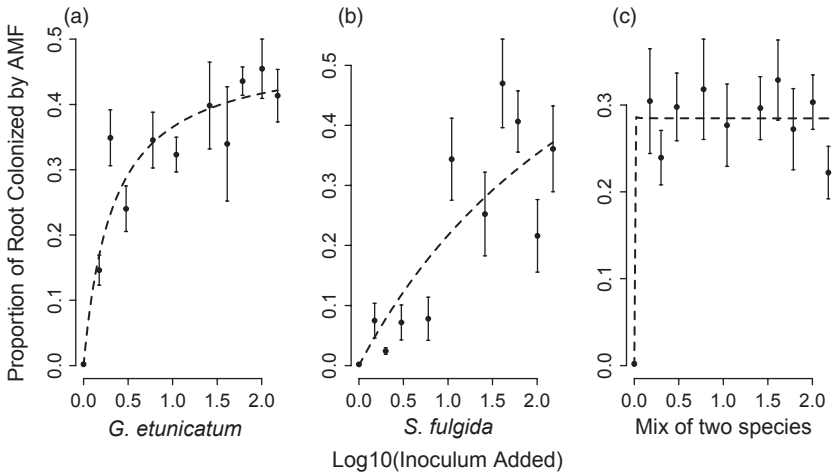

Fig. 1. Best-fit relationships between the abundance of arbuscular mycorrhizal fungi (AMF) inoculum added and the subsequent degree of mycorrhizal colonization in the roots of *Asclepias syriaca* after three months of growth with (a) *Glomus etunicatum*, (b) *Scutellospora fulgida* and (c) a mix of the two species. Best-fit lines were chosen using AICc (Table S2 in Supporting Information). Points represent the mean of 10 replicates ± 1 SE.

eclosion, which represents about 50% of the larval lifespan. Milkweed traits generally have their greatest impact on the performance of monarch caterpillars during early instars (Zalucki *et al.* 2001). Caterpillars were collected, allowed to void their guts, frozen and subsequently freeze-dried and weighed using a microbalance (Mettler Toledo, Columbus, OH, USA). Mean caterpillar growth rate per day was calculated by dividing total caterpillar biomass by five; the number of days all caterpillars had fed following eclosion.

STATISTICAL ANALYSIS

To explore relationships between AMF inoculum availability and the subsequent levels of AMF colonization that resulted in milkweeds, we fit linear, saturating and logistic models to the colonization data from the 'plant phenotype' experiment and compared model fits to that of an intercept-only model using AICc values as models were non-nested. Separate analyses were conducted for each AMF species treatment (Fig. 1).

From previous work (Vannette & Hunter 2011a), we hypothesized that increasing mycorrhizal abundance would affect the expression of plant resistance nonlinearly, based on the carbon costs and nutrient benefits transferred within the mycorrhiza (Smith & Read 2008). To test this hypothesis, we examined a series of linear and nonlinear model fits (Motulsky & Ransnas 1987) between plant traits and AMF abundance manipulated in the plant phenotype experiment. We compared the following full regression equations, fit separately for each plant trait and AMF species, using R v. 2.15.0 (R Development Core Team 2012). Linear (Eqn 1), quadratic (2) and saturating (3) models were fit to relationships between AMF abundance and plant traits (saturating models were not fit for decreasing relationships).

$$R = \beta_o + \beta_1 A \quad \text{eqn 1}$$
$$R = \beta_o + \beta_1 A + \beta_2 A^2 \quad \text{eqn 2}$$
$$R = \alpha * A/(K + A) \quad \text{eqn 3}$$

where R, plant resistance trait and A, AMF abundance.

Non-nested models were first compared using AICc. Saturating models (Eqn. 3) were never within 4 AICc values of the best-fit model, so the null, linear and quadratic models were compared using likelihood ratio tests (LRT) (Pinheiro & Bates 2000). From the best-fit model for each AMF species x trait combination (Fig. 2), measures of model fit, including adjusted R^2 and P-values were extracted (Table 1).

While AICc and the LRT allowed us to choose among alternative models (= response curves) between AMF abundance and plant traits for each AMF species/mix alone, we were also interested in estimating how much of the variation in each plant trait was explained by our predictor variables. This is a much broader analysis that examines the relative power of the various treatments to influence plant trait expression. To examine the relative contribution of AMF species, abundance and plant genotype identity to the expression of plant traits, we used ANCOVA to partition the variance in resistance traits explained by each predictor (Hunter, Varley & Gradwell 1997) by calculating the Explained Sums of Squares/Total Sums of Squares for each plant trait. We also fit each ANCOVA model to include the interactions between milkweed genotype and other predictor variables. These interactions explained little (< 2%) of the total variation in each trait and are therefore not discussed in the main text (but see Fig. S2 in Supporting Information for full details). Variance explained was assessed by removing each variable from the model and assessing the change in model fit (adj. R^2). We modified Eqn. 1 above to include AMF species, plant genotype, AMF abundance and a dummy variable (0/1) for the presence or absence of AMF fungi. Variance explained by AMF abundance is the sum of the linear and quadratic terms.

$$R = A + A^2 + G_i + S_j + P + E \quad \text{eqn 4}$$

where R, plant resistance trait, G_i, each plant genotype, A, AMF abundance, S, AMF species (e.g. *Glomus etunicatum*, *Scutellospora fulgida*, or the mix), P, presence of AMF inoculum (0 or 1) and E, Error.

We tested the hypothesis that variation in AMF abundance would influence herbivore performance (Vannette & Hunter 2011a) by examining its effect on *D. plexippus* larval growth rates. Because plant traits and herbivore performance were measured from different groups of plants, we calculated average values of plant traits and caterpillar growth rates for each plant genotype and AMF abundance treatment for each of the AMF species treatments. This yielded 30 data points (6 AMF abundance treatments for each of five plant genotypes) for each of three AMF species treatments. First, we fit linear and quadratic model fits to the relationship between AMF abundance and log-transformed larval growth rates for each AMF species, using genotype-level means (Fig. 3). Next, we used multiple regression to assess the effects of measured plant traits on log-transformed caterpillar growth rates. We fit full models relating caterpillar growth rate to all measured plant traits for each AMF species treatment and used a

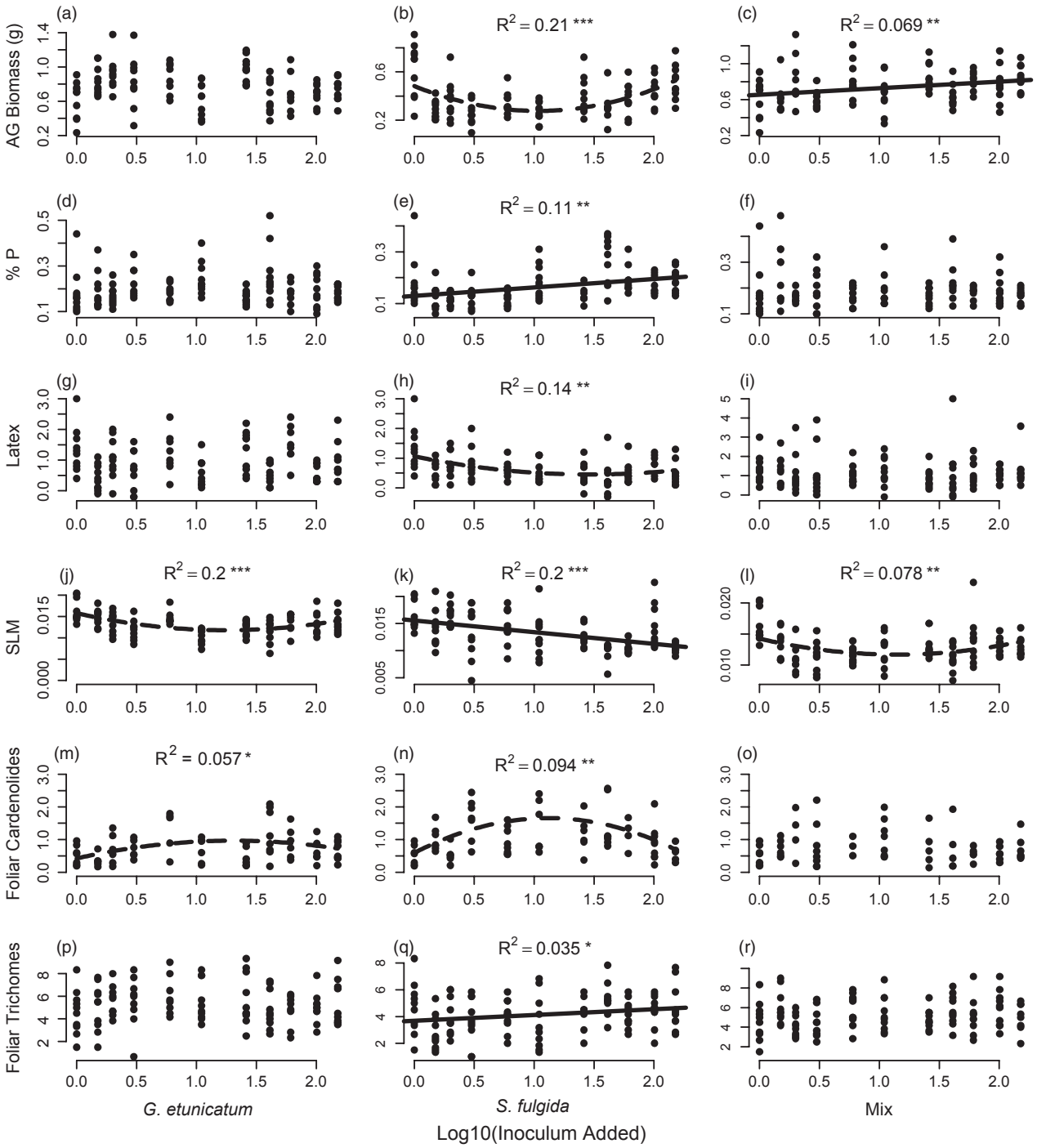

Fig. 2. Best-fit lines relating arbuscular mycorrhizal fungi (AMF) abundance of *Glomus etunicatum*, *Scutellospora fulgida* or a mix of the two species to the expression of plant nutrition and resistance traits in *Asclepias syriaca* plants. Each point represents a plant individual. Traits include (a–c) above-ground (AG) biomass, (d–f) % foliar phosphorus, (g–i) latex exudation (mg), (j–l) specific leaf mass (SLM), (m–o) total foliar cardenolides and (p–r) the density of foliar trichomes on the bottom surface of the leaves. Model significance was assessed using *F*-tests $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

combination of forward and reverse selection based on AICc to select the most parsimonious model for each AMF species treatment (Table 2). Finally, we used an ANCOVA to further examine the relative power of plant genotype and AMF presence, species and abundance to explain variation in log-transformed caterpillar growth, as for plant traits (Eqn. 4), using all data (Fig. 4).

Results

INOCULUM AVAILABILITY AND AMF COLONIZATION

Mycorrhizal inoculation was successful, and no evidence of AMF colonization of roots was found in the control

Table 1. AICc values for model fits, examining the effect of AMF abundance on the expression of traits by *Asclepias syriaca*. The saturating model was not fit for SLM because this trait decreased with AMF abundance. The AICc value for the best-fit model is in bold print, and the best-fit model is the line displayed in Fig. 3

		Model fits			
Trait	AMF	Null	Linear	Quadratic	Saturating
Above-ground Biomass	GE	**−16.4**	−15.9	−16.2	39.9
	SF	−78.5	−77.2	**−102.2*****	1.0
	Mix	−44.2	**−50.8****	−48.8	25.7
% P	GE	**−242.5**	−240.5	−240.9	−192.9
	SF	−263.7	**−275.6*****	−273.9	−219.9
	Mix	**−270.9**	−269.8	−267.7	−217.0
Latex	GE	**213.4**	215.5	217.6	245.4
	SF	161.5	151.6	**147.4***	203.9
	Mix	**276.0**	278.0	279.2	300.5
SLM	GE	−933.3	−938	**−954.3*****	NF
	SF	−907.1	**−930*****	−929.3	NF
	Mix	−971.8	−970	**−978.3****	NF
Cardenolides	GE	100.3	100.8	**98.1***	106.2
	SF	136.6	138.3	**127.9*****	138.3
	Mix	**95.3**	97.1	96.6	103.6
Trichomes	GE	**420.9**	423.0	423.3	473.4
	SF	405.8	**403.1***	404.6	466.6
	Mix	**413.6**	414.1	416.2	638.2

AMF, arbuscular mycorrhizal fungi; NF, Model Not Fit.
Best-fit models were chosen using first a model selection approach (because not all models were nested), and the best-fit model was verified using the LRT (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for improvement over next best-fit model).

treatment. Inoculation with either *Glomus etunicatum* or *Scutellospora fulgida* generated a wide range of AMF colonization intensities (0–69% and 0–78% of plant root colonized respectively), whereas the range of colonization intensities generated by the mixed species inoculum was slightly narrower (0–63%). Root colonization by arbuscules was highly correlated with colonization by all fungal structures ($P < 0.0001$, $R^2 = 0.79$).

Mycorrhizal colonization in the single-species treatments increased with inoculum abundance in the soil (Fig. 1). Specifically, increasing the abundance of *G. etunicatum* and *S. fulgida* inoculum increased mycorrhizal colonization in a relationship best represented by a saturating function (Fig 1, Table S2 in Supporting Information). In contrast, the abundance of inoculum in the mixed species treatment was poorly related to mycorrhizal colonization, and the best-fit saturating function was no longer a good fit to the data when the zero values were removed from the analysis (data not shown).

PLANT PHENOTYPE EXPERIMENT

Figure 2 illustrates the relationships between experimental AMF inoculum abundance and milkweed traits. The best-fit models (Table 1), as determined by LR tests to be statistically significant ($P < 0.05$), are indicated by the presence of regression lines. Increasing abundance of *G. etunicatum* had little effect on above-ground biomass, while *S. fulgida* abundance affected biomass in a u-shaped fashion, first decreasing and then increasing above-ground biomass (Fig. 2a–b). In contrast, increasing abundance of the mix of AMF was weakly, but positively related to above-ground plant biomass (Fig. 2c). Increasing abundance of *S. fulgida* increased foliar P concentration (Fig. 2d–f), but neither *G. etunicatum* nor the mix of species were related to foliar P concentration. Increasing abundance of *S. fulgida* decreased latex exudation in a nonlinear (U-shaped) fashion, whereas abundance of *G. etunicatum* and the fungal mix were unrelated to latex exudation (Fig. 2g–i). Increasing AMF abundance generated decreasing or nonlinear (U-shaped) responses in specific leaf mass (SLM) in all fungal treatments (Fig. 2j–l). Increasing abundance of *S. fulgida* and *G. etunicatum* increased foliar cardenolide concentration in a unimodal relationship (Fig. 2m–n), whereas AMF abundance in the mix treatment was unrelated to cardenolide concentration (Fig. 2o). Foliar trichome density was positively associated with increasing abundance of *S. fulgida*, but not significantly related to AMF abundance in the other treatments (Fig. 2p–r). We also examined how increasing AMF colonization was related to trait expression (Fig. S1 in Supporting Information): the shape and direction of the curve fits are largely concordant with trait responses to AMF abundance.

HERBIVORE PERFORMANCE EXPERIMENT

Mycorrhizal abundance strongly influenced caterpillar performance. Specifically, increasing inoculum abundance of *G. etunicatum* or *S. fulgida* increased caterpillar growth rate (Fig. 3a,b). Increasing abundance of the mix of fungal species also increased caterpillar growth rate in a hump-shaped fashion (Fig. 3c). Multiple regression revealed that SLM was strongly and negatively associated with the rate of caterpillar growth in all AMF species treatments (Table 2). Latex exudation was also negatively associated with caterpillar performance in both single-species inoculations (Table 2). Foliar cardenolide concentration was negatively associated with larval growth in the *S. fulgida* treatment. In addition, an increasing level of mycorrhizal abundance was positively associated with increased caterpillar performance independently of the measured milkweed traits in plants associated with *Glomus etunicatum* and the mix of species (Table 2).

VARIANCE PARTITIONING

Overall, the relative contributions of AMF species, presence/absence and AMF abundance to variation in phenotype expression depended on the trait examined. Of all experimental variables, AMF species identity explained the most variation in plant phenotype (Fig. 4). However, mycorrhizal abundance also explained a large proportion of variation in plant phenotype and more than the presence or absence of AMF in most traits (Fig. 4). Variation in above-ground

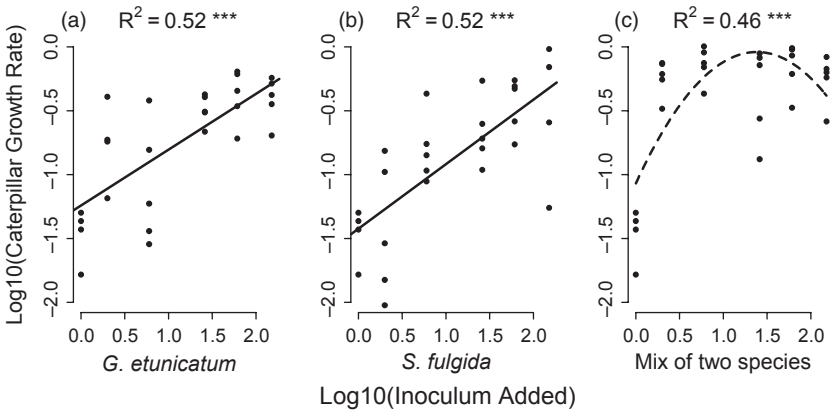

Fig. 3. Relationships between the growth rate (mg day^{-1}) of monarch larvae (*Danaus plexippus)* on *Asclepias syriaca* plants and the availability of fungal inoculum of (a) *Glomus etunicatum*, (b) *Scutellospora fulgida* or (c) a mix of the two species. Each point represents the average larval growth rate on a given plant genotype, regressed against the experimental levels of inoculum provided. Some caterpillars were lost during the experiment, and therefore, one point is missing from each of the GE and SF treatments. Model significance was assessed using *F*-tests *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 2. Partial unstandardized regression coefficients for variables retained in a stepwise regression relating log-transformed *Danaus plexippus* growth rate (mg day^{-1}) to *Asclepias syriaca* phenotype predictor variables. Value in cell indicates the coefficient values of predictors significant at $\alpha < 0.1$. Columns contain values for each mycorrhizal species treatment

Predictor	*Glomus etunicatum*†	*Scutellospora fulgida*‡	Mix§
AG Biomass	–	–	–
% P	–	–	–
Latex	−0.23^{+}	−0.93***	–
Total Foliar Cardenolides	–	−16^{+}	–
SLM	−96.25*	−78.26*	−0.15***
Trichome Density	–	–	–
Plant Genotype¶	–	–	+
AMF abundance	0.32***	–	0.26**

†*Glomus* Model fit: $F_{1,24} = 16.21$, $P < 0.0001$, Adj. $R^2 = 0.63$.
‡*Scutellospora* Model fit: $F_{1,24} = 16.39$, $P < 0.0001$, Adj. $R^2 = 0.67$.
§Mix Model fit: $F_{1,21} = 7.14$, $P = 0.0001$, Adj. $R^2 = 0.70$.
¶Parameter estimate not listed because predictor is a factor.
Symbols indicate significance of predictors, where + indicates $P < 0.10$, *$P < 0.05$, **$P < 0.01$. Regressions were conducted on the genotype means for each AMF × Genotype level ($n = 29$). Coefficients and their significance are reported from the stepwise best-fit model. All regression analyses were performed in R v.2.15.

biomass was overwhelmingly explained by AMF species identity, with the remaining variation explained by AMF abundance (Fig. 4a). Variation in foliar phosphorus concentration was explained in large part by AMF species identity and AMF abundance (Fig. 4b). In contrast, the presence of AMF, species identity and plant genotype, rather than AMF abundance, explained much of the variation in latex exudation (Fig. 4c). AMF abundance accounted for the largest proportion of variance in SLM, while the other AMF variables and plant genotype explained a small amount (2–4%) of variation in SLM (Fig. 4d), although a large proportion of its variation remained unexplained by the variables included in this analysis. Variation in foliar cardenolide concentration was largely explained by AMF species identity, followed by AMF abundance and plant genotype (Fig. 4e). Plant genotype and species identity explained a large proportion of variation in trichome density (Fig. 4f). Herbivore performance was explained largely by AMF presence, followed by the species identity and abundance of AMF (Fig. 4g).

Discussion

In this study, we demonstrate that increasing the abundance of AMF inoculum affects the expression of plant nutrition and resistance traits, in turn, increasing the performance of a specialist insect herbivore. Mycorrhizal enhancement of insect performance was associated with a decrease in physical resistance traits of plant leaves. Additionally, the availability of AMF inoculum explained a large proportion of variance in the expression of plant traits and herbivore performance. Depending on the trait, AMF abundance explained a similar amount of variation to that explained by plant genotype or the presence or identity of AMF. Taken together, these results suggest that AMF abundance in the soil can be an important determinant of multitrophic interactions between plants and herbivores. More broadly, our results suggest that fungal abundance may be an overlooked, but key aspect of the mycorrhizal mutualism (Gange & Ayres 1999; Violi *et al.* 2007; Garrido *et al.* 2010) and explain a similar amount of variation in plant phenotype as other factors commonly manipulated in experiments (Karst *et al.* 2008; Hoeksema *et al.* 2010).

Our results suggest that the composition of the mycorrhizal community determines the relationship between AMF abundance in the soil and the proportion of root colonized by AMF. Specifically, increasing abundance of either species of fungi alone increased mycorrhizal colonization of *Asclepias syriaca* roots (Fig. 1a,b), a result largely concordant with previous studies (Carling, Brown & Brown 1979;

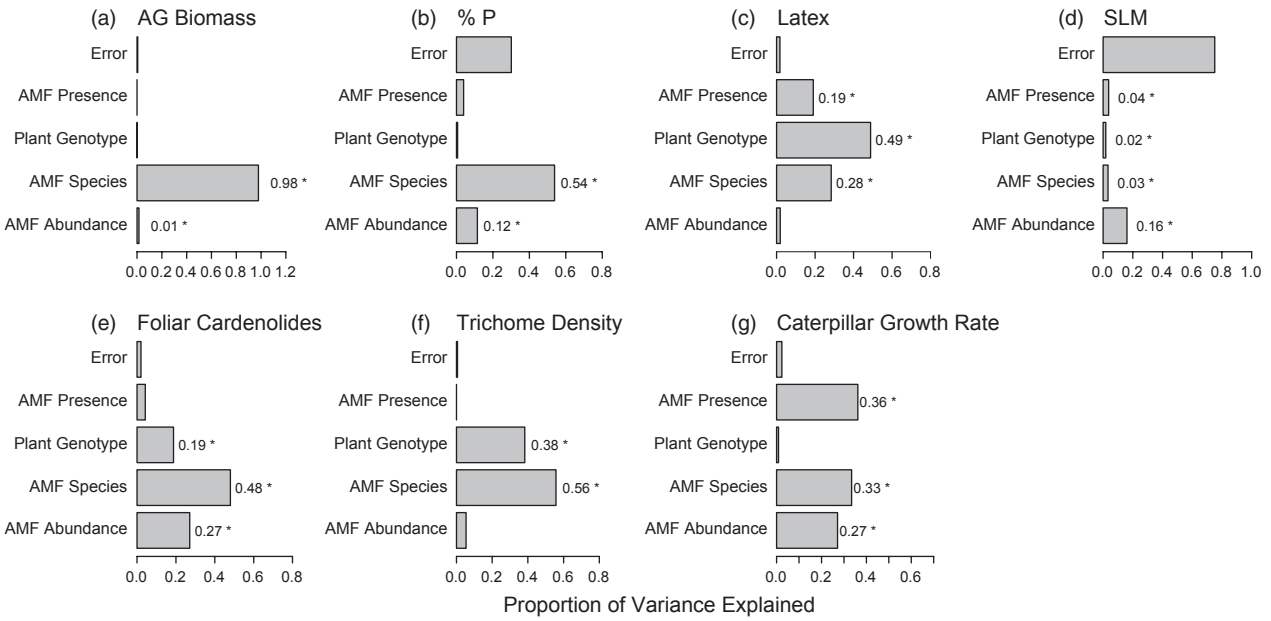

Fig. 4. Proportion of total variance in the expression of *Asclepias syriaca* growth, nutrient and resistance traits explained by the availability of arbuscular mycorrhizal fungi (AMF) inoculum (AMF Abundance), AMF species identity, plant genotype, the presence or absence of AMF or the variance remaining (error). Numbers indicate the proportion of variance explained by each significant predictor ($P < 0.05$), calculated by dividing the sums of squares explained by each factor by the total variance in each trait, among all phenotype assay plants (a–f) or herbivore assay plants (g).

Khan 1988). In contrast, increasing abundance of a mix of AMF did not predict mycorrhizal colonization of *A. syriaca* roots (Fig. 1c). We suggest that biotic interactions among fungal species (e.g. Cano & Bago 2005; Bennett & Bever 2009) may over-ride the importance of inoculum abundance in determining mycorrhizal colonization by some AMF communities. Further work would be required to determine which combinations of AMF interact in such ways. We did not examine the relative proportion of root colonized by each species with increasing inoculum density in the mixed treatment and can only speculate that competitive interactions between fungi may have influenced the proportion of plant root colonized by each species or their relative effects on plant phenotype.

AMF AFFECT PLANT DEFENCE

In our experiment, AMF abundance altered plant growth and resistance phenotype in a species-specific fashion. For example, increasing abundance of *S. fulgida* increased foliar phosphorus and trichome density, affected unimodally foliar cardenolide concentration, but decreased specific leaf mass and latex exudation. In contrast, *G. etunicatum* had little effect on plant traits, save specific leaf mass and cardenolides. Because these results differ somewhat from our preliminary studies (Vannette & Hunter 2011a), we suggest that different plant genotypes or soil properties may affect the outcome of interactions between AMF and their plant partners. However, *A. syriaca* genotypes responded in a similar fashion to increasing AMF abundance (Fig. S2 in Supporting Information), in contrast to the striking genetic variation in response to AMF found among genotypes of *Datura stramonium* (Garrido *et al.* 2010). Our findings are consistent with previous work that describes differential effects of AMF species on direct and induced resistance to herbivory (Bennett, Bever & Bowers 2009) and multitrophic effects on the rate of aphid parasitism (Hempel *et al.* 2009). We hypothesize that the difference in the effects of AMF species on plant phenotype may be attributed to species-specific carbon requirements and efficacy of nutrient foraging (Hart & Reader 2005; Lendenmann *et al.* 2011), traits which exhibit phylogenetic conservatism (Hart & Reader 2002; Powell *et al.* 2009). Taken together, these lines of evidence suggest that fungal species which are more effective at increasing host P may have more marked effects on plant resistance traits that depend closely on P concentration. Future work should examine whether differential effects of AMF species on plant defence are tied to plant C allocation patterns following herbivory (Klironomos, McCune & Moutoglis 2004).

EFFECTS ON LARVAL PERFORMANCE

Consistent among all fungal species treatments, AMF abundance increased caterpillar performance. Also consistent with previous work in our system, the positive effect of AMF on *D. plexippus* was strongly correlated with a decline in specific leaf mass and to a lesser extent to a decline in latex exudation (Vannette & Hunter 2011b; Tao & Hunter 2012). More broadly, AMF colonization increased the performance of a specialist herbivore, which aligns with the results of a recent meta-analysis (Koricheva, Gange & Jones 2009). AMF-mediated changes in cardenolide content may also affect oviposition by specialist herbivores, including Monarch

butterflies (de Roode *et al.* 2011). Although we only examined the effects of AMF on a specialist herbivore, the AMF-induced changes in cardenolides that we observed may be more effective against generalist herbivores such as deer, which are also important in our study system. Future work should compare the effects of AMF on specialist and generalist herbivores that share a host plant to clearly distinguish whether such differential effects on plant resistance are ecologically relevant or feedback to affect fungal performance. Additionally, in our experiment, *D. plexippus* larvae were protected from predators, but we suspect that AMF abundance may also affect the expression of indirect defence (Fontana *et al.* 2009; Hempel *et al.* 2009). We predict that increasing AMF abundance may also affect allocation to volatile organic carbon emission and predator attraction in a nonlinear fashion.

IMPORTANCE OF AMF ABUNDANCE AND COLONIZATION

The importance of mycorrhizal abundance and colonization as meaningful metrics of AMF function has been disputed (Gange & Ayres 1999; McGonigle & Miller 2000), and current research has not yet resolved this debate. For example, recent studies indicate that in some cases, the proportion of root colonized by AMF does not correspond well to C costs or P benefits and eventual 'benefit' gained by the plant (Smith, Grace & Smith 2009; Lendenmann *et al.* 2011). On the other hand, mycorrhizal abundance and resulting colonization levels have been shown to influence plant performance, tolerance to herbivory and survival in the presence of herbivores (Garrido *et al.* 2010; Vannette & Rasmann 2012). This study adds the expression of plant resistance and subsequent herbivore performance to the list of factors influenced by mycorrhizal abundance and associated with AMF colonization. Furthermore, we demonstrate that the magnitude of variance in plant traits explained by AMF abundance is comparable with that explained by the presence and identity of AMF species and greater than that explained by plant genotype. This is noteworthy given that plant genotype is known to have important effects on trait expression by *A. syriaca* (Agrawal 2005; Vannette & Hunter 2011b). Although the mechanisms by which levels of mycorrhizal abundance affect plant performance are not yet clear (Smith, Grace & Smith 2009) and these effects seem to vary with AMF species identity and community composition, the proportion of root colonized by fungi is of significant ecological importance. Future work on the physiological mechanisms by which levels of AMF abundance and resulting colonization affect plant phenotype, including defence signalling, nutritional change and other pathways are clearly warranted.

Conclusions

Our results emphasize that mycorrhizal abundance can profoundly influence plant resistance phenotype and increase the performance of specialist herbivores. Mycorrhizal abundance can be a key factor in determining the outcome of plant–herbivore interactions, and our results suggest that it may also strongly influence the outcome of mycorrhizal interactions more broadly.

Acknowledgements

The authors would like to thank the Matthaei Botanical Gardens for greenhouse space and help with plant care, Mike Grant for analysing phosphorus samples and Hannah DeRose-Wilson and Susan Kabat for help setting up and harvesting the experiment. We thank two anonymous reviewers who provided helpful suggestions that have improved the manuscript. We would also like to acknowledge funding from NSF DEB 0814340 to MDH and RLV and an NSF DDIG to RLV.

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Received 17 September 2012; accepted 22 February 2013
Handling Editor: Marcel van der Heijden

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. AMF treatments consisted of live and autoclaved whole fungal inoculum (homogenized root pieces, spores, hyphae) from *Glomus etunicatum*, *Scutellospora fulgida*, or a 1:1 mix of the two species. Phenotype experiment plants describe the total number of seedlings used to assess plant phenotype.

Table S2. AICc values comparing linear, saturating, and logistic models to root colonization of *Asclepias syriaca* by arbuscular mycorrhizal fungi *Glomus etunicatum*, *Scutellospora fulgida*, and a mix of the two species. Inoculum densities follow those given in Supplementary Table 1.

Figure S1. Best-fit lines relating the degree of AMF colonization of *Glomus etunicatum*, *Scutellospora fulgida*, or a mix of the two species to the expression of plant nutrition and resistance traits in *Asclepias syriaca* plants.

Figure S2. Proportion of total variance in the expression of *Asclepias syriaca* nutrient and resistance traits explained by AMF abundance, AMF species identity, plant genotype, the presence or absence of AMF, and interactions between genotype (G) and all other AMF predictors, or the variance remaining (error).