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RESEARCH ARTICLE

Trait differences in responses to arbuscular mycorrhizal fungi are stronger and more consistent than fixed differences among populations of *Asclepias speciosa*

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PREMISE OF THE STUDY: Arbuscular mycorrhizal (AM) fungi can promote plant growth and reproduction, but other plant physiological traits or traits that provide defense against herbivores can also be affected by AM fungi. However, whether responses of different traits to AM fungi are correlated and whether these relationships vary among plants from different populations are unresolved.

METHODS: In a common garden experiment, we grew *Asclepias speciosa* plants from seed collected from populations found along an environmental gradient with and without AM fungi to assess whether the responses of six growth and defense traits to AM fungi are correlated.

KEY RESULTS: Although there was strong genetic differentiation in mean trait values among populations, AM fungi consistently increased expression of most growth and defense traits across all populations. Responses of biomass and root to shoot ratio to AM fungi were positively correlated, suggesting that plants that are more responsive to AM fungi allocated more biomass belowground. Responses of biomass and trichome density to AM fungi were negatively correlated, indicating a trade-off in responsiveness between a growth and defensive trait.

CONCLUSIONS: Our results suggest that while there is substantial population differentiation in many traits of *A. speciosa*, populations respond similarly to AM fungi, and both positive and negative correlations among trait responses occur.

KEY WORDS antagonism; arbuscular mycorrhizal fungi; *Asclepias*; environmental gradient; genotype; herbivory; mutualism; plant defense; resource acquisition; species interactions.

Associations between plants and arbuscular mycorrhizal (AM) fungi are widespread and can substantially increase plant growth by enhancing nutrient uptake in exchange for photosynthate (Smith and Read, 2008). AM fungi can provide other benefits to plants besides nutrient acquisition, such as increased drought tolerance (Augé, 2001; Aroca and Ruiz-Lozano, 2009) and protection from antagonists (Newsham et al., 1994; Bennett et al., 2006; Maherali and Klironomos 2007; Vannette and Hunter, 2013; Kos et al., 2015). Despite the varied benefits of AM fungi to plants, plant responses to—and investment in—AM fungi are highly variable among species (Wilson and Hartnett, 1998; Klironomos, 2003). Within plant species, recent work has revealed that there can be genetically based differences in the extent of plant responsiveness to AM fungi (i.e., different genotypes or populations have evolved differences in their plastic responses to AM fungi) (Schultz et al., 2001; Seifert et al., 2009; Johnson et al., 2010). Such population-level differences in responsiveness are likely to develop in geographically isolated populations that are exposed to unique environmental conditions (Rúa et al., 2016). While there is substantial evidence for population-level differentiation in the mean value of many plant traits (Clausen et al., 1941; Joshi et al, 2001; Kawecki and Ebert, 2004), whether those involved in mutualisms co-vary in their responsiveness, and whether they exhibit substantial population-level differentiation in responsiveness has not been well studied.

Typically, studies examining responsiveness to AM fungi focus on (plastic) increases in biomass production in the presence vs. absence of AM fungi (Wilson and Hartnett, 1998; Klironomos, 2003). Recent evidence, however, has shown that AM fungi can increase the expression of a variety of plant functional traits beyond biomass. For example, AM fungi can increase leaf nutrients, resulting in increased attractiveness to herbivores (Bennett et al., 2006; Babikova et al., 2014). One the other hand, AM fungi can also increase herbivore resistance (Bennett et al., 2006; Vannette and Hunter, 2013; Kos et al., 2015) by alleviating nutrient limitations to

allow greater allocation to defense or upregulating jasmonate signaling (Pozo and Azcón-Aguilar, 2007; Vannette and Hunter, 2013; Tao et al., 2016). The limited amount of work in this area has primarily documented plastic responses of plant resistance traits to the presence of AM fungi. Whether plant populations show differentiation in how plant defense traits respond to AM fungi has not to our knowledge been tested (reviewed by van Geem et al., 2013).

Variation in abiotic environmental conditions may lead to fixed differences in plant traits, as well as among-population differences in how plant traits respond to AM fungi (Johnson et al., 2010; Revillini et al., 2016). For example, herbivore density varies spatially, typically increasing with resource availability and productivity (Pennings et al., 2009; Joern and Laws, 2013). Since herbivores often select for higher constitutive resistance in high-resource environments (Pennings et al., 2009; Hahn and Maron, 2016), plants originating from populations in these environments may preferentially allocate resources derived from AM fungi to resistance traits over resource acquisition traits. On the other hand, in locations where abiotic stress constrains plant growth and herbivore density, plants may allocate resources derived from AM fungi toward the expression of growth traits over defense. In this scenario, growth responses and resistance responses would be negatively correlated. An alternative scenario is that environmental stress may cause plants to be more dependent on AM fungi for multiple services (Pineda et al., 2010, 2013). Thus, growth and resistance traits may both respond more strongly to AM fungi in low rather than high-resource environments. In this case, growth-responses and resistance-responses would be positively correlated (Vannette and Hunter, 2011). However, how the responses of multiple plant traits are correlated has not yet been experimentally evaluated.

Asclepias speciosa is a perennial forb widely distributed in western North America that benefits greatly from associating with AM fungi (Busby et al., 2011). It is highly defended from herbivores physically with trichomes and latex and chemically with cardenolides (Agrawal and Fishbein, 2008). We investigated the degree to which multiple traits associated with resource acquisition (e.g., biomass production, specific leaf area) and resistance (e.g., latex or trichome production) respond to AM fungi, exhibit fixed differentiation among populations, and differ in their responsiveness among populations using seven spatially distributed populations of *A. speciosa*. To accomplish this, we performed a common garden experiment with *A. speciosa* plants grown from seed collected from populations across the species' natural distributional range (~1200 km), with and without AM fungi. Environmental conditions at the source populations varied in 30-year climate averages, soil conditions (Appendix S1, see the Supplemental Data with this article) and herbivore densities (P. G. Hahn, unpublished data), and thus increase the likelihood that populations would diverge in responsiveness to AM fungi. We addressed the following questions: Do plant growth and herbivore resistance responses to AM fungi vary among plant populations? Are responses of traits to AM fungi correlated?

MATERIALS AND METHODS

Plant populations and AM fungal inoculum

We collected seed from seven *Asclepias speciosa* populations that were separated by a minimum of 7 km from each other and in total spanned most of the east–west distribution of this species (1200 km, Fig. 1, Appendix S1). By sampling populations from across the range of environmental conditions experienced by this species, we maximized the potential trait variation among populations and our ability to detect differences in responsiveness among the populations (Sexton and Dickman, 2016). Within each population, we haphazardly collected one seed pod (i.e., fruit) from each of 4–6 different ramets. *Asclepias* species are self-incompatible and typically pollinated by insects (Hymenoptera and Lepidoptera) (Wyatt and Broyles, 1994). Pollen grains are transferred in units (i.e., pollinium), which results in all the seeds within a fruit sharing a single father (i.e., seeds are full siblings within a fruit).

Seeds were germinated in water and transplanted into 1:1:1 mixture of autoclaved field soil, sand, and clay (Turface, Buffalo Grove, IL, USA) in the University of Montana greenhouse. Plants were grown in 0.6 L Deepots (Stuewe and Sons, Tangent, OR, USA) and watered every 2–3 days. Light was on a 12:12 h cycle, and temperatures ranged from 16–30°C.

Since we were primarily interested in comparing the extent of population-level responses of plants to AM fungi and not coevolutionary relationships in the AM symbiosis, we inoculated plants with the same AM fungal species from a standardized culture collection rather than fungi isolated along the distribution gradient. While using a standard inoculum may dampen overall responses (Klironomos, 2003), most previous studies have shown that plants and AM fungi are not strongly adapted each other (Johnson et al., 2010; Rúa et al., 2016; Koyama et al., 2017). Thus, this approach should allow us to detect differences in responsiveness to AM fungi among the populations, as shown in previous studies (Schultz et al., 2001). Half of the plants received an individual dose of fungal inoculum containing a mixture of nine morphospecies from six genera and approximately 200 AM fungal spores (see Appendix S2 for more details on fungal inoculum). By using representatives from six

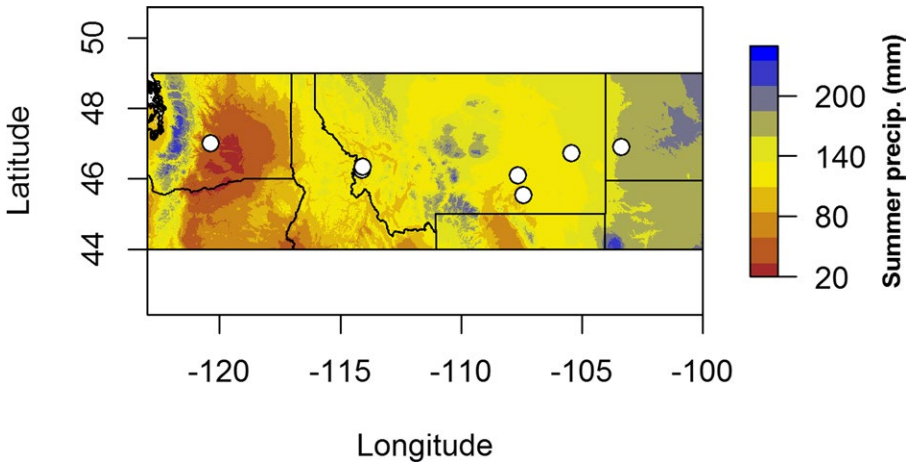

FIGURE 1. Map of the seven study populations of *Asclepias speciosa* (white dots). Note that there are two sites ~7 km apart in western Montana. Heat map shows spatial variation in climate (summer precipitation).

fungal genera, we increase the likelihood that the divergent populations would respond to the inoculum (van der Heijden et al., 1998). We paired plants by full-sib families, such that one plant would receive the +AM fungi treatment and its full sib would receive the –AM fungi treatment. All plants were fertilized with 20 mL of a half-strength Hoagland's solution every 2 wk. This regime was staggered so that every other application lacked P (i.e., P was only added to the solution once per month) to maintain low levels of P to facilitate root colonization of AM fungi (Smith and Read, 2008). Plants were grown for approximately 3 months before harvesting. For each of the seven populations, we grew 12 replicate plants representing 3–6 full-sib families in both AM fungal treatments (7 populations × 2 AM fungi treatments × 12 replicates = 168 plants total). To ensure that the AM fungal inoculations were successful and that plants in the –AM fungi treatment remained nonmycorrhizal, we assessed AM colonization on eight plants from each of three populations across the gradient. Percentage AM colonization was assessed using the gridline intersect method based on approximately 50 intercepts per sample (McGonigle et al. 1990) on rehydrated roots that had been cleared and stained in trypan blue (Brundrett et al. 1996). These analyses showed that AM fungal inoculations resulted in 27% (± 7.7 SE) of the root area of each plant being colonization and that –AM fungi plants remained uncolonized.

Trait measurements

Our goal was to evaluate differences in AM responsiveness of a suite of plant traits related to growth or resource acquisition and defense among populations from divergent resource environments. We measured six plant functional traits, four traits related to resource acquisition (total biomass [biomass], stem height growth rate, ratio of belowground to aboveground biomass [root:shoot], specific leaf area [SLA]) and two traits related to herbivore resistance (latex production and trichome density). We measured stem height three times at approximately 1-month intervals during the experiment. Increases in height began to slow near the end of the experiment, so we used stem growth (cm/day) measured during the second month of the experiment. At the end of the experiment, we harvested one of the top fully expanded leaves from each plant. We collected the latex that exuded from the stem on a pre-weighed 1-cm-diameter filter paper and placed this into a pre-weighed centrifuge tube. The tubes were frozen until they could be weighed on to the nearest 0.1 mg. Latex production was then quantified as fresh mass (Agrawal and Fishbein, 2008; Vannette and Hunter, 2013). We counted trichome density in a 33-mm^2 area on the abaxial surface of the leaf. Trichomes and latex are herbivore resistance traits that can decrease herbivore performance (Agrawal and Fishbein, 2008). Immediately after harvesting leaves, each leaf was scanned and later dried at 60°C for 48 h. Specific leaf area (SLA) was calculated as the area (cm^2) per unit mass (g). Plant biomass was also harvested, separated into above and belowground parts, dried at 60°C for 48 h, and then weighed.

Statistical analyses

Several plants died or were severely damaged by thrips and were removed before analysis. The resulting sample size was 155 plants (–AM fungi plants: $n = 74$; +AM fungi plants: $n = 81$). Before analysis, we examined the distribution of each trait, and latex and trichomes were natural-log-transformed to improve normality. All trait values were centered and scaled to a mean of zero and a standard deviation of one. Centering and scaling was necessary to ensure that all effect sizes were comparable among the six traits. Seed mass can influence early seedling performance, and this trait can be greatly influenced by maternal environment (Roach and Wulff, 1987). Seed mass differed among populations (random effect: $\chi^2 = 76.2$, $P < 0.0001$) but not with summer precipitation (fixed effect: $F_{1,5} = 1.4$, $P = 0.30$; Fig. 1), so we included seed mass as a covariate to account for potential maternal effects on the measured trait values.

To evaluate how responsiveness of growth and resistance traits to AM fungi vary among populations, we initially conducted a multivariate analysis using a multilevel model to account for nonindependence of traits (Appendix S3). The multivariate model containing all traits showed a significant interaction between the trait and AM fungi treatment ($F = 2.6$, $P = 0.031$), suggesting that traits differed in the magnitude of response to AM fungi. Correlations among the traits conducted on trait values separately in the +AM fungi and –AM fungi treatments were also relatively weak (Appendix S3). Therefore, for simplicity, we present the results of univariate analyses conducted separately on each trait.

For the univariate models, the (centered) trait value was the response variable. Fixed-effect variables included AM fungi treatment and seed mass. Population, and population × AM fungi were included as random effects. The main effect of AM fungi tests for responses of the plant trait to AM fungi (i.e., how plastic that trait is in its response to AM fungi). The population term tests for fixed differentiation of a trait among the populations, whereas the population × AM fungi interaction term tests for differences in trait responses to AM fungi among the populations (i.e., differences in plasticity among the populations). Seed mass was included in the model to account for potential maternal effects (i.e., differences in seed masses based on the maternal environment may affect trait values). We originally tested for interactions between seed mass and AM fungi, but this interaction term was never significant. Because we did not have specific a priori hypotheses related to how seed mass might influence trait responses to AM fungi, and because this interaction term was never significant in preliminary analyses, we did not include the seed mass × AM fungi term in any analysis. We initially included family nested with population as a random effect. However, family explained no additional variation beyond what was explained by populations (the estimated variance component was zero), so we did not include family in the model. Analyses were conducted using the lmer function in the lme4 package (Bates et al., 2013). *F*-values and *P*-values were calculated with the anova function in the lmerTest package (Kuznetsova et al., 2016) using the Satterthwaite technique to estimate degrees of freedom. Random effects were tested with the rand function in the lme4 package.

To evaluate how AM responsiveness would be correlated among traits, we first calculated the AM responsiveness of each trait as the percentage responsiveness (Wilson and Hartnett, 1998) as follows:

$$[(T_{i+\mathrm{AMF}} - T_{i-\mathrm{AMF}})/T_{i+\mathrm{AMF}}] \times 100, \quad (1)$$

where $T_{i+\mathrm{AMF}}$ is the value for the *i*th trait in the presence of AM fungi and $T_{i\text{-}\mathrm{AMF}}$ is the value of the *i*th trait in the absence of AM fungi. We used full sibling pairs within a population to calculate the AM fungal response. We then correlated AM fungi responses for all six traits. Correlations were conducted using means of full-siblings families ($n = 38$ full-sibling families).

RESULTS

Do plant growth and herbivore resistance responses to AM fungi vary among plant populations?

Although there was some variation in the magnitude of trait responses to AM fungi, this variation was not significant among populations for any trait (i.e., the Population × AM fungi random effect was not significant for any trait; Table 1, Fig. 2). There was significant variation among populations for mean values of stem height and trichomes and marginally significant variation among populations for root:shoot ratio and SLA (Table 1). All traits except SLA increased in the presence of AM fungi (Table 1). The response to AM fungi was greatest for biomass, followed by latex, height, trichomes, and root:shoot (Table 1, Fig. 2). Biomass was weakly and positively associated with seed mass, but seed mass had no statistical influence on any of the other traits we measured (Table 1).

Are responses of traits to AM fungi correlated?

The responsiveness of biomass to AM fungi was positively correlated with the responsiveness of root:shoot (Fig. 3A) and negatively correlated with the responsiveness of trichomes (Fig. 3B). In other words, the increase in biomass from AM fungi appeared to be largely due to greater root growth, and genotypes that responded most in growth responded the least in terms of shifts in trichome densities. Responses of root:shoot and trichomes were not correlated ($r = -0.20$, $P = 0.23$). The responsiveness of several other traits was weakly or not correlated (Appendix S4).

DISCUSSION

We examined how AM fungi affected traits associated with nutrient acquisition and herbivore resistance, how trait responses varied among populations, and how responses of the traits to AM fungi were correlated. We found population-level differentiation in the mean trait values for four of the six resource acquisition (stem height, root:shoot, and SLA) and resistance traits (trichomes), suggesting the populations represent distinct ecotypes (Turesson 1922). However, the plastic responses of these traits to AM fungi were generally stronger and more consistent than the fixed variation in the mean trait values among populations. Interestingly, for the five traits that responded to AM fungi, all trait values were positively influenced by AM fungi. Although there was no substantial variation among populations in the extent to which traits responded to AM fungi, some of the trait responses to AM fungi were correlated, suggesting predictable patterns in how plants allocate AM fungal-derived resources to various functional traits. Collectively, these results suggest that although plant populations differed in their mean trait values, plastic responses to biotic interactions such as AM fungi were consistent among populations and were stronger than fixed (i.e., genetically based) differentiation among populations.

Trade-offs between growth and defense are a common assumption in plant defense theory (e.g., Herms and Mattson, 1992), although empirical evidence for these trade-offs within a plant species is mixed (Cippolinni et al., 2014; Hahn and Maron, 2016). The strong negative correlation between biomass and trichome density responses to AM fungi suggests that plants may trade-off growth with defense in terms of how they respond to AM fungi (Fig. 3B) rather than a trade-off in the fixed trait values. Similarly, there was also a weaker negative relationship between the responsiveness of SLA and trichomes (Appendix S4). SLA can be associated with resource acquisition (Westoby et al., 2002; Shipley, 2006), herbivore resistance (Schadler et al., 2003; Hahn et al., 2011), or growth and tolerance (Meyer, 1998), making the interpretation of a negative relationship between SLA and trichomes more nuanced. Plants often upregulate photosynthesis when colonized by AM fungi, which can offset the increased cost of hosting the symbionts (Wright et al., 1998; Kaschuk et al., 2009). Thus, we cannot disentangle whether the plant benefited via increased carbon assimilation or via nutrient acquisition from AM fungi. Nevertheless, mycorrhizal plants appear to make gains in biomass or defense, but not necessarily both.

In contrast to the negative growth-defense response to AM fungi, we found a positive relationship between biomass and root:shoot responses. Biomass allocation to roots is a very plastic trait, sensitive to nutrient limitations and mycorrhizal colonization (Kong et al., 2014; Kramer-Walter and Laughlin, 2017). However, the increased allocation to roots when mycorrhizas were present was unexpected, since AM fungi often provide a greater surface area for nutrient uptake and can thus substitute for roots (Smith and Read, 2008). Indeed, herbaceous plants often respond to AM fungi by allocating more biomass above than belowground (Veresoglou et al., 2012). It may be that *A. speciosa*, a clonal plant attacked by highly specialized herbivores (Agrawal and Fishbein, 2008), invest more in roots to increase its ability to regrow following herbivory as a defensive tolerance strategy (Hochwender et al., 2000; Tao et al., 2016). AM fungi may also benefit from this strategy, as increased root growth provides more habitat for colonization. From this perspective,

TABLE 1. Parameter estimates for AM fungi and population effects on values of the six traits. Trait values were centered before analysis so that the effects are comparable across traits. R^2 values are shown for fixed effects (conditional) and both fixed and random effects (marginal).

Effect	Biomass	Height	Root:Shoot	SLA	Latex	Trichomes
AM fungi [a]	0.99***	0.33•	0.26•	−0.15	0.63*	0.30*
Seed mass [b]	0.26**	0.15	−0.01	−0.12	0.06	0.07
Population [c]	0.03	0.28*	0.07•	0.12•	0.06	0.17*
Pop × AMF [c]	0.00	0.01	0.00	0.00	0.06	0.00
R^2 conditional	0.31	0.04	0.02	0.02	0.10	0.03
R^2 marginal	0.34	0.30	0.09	0.13	0.21	0.18

[a]Effect size of +AM fungi relative to -AM fungi (fixed effects)
[b]Slope (fixed effects)
[c]Values are random effect variance components (i.e., variance among intercept estimates for populations)
Notes: •$P < 0.1$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

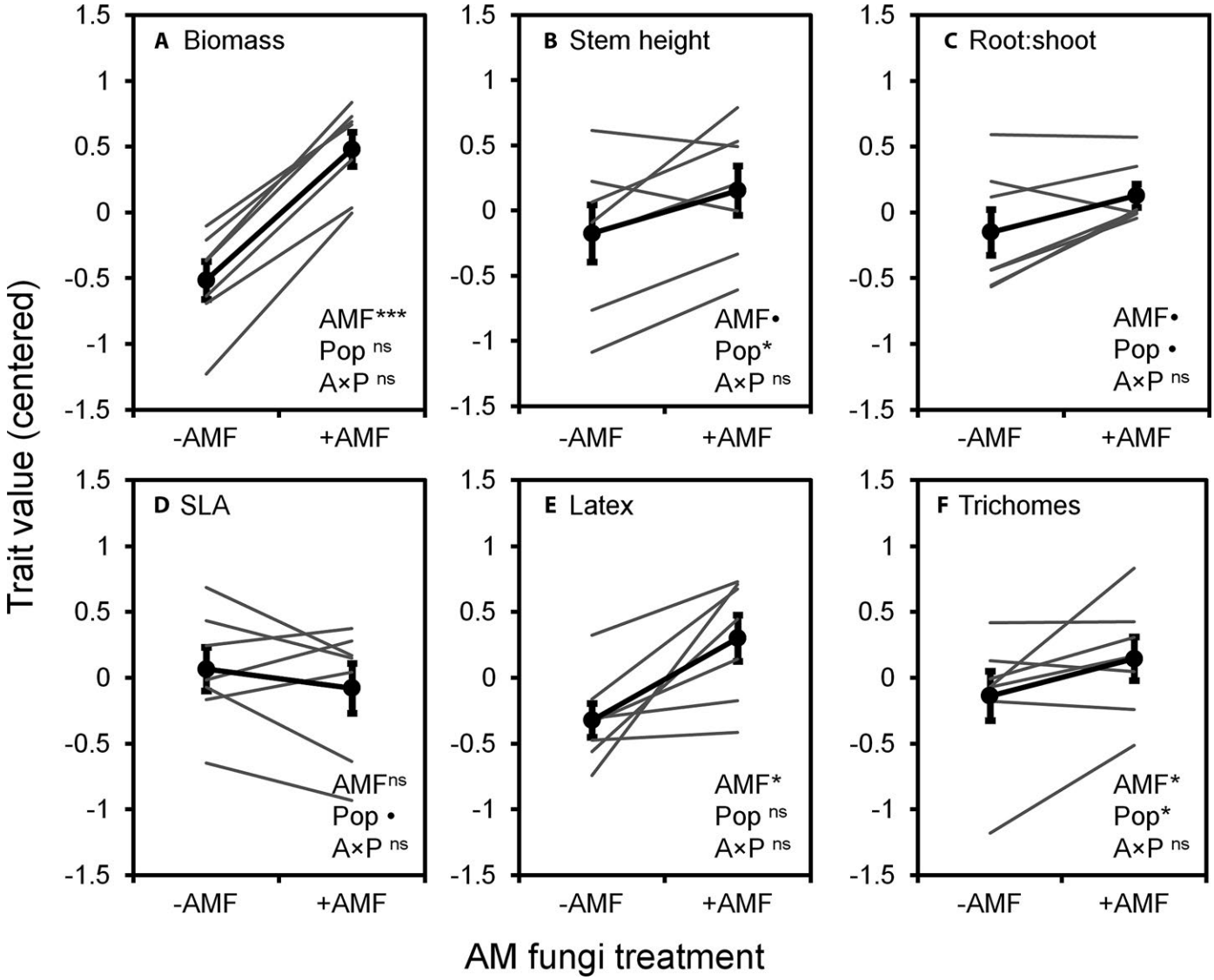

FIGURE 2. Effect of AM fungal inoculation on trait values for *Asclepias speciosa*. Gray lines show population means (n = 12 replications per population), and black dots/lines show overall means calculated from the seven population values, by AM fungi treatment, and are centered and scaled ($\mu = 0$, $\sigma = 1$). See Methods in the main text for a description of trait measurements and units. See Table 1 for a list of parameter estimates from the full model. Significant or marginally significant effects are indicated on panels: •$P \leq 0.1$, *$P \leq 0.05$, **$P \leq 0.01$; ns = not significant.

mycorrhizal plants that made greater gains in biomass allocated more resources to a tolerance trait (i.e., root:shoot), compared with mycorrhizal plants that gained less biomass, but allocated more resources to resistance (i.e., trichomes). Thus, in addition to the trade-off between growth and resistance we describe above, it could be that mycorrhizal plants are also incurring a trade-off between resistance and tolerance responsiveness.

One potential explanation for finding consistent responses to AM fungi among populations, rather than differences in responsiveness as we expected, is that the focal traits are highly plastic and differences might only be detectable if we matched the plant populations to their local AM fungal communities (Pineda et al., 2013). If local adaptation between plant populations and AM fungi occurs (Johnson et al., 2010; Revillini et al., 2016), or if there is variation among the populations in response to different fungal species coupled with spatial variation in fungal community composition (Lekberg et al., 2007; Ji et al., 2010), we would only be able to detect differences in responsiveness among populations if we had collected AM fungal communities originating from each of our seven field populations. Furthermore, we may have observed population-level differences in responsiveness had we grown these plants in the local field environments where the seeds originated (Pánková et al., 2011, 2014) or manipulated resources (e.g., water or nutrients), instead of growing the plants under common light, water, and nutrient conditions. Thus, the fact that we used a standard AM fungal inoculum for all plants and grew plants under controlled greenhouse conditions might have influenced our ability to detect population-level differentiation in the extent of AM responsiveness. We recognize that using a generic inoculum can dampen overall responses (Klironomos, 2003; Rúa et al., 2016). Some evidence suggests that plants or AM

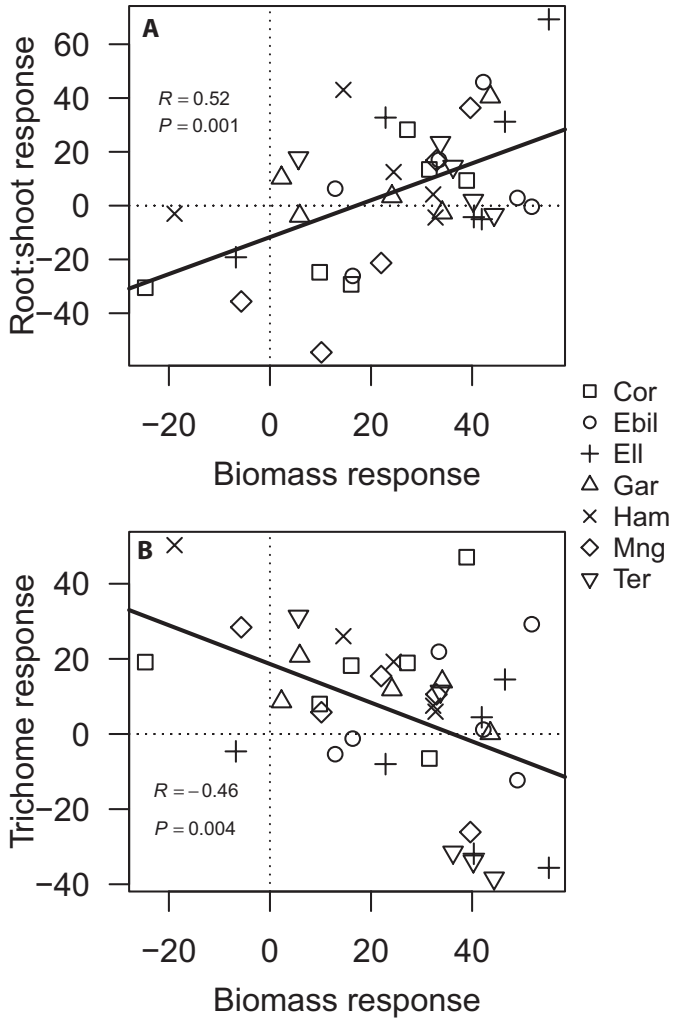

FIGURE 3. Correlations between percentage AM fungi responsiveness of (A) biomass and root:shoot and (B) biomass and trichome density. Values above zero indicate an increase in that trait in the presence of AM fungi, whereas values below zero indicate a reduction in that trait in the presence of AM fungi. AM fungi responsiveness was calculated between full sibling pairs. Symbols represent different populations (see Appendix S1 for population codes).

fungi adapt to local soil conditions, rather than adapting strongly to each other (Johnson et al., 2010; Rúa et al., 2016; Koyama et al., 2017), whereas other evidence points to coevolution between plants and AMF (Merckx et al., 2008). Nonetheless, given that AM fungi can provide a multitude of services (Smith and Read, 2008; Pineda et al., 2013) that may change across environmental gradients, future studies addressing specific pairings of local AM fungi genotypes or communities and plants under different environmental conditions should be informative.

Our results comparing intraspecific trait variation among populations of *A. speciosa* suggest that although plant growth and resistance traits can show genetically based differentiation, interactions with AM fungi may have greater influences on these traits. AM fungi consistently increased growth and defense expression in all plants, regardless of where they originated, which may have particular importance in the context of global change. Drought can have strong negative effects on plant biomass, particularly in plants highly dependent on AM (Kivlin et al., 2013). AM fungi not only ameliorate drought stress for plants (Auge, 2001; Kivlin et al., 2013), but may also increase resistance traits in stressful environments where defense is costly. Finally, our work highlights a unique growth-defense trade-off in terms of responsiveness of these traits to AM fungi. As such, mutualisms may play an important role in mediating growth-defense strategies in plants.

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DATA ACCESSIBILITY

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.8jr73 (Waller et al., 2018).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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