Received: 4 October 2021 | Revised: 17 December 2021 | Accepted: 20 December 2021

DOI: 10.1002/ecs2.4052

ARTICLE

ECOSPHERE
AN ESA OPEN ACCESS JOURNAL

Native mycorrhizal fungi improve milkweed growth, latex, and establishment while some commercial fungi may inhibit them

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Funding information
Division of Environmental Biology, Grant/Award Numbers: 0919434, 1556664; Division of Industrial Innovation and Partnerships, Grant/Award Number: 2016549; Malone Family Foundation Perennial Agricultural Project; Office of Integrative Activities, Grant/Award Number: 1656006; USDA SERDP, Grant/Award Number: RC-2330

Handling Editor: Michael F. Allen

Abstract

Arbuscular mycorrhizal (AM) fungi are root symbionts that can facilitate plant growth and influence plant communities by altering plant interactions with herbivores. Therefore, AM fungi could be critical for the conservation of certain rare plants and herbivores. For example, North American milkweed species are crucial hosts for monarch butterflies (*Danaus plexippus*). Understanding how mycorrhizal composition affects milkweeds will have direct impacts on the conservation and restoration of both increasingly threatened guilds. We present data from three studies on the effect of AM fungal composition on milkweed growth, latex production, and establishment. First, we grew seven milkweed species with and without a mixture of native mycorrhizal fungi. We assessed how important fungal composition is to milkweed growth and latex production by growing four milkweed species with seven fungal compositions, as single-species inoculations with four native fungi, a mixture of native fungi, a single commercial fungus of presumably non-native origin, and noninoculated controls. Finally, we assessed the field establishment of two milkweed species with and without native mycorrhizal inoculation. Milkweed species grew 98% larger and produced 82% more latex after inoculation with native mycorrhizae. Milkweeds were strongly affected by fungal composition; milkweeds were inhibited by commercial fungi (average of −14% growth) and showed variable but positive responses to native fungal species (average of +3% to +38% biomass). Finally, we found that restoration establishment was dependent on inoculation with native fungi and milkweed species. Overall, our findings indicate that some milkweed species (i.e., *Asclepias syriaca* and *A. incarnata*) are not responsive to mycorrhizal fungal presence or sensitive to mycorrhizal composition while others are, including endangered species (*A. meadii*) and species of high conservation value (*A. tuberosa*). We conclude that the reintroduction of native AM fungi could improve the establishment of desirable milkweed species and should be considered within strategies for plantings for monarch conservation.

KEYWORDS
AMF, arbuscular mycorrhizal fungi, *Asclepias*, chemical defense, endangered species, fungal community composition, fungal inoculation, microbes, plant and fungal interactions, restoration, tritrophic interactions

INTRODUCTION

Milkweeds, or plants from the subfamily Asclepiadoideae, are an important functional group in North American grasslands and other biomes across the world. Like most grassland species, milkweeds have suffered from anthropogenic changes resulting in habitat loss and increased pesticide use, where milkweed populations are estimated to have declined 58% in a single decade from 1999 to 2010 (Pleasants & Oberhauser, 2013). This has led to two milkweeds being listed as federally threatened species, and more than 20 *Asclepias* species being protected in one or more states (USDA, 2006). Milkweed decline has cascading effects on a suite of other species, including reduced abundance of the obligately dependent monarch butterfly (*Danaus plexippus*) (Pleasants & Oberhauser, 2013) and milkweed tussock moth (*Euchaetes egle*) (Holdrege, 2010) and less host availability for the dozens of birds, bees, moths, and other insects that use milkweeds for food or shelter (Betz et al., 1994; Holdrege, 2010; Nickell, 1958). For monarchs alone, current conservation efforts are not enough. It was estimated that an additional 1.6 billion milkweeds need to be restored to support a resilient monarch population (Pleasants, 2017). Thus, the improved establishment of milkweeds in conservation plantings will not only reduce milkweed population decline but will aid the conservation of many other milkweed-dependent pollinators and herbivores.

A key to improving milkweed establishment in restoration may be to utilize the milkweed microbiome including arbuscular mycorrhizal (AM) fungi. Past work has identified that AM fungi improve plant local adaptation (Johnson et al., 2010; Schultz et al., 2001), diversity (van der Heijden et al., 1998; Vogelsang et al., 2006), succession (Bauer et al., 2015; Kardol et al., 2007; Koziol & Bever, 2019), and seedling growth and establishment (Grman et al., 2020). Milkweeds and many other grassland species can benefit from AM fungal inoculation (Bauer et al., 2018; Wilson & Hartnett, 1998), including *Asclepias tuberosa*, *A. verticillata*, *A. incarnata*, and *A. syriaca*. Beyond influencing milkweed growth, AM fungi may influence a plant's ability to provide biotic resistance to plant pathogens and herbivores (Malik et al., 2016; Middleton et al., 2015; Sikes et al., 2009) and to increase milkweed defense chemicals (Vannette et al., 2013). Milkweeds produce a variety of plant defense chemicals, most notably latex and cardenolides, which both protect the plant from herbivory and support milkweed-dependent insect associates (Betz et al., 1994).

Although mycorrhizal fungal use as inocula in the nursery and restoration industry is becoming increasingly more common (Koziol et al., 2018), some studies indicate that mycorrhizal inoculation is not always beneficial for milkweeds. For example, past work has shown that AM fungi can improve milkweed production of latex (Waller et al., 2018), cardenolides (Vannette et al., 2013), trichomes (Waller et al., 2018), and volatile organic compounds (Meier & Hunter, 2019). On the contrary, other studies have shown that AM fungi do not alter or reduce the concentration of cardenolides (Vannette et al., 2013; Vannette & Rasmann, 2012) or latex (Tao et al., 2016). Variation in responsiveness is expected between plant species, as late-successional plant species of greater conservation value have been found to be more responsive to AM fungi (Bauer et al., 2018; Koziol & Bever, 2015). However, mycorrhizal responses have varied for a single milkweed species across studies. For example, *A. verticillata* growth ranged from being inhibited by to strongly benefited from mycorrhizal inoculation depending on the study (Bauer et al., 2018; Tao et al., 2016; Vannette & Rasmann, 2012; Wilson & Hartnett, 1998). Identifying the root cause of the variation in milkweed mycorrhizal response is needed to better understand the role mycorrhizal fungi may play in milkweed conservation and restoration.

Variation in milkweed response to mycorrhizal inoculation may be due to differences in plant germplasm, which has been shown to vary for other plant species (Schultz et al., 2001; Seifert et al., 2009). Variation in milkweed response may also be driven by inconsistent mycorrhizal inoculum sources between studies. Past milkweed studies (previously mentioned) have utilized various inoculum sources, including native mycorrhizal cultures, whole soil field inoculum, and several varieties of commercial inocula of presumably non-native origin. The mycorrhizal composition may matter because AM fungal species are ecologically and functionally distinct and can vary in their effect on plant–fungal nutrient exchange and plant growth (Aggangan et al., 2010; Ji & Bever, 2016), plant secondary chemical production (Bennett et al., 2009), and pathogen resistance (Malik et al., 2016). Plants with strong dependencies on mycorrhizae

demonstrate highly specific beneficial responses to particular native fungal species (Cheeke et al., 2019; Koziol & Bever, 2016; Pringle & Bever, 2008). Generally, commercially available mycorrhizal strains have been shown to be less effective for native plant establishment, cover, or soil stability (Emam, 2016; Maltz & Treseder, 2015; Middleton et al., 2015; Ohsowski et al., 2017; Vogelsang & Bever, 2010; White et al., 2008), sometimes even inhibiting native plants. To resolve how sensitive milkweeds are to inoculum source and fungal diversity, a study is needed that compares milkweed response across multiple mycorrhizal inocula.

Here, we present data from two growth chamber experiments and one field study on the effect of mycorrhizal fungi on milkweeds. In the first study, we choose seven milkweed species and assessed their growth and latex production when grown with and without a mixture of native mycorrhizal fungi. In the second study, we assess the importance of fungal composition to milkweed growth and latex production. We grew four milkweed species with seven fungal compositions, as single-species inoculations with four native mycorrhizal species, a mixture of native mycorrhizae, a single commercial mycorrhiza, or controls. For these studies, we assess whether there are growth versus defense trade-offs in milkweeds due to mycorrhizal composition. Finally, we assess field data of two milkweed species when grown with and without native mycorrhizal addition in a restoration context and assess plant establishment. Given past work in this subject area, we hypothesized that (1) most milkweed species would demonstrate positive growth and establishment responses to native mycorrhizal inocula, (2) milkweeds would not be responsive to commercial inocula, and (3) milkweeds would vary in which mycorrhizal species alter growth or latex production.

MATERIALS AND METHODS

Growth chamber experiments

Experimental designs

Growth study, Experiment 1

We chose seven milkweed species with readily available seed (*Asclepias asperula*, *A. speciosa*, *A. syriaca*, *A. tuberosa*, *A. viridis*, *A. incarnata*, and *A. meadii*). Seedlings were transplanted into inoculation treatments arranged in eight randomized replicated blocks. Plants were grown in 500-cm^3 pots inoculated 10% by volume with a diverse native fungal mixture that was living or autoclaved depending on treatment (see fungal material, below). The total number of pots was 112.

Composition study, Experiment 2

We chose four milkweed species (*A. incarnata*, *A. tuberosa*, *A. syriaca*, and *A. viridis)*. Twelve replicates of seedlings were planted into inoculation treatments, with the exception of *A. syriaca*, which had 10 replicates due to low germination. Plants were grown in 500-cm^3 pots. Each inoculated pot received 50 cm^3 of the diverse native fungal mixture, one of four native AM fungal species, or a commercial fungal inocula (Pro-Mix BX Mycorrhizae, Premier Tech Horticulture, USA). Due to differences in consistencies of inoculation material, (i.e., Pro-Mix vs. laboratory-grown cultures), pots inoculated with native fungi and the control received 50 cm^3 of sterilized commercial inocula. Similarly, pots inoculated with commercial fungi and the control received 50 cm^3 of sterilized native inocula. The total number of pots was 322.

Growth chamber

Seed germination and both the growth and composition study occurred in a growth chamber (Conviron BDR16; Controlled Environments Inc., Pembina, ND, USA) beginning in September 2016. The growth study occurred in a single growth chamber, while the composition study occurred in three growth chambers, each containing four replicates. Conditions in all chambers were set to mimic late spring conditions, with 14:10-h (light:dark) day length, 24:20°C temperatures, and 60% relative humidity. Light intensity ranged from 400 to 600 par on a 2-h step-up/down period during the light phase and 0 par during the dark phase. All pots were well watered via drip irrigation and received 66 ml of water twice daily.

Seed germination

Seeds were hand-collected from nearby remnant prairies at or within 20 km of Rockefeller Native Prairie in Lawrence, KS (39°02′43.4″ N, 95°12′07.6″ W). *Asclepias incarnata* seeds were purchased from Prairie Moon (Winona, MN, USA). Seeds were cold moist-stratified at 4°C in flats of sterilized potting mix for 1 month prior to germination in a growth chamber. Seedlings were grown in well-watered conditions for 2 weeks prior to transplanting in experimental pots.

Background soil

Background soil was collected from the University of Kansas Field Station (Lawrence, KS, USA, 39°05′00.6″ N,

95°18′68.9″ W) and was mixed 1:1 with sand. The background soil mix was 10.15 P ppm via Mehlich extraction, 7.375 NO_3-N ppm, and 22.2 NH_3-N ppm via KCl extractions. The background soil mixture was autoclaved twice for 2 h with a 1-day rest period between sterilizations.

Fungal material

Native AM fungal inocula were originally created with spores isolated from tallgrass prairies near the Kankakee Sands Nature Preserve (Morocco, IN, USA, 41°05′11.1″ N, 87°25′05.2″ W). During 2016, single-species mycorrhizal cultures were grown in the glasshouse with sorghum grass in a sterile mixture of sand and Kansas soil (Vogelsang et al., 2006). The native AM fungi included in these experiments were as follows: *Claroideoglomus claroideum*, *Funneliformis mosseae*, *Cetraspora pellucida*, and *Entrophospora infrequens*. The diverse native fungal mix (henceforth called diverse fungal mix) was a mixture of these four fungal species in equal volumes. A mean infection percentage indicated that similar initial infection levels occurred among these fungal species (Appendix S1). The commercial inoculum, Pro-Mix BX Mycorrhizae (Premier Tech Horticulture, multiple production plants, USA), was purchased just prior to the start of the experiment. The commercial product was reported to contain a single mycorrhizal species, *Glomus intraradices* (now known as *Rhizophagus irregularis*).

Plant growth measurements

Plant initial size (as height, in millimeters) was measured immediately after planting the experiments in September 2016, and these initial size measurements were used as covariates in the statistical analyses to account for initial size variation. After 15 weeks, plants were harvested, and dry weights were collected for roots and shoots (Experiment 1). Roots of only three randomly selected replicates of *A. meadii* were collected, as donating living rootstock of the remainder of these plants to restoration was a condition of being able to use seeds of this threatened plant species. After harvesting and weighing, a subsample of roots from each plant was reconstituted in water for 24 h, stained with trypan blue, and analyzed to confirm AM fungal colonization (McGonigle et al., 1990) (Appendix S1). After 18 weeks, shoots were collected (Experiment 2). We did not harvest roots in the composition Experiment 2, as we were allowing the shoots to regrow for a monarch larval feeding experiment, which did not ultimately occur due to egg hatching asynchrony.

Latex production

Latex production was measured by clipping six holes (three per each of the third pair of two leaves) with a standard hole punch along the center vein of each leaf. After leaf damage, the volume of exuded material, namely, latex and henceforth referred to as latex, was collected using a capillary tube (60-mm microhematocrit capillary tube, red-tip heparinized pre-calibrated capillary tubes with an internal diameter of 0.55 ± 0.05 mm, Fisher Scientific, Hampton, NH, USA). The total length of the exuded material was assessed in millimeters. See Appendix S1 for additional details on latex collection methods.

Field study, Experiment 3

During 2015, we planted plugs of *A. incarnata* and *A. tuberosa* into a restoration experiment that was initiated during 2014. Full details of the 2014 restoration design can be found open access, and experimental details, such as AM fungal culturing, site preparation, and harvest, can be found in Koziol and Bever (2017). Briefly, during 2015, seedlings of *A. tuberosa* and *A. incarnata* were cold moist-stratified for 4 weeks in sterilized sand and then allowed to germinate in the glasshouse for 2 weeks. Seedlings were transplanted into 150-cm^3 conetainers that were inoculated 10% by volume with native AM fungi *E. infrequens*, *C. lamellosum*, *C. claroideum*, or *A. spinosa*, their mixture, or sterilized inocula. Seedlings were transplanted in the field after 20 days during the last week of May 2015. Four months after transplanting, we monitored the survival of the seedlings.

Statistical analyses

All data were log(x + 1) transformed prior to analysis. For the growth Experiment 1, we analyzed plant growth response of dry total plant weight, shoot mass, and root mass using Proc GLM in SAS 9.4 (SAS, 2015) with block, plant species, inoculation treatment, initial size by plant species, and plant species by inoculation treatment as predictors. For the composition Experiment 2, we used the same model to assess shoot biomass. To assess the effects of fungal composition, we designed a priori contrasts to compare growth and latex production when plants were inoculated versus noninoculated, inoculated with native fungi versus noninoculated, inoculated with commercial fungi versus noninoculated, differences among inoculation with each native fungal species, differences after inoculation with commercial fungi versus native fungi single-species isolates, and these contrasts by plant species. We used these same models to assess latex production. We used the Bonferroni

corrections on the nonorthogonal contrasts to adjust for multiple comparisons. To assess whether latex production was a direct effect of mycorrhizae or an indirect effect of changes in plant size due to mycorrhizae, we designed a model using milkweed species by total plant size log(1 + mass [g]) at harvest as an additional predictor of latex production.

For both Experiments 1 and 2, mycorrhizal responsiveness (MR) was evaluated using average mass or latex production for each plant by fungal species combination as follows:

$$\mathrm{MR} = \frac{\log(1 + \text{average plant biomass or latex with inoculation})}{\log(1 + \text{average plant biomass or latex without inoculation})}.$$

Pearson's correlation tests were conducted on the MR of plant growth and latex production for each inoculation treatment for both Experiments 1 and 2. To further resolve the role of fungal composition on the growth versus defense allocation in Experiment 2, we designed two mixed models comparing MR in growth and latex with MR in growth, nativeness (either locally sourced or commercial) of inoculum, and their interaction as main effects. First, we grouped fungi into categories of native versus commercial (intercept as the random effect). Second, we treated all inocula as random, using nativeness of fungi isolate and nativeness of fungi by isolate by MR in growth as random effects. We used a chi-squared test to test whether plant establishment was predicted by inoculation with native mycorrhizal fungi for each milkweed for the field study, Experiment 3. With the limited number of replicates in the field study, we were not able to run more complicated models that tested differences among AM fungi inocula.

RESULTS

Growth chamber experiments

Experiment 1

We found that inoculation with native fungi was the strongest predictor of plant total mass, shoot mass, root mass, and latex production (Table 1). Plant shoot mass was 98% greater after inoculation with the native AM fungal mix (Table 1, Figure 1a; $F_{1,74} = 106.25$, $p < 0.0001$), and inoculated plants produced 82% more latex (Table 1, Figure 1b; $F_{1,71} = 62.54$, $p < 0.0001$). Inoculated milkweeds also produced more latex in the model that controlled for final plant size ($F_{1,64} = 6.26$, $p = 0.014$), indicating a direct effect of AM fungal inoculation on improved latex production.

We found a significant milkweed species by inoculation interaction (Table 1, Figure 1c; $F_{6,74} = 3.73$, $p = 0.003$), where although all species grew larger with native mycorrhizal inoculation, some species were found to be more responsive to mycorrhizae than others. Specifically, the shoot masses of *A. incarnata* and *A. syriaca* benefited less from inoculation (MR ≈ 1.2), while *A. asperula*, *A. meadii*, *A. speciosa*, *A. tuberosa*, and *A. viridis* benefited more strongly from mycorrhizal inoculation (MR ranged from 3.3 to 14.6). All milkweeds produced more latex after inoculation with native AM fungi (Table 1, Figure 1d; $F_{6,74} = 1.52$, $p = 0.191$), with *A. syriaca* responding the least and *A. asperula* responding the most to inoculation.

Experiment 2

Similar to what was reported for Experiment 1, inoculation was a strong predictor of plant total mass (Figure 2a) and total latex production (Figure 2b) in the fungal composition Experiment 2 (Table 2). However, this effect was dependent on inoculation source. Average plant mass (13.9% greater; Table 2, Figure 2a; $F_{1,268} = 6.12$, $p = 0.042$) and latex production (21.2% greater; Table 2, Figure 2b; $F_{1,268} = 7.18$, $p = 0.025$) were significantly greater after inoculation with native AM fungi relative to the control. Native fungal species were significantly different from each other in their effect on plant growth (Table 2) and were marginally significantly different in how well they influenced latex production (Table 2, Figure 2b; $F_{3,133} = 2.31$, $p = 0.079$). As was found in Experiment 1, we also found evidence of a direct effect of

TABLE 1 Experiment 1: Growth of seven milkweeds with and without a native mycorrhizal fungal mixture

		Log(1 + shoot mass [g])		Log(1 + total mass [g])		Log(1 + root mass [g])		Log(1 + latex [mm])	
Model predictors	df	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Initial size x milkweed	7	1.17	0.3284	0.64	0.7227	0.42	0.8872	0.38	0.9126
Block	7	1.62	0.1428	1.54	0.1697	1.16	0.3366	1.03	0.4163
Milkweed	6	0.77	0.5991	0.98	0.4487	0.67	0.6779	0.44	0.8526
Inoculation	1	106.25	<0.0001	156.8	<0.0001	137.49	<0.0001	62.54	<0.0001
Milkweed x inoculation	6	3.73	0.0027	8.93	<0.0001	7.76	<0.0001	1.5	0.1912

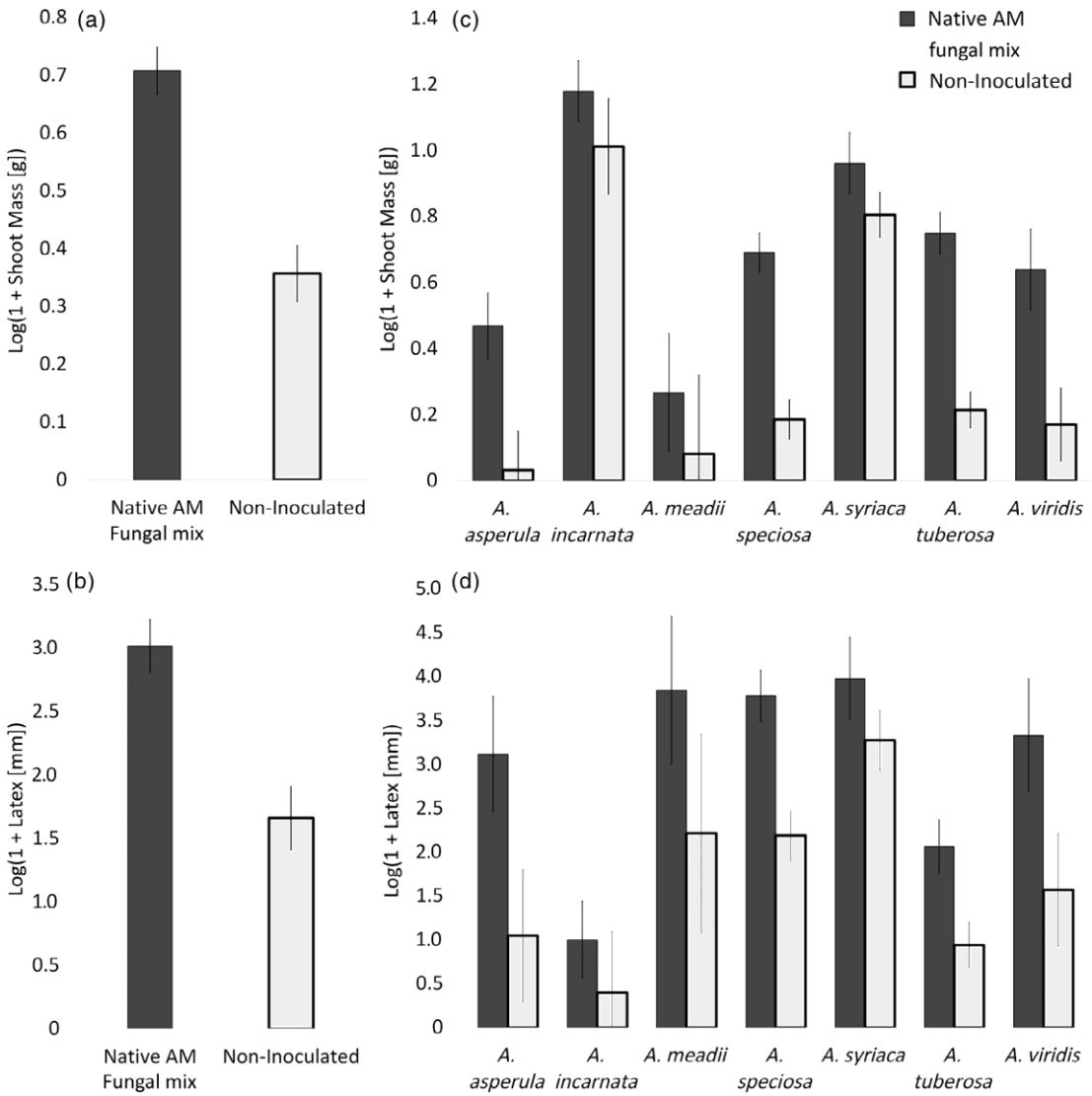

FIG URE 1 Average effect of inoculation on milkweed growth (a) and latex production (b) for all species, the growth (c) and latex production (d) of each individual milkweed species. Bars represent the least squares means of plant growth or latex production, and error bars are standard error from the Proc GLMs. Shading on bars represents the different inoculation treatments of native AM fungi (black) and noninoculated (white)

native AM fungi improving latex production that was not dependent on final plant size ([$F_{1,129}$ = 6.16, p = 0.043], inoculated with native vs. noninoculated contrast).

Generally, commercial inocula affected milkweed growth and latex production differently than native fungi. Commercial inocula reduced plant mass relative to the control (13.8% reduced; Table 2, Figure 2a; $F_{1,268}$ = 3.58, p = 0.2), although this effect was not significant after multiple comparison corrections. Commercial fungi did not alter latex production relative to the noninoculated control (Table 2, nonsignificant), even when controlling for final plant size ($F_{1,129}$ = 0.88, p = 1.0). Plants inoculated with single species of native fungi grew 32.5% larger on average than plants inoculated with the commercial inocula ($F_{1,268}$ = 24.02, p < 0.0001). Because we controlled for differences in background media across the different inocula, these findings can be attributed to the fungal species composition of the native and commercial inocula.

Milkweed species growth significantly differed among the various fungal compositions (Table 2, Figure 3a–d; $F_{18,268}$ = 2.75, p = 0.0002). Similar to growth Experiment 1, the shoot masses of *A. syriaca* and *A. incarnata* were largely unaffected by any inoculation (MR of ≈0.8–1; Figure 3a,b), whereas average *A. tuberosa* and *A. viridis* growth was 48% and 56% larger on average by inoculation with native fungi relative to the control (Figure 3c, d). There was a marginally significant trend for milkweed species to differ in their response to individual native AM fungal species (Table 2, Figure 3a–d; $F_{9,268}$ = 1.82,

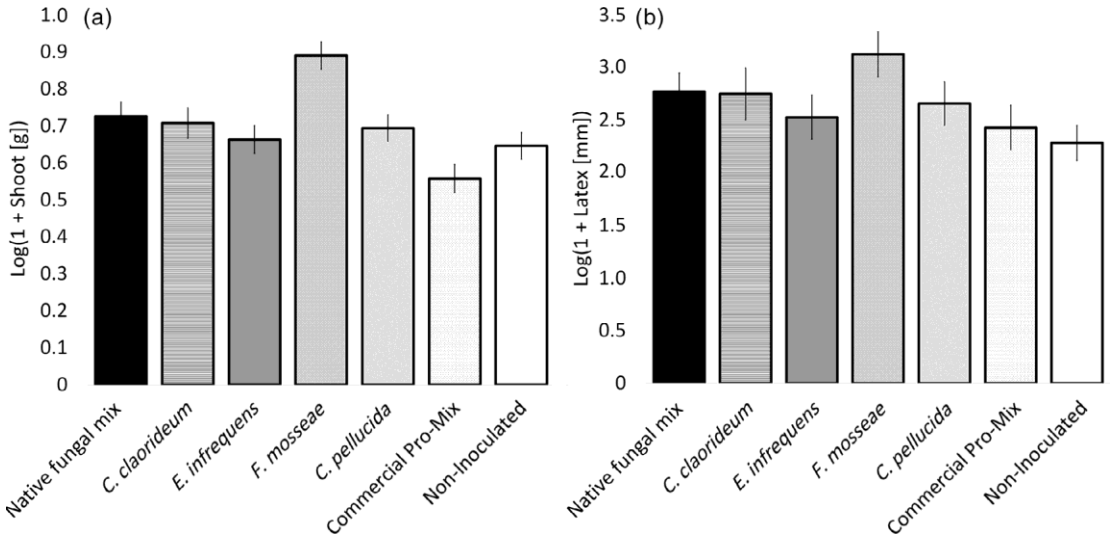

FIG URE 2 Average effect of fungal composition on milkweed shoot growth (a) and latex production (b) for the four milkweed species. Bars represent the least squares means of plant growth or latex production, and error bars are standard error from the Proc GLMs. Shading on bars represents the different inoculation treatments

TABLE 2 Experiment 2: Growth and latex production for four milkweed species with seven different fungal compositions

		Log(1 + total mass [g])		Log(1 + latex [mm])	
Model predictors and contrasts	df	F	p/Bonferroni-corrected p	F	p/Bonferroni-corrected p
Initial size x milkweed	4	5.32	0.0004	2.30	0.0623
Block	11	3.14	0.0005	0.69	0.7491
Milkweed	3	1.74	0.1582	1.16	0.3278
Inoculation	6	9.52	<0.0001	2.59	0.0207
Inoculated versus noninoculated	1	2.8	0.0956	5.79	0.0175
Native fungi versus noninoculated	1	6.12	0.014/0.042	7.18	0.0083/0.0249
Commercial fungi versus noninoculated	1	3.58	0.0594/0.1789	0.35	0.556/1.0
Differences among native fungi	3	9.66	<0.0001	2.31	0.0791
Commercial versus average native single	1	24.02	<0.0001	2.51	0.1154
Milkweed x inoculation	18	2.75	0.0002	1.25	0.2337
Inoculated versus noninoculated x milkweed	3	2.81	0.0398	0.66	0.5804
Native fungi versus noninoculated x milkweed	3	3.71	0.0121/0.0363	0.67	0.5746/1.0
Commercial fungi versus noninoculated x milkweed	3	1.28	0.2813/0.8439	0.35	0.7926/1.0
Differences among native fungi x milkweed	9	1.82	0.0645	1.01	0.4387
Commercial versus average native single x milkweed	3	4.17	0.006	0.30	0.8251

p = 0.065), with growth variation among native fungi ranging from ~20% to 40% for *A. incarnata* and *A. syriaca* and from 100% to 130% for the more mycorrhizally responsive *A. tuberosa* and *A. viridis*. We found significant differences in the commercial fungi versus native fungi contrast by milkweed species ($F_{3,268}$ = 4.17, p = 0.006), which was likely driven by *A. syriaca*, *A. tuberosa*, and *A. viridis* growing smaller with the commercial inoculant relative to inoculation with single native fungal species and *A. incarnata* showing variable growth responses. Inoculation with commercial fungi relative to the control did not differ among the different milkweed species, and most milkweeds were inhibited by commercial fungi to some degree (0% to −42% biomass reduction with commercial inocula; Table 2, Figure 3a–d).

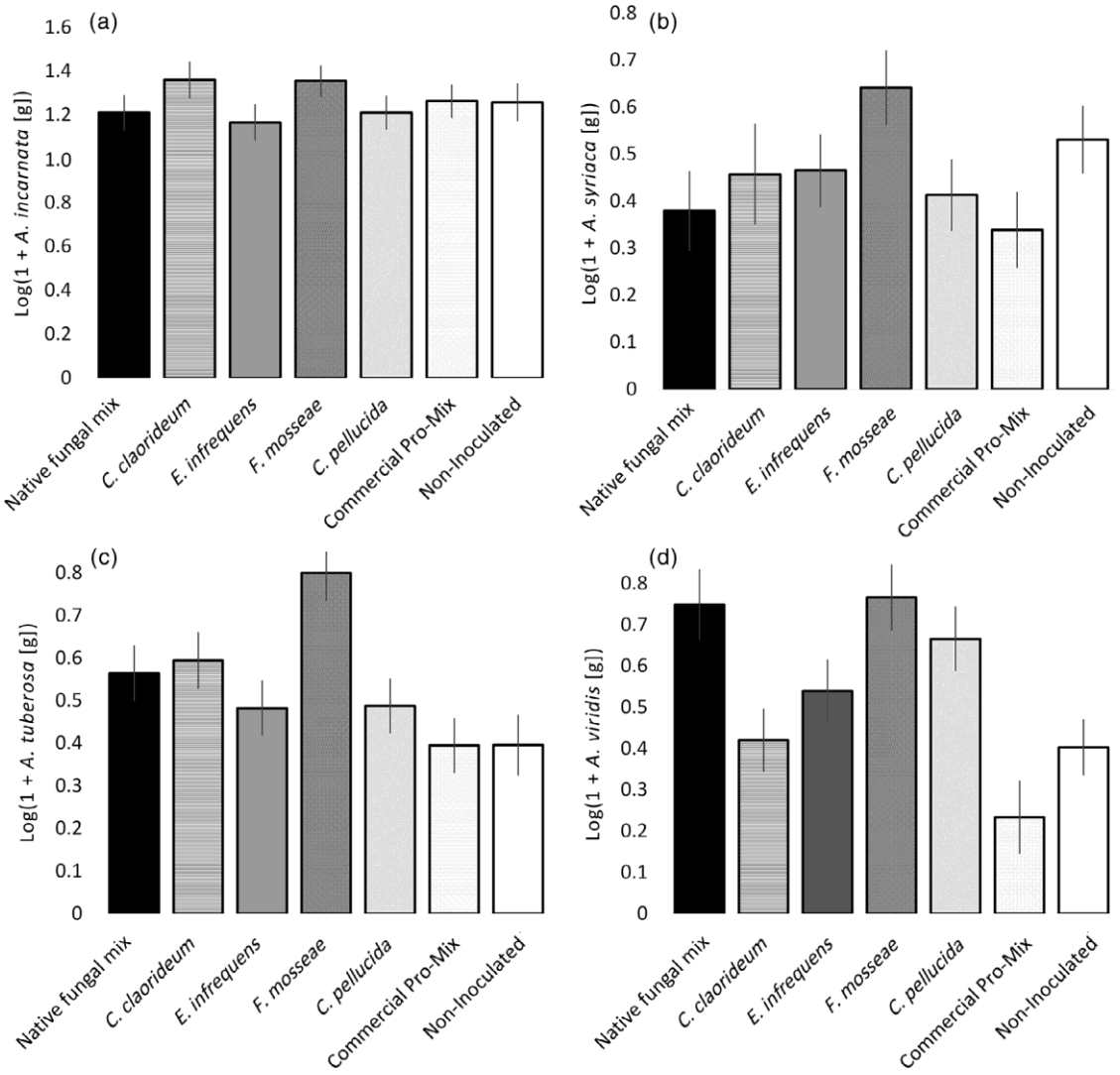

FIG U RE 3 Effect of inoculation on milkweed growth for *Asclepias incarnata* (a), *A. syriaca* (b), *A. tuberosa* (c), and *A. viridis* (d). Bars represent the least squares means of plant growth, and error bars are standard error from the Proc GLMs. Shading on bars represents the different inoculation treatments

Correlation tests of mycorrhizal responsiveness in growth and latex production

Mycorrhizal responsiveness in plant growth was positively correlated with latex production in Experiment 1 (Figure 4a; $r^2 = 0.88$, $t = 5.99$, $p = 0.002$), where plant species that grew larger with native fungi also produced more latex with native fungi relative to controls. This pattern was consistent in the fungal composition Experiment 2 (overall correlation, $r^2 = 0.41$, $t = 3.50$, $p = 0.003$). Our mixed model regressions revealed that MR in latex production was marginally predicted by the nativeness of the fungal inoculation ($F_{1,20} = 3.04$, $p = 0.096$), with an interaction between fungal nativeness and MR in growth ($F_{1,20} = 3.84$, $p = 0.064$). We found that all individual native fungal species showed a positive slope between MR growth and latex, while commercial fungal inoculation resulted in a negative correlation (Figure 4b).

Field Experiment 3

Milkweed survival in the field varied with inoculation and milkweed species (Figure 5). We found that the growing season survival of *A. tuberosa* seedlings was strongly dependent on native AM fungal inoculation, where 50% of 26 inoculated plants survived with native fungal inoculation and 0% of the six noninoculated plants survived the growing season ($\chi^2 = 5.05$, $p = 0.02$). *Asclepias incarnata* had much better

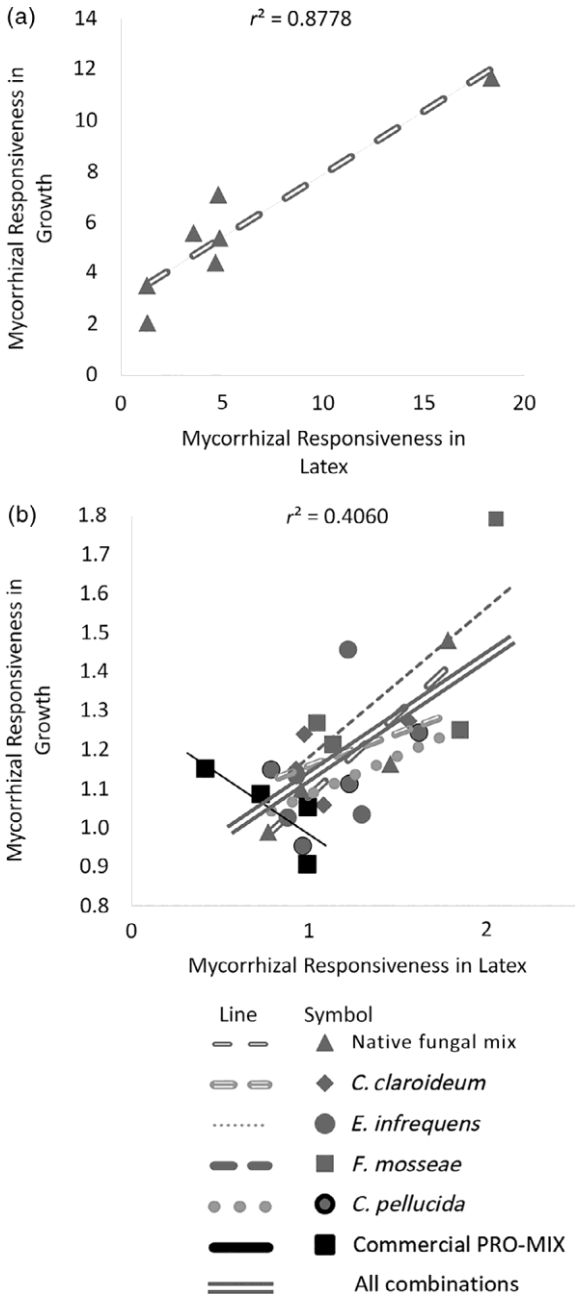

FIG URE 4 (a) Mycorrhizal responsiveness (MR) in growth was strongly correlated with the MR in latex production for each of the seven milkweed species in Experiment 1. Here, each point represents the MR of a plant species with the native fungal mix. (b) The MR in growth was strongly correlated with the MR in latex production for the four milkweed species in Experiment 2. Here, each point represents MR of a plant species when grown with a particular inoculum. The double solid line represents the best-fit line for the overall pattern and the overall r^2. Each fungus is demarked with a unique symbol and the best-fit slope of the relationship between MR in growth and MR in latex production

survival overall (~69%), and its survival was not dependent on mycorrhizal inoculation (χ^2 = 0.01, p = 0.9; Figure 5).

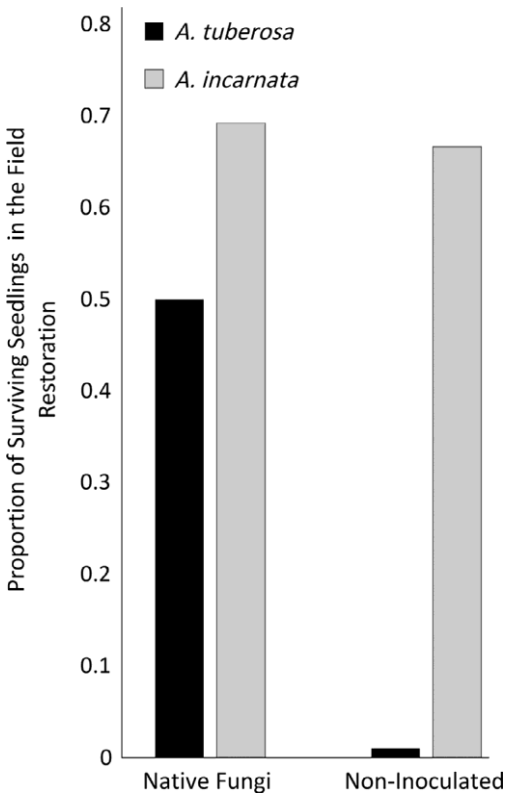

FIGURE 5 Field survival of two milkweed species with native mycorrhizal inocula or noninoculated. Bars represent the proportion of plants surviving

DISCUSSION

We present data from three different studies highlighting that native AM fungi can improve *Asclepias* spp. growth, latex production, and establishment in restoration. Our first experiment assessed mycorrhizal response to a native inoculum mixture for a wide selection of milkweed species including seven species that ranged from the very common milkweed (*A. syriaca*) to the federally threatened Mead's milkweed (*A. meadii*). We found that each of the seven milkweed species grew larger and produced more latex with native mycorrhizal inoculum, a finding consistent with past work comparing native mycorrhizal mixture (either laboratory-derived inoculum comprising only native mycorrhizal cultures or whole soil collections) with noninoculated controls (Bauer et al., 2018; Waller et al., 2018; Wilson & Hartnett, 1998). Similar to those past studies, we found a range of MR from small (i.e., *A. incarnata* and *A. syriaca*) to more than three times growth improvement with native mycorrhizae (i.e., *A. meadii* and *A. tuberosa*). For two of these species, the weakly mycorrhizally responsive *A. incarnata* and the strongly responsive *A. tuberosa*, we showed that mycorrhizal responses in a controlled environment mirrored mycorrhizal response in the field, a pattern that

has been shown previously for other plant species (Koziol & Bever, 2019; Pringle & Bever, 2008). Together, these results suggest that the reintroduction of native mycorrhizal fungi can facilitate re-establishment of milkweeds of conservation interest and has potential value for monarch butterfly conservation strategies.

Fungal composition on milkweed growth

In the study comparing the effects of mycorrhizal composition, the presence of native mycorrhizae was generally a strong predictor of beneficial plant growth. Variation in milkweed response to native fungal isolates ranged from slightly inhibited by to greatly benefited from individual native species. Past work has identified that plants with strong dependencies on mycorrhizae demonstrate greater specificity in growth response to particular fungal species (Cheeke et al., 2019; Koziol & Bever, 2016; Pringle & Bever, 2008). Our study on milkweed mycorrhizal response is consistent with these findings, as the plants that were more strongly benefited from mycorrhizae overall (*A. tuberosa* and *A. viridis*) demonstrated greater variation in growth due to fungal composition and plants that were less benefited from or even inhibited by mycorrhizae (*A. syriaca* and *A. incarnata*) demonstrated less sensitivity to mycorrhizal species composition.

Commercially available mycorrhizal strains have been shown to be less effective for native plant establishment and growth (Emam, 2016; Maltz & Treseder, 2015; Ohsowski et al., 2017; Vogelsang & Bever, 2010; White et al., 2008), sometimes even inhibiting native plants, including milkweeds (Tao et al., 2016; Vannette & Rasmann, 2012). Our study supports these findings, as we found that milkweed growth and latex production were not responsive to this commercial fungus and that this commercial fungus failed to promote milkweed growth and the native fungal species for most milkweed species assessed. The mechanism behind this pattern was not assessed in this study, but a recent review has highlighted that the in vitro cultivation process often utilized in commercial mycorrhizal production can result in mycorrhizal fungi with reduced genetic variation, reduced symbiotic capabilities (including reduced plant benefit), and failed field establishment (Kokkoris & Hart, 2019). It should be noted that the commercial inoculum sources differed among past milkweed studies (i.e., Tao et al., 2016; Vannette et al., 2013). Those studies found the commercial isolates of the fungus *F. mosseae* to be nonbeneficial, whereas the native isolate of *F. mosseae* used in this study was found to be generally beneficial in single-species inoculations and as part of a mixture. An alternative explanation is that many commercial fungi are non-native and nonlocally adapted. Few studies have investigated local adaptation in mycorrhizae, yet past work has indicated that both plants and fungi can be locally adapted to soils and environments (Bauer et al., 2020; Johnson et al., 2010; Rúa et al., 2016; Schultz et al., 2001; Stahl & Smith, 1984). Future work should aim to assess how domestication and commercial cultivation of mycorrhizae may alter their ecological interactions before widespread use in restoration and conservation efforts.

Growth versus defense trade-offs

Given that the variation in milkweed response to mycorrhizae in past work is likely attributed to variable mycorrhizal sources utilized among past studies, the influence of mycorrhizae on the growth versus defense relationship in milkweeds remains unresolved. Reduced dependence on AM fungi may facilitate milkweed colonization of disturbed landscapes where the abundance and infectivity of mycorrhizal fungi can be reduced (Abbott & Robson, 1991; Jasper et al., 1991). However, should mycorrhizally mediated improvements in growth result in less defended plants, reduced latex production could negatively impact the community of biota with adaptive mechanisms that benefit from milkweed latex and cardenolide production, such as the monarch butterfly, milkweed tussock moth, and others (Betz et al., 1994; Holdrege, 2010; Pleasants & Oberhauser, 2013). We found that plant MR in growth was positively correlated with MR in latex production with native mycorrhizal inoculation, but not with commercial fungal inoculation. Our results support past work using commercial fungal mixtures that found no relationship or a negative relationship between growth response to commercial fungi and cardenolides and latex production (Tao et al., 2016; Vannette et al., 2013), as well as past work using native whole soil inoculum, which found a nonsignificant positive correlation between MR and latex production (Waller et al., 2018). Although we include only four native fungal species, each species consistently demonstrated a positive correlation in growth and latex improvements due to fungal composition. Furthermore, the effect of mycorrhizae on improved latex production was maintained when total plant size at harvest was included in the model, indicating a direct effect of increased latex due to native mycorrhizal inoculation. Therefore, we suggest that future work on milkweed growth and defense relationships mediated by mycorrhizae aim to include mycorrhizae native to that system.

Conservation and restoration implications

Milkweed species demonstrated a range of responses to native AM fungal inoculation across these studies. Generally, milkweeds with high conservation value, such as the endangered mead's milkweed (*A. meadii*) and the butterfly milkweed (*A. tuberosa*), consistently benefited from inoculation with native AM fungi. Species of lower conservation value demonstrated variable responses, with several being unresponsive. For instance, in each of the three studies presented, *A. incarnata* was generally unresponsive to mycorrhizae and inoculation is likely not a useful tool to improve the restoration success of this species. Past work across grassland species from other plant families has demonstrated that more conservative species are generally more responsive to their native fungi and demonstrate greater specificity toward specific fungal compositions than less conservative species and that native inoculations can improve field establishment of conservative late-successional plants (Cheeke et al., 2019; Koziol & Bever, 2017). Therefore, native fungal inoculation could be an important tool in establishing critically endangered and highly conservative species such as *A. tuberosa* and *A. meadii* in conservation and restoration on a landscape scale. Given that we found native mycorrhizal fungi to improve latex production of milkweed species and that past work has indicated that latex production has impacts on monarch success (Zalucki et al., 2001), future work should assess the potential value of native AM fungal inoculations for both conservative milkweed establishment and monarch population resilience and restoration.

Overall, we identify that re-establishment of milkweeds of high conservation value can be improved by inoculation with native mycorrhizal fungi. While many milkweeds benefit from mycorrhizal inoculation, care should be taken regarding mycorrhizal source and composition in future restoration efforts. When possible, a species-diverse native mycorrhizal inoculum should be targeted for restoration efforts, as both in this study and others (Bauer et al., 2018; Waller et al., 2018; Wilson & Hartnett, 1998), diverse native inoculum is more consistently beneficial to milkweed growth and field establishment. Native inoculum availability can include sourcing native mycorrhizal cultures, trap cultures, or whole soil harvesting methods that range in cost and current local availability (Koziol et al., 2018). Past work on milkweed response to mycorrhizae has predominately included inoculation solely with commercial inoculum, which we find often performs differently than, even oppositely to, native inoculum. Future work on milkweed–mycorrhizal interactions should include native inoculum to better contribute to a predictive framework for understanding how mycorrhizae influence milkweed growth and defense and can contribute to milkweed restoration and monarch butterfly conservation practice.

ACKNOWLEDGMENTS

We would like to thank Helen Alexander and the Monarch Watch, especially Ann Ryan and Chip Taylor, for donating seeds and providing advice, undergraduate assistants Grace Parker, Austin Yoder, and Josh Schmanski, and the Bever-Schultz laboratory group. Liz Koziol is the owner of MycoBloom LLC. MycoBloom did not participate in this research. We acknowledge support from the National Science Foundation (DEB 0919434 and 1556664, OIA 1656006, PFI 2016549), SERDP (RC-2330), and Land Institute and Malone Family Foundation Perennial Agricultural Project.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data (Koziol, 2022) are available from Dryad: https://doi.org/10.5061/dryad.hdr7sqvk4.

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REFERENCES

Abbott, L., and A. Robson. 1991. "Factors Influencing the Occurrence of Vesicular-Arbuscular Mycorrhizas." *Agriculture, Ecosystems & Environment* 35: 121–50.

Aggangan, N. S., H. K. Moon, and S. H. Han. 2010. "Growth Response of *Acacia mangium* Willd. Seedlings to Arbuscular Mycorrhizal Fungi and Four Isolates of the Ectomycorrhizal Fungus *Pisolithus tinctorius* (Pers.) Coker and Couch." *New Forests* 39: 215–30.

Bauer, J. T., L. Koziol, and J. D. Bever. 2018. "Ecology of Floristic Quality Assessment: Testing for Correlations between Coefficients of Conservatism, Species Traits, and Mycorrhizal Responsiveness." *AoB Plants* 10: plx073.

Bauer, J. T., L. Koziol, and J. D. Bever. 2020. "Local Adaptation of Mycorrhizae Communities Changes Plant Community Composition and Increases Aboveground Productivity." *Oecologia* 192: 735–44.

Bauer, J. T., K. M. Mack, and J. D. Bever. 2015. "Plant-Soil Feedbacks as Drivers of Succession: Evidence from Remnant and Restored Tallgrass Prairies." *Ecosphere* 6: 158.

Bennett, A. E., J. D. Bever, and M. D. Bowers. 2009. "Arbuscular Mycorrhizal Fungal Species Suppress Inducible Plant Responses and Alter Defensive Strategies Following Herbivory." *Oecologia* 160: 771–9.

Betz, R. F., R. D. Struven, J. E. Wall, and F. B. Heitler. 1994. "Insect Pollinators of 12 Milkweed (*Asclepias*) Species." In *Proceedings of the Thirteenth North American Prairie Conference*. 45–60. Windsor: Department of Parks and Recreation.

Cheeke, T. E., C. Zheng, L. Koziol, C. R. Gurholt, and J. D. Bever. 2019. "Sensitivity to AMF Species Is Greater in Late-Successional Native than Early-Successional Native or Non-Native Grassland Plants." *Ecology* 100: e02855.

Emam, T. 2016. "Local Soil, but not Commercial AMF Inoculum, Increases Native and Non-native Grass Growth at a Mine Restoration Site." *Restoration Ecology* 24; 35–44.

Grman, E., J. Allen, E. Galloway, J. McBride, J. T. Bauer, and P. A. Price. 2020. "Inoculation with Remnant Prairie Soils Increased the Growth of Three Native Prairie Legumes but Not Necessarily Their Associations with Beneficial Soil Microbes." *Restoration Ecology* 28: S393–9.

van der Heijden, M. G. A., J. N. Klironomos, M. Ursic, P. Moutoglis, R. Streitwolf-Engel, T. Boller, A. Wiemken, and I. R. Sanders. 1998. "Mycorrhizal Fungal Diversity Determines Plant Biodiversity, Ecosystem Variability and Productivity." *Nature* 396: 69–72.

Holdrege, C. 2010. "The Story of an Organism: Common Milkweed." The Nature Institute. http://www.natureinstitute.org/text/ch/Milkweed.pdf.

Jasper, D., L. Abbott, and A. Robson. 1991. "The Effect of Soil Disturbance on Vesicular—Arbuscular Mycorrhizal Fungi in Soils from Different Vegetation Types." *New Phytologist* 118: 471–6.

Ji, B., and J. D. Bever. 2016. "Plant Preferential Allocation and Fungal Reward Decline with Soil Phosphorus: Implications for Mycorrhizal Mutualism." *Ecosphere* 7(5), e01256.

Johnson, N. C., G. W. T. Wilson, M. A. Bowker, J. A. Wilson, and R. M. Miller. 2010. "Resource Limitation Is a Driver of Local Adaptation in Mycorrhizal Symbioses." *Proceedings of the National Academy of Sciences* 107: 2093.

Kardol, P., N. J. Cornips, M. M. van Kempen, J. T. Bakx-Schotman, and W. H. van der Putten. 2007. "Microbe-Mediated Plant-Soil Feedback Causes Historical Contingency Effects in Plant Community Assembly." *Ecological Monographs* 77: 147–62.

Kokkoris, V., and M. M. Hart. 2019. "In Vitro Propagation of Arbuscular Mycorrhizal Fungi May Drive Fungal Evolution." *Frontiers in Microbiology* 10: 2420.

Koziol, L. 2022. "Data for Native Mycorrhizal Fungi Improve Milkweed Growth, Latex, and Establishment While Some Commercial Fungi May Inhibit Them." Dryad. Dataset. https://doi.org/10.5061/dryad.hdr7sqvk4.

Koziol, L., and J. D. Bever. 2015. "Mycorrhizal Response Trades off with Plant Growth Rate and Increases with Plant Successional Status." *Ecology* 96: 1768–74.

Koziol, L., and J. D. Bever. 2016. "AMF, Phylogeny and Succession: Specificity of Response to Mycorrhizal Fungi Increases for Later Successional Plants." *Ecosphere* 7: e1555.

Koziol, L., and J. D. Bever. 2017. "The Missing Link in Grassland Restoration: Arbuscular Mycorrhizal Fungi Inoculation Increases Plant Diversity and Accelerates Succession." *Journal of Applied Ecology* 54: 1301–9.

Koziol, L., and J. D. Bever. 2019. "Mycorrhizal Feedbacks Generate Positive Frequency Dependence Accelerating Grassland Succession." *Journal of Ecology* 107: 622–32.

Koziol, L., P. A. Schultz, G. L. House, J. T. Bauer, E. L. Middleton, and J. D. Bever. 2018. "The Plant Microbiome and Native Plant Restoration: The Example of Native Mycorrhizal Fungi." *Bioscience* 68: 996–1006.

Malik, R. J., M. H. Dixon, and J. D. Bever. 2016. "Mycorrhizal Composition Can Predict Foliar Pathogen Colonization in Soybean." *Biological Control* 103: 46–53.

Maltz, M. R. & Treseder, K. K.. 2015. "Sources of Inocula Influence Mycorrhizal Colonization of Plants in Restoration Projects: a Meta-analysis." *Restoration Ecology* 23; 625–34.

McGonigle, T., M. Miller, D. Evans, G. Fairchild, and J. Swan. 1990. "A New Method which Gives an Objective Measure of Colonization of Roots by Vesicular—Arbuscular Mycorrhizal Fungi." *New Phytologist* 115: 495–501.

Meier, A. R., and M. D. Hunter. 2019. "Mycorrhizae Alter Constitutive and Herbivore-Induced Volatile Emissions by Milkweeds." *Journal of Chemical Ecology* 45: 610–25.

Middleton, E. L., S. Richardson, L. Koziol, C. E. Palmer, Z. Yermakov, J. A. Henning, P. A. Schultz, and J. D. Bever. 2015. "Locally Adapted Arbuscular Mycorrhizal Fungi Improve Vigor and Resistance to Herbivory of Native Prairie Plant Species." *Ecosphere* 6: 276.

Nickell, W. P. 1958. "Variations in Engineering Features of the Nests of Several Species of Birds in Relation to Nest Sites and Nesting Materials." *Butler University Botanical Studies* 13: 121–39.

Ohsowski, B. M., Dunfield, K., Klironomos, J. N. & Hart, M. M.. 2017. "Plant Response to Biochar, Compost, and Mycorrhizal Fungal Amendments in Post-mine Sandpits." *Restoration Ecology* 26; 63–72.

Pleasants, J. 2017. "Milkweed Restoration in the Midwest for Monarch Butterfly Recovery: Estimates of Milkweeds Lost, Milkweeds Remaining and Milkweeds that Must Be Added to Increase the Monarch Population." *Insect Conservation and Diversity* 10: 42–53.

Pleasants, J. M., and K. S. Oberhauser. 2013. "Milkweed Loss in Agricultural Fields because of Herbicide Use: Effect on the Monarch Butterfly Population." *Insect Conservation and Diversity* 6: 135–44.

Pringle, A., and J. D. Bever. 2008. "Analogous Effects of Arbuscular Mycorrhizal Fungi in the Laboratory and a North Carolina Field." *New Phytologist* 180: 162–75.

Rúa, M. A., A. Antoninka, P. M. Antunes, V. B. Chaudhary, C. Gehring, L. J. Lamit, B. J. Piculell, J. D. Bever, C. Zabinski, and J. F. Meadow. 2016. "Home-Field Advantage? Evidence of Local Adaptation among Plants, Soil, and Arbuscular Mycorrhizal Fungi through Meta-Analysis." *BMC Evolutionary Biology* 16: 122.

SAS. 2015. *SAS 9.4 User's Guide: Survey Data Analysis*. Cary, NC: SAS Institute Inc.

Schultz, P. A., R. M. Miller, J. D. Jastrow, C. V. Rivetta, and J. D. Bever. 2001. "Evidence of a Mycorrhizal Mechanism for the Adaptation of *Andropogon gerardii* (Poaceae) to High- and Low-Nutrient Prairies." *American Journal of Botany* 88: 1650–6.

Seifert, E. K., J. D. Bever, and J. L. Maron. 2009. "Evidence for the Evolution of Reduced Mycorrhizal Dependence during Plant Invasion." *Ecology* 90: 1055–62.

Sikes, B. A., K. Cottenie, and J. N. Klironomos. 2009. "Plant and Fungal Identity Determines Pathogen Protection of Plant Roots by Arbuscular Mycorrhizas." *Journal of Ecology* 97: 1274–80.

Stahl, P. D., and W. K. Smith. 1984. "Effects of Different Geographic Isolates of *Glomus* on the Water Relations of *Agropyron smithii*." *Mycologia* 76: 261–7.

Tao, L., A. Ahmad, J. C. de Roode, and M. D. Hunter. 2016. "Arbuscular Mycorrhizal Fungi Affect Plant Tolerance and Chemical Defences to Herbivory through Different Mechanisms." *Journal of Ecology* 104: 561–71.

USDA. 2006. *The PLANTS Database*. Baton Rouge, LA: National Plant Data Center.

Vannette, R. L., M. D. Hunter, and S. Rasmann. 2013. "Arbuscular Mycorrhizal Fungi Alter Above- and Below-Ground Chemical Defense Expression Differentially among *Asclepias* Species." *Frontiers in Plant Science* 4: 361.

Vannette, R. L., and S. Rasmann. 2012. "Arbuscular Mycorrhizal Fungi Mediate Below-Ground Plant–Herbivore Interactions: A Phylogenetic Study." *Functional Ecology* 26: 1033–42.

Vogelsang, K. M., H. L. Reynolds, and J. D. Bever. 2006. "Mycorrhizal Fungal Identity and Richness Determine the Diversity and Productivity of a Tallgrass Prairie System." *New Phytologist* 172: 554–62.

Vogelsang, K. M. & Bever, J. D.. 2010. .. *The Use of Native Plants and Mycorrhizal Fungi for Slope Stabilization and Topsoil Management*. California Department of Transportation, Roadside Research and Innovation, Landscape Architecture Program.

Waller, L. P., P. G. Hahn, J. L. Maron, and Y. Lekberg. 2018. "Trait Differences in Responses to Arbuscular Mycorrhizal Fungi Are Stronger and More Consistent than Fixed Differences among Populations of *Asclepias speciosa*." *American Journal of Botany* 105: 207–14.

White, J. A., Tallaksen, J. & Charvat, I. 2008. "The Effects of Arbuscular Mycorrhizal Fungal Inoculation at a Roadside Prairie Restoration Site." *Mycologia* 100; 6–11.

Wilson, G. W., and D. C. Hartnett. 1998. "Interspecific Variation in Plant Responses to Mycorrhizal Colonization in Tallgrass Prairie." *American Journal of Botany* 85: 1732–8.

Zalucki, M. P., S. B. Malcolm, T. D. Paine, C. C. Hanlon, L. P. Brower, and A. R. Clarke. 2001. "It's the First Bites that Count: Survival of First-Instar Monarchs on Milkweeds." *Austral Ecology* 26: 547–55.

SUPPORTING INFORMATION

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How to cite this article: Koziol, Liz, Peggy A. Schultz, Sheena Parsons, and James D. Bever. 2022. "Native Mycorrhizal Fungi Improve Milkweed Growth, Latex, and Establishment while Some Commercial Fungi May Inhibit Them." *Ecosphere* e4052. https://doi.org/10.1002/ecs2.4052