RURALS: Review of Undergraduate Research in Agricultural and Life Sciences

Volume 15 | Issue 1 Article 4

October 2022

The Effect of Arbuscular Mycorrhizae on Milkweed Growth and its Implications for Western Monarchs

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1. Introduction

Western monarch butterflies (*Danaus plexippus plexippus*) are one of two subspecies of monarch butterfly found in North America (Jepsen et al. 2015). The western monarch subspecies is distinguished from their counterparts (the eastern monarch) by their distinct migration pattern, breeding west of the Rocky Mountains and migrating to coastal California and parts of northern Mexico to overwinter (Morris et al. 2015). Populations of these two North American subspecies have seen dramatic declines in the last several decades, especially the western monarch with its numbers plummeting by over 99% since 1990 (Pelton et al. 2019; Schultz et al. 2017; The Xerces Society of Invertebrate Conservation 2021b). Despite nearing extinction, western monarchs have been historically understudied even though they make up a large proportion of the monarch's North American distribution (Pelton et al. 2019; Schultz et al. 2017).

The precipitous population decline is attributable to several causes, including 1. overuse of pesticides (Crone et. al 2019); 2. loss of overwintering sites (Crone et al. 2019); 3. the effects of climate change and extreme weather conditions (Brower et al. 2012; Crone et al. 2019); and 4. exposure to the parasite *Ophryocistis elektroschirrha*, which reduces fecundity in female monarchs (Jepsen et al. 2015; Pelton et al. 2018). The most striking reason for this population decline however is the loss of milkweed (*Asclepias* spp.) habitat due to urbanization and invasive agricultural practices, including the use of glyphosate herbicides (Jepsen et al. 2015; Brower et al. 2012). The name milk*weed* also has a negative connotation as being a weed, despite the majority of *Asclepias* species not fitting the characteristics of a noxious weed (The Xerces Society of Invertebrate Conservation n.d.). There are still, however, a handful of species that are in fact toxic to livestock and can even be lethal in varying quantities (Borders & Lee Mäder 2014). Despite these limited cases, this overgeneralization of the toxicity of the very diverse *Asclepias* genus has likely resulted in a generally negative public image of milkweed, possibly contributing to its neglect and destruction.

Milkweeds are an essential part of the monarch's life cycle and survival because it is one of the only plant genera on which monarchs lay their eggs and upon which the larvae feed (Fallon et al. 2015). There are over 200 different species of milkweed in the genus *Asclepias*, belonging to the family Asclepiadaceae (Pelton et al. 2018). Milkweeds grow in a wide variety of habitat types and secrete a white substance when plant tissue is damaged (hence the common name *milk*weed). All species produce a secondary metabolite known as cardenolide that monarchs are able to sequester in their wings as a defense against predators (Jepsen et al. 2015; Brower & Moffitt 1974). Restoring vital milkweed habitat is crucial in protecting monarch butterflies but little is known on best practices for its reintroduction and growth (Pelton et al. 2018; The Xerces Society of Invertebrate Conservation 2021a & 2021b).

Arbuscular mycorrhizal fungi (AMF) are one potential way to aid milkweed reestablishment. AMF colonize plant roots and are considered mutualists because plant growth parameters, a common measure of the effects of AMF colonization, often increase (Jones & Smith 2014). AMF provide mineral nutrients from the soil in return for plant-assimilated sugars from plant photosynthesis (Adesemoye et al. 2008; Van Geel et al. 2016). Mycorrhizal networks between the plant roots also improve soil formation, water relations, and can provide protection against pathogens (Jones & Smith 2004). However, some research suggests that these benefits begin to decline at increasingly high levels of AMF colonization, turning the symbiotic relationship parasitic (Gange & Ayres 1999). These authors found that the benefits of AMF to plants initially increased as colonization increased, but plateaued at moderate levels of colonization and then decreased around 80% colonization, suggesting a curvilinear relationship (Gange & Ayres 1999). Thus, the use of AMF at moderate levels of colonization may be a helpful tool in effectively reestablishing milkweed habitats.

Our study set out to examine the effects of AMF on the growth of two milkweed species native to the Willamette Valley of Oregon state, USA: showy milkweed (*A. speciosa*) and narrow-leaf milkweed (*A. fascicularis*). We hypothesized that AMF colonization would affect milkweed growth. We predicted that milkweed plants with moderate amounts of AMF colonization (40-60%) would show the greatest amount of growth compared to those with lower (0-20) and higher (80-100) percentages of AMF colonization.

2. Methods

Greenhouse Set-Up and Larger Study Treatments:

A total of 350 plants (167 showy and 183 narrow-leaf milkweed) were included in our analysis. Showy and narrow-leaf milkweed were chosen because they are common milkweed species found across Oregon, as well as the only two species of milkweed native to the Willamette Valley where this research was conducted and where the results will be applied (The Xerces Society of Invertebrate Conservation 2012). Both species thrive in dry to moist soil, in habitats ranging from along roads and waterways to open woods, fields, and meadows. Both begin flowering in June, while showy ends its flowering period in August and narrow-leaf lasts a little longer into September.

Milkweeds were grown from seedlings during three summers (2019-2021) in the University of Portland's greenhouse at 27^{o}C for 16 hours of light and 20^{o}C for 8 hours of darkness. Seedlings (≥ 1cm) were grown from roots harvested the previous fall and all had sprouted by the beginning of each summer's research. Seedlings were transplanted into plastic planter boxes (35 x 35 x 12.5cm), with four seedlings spaced evenly per box. Boxes were randomly assigned placement in the greenhouse. Seedlings were grown in 8 cm of a commercially purchased all-natural gardening soil (Kellogg Garden Organics) and were watered twice weekly. The soil was not sterilized before planting.

This study was part of a larger project analyzing the effects of Roundup and spraying AMF on the roots (inoculation) on milkweed growth. Although the number of boxes varied slightly across the years, during each summer there were equal numbers of boxes treated four different ways: 1. soil sprayed with Ready-To-Use Roundup (Monsanto Company, Marysville, OH); 2. roots sprayed with an arbuscular mycorrhizal fungi (AMF) inoculant (28.35g AMF / 2000ml water); 3. a combination of both Ready-To-Use Roundup and AMF inoculant; or 4. no treatment as a control. The AMF inoculant consisted of a powdered mixture of 11 species of common mycorrhizal fungi (Mycorrhizal Applications LLC, Grants Pass, OR).

Three growth parameters were measured at the time of planting: height (cm), number of leaves, and leaf area (average length of the largest three leaves x average width of the largest three leaves x 0.7196 adjustment for shape [per Shi et al. (2019)]). Plant height was measured from the base of the stem to the top of the tallest leaf. Leaves that were yellowed, shriveled, and / or fell off when touched lightly were not included in the leaf number count. After 10-11 weeks, the growth measurements were taken again before the milkweeds were harvested. The plant material (including stems, leaves, and roots) was rinsed and separated into above- and below-ground portions, dried at 100ºC for 72 hours, and weighed for above-ground and below-ground biomass (g). Since plants were not the same size initially, changes in height, number of leaves, and leaf area were quantified as a % change ([(final – initial measurement) / | initial |] x 100).

AMF Colonization Analysis:

Unexpectedly, we found that spraying the roots with AMF did not result in higher levels of AMF actually colonizing the roots. In fact, the AMF-treated plants only averaged 46% colonization compared to 49% for the control, 57% for the Roundup treatment, and 61% for the combination Roundup and AMF treatment ($p = 0.013$). While we did not identify the species of AMF that colonized the milkweed roots, the above results suggest that the AMF colonization we observed did not result from the spraying of roots with AMF inoculant but likely occurred from AMF found naturally in the soil. Regardless of how AMF colonization occurred, we compared plants with varying levels of AMF colonization (0-100%) based on direct measurement as described below rather than comparing plants among treatments.

Five root tips measuring 1 cm were cut from varying areas of the root mass of each milkweed plant, rinsed thoroughly to remove excess dirt, then cleared and stained using the procedures of Vierheilig et al. (1998). Root tips were then examined under a microscope at 40x magnification for AMF colonization presence or absence. Each root tip exhibiting AMF colonization was counted as 20%, giving total colonization values / percentages of 0, 20, 40, 60, 80, or 100% for each plant. Despite having six total AMF colonization percentage categories, data were analyzed in three bins (0-20%, 40-60%, and 80-100% colonization) due to the fact that some of the AMF colonization percentages had too few data points for meaningful analysis. Data were analyzed using single-factor ANOVAs and post-hoc Tukey's HSD test for pairwise comparisons with $\alpha = 0.05$.

3. Results

Our results showed differences between the two focal species in the effects of AMF colonization on their growth. For showy milkweed, both the height (**Figure 1A**) and number of leaves (**Figure 1B**) increased at higher levels of AMF colonization. The average % change in height was 25, 64, and 92 at 0-20, 40-60, and 80-100% colonization respectively ($F = 7.5$, $p = 7.64 \times 10^{-4}$). Similarly, the average % change in the number of leaves increased from 165 to 325 and 508 as colonization increased ($F = 6.2$, $p = 0.003$). There was no significant difference in the % change in leaf area among colonization levels ($F = 2.0$, $p = 0.135$).

The average above-ground (**Figure 2A**) and below-ground (**Figure 2B**) biomass of showy milkweed showed the opposite trend, with higher biomass on average at the lowest levels of AMF colonization. At 0-20% colonization, showy milkweed produced an average of 0.87g of above-ground biomass, 0.44g at 40-60% colonization, and 0.43g at 80-100% colonization ($F = 6.6$, $p = 0.002$). For below-ground biomass, showy milkweed produced an average of 1.46g at 0-20% colonization, 0.78g at 40-60% colonization, and 0.90g at 80-100% colonization ($F = 3.9$, $p = 0.023$).

In narrow-leaf milkweed, only average above-ground biomass showed significant differences among AMF colonization levels among the five growth parameters analyzed (**Figure 3**). The average above-ground biomass was 2.61g, 2.51g, and 1.66g for 0-20%, 40-60%, and 80-100% colonization respectively ($F = 3.4$, $p = 0.036$). No other growth measure was significantly different among the three AMF colonization levels ($p > 0.144$ for all).

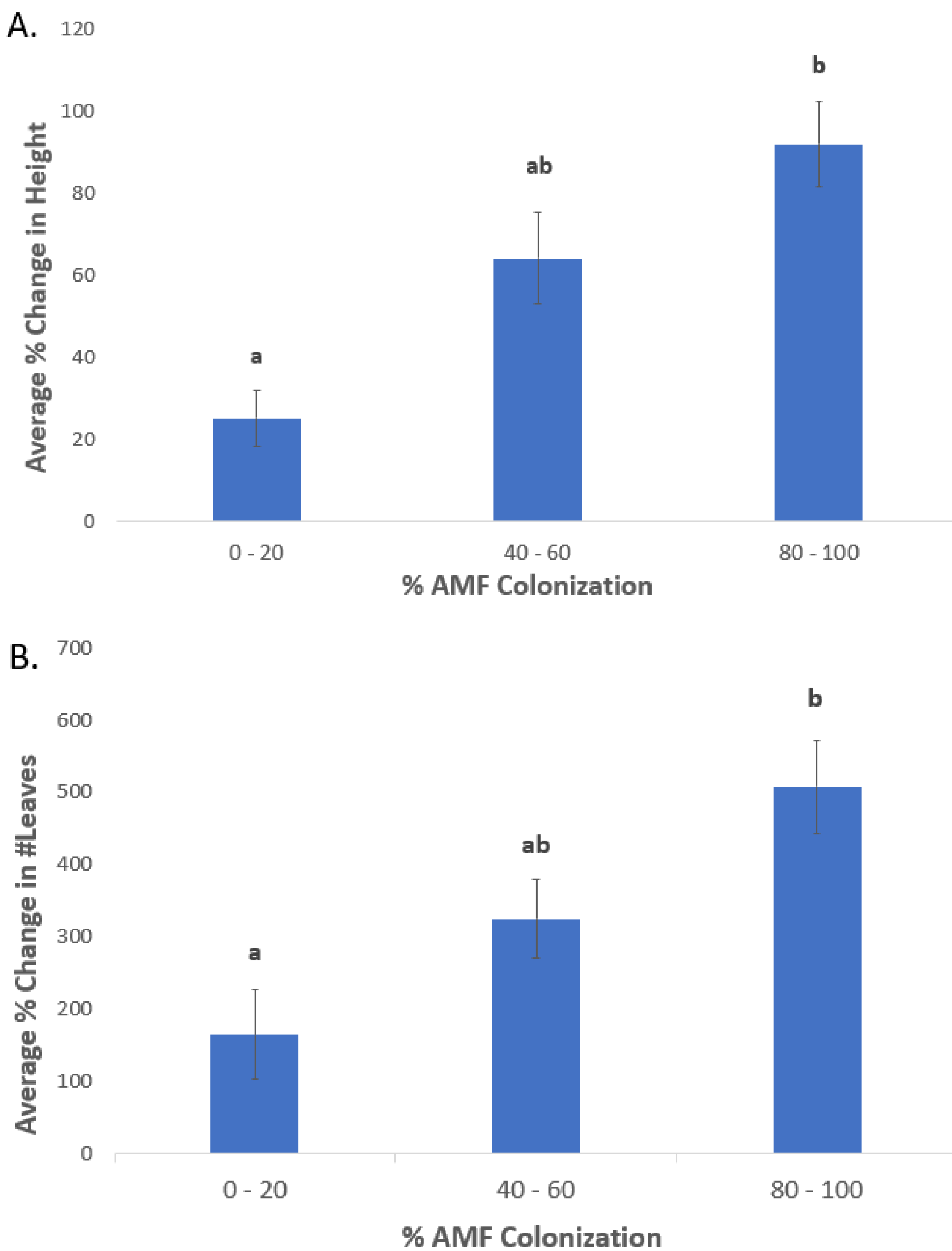

Figure 1. Average % change in height (A) and number of leaves (B) in showy milkweed between three increasing percentages of AMF colonization. Both growth measurements increased significantly with the highest level of colonization ($p = 7.64x10^{-4}$ and 0.003, respectively). Bars represent SEM.

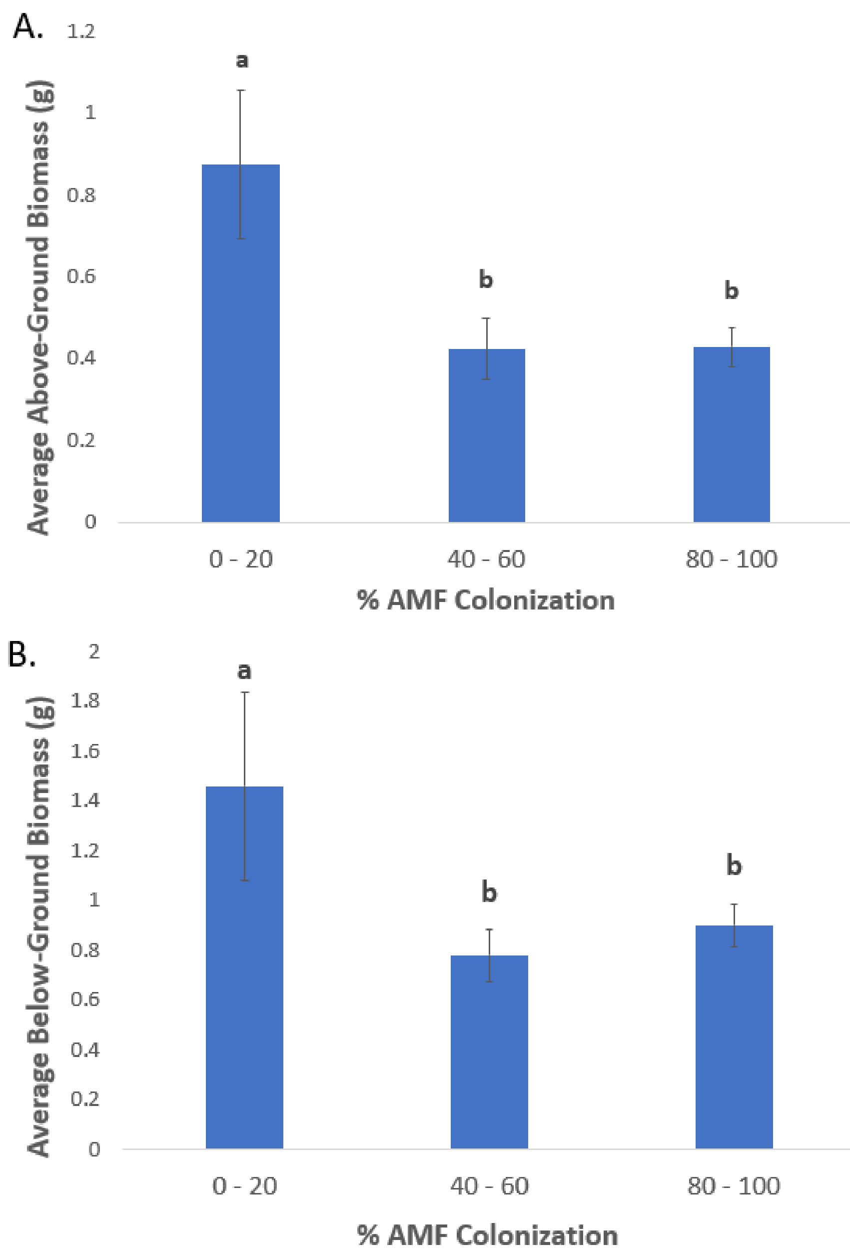

Figure 2. Average above-ground biomass (A) and below-ground biomass (B) in showy milkweed between three increasing percentages of AMF colonization. Both above-ground and below-ground biomass were significantly greater at low levels of AMF colonization compared to both moderate and high levels ($p = 0.002$ and 0.023, respectively). Bars represent SEM.

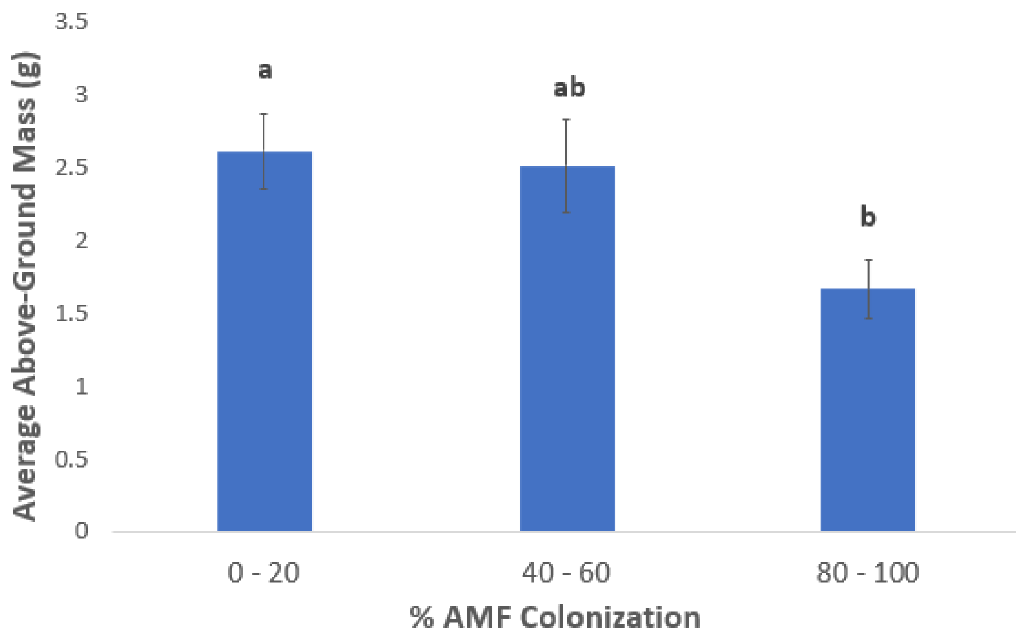

Figure 3. Average above-ground biomass in narrow-leaf milkweed between three increasing percentages of AMF colonization. Above-ground biomass was significantly lower at high levels of AMF colonization compared to both low and moderate levels ($p = 0.036$). Bars represent SEM.

4. Discussion

Although our findings suggest that AMF colonization does have an effect on milkweed growth as hypothesized, they did not support our prediction that milkweed plants with moderate amounts of AMF colonization would have the greatest growth compared to those with lower and higher amounts of colonization. Instead, our results suggest that the effects of AMF on milkweed growth differ among species and parameters, though there may be overlapping patterns in some measurements. While increasingly high levels of AMF colonization had an increasingly positive effect on height and number of leaves in showy milkweed (Figure 1), there was no effect on either measure in narrow-leaf milkweed, as well as no effect on the leaf area for either. However, for both species, above-ground biomass was negatively affected by increasingly high levels of AMF colonization (Figures 2 and 3).

Some studies suggest that AMF becomes parasitic at high levels of colonization (Gange & Ayres 1999; Jones & Smith 2014). Our results are mixed in support of these findings. For showy milkweed, two out of five growth parameters continued to increase at higher levels of AMF colonization (Figure 1), implying the relationship might not *always* become parasitic. However, above-ground biomass decreased in both milkweed species at increasingly higher levels of AMF colonization (Figures 2 and 3), as well as below-ground biomass for showy milkweed (Figure 2), suggesting that the relationship may have become parasitic, specifically in how much mass the milkweed could accumulate during its growth. Overall, the mixed results indicate that the effects of AMF colonization are species and parameter specific.

More studies investigating the effect of different species of AMF on different species of milkweed's growth would be beneficial. Van Geel et al. (2016) also found that benefits from AMF are species-specific for both plants and AMF, and that a single species AMF inoculation was the most beneficial. Our inoculant was a mixture of 11 species of AMF known to colonize a variety of plant species, though none were specifically associated with *Asclepias*. This might explain why, in our study, spraying plant roots with the AMF inoculant did not result in increased AMF colonization. Studies exploring inoculants of increasing AMF concentration might elucidate treatment levels where added inoculant does increase colonization.

Other studies suggest that AMF may not just aid in milkweed establishment but may also be an important tool in alleviating the impacts of climate change. For example, the addition of mycorrhizal fungi to the soil was shown to counteract the detrimental effects of increased amounts of CO_2 on plant mass and root morphology of certain species of milkweed (Malik & Bever 2021). Additionally, AMF inoculation of milkweed seedlings significantly increased drought resistance (Bahmani et al. 2018).

Increasing our understanding of different means to improve milkweed growth overall is a crucial step in the fight to save the highly imperiled western monarch.

These findings are a start in establishing the best practices for milkweed habitat restoration, thus ensuring the survival of these beloved and iconic butterflies.

5. References

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