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The Effect of Residual Roundup on Showy Milkweed Growth and Cardenolide Production

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Cover Page Footnote
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Introduction

The migratory population of Western monarch butterflies (*Danaus plexippus plexippus*) has declined by 99% since the 1980s due to development of coastal overwintering areas and broad-spectrum pesticide use in central California (Pelton et al. 2019). While overwintering sites are characterized by protective trees and vegetation on which butterflies can roost, breeding sites are characterized by the abundance of milkweed (*Asclepias* spp.) upon which adult monarchs lay their eggs (Pelton et al. 2016). There are 13 milkweed species native to the Western United States, each adapted to its own specific habitat (Dilts et al. 2019). While monarch butterflies are habitat generalists, they are host specialists; they almost exclusively oviposit on milkweed plants. Milkweeds produce a cardenolide toxin that monarch larvae ingest while feeding, then sequester and repurpose for their own chemical defense (Brower et al. 1968). Much of the land historically occupied by milkweed has been developed or converted to agriculture (Malcolm 2018). Reestablishing milkweed habitat across the Western monarchs' migratory paths and breeding grounds is integral to the butterflies' survival and population stabilization (Pleasants & Oberhauser 2013).

The aim of our study was to assess the effects of various environmental conditions on milkweed growth and cardenolide production. Roundup, a glyphosate-based broad-spectrum herbicide, is one of the most widely used weed-control methods for both agriculture and small-scale yard use and the widespread loss of milkweed habitat has been attributed to its use (Pleasants & Oberhauser 2013). The recommended instructions are to apply Roundup directly to foliage, but the herbicide often lingers in soil after use. Trace amounts of glyphosate in soil have been shown to increase the growth of some crops (Belz & Sinkkonnen 2019, Helander et al. 2019). Furthermore, increased plant growth has been associated with increased cardenolide production since the plant has more energy available to allocate not only to foliar growth but also secondary metabolites for plant defense (Züst et al. 2015).

The presence of root symbionts, such as arbuscular mycorrhizal fungi (AMF), has also been shown to impact secondary metabolite production in milkweeds (Vannette et al. 2013). AMF are common symbiotic fungi that colonize the root systems of plants, using their wide system of hyphae to extend the plant's nutrient uptake in exchange for carbohydrates from the host plant (Smith & Read 2008). Because elevated overall nutrition has been shown to correlate with higher production of plant defense chemicals, the presence of AMF colonization in milkweed roots is likely to lead to a higher concentration of cardenolide throughout the plant (Vannette & Hunter 2011).

We predicted that Roundup residues in soil will lead to (1) increased growth and (2) increased cardenolide concentration in milkweed. We also predicted that (3) AMF inoculation will enhance the effects of Roundup and therefore milkweeds treated with both AMF and Roundup will show the highest levels of growth and cardenolide production.

Methods

Growing Conditions:

We evaluated showy milkweed (*A. speciosa*), which is widespread across the North American West coast states (Skinner 2008) and is one of the few milkweed species native to the Willamette Valley where our research was conducted. Showy milkweed seedlings (n=96) were grown in the University of Portland's greenhouse during the summer of 2021 and divided evenly into four treatments. Treatment 1 was a combination of Roundup addition to the soil and AMF inoculant of roots. For treatment 2, Roundup alone was mixed into the soil. For treatment 3, the roots were inoculated with only AMF. Treatment 4 was the control. Roundup treatments were prepared by spraying Ready-To-Use Roundup (Monsanto Co., Marysville, OH) five times into the soil and mixing 5 times by hand 30 minutes before planting. AMF treatments were prepared by spraying seedling roots 5 times with a solution of 11 common AMF species at a concentration of 28.35 g AMF/2000 ml water (Mycorrhizal Applications, LLC, Grants Pass, OR) prior to planting. Initial measurements of plant height, leaf number and leaf area were taken from each plant. Plant height (cm) was measured from the base of the central stem to the top of the tallest leaf. All leaves were counted except for those that were yellowed or fell off when touched lightly. Leaf area was based on the three largest leaves and was determined by multiplying their average length and width by a 0.7196 adjustment factor for shape (Shi et al. 2019). The seedlings were grown at 27°C for 16 hours of light and 20°C for 8 hours of darkness for two months with bi-weekly watering. Seedlings were spaced evenly (4 per box) in 35 x 35 x 12.5 cm plastic planter boxes filled with 8 cm of commercially purchased all-natural gardening soil (Kellogg Garden Organics, The Home Depot). Directly before the plants were harvested, second measurements were recorded. Because plants were not initially the same size, growth parameters of each plant were quantified as percent changes [(final measurement - initial measurement)/initial measurement] x100). The plants were harvested by collecting all plant material (stems, leaves, and roots), washing off dirt residue with water and separating the plants into above- and below-ground portions. The plant samples were dried at 100°C for 72 hours, then weighed to determine biomass (g).

Cardenolide Extraction:

Cardenolide extraction was adapted from the methods of Rafter et al. (2017). The dried milkweed samples were ground through the course screen of a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ). If possible, 0.10g below-ground material and 0.05g above-ground material was collected for each plant. Some plants did not have this much biomass: in these cases, all plant material was used, the available mass was recorded, and the reagents adjusted proportionally. Each sample was placed in a microcentrifuge tube (1.5 ml) and ethanol (95%) was added to at a ratio of 1mL ethanol/0.10g plant material. The samples were kept at 2°C and vortexed for 5 seconds twice per day for two days (48 hours) to allow cardenolide to extract from plant tissue.

Tubes were centrifuged at 9500 rpm for 15 minutes before extracting 120μL of supernatant. To create a standard curve, a serial dilution of digitoxin (Sigma Aldrich, St. Louis, MO) and 95% ethanol was made (0.07g/100mL 95% ethanol, prepared the day of use). A 120-μL aliquot of 2% 3,5-dinitrobenzoic acid, prepared in 100% ethanol, was added to all milkweed, standard and control (water) samples. Solutions were vortexed for 5 seconds, then centrifuged for 5 seconds. A 95-μL portion of each sample was then added to a 96 well microplate (Greiner Bio-One, Frickhausen, Germany), testing all samples in duplicate. Lastly, 100μL of 3%

NaOH prepared in 100% ethanol was added to each well. After 7 minutes had elapsed from the first 3% NaOH addition, the plate was read at 540 nm. Duplicate samples were averaged to generate a standard curve and determine cardenolide concentrations of the milkweed samples.

Data were analyzed using single-factor ANOVAs and post-hoc pairwise t-tests with Tukey's HSD with $\alpha = 0.05$.

Results

Plants treated with Roundup + AMF experienced a significantly greater change in height than any other treatment (Figure 1: $F=7.23$, $p=2.07 \times 10^{-4}$). Plants treated with only Roundup showed significantly less change in height than control plants (Figure 1: $F=7.23$, $p=2.01 \times 10^{-4}$).

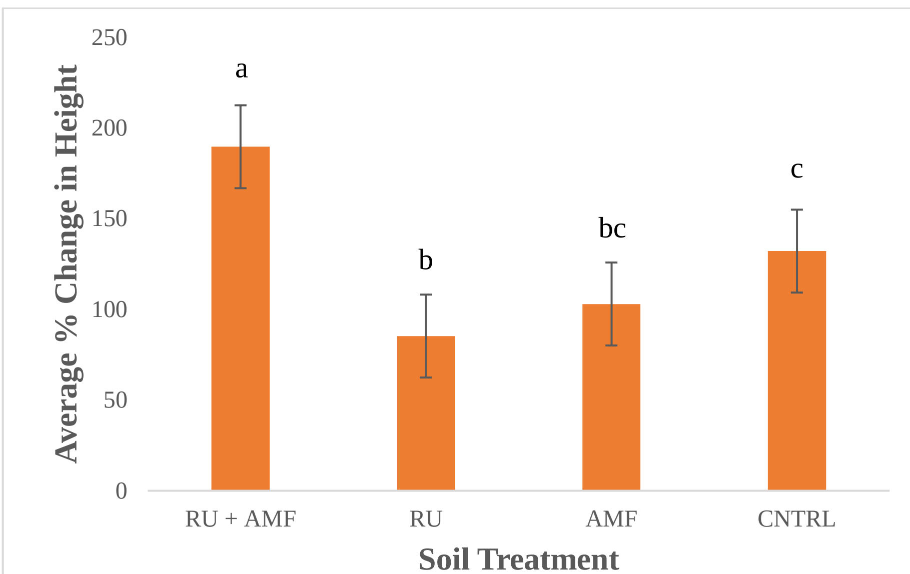

Figure 1. The average percent change in height was greatest in plants exposed to RU + AMF. Plants exposed to RU alone showed less change in height compared to RU+AMF and control. Plants inoculated with AMF alone were not significantly different from RU or control (ANOVA: $F = 7.23$, $p = 2.07 \times 10^{-4}$). RU = Roundup, AMF = arbuscular mycorrhizal fungi.

The average percent change in leaf number was greater with all soil amendments (Roundup, AMF, and both) compared to the control (Figure 2: F=15.26, p=3.86x10^{-8}). However, none of the treatments were significantly different from one another (Figure 2: F=15.26, p=3.86x10^{-8}). Treatment with Roundup, AMF, or a combination of the two did not affect percent change in leaf area (F = 1.78, p = 0.16), above-ground biomass (F= 0.93, p = 0.43), or below-ground biomass (F = 2.29, p = 0.08).

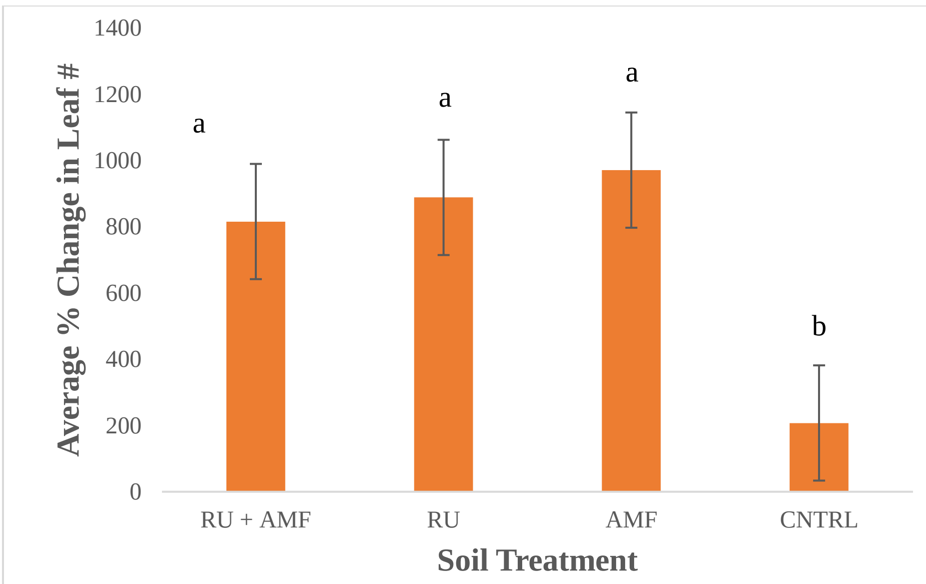

Figure 2. The average percent change in leaf number was significantly greater in all soil treatments than the control although treatments did not differ from one another (ANOVA: F= 15.26, p = 3.86x10^{-8}). RU = Roundup, AMF = arbuscular mycorrhizal fungi.

Plants that were treated with AMF alone showed a significantly lower above-ground cardenolide concentration than control plants (Figure 3: F = 2.71, p = 0.049). Roundup + AMF and Roundup alone did not significantly affect above-ground cardenolide concentration (Figure 3). Below-ground cardenolide concentration was not significantly affected by any treatments (F = 1.89, p = 0.14).

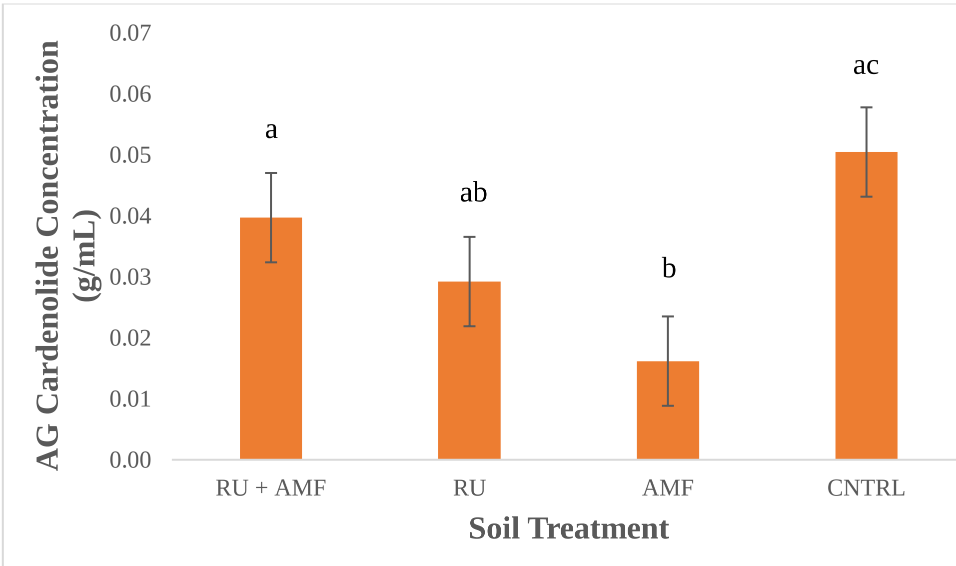

Figure 3. Above-ground cardenolide concentration did not significantly differ among RU, RU + AMF, and control treatments, but plants in the AMF alone treatment produced significantly less above-ground cardenolide compared to RU+AMF and the control (ANOVA: $F= 2.71$, $p = 0.049$). RU = Roundup, AMF = arbuscular mycorrhizal fungi.

Discussion

We found little support for our hypotheses that Roundup in the soil enhances the growth of showy milkweed. Of five measured growth parameters, only % change in leaf number was significantly higher in plants treated with Roundup alone compared to the control (Fig. 2), while % change in height was significantly lower (Fig. 1). The number of monarch eggs laid on a host plant correlates positively with the total leaf area of that plant (Cohen and Brower, 1982). Though plant height is important, a milkweed plant supporting more leaves will be able to host more oviposition, and better shield monarch larvae from predators and weather via overlapping stems and leaves (Cohen and Brower, 1982). Therefore, though the plants grown in Roundup-treated soil showed a smaller increase in height, they produced a greater increase in leaf number which in turn may support increased monarch oviposition.

We found no support for our second hypothesis that Roundup would increase cardenolide concentration in milkweed, as plants grown in Roundup-treated soil produced cardenolide concentrations similar to the control (Figure 3). These results suggest that Roundup residues lingering in soil will not affect the toxicity of showy milkweed plants. Interestingly, the AMF-only treatment showed a significantly lower above-ground cardenolide concentration than control plants (Figure 3). This pattern has been observed in other milkweed species, which showed a decrease in foliar cardenolide concentration when inoculated with AMF, while the toxin concentration in the roots remained unaffected (Vanette et al., 2013). Foliar cardenolide concentration is critically important because female monarchs prefer to lay eggs on milkweeds with moderate levels of the toxin to minimize metabolic costs on larvae (Agrawal et al. 2021).

The effects of AMF inoculation on toxin production varied greatly among milkweed species evaluated (Vanette et al., 2013). Showy milkweed was not included in the study, making our results an important contribution to the overall knowledge of milkweed cardenolide response to mycorrhizal fungi associations. Our results suggest that Roundup in the soil, with or without the addition of AMF, does not affect cardenolide concentration in showy milkweed, and therefore will not affect ovipositing female preference.

The addition of AMF to Roundup-treated soil positively affected plant height (Figure 1), partially supporting our third hypothesis that AMF would enhance the effects on growth from Roundup in the soil. However, all other growth parameters and cardenolide concentrations were similar between plants grown in Roundup + AMF compared with plants grown in Roundup alone. Because glyphosate, the active ingredient in Roundup, can influence the proportion of mycorrhizal fungus species colonizing the roots of plants growing in soil, the variation in growth could depend on which fungal species are dominant in soil with vs. soil with Roundup (Sheng et al. 2012). Though the roots were sprayed with a mixture of 11 AMF species, the differences in species diversity between treatments was not assessed and therefore its effect is unknown in our plants.

This study is a first attempt at determining the impacts of glyphosate-based compounds like Roundup on milkweed growth and cardenolide production. Though our results indicate that Roundup soil residues do not appear to affect showy milkweed plants in a way that would cause harm to Western monarch butterflies, greater amounts of Roundup residue or associations with other species of arbuscular mycorrhizal fungi could cause variation in these outcomes. More research on the interaction between glyphosate and arbuscular mycorrhizal fungi is needed in order to fully understand their combined impact on milkweed which may in turn help the long-term survival of the imperiled Western monarch butterfly.

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