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Article

Application of arbuscular mycorrhizal fungi to inhibit nitrogen uptake of weeds within crop fields

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SUMMARY

Invasive species cost the agriculture industry billions of dollars each year in lost revenue and control costs. This research addresses the potential neutralizing effects of arbuscular mycorrhizal fungi (AMF) on the invasive species and aggressive agricultural weed, *Cirsium arvense*, by limiting its nitrogen uptake from the soil. A total of 36 *C. arvense* plants and 24 Glycine max soybean plants were grown in a controlled laboratory environment, with G. max being used to create a simulated crop field. We hypothesized that introducing AMF to *C. arvense* would reduce its ability to absorb nitrogen, therefore reducing the plant's growth. If AMF significantly reduces *C. arvense*'s capability to thrive, it is possible that AMF could be incorporated into commercial and private crop fields to serve as a natural herbicide against *C. arvense*. Growth data were collected for every plant including height, root length, and dry mass. Each sample was tested for its usable total nitrogen content. *C. arvense* plants grown with AMF displayed no significant difference in growth compared to those plants grown without AMF. No significant difference in the cohabitated groups was found. However, a significant difference in nitrogen content was found in all experimental groups. These results demonstrate that AMF has an effect on the nitrogen content of *C. arvense*, but does not affect the growth in the seedling stage.

INTRODUCTION

Cirsium arvense, known commonly as the Canada thistle (**Figures 1,2**), is a highly invasive species native to the Mediterranean that was accidentally introduced to America in the 1600s (1). The introduction of the *C. arvense* into North America has produced numerous problems, including destroying native ecosystems and reducing crop yields. It is considered one of the most economically damaging weeds, costing farmers tens of millions of dollars in direct crop loss and even more in herbicide and other control measures (1,2). In Missouri, *C. arvense* is described as an "alien exotic species capable of crowding out and replacing native grasses and forbs" (3). Additionally, *C. arvense* is listed as a noxious weed in Missouri (4). Current methods of eradication and control, such as burning, digging, or mowing, are time-consuming and not 100% effective(5). Tillage only provides an ideal environment for spreading, and the thistle's spines prevent livestock from grazing (3). Prescribed burns effectively eliminate *C. arvense* infestations, but only at a certain growth stage. Current herbicides, although effective against *C. arvense*, can have negative effects on local water quality and aquatic ecosystems (6). It is necessary for a new approach to the control of *C. arvense* to be found.

One reason the *C. arvense* has become rampant in North America is its ability to reproduce through wind-borne seeds as well as root offshoots (1). These dual reproduction methods allow *C. arvense* to reproduce quickly and crowd out native species. The plants get their energy to grow and reproduce through photosynthesis. Chlorophyll, a key component in the photosynthetic process, is primarily made of the element nitrogen (7). Therefore, when a plant's access to nitrogen is restricted, lack of sugars causes growth and reproduction to slow down.

Recent research into the control of other aggressive agricultural weeds suggests that arbuscular mycorrhizal fungi (AMF) may reduce the growth of different weed families (8-10). AMF builds symbiotic relationships with certain plant species. These relationships extend the root system of the plant,

Figure 1: *C. arvense* flower.

Figure 2: *C. arvense* leaves and spines.

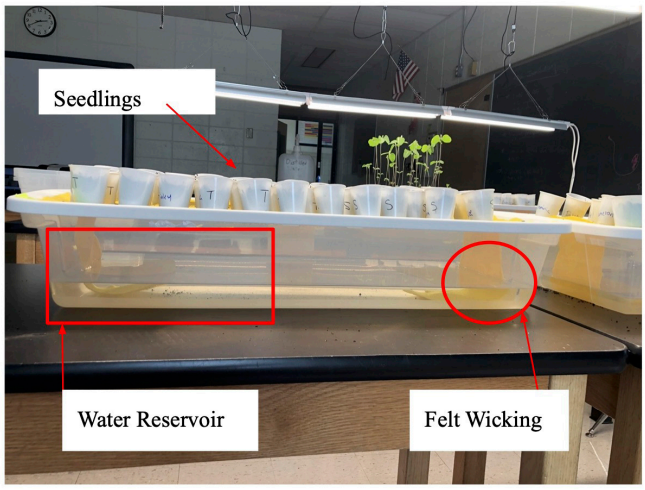

Figure 3: The self-watering system used to germinate and grow seedlings. Felt wicking extended from each individual plant to the water reservoir below. The water was drawn up from the reservoir by capillary action.

allowing it to gain access to an increased amount of water and nutrients from the soil (11). These relationships can be hugely beneficial to *Glycine max* (soybean), as well as other crops. AMF inoculation can increase *G. max´*s nitrogen absorption by 20% in field conditions (12). AMF can be indirectly harmful to plants with which it does not have a relationship (13). By funneling water and nutrients to the plants it has partnered with, AMF reduces the amount of available nutrients in the soil (13). The plants that do not have a symbiotic relationship with AMF, therefore, have a limited supply of the nutrients they need to thrive.

We hypothesized that introducing AMF to *C. arvense* will reduce its ability to absorb nitrogen, reducing the plant's growth. If AMF significantly reduces *C. arvense's* capability to thrive, it could be incorporated into commercial and private crop fields to serve as a natural herbicide against *C. arvense.* This research aimed to determine the effect of AMF application on the nitrogen absorption of *C. arvense*. In addition to this, we determined whether or not the potential change in nitrogen uptake will have a significant effect on the growth and biomass of *C. arvense* to be beneficial to crop yields. Thus, this experiment strived to understand AMF's effect on the nitrogen uptake of *C. arvense.* The results of this study indicated that inoculated seedlings had significantly less nitrogen than uninoculated seedlings. However, there was no significant difference in growth observed between the two groups.

RESULTS

To determine the effect of AMF on the nitrogen absorption of *C. arvense*, we conducted two experiments, named X and Y. In the first, 12 *C. arvense* seedlings were inoculated with AMF and monitored for 38 days (Group Xt). An additional 12 seedlings were left uninoculated as a control (Group Xc). The second group consisted of 6 inoculated *C. arvense* seedlings coexisting with 12 inoculated *G. max* seedlings of the same age (Group Yt). These seedlings were grown for 38 days after germination, and an equal number of uninoculated seedlings were grown as controls (Group Yc). All plants were grown in a controlled indoor environment under LED grow lights. The plants were watered using a capillary self-watering system (**Figure 3**). We measured the height of each plant every 5 days and averaged each group (**Table 1**).

Days After Germination	Average Size Xt and Yt (cm)	Standard Deviation (cm)	Average Size Xc and Yc (cm)	Standard Deviation (cm)
10	1.60	0.50	2.11	0.37
15	2.00	0.46	2.44	0.45
20	2.37	0.78	2.85	0.97
25	2.53	1.02	3.12	1.17
30	2.70	1.11	3.37	1.24
35	2.89	1.25	3.46	1.34
38	3.20	1.42	3.68	1.37

Table 1: Inoculated v. uninoculated *C. arvense* seedlings. This table displays the average sizes of inoculated and uninoculated *C. arvense* seedlings throughout the growth period. n=12.

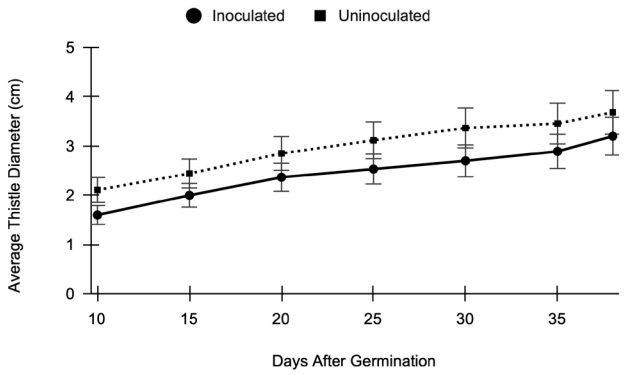

Figure 4: Growth curves of inoculated and uninoculated *C. arvense* plants. These results were collected from *C. arvense* plants grown alone. The diameter of each seedling was measured every 5 days for 38 days after sprouting. No significant differences recorded. n=12. Error bars represent standard deviation.

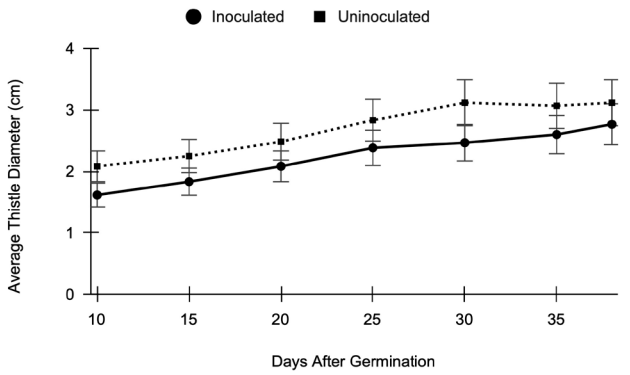

Figure 5: Growth curves of inoculated and uninoculated *C. arvense* plants grown with *G. max*. These results were collected from ***C. arvense*** plants transplanted together with ***G. max*** 10 days after germination. The diameter of each seedling was measured every 5 days for 38 days after sprouting. No significant results recorded. n=12. Error bars represent standard deviation.

Throughout the growth period, the size measurements of the inoculated groups, Xt and Yt, remained consistently smaller than those of the uninoculated groups, Xc and Yc, (**Figures 3,4**). However, when we performed statistical analysis on the data, there was no significant difference in size between the test and control groups. *C. arvense* plants that were inoculated with AMF displayed no significant difference in growth rate or size compared to those grown without AMF (p = 0.4805). *C. arvense* plants that were cohabitated with *G. max* also displayed no significant differences in growth (p = 0.6829). This data is also displayed in **Table 2** as well as the growth curves, **Figures 4 and 5**.

At the conclusion of the experiment, we uprooted each seedling and dried them completely at room temperature. Then, we carefully separated and discarded the roots. The above-ground plant matter was then tested for functional nitrogen content using the wet-ash method. Experimental groups were tested together. For example, we tested all the plants in group Xt together, but separately from all other groups.

The nitrogen contents of groups Xc and Xt were determined to be significantly different through statistical analysis, and the same can be said for groups Yc and Yt (**Table 3**). When grown alone, inoculated *C. arvense* seedlings had 0.5% less functional nitrogen than the uninoculated seedlings. This was a 30% decrease in nitrogen content from the control. When grown with *G. max*, a nearly 4% difference in nitrogen content was recorded, which was a 54% difference from the control (**Table 3**). The nitrogen contents of inoculated and uninoculated *G. max* plants were found to be not significantly different (**Table 3**).

When the average heights of the *G. max* seedlings were compared in a two-tailed T-test, there was no significant difference (p = 0.861). Based on this, we determined that AMF inoculation did not appear to make any significant difference in the growth of *G. max* (**Table 4**). The nitrogen contents of the *G. max* plants were also compared using a T-test and the analysis yielded a p-value of p = 0.072. From these results, we concluded that there was a trend towards inoculated *G. max* plants having reduced nitrogen, but the results were not significant. It must be acknowledged that the plants in all experimental groups were not allowed to grow to maturity before being uprooted for nitrogen testing. Past

Trial Group	Size at Day 0	Size at 38 days	Standard Deviation	Average Change in Size	Number of Plants	P-value from T-test
***C. arvense* plants grown alone with AMF**	0.49 cm	3.20 cm	1.42 cm	2.71 cm	12	0.4805
***C. arvense* plants grown alone without AMF**	0.55 cm	3.68 cm	1.37 cm	3.13 cm	12	
***C. arvense* grown with *G. max* and AMF**	0.62 cm	2.77 cm	0.71 cm	2.15	6	0.6829
***C. arvense* grown with *G. max* and without AMF**	0.52 cm	3.12	1.91	2.60 cm	6	

Table 2: Statistical analysis of the growth of *C. arvense*. This table displays the data used to perform two-tailed t-tests for each group of *C. arvense* seedlings. *C. arvense* alone groups - n=12, Cohabitation groups - n=6.

Experimental Group	Percent Nitrogen	*p*-value	Statistically Significant?
***C. arvense* grown alone with AMF**	1.13%	0.023	Yes
***C. arvense* grown alone without AMF**	1.63%		
***C. arvense* grown with *G. max* and AMF**	7.14%	0.0001	Yes
***C. arvense* grown with *G. max* and without AMF**	3.28%		
***G. max* with AMF**	0.89%	0.072	No
***G. max* without AMF**	1.28%		

Table 3: Results and statistical analysis of nitrogen tests. Percent usable nitrogen in the plant matter of each test group, including soybeans. n=12.

Days after Germination	Average Height of test group (cm)	Standard deviation	Average Height of control group (cm)	Standard Deviation	Final *p*-value
10	22.42	4.56	27.83	13.50	**0.8160**
15	31.21	4.82	35.75	16.94	
20	43.92	7.13	47.83	21.10	
25	52.50	7.80	59.67	22.87	
30	58.42	8.90	68.25	17.35	
35	68.92	10.36	70.08	16.09	
38	73.08	12.27	71.75	15.24	

Table 4: Growth data for *G. max* seedlings. Average sizes of inoculated and uninoculated *G. max* seedlings throughout the growth period. n=12.

studies involving many more *G. max* plants grown to maturity were aimed at proving AMF's benefits towards *G. max*.

DISCUSSION

C. arvense is an invasive species that not only impacts the global agricultural economy to the tune of millions of US dollars every year, but has a rapid life cycle and is resistant to most modern control measures including controlled burns and livestock grazing. AMF has been shown in previous studies to have a negative effect on invasive species by allowing the native plants greater access to water and nutrients from the soil. This study examined *C. arvense's* response to AMF inoculation when grown in a solitary environment and when grown in a simulated crop field. The goal of this study was to determine the viability of AMF to be used as a preventative measure for *C. arvense* infestations.

Considering the results of the data analysis, two conclusions can be drawn. Firstly, the inoculation of AMF has no significant effect on the growth of *C. arvense* within the seedling stage of the plant and therefore is not a feasible preventative natural herbicide. Secondly, the inoculation of AMF has some effect on the nitrogen absorption of *C. arvense*, but the immediate effect of this on the actual plants is not observable in the seedling stage.

These conclusions do not fully support our original hypothesis that AMF inoculation would slow the growth of *C. arvense* plants by decreasing the amount of nitrogen they could draw from the soil. The growth curves of the data suggest that AMF inoculation does not significantly affect the growth of *C. arvense* (**Figures 4, 5**). When we performed statistical analysis on the average sizes of each group, the results proved insignificant (**Table 2**). However, further studies are required to confirm this data. It is possible that different methods, concentrations, or times of AMF inoculation could affect *C. arvense* and therefore be a possible method of eradication.

The results of the nitrogen tests indicate that AMF may have an effect on the nitrogen uptake of *C. arvense*. However, the results are contradictory to themselves. When grown alone, inoculated seedlings absorbed less nitrogen, yet when grown with *G. max*, the inoculated group absorbed more nitrogen compared to the control (**Table 3**). The discrepancy

leads one to believe that the small sample size may have led to inaccurate results. It may be possible that AMF has a more long-term effect on *C. arvense* that was not yet observable in the seedling stage. In addition to this observation, *C. arvense* seedlings grown with *G. max* had more nitrogen overall, regardless of inoculation status (**Table 3**). *G. max* has a natural ability to fix nitrogen in the soil, which could have contributed to the overall higher nitrogen content. The *G. max* likely increased the overall nitrogen content of the soil, which in turn increased the overall nitrogen content of the seedlings grown in it. More research is needed to determine the long-term effects of AMF.

This avenue of research needs to be continued, as a natural herbicide is vital to modern agriculture. We could pursue many possible ways to further this research. We could observe the long-term effects of AMF inoculation on *C. arvense* plants, possibly across multiple generations. Another way could be to test varying concentrations of AMF inoculation in the soil or varying methods of inoculation, including soil-mixing and root-dips, as well as inoculation of AMF at varying stages of plant growth, such as pre-germination, immediately post-germination, and post seedling stages.

Based on the results of growth data and nitrogen testing, we determined that AMF inoculation is an unreliable way to prevent *C. arvense* growth in the seedling stage. Any significant growth decrease would have indicated a possible growth deterrent for *C. arvense*; however, the lack of significant difference in seedling growth between inoculated and uninoculated seedlings suggests that AMF does not affect their growth. Therefore, AMF is not a reliable option for a preventative herbicide against *C. arvense* at the seedling stage. However, the nitrogen difference found in inoculated and uninoculated seedlings indicate that it may be useful as a post-germination or post-seedling herbicide. While our original hypothesis was not supported, the results of nutrient testing open up new research opportunities to further explore AMF inoculation as a weed prevention technique. Discovering organic opportunities to effectively control agricultural weeds and invasive species like *C. arvense* is vital to modernizing the agriculture industry.

MATERIALS AND METHODS

Materials

The soil used for this study was Miracle-Gro Garden Soil, All-Purpose, In-Ground Use. This soil was chosen for its similarity to local soil. Four cubic feet of soil were sterilized in an oven until the internal temperature reached 180ºC. The *G. max* seeds were bought from Flinn Scientific, and the thistle seeds were collected from local plants after they were identified as *C. arvense.* The AMF used in this study was acquired from Sustainable Agriculture Technologies and consisted of four species of AMF, all from the genus *Glomus.*

Growth of Seedlings

We started the seeds in 3-ounce cups under Monios-L T8 LED Grow Lights in a controlled, indoor environment. The temperature averaged 22.2ºC, and the first sprouts of both plants were seen six days after planting. In order to provide all seedlings with sufficient water, a simple self-watering system was set up. A reservoir of deionized water sat below the shelf where the plants were growing. The shelf was covered in a layer of felt and each planter had a 4 cm felt wick extending out of the bottom of the soil. A 30 cm felt wick extended from the water in the reservoir to the felt on the shelf. Capillary action through the felt wicking allowed water to be pulled from the reservoir by the plant roots (**Figure 3**).

AMF inoculation and seedling growth measurements

To test the effects of AMF on *C. arvense,* we inoculated twelve seedlings with 0.25 g of AMF within 24 hours of sprouting. The seedlings were inoculated by evenly sprinkling the AMF powder onto the surface of the soil around the seedlings and then watering well. Twelve seedlings that sprouted on the same day were not inoculated and compared to the inoculated test group. Beginning when the seedlings were ten days old, the diameter of each seedling was measured at its widest point. We took measurements every five days for the duration of the experiment; the final measurement was taken 38 days after sprouting.

To test the effects of AMF on *G. max* and thistles when grown together, we inoculated 12 *G. max* plants and 6 thistles with 0.25 g of AMF within 24 hours of sprouting. The same procedure was used to inoculate the plants in other trials. After ten days, we transferred the seedlings into two 6-quart containers full of sterilized soil. Each container had six *G. max* seedlings planted in two evenly spaced rows of three. Three thistle seedlings were planted randomly around the *G. max* plants. We selected this arrangement to best mimic how each plant would grow in a crop field setting. The seedlings were not planted together until they were ten days old to ensure that each seedling received the same amount of AMF inoculation. We followed the same procedure for two more groups of the same size and formation; the seedlings in these groups received no AMF in order to serve as a control. Measurements were taken in the same manner as the groups with only one type of plant.

Nitrogen Content Analysis

After we conducted each experiment, we uprooted the plants to measure the root length of each. Each seedling was laid flat and measured from the base of the stem to the longest root tip. After the measurements were taken, each plant was air-dried. We then removed the roots from each seedling, and each experiment group was tested for nitrogen together at Custom Laboratories in Monett, Missouri. The dried plant samples were ground evenly and placed in individual killball flasks. We then digested and diluted the samples with NaOH and deionized water. Then, we distilled the samples into a weak acid solution. After the samples cooled, we titrated them until the indicator changed from green to pink and the color did not change for a few seconds. We calculated the total percentage of nitrogen in each sample through the following equation: **$\%N = mL_{titrated} * (0.1142 \times 1.401) / \text{sample weight}$.** Where mL titrated is the amount of solution used to stabilize the indicator color, 0.1142 is the constant, and 1.401 is the standard nutrient content of plants. After we collected all the final data, we calculated two-tailed t-tests in Google Sheets on all data sets to determine if there was a significant difference between inoculated and uninoculated seedlings.

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