

MIXTURES OF MYCORRHIZAL FUNGI IMPROVE GROWTH OF *LACTUCA SATIVA* AND REDUCE LEVELS OF ZINC IN CONTAMINATED SOIL

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ABSTRACT

Bioremediation is currently under investigation as a viable way to remove many environmental pollutants and most commonly involves the use of microorganisms to extract organic pollutants or heavy metals from water or soil. One of the most abundant heavy metals found in industrially polluted sites is zinc (Zn); it is often found alongside metals like lead (Pb), arsenic (As), and mercury (Hg). This experiment investigated the potential bioremediation of pasteurized soil contaminated with zinc using different vesicular arbuscular mycorrhizal fungi (VAM) species and lettuce plants (*Lactuca sativa*). Soil was amended with 0.4 g of zinc chloride ($ZnCl_2$) per kg of soil. Amended and unamended soils were inoculated with two different mixes of VAM, BioAg VAM-Endo™ and MycoBloom. For each treatment, *L. sativa* plants (15 pots per treatment) were grown in a greenhouse setting. Plant diameter was measured weekly. Plants were harvested after 55-days and the wet weight of leaf tissue was measured before the tissue was sent for analysis of zinc levels. Roots were assessed for mycorrhizae using a trypan blue staining procedure. The BioAg VAM-Endo™ mix was the most successful at removing $ZnCl_2$ from the soil. *L. sativa* inoculated with VAM mixes formed mycorrhizae, grew healthier and removed more zinc from the soil than the non-inoculated group. We propose further investigation into the use of mycorrhizal fungi paired with other plant species to remove zinc from contaminated sites with harmful levels of zinc.

KEYWORDS

- Bioremediation
- Mycorrhizal Fungi
- VAM
- *Lactuca sativa*
- Zinc

INTRODUCTION

Bioremediation uses an introduced or naturally present organism to break down environmental pollutants. Several subcategories of bioremediation exist, three of which are phytoextraction, rhizosphere degradation, and mycoremediation. Phytoextraction uses plants to take up a contaminant to be stored within its tissue and the tissue is then harvested and typically destroyed. Rhizosphere degradation takes advantage of the soil microbiome to help breakdown the contaminant. In mycoremediation fungi are used to degrade or capture contaminants (9, 14).

Phytoextraction, alone, has not been determined to be an effective method of removing heavy metals from contaminated sites, as of yet (29). However, when multiple bioremediation methods are used in concert, contaminant uptake can be increased. When vesicular-arbuscular mycorrhizal fungi (VAM) are associated with a plant, zinc uptake and biomass are significantly increased, in lab and field studies (17, 21).

Many fungi have powerful enzymes that can break down pollutants directly and they also contain heavy metal chelators, making them particularly suited to remediate areas contaminated with heavy metals (4, 14, 30). Fungi can bind these metals to chelators anchored within their cell wall, intracellular chelators within the cytosol, or bind them to extracellular chelators. Each of these methods render the metals inactive which can ultimately save organisms, like plants, from bioaccumulation of these toxic agents in tissues, or humans from ingestion of food crops containing toxic quantities (16, 30). Fungi have successfully been used to remediate areas of concern, but more research needs to be done in this area to make them more effective. *Glomus intraradices* is one such species of fungus that has been studied extensively. *G. intraradices* is a species of

VAM that associates with plant roots in a symbiotic relationship. A few studies have showed *G. intraradices* has a higher tolerance for heavy metals when compared to other VAM grown under the same conditions (11, 20).

Heavy metals are released into the environment through a number of processes. They often accumulate, naturally, through erosion of parent material, such as exposed bedrock and other parts of the earth's crust. Human activity has caused a sharp increase in release of heavy metal pollutants within the environment. One example of a heavy metal is zinc, which can be found in toxic concentrations in many anthropogenically contaminated sites (6). In 2005, 985 of the 1,662 hazardous waste sites that were proposed for inclusion on the EPA National Priorities List contained zinc, although it is unknown how many of those sites were evaluated for toxic quantities of zinc (1).

Zinc has many important industrial uses (e.g., manufacturing of batteries, use in paint and rubber products, and zinc oxide). Another major source of zinc contamination is through mining, which includes left-over tailing piles, by-products of smelting, and hydraulic fracturing (7). Hydraulic fracturing, otherwise known as fracking, is a mining technique used to extract oil and natural gas. Since natural gas has gained popularity as a transitional fuel in the race to find a sustainable source of energy, hydraulic fracturing has increased in popularity. In 2015, there were 623 reported fracking fluid spills in the United States, according to the Colorado Oil and Gas Conservation Commission (27). This is down from the highest recorded number of spills in 2014, which is 786, also reported by the Colorado Oil and Gas Conservation Commission (27). The use of hydraulic fracturing, like any other form of mining, generates waste. In this case, it is the contaminated water expelled from the hydraulic fracturing wells along with the contents of whatever was in the ground (12). This waste often contains high concentrations of zinc brought up from deep within the earth. Inevitably when this water is expelled into the area around the well, zinc as well as other pollutants can accumulate in the soil often in the form of zinc chloride and other organic materials (12, 15).

In plants, zinc is used as a constituent in many enzymes and proteins and it is vital to the production of chlorophyll, to the development of cold resistance, and to the conversion of starches to sugars (5, 25). The normal concentration of zinc in healthy leaf tissue is measured to be in the range of 10–15 ppm (5, 19). In toxic concentrations of zinc, plants experience stunted growth, chlorosis of the leaves, and changes in root

growth (22). High zinc concentrations also interfere with the uptake of other nutrients such as phosphorus, iron, manganese or copper, ultimately leading to their deficiency. If the toxicity is severe enough, partial or whole plant death will occur (5). Noticeable symptoms of toxicity generally occur when zinc levels accumulate to greater than 200 ppm (5, 19). The presence of elevated zinc levels in the environment by things like hydraulic fracturing wastewater has an indirect effect on us. Contamination can have detrimental effects on many staple crops, as well as native species. Zinc accumulation can be detrimental to a multitude of ecosystems and human well-being.

Knowing that VAM often form large multi-species complexes in nature, mixtures were chosen for this current study rather than using only a single species. These mixes were paired with a popular variety of *L. sativa*. *L. sativa* is a common crop grown around the world with a fast maturation rate. *L. sativa* is also susceptible to different types of environmental pollution, making it a good candidate for study on the effects of soil contamination on plants. This experiment used the same variety of *L. sativa* but with different mixes of VAM, one containing species native to the great lakes region (MycoBloom) and another sold for commercial agricultural species (BioAg VAM-Endo™). It was hypothesized that the presence of the VAM mixes would reduce zinc concentrations within the soil and that different types of VAM will have a different impact on the uptake and bioaccumulation of zinc.

MATERIALS AND METHODS

SOIL PREPARATION

Approximately 155 kg of agricultural soil was gathered from Marquette, Michigan. This soil was pasteurized, in a single batch at Northern Michigan University, twice at 75 °C for 1-hour, with a 24-hour resting period in between pasteurizations. A soil sample was then sent to A&L Great Lakes Laboratory, Inc. to determine the nutrient profile of the soil before amending.

SOIL TREATMENTS

Six treatment groups were created, each consisting of 15 pots with 1.78 kg of soil in each pot (5.5 inch Square Jumbo Dillon Greenhouse Pots, Growers Solution, Cookeville, TN). This pot size and volume of soil allowed for proper *L. sativa* plant growth (Whitacre and Neumann, unpublished). The first treatment group, T1, consisted of unamended pasteurized soil. The second and third treatment groups, T2 and T3, consisted of pasteurized soil amended with the mycorrhizal inoculants. T2 was amended with BioAg VAM Endo-Mix™. T3 was amended with MycoBloom. BioAg VAM Endo-Mix™ consists of several different species of VAM, the predominant one being *Glomus intraradices* (65.8% of the species mix). Smaller amounts, each comprising 5.7% of the species mix, of *Glomus claroidium*, *Glomus deserticola*, *Glomus mosseae*, *Glomus etunicatum*, *Gigaspora albida* and *Glomus clarum* were also included. MycoBloom consists of seven different VAM species native to the great lakes region. The species of VAM include: *Claroideoglomus claroideum*, *Funnelformus mosseae*, *Cetraspora pellucida*, *Claroideoglomus lamellosum*, *Acaulospora spinosa*, *Racocetra fulgida* and *Entrophospora infrequens*. The other three treatment groups, T4, T5, and T6, used the remaining half, 38.75 kg, of the pasteurized soil. This soil was moved to a separate container where a 1 L solution of water containing 31.14 g of ZnCl₂ was mixed in using a shovel. This was done for approximately 10-minutes to ensure uniformity mixing of the ZnCl₂. This process added approximately 0.4 g of ZnCl₂ per kg of soil. The pasteurized soil with ZnCl₂ added was then split up into the other 45 pots. This amount of ZnCl₂ was chosen due to the results of previous trials consisting of

0.5 g of ZnCl₂ per kg of soil (Whitacre and Neumann, unpublished), where plants across all zinc amended groups died in significant amounts. T4 consisted of the pasteurized soil amended with 0.4 g ZnCl₂ per kg of soil. T5, in addition to ZnCl₂, was mixed with BioAg VAM Endo-Mix™. T6 was mixed with MycoBloom™. The mycorrhizal inoculants for all respective treatment groups were mixed in by hand at 5.6 g per pot. Each pot contained approximately 1.78 kg of soil or soil with inoculate. All measurements made were made separately on a Taylor digital kitchen scale to the nearest 100th decimal place.

SEED GERMINATION AND PLANTING

Once each of the treatment groups were properly amended, the pots were immediately moved to Northern Michigan University's greenhouse. *L. sativa* seeds (Black Seeded Simpson, Johnny's Selected Seeds Co.) were germinated using a wet paper towel placed inside a plastic bag, for a period of 48-hours. Once germinated, two seeds were planted in each of the 90 pots to ensure survival of at least one plant. The pots were covered with plastic wrap and left for 10-days until the seedlings were culled to one seedling per pot. After 2-weeks since planting, the plastic wrap was removed. Another 2-weeks from this point, the plant diameter was measured every week for 4-weeks. A growth period of 55-days was chosen to ensure full maturity was reached, according to Johnny's Selected Seeds description. Plants were watered as needed throughout the experiment. The computer control system of the greenhouse conditions were set to not exceed 29 °C or fall below 16 °C. However, 3-days of the experiment temperatures had reached 38 °C due to a malfunction of the greenhouse cooling system. The light conditions consisted of 14-hours of sunlight, and 10-hours of dark.

SOIL AND TISSUE SAMPLING

After a total growth period of 55-days the *L. sativa* leaf and stem tissues were harvested. The harvesting was done by cutting the stem just below the basal rosette, at roughly the same place on each plant. Wet weight was taken for each plant to create an average, and then all the plants in each group were weighed together for a total group biomass. The soil from each treatment group was then bulked, taking care to keep each treatment group separate. Soil samples were then taken for each separate treatment group.

The soil and tissue samples were stored overnight in a cold room at 4 °C. The next day the samples were shipped overnight, unrefrigerated to A&L Great Lakes Laboratory (Fort Wayne, Indiana) and tested for their nutrient profile. The plant tissue was tested for total nitrogen using the Dumas method using an Elemental rapid-N cube, while mineral analysis was conducted using inductively coupled argon plasma using a Thermo iCAP 6500. The soil analysis procedures were done in accordance with “Recommended Chemical Soil Test Procedures for the North Central Region.”

STAINING OF ROOTS FOR VAM

The roots were stained and analyzed for VAM colonization. They were first washed of any remaining soil with deionized water (DI water) and placed in separate beakers. Each group was then placed in 5 % potassium hydroxide (KOH) (w/v) and allowed to soak for a period of 2-days, until they appeared translucent and white (or as some would say, looked like “rice noodles”). The roots were rinsed with DI water in between soaking periods. The roots were then rinsed with DI water again and placed in a 5% hydrochloric acid (HCL) (v/v) solution to soak for 1-minute. A trypan blue stain was then created using 500 mL of glycerol (C₃H₈O₃), 475 mL of deionized water, 25 mL of acetic acid (CH₃COOH) and 0.1 g of trypan blue. The roots were allowed to sit in the trypan blue stain (C₃₄H₂₈N₆O₁₄S₄) for 7-days and were then observed under a microscope.

RESULTS

PLANT GROWTH AND BIOMASS DATA

The relationship between treatment groups and mean weekly plant diameter can be seen in Figure 1. T4, which contained zinc only (no VAM present) had stunted plant growth compared to all other treatments for all 4-weeks of measurements, however these plants had a clear linear growth rate throughout the 4-weeks. With all other treatments (either the control containing no zinc, or treatments with zinc plus VAM), plant diameter measurements were similar as shown by the clustering of the data (Fig. 1.). T5 had the greatest plant diameter at the end of the experiment, while T4 had

the smallest diameter. The final plant diameters of T4 significantly differed from T1 (p<0.005), T2 (p<0.05), T5 (p<0.00005) and T6 (p<0.0005). Other treatments that differed significantly from each other, are T5 when compared to T3 (p<0.05), as shown in Figure 2.

The mean final biomass of *L. sativa* tissue is shown in Figure 3; the final biomass correlates similarly with the results of the mean final diameter. T3 and T4 had statistically significant lower biomass values than all other treatments (p<0.005), but they were not significantly different from each other. All the other treatments (T1, T2, T5, and T6) were not significantly different from each other.

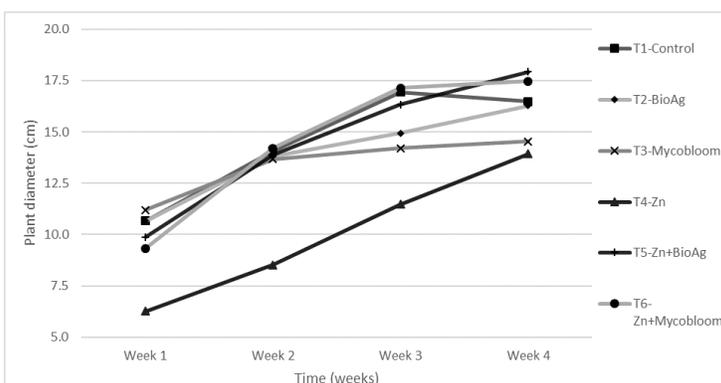


Figure 1. Mean *L. sativa* diameter (cm) at the end of each week of growth.

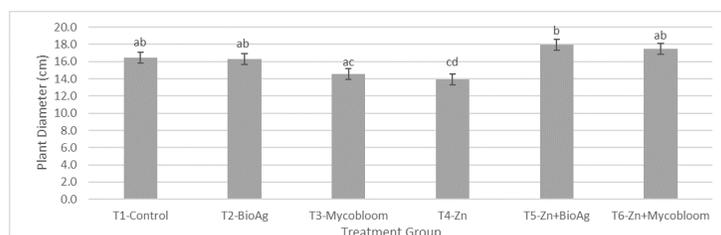
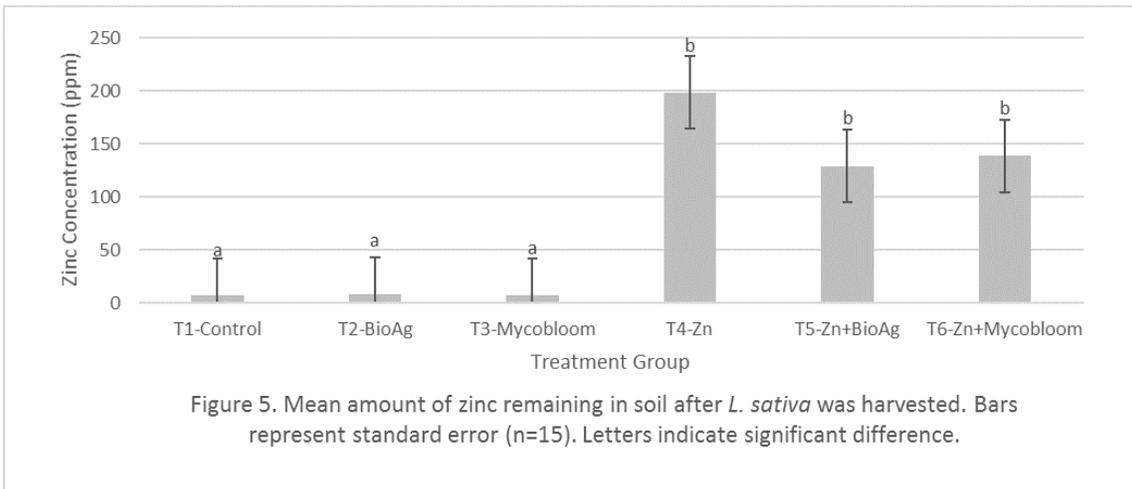
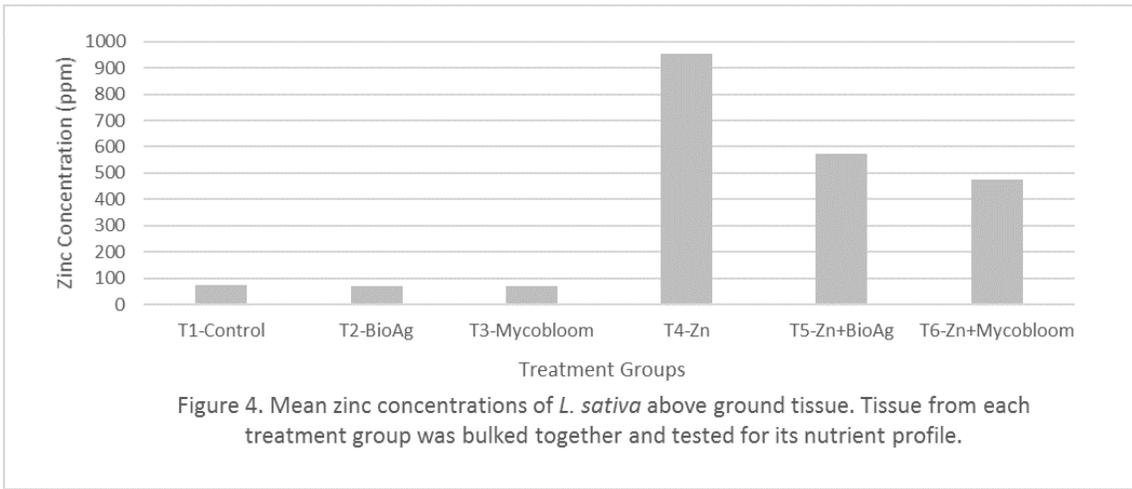
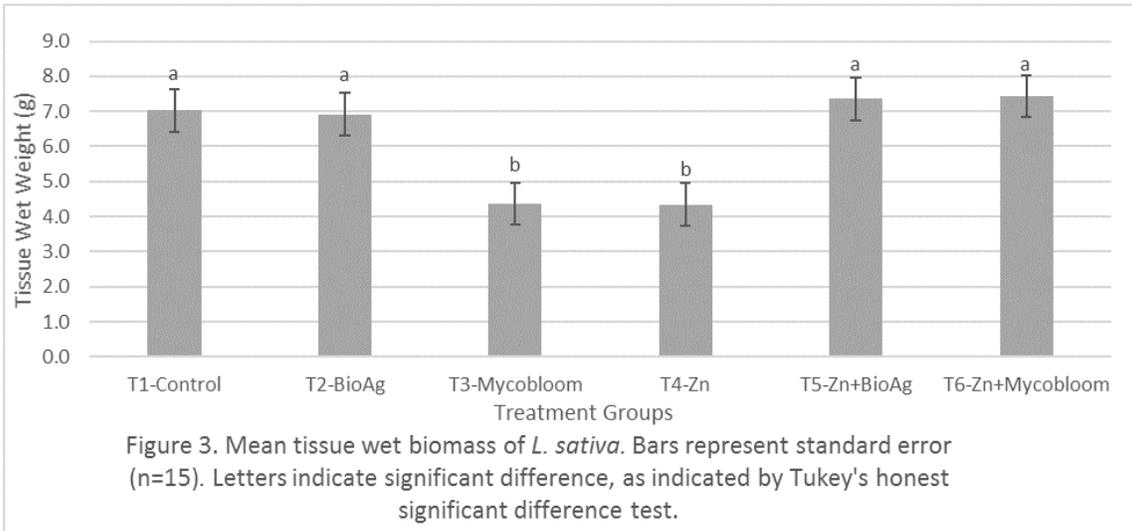


Figure 2. Mean final diameter of *L. sativa*. Bars represent standard error (n=15). Letters indicate significant difference, as indicated by Tukey's honest significant difference test.

ZINC CONCENTRATION COMPARISONS

The zinc concentrations in the above ground tissue of *L. sativa* are shown in Figure 4. Plant leaves from all 15 pots were bulked for zinc analysis (due to cost constraints) thus no statistical tests could be run. However, the tissues of the T4 samples contained a considerably larger amount of zinc, 952 ppm, than any of the other zinc amended groups. The tissues of the treatments treated with mycorrhizae and zinc, T5 and T6, contained 573 ppm (60.2% of the amount of zinc as T4), and 476 ppm (50% of the amount present in T4) of zinc respectively. The tissues of the treatments that were not amended with zinc, T1-T3, all contained similar small amounts of zinc at the end of the experiment.



DISCUSSION

The concentrations of zinc remaining in the soil are displayed in Figure 5. Soil from all 15 pots were bulked for zinc analysis (due to cost constraints) thus no statistical tests could be run. The treatment groups that were not amended with zinc all displayed similarly low levels of zinc. T4 clearly had the highest amounts of zinc in the soil. While the zinc concentrations of T5 and T6 had higher levels of zinc than unamended treatments (T1-T3), the levels of zinc are clearly lower than T4 that did not have any VAM treatment.

To ensure mycorrhizae had formed between the fungi and the roots in treatments T2, T3, T5 and T6 and that T1 and T4 were not colonized by VAM, root tissue was stained and observed under microscope. Figure 6 serves as a representative image of roots colonized by VAM, which were evident in all treatments inoculated with the mycorrhizal fungi mixes. T1 and T4 had an absence of mycorrhizae (data not shown).

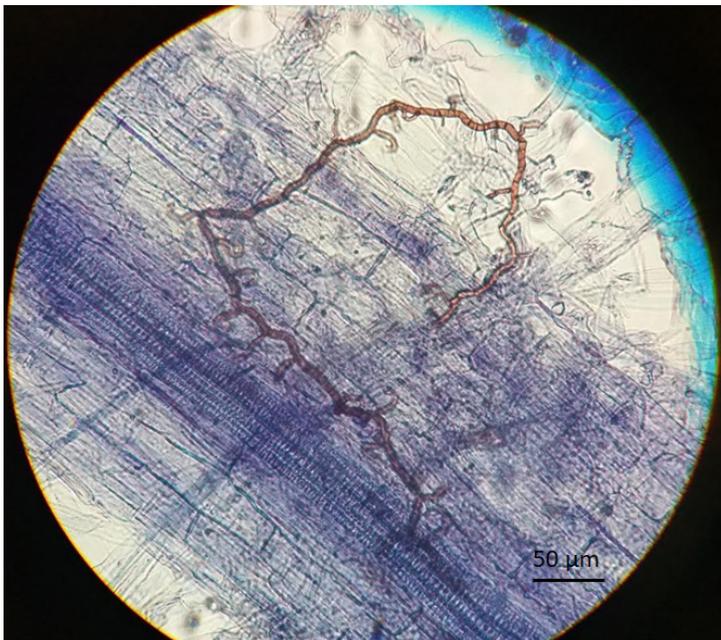


Figure 6. A representative image of *L. sativa* root tissue colonized by VAM.

STATISTICAL ANALYSIS

The results of this experiment were analyzed using the Tukey's honest significant difference test, conducted in the program R, to assess the diameters and biomasses of the various test groups.

The *L. sativa* plants, which were treated with only zinc, experienced stunted growth initially and throughout the experiment (Fig. 1). But, plants treated with VAM (T5, T6) in zinc amended soils had similar weekly plant diameters and biomass as the treatment groups that had no zinc present (Fig. 1). Taken together, this suggests the presence of the VAM aided in plant growth, likely by protecting the plant from growth-inhibitory or toxic levels of zinc. Previous studies mentioned plant biomass had significantly increased due to additions of zinc and VAM, but those studies applied lower concentrations of amended zinc (2, 16, 20). At the end of this experiment, T4 (treated only with zinc) exhibited significantly lower growth, biomass, and mean diameter compared to every other treatment group, besides T3 (Fig. 2 and Fig. 3). There were also two plant deaths that occurred in T4. During statistical analysis, the size measurements for the dead plants were counted as values of zero. The values were used in the analysis, because the deaths were believed to be caused by zinc, due to leaf chlorosis. Since this occurred, the treatments amended with zinc were on the threshold of zinc toxicity. Unexpectedly, without the addition of zinc, the plants treated with Mycobloom (T3) at the completion of the study were affected negatively to the point where they had similar biomass and plant diameter data to the plants in soils treated with zinc only (T4) (Fig. 2 and Fig. 3). This could be due to non-optimal conditions for one or more of the VAM species in the mix, perhaps causing a competitive or parasitic relationship to develop with the plants (10). The BioAg VAM Endo-Mix™, however, had a positive effect on plant growth, in both the zinc treated and untreated soils, when compared to their respective controls. This supports data found, in earlier experiments, on the ability of various mycorrhizal species to prevent heavy metal stress in *L. sativa* (13). The fungal species composition and ratios differed between the two VAM mixes, which could help explain the different impacts on *L. sativa* plant growth. Excessive heat in the greenhouse may have also played a role, although visible signs of widespread plant heat stress (e.g., chlorosis, wilting) were not evident through the course of the experiment.

Plant tissue grown in zinc amended soil without VAM (T4) had very high levels of zinc (952 ppm) (Fig. 4). In contrast, T5 and T6, also grown in zinc amended soil but with inoculated VAM mixes, had

substantially lower levels of zinc compared to T4 (573 ppm and 476 ppm respectively) (Fig. 4). This suggests the VAM were either sequestering some of the zinc in their fungal tissues, or possibly had chelated the zinc through release of extracellular chelators. Although the zinc levels in the soil remained high (according to the standards followed by the A&L Great Lakes Laboratories) (Fig. 5), the quantity of zinc in the soil of T5 was substantially lower than in T4, and zinc levels in the T6 soil was also lower than T4 (Fig.5) . This suggests that with longer VAM treatment times zinc concentrations may be brought back to within a normal range, if the fungi were sequestering the zinc from the soil into their tissues. The soils of T5 and T6 may also be considered nontoxic according to some standards that report a 300 ppm toxic threshold of zinc and the T5 and T6 values are below that 300 ppm (19). While the average value of T5 and T6 did measure below 300 ppm, health hazards may still exist (2). It is possible that treatments of *L. sativa* only, without the addition of mycorrhizae, could bring the soil to acceptable levels of heavy metal concentrations. However, using mycorrhizae would be preferential, as the lettuce would likely experience less strain from zinc toxicity (3, 13). Quantities of zinc remaining in the soil and tissues of T5 and T6 were different from each other (though not dramatically so); this, along with the unusually low plant biomass seen in T3 (which also had VAM treatment), suggest that different VAM mixes and pairing with proper plant species should be formulated to accommodate specific heavy metal contaminations more appropriately. Choosing VAM species that readily form mycorrhizae with the desired plants is important for this process. In this study, visual evidence of mycorrhizae formation was confirmed (Fig. 6). The results from this study support other published findings that combinations of VAM and their symbiotic plant species can be effective at remediating polluted soils (18, 24). Further, we believe combined remediation techniques such as using specific plant species, tailored VAM mixes, beneficial bacteria, and addition of chelators could be a highly successful strategy for bioremediation of polluted environments (9, 14, 16, 20, 21).

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