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The combined effects of soil fertility and soil amendments on the growth and mycorrhizal associations of American Ginseng (Panax quinquefolius)

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The combined effects of soil fertility and soil amendments on the growth and mycorrhizal associations of American Ginseng (*Panax quinquefolius*)

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Dedication

This work is dedicated to all the intelligent and strong woman who inspired me and cultivated my love for science. This list includes teachers, mentors, and college professors. I would have never ended up here without all of you.

Sarah Meiss Ph.D.

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Kelli Dellarose

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Thank you for showing me how amazing women in science are.

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Abstract

Arbuscular mycorrhizal fungi (AMF) are fungi that form symbiotic associations with 70-90% of plant families. They are known to allow for the extension of the root system as well as an increase in plant size by assisting with the uptake of nutrients such as nitrogen and phosphorus. The role that AMF play in plant health and success has led to the development of commercial inoculum, which is used in agricultural settings. However, soil fertility, and soil amendments are known to affect AMF and plant associations. This study intends to look at how cultivated American Ginseng seedlings are affected by commercial inoculum. This greenhouse study examines how soil type (2 levels), liming (2 levels), and inoculation (2 levels), affect plant growth. Two distinct soil types were collected from the field. Stratified American ginseng seeds were planted in cone-tainers in a regulated greenhouse system in a factorial design, with fifteen cone-tainers for each treatment combination. At seven months, seedlings were measured for root length, stem length, leaflet width, above-ground biomass, and percent infection Additionally, roots of American Ginseng plants planted in Rowlesburg, WV in a field plot design were examined for arbuscular mycorrhizal association while spores from this field site were counted and quantified. Liming had greatest effect on most parameters including root length, stem length, leaf width, and leaf length ($p<.001$). Soil fertility (high) had a positive effect on root length ($p<.01$), but a negative effect on below ground biomass ($p<.001$). Additionally, inoculum (+) had a positive effect on average leaf width ($<.05$) and infection (%) ($p<.01$). All American Ginseng plants growing in planted field plots had formed associations with AMF, and spore counts did not differ across soil types within the design. Appropriate pH appears to be a large factor in cultivation successes.

pH combined with AMF show a trend of positive effects on growth parameters of the plant. This could be useful when cultivating American Ginseng for both commercial and reintroduction purposes.

Introduction

American Ginseng (*Panax quinquefolius)* is an excellent model species to use to determine the importance of mycorrhizae in plant growth relative to soil conditions. This herbaceous species has been described as both a specialist and a generalist. They have specific soil nutrient requirements, but requirements but are known to grow across many soil types. Mycorrhizal associations may explain how ginseng can thrive in a variety of soil conditions. This is an important concept to explore because of both reintroduction and commercially-grown programs with this species.

Arbuscular mycorrhizae (AM) are symbioses between plant roots and fungi. The symbiosis is very common and is found in 70-90% of all plants, ranging from those living in tropical rainforests to temperate grasslands. The fungus penetrates the outer layers of the plant root and grows through the soil via a network of hyphae. Plants provide the fungi with carbohydrates from photosynthesis, while the fungi provide the plants with phosphorous and nitrogen from the soil. These associations have also been shown to be able to increase the plant's resistance to biotic and abiotic stressors (Yue and Liu, 2016; Genre, 2005). Such symbioses can be essential for plant survival. The arbuscular mycorrhizal fungi (AMF) are completely reliant on the relationship with the plant and cannot complete their life cycle without the symbiosis. AMF are unique in that they are not species specific, and the fungi can often invade more than one plant. Plants can also be colonized by more than one species of AMF at a time. Different plant species may become interconnected through their mycorrhizal symbionts (Yue and Liu, 2016).

All AMF belong to the phylum Glomeromycota and the class Glomeromycetes. Members of this class are only able to complete their life history with their symbiotic partner (Yue and Liu, 2016). Colonization usually occurs when essential plant nutrients in the soil, especially phosphorus, are limiting; and the plant needs the aid of the fungi to sequester necessary nutrients from the surrounding environment (Rillig, 2004). AMF form their spores in the soil. The AMF have hundreds of nuclei in one cytoplasm and propagate through asexual reproduction. After spores germinate, the hyphae invade roots, and then penetrate the cortex of the root when conditions allow. After the hyphae enter the root, they can form hyphal structures within the cells. The two characteristic structures are arbuscules, the point of nutrient exchange between the plant and fungi, and vesicles that store nutrients. There are some species that do not form vesicles. Internal hyphae and spores can also be formed once the fungi enter the plant (Yue and Liu, 2016). Before infection can occur, the host root must release a chemical into the soil (strigolactone), which signals the AMF to switch to a presymbiotic growth phase. This process results in branching hyphae near the root; once they contact the root cortex, the AMF develop appressoria, structures that use turgor pressure, and penetrate the root tissues (Genre et al., 2005) (Figure.1).

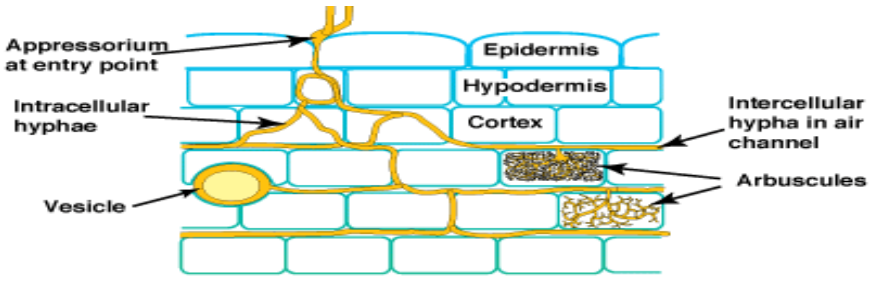

Figure 1.This image depicts the how the association between plants and AMF form. It shows all structures including vesicles, hyphae, and arbuscules within plant cell. Hyphae are the body of the fungi that penetrate the root system, and entre the cells, vesicles form as a location for nutrient storage and arbuscules form as the point of nutrient exchange between AMF and root cells. (University of Minnesota Botany Lab 2015).

Studies have shown that mycorrhizal associations can significantly increase plant growth and health. They have been known to not only result in extension of the root system, but also, increase number of leaves, height, and leaf area. They have also significantly increased phosphorous concentrations in plants (Frosi et al., 2016). In one study, AMF inoculated plants had significantly higher concentrations of N, K, P, and B in their shoots; and N, S, Cu, Fe, Mn, Mo, and Ti in the roots (Gastol et al., 2015). Magnesium and zinc concentrations facilitate these associations (del Mar Alguacil et al., 2016).

Plants are the partner in the symbiont that signal for the association to form. If nutrients are available to the plant they do not need to form associations. Soil texture and pH can affect how well plants become inoculated with AMF. Root colonization can be reduced in clayey soils and in soils amended with lime (Carrenho et al., 2007). Mycorrhizal colonization has also been reduced when phosphorous is added to soils (Carrenho et al., 2007).

The role of AMF in increasing plant health has led to the production of commercial inoculum. The idea is that this inoculum of fungi can be used to increase crop yield. This could be useful to reduce the use of pesticides (Hui and Ying, 2016). However, the complexity of the soil environment may change the success rate of commercial inoculum. AMF inoculant may be less successful in soils with high nutrients (Herrara-Peraza et al., 2011).

The cultivation of American Ginseng *(Panax quinquefolius*) may benefit from the incorporation of commercial inoculum. American Ginseng is a perennial herbaceous species in the Araliaceae family. This species occurs in North America with a large geographic range in the Midwest and on the East coast (Figure 2).

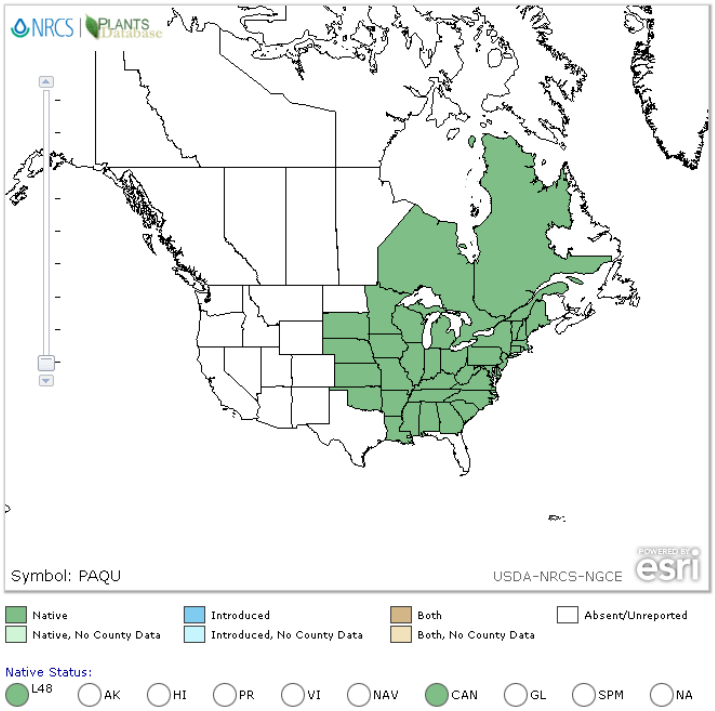

Figure 2. USDA distribution map of American Ginseng (*Panax quinquefolius*) created by the NRCS and USDA Plant Data Team (2017)

Although American Ginseng is commonly associated with forests dominated by Sugar Maple, it has also been found in drier oak-hickory forests and in walnut-sycamore dominated forests (USDA, 2017). They are often found in micro-habitats with rich soils in mountainous regions (Charron and Gagnon, 1991; U.S. Fish and Wildlife Service, 2016). However, they can grow on a variety of soils, ranging in texture from sandy soils to silty soils to moderately high clay soils (Anderson et al., 1993), suggesting that ginseng is a generalist.

American Ginseng has several large and thick taproots that are forked and grow from a narrow rhizome, with the largest taproot at the distal end. The root has multiple parts including the bud for next year's growth, and the rhizome, which consists of the main root and thinner tail roots (Sokhansanj et al., 1999). The roots are harvested for their ginsenoside content, believed to correct human physiological imbalance (Proctor and Bailey, 1987; Sokhansanj et al., 1999).

The rhizome of American Ginseng is identified by large scars that form as the result of annual abscission of the aerial stem. Due to these scars and the long-lasting rhizome, plants can accurately be aged and have been shown to be able to live longer than 50 years. One aerial stem is produced each year, normally emerging after the forest canopy closes. The height of the aerial stem ranges anywhere from 7 cm to 40 cm (Charron and Gagnon, 1991; Lewis and Zenger, 1982) (Figure 3.).

Mature American Ginseng plants have leaf structures that appear whorled, but are known as prongs. These prongs are not directly comparable to leaves because they do not possess axillary buds at their intersection with the aerial stem. Each prong possesses a petiole and can have anywhere from 3-5 palmately compound leaflets. Young American Ginseng can vary in size and number of prongs. Formation of additional prongs is not an annual event (Lewis and Zenger, 1982). The plant is typically able to reproduce after three years, but can sometimes take from 4-7 years based on conditions and nutrient availability. Additionally, when seeds enter the seed bank they are in a state of dormancy, the seeds must be stratified or exposed to the elements over winter to soften their seed coat (DCNR Ginseng Fact Sheet, 2017; Charron and Gagnon, 1991).

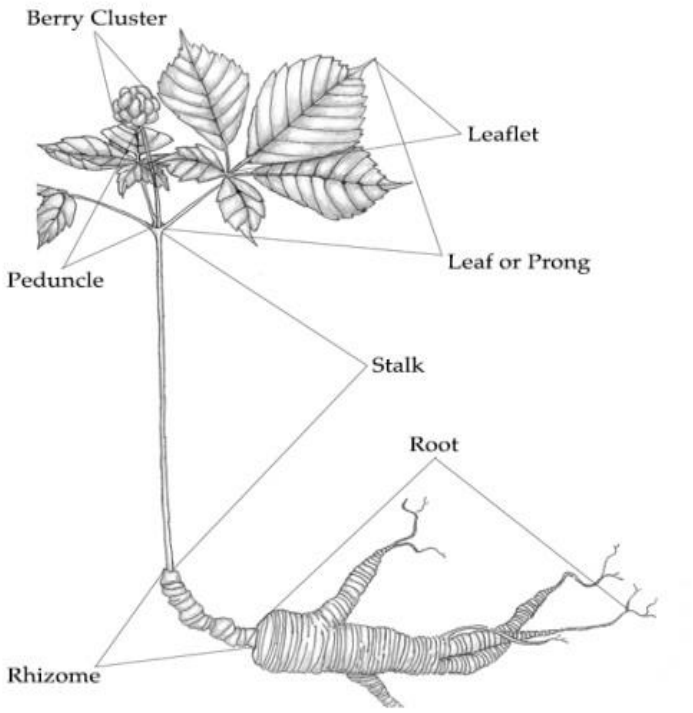

Figure 3. Adult American Ginseng plant morphology (Van Der Voort, 2003 as cited in Mcgraw, et al., 2003) including its large tap root attached at the stem. Each year there is abscission of the aerial stem that leaves a scar on the rhizome, allowing for plant aging.

The fruits mature in the fall; the seeds are dispersed and remain dormant for 20 months before germinating (Charron and Gagnon, 1991; Lewis and Zenger, 1982; Fiebig et al.; 2001).

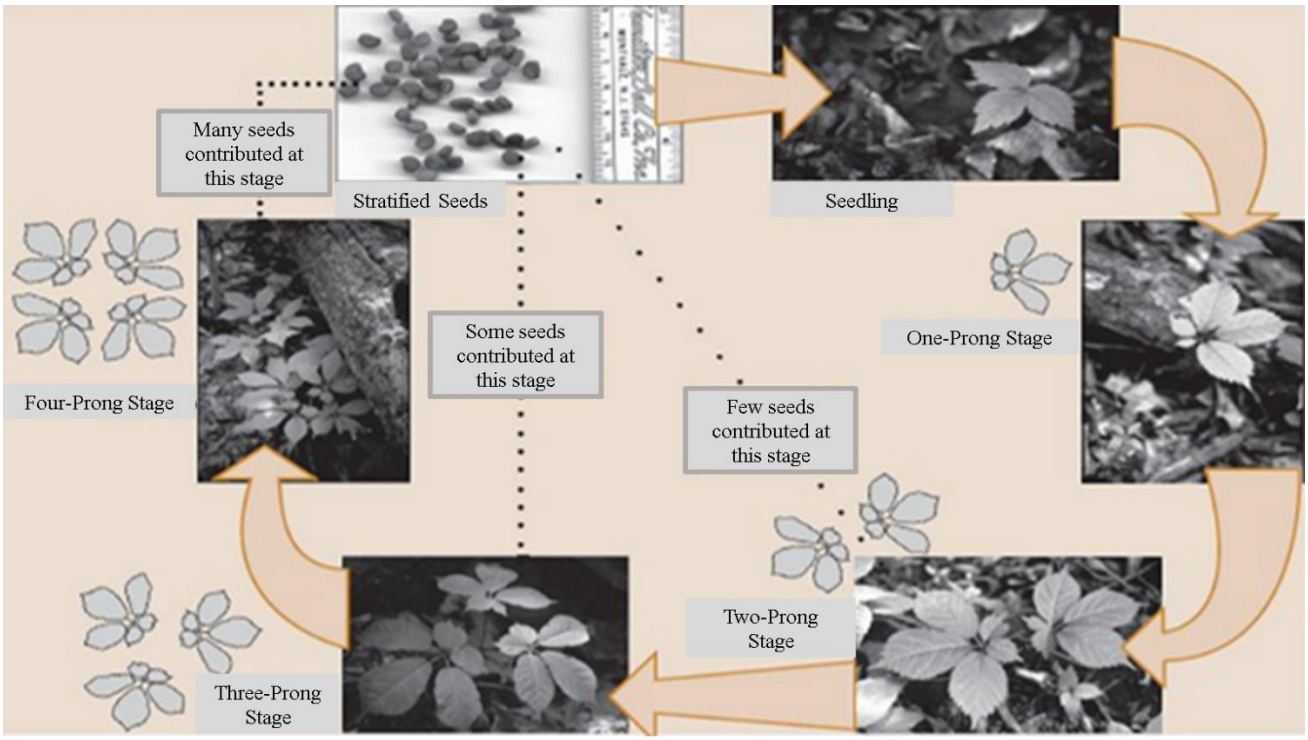

Figure 4. This figure depicts the American Ginseng lifecycle. Initially, seeds are stratified within the soil and as they germinate enter the seedling stage. During this time, they are not contributing any genetic material to the seed bank. As the plants age and develop additional prongs they can contribute more seeds for regeneration. (DCNR Pennsylvania, 2017).

A study by Lewis and Zenger (1982) in Missouri suggested that age, structure, and morphology are correlated. One-pronged plants ranged in age from one to six years old, two pronged were three to sixteen years old, three-pronged were four to seventeen years old (but were clustered in the age range from six-ten years old), and four pronged plants ranged from eleven to eighteen years old. There are stable periods of no prong growth once the plant reaches one of the classes. Their intervals vary, but these stable periods increase in length as the plant develops more prongs (Figure 4). Prong numbers help determine the age of the plant.

The plant has been used in traditional medicinal practices by both Native Americans and Asian countries since the mid-1700s. The majority of harvested American Ginseng is currently exported to China, primarily Hong Kong. It accounts for approximately 80% of purchases of unprocessed roots. The plant's roots are sold for $330 per kilogram to $1000 per kilogram (McGraw et al., 2003; Hankins, 2009). Cultivated Ginseng sells for much less than forest-grown Ginseng (Hankins, 2009).

Although American Ginseng has a wide distribution and can be found in different forest and soil types, natural populations are becoming more difficult to find (McGraw et al., 2003). Populations may be declining due to overharvesting, habitat destruction, climate change, and/or vertebrate herbivory. Overharvesting, most likely the leading cause of declining population numbers, also reduces genetic diversity. This in turn may decrease the resilience of this species to climate change (Souther et al., 2012).

Ginseng populations may also be declining due to herbivory. White-tailed deer (*Odocoileus virginianus*), the most abundant wild ungulate in the U.S., often browse and consume American Ginseng. Voles (family: Cricetidae) are believed to cause damage to the roots of the plant. The above ground structures of the plant often fall prey to aphids, stink bugs, slugs, and grasshoppers. Disease such as *Alternaria* blight can lead to lesions on the leaves (Vaughn et al., 2011)

American Ginseng is protected under the Convention on International Trade in Endangered Species (CITES) of Wild Fauna and Flora under Appendix II, which states that the species could become threatened if trade is not controlled. This species is exported in larger volumes than any other native plant listed in CITES. Although it is listed, American Ginseng can still be harvested in 19 U.S. states. Most states will only allow

legal exports if the plant is six years of age or older, and if it was collected during designated state harvest seasons. It is typically illegal to harvest on state lands and national forests, although some do issue harvest permits (U.S. Fish and Wildlife Service, 2017).

Due to the large interest in American Ginseng in both traditional medicine and in the health food 'craze', American Ginseng cultivation is on the rise in the United States, Canada, and China. It can be grown in open fields with artificial shade to mimic the forest canopy. Roots are typically able to be harvested after four years due to lack of competition with other forest plants. *Alternaria* blight is one major challenge in cultivating American Ginseng. It is a widespread fungal disease that can destroy an entire crop due to proximity between plants. It often occurs if soil does not have adequate drainage (Hankins, 2009). American Ginseng can also be cultivated in a manner called 'Wild Simulated Ginseng'. This involves planting American Ginseng in the understory of a naturally forested area. A major threat to wild simulated American Ginseng is poaching. This ginseng is just as marketable as wild American Ginseng (Hankins, 2009).

The interest in ginsenosides has led to research in how to increase Ginseng size and concentrations of ginsenoside. Concentrations of root tissue nutrients such as N, P, K, Ca, Mg, Mn, Cu, Zn, and Fe have positive correlations with increased ginsenosides. Likewise, soil fertility nutrients such as P, K, Ca, Mg, Mn, Zn, and Na, are positively correlated with increased ginsenosides in the leaves of the plant (Konsler et al., 1990).

Colonization of AMF in American Ginseng is the Paris-series type, which is characterized by extensive intracellular hyphae in the cortical cells. The fungi also form sections of coiled hyphae which can remain long after the arbuscules deteriorate. Vesicles have rarely been recorded in American Ginseng plants (Whitbread et al., 1996). In the

Asian species of Ginseng *(Panax Ginseng*), AMF inoculation has been recorded as successful, and has increased nutrient uptake and growth of seedlings (Cho et al., 2009).

American Ginseng typically grows in slightly acidic soils (5.5 to 6.0), ranging in texture from sandy loam to loam (Kim et al., 2014; Charron and Gagnon, 1991; Hankins, 2009).

However, American Ginseng also has been found in highly acidic soils (pH = 5-.7) (Beyfuss 1997; Hankins, 2009). The soils within this study fall within that range at 3.51 (high fertility soil) and 3.45 (low fertility soil). American Ginseng is also more abundant in soils high in Ca^{2+} (4,000 lbs per acre or 2,000 ppm), (Hankins, 2009). Their tolerance of a wide range of soils in their native habitat could be determined by associations with mycorrhizae. If this is the case, adding commercial inoculum may increase the success of cultivation and reintroduction programs with American Ginseng.

Study Objectives

This study examined how soil fertility and pH alters the effect of inoculum success (i.e., percent infection) as well as plant growth. The hypothesis was that soil fertility and pH would affect the natural inoculation of American Ginseng as well as percent inoculation when commercial mycorrhizae is added. This in turn would affect above and below ground growth parameters. The prediction was that soils higher in fertility and pH would result in less infection. Conversely, ginseng grown in soils with lower fertility and pH would benefit the most from commercial mycorrhizae additions. Research on commercial inoculum in different field soils will help inform both cultivation practices and reintroduction of American Ginseng

Methods

Greenhouse Study

The design for this experiment was a factorial model with three independent factors and all possible interactions: soil fertility (high or low), commercial inoculation (addition or no addition) and liming (addition or no addition). Autoclaved treatments were used as a control. The dependent factors included average percent infection, biomass (below and above-ground), stem length, root length, and leaflet length and width.

This study was completed in the greenhouse at James Madison University. Two soil types (high and low fertility) were collected from the two field sites where soil was collected for the field experimental study in the Monongahela National Forest (N39.29649^{o} , W79.63939^{o}) (Figure. 5). Soils differed in texture as well as nutrients such as phosphorous, calcium and potassium (Table 1, Table 2). Dolomitic pelletized lime was added to each of the soils to increase calcium levels and to raise the pH to 6.4. The initial pH of the High Fertility soil was 3.51 and the initial pH of the Low fertility soil was 3.45. The pelletized lime also included magnesium. Pellets were added to homogenized soil, water was the added to allow the lime to make its way into the soil. After 24 hours, soil was mixed thoroughly to ensure lime was spread throughout. The

amount of lime added was based on calcium and pH levels in soils.

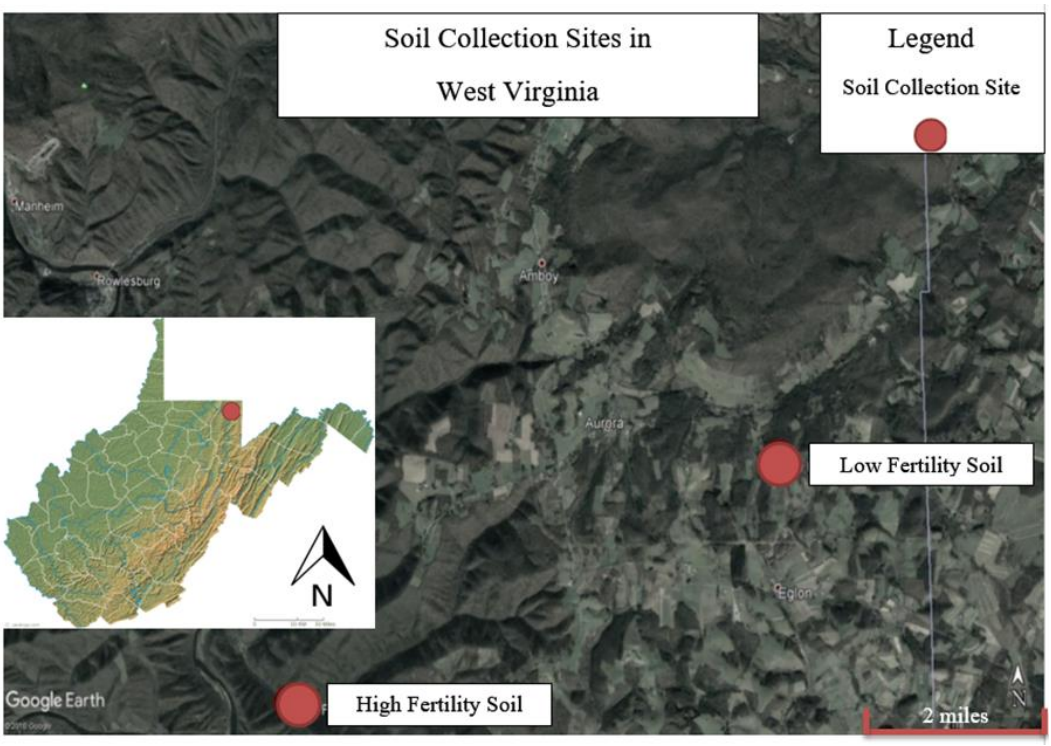

Figure 5. These are the locations of the sites from which soil was collected in Rowlesburg, West Virginia. Soil was collected from site 1 (39.283702, -79.622852) and is known to be a higher fertility soil (Macove). Soil was collected approximately 8.5 miles from site 1 at site 2 (39.319244, -79.518418) and is known to be a lower fertility soil (Dekalb). The collection sites are both found within the Appalachian Mountains in Preston County, West Virginia.

Table 1. Results from 2013 soil analysis from collection sites in West Virginia. Soils collected include a low fertility soil (Dekalb Soil Series) and a high fertility soil (Macove Soil Series). The University of Georgia completed soil analysis.

	Low Fertility Soil	High Fertility Soil
C%	4.36	2.91
N%	0.27	0.26
Sand%	67.9	49.8
Silt%	18	32.1
Clay%	14.1	18.1
Soil Type	Sandy Loam	Loam
Al (ppm)	278.9	415.1

Table 2. Results from 2013 soil analysis from the study sites in West Virginia. The University of Georgia completed soil analysis. There are noticeable differences between phosphorous, potassium, and calcium content. These nutrients determined the low/high fertility differentiation. In addition, Lime buffer capacity is provided (LBC) which indicates the amount of soil acidity that must be neutralized to raise soil pH.

Location	Low Fertility Soil	High Fertility Soil
LBC 1 (ppm CaCO3/pH)	864	920
pH CaCl2	3.45	3.51
Equiv. H2O pH	4.05	4.11
Base Saturation	5.24	9.28
CEC (meq/100g)	15.6	17
Ca (mg/kg ppm)	**98.2**	**199.2**
Cd (mg/kg ppm)	<0.01	<0.01
Cr (mg/kg ppm)	<0.03	<0.03
Cu (mg/kg ppm)	0.43	0.51
Fe (mg/kg ppm)	62.04	74.76
K (mg/kg ppm)	**41.43**	**87.97**
Mg (mg/kg ppm)	20.48	35.57
Mn (mg/kg ppm)	4.62	19.01
Mo (mg/kg ppm)	<0.03	<0.03
Na (mg/kg ppm)	11.32	13.59
Ni (mg/kg ppm)	0.13	0.2
P (mg/kg ppm)	**5.96**	**10.68**
Pb (mg/kg ppm)	3.05	2.6
Zn (mg/kg ppm)	4.11	3.11

Stratified American Ginseng seeds were purchased from Harding's Wild Mountain Herbs in Maryland. It was important to ensure the seeds were stratified (which mimics natural processes that soften the seed coat to allow for germination) so that the seeds would germinate. Commercial mycorrhizal inoculum was ordered from BioOrganics (https://bio-organics.com/), a company that sells the inoculant in a micronized form. It contains a blend of nine endomycorrhizal spores including: *Glomus aggregatum, G. etunicatum, G. clarum, G. deserticola, G. intraradices, G. monosporus, G. mosseae, Gigaspora margarita, and Paraglomus brasilianum.* In addition to spores,

proprietary nutrients were added to the mixture to allow for the survival of the spores and a two year shelf-life.

The experiment was set up in the greenhouse under shade cloth using cone-tainers and a regulated irrigation system. Three seeds were planted in cone-tainers in the treatments described in Figure 6. If multiple plants germinated in one container, seedlings were removed to prevent competition. There was a total of fifteen replicates for each treatment for both low and high fertility soils. Containers were rotated on a weekly basis. Soil was autoclaved on a 15-minute cycle for the control.

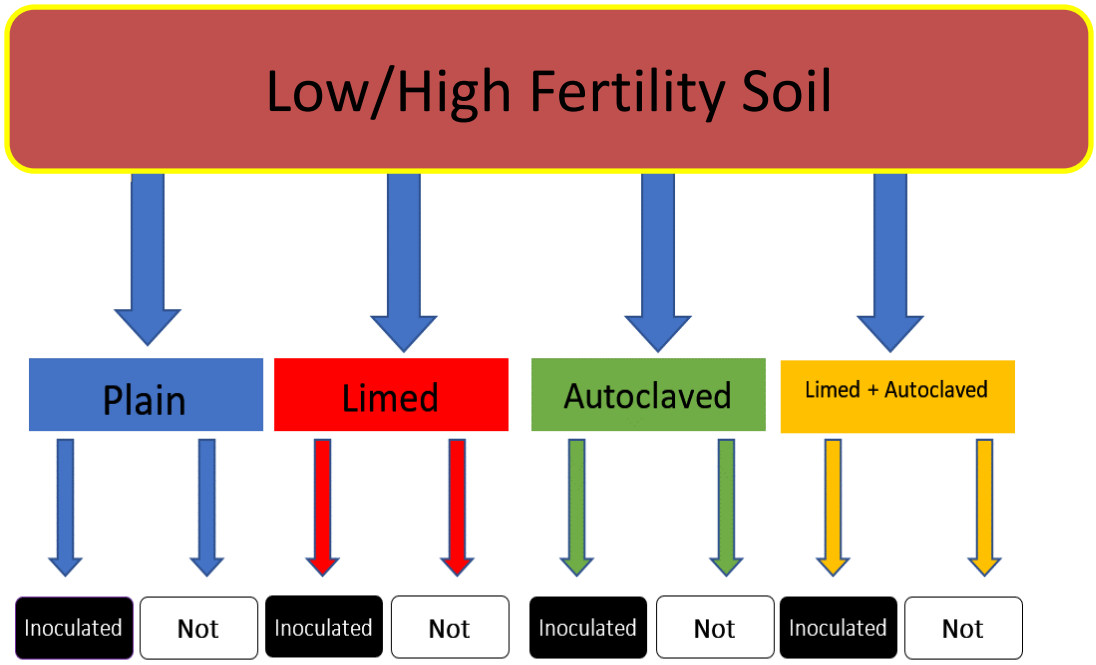

Figure 6. Treatments used in the greenhouse experiment with fifteen replicates planted for each treatment. Seedlings were planted in cone-tainers, placed under shade cloths with a regulated watering system and allowed to grow within these treatments for 7 months. Cone-tainers were rotated weekly to avoid edge effects. Autoclaved soil versions of each of these treatments were also set up as a control.

Seven months after planting, cone-tainers were dismantled, plants were harvested, and data was collected from germinated seedlings. Immediately following harvest, stem

length (mm), average leaflet length and width (mm) and root length (mm) was measured using a ruler. Above and below ground material were then stored in glass vials. Afterward, above ground biomass (mg) was determined when plant stems, and leaflets were placed in a drying oven at 100 degrees Celsius for 24 hours and then weighed on a calibrated scale. Roots were stored in a 1:1:1 Glycerol, Lactic Acid, DI water solution to preserve them before visualization. Lateral roots of the plant were cut into 1cm pieces and prepared for visualization by being placed into a 10%KOH solution for 24 hours for clearing. Roots were rinsed three times with DI water and then placed into a 1% HCL solution for 5 minutes to neutralize them. After, roots were placed into a Trypan Blue stain (.05%) prepared using a 1:1:1 solution of Glycerol, Lactic Acid, DI water, and 0.5 grams of trypan blue for 24 hours. Roots were de-stained for 24 hours in DI water (Bevege, 1968; Phillips & Hayman 1970, Kormanik & McGrawn 1982, Brundrett et al. 1984 Vierheilig et al, 1998, Koke and Gemma, 1989 as cited by Gaston et al, 2016) (Gastol, Domagala-Swiatkietewicz, & Bijak, 2015). Roots were visualized using a Zeiss Axio compound microscope at 40x. Each plant had 1-20 lateral root segments visualized, with each segment being passed through five times. At each pass through, mycorrhizal structures were described as: no structures present, hyphae present, vesicles present, and arbuscules present. Percent infection was determined by calculating the number of pass throughs that showed inoculation divided by the total number of pass throughs multiplied by 100**.**

Field Study

The greenhouse study was modeled after existing field beds growing in Rowlesburg, West Virginia that were planted in 2013 by the Griscom Lab (James Madison University). They quantified how slope and soil type affect the growth of American Ginseng plants. A total of six field beds were planted, three on the northern facing slope (top, middle, and bottom); and three on the southern facing slope (top, middle, and bottom). The same low and high fertility soils mentioned above were used in these beds. Additionally, the high fertility soil was limed as another treatment. This resulted in a total of three soil treatments. Six subplots were planted in each field bed duplicating each soil treatment in a randomized fashion. For the purposes of this study, data was collected from these field beds to gain an understanding of how the soils affects infection of ginseng plants, as well as the number of spores within the soil.

In September 2017, root samples were collected from one plant in each bed for visualization of mycorrhizal association, the number of plants in each plot varied based on survival rate. The largest plant based on height and leaf area was chosen for partial root collection. The same method as mentioned above was used to stain and visualize associations. The first 10 cm of soil was collected in triplicate using a one-inch soils auger and placed into pre-labeled bags for spore count. Soil was homogenized before counts took place. Spore count was determined using the sieving and decanting technique (Gerdmann and Nicholson, 1963). Ten grams of homogenized soil was mixed with 200 mL of DI water and disturbed using a Hamilton blender for one minute. Blending allowed for spores to break away from soil particles. After, the mixture was placed in a beaker for 24 hours to allow for soil particles to settle out into the bottom and spore to

raise to the top. The supernatant was then poured through a series of sieves ranging from 500 um to 20 um. Sieves were then viewed under a dissecting microscope, and spores were counted.

Analysis

Data was analyzed using six separate multi-way ANOVAs. Appropriate post hoc tests were completed to determine significant differences between treatments. All statistics were completed in R (R Core Team, 2016). Autoclaved treatments were removed from the analysis due to poor survival after the soil was altered.

Results

Multiple plant growth parameters and infection percentages were measured to determine how the three main effects, liming, soil fertility, and inoculum, affected the growth of seven-month-old American Ginseng seedlings. Two and three-way interactions between the main effects were also examined and analyzed. All main effects influenced at least one parameter.

Liming the soil, which increased pH, Ca^{2+} and Mg^{2+}, had the greatest effect on the most growth parameters. Liming (+) had a significant, positive effect on average root length, stem length, average leaflet width, average leaflet length, and below ground biomass ($p<0.001$) (Table 3). Root length was most affected by this treatment, with a median of 119.5 mm in limed soils and 60 mm in soils that had not been limed (Figure 7a) ($F = 60.91$; $p =<0.001$). Average leaflet length was the second most affected by liming of the soil with a median of 36.33 mm in limed soils and 28.33 mm in soils with

no lime (Figure 7d) ($F = 48.49$; $p = <0.001$). Stem length also increased with the addition of lime to the soil, limed soils resulted in a stem length median of 59 mm, while the stem length median in soils with no lime was 44 mm (Figure 7b) ($F = 14.85$; $p = 0.002$). The fourth most affected parameter was leaflet width, in limed soils the median was 14.75 mm while in soils without lime it was 12.25 mm (Figure 7c) ($F = 6.70$; $p = 0.002$). Finally, below ground biomass was also positively affected by lime amendments within the soil. The median below ground biomass weight was 44.45 mg in limed soil and 22.60 mg in un-limed soil (Figure 7e) ($F = 5.94$; $p = 0.020$).

Soil fertility affected two growth parameters and had no effect on percent inoculation. High soil fertility had a significant, positive effect on root length ($F = 8.93$; $p=0.004$) (Figure 8a; Table 3), and a negative effect on below ground biomass ($F = 14.29$; $p<0.001$) (Figure 8b ; Table 3). The median root length in high fertility soils was 97 mm compared to 70 mm in low fertility soils. Interestingly, the median below ground biomass was only 26.75 mg in high fertility soils, but 52.70 mg in low fertility soils.

Inoculation with commercial AMF also affected two parameters, including average leaflet width ($F = 5.07$; $p < 0.05$) (Figure 9a) and percent infection of the lateral root ($F = 9.61$; $p < 0.01$) (Table 3). It had the greatest effect on percent infection with an increase from a median of 12% infection in not inoculated soils, to 16% infection in uninoculated soils (Figure 9b). Additionally, 96% of plants in inoculated soils showed presence of AMF while only 84% of plants in uninoculated soils showed infection with AMF. In addition to these three main effects, interactions between the three factors occurred and affected growth parameters.

The two-way interactions that had the greatest effect on growth parameters was between liming and soil fertility. This interaction affected three parameters including stem length (F-value=4.15; $p<.05$), leaflet width (F-value= 4.85; $p<.05$), and leaflet length (F-value= 10.14; $p=<.001$) (Figure 10; Table 4.) The effect of liming changed depending on soil type. Stem length had a median increase of 12 mm in low fertility soil amended with lime, and a 6.2 mm increase was seen in high fertility soil amended with lime (Figure 10a). Leaf width in low fertility soil amended with lime increased by 3.24 mm while leaf width in high fertility soil amended with lime only increased by 1.75 mm (Figure 10b). Likewise, leaf length in low fertility soil amended with lime increased by 18.33 mm while high fertility soil with the same treatment did not show an increase (Figure 10c). Liming effectively closed the gap in seedling performance between low and high fertility soils. In some cases, such as with average leaflet length, liming the soil resulted in seedlings grown in low fertility soil to surpass seedlings grown in high fertility soil (Figure 10).

There was a significant two-way interaction between inoculum and soil fertility treatments for average leaflet length (F= 11.76; $p<0.01$) (Figure 11; Table 4). Inoculation was more effective in low fertility soil with an increase in leaf length of 7 mm opposed to no increase in high fertility soil. As with liming the soil, adding inoculum closed the gap in seedling performance within low and high fertility soils. There was no significant two-way interaction with liming and inoculum.

Although interactions were not significant for all growth parameters and infection percent, it is still informative to look at the interaction plots and identify how the parameters are affected regardless of significance. For the interaction between soil type

and liming, although only stem length and average leaflet width and length were significant, there was a positive increase for all growth parameter and infection percent in both soil types when lime is added to the soil (Figure 12). A similar trend was found with the interaction between inoculation and liming. Although there was no significance for this interaction, there was an increase for all growth parameters and infection percent when lime in both inoculated and not inoculated soil (Figure 13). For most parameters the increase was steeper in uninoculated soils.

From the interaction plots between soil type and inoculum, although only significant for average leaflet length, inoculation had varying effects on growth parameters based on soil fertility. For all parameters, except for percent infection and below ground biomass, there was an increase in both soil types when treated with inoculum. Infection does not show that great of increase in low fertility soils, and below ground biomass does not show an increase in high fertility soils (Figure 14).

There was a three-way interaction between soil fertility, liming, and inoculum. The three-way interaction between liming, soil, and inoculum was significant for average leaflet length (F-value= 5.25; $p<0.05$) and below ground biomass (F-value= 4.22, $p<0.05$) and the ratio between below and above ground biomass (F-value= 5.65; $p<0.05$) (Figure 15; Table 4). Low fertility soil alone had a median average leaflet length of 12 mm, when amended with inoculum, a median length of 31.33 mm, when amended with lime a median length of 30.33, and when amended with both inoculum and lime, an average length of 38.166. High fertility soil alone had a median average leaflet length of 30 mm, with inoculation 29.83 mm, with lime 34.4, and with both liming and inoculum 34.16.

Liming and inoculation treatments in low fertility soil allowed for a significantly larger average leaf length compared with high fertility soil treatments (Figure 15a).

A similar trend was seen for below ground biomass. In low fertility soil, the median weight of below ground biomass was 2.6 mg, when inoculum was added the median was 69.6 mg, when lime was added 39.5 mg, and when both lime and inoculum were added, the median below ground biomass weight was 63.8 mg. In high fertility soil, the median weight of below ground biomass was 25.5 mg, when inoculum was added it was 14.7 mg, when lime was added it was 43.4 mg, and when both lime and inoculum were added the median below ground biomass was 37.1 mg. Soil amendments allowed for low fertility soils to compete with high fertility soils (Figure 15b).

The interaction was also significant for the ratio of below to above ground biomass. In the low fertility soil the ratio was 0.16, when the soil was inoculated it was 1.04, when it was limed it was 1.94, and when both amendments were added it was 1.27. An opposite trend was seen with the high fertility soil where the ratio was 1.70 in untreated soil, .49 in inoculated soil, 1.37 in limed soil, and 1.08 in limed and inoculated soil.

Table 3. Pairwise comparisons of main effect treatments and their significant effects (p-value<.05) on growth parameters and infection percentages of American Ginseng determined using multi-way ANOVA and Post-hoc Tukey HSD tests. There is one degree of freedom for each comparison.

Independent Variables	Response Variables	Difference	p-value	F-Statistic
Limed (+/-)				
	Root Length (mm)	55.84	<.001	60.91
	Stem Length (mm)	11.26	0.002	14.85
	Average Leaflet Width (mm)	2.28	0.002	6.70
	Average Leaflet Length (mm)	8.98	<.001	48.49
	Infection (%)	3.60	0.411	0.68
	Below Ground Biomass (mg)	14.38	0.020	5.94
	Above Ground Biomass (mg)	-	0.104	2.72
	Ratio of Below to Above Ground Biomass	-0.30	.156	0.36
High Fertility vs. Low Fertility				
	Root Length (mm)	21.06	0.004	8.93
	Stem Length (mm)	-4.97	0.090	2.88
	Average Leaflet Width (mm)	.146	0.604	0.28
	Average Leaflet Length (mm)	1.21	0.350	0.88
	Infection (%)	6.57	0.167	2.4
	Below Ground Biomass (mg)	-22.62	<.001	14.29
	Above Ground Biomass (mg)	-	0.128	2.39
	Ratio of Below to Above Ground Biomass	-0.30	.286	1.16
Inoculated (+/-)				
	Root Length (mm)	10.77	0.131	2.35
	Stem Length (mm)	0.88	0.760	0.10
	Average Leaflet Width (mm)	1.95	0.027	5.07
	Average Leaflet Length (mm)	2.03	0.110	2.60
	Infection (%)	15.69	0.003	9.61
	Below Ground Biomass (mg)	7.92	0.170	1.90
	Above Ground Biomass (mg)	-	0.104	2.18
	Ratio of Below to Above Ground Biomass	-0.40	0.085	2.88

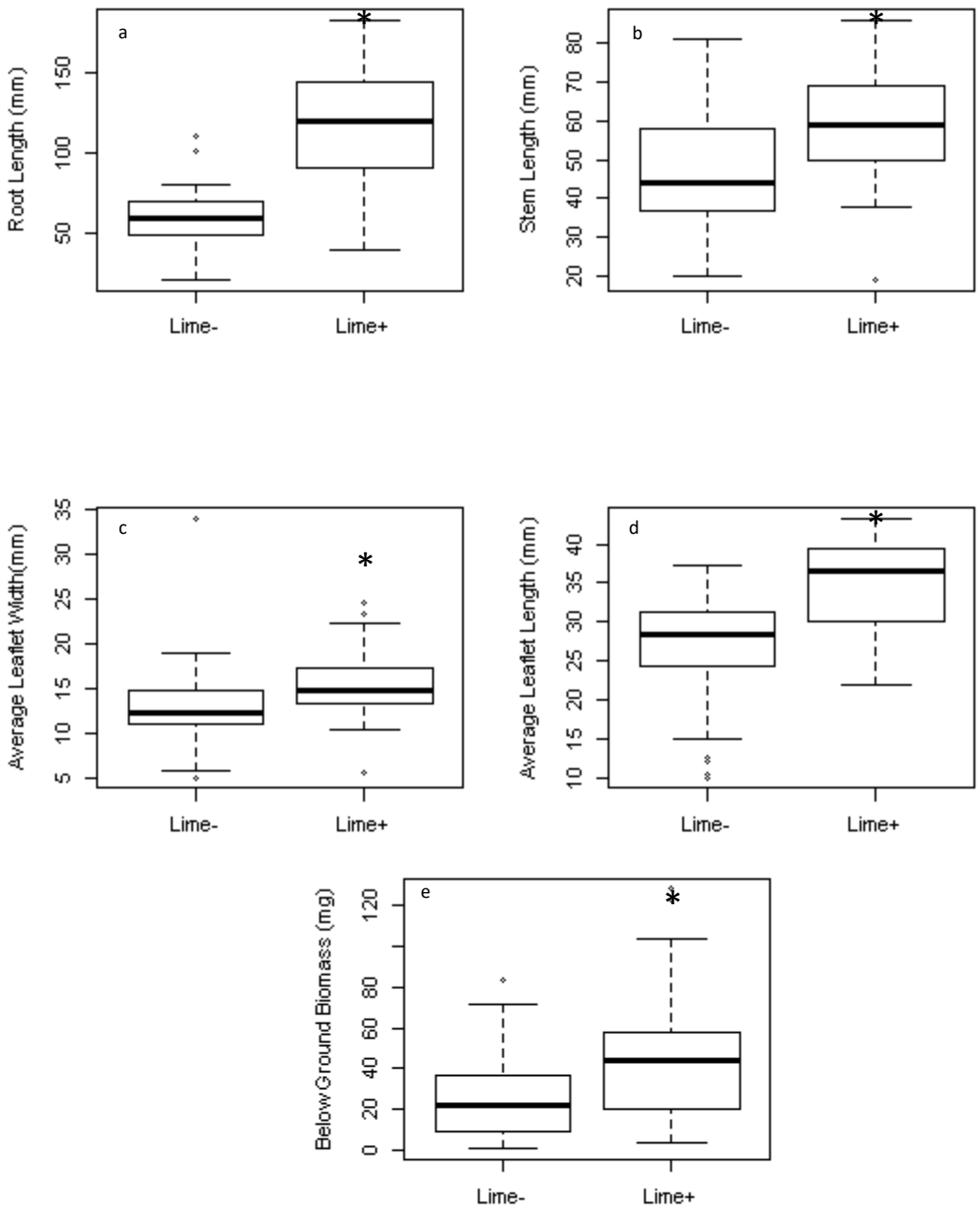

Figure 7. Box-plots a-e illustrate differences between not limed and limed soil treatments for six dependent variables in American Ginseng seedlings grown for 7 months in the greenhouse. Significance ($p<0.05$) is indicated with an asterisk on the treatment with the significantly higher value. The solid bolded line in the boxplot represents the mean measurement for each treatment.

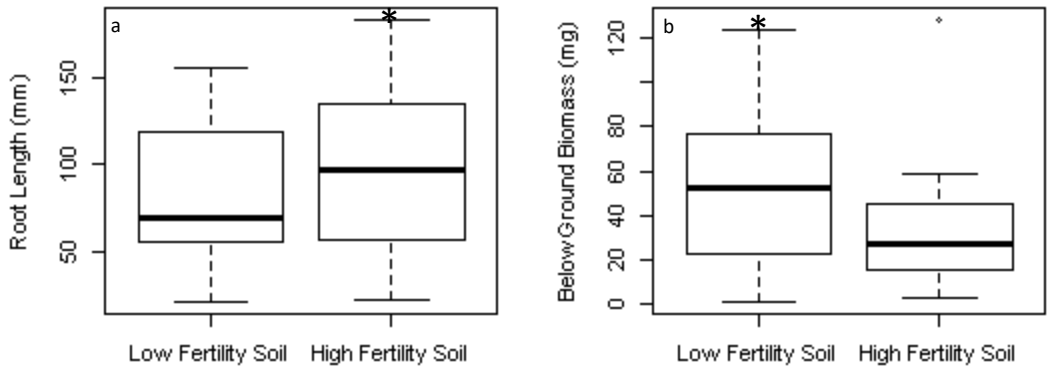

Figure 8. Box-plots a and b illustrate significant differences in American Ginseng seedlings grown for 7 months in the greenhouse in low and high fertility soil treatments, determined by soil nutrient content Soil was collected from two field sites in West Virginia. Significance ($p<.05$) is indicated with an asterisk on the treatment with the significantly higher value. The solid bolded line in the boxplot represents the mean measurement for that treatment.

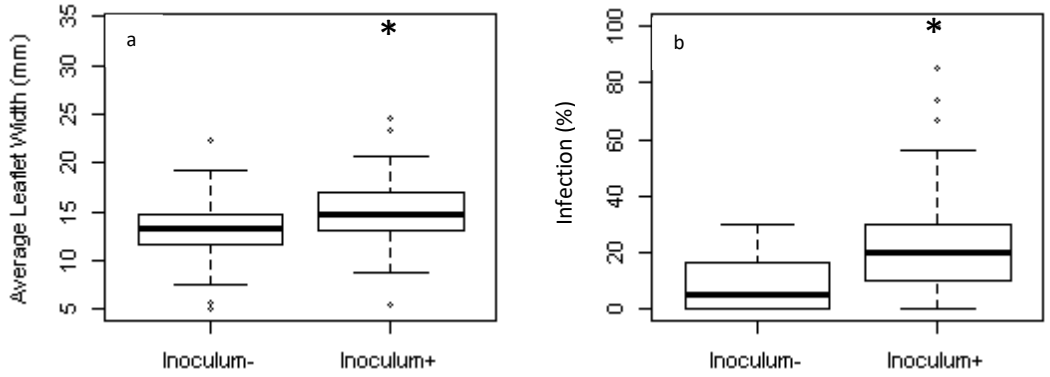

Figure 9. Box-plots a and b illustrate significant differences between inoculated (+/-) treatments on average leaflet width and inoculation percent of American Ginseng seedlings grown for 7 months in the greenhouse. Significance ($p<.05$) is indicated with an asterisk on the treatment with the significantly higher value. The solid bolded line in the boxplot represents the mean measurement for that treatment.

Table 4. Significant interactions between the three treatments of liming (+/-), soil type (high fertility/low fertility), and Inoculum (+/-) (p-value<.05) on growth parameters and infection percentages of American Ginseng greenhouse grown seedlings determined using multi-way ANOVA. The degrees of freedom for each of these comparisons is one.

Independent Variables	Response Variables	p-value	F-Statistic
Limed (+/-) x Soil Type(H/L)	Stem Length (mm)	0.045	4.15
	Average Leaflet Width (mm)	0.030	4.85
	Average Leaflet Length (mm)	0.002	10.14
Soil Type(H/L) x Inoculum (+/-)	Average Leaflet Length (mm)	<.001	11.76
Limed (+/-) x Soil Type(H/L) x Inoculum (+/-)	Average Leaflet Length (mm)	0.025	5.25
	Below Ground Biomass (mg)	0.040	4.22
	Below Ground Biomass: Above Ground Biomass	0.020	5.65

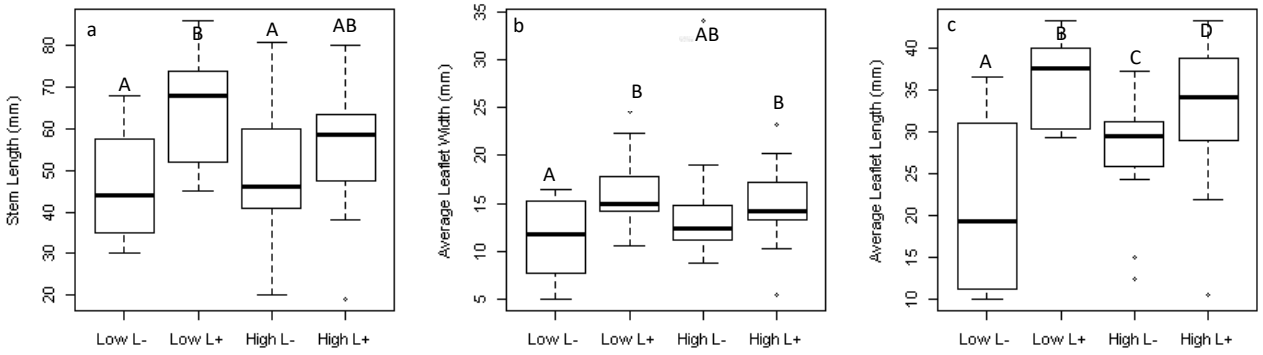

Figure 10. Box-plots a-c illustrate significant interactions between lime (+/-) and soil type (low fertility and high fertility) on stem length (a), average leaflet width (b), and average leaflet length (c). Significance (p<.05) is indicated by lower-case letters. Boxes with the same letters are not significantly different from one another. L- indicates no liming and L+ indicates liming.

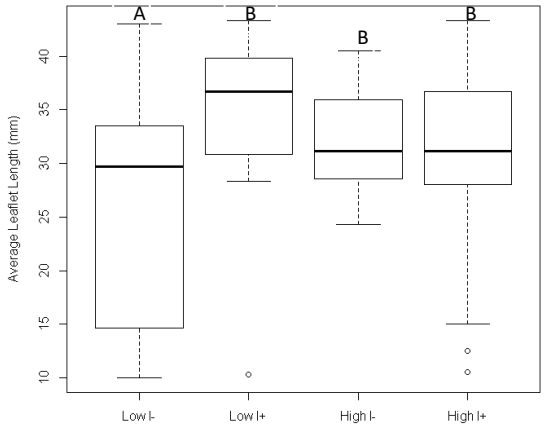

Figure 11. This figure shows the interaction between soil type (low fertility and high fertility) and inoculum (+/-). Significance (p-value<.05) is indicated by letters a and b. Treatments with the same letter are not significantly different from one another. I- indicates no inoculum and I+ indicates inoculum.

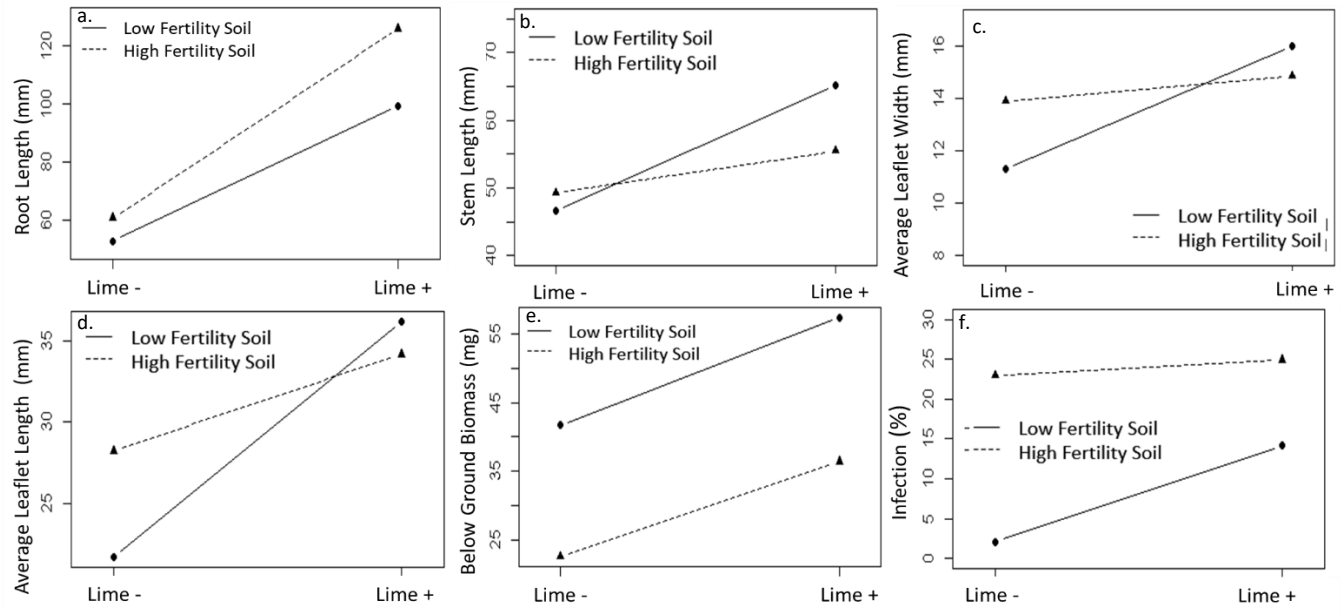

Figure 12. a-f are interaction plots between soil type (low fertility soil and high fertility soil) and liming (+/-) and their effect on five growth parameters and percent inoculated. Solid symbols represent the means of each combination. Solid circles represent the means in ow fertility soil and solid triangles represent the means for high fertility soils. Solid lines represent low fertility soil and dotted lines represent high fertility soil.

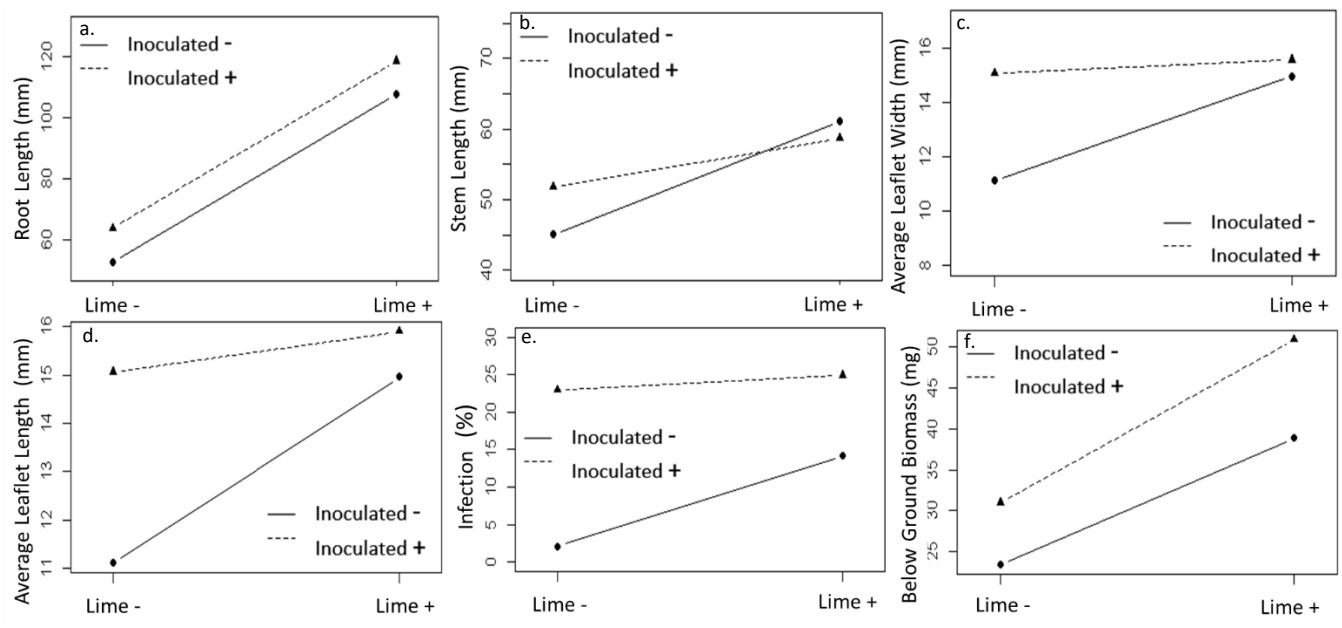

Figure 13. a-f are interaction plots between inoculation (+/-) and liming (+/-) and their effect on five growth parameters and percent inoculation of American Ginseng seedlings. Solid symbols represent the means of each combination. Solid circles represent the means in inoculated + soil and solid triangles represent the means for inoculated-. Solid lines represent inoculum- soil and dotted lines represent inoculum- soil.

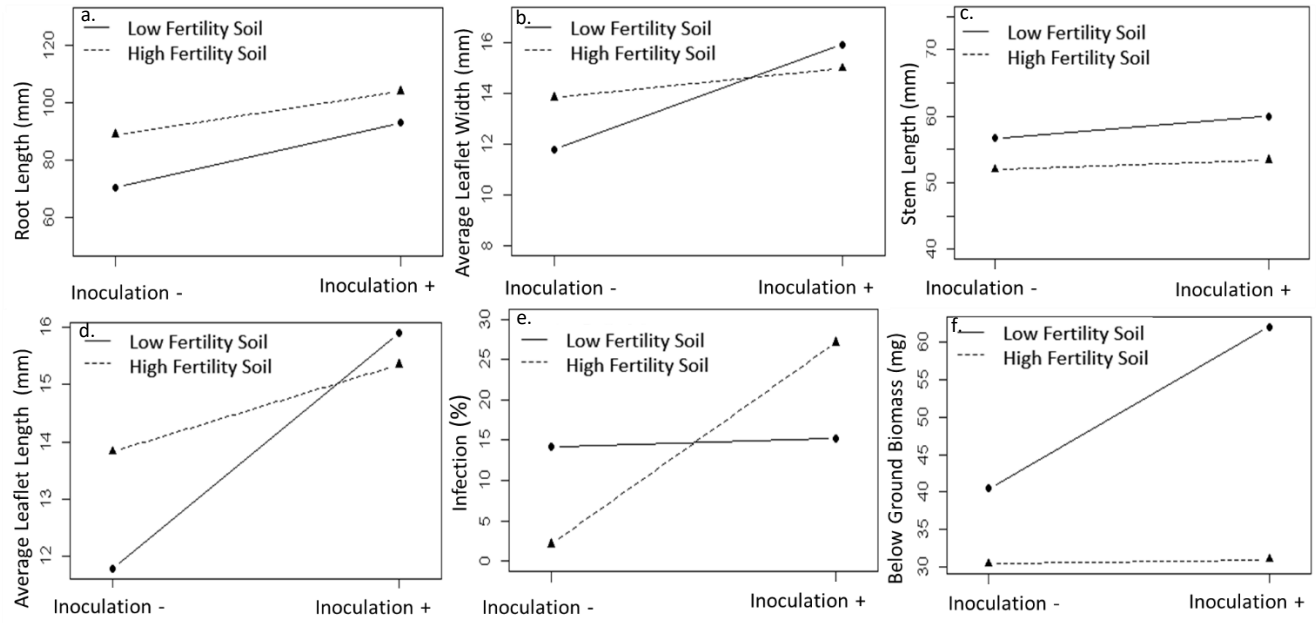

Figure 14. a-f are interaction plots between inoculation (+/-) and soil type (low fertility soil and high fertility soil) and their effect on a growth parameters and inoculation of American Ginseng. Solid circles represent the means in low fertility soil and solid

triangles represent the means for high fertility soil. Solid lines represent low fertility soil and dotted lines represent high fertility soil.

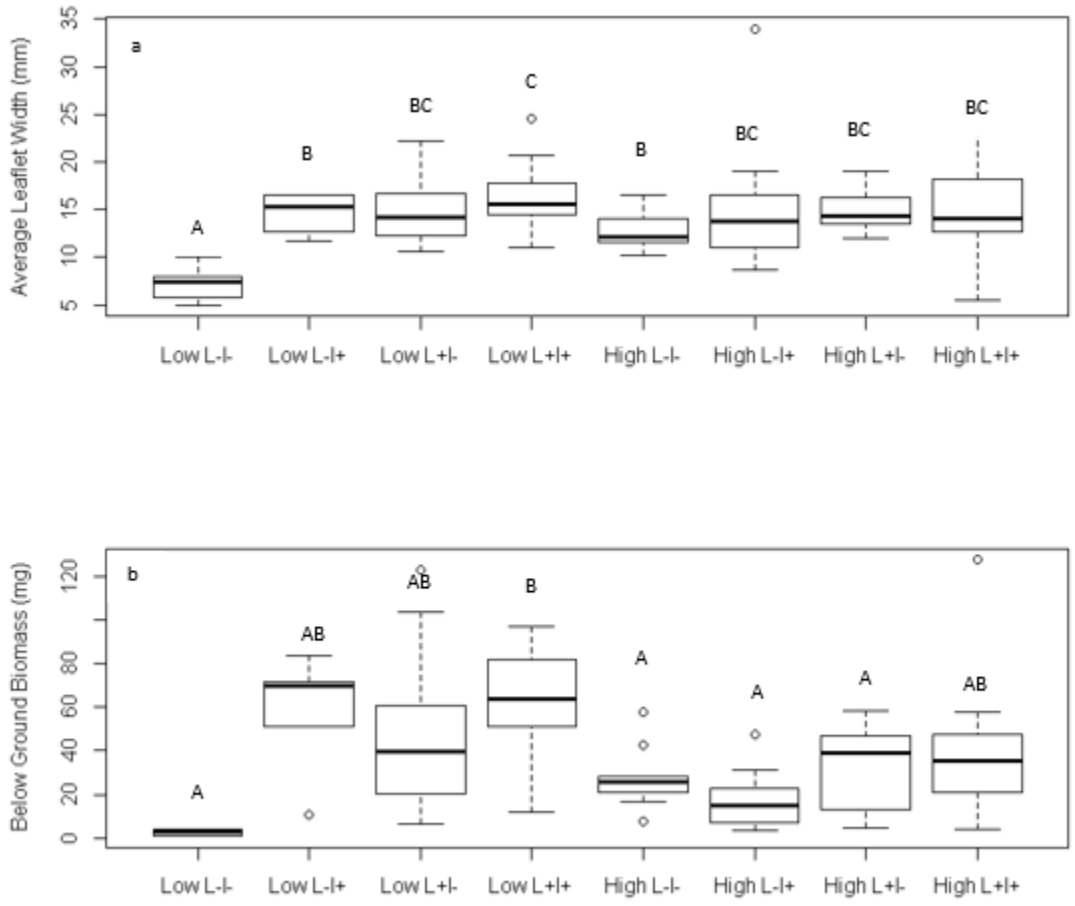

Figure 15. Box-plots a and b show all possible combination for treatments (Liming (+/-), inoculum (+/-) and soil type (low and high fertility) on leaflet length (a) and below ground biomass (b). Significance (p-value<.05) is indicated by a letter. Treatment combinations with the same letters are not significantly different from one another.

Field Component

In field seedbeds, American Ginseng formed similar associations with existing fungi symbionts in high and low fertility soils (Table 6.). This was true even for soils with

where lime had been added. Additionally, there was no difference in spore count between soil treatments (Table 5; Figure 16).

Table 5. These are average spores counts for each soil type within the field study sites in Rowlesburg, West Virginia. A Kruskal Wallis Test was used to compare these data from these sites. There were no differences between the number of spores by soil type (Chi square value=.251, p-value>.05,df=2)

Treatment	Average Spore Count/10 grams of soil
Low Fertility	2.6
High Fertility	2.9
High Fertility Lime+	2.3

Table 6. This table refers to field seedbeds planted in 2013 in Rowlesburg, WV. American Ginseng was grown in three different soil treatments. These soils were collected from nearby locations within the Monongahela region. In 2017 two cm root segments were collected from plants growing within the field beds. All observation showed that American Ginseng roots growing in field beds formed an association with AMF. All fungal structures were observed within the roots.

Treatment	AMF	Hyphae	Arbuscules	Vesicles
Low Fertility	+	+	+	+
High Fertility	+	+	+	+
High Fertility Lime+	+	+	+	+

Discussion

Overharvesting, harvesting regulations, and high demand for the root has led to the cultivation of American Ginseng. American Ginseng is known to form associations with arbuscular mycorrhizal fungi. AMF inoculation has been shown to increase nutrient uptake and growth in Asian Ginseng (*P. Ginseng*) (Cho et al., 2009). AMF are available in a micronized form and sold commercially. Commercial inoculum has been shown to

increase plant health parameters in other agricultural crops, such as maize and soy (Herrara-Peraza et al., 2011). However, soil characteristics may determine the effectiveness of commercial inoculum. Arbuscular mycorrhizal fungi commercial inoculants have been unsuccessful in high nutrient soils (Herrara-Peraza et al., 2011). Plants living in soils with high nutrients may not need to form associations because they do not require assistance in up taking, necessary nutrients from the environment.

This study addressed the hypothesis that American Ginseng growing in different soil types (fertility and pH) would be infected with different quantities of mycorrhizae and that both soil characteristics (fertility and pH) and commercial mycorrhizae would affect height, leaf width, root length, and biomass.

Seedlings in the higher fertility loam soils were expected to have greater growth and lower rates of infection percent along the lateral root. Only root length and below-ground biomass differed between soil types. Plants grown in the low fertility sandy loam soil (50% less potassium and phosphorous) had greater below-ground biomass (but shorter root lengths) compared with high fertility loam soils. This finding conflicts with other studies that have found that soil low in potassium and phosphorous can result in poorly developed root systems and reduced root length (Crouse, 2018). This is explained by soil treatments (liming and inoculum) affecting performance differently in high and low fertility soils. The interaction between soil amendments and soil fertility resulted in greater below ground biomass in low fertility soils.

Soil pH (manipulated by liming the soil) was predicted to have the greatest effect on growth parameters, especially within low fertility soils. The rationale for this treatment was to assess the ability of ginseng to grow in more acidic soils if lime is

added. Liming increased root length, stem length, leaflet width and length and below ground biomass compared to soils that had not been limed (Figure 6). Liming had the greatest effect on root length with a difference of 55.8 mm. This is likely because American Ginseng plants thrive in environments with calcium. The interaction between soil type and liming was significant for stem length, leaflet width and leaflet length. Liming was more effective in low fertility soil for all three parameters.

Soil pH, as well as levels of magnesium and zinc, has the potential to drive arbuscular mycorrhizal communities (del Alguacil et al., 2016). It has been argued that higher pH has been shown to limit the dependence on AMF by increasing the mobilization of nitrogen (Carrenho et al., 2007). However, it has also been found that amending soil with lime has been shown to increase the colonization of AMF in root systems (Heyburn, 2017). This may occur because liming soils can immobilize phosphorous within the soil as it binds with calcium components of the lime (Heyburn, 2017). The decrease in available nutrients may lead to the association being formed. Within this study, liming did not have a significant effect of percent infection, suggesting that the addition of lime along with commercial inoculum will not prevent mycorrhizal associations from forming with American Ginseng seedlings.

Soil treated with commercial inoculum was also predicted to improve performance of ginseng seedlings. Arbuscular mycorrhizae fungi associations in other plant species have been shown to increase leaf area and stem length through the extension of the root system, allowing access to necessary nutrients (Frosi, 2016). In this experiment, American Ginseng seedlings' average leaflet width was significantly increased when inoculum was added. There were no other significant effects on other

plant growth parameters. Inoculating plants also increased the infection percent (+3.8%) within American Ginseng plants, indicating that American Ginseng incorporated commercial inoculum within its first seven months of growth. The addition of commercial inoculum in low fertility soils was predicted to increase growth of American Ginseng, comparable to growth measurements in high fertility soils. Inoculum alone had no effect on seedling growth in low fertility soils. However, with the addition of liming, growth parameters, such as leaflet length, were comparable (and sometimes greater) than seedlings grown in high fertility soils with similar treatments.

American Ginseng seedlings may not be affected by commercial inoculum in low fertility soils as infection was similar in both treated and untreated soil. However, there was an increase in average leaflet length when commercial inoculum was added. This may be due to nutrients added into the commercial inoculum intended for use by AMF. The nutrients added are considered proprietary information by BioOrganics, although it was stressed that the inoculant itself could would not be an effective fertilizer.

The field study revealed that even without the use of commercial inoculant, American Ginseng plants will form associations with naturally occurring AMF within the soil. It also indicates that regardless of soil fertility and liming, associations formed.

Conclusions

Soil texture, nutrients, and pH are all important to consider in the cultivation of American Ginseng. Overall, results from this study support using lime and commercial inoculum to amend soils for American Ginseng. From the literature, it was unclear how liming the soil would affect mycorrhizal associations with American Ginseng. The results from this

study suggest that liming did not have a significant effect on infection percent. Additionally, commercial inoculum combined with appropriate soil pH amendments show a trend of positively affecting American Ginseng in low fertility soils, although it is important to consider that this trend could be due to added nutrients within the commercial inoculum rather than increased infection. Greater success in cultivating American Ginseng may reduce the number of individuals collected from its natural habitat. There may also be implications for utilizing commercial inoculum for American Ginseng reintroduction programs. A longer-term study further investigating effects of commercial inoculum on below ground biomass in low fertility soils is recommended.

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Appendix

Raw data will be stored and made publicly available through Open ScienceFramework (OSF). This link will lead to the data : https://osf.io/p9j8c/?view_only=8e59cb01174e4f57a84dc9e841ac3807