EFFICACY OF MYKE® ON *IN VITRO* GROWTH OF AMERICAN ELM (*Ulmus americana*) SEEDLINGS

by

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Partial Fulfillment of the Requirements for the
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Lakehead University

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Major Advisor Second Reader

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Abstract

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Keywords: elm, *Glomus intraradices,* MYKE®, *Ulmus americana*, urban forestry, urban soils, vesicular-arbuscular mycorrhizas.

This thesis is an examination of the commercial product MYKE which is a perlite-peat mixture containing 7 spores/g of *Glomus intraradices* N.C Schenk & G.S. Sm., a vesicular-arbuscular mycorrhiza-forming fungus.

Seeds of American elm (*Ulmus americana* L.) were aseptically geminated and forty seedlings planted in Erlenmeyer flasks containing a vermiculite-peat mixture (10:1) and supplemented with different amounts of MYKE. Ten flasks received no MYKE, ten flasks received 1 gram, ten flasks received 5 grams, and ten flasks received 25 grams. All flasks were incubated under fluorescent white lights for 3 months at 25°C. When seedlings were harvested, they were grouped according to treatment and photographed, then bagged, dried and weighed. A representative seedling from each group was stained with Trypan Blue. Results revealed that no spores had germinated as there was a lack of mycelium in the flasks and no colonization of the roots had occurred. It was found that increasing the amount of MYKE per flask caused a reduction in seedling growth. It was hypothesized that several factors may have resulted in lack of spore germination such as low light intensity affecting seedling growth and subsequently affecting quantity and quality of root exudates that would normally stimulate spores to germinate. The results of this experiment do not negate the potential benefits of vesicular-arbuscular mycorrhiza-forming fungi, especially in the urban landscape.

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Introduction

This thesis is an examination of the commercial product MYKE® using American elm (*Ulmus americana* L.) seedlings as the host tree. MYKE® is a soil additive that contains spores of *Glomus intraradices* N.C. Schenk & G.S. Sm., a fungus that creates a symbiotic relationship with the roots of a host tree known as a vesicular-arbuscular mycorrhiza (VAM). The fungus receives simple carbohydrates from the tree and the tree receives an increase of water and nutrients from the soil that the fungus hyphae is able to extract. In the hostile urban environment where there is often little growing room and no other forms of life in the soil this association could make the difference for survival.

The term mycorrhiza is given to permanent associations of roots with hyphal fungi and was coined by Albert Bernhard Frank in 1885, and the term symbiosis was first used by Anton de Bary in 1887 to signify the common life of a parasite and host (Smith and Read 1997). Mycorrhizas are usually defined as mutualistic symbioses between fungi and plants in which both partners can benefit from the association (Smith and Read 1997). An important evolutionary trait was the development of a specialized interface zone for bidirectional nutrient exchange (Brundrett 2002). The term “balanced mycorrhizas” has been proposed to denote situations in which both organisms receive essential materials through reciprocal exchange (Brundrett 2002). Not all plant-fungus relations are mutualistic. The term “exploitive mycorrhizal association” is used when there is an unidirectional flow of nutrients with the main benefactor being the plant (Brundrett 2002). The importance of mycorrhizal associations cannot be overstated as plants with VAM associations dominate nearly all terrestrial habitats (Malloch 1987). The colonization of land, and the evolution of terrestrial plants was possible because of the establishment of early symbiotic associations of certain aquatic fungi with aquatic plants. Terrestrial plants of the

present are the product of this ancient and continuing partnership (Pirozynski and Malloch 1975). The first appearance of fossil fungi in association with algae goes back to the late Precambrian and the association was between *Eomycetopsis* which is the earliest named fungus and the earliest forms of eukaryotic green algae (Schopf 1970). *Eomycetopsis* could be a member of the Oomycota, but it is difficult to fully understand the evolutionary processes of the origin of mycorrhizal associations due to limited fossil evidence (Brundrett 2002). Vesicular-arbuscular mycorrhizas are the oldest type and make up 80% of the associations formed by land plant species. Even today, plant nutrient transportation systems are closely bound to mycorrhizas (Peterson & Massicotte 2004). The interaction between mycorrhiza-forming fungi and roots involves a series of events ultimately leading to an integrated, functioning structure (Peterson & Farquhar 1994). First, hyphae have to be attracted to the root surface and then respond in a recognition event. Following this, a close association (adhesion) occurs and in some instances a specialized structure forms known as an appressorium. Subsequent to this the root must be penetrated by hyphae either intracellularly or intercellularly (sometimes both) and barring a resistance reaction on the part of the plant root (Peterson & Farquhar 1994), the mycorrhizal association will be formed.

Simple carbon compounds suitable as the main substrates for fungal growth are not plentiful in the soil, but areas such as the root surface provide a supply of available carbon compounds which allows for the germination of resting fungal propagules. Fungi are heterotrophic and are unable to utilize energy from the sun, but they have the ability to easily absorb life essential elements such as phosphorus and nitrogen (Pace 2003). Plants are autotropic and are able to produce carbohydrates through the process of photosynthesis, but struggle to obtain sufficient amounts phosphorus and nitrogen. A parasitic fungus may evolve into a

mutualistic relationship when it is energetically less costly to supply the host with nutrients rather than to overcome the hosts defences (Cooke and Whipps 1980). It is a cost-benefit trade off in which the plant is likely to fall prey to the fungus when environmentally stressed tipping the scale occurs from mutualism to parasitism (Egger and Paden 1986). The colonization of fungal hyphae on the plant roots greatly increases the surface area in which water and nutrients can be absorbed maximizing the plant's access to the life essential compounds (Pace 2003).

Mycorrhizal fungi have evolved independently from one another in many cases and because of this, they function in a great variety of ways (Lewis 1973). Each of the seven main types of mycorrhiza has advantages and disadvantages and because of this, not one form will dominate in every situation (Read 1983). The disadvantages of each form of mycorrhiza is related to the cost of photosynthate required from the plant in which fungi that are best suited to stressed environments (*e.g.* ericoid and ectomycorrhizal) produce the largest amount of fungal tissue and therefore require large amounts of photosynthate. The advantages of various mycorrhizas become apparent in sites of varying soil conditions usually related to the availability and chemical nature of phosphorus and nitrogen (Malloch 1987).

The majority of fungal species that are involved in ectomycorrhizal associations belong to families in the Basidiomycota, (*e.g.* gilled mushrooms, boletes, false truffles) with a few belonging to the Ascomycota (*e.g.* true truffles) and one genus in the Zygomycota (*Endogone*) (Smith and Read 1997). There are approximately 5 500 known species of ectomycorrhiza-forming fungi, 80% of which are epigeous, forming reproductive structures above ground (Peterson *et al*. 2004). Although there is considerable variation in morphological and structural characteristics of ectomycorrhizas, three features are typical of this association: the formation of a mantle or sheath of fungal hyphae that covers a considerable portion of the lateral roots, the

development of hyphae between root cells to form a branched structure called the Hartig net, and hyphae that emanate from the mantle and grow into surrounding soil (Peterson *et al.* 2004). Considerable attention has been given to ectomycorrhizas because many tree species in association are also commercially important for lumber and paper products (Peterson *et al.* 2004). Recent fossil evidence shows that the association between conifers and ectomycorrhizas has existed for over 50 million years as dichotomous branching typical of this association in pines is evident in fossil material (LePage *et al.* 1997). Because most ectomycorrhiza-forming fungi can be grown *in vitro*, it is possible to control the colonization process more precisely (Peterson *et al.* 1994).

Orchid mycorrhizas are unique to the family Orchidaceae, one of the largest plant families on earth comprising 10% of all flowering plants (Dressler 1981). Much of the diversity and unique characteristics of the orchid family may be attributed to its distinctive relationship with mycorrhizal fungi (Benzing 1981). Orchid mycorrhizal fungal associations are essential for both seed germination, and seedling establishment of orchid plants in nature (Rasmussen 1995). For orchids that require specific fungi, the availability of the appropriate symbionts may determine which habitats allow orchid growth (McCormick *et al.* 2004). It is often presumed that orchid mycorrhizas are a mutualistic symbiosis but there is no evidence that the fungus benefits from this association (Peterson *et al.* 2004). The suggestion is that orchids have evolved to form a unique relationship that involves parasitism of their associated fungi (Smith and Read 1997). The main defining characteristic of orchid mycorrhizas is the formation of complex hyphal coils, known as pelotons, within the host plant cells (Peterson *et al.* 2004). The initial development stage of all orchids is a non-photosynthetic protocorm that is myco-heterotropic (Rasmussen and Whigham 2002). All orchids are initially myco-heterotrophic but most will develop leaves and

become photosynthetic later in their growth (Leake 1994). Fungi associated with photosynthetic orchids are often easy to culture in contrast to fungi in association with non-photosynthetic orchids, but these fungi often remain asexual and lack many features used to identify and differentiate fungi (Warcup 1981). Sexual stages known as teleomorphs have been cultured after several asexual anamorph stages (Rasmussen 1995). Because of the lack of features in the asexual stages, orchid mycorrhizas are often characterized as belonging to one of several form genera: *Epulorhiza, Ceratorhiza,* and *Moniliopsis* but even classification at this level is questionable (Warcup 1981). Use of the molecular method has enhanced the identification of fungal symbionts in orchid mycorrhizas (McCormick *et al.* 2004).

Ericoid mycorrhizas form a distinctive association with plants in the Ericaceae family (Tian *et al,* 2011). These plants rely on ericoid mycorrhizal fungi to trap the broad range of different forms of nitrogen and phosphorus found in the soil (Mitchell & Gibson 2006). Ericoid mycorrhizal associations are found in mor humus heathlands in the northern hemisphere where the growing season is short and litter decomposition is slow (Mitchell & Gibson 2006). They are typically confined to soils which are either peaty/highly organic or sandy, where nutrients are low in availability (Read 1983). Ericoid mycorrhizal associations are also able to regulate the uptake of harmful metals (Mitchell & Gibson 2006). *Hymenoscyphus ericae* (D.J.Read) Korf & Kernan in particular has a high affinity for a number of metals such as iron, copper, and zinc reducing the passage of these metals to the host plant (Shaw & Read 1989). Ericoid mycorrhizal fungi have also been associated with ectomycorrhizas of conifer and deciduous trees in forests of north temperate and boreal zones (Bergero *et al.* 2000). The fungi forming ericoid mycorrhizal associations are found within the Order Helotiales and Order Onygenales of the Ascomycota. The majority of these fungi can be cultured on artificial media (Mitchell & Gibson 2006).

Microfibrils rich in polysaccharides anchor the fungus to the root which is the first step of mycorrhizal colonization, followed by the formation of hyphal coils which can be found in cortical cells of root hairs (Bonfante-Fasolo & Gianinazzi-Pearson 1982). The external mycelium extends no further than 1cm from the surface of the root hairs (Mitchell & Gibson 2006). The ericoid mycorrhizal association is short lived, lasting no more than 11 weeks (Mitchell & Gibson 2006).

Vesicular-arbuscular mycorrhizas (VAM) contain three important components: the roots themselves, the fungal structures within the cells of the root and the extramatrical mycelium in the soil (Smith & Read 1997). VAM plays an important role in mineral nutrient uptake as well as protecting against pathogenic attack and drought (Field *et al.* 2012). It was the fungi that form VAM that allowed the first terrestrial plants to establish themselves on land. These fungi are considered to be primitive due to their relatively simple spore, their lack of sexual reproduction, and because they associate with a wide diversity of plants (Morton 1990). Vesicular-arbuscular mycorrhizal fungi are highly adapted to mobilize phosphorus towards nutrient depletion zones around roots and also from pores too small for the root hairs to access. They have physiological adaptions that include phosphate transporters that contain membranes with very high affinities for soil phosphorus (Field *et al.* 2012). VAM fungi are found within the Order Glomales of the Zygomycota and they are traditionally classified into six genera: *Acaulospora, Entrophospora, Gigaspora, Glomus, Sclerocystis*, and *Scutellospora* (Yao *et al.* 1996). Hyphal growth can be achieved *in vitro* by use of flavonols and CO_2, but a link between the production of these substances by roots and the establishment of mycorrhizas has not been made (Becard *et al.* 1992). Mycorrhizal associations may be initiated by spore germination or hyphae may originate from fragments of roots, but in many cases there is already a network of hyphae from previous

root activity. If hyphae resulting from spore germination do not find a susceptible root within a week they will die (Brundrett 2002). At the site of penetration, the fungus produces an appressorium which is a pre-penetration swelling to help the fungus attach to the host's roots. In normal VAM associations, penetration hyphae initiate the spread of the fungus into root tissues ultimately leading to the formation of highly branched structures known as arbuscules in cortical cells (Peterson & Farquar 1994). The organization of the hyphal wall changes dramatically from the time the fungus enters the root until the fine branches of the arbuscule form (Bonfante-Fasolo 1992). It is thought that the alteration in fungal wall structure is important in a functional way as this is the main interface for uptake of sugars by the fungus and ions by the plant's cortical cells.

Urban soils are materials that have been manipulated, disturbed or transported by human activities used as a medium for plant growth. It is estimated that 50% of all land has been transformed by humans (Vitoussek *et al.* 1997). Urban ecosystems are recognized as spatially heterogeneous areas of land use and cover under significant human activity (Grimm *et al.* 2000). These areas differ further from undeveloped surroundings in having larger areas of impervious surfaces, increased pollution and heat island effects that alter local climate (Vitoussek *et al.* 1997). Urban development may entail disturbance and/or removal of topsoil, and similar disturbance resulting from agriculture or mining activities has been shown to decrease vesicular-arbuscular mycorrhizal fungal species richness and infectivity (Helagson *et al.* 1998, Stabler *et al.* 2001). After development, the new urban landscape may have increased vegetative land cover (as with turf), potentially favoring arbuscular mycorrhizal fungi, or there could be a decrease of vegetative cover which is the case with largely paved urban surfaces that eliminates microbial communities in the underlying soil. Fungal species composition is closely tied to the vegetation species at each site (Cousins *et al.* 2003). It is common for urban environments to contain a high

proportion of non-native plant and animal species which alters the species composition and richness compared with non-urbanised surroundings (McDonnell and Pickett 1990). Common anthropogenic pollutants found in urban areas, such as nitrogen deposition, toxic metals and ozone, have been shown to decrease arbuscular mycorrhizal colonization and impact community composition (Cairney & Meharg 1999). Soil additives can be used in the urban environment to help form mycorrhizal communities and promote root colonization.

MYKE® is a soil additive containing spores of *Glomus intraradices* that can be used in the urban setting to promote vesicular-arbuscular mycorrhizal associations with tree roots (Premier Tech 2016). Adding MYKE® to urban soils helps to create the fungal communities that may have been destroyed in the development of the urban planting location. Premier Tech is the creator of MYKE® and they state that the association created will provide the tree with easier access to nutrients, which will make the tree stronger and healthier and able to fight off pathogens and diseases (Premier Tech 2016). They also state that vesicular-arbuscular mycorrhizas provide the tree with better tolerance to drought, salinity, soil compaction and other environmental stresses commonly found in the urban setting. Premier Tech (2016) state that roots colonized with VAM are able to spread over their available area more rapidly resulting in faster root development, an increased survival rate, and better maintenance of soil structure. Because mycorrhizas are able to convert phosphorus into a soluble state, the tree is able to directly use more of the phosphorus contained in the soil (Premier Tech 2016).

An experiment was set up to test whether some of these claims about MYKE® are actually true. Elm seedlings were chosen as the host plant because of the high germination rate of seeds without stratification, rapid seedling growth, and because they can be colonized by vesicular-arbuscular mycorrhizal fungi (Harley & Harley 1987). Four treatments were set up:

uninoculated controls, one gram (7 spores) of MYKE® added to flasks, five grams (35 spores) of MYKE® added to flasks, and twenty-five grams (175 spores) of MYKE® added to flasks. The null hypothesis is that there will be no change in growth of the elm seedling under the different treatments.

Methods and Materials

Seeds of American elm (*Ulmus americana*) (lot #9810006.3) were obtained from the National Tree Seed Centre, Fredericton, New Brunswick. On October 19th 2016 the seeds were submerged in water and left in a cool refrigerator for three days. After the three-day soaking period the seeds were placed into a beaker containing 30% hydrogen peroxide in order to surface sterilize the seed coats . The beaker contained a stir bar and was placed on a stirrer for 30 minutes. After the surface sterilization of the seeds, the contents of the beaker were poured through a sterilized crucible containing minute pores so that most of the seeds were separated from the hydrogen peroxide. In a transfer hood sterile distilled water was added to the crucible containing the seeds to wash off the hydrogen peroxide. When the seeds were thoroughly washed, forceps were flamed, allowed to cool and then used to pick seeds aseptically from the crucible to be placed with the pointed end of each seed down into the 1.0% water agar in 400mL beakers which were covered by glass Petri dishes. Seeds were planted in rows of 5 x 5 to achieve a total of 25 seeds per beaker providing a total of 100 seeds planted across the 4 beakers. Tests conducted by the National Tree Seed Centre claimed viability of 99%. The beakers containing the seeds were incubated @25°C under fluorescent white lights (45/umol/m^2/sec) for approximately 12 hrs light/ 12 hr dark per day. Despite best efforts 1 of the 4 beakers became contaminated. Seeds began to germinate within a week and were grown until enough roots were developed in the agar media. (Figure 1)

Figure 1. Seedlings in 1% water agar

Separately, a vermiculite-peat mixture (10:1) was mixed in a large bowl and 120mL of this this mixture was added to forty 250mL Erlenmeyer flasks. To add obtainable nutrients for the fungi and elm seedlings and also to add moisture to the mixture, 70mL of a ¼ strength modified Melin-Norkrans solution (Marx 1969) without glucose was also added to each flask. Cotton plugs wrapped in cheese cloth were inserted into the opening and covered with aluminum foil. The aluminum foil was then numbered to correspond to the number of the flask and then all of the flasks were put into an autoclave to sterilize for 60 minutes at 121°C and 1.7Kg/cm^2 pressure.

Before the seedlings were planted into the 250mL flasks a varying amount of MYKE® was added to the 40 flasks (Figure 2). Ten flasks became the control group receiving no MYKE®,

ten received 1 gram of MYKE®, 10 received 5 grams of MYKE®, and 10 received 25 grams of MYKE®. After the MYKE® was added, utilizing aseptic techniques in the transfer hood, a small pit was dug into the middle of the vermiculite: peat mixture in each flask prior to the addition of a seedling. (Figure 3)

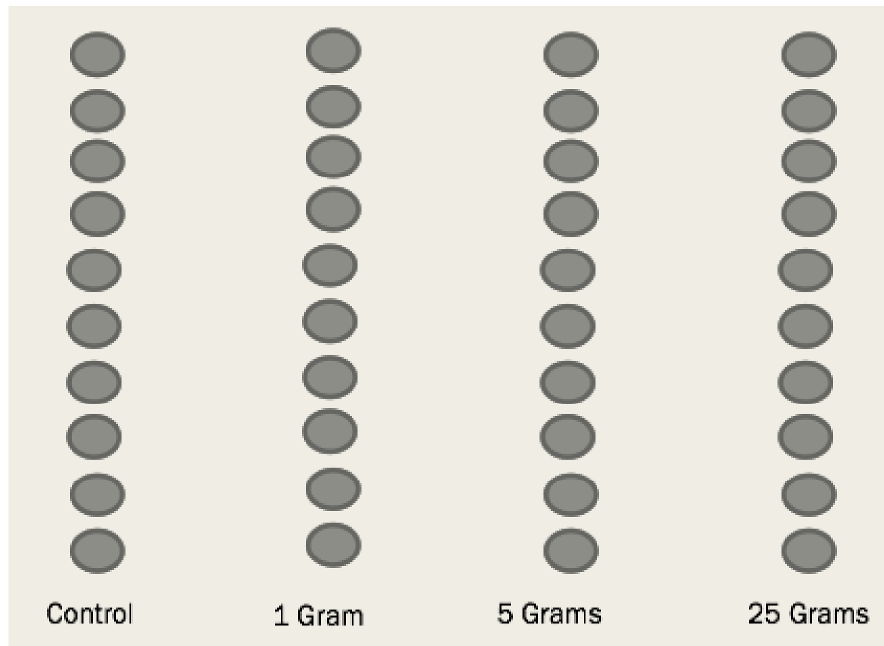


Figure 2. Sample Layout and Concentrations

Figure 3. Replanting Seedlings to Growing Flasks

After each 250mL flask had a seedling planted inside, the cotton plug was replaced and covered by the aluminum foil. The flasks were placed into a growing chamber that has a light set up (45/umol/m^2/sec) and a controlled temperature at 25°C (Figure 4). The flasks were randomly moved around inside the growing chamber on a regular basis to allow for each seedling to receive the same amount of light.

Figure 4. Growing Chamber

Some of the seedlings died while they were growing in their flasks and on December 6th 2016 new seedlings were planted from the seedlings remaining in the beakers containing 1% water agar (flasks 35,37-39). Some of the seedling required the leaf codyledon be removed because of mould threatening to overtake the seedling (flasks 22,31-34,36,40).

On March 2nd 2017 the harvest of the seedlings was conducted. Each seedling was carefully removed and placed in a Petri dish of water. Utilizing a dissecting microscope, the roots were carefully cleaned of any adhering vermiculite and peat. Each set of treatments were photographed (Appendix I) and then each seedling was placed in a numbered paper bag (corresponding to the numbered flask) and dried at 100°C for three days. The dried seedlings were then weighed. In order to determine if the *Glomus intraradices* had colonized the roots, one representative from each of the set of treatments were set aside and the roots were stained with trypan blue utilizing a modified technique outlined by Phillips and Hayman (1970)(Figure 5)(Appendix II). Once stained, each root sample was mounted in lactophenol on a microscope slide and then gently crushed under the cover slip. Cortical root cells were examined microscopically for signs of fungal colonization (i.e. arbuscules).

Figure 5. Staining the roots with Trypan Blue

In a petri dish, water was added to one gram of MYKE®, mixed, and this was then examined using a dissecting microscope in order to determine the number of spores per gram. Pins were used to move the substrate when searching for spores (Figure 6). When spores were located, an eye dropper was used to remove them from the Petri dish to eliminate the chance of double counting the same spore. A total of 10 grams of MYKE® were examined.

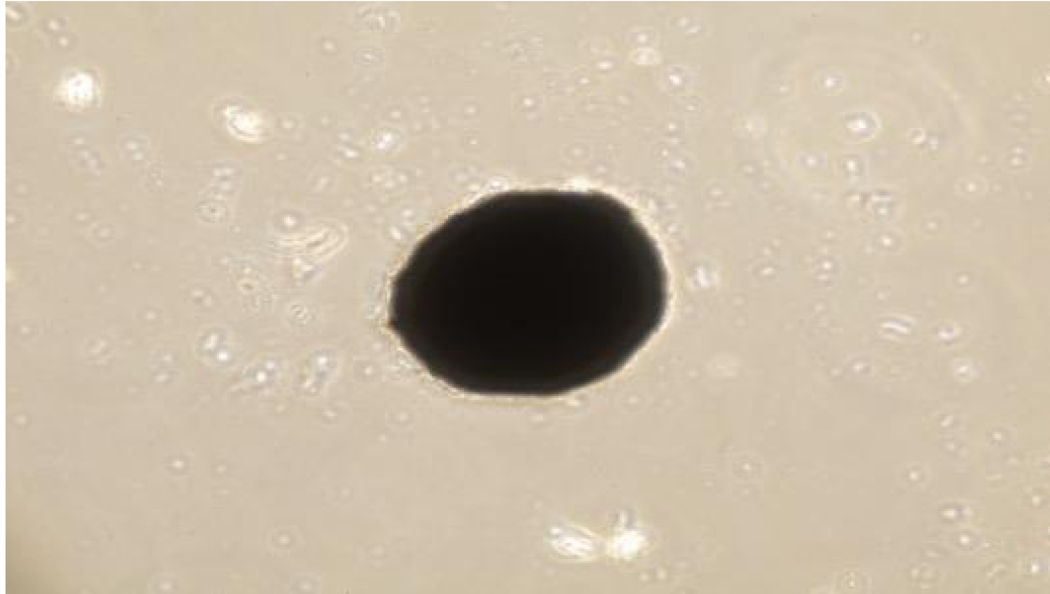


Figure 6. Spore of *Glomus intradacies*

The dried weight data (Appendix III) was then used in a one way ANOVA statistical analysis (Appendix IV) to determine if there was any significant change in growth between different treatments.

Results

The results of the 10 spore counts confirmed the claim of 7 spores per gram of MYKE® (Table 1). The average of the ten trials was found to be 7 with a standard deviation of 0.471.

Table 1. Results from 10 spore count trials

Trial #	# of Spores
1	6
2	7
3	7
4	7
5	7
6	8
7	7
8	7
9	7
10	7

Upon harvest of the seedlings and the washing of the roots, it became apparent that there was a complete lack of extramatrical mycelium emanating from the roots. Staining of the roots systems of selected seedling from each treatment revealed a complete lack of fungal infection (Figure 7). It was clear that there had been a lack of spore germination and subsequent colonization of the elm seedling roots. Despite this result, an ANOVA (Appendix IV) did find differences in the dry weights of the seedlings for the different treatments, but as there was no fungal germination the rejection of the null hypothesis cannot be related to fungal influence.

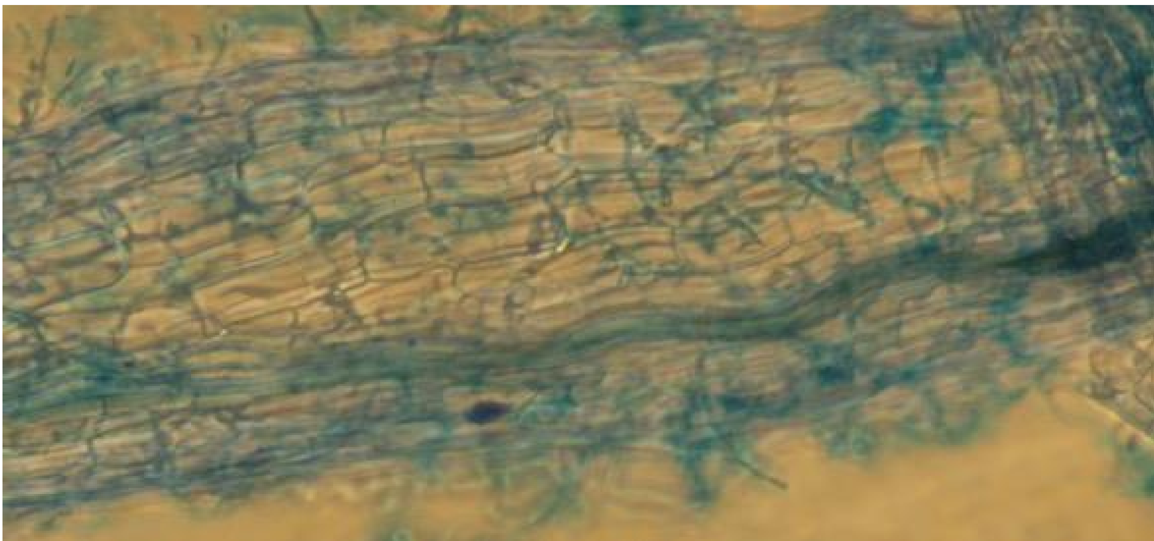
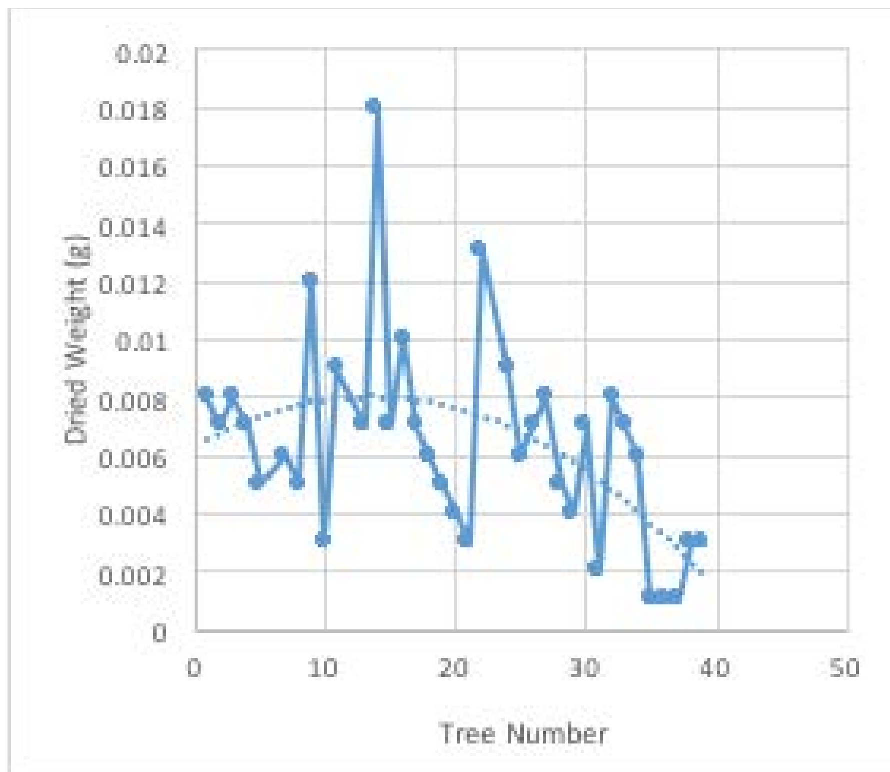


Figure 7. Cortical Root Cells Devoid of Fungal Colonization

Discussion

Although the ANOVA forces the rejection of the null hypothesis there is no definitive reason as to why the dried weights between the test groups would be significantly different. One trend that becomes apparent, as seen in figure 8, which is contrary to what may have been expected if the spores were to germinate is that the highest concentration of MYKE® corresponds to the lowest dried mass. The reason for this could be that the addition of greater amounts of MYKE® dried out the soil. Water was added to the flasks with the higher concentrations in an attempt to compensate for this moisture loss. The nutrients added to the soil could have been diluted by the greater volume of growing medium.



Scatter plot of seedling dried weight (g) related to tree number with

It is impossible to say with absolute certainty what caused the failure of the spores to germinate, but some possible hypothesies as to why this may have occurred will be explored:

Hypothesis A: Nutrient suppression of fungal spore germination

The addition of the dilute Melin-Norkrans solution (Marx 1969) was necessary to add nutrients to an otherwise nutrient free growing medium for uptake by the elm seedlings. Without the addition of nutrients, the seedling would have entered a chlorotic state, withered, and died. There is a balance that must be achieved when supplying nutrients to the growing stock because varying nutrient concentrations can have adverse effects on fungal spore germination. Increased levels of phosphorus applied to soil generally decreases the colonization of plant roots by vesicular- arbuscular mycorrhizal fungi (Abbott and Robson 1984). Nitrogen added to soils is proven to stimulate root colonization (Furlan and Bernier-Cardou 1989). When studying VAM systems it is of paramount importance to consider the ratio of applied nitrogen to phosphorus. A better understanding of optimal nutrient conditions would increase the production of spores in the rhizosphere and ensure a high potential source of VAM inoculum for improved plant growth (Furlan and Bernier-Cardou 1989).

Hypothesis B: Lack of photosynthate root excretions

The elm seedlings were in the early stages of their life and because of this were not releasing root exudates at a quantity high enough to stimulate fungal spore germination. Roots produce a wide variety of compounds, which may serve as stimulants, attractants, inhibitors, nutrients or genetic regulatory signals for vesicular-arbuscular mycorrhizal fungi prior to colonization (Vierheilig *et al.* 1998). It has been shown *in vitro* that volatile root exudates cause the growth of germ tubes of *Gigaspora gigantea* (Nicol. & Gerd.) Gerd.& Trappe towards roots of vesicular-arbuscular mycorrhizal host plants (Gemma and Koske 1988). Root exudates of have

also been shown to stimulate hyphal spreading of arbuscular mycorrhizal fungi (Vierheilig *et al.* 1995).

Hypothesis C: Poor spore quality

MYKE® is not fully aseptic and it is possible that a mycoparasite could have infected the spores; however, no hyphae were observed colonizing the spores of *Glomus intradices* when examined microscopically. Low spore quality could have also been a manufacturing fault.

Hypothesis D: Temperature and light fluctuations outside fungal germination range

During the experiment the light conditions varied. Over a period in December the plants were relocated into an alternate growing chamber which may have altered the light and temperature conditions. The light reading of (45/umol/m^2/sec) could have also been below the light intensity required to maximize growth. The light intensity beside a window can be up to 100 (umol/m^2/sec) and on a sunny day outside it can be up to 1000. Adding more lights to the growing chamber is risking the creation of an environment that is too hot which may kill the seedlings.   VAM infection is influenced considerably by light and temperature (Hayman 1974). Temperature and light affect plant growth more than they affect mycorrhizal infection. It has been observed that more VAM infections occurs on sunny sites than in shady sites (Peyronel 1940). Tobacco plants grown under full light were found to have 85% VAM infection whereas those in half-light had only 31% infection (Peuss 1958). The decreased stimulation of plant growth by VAM under poor light and temperature conditions could be explained by a deficiency of functional arbuscules (Hayman 1974). Light and temperature affect plant growth more than mycorrhizal infection because light intensity, day length and temperature have a very marked effect on plant growth (Hayman 1974).

Hypothesis E: Spore concentration insufficient

It is possible that the spores were not at a high enough concentration to cause the colonization of the roots. At the highest intensity (25 grams of MYKE®) there should have been enough as there were 175 spores in each flask of this concentration. It is recommended that 300-500 spores be present per 500 grams of soil and approximately 10,000 spores be present in 1m^2 of soil (Ferguson and Woodhead 1982).

Cumulative Effect Among Hypotheses

It is likely that the lack of fungal germination was caused by the relation and cumulative effects among some of the different hypotheses. The insufficient light and dilute nutrients may have put the elm seedling into a state of dormancy which would then reduce the supply of root exudates inhibiting spore germination.

Conclusion

Although it is uncertain as to why the elm seedlings failed to stimulate spore germination and subsequent root colonization by MYKE®, the results do not in any way whatsoever negate the potential benefits that VAM fungi have on plant growth and survival. Future studies may want to employ pots of soil in the greenhouse rather than the use of flasks in incubators in order to stimulate a more representative situation in the rhizosphere of plants. In a greenhouse it is easier to get the correct balance of temperature and light intensity without the chance of overheating the seedlings. Moisture levels are also easier to control as the seedlings can be watered when required

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APPENDICIES

Appendix I: Seedlings Harvest Photographs

Figure 9. Harvested American elm Seedlings after growth with different concentrations of MYKE®

Appendix II: Modified Phillips & Hayman Staining Protocols for Unpigmented Roots

1. Heat for 1 hour in 10% KOH at 90°C
2. Water rinse for 5 minutes
3. Water rinse for 5 minutes
4. 5% HCL rinse for 5 minutes
5. 5% HCL rinse for 5 minutes
6. .05% trypan blue-lactic acid (875mL lactic acid, 63 mL glycerin, 62 mL water, 0.5g trypan blue) for 3-5 minutes at simmer
7. Clear in clear lactic acid solution rinse (875 mL lactic acid, 63 mL glycerin, 62 mL wate)
8. Mount in clear lactic acid solution
9. Gently crush roots

Appendix III: Dry Weights of harvested American elm seedlings

Tree #	Mass (g)
1	0.008
2	0.007
3	0.008
4	0.007
5	0.005
6	xxxxxxxxxxx
7	0.006
8	0.005
9	0.012
10	0.003
11	0.009
12	xxxxxxxxxxx
13	0.007
14	0.018
15	0.007
16	0.01
17	0.007
18	0.006
19	0.005
20	0.004
21	0.003
22	0.013
23	xxxxxxxxxxx
24	0.009
25	0.006
26	0.007
27	0.008
28	0.005
29	0.004
30	0.007
31	0.002
32	0.008
33	0.007
34	0.006
35	0.001
36	0.001
37	0.001
38	0.003
39	0.003
40	xxxxxxxxxxx

Appendix IV: ANOVA

Anova: Single Factor						
SUMMARY						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Control	9	0.061	0.00677778	6.4444E-06		
1g	9	0.073	0.00811111	1.7111E-05		
5g	9	0.062	0.00688889	8.8611E-06		
25g	9	0.032	0.00355556	7.5278E-06		
ANOVA						
rce of Variat	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Grc	0.00010244	3	3.4148E-05	3.41956421	0.02886305	2.90111958
Within Grou	0.00031956	32	9.9861E-06			
Total	0.000422	35				