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PHOSPHORUS UPTAKE IN SORGHUM SEEDLINGS COLONIZED WITH ARBUSCULAR MYCORRHIZAL FUNGI

BY

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APPROVAL FORM

This is to certify that the project entitled “Phosphorus uptake in sorghum seedlings colonized with Arbuscular Mycorrhizal Fungi”, submitted in partial fulfilment of the requirements for Bachelor of Science Honours Degree in Applied Biosciences and Biotechnology at Midlands State University, is a record of the original research carried out by Jacqueline Tafadzwa Chirambaguhwa R144705Q.

Name of supervisor……

Signature…….

Chairperson signature……

DECLARATION

I, **JACQUELINE TAFADZWA CHIRAMBAGUHWA**, do hereby declare that this research project is a product of my own work and has never been submitted to any other University or any other institution of tertiary learning for any award.

Signed..... Date.....

This research project is being submitted to the Midlands State University for the award of a Bachelor of Science in Applied Biosciences and Biotechnology Honours Degree with the approval of an academic supervisor.

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ABSTRACT

The process of re-establishing the natural level of arbuscular mycorrhizae (AM) fungi richness can represent a valid alternative to conventional phosphate based fertilization practices, with a view to sustainable agriculture. The main strategy that can be adopted to achieve this goal is the direct re-introduction of AM fungi inoculum into a target soil. AM fungi have a high affinity for phosphorus, an essential macronutrient that participates in the skeleton of the nucleic acids DNA and RNA. The aim of the study was to determine the effect of AM fungi on sorghum growth by measuring phosphorus uptake by the plant and the plant height. The AM fungi propagules were collected from the starter soil in Matopos. The propagules were identified by clearing the roots in 10% KOH and then staining in Methylene blue prior to microscopic analysis. After positive identification propagules were multiplied in greenhouse pot plants using the bait plant sorghum for 6 weeks. The inoculum was then transferred to the pot trials, using three different soil types red, black and sandy. After 6 weeks the sorghum seedlings were measured for phosphorus and plant height. The soil types were measured for phosphorus before and after the trials. AM fungi significantly increased plant phosphorus ($p=0.0$) and significantly increased plant height ($p=0.013$). There was no interaction between AM fungi and soil type in influencing plant phosphorus as there was no significant difference ($p=0.073$) and no interaction in influencing plant height as there was no significant difference ($p=0.534$). AM fungi inoculum has significant potential to be used as a natural bio-fertilizer. However, more field experiments need to be done to see the performance of AM fungi in an uncontrolled environment.

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DEDICATION

To my beloved mother

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CHAPTER 1

1.0. INTRODUCTION

1.1. Background

It is estimated that the world's population will exceed 9 billion by the year 2050 (Rodriguez and Sanders, 2015). As a result of this population boom, global agriculture will have to double the current food production, at the same time decreasing the dependence on agro-chemical inputs to protect human and environmental health. This forecasted food increase exceeds the current global capacity to produce food (Rodriguez and Sanders, 2015). Hence it has become essential to find methods that ensure a food-secure future; methods that are reasonably priced, environmentally safe and easily accessible.

One of the methods that can be harnessed is the implementation of eco-friendly technologies, such as the mutualistic symbiotic relationships with arbuscular mycorrhizae fungi (AM fungi) so as to produce AM fungi-based fertilizers. The process of re-establishing the natural level of AM fungi richness can represent a valid alternative to conventional fertilization practices, with a view to sustainable agriculture (Barr, 2010). In order to maintain crop yields, modern agricultural systems are highly dependent on continual inputs of phosphate based fertilizers.

Phosphorus is not a rare element on earth, but it is often found in low concentrations and it is an essential macronutrient required by plants in large quantities (Marschner, 1995). Plants absorb phosphorus in the form of orthophosphate. This is the simplest phosphate available in soil and water. However, it is found in small quantities resulting in a deficiency for plants. This is so because the solution P pool in which orthophosphate is found is very small and it is maintained by the release of phosphates from the active P pool. The active P pool contains inorganic phosphates and because of its particular chemistry, it reacts readily with positively charged iron (Fe^{2+}), aluminum (Al^{3+}), and calcium (Ca^{2+}) ions to form relatively insoluble substances (Khan, Marwat, Amin, Nawaz and Khan, 2012). When this occurs, the phosphorus is considered fixed or tied up.

Phosphorus has a wide range of functions. It participates in the skeleton of the plasma membrane (phospholipids), nucleic acids (DNA and RNA), and organic molecules such as ATP. Pi is also involved in controlling key enzyme reactions and in the regulation of metabolic pathways (Marschner, 1995). It also promotes healthy root growth by aiding in the translocation of carbohydrates. Its deficiency will result in stunted growth, prolonged dormancy, pre-mature leaf fall, reduced tillering, dead patches and blue-purplish leaves (Marschner, 1995).

Exploitation of new plant germplasm in new environments may depend on the natural presence of a suitable micro-symbiont or its simultaneous introduction (Barea, Palenzuela, Cornejo, Sanchez-Castro, Navarro-Fernandez, Lopez-Garcia, Estrada, Azcon, Ferrol and Azcon-Aguilar, 2011). Mycorrhizas are the most important universal type of symbiotic mutualistic associations between soil, fungi and plants (Kang, Khan, Hamayun, Shinwari, Kim, Joo and Lee, 2012). They are essential in improving plant fitness and soil quality. They improve the resilience of plant communities against environmental, nutritional and drought stresses (Barea *et al.*, 2011).

Mycorrhizal fungi can be divided into two major groups: aseptate endophytes such as Glomeromycota, or septate Ascomycota and Basidiomycota. More commonly, mycorrhiza classifications reflect anatomical aspects and identify two broad categories, referred to as ectomycorrhizas and endomycorrhizas, depending on whether the fungus colonizes the root intercellular spaces or develops inside cells. Endomycorrhizas are further divided into orchid, ericoid and arbuscular mycorrhizas (Smith and Read, 2008). They are the most common biotic factor of ecosystems which establish symbiotic relationship with terrestrial plants (Willis, Rodrigues and Harris, 2013). This type of fungus has a very high affinity for phosphorus. The mycelia are smaller in diameter than the smallest root and can explore a greater volume of soil, increasing the surface area for absorption (Moore, Robson and Trinci, 2011).

The hyphae penetrate the root cell walls and become enclosed in the cell membrane. This creates a greater contact surface area, as well as protection to the plant roots (Roy-Bolduc and Hijri, 2011). AM fungi are known to be of great importance due to their great capability to increase growth, yield and crop quality, through efficient nutrient acquisition in infertile soils and therefore lessen the use of phosphate based fertilizers (Roy-Bolduc and Hijri, 2011). In turn, the

fungi get carbon from the host plant. AM fungi are able to absorb and transfer all of the 15 major macro- and micro-nutrients essential for plant growth (Bonfante and Genre, 2010).

It is crucial to gain a more comprehensive picture of what AM fungi taxa are present, how they react to different host crops and to various soil treatments, and how they interact with other soil microbes (Rilling and Mummy, 2006). The colonization of sorghum with AM fungi under different soil types will not only increase the rate of phosphorous uptake as well as other essential elements but will also improve the drought tolerance capabilities of sorghum (Dahlberg, 2000).

1.2. Problem statement

The continuous change in the climatic conditions, has had a negative impact on the agriculture sector globally and particularly in the semi-arid tropics (SATs) (Balemi and Negisho, 2012). Given the predicted increase in global demand for food and the current rate of phosphorus extraction, the resource could be depleted in 50 to 125 years (Sharma, Sayyed, Trivedi and Gobi, 2013). There will be no soil phosphorus reserves by the end of 2050 for sustainable crop production especially in the tropical and subtropical regions of the world (Balemi and Negisho, 2012). It has become essential to find different approaches to address the issue of phosphorus scarcity so as to ensure a food secure future. One of the approaches is the consideration of soil microorganisms, such as AM fungi. The mutualistic symbiotic relationships with AM fungi can improve the efficient use of phosphorus and could be a very effective approach for a more efficient resource use. In order to overcome the phosphate crisis, it is important that all potential solutions be considered, especially solutions that are reasonably priced, easily accessible as well as environmentally safe.

1.3. Justification

The household food security in Zimbabwe, which falls under the SATs has declined due to drastic reduction in food and agricultural production following erratic rainfall and the gross lack of key farming inputs (Mgonja, 2010). The FAO /WFP (2008) Crop and Food Supply assessment mission to Zimbabwe established that production decline in agriculture has been the main cause of household food insecurity in communal areas. The worst affected provinces are those that lie

in agro-ecological regions IV and V, which are provinces of Masvingo and Matebeleland. FAO (2006) estimates that about 70% of Zimbabwe's communal lands lie in regions IV and V.

One of the reported challenges of the smallholder farmers is access to key inputs such as fertilizer (Monyo and Mgonja, 2001). Fertilizer recommendation and application rate for compound D is 250kg/ha (Zimbabwe fertilizer Company Limited, 2016). Compound D contains nitrogen, phosphorus and potassium in the ratio 7:14:7, respectively. Among these nutrients, phosphorus constitutes a particularly critical component because, on one hand, it is limiting for crop yield on a large proportion of global arable land and, on the other hand, it is a non-renewable resource. At present, modern agricultural systems are highly dependent on continual inputs of phosphate-based fertilizers (Pizzeghello, Berti, Nardi and Morari, 2011). The use of phosphate-Based fertilizers has increased from 9 million tons per year in 1960 to 40 million tons in 2000 (Pizzeghello *et al*., 2011).

Soil microorganisms such as AM fungi represent a key link between plants and soil mineral nutrients. Thus, they are collecting growing interest as natural fertilizers (Lehman, Taheri, Osborne, Buyer, and Douds, 2012). AM fungi have a very high affinity for phosphorus. A recent molecular study (Chu, Wang, Yang, Chen, Zhang and Feng, 2013) identified the gene *GvPT,* which encodes for a high affinity fungal phosphate transporter in external hyphae. An extensive network of hyphae extends from the root, enabling the plant to explore a greater volume of soil. This enables the plant to overcome limitations imposed by the slow diffusion of Pi in the soil.

1.4. Objectives

1.4.1. Main objective:

- to determine the effect of AM fungi on the growth of sorghum.

1.4.2. Specific objectives:

- to determine the effect of AM fungi on phosphorus uptake by the plant.
- to determine the effect of AM fungi on the plant height of sorghum, and
- to determine the effect of different soil types (red, black and sandy) on the colonization of AM fungi and the subsequent effects.

CHAPTER 2

2.0. LITERATURE REVIEW

2.1. Sorghum

Sorghum [*Sorghum bicolor* (L.) Moench] has been cultivated in arid and semi-arid regions of Africa for thousands of years (Dahlberg, 2000). It originated from North Africa and it was domesticated in present day Ethiopia between 4,000 and 3,000 B.C (Dillon, Shapter, Henry, Cordeiro, Izquierdo and Slade, 2007). Sorghum later spread to other parts of Africa and eventually to India, the Middle East, and China (Dahlberg, 2000). Grain sorghum is the fifth most important cereal crop in world (Dillon *et al*., 2007).

2.2. Taxonomy and Characteristics

All sorghums belong to the *Poaceae* grass plant family, and in the *Sorghinae* sub-tribe within the *Andropogoneae* plant tribe (Dahlberg, 2000). The genus *Sorghum* includes three species: *S. bicolor, S. propinquum,* and *S. halepense*, with *Sorghum bicolor* containing all of the cultivated sorghums. Both *Sorghum propinquum* and *Sorghum halepense* are rhizomatous wild, weedy perennials that are able to cross with *Sorghum bicolor*. *Sorghum bicolor* (L.) Moench is further divided into three subspecies, *Sorghum bicolor* subsp. *bicolor, S. b. drummondii*, and *S. b. verticilliforum* (formerly known as *Arundinaceum*). The latter two subspecies are annual weeds (Dahlberg, 2000).

The cultivated subspecies *S. bicolor* consists of five different races, which can combine with each other to produce ten hybrid intermediate races, totaling 15 races all together. The race *S. bicolor* is large and complex, consisting of many different sub-races that produce small amounts of grain and that are mainly used as forage. One of the most important sub-races of the race *bicolor* is *sorgo,* which contains many of the sweet sorghum cultivars that are used for syrup production (Dahlberg, 2000).

Another race, *S. b. guinea*, is well adapted to high-rainfall environments and is very important in West Africa where it is widely grown for human consumption (Dillon *et al*., 2007). The race *S. b. durra* contains the most drought tolerant cultivars that are grown in the Near East, Ethiopia,

and India. A fourth race, *S. b. caudatum,* is known for its high grain yield potential and high seed quality. The last race, *S. b*. *kafir*, is agronomically important due to its high yield potential and closed to semi-open panicle structure (Dahlberg, 2000).

Sorghum grows in a wide range of soil types from sandy to heavy clay soils, although in dry climates, soils with sand are desirable (Monyo and Mgonja, 2001). It is not tolerant of acid soils, but sorghum has moderate tolerance to saline soils and will grow in soils that range in pH from 5 to 8.3, although the optimum pH range is 6.2 to 7.8. Nutrient requirements for grain sorghum are similar to corn, and in a growing season a sorghum grain crop yielding 6.3 metric tons ha-1 will use around 38, 19, and 10 kg ha-1 of nitrogen, phosphorus, and potassium, respectively, (Cothren, Matocha and Clark, 2000).

2.3. Taxonomy of arbuscular mycorrhizae fungi

The fungi forming arbuscular mycorrhizae were identified from sporocarps produced near mycorrhizal roots and they were recognized initially as one or other species of *Endogone*. The AM fungi represent a convergent evolution of unrelated fungi over millions of years, all becoming physiologically and morphologically adapted to the living roots so that they now appear anatomically and functionally similar (Hayman, 1983).

The classification of arbuscular mycorrhizal fungi was revised. They replaced the term vesicular arbuscular mycorrhiza by arbuscular mycorrhiza (AM) since the two genera, *Scutellospora* and *Gigaspora*, do not form vesicles. AM fungi cannot be cultured axenically without their host system and are therefore obligate associates of host plants (Willis *et al*., 2013). As research on mycorrhizae is in progress all over the world, new species are added to the list of AM fungi and at present the total number of species is about 200 (Morton, Bentivenga and Wheeler, 1993).

2.4. The root colonization process by AM fungi

Root colonization is vital to AM fungi. Their spores feed germinating hyphae through the catabolism of storage lipids for a few days. During this period, hyphae explore the soil in search of a host, if no host is found the hyphae arrest their growth and retract their cytoplasm back into

the spore, which may become dormant and restart the germination process over and over (Smith and Read, 2008).

However, if a host is found, the germination of a resting spore is followed by the production of a short explorative mycelium (Harrison, 2005). The perception of plant exudates, released by the host root, induces recursive hyphal branching, increasing the probability of a direct contact between the symbionts (Willis *et al*., 2013). In the meantime, fungal exudates are perceived by the root, where they trigger calcium spiking. Signal transduction leads to the activation of cellular and transcriptional responses (Harrison, 2005).

The contact between the plant and fungus is followed by the adhesion of a hyphopodium to the root surface. This triggers the assembly of a broad aggregation of cytoplasm named prepenetration apparatus (PPA) in the contacted epidermal cell and underlying outer cortical cell. Subsequent intracellular fungal colonization strictly follows the route of PPAs from the epidermis to the inner cortex. Here, intercellular hyphae can develop along the root axis. The PPA mechanism is then replicated in the contacted inner cortical cells. Eventually a highly branched arbuscule occupies most of the cell volume, forming an extensive surface for nutrient exchange (Harrison, 2005).

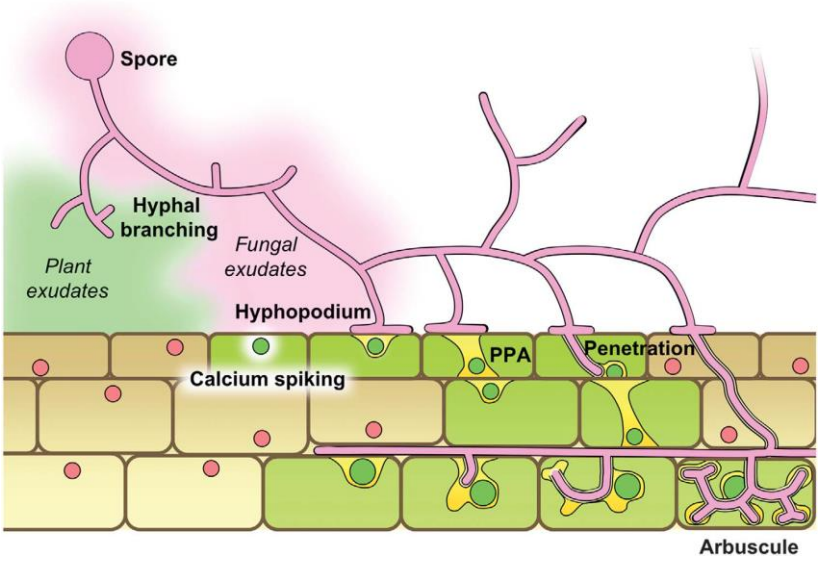

Figure 1. The root colonization process by AM fungi.

2.5. The mechanisms of AM fungi

The characterization of a high-affinity inorganic phosphate (Pi) transporter in AM fungi has provided a breakthrough in the understanding of fungal function (Smith and Smith, 2012). The nutritional aspects of AM symbiosis have been studied extensively from both a physiological and a molecular perspective (Chu *et al*., 2013). AM fungi are capable of significantly improving plant mineral nutrient acquisition, mainly in low-nutrient conditions, and it has been demonstrated that plants possess a symbiotic Pi uptake pathway (Smith and Smith, 2012).

After the identification of a complementary DNA that encoded a transmembrane Pi transporter from *G. versiforme*, the function of the protein was confirmed by complementation of a yeast mutant affected in Pi transport (Harrison and van Buuren, 1995). The expression of this Pi transporter was localized to the extraradical hyphae of *G. versiforme*, the site of phosphate uptake from the soil. Accumulated as polyphosphate, Pi is then rapidly translocated along the aseptate mycelium to the host plant. The radiotracer experiments have made it possible to verify the relative amount of Pi that enters a plant via AM fungi and directly through the root transport system, and have revealed that the fungus can transfer the Pi to the plant even without an evident growth effect (Smith, Smith and Jakobsen, 2004).

It has been proposed that the nutrient dependent regulation of AM fungi colonization provides an important feedback mechanism for plants to promote or limit fungal colonization according to their needs (Nouri, Breuillin-Sessoms, Feller and Reinhardt, 2014). It has already been demonstrated that phosphorus availability represents an environmental factor that can disturb the symbiotic interaction of AM fungi. In fact, the suppression of AM fungi colonization due to high Pi levels has been reported in several experiments. According to a study by Kowalska, Konieczny, Gastol, Sady and Hanus-Fajerska (2015) the highest level of mycorrhiza colonization was found in plants that received a nutrient solution with a lower concentration of P.

2.6. Role of AM fungi in other mineral absorption

Nitrogen is another important element taken up by AM fungi, and genes involved in the transport of ammonium and amino acids have been identified (Schussler, 2006). Although carbon transfer from plants to AM fungi was demonstrated in the 1960s, its molecular mechanisms are still

unclear, no hexose transporters responsible for carbon uptake from host cells have so far been characterized in Glomeromycota (Schussler, 2006). Increased uptake of Zn, Mn and Fe in peanut has been reported on inoculation with AM fungus *Glomus fasciculatum*, as well as enhanced AM fungi mediated K accumulation in host plants (Ryan and Angus, 2003).

AM fungi can deliver up to 80 % of plant P, 25% of plant N, 10% of plant K, 25% of plant Zn and 60% of plant Cu requirements (Marschner and Dell, 1994). Promotion of plant growth with the help of mycorrhizal infection may be through direct acquisition of nutrients by the fungus or indirectly by modifying transpiration rates and the composition of rhizosphere microflora (Marschner and Dell, 1994).

AM fungi contribute substantially to Cu, Zn and Cd uptake in bean and maize and mycorrhiza assisted enhanced Zn uptake has also been noticed in subterranean clover and in pea (Oehl, Sieverding, Mader, Dubois, Ineichen, Boller and Wiemken, 2003). AM fungi isolated from metal tolerant *Viola calaminaria* was found to accumulate heavy metals in clover roots without significantly affecting the concentrations of the metals in the shoot and plant growth. Such AM fungi may be used for phytoremediation process, by preventing elevated concentrations of heavy metals in shoot system (Ryan and Angus, 2003).

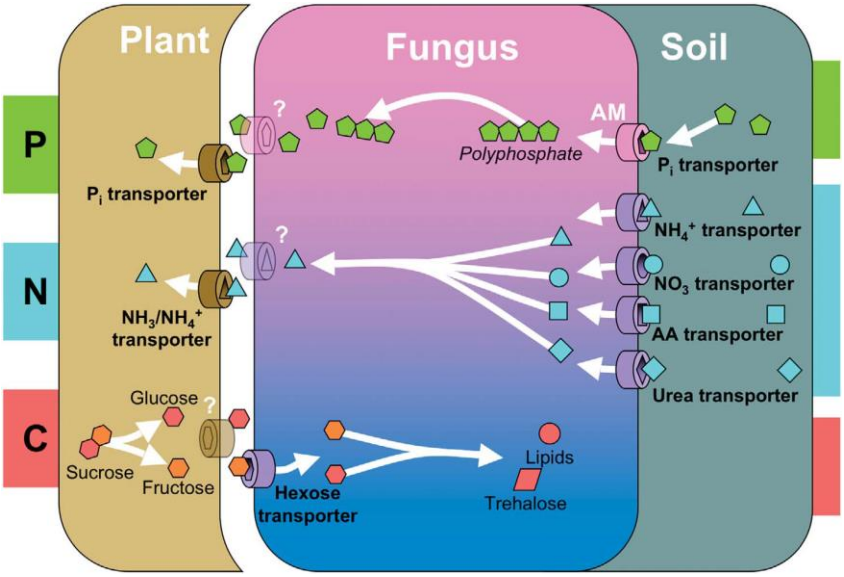

Figure 2. The main (Phosphorus, Nitrogen and Carbon) nutrient exchange processes in AM symbiosis.

2.7. Factors influencing AM fungi development

There are reports of various factors that influence AM fungi development and expression.

2.7.1. General Soil Characteristics and AM fungi

AM fungi population varies from soil to soil. Among different soil parameters, soil texture influences spore germination and hyphal growth so that any specific AM fungi population develops according to the prevailing soil texture. The glycoprotein glomalin, produced by AM fungi helps water stable soil aggregation (Oehl *et al.*, 2003). Cultivation of host plants favour increased sporulation, while non host plants decrease them. Soil moisture influences the AM infection in roots and the distribution of spores. Poor sporulation and root colonization have been noticed in high moisture availability (Xiong, Lu and Truong, 1994).

Spore count varies significantly with soil aeration and porosity. It has been observed that the spore count of *Glomus* sp is higher in sandy soils than in clay soils. Altitude and seasons play significant roles in spore formation in *Glomus mosseae* and *Glomus aggregatum*, spores decrease in soil samples with increasing altitude. Soils under low input management have higher AM fungal spores than soils under conventional management. Increased land use intensity is correlated with a decrease in AM fungal species richness and there is a preferential selection of species that colonize roots slowly and form spores rapidly in such soils (Oehl *et al.*, 2003).

2.7.2. Soil temperature and carbon dioxide

At low temperatures the host-endophyte balance changes from beneficial to mildly harmful to the plant. Storage temperature also affects spore viability, which varies with AM fungal types. High soil temperature favours root colonization by AM fungi in spring barleys (Xiong *et al.*, 1994). The percentage root colonization of AM fungi, soil hyphal length and soil concentration of glomalin shows linear increase with CO_2 gradient in a grassland. Artificial climate warming positively affects AM fungi but decreases soil aggregate water stability. Studies suggest that temperature influences AM fungi development than CO_2 (Rillig, 2004).

2.7.3. Fertilizers and organic substances

Application of super phosphate to mycorrhizal plants shows reduction in sporulation. Soil fertility affects soil-plant interaction, which in turn affects plant-fungus interaction and subsequently affects mycorrhizal colonization and spore numbers. Increased P and N fertilizer application of the soil leads to establishment of inferior mutualistic AM fungi and hence reduced benefits to the host plant. The functioning of AM fungi association is also affected by chemicals in the environment, especially fungicides as they cause inhibition of sporulation in AM fungi (Rillig, 2004).

2.7.4. AM fungi and cultivated non leguminous crops

AM fungi have been reported in many cultivated crops (Jakobsen and Rosendahl, 1990). Cassava is extremely dependent on an effective mycorrhizal association for normal growth in low P soils. Mycorrhizal infection in annual crops shows that P uptake is affected significantly by AM fungi only if infection is well established shortly after seedling emergence. However, the concept that infection susceptibility is similar in various crops is controversial and needs further testing (Jakobsen and Rosendahl, 1990).

2.8. Challenges related to AM fungi inoculum production

The need to benefit from AM fungi as a bio-fertilizer, with a view to sustainable agriculture, has become increasingly urgent since the appropriate management of these symbiotic fungi could potentially decrease the use of agrochemicals. The main strategy adopted to achieve this goal is the inoculation of AM fungi propagules (inoculum) into a target soil. Unfortunately, AM fungi are obligate symbionts and cannot be cultivated in pure cultures, away from their host plants. This constraining feature makes the large-scale production of AM fungi inocula very challenging and complex (IJdo, Cranenbrouck and Declerck, 2011).

There are three main types of AM fungi inocula. First, soil from the root zone of a plant hosting AM fungi can be used as inoculum as it normally contains colonized root fragments, AM fungi spores, and hyphae. However, unless precise information about the propagule abundance, diversity, and infectivity are available, soil inocula can be unreliable and carry the possible risk of transferring weed seeds and pathogens (IJdo *et al.*, 2011).

Spores extracted from soil can instead be used as starters for crude inoculum production (Willis *et al*., 2013). Crude inoculum can be obtained after a known isolate of AM fungi and a host trap plant are grown together in an inert medium optimized for AM fungi propagation. This is the most commonly used type of inoculum for large-scale crop inoculation as it usually contains a more concentrated set of the same kind of propagules found in soil inocula. Finally, infected root fragments alone of a known AM fungi host that have been separated from a trap plant culture can also serve as a source of inoculum (IJdo *et al.*, 2011).

The production of AM fungi crude inoculum on a large-scale remains very challenging even though new methods for massive production and seed coating technology have been developed in recent years (Willis *et al*., 2013). The main obstacle to the production of an AM fungi inoculum lies in the obligate symbiotic behavior of AM fungi, that is, their need to have a host plant for growth and completion of their life cycles. This means that the propagation step must include a phase of cultivation with the host plant that is usually time and space demanding. As a consequence, the setting up of AM fungi reference collections also requires methodologies that are rather different and more binding than those used for other microbial collections (Vosátka, Látr, Gianinazzi and Albrechtová, 2013).

Moreover, the absence of a prompt method for assessing whether and to what extent the host plant is colonized by AM fungi also contributes to making AM fungi agricultural usability challenging. The management of the high amount of inoculum necessary for large-scale application is also a demanding process. However, AM fungi inoculation is carried out more easily for plant production systems that involve a transplant stage, since smaller amounts of inoculum are needed. At a first glance, carrying out an open-field, extensive inoculation treatment could seem technically impractical and economically prohibitive (van der Heijden, Martin, Selosse and Sanders, 2015).

However, once AM fungi biodiversity is restored and well-established, and if an AM fungi-friendly management, such as fall cover cropping and conservation tillage is put in place, the AM fungi community will persist. If no detrimental practices are carried out before and after cultivation, it is known that the biodiverse mycorrhizal hyphal network will remain unaltered and

infective in the future. As an alternative to large-scale inoculation, a small-scale approach is also feasible (Säle, Aguilera, Laczko, Mäder, Berner and Zihlmann, 2015).

Taking inspiration from the idea of creating the so-called “fertility islands” (Lehman *et al.*, 2012) AM fungi inoculation could be limited to small portions of a field, and this would gradually lead to the establishment of a healthy AM fungi mycelial network, but with reduced costs. This technique would be particularly indicated when the AM fungi inoculation is aimed at assisting the revegetation of a degraded land, since inoculated fertility islands likely allow native plant species to recover the nutrient impoverished land faster (Lehman *et al.*, 2012).

Hence, AM fungi restoration only represents an initial cost that, if the persistence of AM fungi is favored in the soil, could be prorated over the years. As already demonstrated (Barr, 2010), AM fungi inoculation can be economically profitable, in comparison to conventional fertilization, providing substantial savings for growers and for degraded land recovery projects. In order to provide further data to assess AM fungi inoculation attractiveness, it is important that the end-users should also cultivate an uninoculated portion of their crop, so as to be able to evaluate the cost-effectiveness and beneficial effects on plant fitness due to AM fungi (Dalpé and Monreal, 2004).

The global economic crisis is now forcing growers to try to understand the potential of sustainable agricultural systems, and of reducing the input of phosphorus using AM fungi inocula. However, solid inoculation practices have yet to be implemented, and applied research focused on defining the best inoculum formulation strategies are being encouraged (Säle *et al.*, 2015). The potential of AM fungi has drawn the attention of the commercial sector, and several companies now produce and sell AM fungi-based inocula (Verbruggen, van der Heijden, Weedon, Kowalchuk and Roling, 2012).

The general tendency is to formulate inocula with only a few AM fungi species as components. Some manufacturers have chosen the single formulation approach, but others produce different products that are supposedly targeted for end-users who are willing to apply the formulation to a range of environmental conditions and host-plant groups. The few species that are used can

easily be routinely propagated and are normally generalist, as they are found in association with a large variety of host plants in different biomes. Although commercial inocula is often advertised as suitable for a wide range of plants and environmental conditions, the real benefits are not always positive (Faye, Dalpé, Ndung'u-Magiroi, Jefwa, Ndoye and Diouf, 2013).

In order to promote AM fungi inoculum market development and improvement, scientists should strengthen the link between research and companies and introduce a series of best practices that can be adopted to solve issues related to the functioning of commercial inocula. One of these issues arises from the need to control the biological composition of a product, due to the possible presence of pathogens and weeds (Tarbell and Koske, 2007), but above all to the need to assess its purity in terms of AM fungi composition. The species list declared in a commercial inoculum label does not always correspond accurately to the actual inoculum composition (Berruti, Borriello, Della-Beffa, Scariot and Bianciotto, 2013).

AM fungi inocula are mostly produced using a containerized-culture, either in greenhouses, growth chambers, or in open fields, and, as a result, cannot possibly be free from external microorganisms. As a result of the increased awareness of the risk of pathogens, many concerned manufacturers now apply agrochemicals in order to avoid contamination of their product (Douds, Nagahashi, Pfeffer, Kayser and Reider, 2005). In order to reduce pathogen carry-over, it is possible not to include host root residues in the inoculum while, as an alternative, the incorporated root fragments can be surface sterilized without jeopardizing the viability of the AM fungi propagules (Mohammad and Khan, 2002).

Over the last few decades, several techniques have been applied to the molecular characterization of entire AM fungi communities in complex matrices, such as soil and AM fungi inocula (Säle *et al.*, 2015). These methodologies also allow the inoculated AM fungi to be monitored inside the host plant during the cultivation cycle (Werner and Kiers, 2015). High-throughput next generation sequencing (NGS) potentially offers the most powerful and sensitive technique to trace the introduced fungus, both temporally and spatially. This set of techniques also makes it possible to verify whether the inoculated AM fungi favour significant levels of colonization, although this may not necessarily be important if the effects on crop production and quality are

indirect via the resident AM fungi community. Finally, NGS also leads to the understanding of how the introduced AM fungi interact and coexist with the local AM fungi community (Rodriguez and Sanders, 2015).

2.9. Challenges related to AM fungi application as a bio-fertilizer

Despite its enormous potential, the application of AM fungi has not been fully adopted by farmers so far (Ceballos, Ruiz, Fernández, Peña, Rodríguez and Sanders, 2013). It has been pointed out that AM fungi inoculation overall produces positive outcomes on plant production in both controlled and open-field conditions, mainly due to the several nutrition-related benefits that this class of soil fungal symbionts is able to provide to their host-plant. In particular, AM fungi inoculation in the field has proven to be as effective as inoculation in the greenhouse (Ceballos *et al.*, 2013).

The next significant step toward the stable use of AM fungi in agriculture is to carry out large-scale multi- location field trials and conduct cost-benefit analyses, in order to increase awareness among the potential end-users of the benefits of AM fungi inocula. Indigenous AM fungi have been demonstrated to be equally or even better performing than commercial or culture collection isolates, farmers are encouraged to autonomously produce their AM fungi inocula, starting from native soils. This makes the bio-fertilization technology more likely to be affordable for farmers, including those in developing countries who need their cropping system to be as highly sustainable as possible (Ceballos *et al.*, 2013).

When considering mycorrhizal symbioses in the context of current global challenges such as environmental change, ecosystem conservation, sustainable agriculture, development of plants for future needs and food safety, it is acknowledged that AM fungi may be crucial in many of these fields. AM fungi mobilize P and N, and are an important C sink in the soil, having therefore an important impact on the cycling of these elements (Sawers *et al.*, 2014).
As bio-fertilizers, they may counteract fertilization excess and thus promote sustainable agriculture. The selection of new crop varieties giving yields on poor soils and in low fertilization conditions should therefore consider new aspects, such as their responsiveness to AM fungi, which has never consciously been taken into account during modern plant breeding.

Food quality and security are strictly linked to the necessity of feeding a constantly growing global population under the threat of climate change; in this frame, unravelling the contribution of AM fungi to the nutritional quality of edible plants is a priority (Hayashi, 2010).

CHAPTER 3

3.0. MATERIALS and METHODS

3.1. Study site

The study was conducted at Midlands State University in Gweru. The climate was characterized by distinct wet and dry seasons with a mean annual rainfall of 684mm. The experimental plants were grown in the green house and all microscopic analysis was conducted in the laboratories.

3.2. Experimental design

The factorial arrangement included two AM fungi treatments (with mycorrhizae fungi and without mycorrhizae fungi) for each soil type (red, black and sand loam). Three replicates were used for each treatment combination, thus the experiment had a total of 18 pots. The experiment was carried out based on a randomized complete block design (RCBD).

3.3. Collection of starter soil

The method used was by Corkidi, Allen, Merhaut, Allen, Downer and Bohn (2008). The soil was collected from virgin soil in Matopos. Matopos is located 28km away from Bulawayo. The climate was characterized by distinct wet and dry seasons with a mean annual rainfall of 570mm. The soils were red to black sand loams derived from basement schist and coarse textured sandy derived from gneissic granite sands. The vegetation was predominantly *Acacia savanna*, dominated by *Acacia karoo* and *Acacia nilotica*. 0.5m^2 of the vegetation was cleared away using a hoe and a shovel. A depth of 25cm was dug to collect the starter soil and as many fine roots.

3.4. Identification of the mycorrhizae

Soil samples were washed and wet sieved through a set of sieves by the wet sieving and decanting technique as described by Gerdeman and Nicolson (1963) The root samples were cut into lengths of 1 cm. The root samples were cleared in 10% KOH at 90 ^oC for 15 min in an autoclave, then stained in Methylene blue at 90 ^oC for 15 minutes (Leck, 1999). From each sample, 5 root segments of 1 cm length were examined for the presence of AM fungi colonization under a compound microscope (Leck, 1999).

3.5. Multiplying the mycorrhizae

Multiplication of AM fungi was archieved in the greenhouse. The bait plant species used was *Sorghum bicolor* L. The starter soil was used to fill 8 pots. The seeds were soaked overnight in distilled water and then planted in the pots. The trap-pots were watered regularly to maintain the moisture and monitored over a period of 6 weeks (Corkidi *et al.*, 2008).

3.6. Utilization of the inoculum

After six weeks, 10 days before the inoculum was used, bait plants were cut off by the base and the roots were mixed with the soil. The media soils; red, black and sandy were measured for phosphorus, before being autoclaved for 60 minutes. The soil media was each added to 3 pots. The inoculum was then added to the 1^{st} pot and the 2^{nd} pot was the control, with no inoculum. The experiment was replicated 3 times and randomized and blocked according to the soil type.

The sorghum seeds were surface sterilized with 1.0% sodium hypochlorite solution, there-after top of paper germination tests were carried out. This was done by moistening the paper with distilled water. The 100 seeds were arranged on top of the paper, in a petri dish and incubated for 72 hours. Germination tests were 80% and above, hence, the seeds were planted. The plants were grown and monitored in the greenhouse for a period of 6 weeks (Corkidi *et al.*, 2008).

3.7. Quantitative determination of Phosphorus from soil

To determine the phosphorus content, 30g of each soil sample was measured (m). Seventy milliliters of 55% nitric acid were added to each sample to form phosphoric acid. The mixtures were heated to boiling point using a hot plate, cooled in open air and then filtered with whatman No. 1 filter paper. Five grams of ammonium molybdate were weighed out (m_1) using an electronic balance and added to twenty milliliters of 25% ammonium. This was then slowly added to the phosphoric acid mixture in a laminar flow. The ammonium phosphomolybdate (Mr) was cooled in a freezer. It was filtered and the filtrate was oven dried. Phosphorus percentages were then calculated using the following formula:

$$\% \text{ of } P = \frac{31 \times m_1 \times 100}{1877(Mr) \times m}$$

3.8. The colorimetric determination of Phosphorus in plant materials

Five grams of each plant material were ashed at 550^{o} overnight. Samples were then moistened with 5 ml of concentrated hydrochloric acid. The acid was evaporated to dryness and the residues were moistened with 5ml of concentrated hydrochloric acid, and gently boiled for 2 minutes. Twenty five milliliters of water were added and boiled. The solutions were filtered and the filtrate and washings were made up to 250 ml to produce solution (A) for each sample. Ten milliliters of A was then pipetted into a 50 ml graduated cylinder and 10 ml of 5M hydrochloric acid was added. This was diluted to 50 ml with water. Five milliliters of this solution were pipetted into a dry glass-stoppered tube and left in a water-bath at 20^{o} for 5 minutes. Five milliliters of mixed reagent (R) were added at 20^{o}. The reagent was prepared by dissolving 5g of ammonium molybdate and 0.25g of ammonium vanadate in warm water. The solution was cooled in open air, diluted to 500 ml and filtered. The solutions A and R were mixed by inverting the tubes several times. The tubes were left to remain in the water-bath at 20^{o} for 5 minutes and then the transmittancy at 400nm was measured using a Spectrophotometer.

3.9. Plant and soil analysis

After the six weeks, soil samples were measured for phosphorus using the quantitative determination of phosphorus from the soil described above. The seedlings were measured for phosphorus, using the colorimetric determination of phosphorus in plant materials described above. Plant height was measured using a measuring tape. Finally, the obtained data was analyzed using IBM SPSS Statistics ver. 21 and a two-way ANOVA was performed so as to compare the plant height and the plant phosphorus.

CHAPTER 4

4.0. RESULTS

4.1. Sorghum plant height

The means plot (SPSS appendix 1) suggests that there was no interaction between the treatment (AM fungi) and the soil type in influencing the plant height (two-way anova: $p = 0.534$) as there was no significant difference. The treatment significantly increased plant height (two-way anova: $p = 0.013$) and the soil type significantly increased plant height (two-way anova: $p = 0.001$).

Table 4.1 Mean values for plant height across all treatment groups

Treatment	Soil type	Mean ± SD
+ AM fungi	Sandy	41.00 ± 5.292
	Red	28.67 ± 4.163
	Black	32.67 ± 3.055
-AM fungi	Sandy	35.33 ± 4.509
	Red	26.33 ± 1.528
	Black	25.67 ± 1.155

AM fungi inoculum increased plant height only in the inoculated sorghum plants as compared to non-inoculated plants. The mean plant height was higher in sorghum plants inoculated with AM fungi. The soil type sandy had the highest (41±5.292 (SD) and red soil had the lowest (28.67 ± 4.163 (SD). The non-inoculated control plants had a lower mean than the treated plants. Sandy soil had the highest mean and black soil had the lowest (Table 4.1, SPSS output appendix 1).

4.2. Sorghum plant phosphorus

The means plot (SPSS appendix 2) suggests that there was no interaction between the treatment (AM fungi) and the soil type in influencing the plant phosphorus (two-way anova: $p = 0.073$) as there was no significant difference. The treatment significantly increased plant phosphorus (two-way anova: $p = 0.00$) and soil type significantly increased plant phosphorus (two-way anova: $p = 0.00$).

Table 4.2 Mean values for plant phosphorus across all treatment groups

Treatment	Soil type	Mean ± SD
+ AM fungi	Sandy	0.357 ± 0.0075
	Red	0.339 ± 0.0035
	Black	0.342 ± 0.0020
-AM fungi	Sandy	0.239 ± 0.0035
	Red	0.216 ± 0.0050
	Black	0.206 ± 0.0115

AM fungi inoculum increased phosphorus only in the inoculated sorghum plants with as compared to non-inoculated plants. The mean plant phosphorus was higher in sorghum plants inoculated with AM fungi. The soil type sandy had the highest (0.357±0.0075 (SD) and red soil had the lowest (0.339 ± 0.0035 (SD). The non-inoculated control plants had a lower mean than the treated plants. Sandy soil had the highest mean and black soil had the lowest (Table 4.2, SPSS output appendix 2).

CHAPTER 5

5.0. DISCUSSION

AM fungi are a potential substitute to replenish the depleting reservoirs of phosphorus, particularly in tropical and subtropical soils (Kang *et al*., 2012). The direct application of low cost cultured AM fungi propagules is an important approach to decrease the use of chemical fertilizers and improve phosphorus supply for sustainable crop production (Saxena, Amita, Indu, Shalini and Veena, 2015).

The response of the inoculated plants was found significantly higher than the non-inoculated plants. AM fungi stimulated sorghum roots to absorb nutrients from soil and thus enhanced the overall plant growth as compared to the treatments having no AM fungi inoculum. The microbial activities stimulated nutrient uptake and plant growth may have been due to hormones such as auxin or gibberellic acid production (Minaxi, Saxena, Chandra and Nain, 2013).

5.1.1. Sorghum plant height

As expected the sorghum seedlings inoculated with AM fungi had the higher mean height in comparison to the non-inoculated control seedlings. In this experiment, the higher growth of sorghum observed was due to plant growth promoting activities of AM fungi in the rhizosphere. This is also attributed to absorbance of more phosphorus from the soil and its accumulation towards shoots, resulting in increased shoot and root dry weight and plant height (Parewa, Rakshit, Rao, Sarkar and Raha, 2010). Sandy soil had the highest mean across all treatment groups, this is probably due to the fact that sorghum grows better in sandy soil, as there was no interaction between the soil type and the AM fungi inoculum.

Black and red soil had lower mean heights, this is probably a result of the clay-loam texture of the soil, as well as the fact that containerized roots stop growing because of constraints imposed by pot boundaries (Gosling, Jones and Bending, 2015).

5.1.2. Sorghum plant phosphorus

The mean plant phosphorus was higher in sorghum plants inoculated with AM fungi. However phosphorus uptake differed across sorghum plants from the different soil types. The soil type sandy had the highest and red soil had the lowest. This could be a result of the different nutrient levels amongst the soil types, as they all had different phosphorus readings before the experiment (sandy-0.7%, red-0.6% and black-0.535%) (Tanwar, Aggarwal, Kadian and Gupta, 2013).

The non-inoculated control plants had a lower mean than the treated plants. Sandy soil had the highest mean and black soil had the lowest. The means plot suggests that there was no interaction between the treatment (AM fungi) and the soil type in influencing the plant phosphorus. The treatment and soil type independently influenced the plant phosphorus.

Optimal benefits are therefore more likely to be obtained from inoculation after a careful selection of the favorable host, niche and fungus combinations. The current general trend is to try one or more species of AMF for individual inoculation (mono species inoculum), as seen in some of the previous experiments (Gosling *et al*., 2015).

Some experiments have been done to compare the synergistic effects of AM fungi and phosphate solubilizing bacteria (PSB) on nutrients solubility and uptake (Zhang, Barberán, Zhu, Zhang and Han, 2014). During interaction and nutrients solubility the PSB produce enzymes and secrete organic acids and biological materials such as auxins, gibberlic acid, vitamins and hormones that increase the dissolution of phosphate (He, Bian and Zhu, 2002). The PSB increases the soil phosphorus pool available for AM fungi.

5.2. Conclusion

It is concluded that the inoculation of AM fungi is a promising approach to manage phosphorus sustainability in phosphorus deficient soils. Moreover, inoculation of plants by AM fungi is beneficial to minimize dependence on phosphate-based fertilizers. Hence, AM fungi can be used as a useful strategy to prepare bio-fertilizers and solving the phosphorus problems in deficient soils. Culturing of the inoculum although lengthy, is feasible for all farmers, smallholder and commercial.

5.3. Recommendations

This study has shown that AM fungi have a positive effect on plant physiology and nutrition uptake particularly phosphorus. The presence of AM fungi can also be checked by quantification of an AM specific fungal biomarker (Baquall and Das, 2010). However, more molecular and technological (High-throughput next generation sequencing) studies need to be carried out to identify which species is more effective on crop plants, after which open-field experiments can be conducted to measure the performance of inoculated plants under uncontrolled conditions. Further research work is suggested to investigate the effects of the use of AM fungi for various crops under different agro-ecological conditions.

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APPENDICES

Appendix 1: Two-way Anova SPSS output for plant height

Descriptive Statistics

Dependent Variable: Height

Treatment	Soil type	Mean	Std. Deviation	N
+ AM Fungi	Sandy soil	41.00	5.292	3
	Red soil	28.67	4.163	3
	Black soil	32.67	3.055	3
	Total	34.11	6.585	9
- AM Fungi	Sandy soil	35.33	4.509	3
	Red soil	26.33	1.528	3
	Black soil	25.67	1.155	3
	Total	29.11	5.278	9
Total	Sandy soil	38.17	5.382	6
	Red soil	27.50	3.082	6
	Black soil	29.17	4.355	6
	Total	31.61	6.335	18

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Height

F	df1	df2	Sig.
1.789	5	12	.190

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

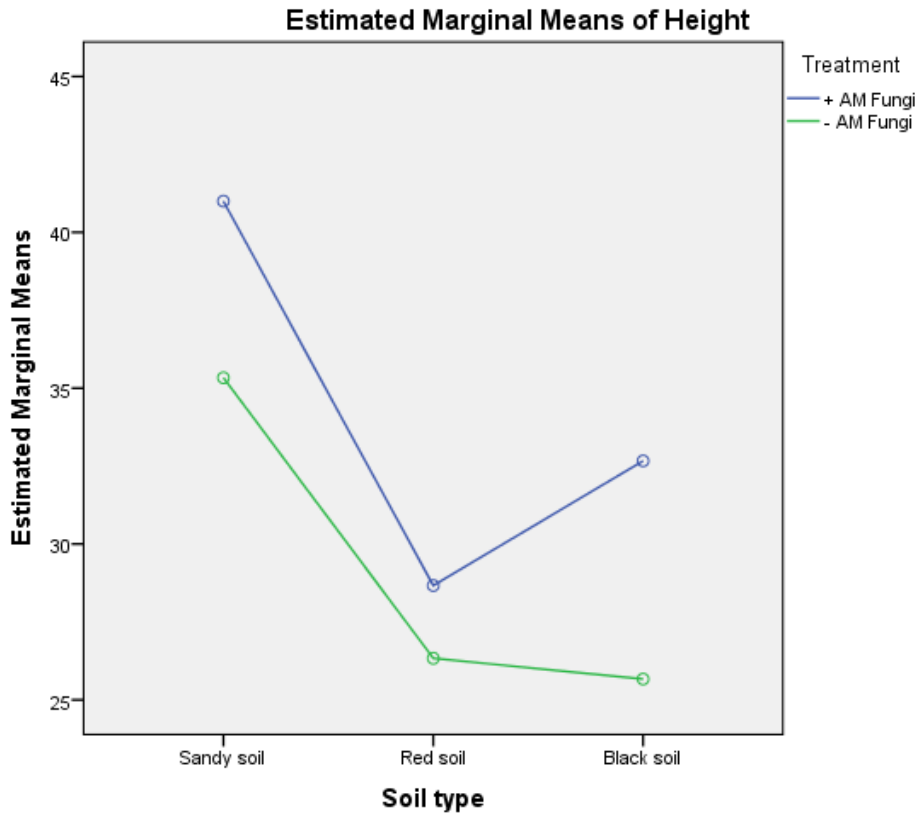
a. Design: Intercept + Treatment + Soil + Treatment * Soil

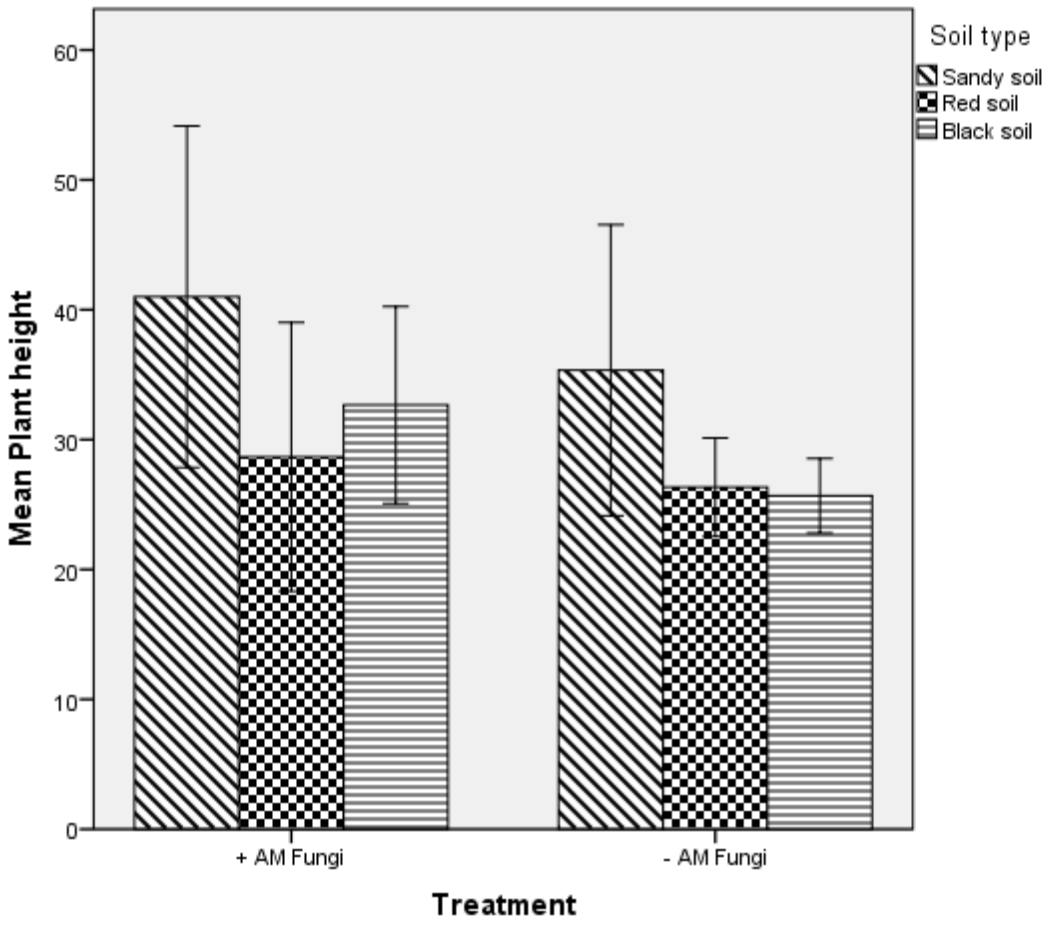
Tests of Between-Subjects Effects

Dependent Variable: Height

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	524.944[a]	5	104.989	8.008	.002
Intercept	17986.722	1	17986.722	1371.869	.000
Treatment	112.500	1	112.500	8.581	.013
Soil	395.111	2	197.556	15.068	.001
Treatment * Soil	17.333	2	8.667	.661	.534
Error	157.333	12	13.111		
Total	18669.000	18			
Corrected Total	682.278	17			

a. R Squared = .769 (Adjusted R Squared = .673)




Soil type
Sandy soil
Red soil
Black soil
Mean Plant height
60
50
40
30
20
10
0
+ AM Fungi
- AM Fungi
Treatment
Error bars: 95% CI

Appendix 2: Two-way Anova SPSS output for plant phosphorus

Dependent Variable: Plant phosphorus

Treatment	Soil type	Mean	Std. Deviation	N
+ AM Fungi	Sandy soil	.35767	.007506	3
	Red soil	.33967	.003512	3
	Black soil	.34267	.002082	3
	Total	.34667	.009381	9
- AM Fungi	Sandy soil	.23933	.003512	3
	Red soil	.21600	.005000	3
	Black soil	.20600	.011533	3
	Total	.22044	.016187	9
Total	Sandy soil	.29850	.065025	6
	Red soil	.27783	.067845	6
	Black soil	.27433	.075221	6
	Total	.28356	.066197	18

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Plant phosphorus

F	df1	df2	Sig.
1.456	5	12	.274

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

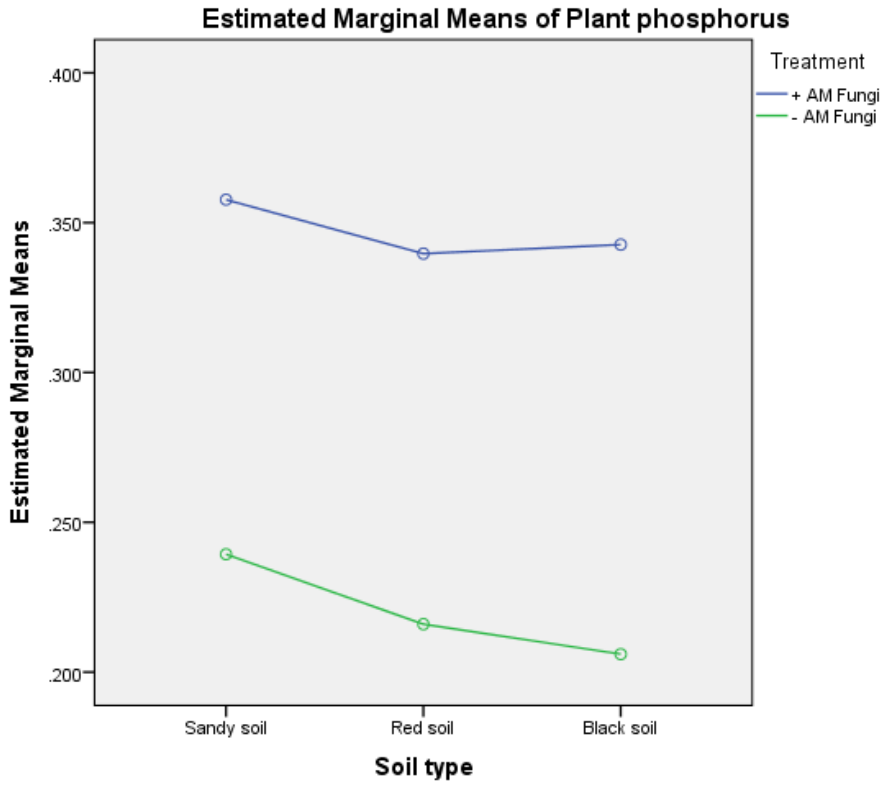
a. Design: Intercept + Treatment + Soil + Treatment * Soil

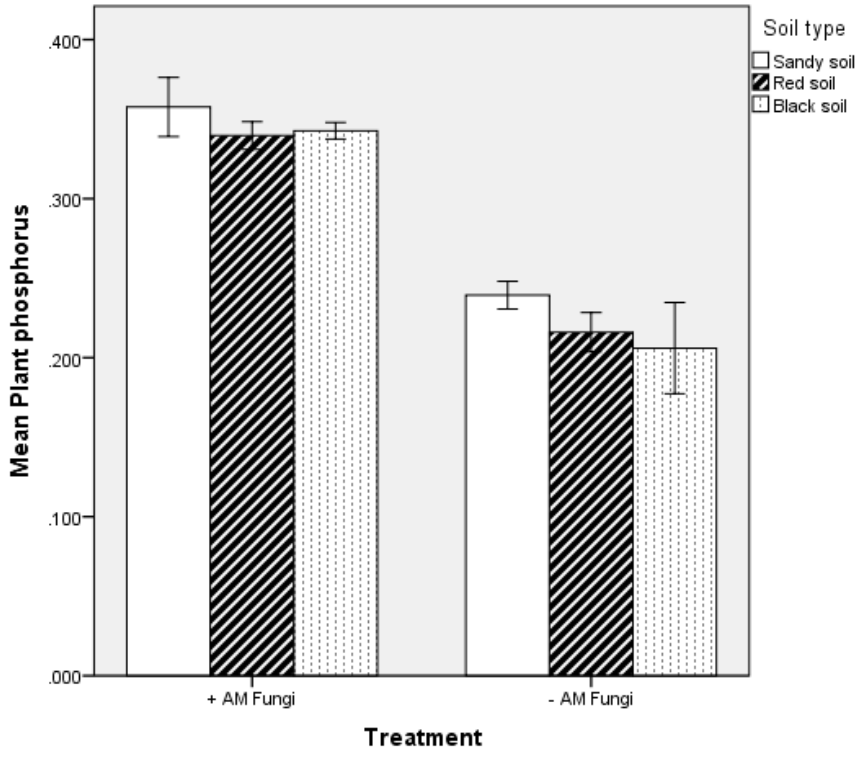
Tests of Between-Subjects Effects

Dependent Variable: Plant phosphorus

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.074[a]	5	.015	364.970	.000
Intercept	1.447	1	1.447	35686.049	.000
Treatment	.072	1	.072	1767.803	.000
Soil	.002	2	.001	25.234	.000
Treatment * Soil	.000	2	.000	3.289	.073
Error	.000	12	4.056E-005		
Total	1.522	18			
Corrected Total	.074	17			

a. R Squared = .993 (Adjusted R Squared = .991)





Error bars: 95% CI

Appendix 3: First and Second soil sample analysis

First soil analysis

Soil Type	Phosphorus %
Sandy	0.7
Red	0.6
Black	0.535

Second soil analysis

Sample	Soil type + treatment	Phosphorus %
1	S+	0.342
2	R+	0.260
3	B-	0.340
4	S-	0.461
5	B+	0.194
6	R-	0.389
7	R+	0.257
8	B-	0.317
9	S+	0.335
10	R-	0.384
11	B+	0.190
12	S-	0.464
13	B-	0.330
14	R+	0.264

15	S-	0.457
16	R-	0.379
17	S+	0.350
18	B+	0.193