

ABSTRACT

SHAH, GARUD. Variation in Levels and Effects of Arbuscular Mycorrhizal Root Colonization Among Maize Lines. (Under the direction of Dr. Peter Balint-Kurti)

Arbuscular mycorrhizal (AM) fungi are found all over the world in natural soil and colonize the majority of land plants, including many crop species. First, we developed a robust assay to find the good AM inoculum (inoculum prepared in the leek and marigold plants) and the pot size, where AM fungi colonizes the maize roots. Next, we studied how the different fertilizer concentrations affect the root colonization in maize and found higher concentrations significantly decreased the root AM colonization in B73 and W22 maize lines. Then, we evaluated root colonization level among 31 diverse maize inbred lines and derived hybrids in replicated trials in two field locations with high and low phosphorus conditions and 30 inbred lines under controlled conditions in the greenhouse. In the greenhouse, we assessed the effect of AM inoculation on various agronomic traits that included plant height, chlorophyll content, shoot and root biomass, and shoot nutrient content. A lower correlation in root colonization between replications in one field locations ($r=0.28$, $p<0.05$, high phosphorus locations) and no significant correlation in the other locations (low phosphorous locations) compared to the greenhouse experiment ($r=0.59$, $p<0.001$) and significant variation in AM colonization among maize lines was observed. However, this variation was not consistent across conditions. In the greenhouse experiment, a positive correlation was observed between root colonization and shoot biomass ($r=0.45$, $p<0.001$), root biomass ($r=0.11$, $p<0.05$), plant height ($r=0.34$, $p<0.001$), chlorophyll content ($r=0.42$, $p<0.001$), and shoot potassium content ($r=0.36$, $p<0.001$), whereas a negative correlation between root colonization and shoot nitrogen ($r= -0.17$, $p<0.01$) and phosphorus content ($r= -0.19$, $p<0.001$). Thus, evaluation of diverse maize lines showed variation in AM root colonization among maize lines and AM symbiosis can be helpful to host plants. We also, evaluated the effect of a mutation in the *CASTOR*

gene, which has been shown to be essential for AM colonization, in two lines: B73 and W22 (wild type and *CASTOR* mutant) in the same two field locations and found that *CASTOR* mutant plant stand and growth were worse compared to wild type in both locations.

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Variation in Levels and Effects of Arbuscular Mycorrhizal Root Colonization Among Maize

Lines

by
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DEDICATION

Dedicated to my parents, Balint-Kurti's lab, Garcia's lab, and researcher working with the Arbuscular Mycorrhizal Fungi.

BIOGRAPHY

Garud Shah, the son of the late Mahendra Bikram Shah and Mrs. Rajeshwori Chand (Shah), was born in a small city in the far-western region of Nepal (a small country). Growing up, he witnessed his parents facing numerous challenges in the agricultural sector, that sparked his interest in agricultural sciences and his desire to become a researcher dedicated to solving farmers' problems. To pursue his aspirations, he successfully completed his Bachelor of Science in Agriculture in 2018 from the Agriculture and Forestry University in Rampur, Chitwan, Nepal. Throughout his undergraduate studies, he gained extensive knowledge in various agricultural science disciplines, including Agronomy, Soil Science, Horticultural Science, Plant Breeding and Genetics, Entomology, and Plant Pathology.

Given that Nepal is a developing country with a limited focus on research, Garud recognized the need to extend his knowledge and research experience. To achieve this, he joined North Carolina State University in Raleigh, North Carolina, USA in 2021 spring, to pursue a master's degree in the Plant Pathology, working under the guidance of Dr. Peter Balint-Kurti and Dr. Kevin Garcia. As a master's student, Garud actively participated in various research projects, that provided him with invaluable research experience and further deepened his understanding of the field.

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Garud Shah

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CHAPTER 1

Literature Review

1.1 Introduction to Different Types of Mycorrhizal Symbiosis

Mycorrhizal fungi are important microorganisms found in nature that form symbiotic relationships with different plant hosts. There are five different types of mycorrhizae (Fig 1.1): (1) ectomycorrhizal (EM) fungi, (2) arbuscular mycorrhizal (AM) fungi, (3) orchid endomycorrhizal fungi, (4) ericoid mycorrhizal (ERM) fungi, and (5) ectendomycorrhizal fungi.

About 5% of land plants form ectomycorrhizal symbiosis. Plants that form symbiotic relationships with ectomycorrhizal fungi are generally woody perennials. They are characterized by the formation of three pseudo-tissues: a sheath or mantle, Hartig net, and extraradical hyphae. A dense mass of fungal tissue that encloses the plant roots is called the sheath or mantle and helps in bidirectional movement of nutrients. Between the cortical and epidermal cells, a labyrinthine inward growth of hyphae occurs, called a Hartig net that provides a large surface area and helps in resource exchange of water and nutrients. The fungal hyphae that grow outside the plant roots are called extraradical hyphae and their function is the mobilization, absorption, and translocation of mineral nutrients and water from the soil substrate to plant roots.

Arbuscular mycorrhizal fungi are the most abundant type of mycorrhizal fungi found in the soil and described in more detail in section 1.2. They form symbiotic relationships with more than 80% of land plants including many crop species. When they internally colonize plant roots, they develop intraradical hyphae, arbuscules, and sometimes vesicles. The fungal hyphae that grow outside of plant roots are called extraradical mycelium whereas fungal hyphae that grows inside of the plant roots are called intraradical mycelium. Fungal hyphae that form bladder-like, swollen

structures are called vesicles. The compact mass of fungal hyphae or branched finger-like structure of hyphae is called arbuscules and they are formed inside the cortical cells. The formation of arbuscules with or without vesicles is considered a functional indication for AM symbioses in the plant roots.

Orchid mycorrhizal fungi exclusively form symbiotic relationships with plants belonging to the Orchidaceae family. Orchid mycorrhizal fungi form extraradical mycelium and pelotons (a dense mass of coiled hyphae) in the plant host. Pelotons increase the interfacial surface area between fungus and orchids to facilitate nutrient exchange.

Ericoid mycorrhizal fungi form associations with plants from the Ericaceae family such as cranberry, blueberry, and huckleberry. They form structures like hyphal mantles/sheaths, pelotons, and extraradical mycelium.

Finally, ectendomycorrhizal fungi show features of both ectomycorrhizae and endomycorrhizae and associate primarily with species in the genus *Pinus* (Pine), *Picea* (Spruce) and, to a lesser extent, *Larix* (Larch). These mycorrhizal fungi form extraradical mycelium, hyphal mantle, and pelotons in plant roots (Smith & Read, 2008).

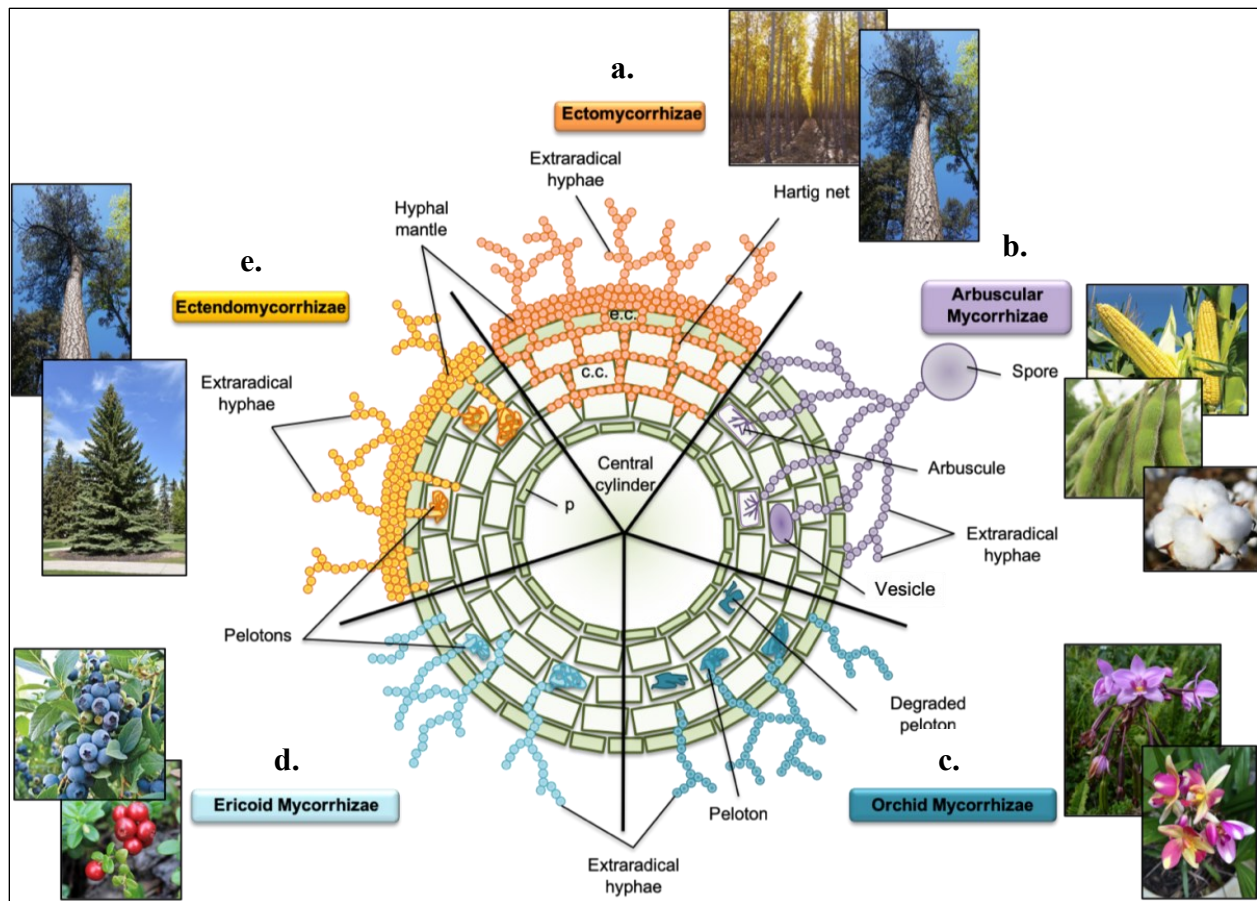


Figure 1.1: Different structures formed in plant roots by various types of Mycorrhizal Fungi. (a) Ectomycorrhizae, (b) Arbuscular mycorrhizae, (c) Orchid mycorrhizae, (d) Ericoid mycorrhizae, and (e) Ectendomycorrhizae and a few of their plant hosts. (a) Ectomycorrhizal fungi form symbiotic relationships with roots of perennial trees and form structures called hyphal mantle, extraradical hyphae, and Hartig net. (b) Arbuscular mycorrhizal fungi form symbiotic relationships with most of the land plants including many crop species and form extraradical hyphae, sometimes vesicles, and arbuscules in the plant roots. (c) Orchid mycorrhizal fungi are unique and colonize the roots of orchid plants only. (d) Ericoid mycorrhizal fungi form symbiotic relationships with plants from the Ericaceae family. Both orchid and ericoid mycorrhizae form extraradical hyphae and peloton in host plant roots. (e) Ectendomycorrhizal fungi show the characteristics of both ectomycorrhizae and endomycorrhizae and form symbiotic relationships with trees like pine and spruce. They form extraradical hyphae, mantle, and pelotons in the plant roots. (Source: Dr. Kevin Garcia at <https://cals.ncsu.edu/crop-and-soil-sciences/news/fungi-fertilize-the-future/>)

1.2 Arbuscular Mycorrhizal Fungi

Arbuscular mycorrhizal (AM) fungi are important beneficial microorganisms found all over the world. AM fungi played a crucial role in colonizing the land habitats by green plants (Helgason & Fitter, 2005). All fungi that form arbuscular mycorrhizae belong to the subphylum Glomeromycotina (Spatafora et al., 2016) and form symbiotic association with most land plants. Some plants belonging to families like Brassicaceae (Mustard, Radish, Cabbage, Cauliflower), Chenopodiaceae (Beets, Spinach, Swiss Chards), Caryophyllaceae (Carnation), Polygonaceae (Buckwheat), Juncaceae (*Juncus*), Proteaceae (*Protea*, Macadamia) are not able to form a symbiotic relationship with AM fungi (Smith & Read, 2008). The plants of these families have lost the orthologs of symbiotic genes (see section 2.2), resulting in their inability to form symbiotic associations with AM fungi (Cosme et al., 2018).

1.2.1 Development of Arbuscular Mycorrhizal Fungi in Plant Roots

AM fungi colonize the plant roots when there is exchange of signaling molecules between both partners. Plant roots exude a class of plant hormones called strigolactones that trigger a change in fungal development after fungal spore germination. Fungal perception of strigolactones induces the pre-symbiotic phase (Fig 1.2) that is characterized by continued hyphal growth, increased physiological activity, and profuse hyphal branching (Besserer et al., 2006; Parniske, 2008).

Hyphopodia, a special type of appressoria (specialized infection structures of fungi), are formed by AM fungi, which is developed from mature hyphae (Fig 1.2). Plant root cells form a prepenetration apparatus (PPA) resulting from sequential chemical and mechanical stimulation by the growing hyphae (Genre et al., 2005). The PPA appears as a cytoplasmic column that contains microtubule and microfilament bundles, dense endoplasmic reticulum cisternae, and a central

membrane thread. Once formation of a column occurs, the fungus enters the cell through column and grows across cortical cells (Siciliano et al., 2007). Fungal hyphae extend from the hyphopodium enter the PPA and guides the fungus towards the cortical cells. The fungus after entering the root cells leaves it and enters the apoplast where branching and lateral growth along the root axis (Genre et al., 2005).

Hyphae branches form an arbuscules inside the inner cortex. Exchange of nutrients take place through arbuscules between fungus and the plant roots (Parniske, 2008). Vesicles that store lipid and carbohydrates are also formed by 80% of AM fungi (Smith & Read, 2008). *Scutellospora* and *Gigaspora* from the family Gigasporaceae do not develop vesicles. Members of other genera develop vesicles either intercellularly or intracellularly in the cortex (Abbott, 1982). New spores are formed at the leading tip of individual fungal hyphae outside of the plant root (Parniske, 2008).

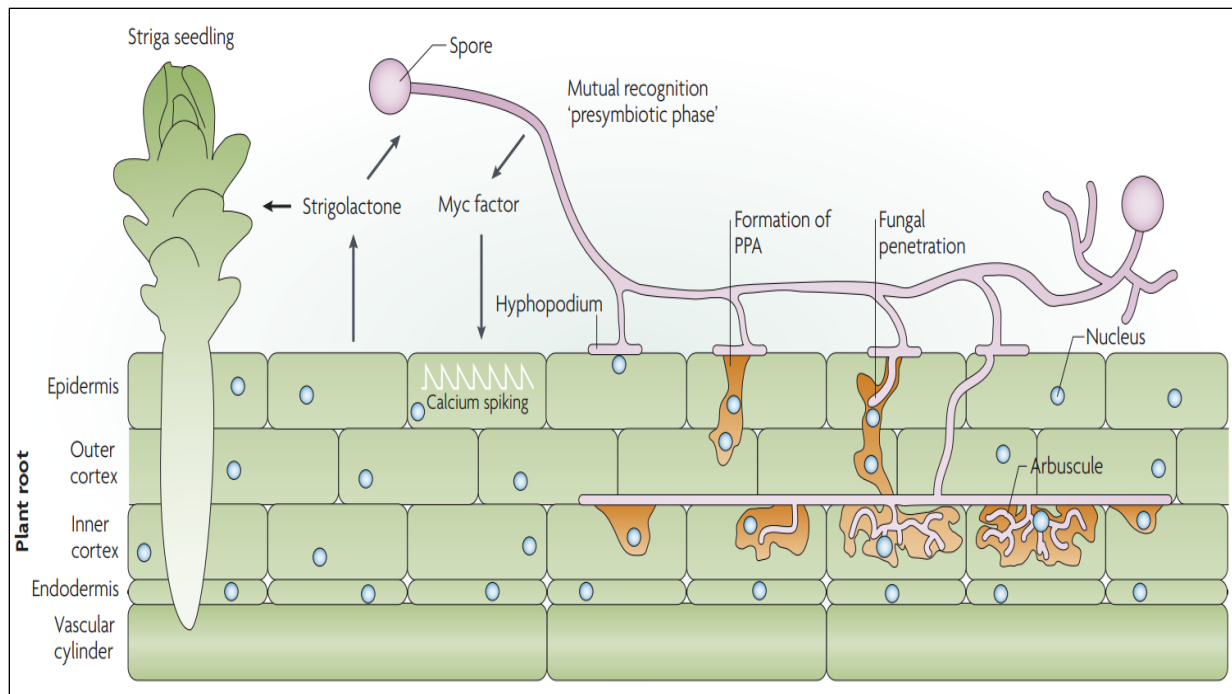


Figure 1.2: Development of AM fungi in plant roots. Plant roots exude strigolactones which triggers hyphal branching, increase its physiological activity, and induces the pre-symbiotic phase. Also, strigolactones trigger the germination of striga plants. From a mature hypha, AM fungi formed a hyphopodium on the root surface. With sequential chemical and mechanical stimulation, pre-penetration apparatus (PPA) will be formed on the root. Then, the fungus enters the root cell through PPA into the cortex where branching and lateral growth of fungus along the root axis takes place forming the arbuscules in the root cortical cells. (Source: Parniske, 2008)

1.2.2 Molecular and Genetic Mechanisms for the Establishment of AM Symbiosis in Plant Roots

AM fungi produces lipo-chitooligosaccharides (LCOs) and chitooligosaccharides (COs) that are perceived by the host plants, resulting into initiation of symbiotic responses (Genre et al., 2013). Molecular mechanisms for AM symbiosis were largely studied in *Medicago truncatula* and *Lotus japonicus*. Fungal LCOs and COs are perceived by LysM receptor kinases present in plasma membrane in combination with SYMRK (DMI2 in *Medicago truncatula* or NORK in *Medicago sativa*). CASTOR and POLLUX are the calcium channels that facilitate calcium movement between the cytoplasm and nucleus resulting into calcium spiking in plant nuclei (Parniske, 2008).

This early stage of symbiotic signal transduction can increase or decrease nuclear calcium concentrations which results in regulation of downstream symbiosis-related gene expression is called calcium spiking (Kim et al., 2019). The calcium-calmodulin-dependent protein kinase (CCaMK) (DMI3 in *Medicago truncatula*) and a phosphorylation substrate - CYCLOPS (IPD3 in *M. truncatula*) form a complex within the nucleus. The formation of this complex decodes the calcium signatures that are induced by AM fungi (Parniske, 2008). Finally, CCaMK activates in an AM-specific mode thereby regulating AM-specific gene expression (Fig 1.4).

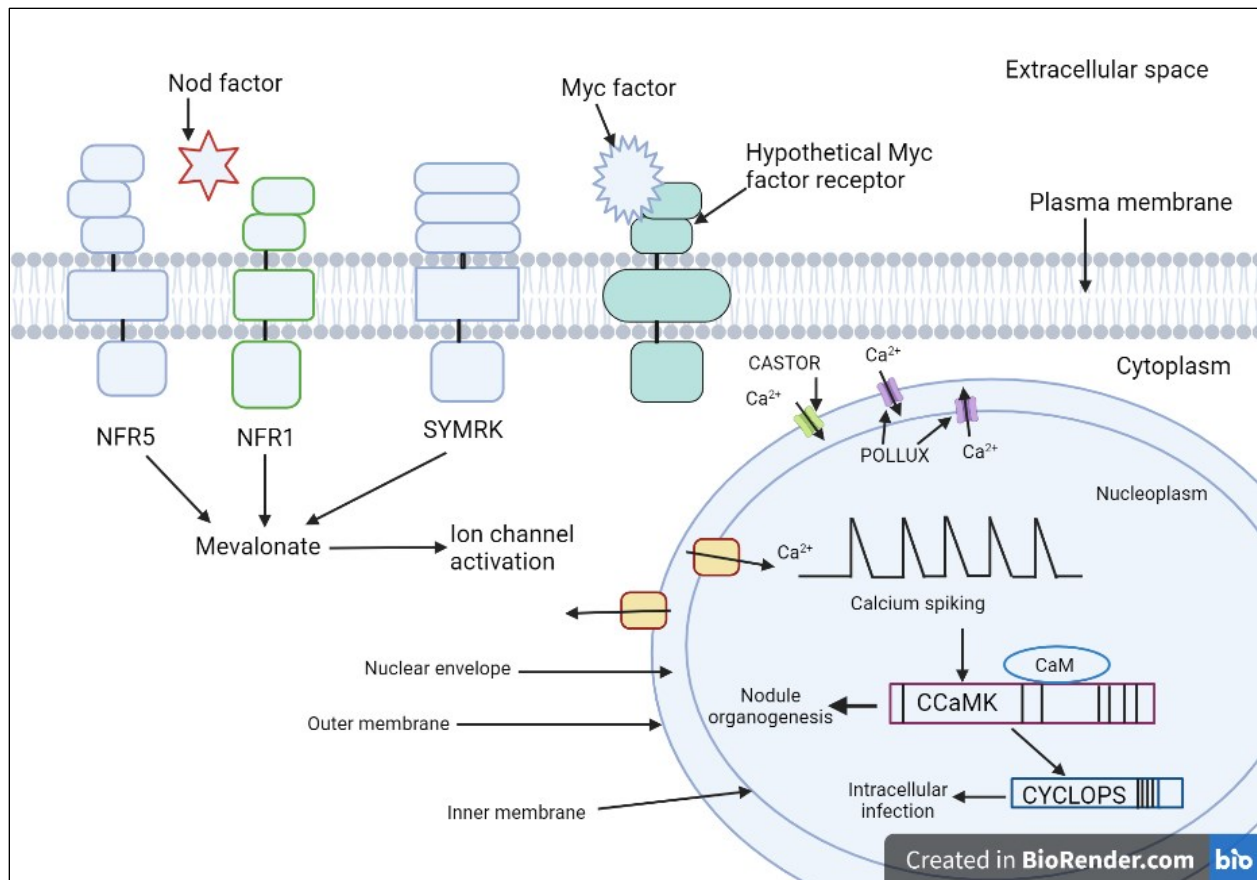


Figure 1.3: Molecular and genetics mechanisms for the AM symbiosis in plants. Myc-factors are perceived by LysM receptor kinases present in plasma membrane in combination with SYMRK (DMI2 in *Medicago truncatula* or NORK in *Medicago sativa*). Similarly, Nod-factors (compounds secreted by Rhizobia - a root nodule bacteria) are perceived by Nod-factor receptor kinase NFR1 and NFR5. “The mevalonate pathway is also necessary for the earliest responses of plants to symbiotic signals produced by nitrogen-fixing rhizobia and AM fungi” (Venkateshwaran et al., 2015). The signal perception induced calcium spiking in the nucleus in presence of calcium ion channel - CASTOR and POLLUX (DMI1 in *Medicago truncatula* and SYM8 in pea). The calcium-calmodulin-dependent protein kinase (CCaMK) (DMI3 in *Medicago truncatula*) and a phosphorylation substrate- CYCLOPS (IPD3 in *M. truncatula*) forms a complex within the nucleus. The formation of this complex might decode calcium signatures induced by AM fungi (Parniske, 2008). Finally, CCaMK activates in an AM-specific mode thereby regulating AM-specific gene expression. If CCaMK does not form a complex within the nucleus, this protein will regulate nodule-specific gene expression in legume plants. (modified the figure from Parniske, 2008).

1.3 Benefits of Arbuscular Mycorrhizal Fungi

Deficiency of mineral nutrients will have detrimental effects on plant growth and development. AM fungi play vital roles in the nutrient uptake of most land plants including many important cultivated crop species. During the formation of symbiotic association, extraradical hyphae extend beyond the rhizosphere and increase root surface area for uptake of soil nitrogen (N), phosphorus (P), potassium (K), or micronutrients (Chen et al., 2017). When nutrients are sufficient or not limiting, AM fungi can negatively impact plant growth (i.e., when the advantage to the host outweighs the expense of maintaining the mycorrhizal fungi) (Kaeppeler et al., 2000). AM fungi improve plant nutrient acquisition under nutrient limiting conditions or prevent nutrients accumulation when present in excess amounts (Merlos et al., 2016).

1.3.1 Phosphorus (P)

Generally, AM fungi in a P limited environment, contributes to inorganic P (Pi) nutrition of their hosts (Smith et al., 2010). AM fungi play a vital role in increasing total P uptake of *Lactuca sativa* (lettuce) and *Abutilon theophrasti* (velvet leaf) and P use efficiency in *Abutilon* and *Beta vulgaris* (beet) (Koide, Goff & Dickie, 2000). P uptake by AM fungi can increase the plant growth and yield (Ibijbijen et al., 1996). For example, tomato plants inoculated with AM have increased leaf area, N, P, Ca, and K content (Balliu, Sallaku & Rewald, 2015). Similarly, plant roots colonized by AM can increase N and P uptake (George, Marschner & Jakobsen, 1995) in AM inoculated plants compared to non-inoculated plants. However, disruption of AM symbiosis significantly reduced the P uptake, growth, and yield in sunflower (Thompson, 1987).

1.3.2 Nitrogen (N)

Nitrogen is an important limiting nutrient primarily due to leaching in land-based ecosystems (Vitousek & Howarth, 1991). Thirkell, Pastok & Field (2020) and Hodge & Fitter (2010), respectively, in three different cultivars of wheat and *Plantago lanceolata* (plantain) reported significant N transfer from fungus to plant in host in AM inoculated plants compared to non-inoculated AM plants. AM fungi can transfer inorganic N to their host in the form of NO_3^- or NH_4^+ (Govindarajulu *et al.*, 2005). Under greenhouse conditions under double the atmospheric carbon dioxide concentration, carbon and N accumulation, N recovery rate, and N use efficiency were significantly higher in AM inoculated wheat plants compared to non-AM plants (Zhu *et al.*, 2016).

1.3.3 Potassium (K)

Potassium is one of the important plant macronutrients involved in processes like photosynthesis, enzyme activation, protein synthesis, and osmotic potential in the plant (Marschner, 2012). AM fungi can play an important role in uptake of K (Garcia & Zimmermann, 2014). AM fungi enhanced the uptake of K in lettuce (Baslam, Garmendia & Goicoechea, 2013), pelargonium (Perner *et al.*, 2007), *Vicia faba* (broad bean) (Shi *et al.*, 2021), and *M. truncatula* (Kafle *et al.*, 2022) in AM inoculated plants compared to non-AM plants in a controlled environment.

1.3.4 Micronutrients

In plants, micronutrients are needed in smaller quantities than macronutrients and are very important for cellular metabolism. The micronutrients copper, iron, and manganese are crucial for protein, lipid, and carbohydrate synthesis (Marschner, 2012). Lack of these nutrients can impact plant growth and development. If these nutrients are present in excess amounts, they can be toxic

to plants. AM fungi play an important role on plant micronutrient status and facilitate macronutrient and micronutrient uptake (Faber et al., 1990; Ruytinx et al., 2020). In presence of high concentrations of zinc, copper, and iron in soil, AM fungi decreased the concentrations of these nutrients in shoots of mycorrhizal plants compared to non-mycorrhizal plants (Dueck *et al.*, 1986; Weissenhorn *et al.*, 1995).

1.3.5 Carbon (C)

In exchange for these macro and micronutrients, plants provide photosynthates i.e., sugars and/or lipid to the fungus for their growth and development (Y. Jiang et al., 2017). Plant hosts may provide up to 20% of C to their AM symbionts (Bago, Pfeffer & Shachar-hill, 2000). The transfer of carbohydrates and fatty acids from plants to fungal partners (Keymer et al., 2017), comprises up to 5 trillion kilograms of C per year worldwide (Bago, Pfeffer & Shachar-hill, 2000). Thus, AM fungi are an important source of soil C. Jones, Nguyen & Finlay (2009) provided evidence that AM fungi play an important role in soil C cycles. More recently, it was reported that approximately 3.93 trillion kilograms of carbon dioxide (CO₂) equivalent are absorbed by land plants and stored for certain period of time in the mycelium of AM fungi. This amount is roughly equivalent to 11% of total CO₂ emissions produced by burning fossil fuels (Hawkins et al., 2023). Therefore, AM fungi play an important role in C pool.

1.3.6 Other benefits of AM fungi

The symbiotic association of AM fungi and plants are good examples of mutualistic relationship that regulates plant growth and development (Begum et al., 2019). AM fungi can enhance lateral root development in angiosperms by triggering initiation of lateral root primordia and/or increasing emergence of pre-formed lateral root primordia (Paszkowski & Orvo, 2022). AM fungi affects the

production of carotenoids and certain volatile compounds that enhanced the dietary quality of tomatoes (Hart et al., 2015). Accumulation of anthocyanins, total soluble phenolics, carotenoids, chlorophyll, tocopherols, and various mineral nutrients are enhanced by AM fungal symbiosis in lettuce (Baslam, Garmendia & Goicoechea, 2011). The AM fungus *Glomus versiforme* enhances fruit quality by increasing sugars, organic acids, vitamin C, flavonoids, and minerals contents in citrus (Zeng et al., 2014). Further, AM fungi can enhance potato yield in commercial production field experiments. Commercial AM fungi inoculant: Myke® Pro Potato-L containing *Rhizophagus irregularis* DAOM 197198 significantly increased potato yield of AM plants compared to non-AM plants (Hijri, 2016).

Nutrient uptake is often the focus for most AM research on AM investigations. But there are other benefits of AM fungi as well in plants. They play an important role in crop pests and disease suppression. Particularly AM limit disease severity caused by soil-borne plant pathogens (Newsham et al., 1995; Whipps 2004). AM fungi alter plant morpho-physiological traits and can improve plant tolerance to increased abiotic stress such as saline conditions and heavy metal contamination (Alqarawi, Abd Allah & Hashem, 2014; Shetty, Hetrick & Schwab, 1995). Moreover, considerable evidence can be found that suggest AM improve drought resistance (Augé et al., 2001) and soil particle aggregation by binding microaggregates into larger macroaggregates with fungal hyphae (Rillig & Mummey, 2006).

1.4 Factors Affecting AM Colonization

AM fungi benefits can be seen in nutrient limiting conditions and the use of high fertilizers, especially high concentration of P, can suppress AM colonization (Olsson et al., 1997; Hetrick et al., 1996; Kaeppler *et al.*, 2000; Sawers *et al.* 2017; Liu *et al.*, 2018), abundance, and diversity (Camenzind et al., 2014; Treseder & Allen, 2002; Wang et al., 2018). Plants strigolactone synthesis

is highly dependent on phosphate nutrition. When phosphate fertilizer is low, strigolactone synthesis in plant roots is high and provides favorable conditions for AM symbiosis (Yoneyama et al., 2008). Further, under the conditions of high soil P, when AM colonization still occurs then it may reduce crop growth (Kahiluoto et al., 2001). There are reports showing that soluble N fertilizers can negatively impact levels of AM colonization (Liu et al., 2000; Albizua et al., 2015), whereas other studies found no significant effect of N on AM symbiosis (Tian et al., 2013). The N:P ratio (Williams et al., 2017), and soil pH (Rousk et al., 2010) can also affect the AM community and AM symbiosis. Therefore, it was hypothesized that selection of wheat lines under high level of fertilization can result in the selection of non-mycorrhizal genotypes (Hetrick, Wilson & Cox, 1993).

In addition to fertilization, other aspects of crop management can also affect symbiotic association between AM fungi and plant roots. Agricultural practices such as use of chemical fertilizers and biocides, tillage, monocultures, and planting of non-mycorrhizal crops from family Brassicaceae and Chenopodiaceae create unfavorable conditions whereas the organic farming without the use of chemical fertilizers, and crop rotation with mycorrhizal crops create favorable environment to AM fungi (Gosling et al., 2006).

1.5 Maize and AM Fungi

Maize is one of the most important food and feed crops in the world. The USA is the leading producer of maize globally in 2021 (<https://downloads.usda.library.cornell.edu/usda-esmis/files/tm70mv177/zk51wt228/rr173695n/crop1222.pdf>). Maize, along with many important agricultural crops, form symbiotic associations with AM fungi. Therefore, it is important to understand how beneficial AM fungi interact with or affect maize growth and development.

A study of AM colonization among different maize lines (141 inbred lines, 38 hybrids, and 76 landraces) and in response to plant breeding programs showed that inbred lines released in specific locations and years had significantly greater colonization of roots with AM fungi than other lines. However, inbred lines and landraces had significantly lower AM colonization than modern hybrids (An et al., 2010). Similarly, in another study of AM symbiosis performance in maize lines (a landrace, two conventional hybrids, and two genetically modified (GM) hybrids - Bt11 and MON 89034), found no consistent effects of AM colonization on growth of GM crops. However, symbiosis was affected depending on maize genotype and AM fungal species. Conventional and GM hybrid showed negative response i.e., decrease in biomass and P concentration while maize landrace showed positive response i.e., increase in biomass and P concentration to soil amendment with *Rhizophagus clarus* (Londoño et al., 2019). Londoño *et al.* (2020) characterized AM fungal communities associated with roots and rhizosphere soil of three maize lines (GM hybrid, a non-modified isoline, and landrace) and found that maize lines or genetic modification had no effect on the AM fungal community, but different growth stages (V3-vegetative, R1-flowering, and R3-grain development) of the crop affected AM fungal communities associated with maize roots. Ramírez-Flores *et al.* (2020) studied the AM response in *CASTOR* maize mutant lines that do not form symbiotic relationship with AM fungi and their AM-compatible counterparts. These authors found that AM fungi contributed to about 33% of the grain production in a medium input field.

Sawers *et al.* (2017) studied the effect of host genotype on growth response to AM inoculation in 30 maize lines and reported that AM colonized plants had greater growth. They reported that shoot dry weight increased from 76.6% to 192.7% in AM inoculated plants compared to non-inoculated plants. They evaluated six lines for AM colonization and reported significant

difference in root colonization among maize lines in low P conditions with significantly higher colonization in Mo18W compared to Mo17 and Oh43. Kaeppler *et al.* (2000) evaluated AM colonization in 12 maize lines in low and high P conditions and reported significant variation in percentage root colonized among lines. Kaeppler *et al.* (2000) also reported 3 QTL that controlled the maize growth based on shoot weight without the presence of AM fungi and 1 QTL that controlled mycorrhizal responsiveness in maize. Also, Ramírez-Flores *et al.* (2019) observed that the AM inoculated plants had both higher biomass and total nutrient content compared to non-inoculated plants. AM colonization in five maize lines (4 landraces and 1 hybrid) were evaluated in greenhouse conditions where Sangabriel-Conde *et al.* (2014) reported significantly higher root colonization (60-80%) in some lines compared to the hybrid (45%)

Several studies reported that in a P limited conditions, AM fungi increased shoot P content in host plants (Liu *et al.*, 2018; Sawers *et al.*, 2017; Smith *et al.*, 2010; Kaeppler *et al.*, 2000). A study that examined the effect(s) of P in Zn, Cu, Mn, and Fe uptake in mycorrhizal and non-mycorrhizal maize, found that Zn uptake in shoots was significantly higher in mycorrhizal plants at low P and no or low micronutrient level. Similarly, Cu and Fe uptake in shoots were significantly higher in mycorrhizal plants at low P and no micronutrient. However, uptake of Mn in maize shoots was significantly lower in mycorrhizal plants at the highest micronutrient level (Liu *et al.*, 2000). Further, AM fungi were used in commercial field production trial of forage maize. Maize seeds treated with commercial product (@ 1 kg/ha) of Micosat F® containing *Glomus spp.* showed significant increase in the dry matter yield and quality of forage maize compared to non-inoculated plants (Sabia *et al.*, 2015).

1.6 Commercial AM Fungi Inoculum

Different AM products are available in the market worldwide. Large scale production and using AM fungi as a commercialized inoculant in the agricultural field is relatively new. However, adoption of these products has been slow by the crop producers because of the quality and efficiency of the commercial products (Salomon et al., 2022). Faye et al. (2013) reported that only 3 of 12 AM inoculants evaluated significantly increased fungal root colonization in maize. Knerr, Paulitz & deToit, (2016) evaluated commercial Bio Terra Plus, Myco-Apply Ultrafine Endo, MykePro Granular, and Mykos Gold Granular AM fungi inoculants in the field experiments, and only fungi in the Mykos Gold Granular product colonized onion roots. Similarly, another study where commercial inocula - liquid, liquid mix, and solid, and native AM spores were used, only solid treatment (solid commercial inoculum) and native AM spores increased AM colonization in the maize roots (Lauriano-Barajas & Vega-Frutis, 2018). Furthermore, two commercial, and two bulk soil inoculum treatments (containing either *Rhizophagus irregularis* or *Glomus mosseae*) when applied to Romaine lettuce, AM fungal colonization was significantly higher in plants treated with *R. irregularis* compared to commercial and other soil inoculants (Garmendia & Mangas, 2014). More recently, Salomon et al. (2022) evaluated more than 25 commercial AM products available in Europe, Australia, and North America and reported that most of the commercial AM products (>80%) were unable to colonize the plant roots. Therefore, it is important to check the quality and viability of commercial AM inoculum before use.

1.7 Rationale of the Study

Climate change, increasing population, and abiotic and biotic stress are factors that have significant effects on agricultural production worldwide. Plant breeders are focused on developing resilient and productive varieties (cultivars) to feed the growing population. However, modern agriculture encourages high fertilizer inputs in which AM fungi may not be beneficial to the host plants (Schmidt, Bowles & Gaudin, 2016). Currently, plant breeding experiments are typically conducted in well fertilized fields and focus on selecting genotypes that produce high yield under these conditions (Rengel, 2002). Researchers hypothesize that artificial selection for high yielding genotypes in high-input environments may have led to selection of genotypes with reduced capacity to form symbiotic association with AM fungi (Rao, Tilak & Arunachalam, 1990). Thus, it is important to understand whether host genetics (i.e., different varieties) influences AM association in newly developed varieties or not. This thesis will discuss the interaction of AM fungi and diverse maize lines in detail in the following chapters (Chapters 2 and 3).

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CHAPTER 2

Developing an Assay for Maize Root Colonization by Arbuscular Mycorrhizal Fungi under Controlled Environment Conditions

Abstract

Arbuscular mycorrhizal (AM) fungi are beneficial microorganisms that form symbiotic relationships with most species of land plants. AM fungi contribute to nutrient uptake and may improve plant tolerance to environmental stresses like drought, high salinity, and heavy metal contamination. Considering these benefits, many commercial AM products are available worldwide to use as an inoculum for crop production. Here, we focus on determining optimal conditions for assessing maize root colonization by AM fungi. Application of a commercial AM product (MycoApply) did not result in colonization of six tested lines whereas we did observe colonization using *in vitro* cultured and bulk soil AM inoculum in two maize lines. We tested AM colonization in different pot size and observed colonization in 4.5-inch and 6-inch diameter pots but not in ‘long’ pots (4-inch x 14-inch). Lastly, five fertilizer concentrations were examined to determine root colonization when non-sterilized field soil was used without the addition of AM inoculum and observed higher concentrations of fertilizer inhibit the AM symbiosis in B73 and W22. Our study demonstrates to take caution while using commercial AM inocula and also, the high fertilizer concentrations and the pot dimensions (especially height of the pots) used can significantly affect colonization in AM experiments.

2.1 Introduction

Arbuscular mycorrhizal (AM) fungi are important plant beneficial soil microorganisms interacting with the roots of most species of land plants, including many cultivated crop. However, plants belonging to some families, such as Brassicaceae, Pinaceae, and Chenopodiaceae, have not evolved an AM fungal symbiosis. AM fungi belong to the subphylum Glomeromycotina (Spatafora et al., 2016), and form different structures inside the host roots called intraradical hyphae, arbuscules, and sometime vesicles. Arbuscule formation inside plant roots is considered as a proxy for AM symbiosis (S.E. Smith & Read, 2008).

AM fungi develop extraradical hyphae that extend beyond the rhizosphere to explore soil for uptake and transfer of nitrogen (N) (Thirkell et al., 2020; Hodge & Fitter, 2010), phosphorus (P) (Koide et al., 2000; Balliu et al., 2015), potassium (K) (Baslam et al., 2013; Shi et al., 2021), and copper (Cu), zinc (Zn), manganese (Mn) to plant roots (Faber et al., 1990; Ruytinx et al., 2020). In return to these nutrients, the plant host provide photosynthates i.e., carbon (sucrose) and/or lipid that are used by the fungus for growth and development (Jiang et al., 2017). AM fungi enhance lateral root development in angiosperms (Paszkowski & Orvo, 2022), improve plant tolerance to abiotic and biotic stress (Alqarawi, Abd Allah & Hashem, 2014). AM fungi can also enhance crop yield in some crops like potato (Hijri, 2016), forage maize (Sabia et al., 2015). Moreover, AM fungi play a crucial role in the suppression of crop pests and disease (Whipps, 2004), tolerance to high salinity and heavy metals (Shetty, Hetrick & Schwab, 1995), and improve soil particle aggregation (Rillig & Mummey, 2006).

The use of AM fungi in agricultural research is increasing because of the multiple benefits of AM fungi in the host plants and several commercial AM products (>25) are available in the market globally (Salomon et al., 2022). Due to lack of suitable AM fungi-free control fields, it is

challenging to study beneficial effects of AM fungi in the field. Many AM species are found in natural soil and their distribution is variable, even at local scales (Cheeke et al., 2015). Also, in the field it is hard to achieve uniform conditions due to local variation in soil chemical and physical properties including soil organic/inorganic matter, nutrient content, and associated microbial communities (Kuila & Ghosh, 2022; Jiang et al., 2021; Lekberg et al., 2007; Boddington & Dodd, 2000). Therefore, experiments in controlled environments such as growth chambers and greenhouses, are often used to examine AM symbiosis. The AM inoculum used is either derived from *in vitro* cultures, bulk soil inoculum, or from commercial products given that AM fungi cannot be grown in pure culture on a nutrient medium.

Several studies have reported that the majority of commercial AM products evaluated were not effective and the fungus was unable to colonize the host plant roots (Salomon et al., 2022; Garmendia & Mangas, 2014; Faye et al., 2013; Knerr, Paulitz & deToit, 2016). Therefore, questions remain concerning the quality and efficacy of commercial AM products.

AM inoculum can be produced using colonization *in vitro* cultures in transformed carrot roots (Chabaud et al., 2006). Salomon et al. (2022) reported that *in vitro* culture of *R. irregularis* prepared on carrot roots resulted in increased root colonization of leek (48%) and tomato (79%) compared to commercial AM inocula. Similarly, Kafle et al. (2022) also reported colonization of up to 70% when *in vitro* culture AM inoculum was used to inoculate *M. truncatula*. Although AM inocula produced using *in vitro* culture are effective, their production is time consuming and may not be feasible for large experiments. Therefore, inocula prepared in the soil of pots planted with leek, alfalfa, or marigold is often the preferred method for experiments (Sawers et al. 2010; Sawers et al. 2017; Ramírez-Flores et al. 2020; Garmendia & Mangas, 2014). Garmendia & Mangas (2014) evaluated two commercial inoculants and bulk soil-produced inocula for colonization of

Romaine lettuce and reported that AM fungal colonization was significantly higher with bulk soil inoculum compared to commercial inoculants. While bulk soil inoculum is easier to produce, the advantage of using *in vitro* inoculum prepared on carrot root in the lab compared to bulk inoculum soil is mainly that it is easier to control the inoculum purity and quantity.

The major aim of our research was to study the interaction between different maize lines and AM fungi and to develop a robust protocol for AM inoculation of maize under controlled environmental conditions. Different types of AM inoculum: commercial products, *in vitro* culture, and bulk soil were used to determine and evaluate efficacy in our experiments. We compared root colonization from commercial products and *in vitro* culture lab spores in two maize lines. Then we evaluated the colonization variation in different pot sizes. After identifying the most effective AM inoculum and pot suitable for root colonization by AM fungi, four maize lines were evaluated for AM root colonization in growth chamber experiments. For these experiments, four maize lines were grown with and without AM inoculum to determine root colonization, shoot and root biomass, and shoot nutrient content among lines. The influence of fertilized on AM colonization was also evaluated in greenhouse using field soil without the addition of AM inoculum in B73 and W22.

2.2 Materials and Methods

2.2.1 Plant, Fungal, and Planting Materials

Nine lines that represent the genetic diversity available within elite maize germplasm were used in this study: B73, CML277, Mo17, Mo18W, NC350, Oh43, Oh7B, P39, and W22 (K. Liu et al., 2003). MycoApply® EndoPrime™ (Valent U.S.A. LLC, 710 NW E Street, Grants Pass, Oregon), *in vitro* cultured, or bulk soil inoculum of *Rhizophagus irregularis* DAOM 197198 was used for inoculating each maize line. Sungro Horticulture MM830 F3B (Product of USA) was used as a planting material. Five hundred ml (4.5-inch diameter) and 1-liter (6-inch diameter) plastic pots were used for optimization assay experiments, and 2 L (8-inch diameter) plastic pots were used to prepare soil inoculum. The composition of MycoApply and Sungro soil is shown below.

Composition of commercial inoculum MycoApply EndoPrime:

- Non-plant food ingredients
- 21.6% total active ingredients
- 15% humic acid derived from leonardite
- 78.4% total inert ingredients
- 4 AM fungi:
 - *Rhizophagus irregularis*
 - *Glomus mosseae*
 - *Glomus aggregatum*
 - *Glomus etunicatum*
- Each species contain approximately 5,625 propagules (resting spores)/g

Composition of Sungro Horticulture Mix Soil:

- Non-plant food ingredients
- 50-60% Sphagnum peat moss, softwood bark, perlite, dolomite limestone, and wetting mix
- pH adjusted for this soilless mix
- 0.25% silicon dioxide from calcium silicate
- 0.12% soluble silicon

2.2.2 *In Vitro* Production of AM Inoculum

Axenic carrot (*Daucus carota*) Ri T-DNA root cultures, colonized by *Rhizophagus irregularis* DAOM 197198 were growing in Petri dishes (10-cm diameter) containing minimal medium (M medium) (Table 2.1). Sub-culturing of AM root were done using previously cultured root segments. To subculture AM roots, 5-6 non-mycorrhizal root segments (2-3 cm long) were transferred to a 10 cm diameter plastic Petri plate containing M medium. Five to six AM root segments (2-3 cm long) were kept in the same Petri plate having non-mycorrhizal root segments. Newly made Petri plates of AM cultures were incubated at 26⁰C for 2-3 months. After 1 month, Petri plates were checked every week under a stereoscope to visually evaluate formation of resting spores. Petri plates with no or minimal spore formation were discarded and Petri plates with relatively high concentrations of spores were retained for extraction and subculturing. New sub-cultures were performed every month to maintain spore viability. Regular sub-culturing of AM fungi provided viable spores that germinate and colonize roots.

Table 2.1: Composition for Minimal medium (M medium) (Becard & Fortin, 1988)

	Stock Solution (1 litre)	Concentration mM
A) Macro elements 100x		
MgSO ₄ ·7H ₂ O	73.1 g	296.6
KNO ₃	8.0 g	79.13
KCl	6.5 g	87.19
KH ₂ PO ₄	0.48 g	3.53
B) 100x		
Ca(NO ₃) ₂ ·4H ₂ O	28.8 g	121.96
C) 200x		
NaFe EDTA	1.6 g	4.36
D) 1000x		
KI	0.75 g	4.52
E) Micro elements 1000x		
MnCl ₂ ·4H ₂ O	6 g	30.32
ZnSO ₄ ·7H ₂ O	2.65 g	9.25
H ₃ BO ₃	1.5 g	24.26
CuSO ₄ ·5H ₂ O	0.13 g	0.52
Na ₂ MoO ₄ ·2H ₂ O	0.0024	9.92
F) Vitamins 200x		
Glycin	600 mg	
Thiamin HCl	20 mg	
Pyridoxin HCl	20 mg	
Nicotinic Acid	100 mg	
Myo-inositol	10000 mg	

Preparation of M medium:

The macroelements (100x), $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (100x), NaFe EDTA (200x), KI (1000x), microelements (1000x), and vitamins (200X) were prepared first. Then, 10 ml of stock solution of macroelements and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (100x), 5 ml of NaFe EDTA (200x), 1 ml of KI (1000x), 1 ml of micro-element stock solution (1000x), and 10 ml of vitamin stock solution (200x) was mixed and made the final volume of 1 litre by adding milli-Q water. In that mixture, 10 g l^{-1} of sucrose was added followed by 3.5 g l^{-1} of Gel Gro/Gellan Gum to rapidly stirring solution to prevent clumping. pH was adjusted to 5.5 with 0.1 M KOH solution and the prepared Minimal medium was autoclaved (121°C at 15 psi for 30 minutes). After autoclaving, the medium was poured into the Petri plates inside a Biosafety cabinet, sealed with parafilm and stored at 4°C until use.

2.2.3 Spore Collection and Extraction from *In Vitro* Carrot Root Culture of AM Fungi

For spore extraction, *in vitro* cultured carrot roots were examined with a stereoscope for AM spores in each Petri plates and colonized roots were removed and cut into 0.5-1 cm pieces. These root segments along with extracted spores would serve as the source of primary inoculum for our experiments. After removing roots, the Petri dishes with M media had spores and some roots attached to it. Then, the M media were kept into the blender (Blender 5010 S Model 5010, Dynamics Corporation of America, New Hartford, Conn. USA) and 200-250 ml of 10 mM sodium citrate buffer was added in a blender and run at half to high speed for 60 seconds. The blended solution was poured into the Büchner funnel containing single filter paper and a vacuum pump was used to filter the solution and spores. Spores on the filter paper were washed 2X in sterile distilled H_2O and collected in a glass beaker for estimating spore concentration. Based on the

average of two spore counts, the suspension was adjusted to approximately 300-400 spores per ml and used for inoculation experiments.

2.2.4 Bulk Soil Inoculum Production of AM Fungi

AM soil inoculum was prepared in the greenhouse and growth chamber. Seeds of leek (*Allium* spp.) and marigold (*Tagetes* spp.) were germinated in a 1:1 mixture of cement sand and sungro soil. Eight to 10 days after germination, seedlings were transferred to 8-inch diameter (20 cm or 2 litre) plastic pots filled with the sand and sungro soil mixture. During the transplanting process, spores and small root segments (see section 2.2.3) were added to the same hole where seedlings were planted. There were 8-10 seedlings planted in each pot. Seedlings were irrigated twice a week and modified Hoagland's solution (see section 2.2.5) low in phosphorus was provided once a week. Six weeks after transplanting, roots from 2-3 plants were sampled to confirm AM fungal colonization of roots. After confirmation, plants were grown for 2.5 months, and harvested. Shoots were discarded and soil associated with roots was dried for 2-3 days in the greenhouse at 25-30⁰C. Dried soil and root segments were stored at 4⁰C for future use as inoculum. Bulk soil inoculum prepared using *in vitro* cultured lab inoculum was used to produce additional soil inoculum, mixed together and stored at 4⁰C.

2.2.5 Preparation of Modified Hoagland's Nutrient Solution Low in Phosphorus

Hoagland & Broyer (1936) developed a nutrient solution low in phosphorus for AM experiments. The composition and steps to prepare this solution is shown below (Table 2.2).

Table 2.2: Composition of Hoagland's nutrient solution

Component	Stock Solution	0.5 strength (ml of Stock Solution per liter)
Macro-elements		
1M KNO ₃	101 g/l	2.5
1M Ca(NO ₃) ₂ .4H ₂ O	236 g/l	2.5
Iron Stock solution	See below.	0.125
0.5M MgSO ₄ .7H ₂ O	123 g/l	2
0.25M NH ₄ NO ₃	20 g/l	2
Micro-elements		0.5
H ₃ BO ₃	2.86 g/l	
MnCl ₂ .4H ₂ O	1.81 g/l	
ZnSO ₄ .7H ₂ O	0.22 g/l	
CuSO ₄	0.051 g/l	
Na ₂ MoO ₄ .2H ₂ O	0.12 g/l	
For AM Fungi experiment, 20 μM phosphate was used.		
1M KH ₂ PO ₄ (pH 6 with 3M KOH)	136 g/l	0.02

Iron Stock solution:

2.61 g EDTA was dissolved in 30 ml H₂O with 1.9 g KOH. FeSO₄.7H₂O (2.49 g) was dissolved in 50 ml H₂O. Iron sulfate solution was added slowly to the potassium EDTA solution, and solution aerated overnight with stirring. If the iron sulfate solution is added too quickly, it can lead to the formation of excess precipitation. Therefore, slowly adding iron sulfate solution minimizes the chances of forming precipitation. The solution pH to 7.1 with wine red in color (very little precipitation occurs). The solution was adjusted to a final volume of 100 ml and stored in a bottle covered with aluminum foil (dark condition) at 4⁰C.

2.2.6 Maize Root Staining for AM Fungal Quantification

Maize roots were harvested and rinsed with de-ionized (DI) H₂O 3-4 times. Roots were submerged in 10% KOH in a 50 ml Falcon tube and placed in a 95⁰C water bath (Lindberg/Blue M, Asheville, NC, USA) for 10 minutes. The KOH was decanted, and roots were rinsed with DI H₂O 3-4 times. Four to 5 ml of 5% Pelikan Black Ink (made by mixing 5 ml of Pelikan Black Ink in 95 ml of distilled white vinegar) was added to roots and incubated at 95⁰C for 10 minutes. Roots were washed with DI H₂O 3-4 times and stored at 4⁰C until root colonization was quantified.

2.2.7 Root AM Fungal Colonization Quantification

AM root colonization was quantified using a gridline intersection method. Stained roots were dispersed in a 10-cm diameter plastic Petri dish using fine forceps and a dissecting needle and examined with a stereoscope and the number of uncolonized (Figure 2.1a) and colonized (Figure 2.1b and 2.1c) roots that intersect the gridlines (1 sq. cm area) was recorded. Percent root colonization was determined using the following equation: $X \times 100 / (X + Y)$ where X= colonized roots (blue) that intersect gridlines and Y= non-colonized roots (black) intersect the gridlines (Fig 2.1d).

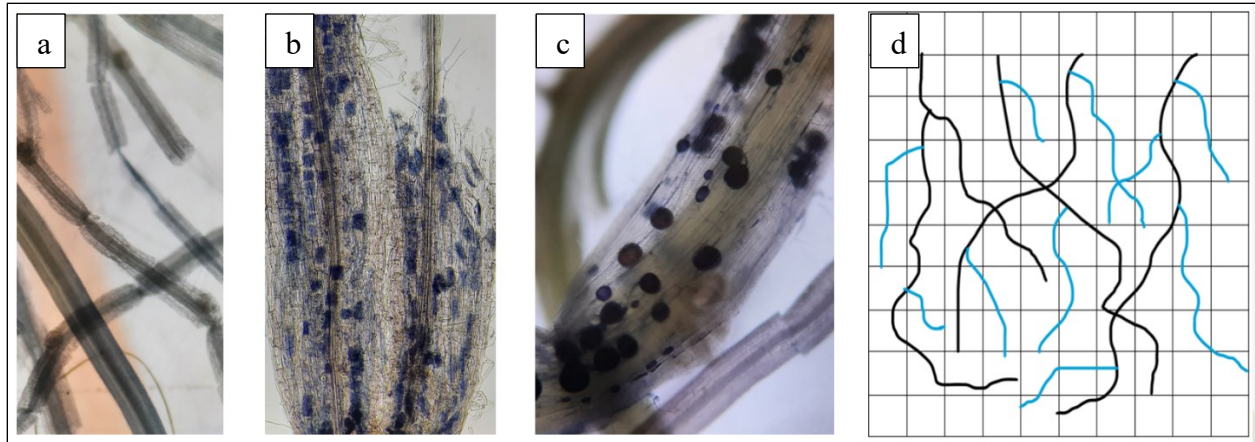


Figure 2.1: Stained roots as seen under microscope and dispersed roots in the gridline. (a) Uncolonized roots as no fungal structure was seen. Colonized roots by the formation of (b) Arbuscules and (c) Vesicles. (d) Black lines indicate the non-colonized roots whereas blue lines indicate the colonized roots.

2.2.8 Evaluation of Colonization through Commercial Inoculum

Six lines (B73, CML277, Mo17, NC350, Oh7B, and P39) representing a wide range of maize genetic diversity (Liu et al., 2003) were inoculated with 0 (control), 50, 75, 100, 200, 300, or 500 spores per ml of the commercial AM product MycoApply. Following the MycoApply label, 1 g of product contains 22500 resting spores of *Rhizophagus irregularis*, *Glomus mosseae*, *G. aggregatum*, and *G. etunicatum*. Spore suspensions were made by mixing MycoApply with DI H₂O. There were 6 replicates per treatment and the experiment was conducted in the greenhouse. Sungro soil was autoclaved at 121⁰C for 1 hour at 15 psi pressure and added to 500 ml (4.5-inch) pots containing a coffee filter at the bottom of each pot. Two seeds of each maize line were sown at the depth of 3-5 cm in each pot. After germination, 1 plant was retained per pot and the spore solutions representing each concentration were added by the help of pipette near plant roots. Plants were watered 2X a week, and 100 ml of modified Hoagland's nutrient solution low in phosphorus (Table 2.2) was applied to soil in each pot once a week (Hoagland & Broyer, 1936). Plants were removed from each pot after 6 weeks and roots harvested to quantify AM root colonization.

2.2.9 Comparison of Colonization Levels between *In Vitro* Lab Spores and MycoApply

Two maize lines B73 and Mo17 were selected to compare the colonization between *in vitro* produced lab spores and MycoApply. Seeds were soaked in 5% NaOCl for 20 min, rinsed with DI H₂O 3 to 4X, soaked in DI H₂O for 60 min, and placed on a germination paper. The germination paper was rolled and placed in a 1L beaker half filled with DI H₂O. After 6-7 days, germinated seeds and seedlings were ready to transplant into the pots. Autoclaved Sungro soil was added to each 6-inch (15 cm) diameter pot with a coffee filter at the bottom of the pots. Soil in each pot was inoculated with either spores in the MycoApply product or spores produced on carrot roots *in vitro*. Approximately 300-400 spores produced on carrot roots and 500 spores in the Mycoapply product were added to each pot. Experiments were conducted in the North Carolina State University Phytotron facility growth chamber. There were six replicates of each treatment arranged in a completely randomized design. The growth chamber was set at 25⁰C day temperature and 22⁰C night temperature with 12 h day:12 h night photoperiod. Plants were irrigated twice a week and 100 ml modified Hoagland's Solution was applied once a week. Plant roots were harvested after 6 weeks of transplanting for AM quantification (see section 2.2.7).

2.2.10 Evaluation of Colonization in Plants Grown in Different Pot Sizes

Three maize lines (NC350, Oh7B, and P39 chosen based on highest number of germinated seeds) were used to examine the effect of pot size on AM colonization. For this experiment, 4.5-inch (10 cm) diameter, 6-inch (15 cm) diameter, and 4-inch x 14-inch (10 cm width x 35 cm depth) (referred to a "long pot") (Stuewe Sons Inc., Corvallis, Oregon) (Fig 2.2) were used. Autoclaved Sungro soil was added to each pot and 6–7 days old seedlings were transplanted into each pot. During the transplanting process, 300-400 spores extracted from *in vitro* AM fungi cultured on carrot roots

were inoculated per pot for AM treatment by the help of pipette. This experiment was conducted in the growth chamber in the NCSU Phytotron facility. There were three replicates of each treatment arranged in a completely randomized design. The growth chamber condition and harvesting time were same as mentioned in section 2.2.9.

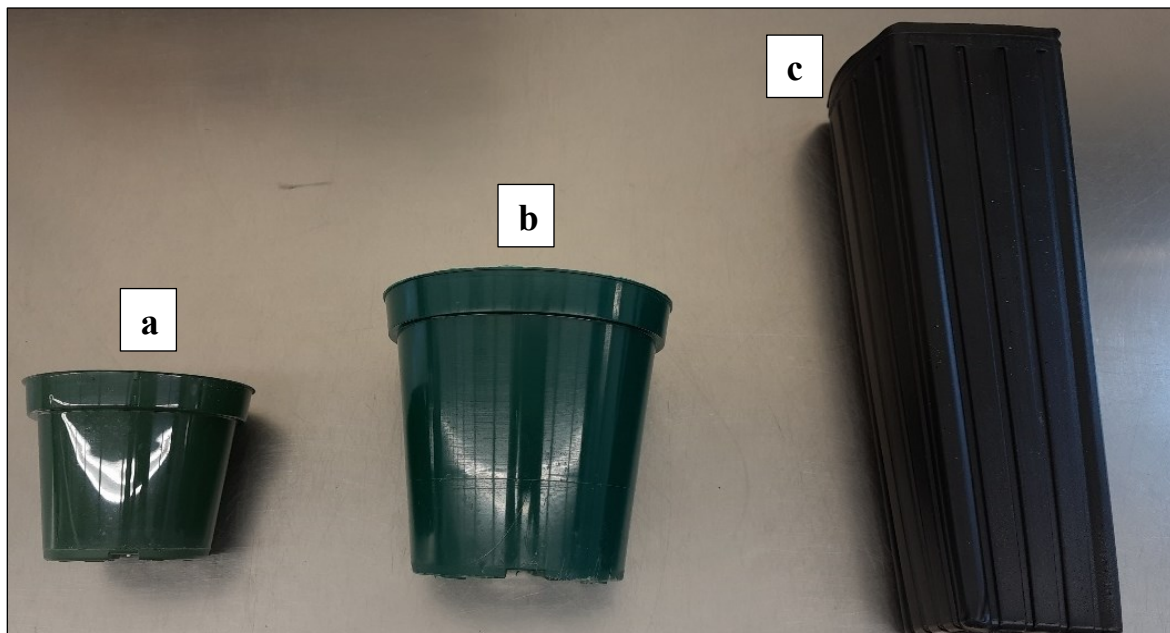


Figure 2.2: Different pot sizes: (a) 4.5-inch pot, (b) 6-inch pot, and (c) long pot.

2.2.11 Responses to AM Colonization in Four Maize Lines

Maize lines B73, Mo17, Mo18W, and Oh43 were used in this experiment. The latter two maize lines were selected based on a prior information suggesting a low and high mycorrhizal response, respectively (Sawers *et al.* 2017). The experimental methods described previously in section 2.2.9 were followed. Plant shoots were harvested after 6 weeks, shoots were excised from plants and kept for dry biomass in oven at 60⁰C for 3-4 days. Some roots from each replicates were kept for AM quantification and the remaining roots were kept for root biomass in oven at 60⁰C for 3-4 days. After measuring the shoot biomass, approximately 0.5 g oven dried shoot samples for each

treatment were submitted to Environmental and Agricultural Testing Service (EATS) Lab, North Carolina State University for determination of phosphorus (P), potassium (K), and sodium (Na) content.

2.2.12 Effect of Fertilizer Concentrations on AM Colonization in Maize Lines

Two maize lines B73 and W22, and the soils from 2 field locations: Central Crops Research Station, Clayton, NC and Lake Wheeler Road Field Laboratory, Midpines, NC were used for the greenhouse experiment. Five fertilizer concentrations were used in this study (Table 2.3). Soil from each location were mixed together in a 1:1 ratio and the 6-inch (15 cm) diameter pots were half filled with mixed soil. Osmocote (slow releasing NPK fertilizer) with the concentrations mentioned in Table 2.4 was sprinkled in the pots. After sprinkling with Osmocote (Table 2.3), each pot was filled with soil. Maize lines B73 and W22 were sown separately in the pots and the pots of B73 and W22 with the same fertilizer concentrations were kept together in the same tray. Soil with no added Osmocote served as the control. Three maize seeds were sown in each pot and after germination only one plant was retained. Two weeks later, different concentrations of Jack's Cal-Mag (Table 2.3) were applied by watering from the top every week until harvesting and no fertilizer was given for control. The composition of Osmocote and Jack's Cal-Mag was shown below (Table 2.4). The different levels of fertilizer provided corresponded to 0, 25, 50, 75 and 100% of the fertilization levels that we routinely apply to maize plants grown in the greenhouse. There were 6 replicates of each treatment of fertilizer concentration arranged in a completely randomized design in the greenhouse. Plant height, stalk width, and chlorophyll content were measured at week 3, 4, 5, and 6 after planting. Plant height was measured from the soil surface to the tip of longest leaf, stalk width was measured 3-5 cm above the soil surface with a digital Vernier Caliper (General Tools and Instruments, New York, NY 10013), and chlorophyll content

was measured as an average of the youngest terminal and penultimate leaves with a SPAD (Soil Plant Analysis Development) meter (CCM-200 plus GPS, Opti-Sciences Inc.). Roots were harvested after 7 weeks of seed sowing for AM colonization quantification (see section 2.2.7).

Table 2.3: Treatment combinations for fertilizer concentrations experiment

Fertilizer as a proportion of routinely used amount	Osmocote (only once)	Jack's Cal-Mag solution (22g/gallon of water)
0%	-	Only 100 ml water
25%	1.16 g/pot	25 ml Jack's Cal-Mag solution + 75 ml water per pot per week
50%	2.33 g/pot	50 ml Jack's Cal-Mag solution + 50 ml water per pot per week
75%	3.49 g/pot	75 ml Jack's Cal-Mag solution + 25 ml water per pot per week
100%	4.65 g/pot (routinely used amount)	100 ml Jack's Cal-Mag solution per pot per week (routinely used amount)

Table 2.4: Nutrient composition of Osmocote and Jack's Cal-Mag

Nutrients	Osmocote	Jack's Cal-Mag
Total Nitrogen	14%	15%
Nitrate Nitrogen	5.8%	12%
Ammoniacal Nitrogen	8.2%	3%
Available Phosphate	14%	5%
Soluble Potash	14%	15%

2.2.13 Statistical Analysis

Data analysis includes the percentage root colonization in each maize lines and shoot tissue nutrient content, shoot and root biomass between AM inoculated and non-inoculated plants in the growth chamber. Data from the fertilizer concentrations and interaction effects include the percentage root colonization, plant height, chlorophyll content, and stalk width in the greenhouse in two maize lines. Figures were made using ggplot2 from R software (version 2022.07.2 Build 576 ©2009-2022 RStudio, PBC). Differences among means were analyzed using a two-way ANOVA followed by Tukey HSD tests.

2.3 Results

2.3.1 AM spores produced *in vitro* in the lab colonized maize roots, but application of the commercial AM product MycoApply did not result in colonization.

We tested the commercial AM product (Mycoapply) to determine whether the fungi in this product could colonize roots of six different maize lines (B73, CML277, Mo17, NC350, Oh7B, and P39) in greenhouse conditions. For this experiment, spore concentrations of 0, 75, 100, 200, 300, and 500 spores per ml of the commercial inoculum MycoApply were applied and evaluated. AM colonization was not observed at any tested concentrations in any maize lines (data not shown).

We next used both MycoApply and *in vitro* cultured AM (*R. irregularis* DAOM 197198) to evaluate the AM colonization in B73 and Mo17 maize lines in the growth chamber. Roots of B73 and Mo17 maize lines, inoculated with *in vitro* lab spores, were colonized by AM fungi. MycoApply application did not result in AM colonization of the roots of either line (Fig. 2.3). The average colonization with *in vitro* cultured AM in B73 (6 plants) was 14.9 % compared to 30.1% in Mo17 (3 plants) (Fig 2.3). In this assay Mo17 had significantly higher root colonization compared to B73 (Tukey HSD, $p < 0.05$). Also, we tested bulk soil inoculum by mixing with the soil and found that AM fungi colonized the maize roots (data not shown).

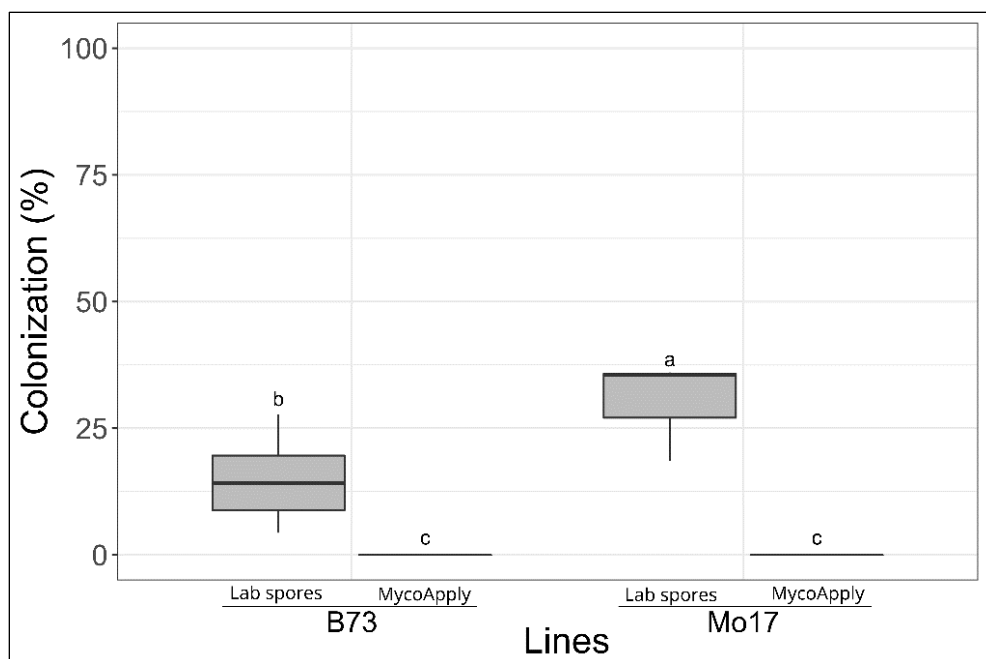


Figure 2.3: Colonization of B73 and Mo17 maize roots inoculated with spores of AM fungi produced on carrot roots and the commercial product MycoApply. Lab spores produced *in vitro* in the lab used as an inoculum colonized maize roots, but MycoApply did not colonize the maize roots. The whisker box plots represent the interquartile range. Six B73 and three Mo17 plants were assayed. Mo17 had significantly higher colonization compared to B73 (Tukey HSD, $p < 0.05$). Levels not followed by the same letter were significantly different.

2.3.2 Maize roots were colonized in 4.5-inch and 6-inch pots but not in ‘long pots’

After determining that AM inoculum derived from *in vitro* culture colonized maize roots, the next step was to determine how pot size influences AM root colonization. For this study, three different pot size: 4.5-inch, 6-inch, and long pot (dimensions of these pots were mentioned in section 2.10 and Fig 2.2) and 3 maize lines (NC350, Oh7B, and P39) were chosen because these were the only lines that germinated out of 6 different lines planted for this experiment (B73, CML277, Mo17 did not germinate). AM derived from *in vitro* cultured inoculum colonized the roots of different maize lines only in the 4.5-inch and 6-inch pots but did not colonize the roots of different lines in long pots (Fig 2.4). The average colonization of NC350 was 36.9 % and 15.3 %, Oh7B was 19.4 % and 23.8 %, and P39 was 26.5 % and 12.6 % in 4.5-inch and 6-inch pots respectively. We observed

that the percentage root colonization in 4.5-inch and 6-inch pots did not vary significantly for any of the three lines (Tukey HSD, $p > 0.05$) (Fig 2.4).

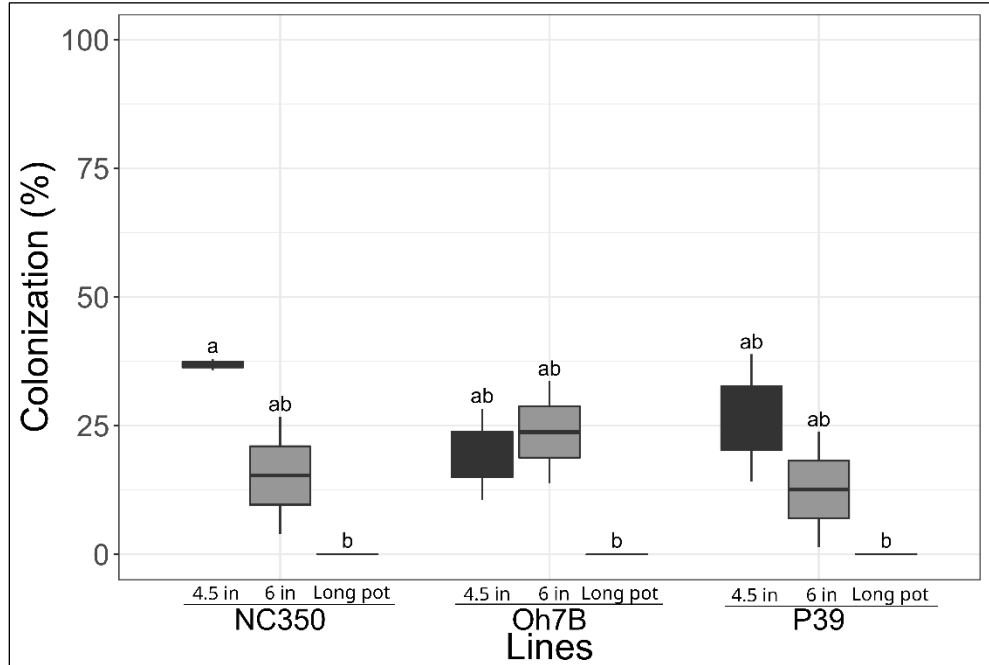


Figure 2.4: Colonization of NC350, Oh7B, and P39 maize lines grown in 4.5-inch, 6-inch, and long pots. *In vitro* culture AM when used as an inoculum was able to colonize maize roots in 4.5-inch and 6-inch pots only but not in long pots. The whisker box plots represent the interquartile range. Box plots showed percentage root colonization of NC350, Oh7B, and P39 lines in 4.5-inch, 6-inch pots, and long pots. There was no significant difference in root colonization of maize lines grown in 4.5-inch, 6-inch pots, and long pots. There was no significant difference in root colonization of maize lines grown in 4.5-inch and 6-inch pots (Tukey HSD, $p > 0.05$). Levels not followed by the same letter were significantly different.

2.3.3 Effects of root colonization on shoot and root biomass, and K, Na, P content

After determining optimum pot size for AM colonization for *in vitro* lab culture inoculum, another preliminary experiment was performed to examine the colonization of maize lines and to examine shoot biomass, root biomass, and nutrient content in relation to AM colonization. For this study, four maize lines (B73, Mo17, Mo18W, and Oh43) were grown in the growth chamber with and without AM inoculum. AM fungi cultured on carrot roots *in vitro* used as an inoculum successfully colonized roots of all lines used whereas no colonization was observed in non-AM inoculated control plants. Root colonization was significantly higher in Mo17 compared to Mo18W (Fig 2.5a). Analysis of variance (ANOVA) for root colonization for maize line, treatment, and interaction effect were shown in Table 2.5. There was a significant effect of maize line ($p < 0.05$) in root colonization (Table 2.5) and greater effect of treatment. There were no significant differences in shoot biomass (Fig 2.5b) between AM inoculated and non-inoculated plants in tested lines and significantly higher root biomass in AM inoculated B73 compared to non-inoculated B73. Root biomass of AM inoculated B73 was significantly higher compared to AM inoculated Mo17 and Oh43 (Fig 2.5c). No difference in K (Fig 2.5d), Na (Fig 2.5e), and P (Fig 2.5f) content of shoot tissues between AM inoculated and non-inoculated conditions in maize lines was observed. There was no correlation between colonization, shoot and root biomass, K, Na, and P shoot content ($p > 0.05$) (data not shown).

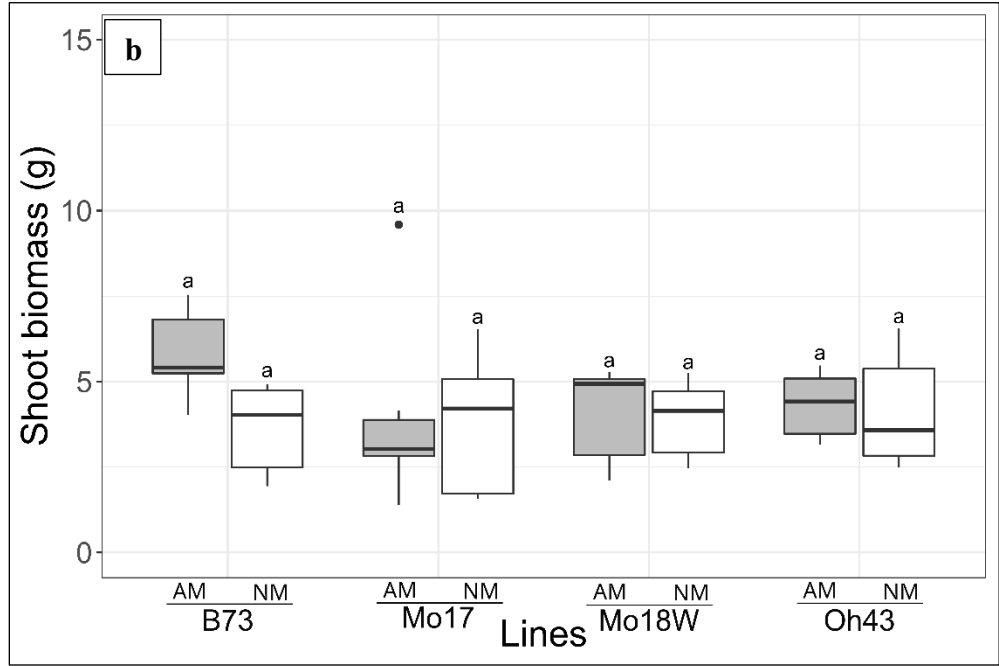
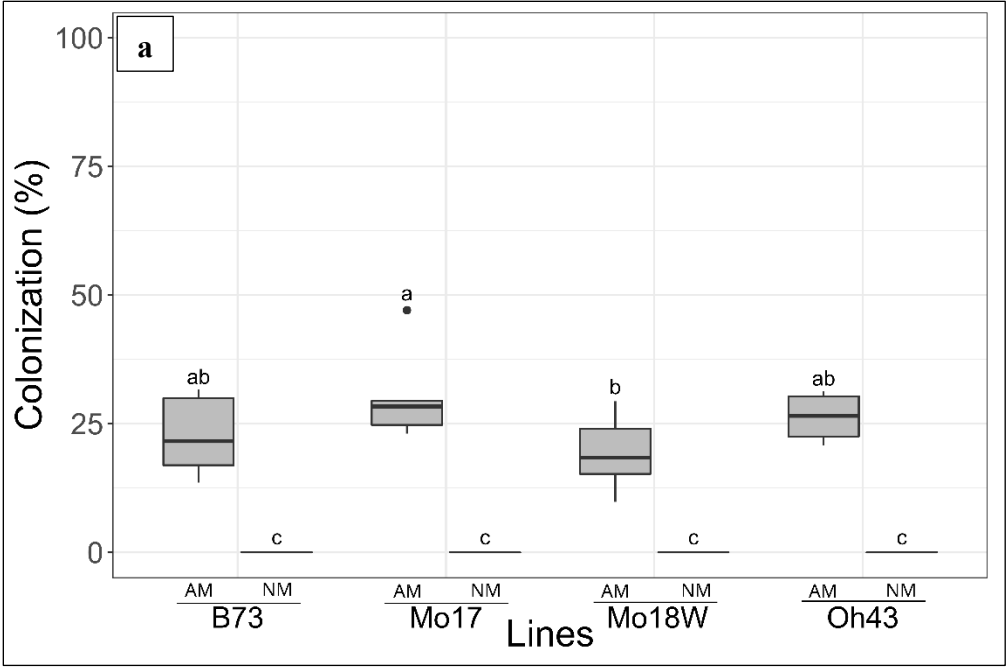
Table 2.5: Analysis and sources of variation for root colonization between maize lines and inoculum treatment

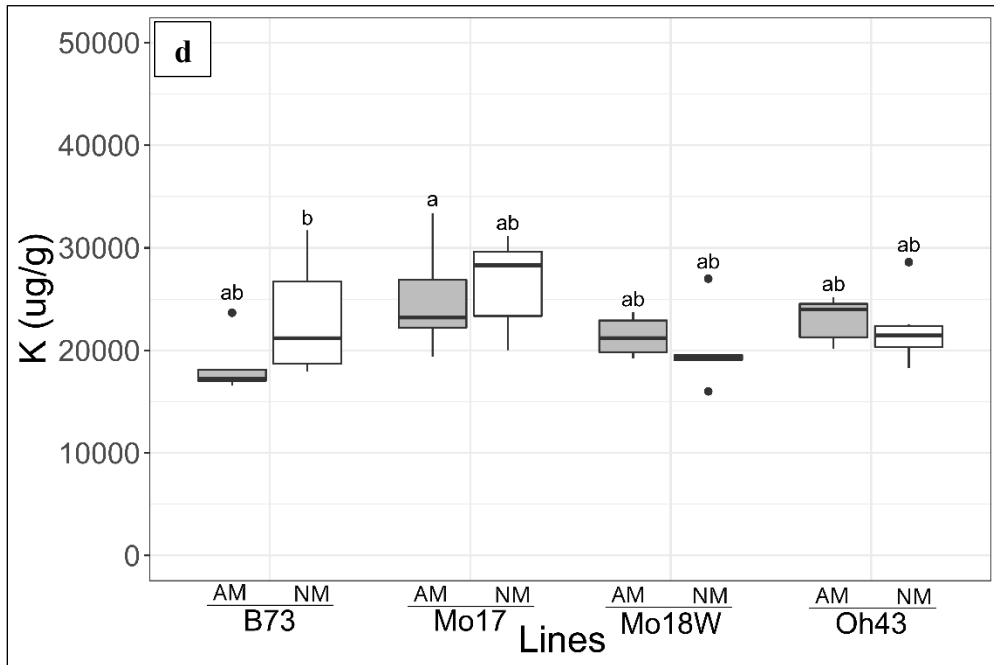
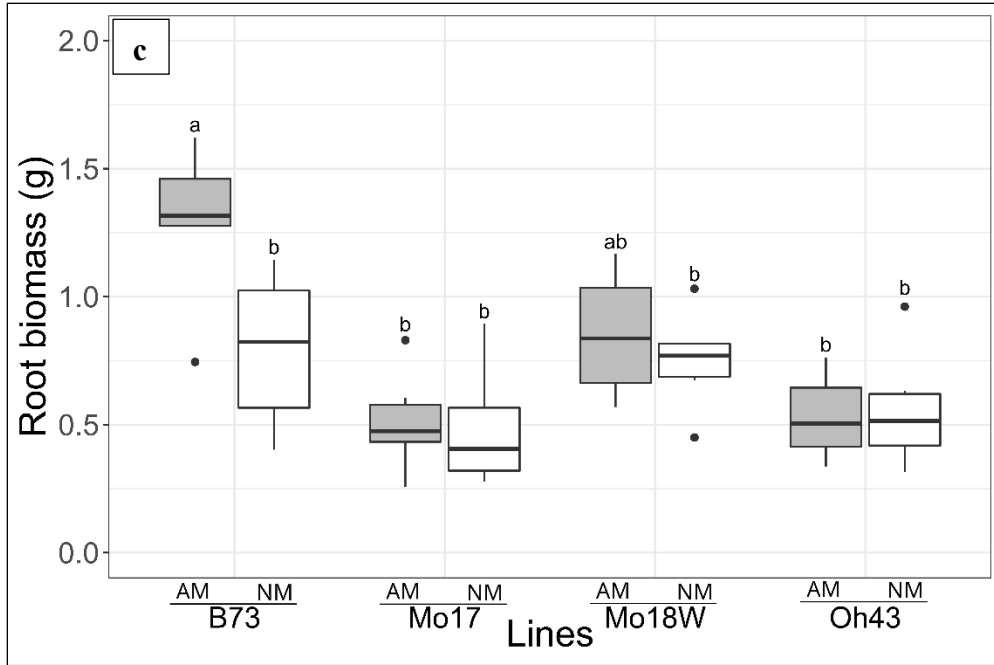
Source	df	Sum of Square	Mean Square	F value	Pr(>F)
Lines	3	317	106	4.04	0.0138*
Treatment	1	6892	6892	262.91	<2e-16***
Lines*Treatment	3	182	61	2.31	0.0919
Residuals	38	996	26		

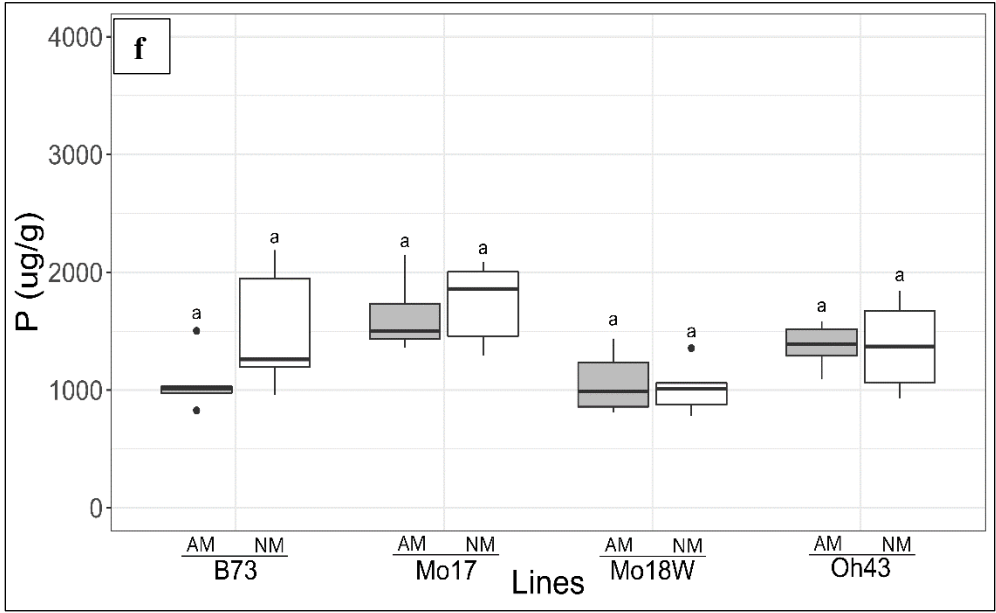
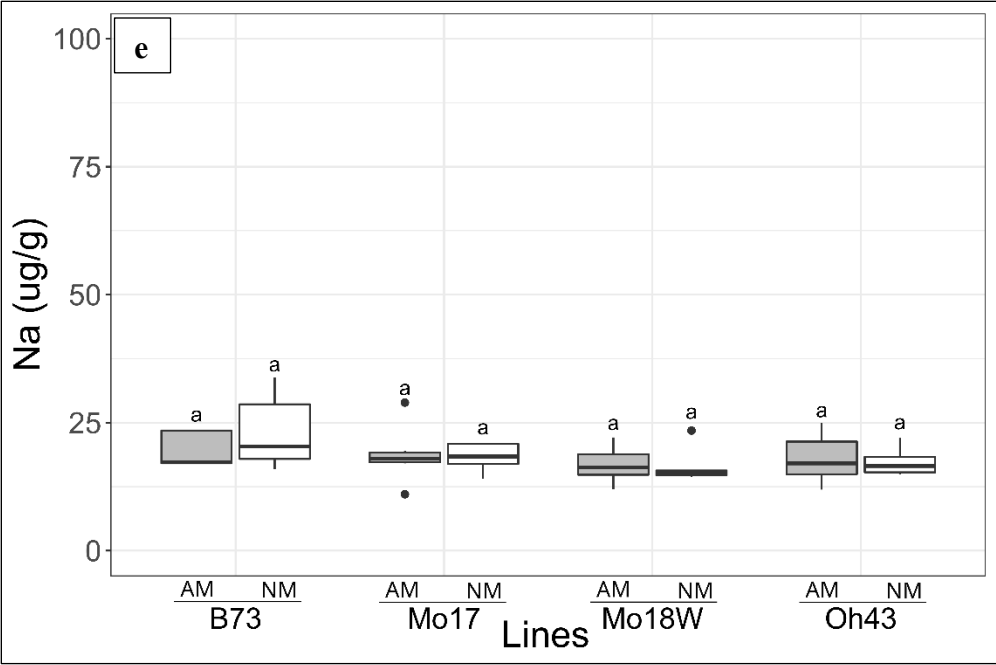
* Significant at p= 0.05 level

*** Significant at p= 0.001 level

Figure 2.5: Effect of root colonization in shoot/root biomass, shoot tissue P, K, and Na content in B73, Mo17, Mo18W, and Oh43 maize lines. Four different maize lines, B73, Mo17, Mo18W, and Oh43 lines grown in 4.5-inch pots amended with AM from *in vitro* produced inoculum. Lines shown on the x-axis and with different plant growth parameters on the y-axis (a) Percentage root colonization of AM (b) Shoot (c) Root biomass and (d) K leaf content, (e) Na leaf content, and (f) P leaf content. The whisker box plots represent the interquartile range. Data points that do not fall in whisker box ranges were considered as potential outlier data and represented by black dot (•). Significant differences were determined by Tukey HSD ($p > 0.05$). Treatments followed by the same letter were not significantly different.







2.3.4 Effect of fertilizer concentration in AM colonization, plant height, and chlorophyll content

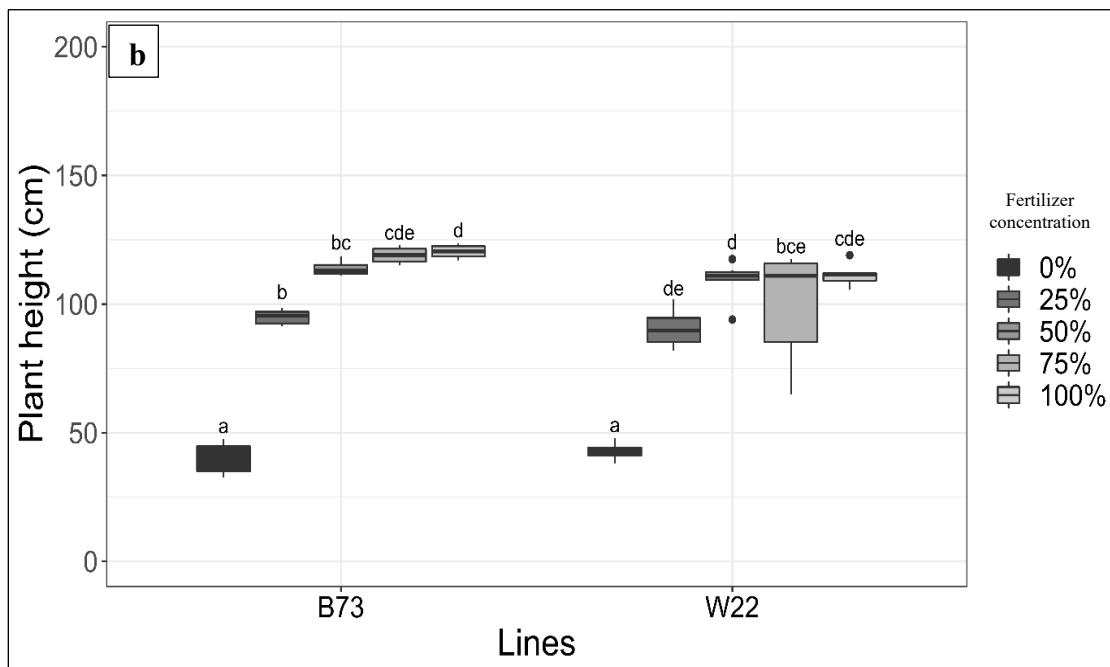
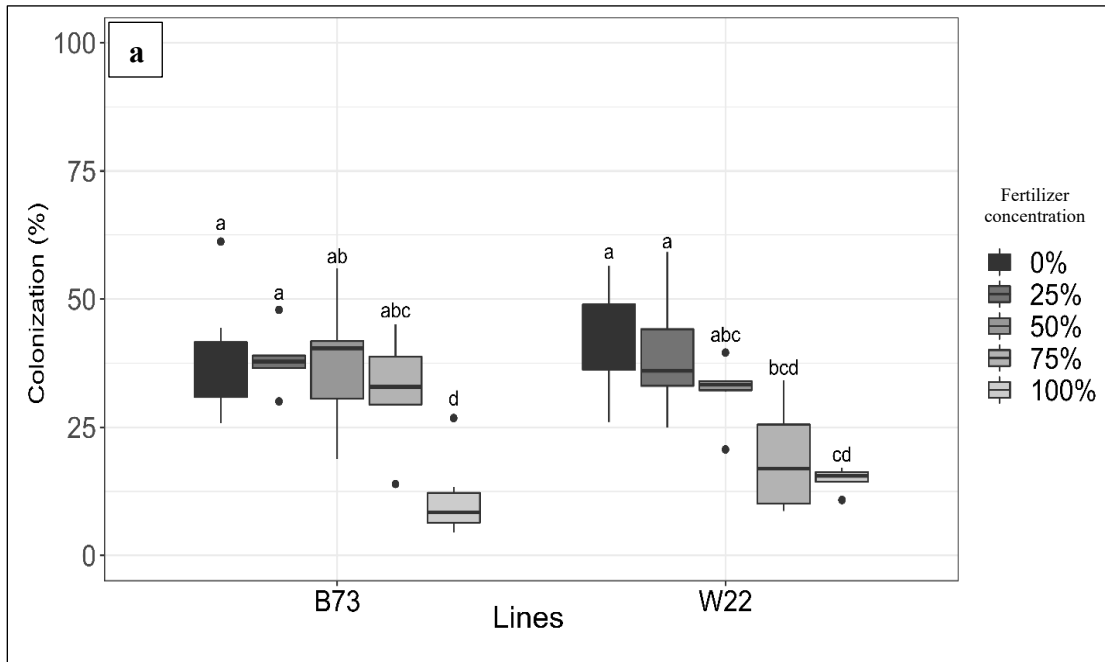
Higher fertilizer concentrations can improve plant growth but may negatively affect AM colonization of roots. To determine the optimal fertilizer concentrations for AM colonization and maize plant growth, concentrations (0, 25, 50, 75 and 100%) of Osmocote and Jack's Cal-Mag (Table 2.3) were applied to maize in the greenhouse experiments. We determined the colonization levels and plant height, stalk width, and chlorophyll content for two maize lines B73 and W22 under the different fertilization regimes. In B73, AM root colonization was not significantly different at 0, 25, 50, and 75% fertilizer concentration but was significantly reduced in 100% fertilizer concentration. Similarly, in W22, AM root colonization was not significantly different at 0, 25, and 50% fertilizer concentration but was significantly lower in 75 and 100% fertilizer concentration compared to other concentration (Fig 2.6a). Plant height and chlorophyll content were almost similar at 50, 75, and 100% fertilizer concentration in B73 and W22 lines.

There was a highly significant root colonization among fertilizer concentration treatments (Table 2.6, $p < 0.001$). At 75% fertilizer concentration, stalk width at 3 and 4 weeks and plant height at 4, 5, and 6 weeks after sowing were significantly correlated with root colonization (Table 2.7). Similarly, chlorophyll content at 0% and stalk width at 100% fertilizer concentration after 6 weeks of sowing was significantly correlated with root colonization (Table 2.7).

There was a highly significant variation of root colonization due to fertilizer concentration treatment (Table 2.6, $p < 0.001$). At 75% fertilizer concentration, we observed that stalk width at 3 and 4 weeks after sowing as well as plant height at 4, 5, and 6 weeks after sowing were significantly correlated with levels of root colonization (Table 2.7). Similarly, chlorophyll content at 0% and

stalk width at 100% fertilizer concentration after 6 weeks of sowing was significantly correlated with root colonization (Table 2.7).

Figure 2.6: Effect of fertilizer concentrations on root colonization in B73 and W22 maize lines. Two different lines B73 and W22 were grown in non-sterilized field soil without the addition of AM inoculum at different fertilizer concentrations. Lines sown on the x-axis and the following different parameters on the y-axis (a) Percentage root colonization of AM (b) Plant height (c) Chlorophyll content. The whisker box plots represent the interquartile range. Data points that do not fall in whisker box ranges are considered as potential outlier data and were represented by black dot (•). Significant differences were determined by Tukey HSD ($p < 0.05$). Treatments followed by the same letter were not significantly different.



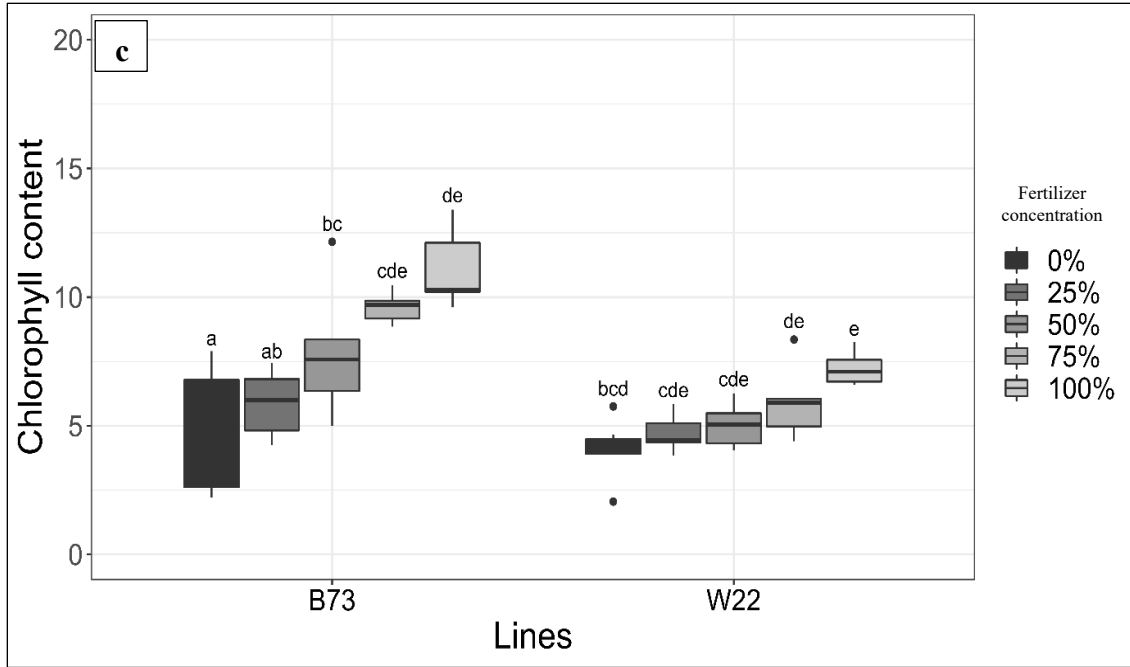


Table 2.6: ANOVA for colonization variation between lines and different fertilizer treatment

Source	df	Sum of Square	Mean Square	F value	Pr(>F)
Lines	1	71	71	0.731	0.397
Treatment	4	5964	1490.9	15.346	2.97e-08***
Lines*Treatment	4	638	159.4	1.641	0.179
Residuals	50	4858	97.2		

*** Significant at p = 0.001 level

Table 2.7: Correlation between AM root colonization and different parameters (plant height, stalk width, and chlorophyll content) at different levels of fertilizer concentration after 6 weeks

	Percentage root colonization of AM fungi at				
	0%	25%	50%	75%	100%
Plant height (3 WAS)	ns	ns	ns	ns	ns
Stalk width (3 WAS)	ns	ns	ns	0.66*	ns
Chlorophyll content (3 WAS)	ns	ns	ns	ns	ns
Plant height (4 WAS)	ns	ns	ns	0.61*	ns
Stalk width (4 WAS)	ns	ns	ns	0.65*	ns
Chlorophyll content (4 WAS)	ns	ns	ns	ns	ns
Plant height (5 WAS)	ns	ns	ns	0.59*	ns
Stalk width (5 WAS)	ns	ns	ns	ns	ns
Chlorophyll content (5 WAS)	ns	ns	ns	ns	ns
Plant height (6 WAS)	ns	ns	ns	0.61*	ns
Stalk width (6 WAS)	ns	ns	ns	ns	0.59*
Chlorophyll content (6 WAS)	0.58*	ns	ns	ns	ns

WAS = Weeks after sowing

* Significant at p= 0.05 level

2.4 Discussion

AM fungi are found all over the world in natural soil. However, due to the lack of suitable AM-free controls in the natural environment, it is challenging to study the consequences of AM-host interactions. Therefore, this beneficial symbiosis can be studied in a controlled environment where the use of *in vitro* cultured AM or soil AM inoculum is necessary. It is important to have a reliable and effective AM inoculum to study the interactions of AM fungi with host plants.

We observed that AM fungi in the commercial product, MycoApply, did not colonize maize roots. Knerr, Paulitz & deToit (2016) also reported that MycoApply Ultrafine Endo used in their experiments did not colonize onion roots, and the Garcia Lab at NC State also demonstrated lack of colonization in three soybean cultivars (Cooney, 2022). Several other papers reported that commercial products did not efficiently colonize multiple host plant species (Salomon et al. 2022; Faye et al. 2013). It might be due to the lack of standardized quality controls for these products or even unreliable products that do not contain viable propagules. Also, we do not know if these commercial products will perform better under specific conditions or with specific plant species. Of course, if this is the case, it is important to mention it on the product label.

Using *in vitro* produced AM inoculum (*R. irregularis* DAOM 197198), we observed that the maize roots were colonized only in 4.5-inch and 6-inch diameter pots but not in long pots (Fig. 2.2). While we watered the plants growing in the 4.5-inch and 6-inch pots from the bottom, we felt that this would not be appropriate for the plants growing in long pots since it would be difficult to achieve sufficient upward movement of water by capillary action in these deeper pots to adequately moisten the upper portions of the soil. In retrospect it is likely that the inoculated spores may have washed out while watering the plants from the top and a plausible reason we did not observe the colonization in long pots.

Root colonization in Mo17 was significantly higher compared to Mo18W when *in vitro* lab culture was used as inoculum. In contrast, Sawers *et al.* (2017) reported that Mo18W had significantly higher total colonization compared to Mo17. This discrepancy might be because of the different AM fungi species used in the two studies or other experimental difference such as the the substrate used. We used SunGro Horticultural Mix substrate and *R. irregularis* (DAOM 197198) in our experiment whereas Sawers *et al.* (2017) used a mixture of loam and quartz sand (v/v) in 1 : 10 mixture and *Funneliformis mosseae* (isolate number 12).

In the growth chamber experiment, we observed a significant difference in root biomass between AM inoculated and non-inoculated plants for B73 but not for Mo17, Mo18W, and Oh43. However, there was no significant difference in shoot biomass, P, K, and Na leaf (shoot) tissue content between AM inoculated and non-inoculated plants in any of the lines. We conducted this experiment in the growth chamber and after a few weeks of growth, maize plants touched the ceiling of the growth chamber. Because of the limited space, plants were unable to grow happily so this may cause stress in the plants thereby compromising the effect of AM fungi on plant growth.

In a high fertilizer concentrations regimes, host plants do not depend on AM symbiosis for sufficient nutrient uptake from the soil and therefore plants do not allocate sufficient carbon to AM fungi for their growth and development (Kaeppler *et al.*, 2000). Several studies reported that the high phosphorus concentrations in the soil reduced AM root colonization in maize plants (Kaeppler *et al.*, 2000; Sawers *et al.* 2017; Liu *et al.*, 2018). In our study, we also observed less AM root colonization in high fertilizer concentration.

We observed that only at 75% fertilizer concentration, there was a significant correlation between plant heights and root colonization. This may be related to the imposed fertilizer concentration not inhibiting AM fungi and they might help host plants in nutrient uptake and in

return plants provide photosynthates to fungi for growth and development. Lower fertilizer concentrations do not inhibit AM fungi, but at these concentrations there are simply not quite enough nutrients for robust plant growth and development without AM (see Fig 2.6c, chlorophyll content). If there are less soil nutrients, plants cannot get enough nutrients for their growth and development. If plants themselves are not getting enough nutrients from the soil and if they have to maintain symbiotic association with AM fungi in low nutrient soil, then symbiosis will be expensive for the plant as plants have to provide carbon to the fungus for their growth and development at the expense of its own growth. So, there is likely that plant growth will be inhibited more in nutrient deprived soil if they have to support AM symbiosis. Root colonization was not significantly decreased in B73 and decreased significantly in W22 at 75% compared to 50% or lower fertilizer concentration. But the stalk width and plant height showed positive correlation at 75% fertilizer concentration. Therefore, the optimal fertilizer concentrations for our purposes might be 75 % of what we used routinely in the greenhouse.

In summary, we observed that the *in vitro* spores produced on carrot root cultures colonized maize roots, but that AM fungi in the commercial product (MycoApply) did not colonize maize roots. Thus, it is important to check the quality and effectiveness of commercial products before using it in an experiment. Also, root colonization was observed in 4.5-inch and 6-inch pots only but not in long pots. Finally, higher fertilizer concentrations inhibited root colonization. The next chapter includes the evaluation of root colonization in additional maize lines in the greenhouse conditions with bulk soil as inoculum and in sterilized mixture of sand and SunGro soil.

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CHAPTER 3

Variation in Levels and Effects of Arbuscular Mycorrhizal Root Colonization Among Maize Lines in the Field and Controlled Environment Conditions.

Abstract

Arbuscular mycorrhizal (AM) fungi are found all over the world in the natural soil and colonize most species of land plants, including many cultivated crop species. We evaluated root colonization in 31 diverse maize inbred lines and hybrids in replicated field experiments at two locations in a soil with a history of high and low phosphorus and 30 inbred lines in the Sungro and sand mixed together in the greenhouse. In the greenhouse experiment, we assessed the effect of AM inoculation on plant height, chlorophyll content, shoot and root biomass, and shoot nutrient content. A lower correlation in root colonization between replications in one field locations ($r=0.28$, $p<0.05$, high phosphorus locations) and no significant correlation in the other locations (low phosphorous locations), but it was somewhat higher in the greenhouse ($r=0.59$, $p<0.001$). We observed significant variation in AM colonization among maize lines in all three conditions: both field locations and in the greenhouse. However, this variation was not consistent across conditions. In the greenhouse, positive correlation was observed between colonization levels and shoot biomass ($r=0.45$, $p<0.001$), root biomass ($r=0.11$, $p<0.05$), plant height ($r=0.34$, $p<0.001$), chlorophyll content ($r=0.42$, $p<0.001$), and shoot potassium content ($r=0.36$, $p<0.001$) whereas negative correlation was observed between colonization levels and shoot nitrogen ($r= -0.17$, $p<0.01$) and phosphorus content ($r= -0.19$, $p<0.001$). Thus, evaluation of diverse maize lines showed variation in colonization among maize lines and AM symbiosis can be helpful to host plants. We also, evaluated the effect of a mutation in the *CASTOR* gene, which has been shown to

be essential for AM colonization, in two lines: B73 and W22 (wild type and *CASTOR* mutant) in the same two field locations and found that *CASTOR* mutant plant stand and growth were worse compared to wild type in both locations.

3.1 Introduction

Arbuscular mycorrhizal (AM) fungi are important beneficial microbes and obligate biotrophs that form symbiotic associations with the roots of more than 80% of land plants (Smith & Read, 2008). Palaeobiological and molecular evidence suggests AM symbiosis originated at least 460 million years ago and contributed to the evolution of land plants (Simon et al., 1993). AM fungi in the roots of host plants, form hyphae, arbuscules and occasionally vesicles. Vesicles act as storage structures while arbuscules function in nutrient exchange with the host plant. AM hyphae extend beyond the rhizosphere and facilitate uptake of water, phosphorus, potassium, nitrogen, and micronutrients (Zn, Cu, Mn, and Fe) (Smith & Read, 2008).

AM fungi are typically beneficial to the host plants under nutrient limiting conditions (S.E. Smith & Read, 2008) and environmental abiotic stress such as drought (Augé et al., 2001), alkaline or heavy metal contaminated soil (Shetty et al., 1995). The use of fertilizers, especially P, can suppress AM colonization (Olsson et al., 1997; Smith & Read, 2008). Modern agriculture encourages high fertilizer input that reduce the beneficial effects of AM fungi on host plant growth and development (Schmidt, Bowles & Gaudin, 2016) and plant breeding programs are focused on selecting genotypes that produce high yield in well fertilized conditions (Rengel, 2002).

Several studies have addressed root AM colonization of host genotypes in a number of cultivated crops including wheat (Hetrick, Wilson & Cox 1992); oat (Koide *et al.* 1988); tomato (Bryla & Koide 1990); and maize (An *et al.*, 2010; Kaeppeler *et al.* 2000; Sawers *et al.* 2017; Londoño *et al.* 2019). In maize, Kaeppeler *et al.* (2000) reported significant difference in percentage root colonized of 12 lines and less colonization in high P conditions compared to low P conditions. In a greenhouse study, two of four landraces of maize showed significantly higher root colonization (60-80%) compared to a hybrid (45%) (Sangabriel-Conde et al., 2014). Sawers *et al.*

(2017) evaluated 30 maize lines and reported that all AM colonized plants had greater growth and six lines were identified that exhibit differential responses to AM root colonization and plant growth. B73 and Mo17 are the widely used reference lines, while Oh43 and Mo18W, had the highest and lowest growth responses, respectively, and HP301 and Pa36- had the highest and lowest shoot dry weights, respectively, when not colonized. They reported significant differences in root colonization among host genotypes, in low P conditions, with significantly higher colonization in Mo18W compared to Mo17 and Oh43. They also reported that higher P levels decrease root colonization in maize. Londoño *et al.* (2019) studied AM colonization in five maize genotypes (a landrace, two genetically modified maize varieties and their associated isolines) under greenhouse conditions and reported that the mean colonization was 5% and ranged from 0.35 to 8.6%. More recently, Londoño *et al.* (2020) evaluated three maize lines in field conditions with native AM fungi, and reported that there was no significant difference in colonization between lines. Kaepler *et al.* (2000) identified three QTL that controlled the maize growth at low P without the presence of AM fungi for shoot weight and a QTL was associated with mycorrhizal responsiveness. However, the genetic basis for AM fungal colonization of maize genotypes is not well understood.

In previous studies of the maize-AM fungi interactions, the number of lines evaluated was relatively small. One of the goals of this research was to study these interactions across a larger sample of maize genetic diversity. In this study, we studied AM root colonization among the parents of the maize nested association mapping (NAM) population. The NAM population consists of 25 independent recombinant inbred line populations derived from crossing 25 diverse donor parents with the common founder parent B73 (Gage *et al.*, 2020). Our experiments used 26 parents

and hybrids derived from NAM population, together with a few other selected lines as detailed below (section 3.2.1).

We performed experiments in two different field locations in North Carolina (in soils with a history of low and high P) by planting seed of maize inbred and hybrid lines to study the AM colonization. We also evaluated two lines wild type of B73 and W22 and *CASTOR* mutant in the same field locations to study how the *CASTOR* mutant will respond compared to wild type in presence of natural AM fungi. It is difficult to study the actual benefit of AM fungi in the field due to lack of suitable AM fungi-free controls. Therefore, controlled greenhouse experiments were performed to study the maize genotype response to AM inoculation. Also, greenhouse study was done to find whether any colonization variation we observed in the field would be reproducible in controlled conditions or not.

3.2 Materials and Methods

3.2.1 Field Sites and Experimental Design

Two field locations; the Central Crop Research Station, Clayton, NC and Cherry Farm, Goldsboro, NC were used for the field experiment. The following 31 inbred lines and hybrids crossed with B73 were grown in both locations: B73, B97, CML103, CML228, CML247, CML277, CML322, CML333, CML52, CML69, H100, HP301, IL14H, Ki11, Ki3, Ky21, M162W, M37W, Mo17, Mo18W, Ms71, NC262, NC304, NC344, NC350, NC358, Oh43, Oh7B, P39, Tx303, and Tzi8. Twenty-six inbred lines (excluding H100, Mo17, NC262, NC304, and NC344) are parents in the NAM population. In Clayton, 10-12 seeds were sown per plot and two replicates of each maize line were arranged in a Randomized Complete Block Design (RCBD). Similarly, in Goldsboro, eight seeds were sown per plot and two replicates of each treatment were arranged in a RCBD. Seeds were sown on April 21, 2022, and April 28, 2022, in Clayton and Goldsboro, respectively.

3.2.2 Soil Samples Collection from the Field

The top 2-3 centimeters of soil were removed, and 15 cm deep was dug with the help of shovel to collect the soil samples. Soils from five to six different arbitrarily selected locations within each experimental plot were collected, placed in a bucket, and mixed together. Soil samples were collected before the experiment. Approximately 500 g of soil from each location were sent to North Carolina Department of Agriculture, Raleigh, North Carolina for nutrient analysis (Appendix A: Table A1).

3.2.3 Maize Root Samples Collection from the Field

Seven weeks after planting, root samples were collected from experimental plots at both field locations for AM colonization quantification. Roots of three arbitrarily selected plants per line were sampled from each replication in each location. For root sample collection, soil around the plant was loosened with the help of shovel and each plant was carefully removed from the soil to minimize root damage and loss. The root system of each was cut into two parts with the help of scissors and one half put in a sealed zip-loc plastic (26.8cm x 27.3cm) bag and placed in a cooler on ice. The root sample was then transferred to a 50 ml Falcon tube with 50% EtOH and stored at 4°C temperature until colonization quantification was done.

3.2.4 Greenhouse Experimental Design

For the greenhouse experiment, 30 inbred lines used for field experiments (see section 3.2.1) were used except for CML228 due to a shortage of seeds. First, sand and Sungro soil were mixed together in 1:1 ratio (v/v) using soil mixture equipment. The mixed soil was treated with hot water steam for 2 hours at 79°C-82°C. A coffee filter was placed in the base of each 6-inch pot to prevent soil from escaping through the drainage holes, and mixed soil was added into each pots. For the non-AM conditions, only sterilized soil mixture was used while for AM conditions, 25% bulk soil inoculum (see section 2.2.3 from chapter 2) was mixed with the sterilized soil mixture.

Three seeds were planted per pot at 1.5-2-inch depth. There were two replicates of each genotype with a total of 360 plants or pots. The AM and non-AM pots for same line were kept side by side in the same tray and arranged in a RCBD. The greenhouse day temperature was around 25-27°C and night temperature around 18-20°C with four hours of photoperiod from 8:00 am to 12:00 pm. After seed germination, only one plant was retained per pot and any remaining seedlings

were discarded. Every week, pots were randomized within the replication. Plants were irrigated twice a week and modified Hoagland's solution low in P (see section 2.2.5 from chapter 2) once per week. After 2, 4, and 6 weeks of seed sowing, plant height and chlorophyll content were measured. Plant height was measured from the soil surface to the tip of the tallest leaf. Chlorophyll content was measured in the middle section of the penultimate leaf using SPAD meter. The penultimate leaf was selected to maintain the same position throughout the experiment. Seven weeks after seed sowing, shoots were cut and processed to determine biomass while a few roots were kept for AM fungal quantification and the rest was kept for biomass determination. Shoots were kept at 50°C for two weeks while roots were kept at 70°C for 72 hours in hot air oven. Dried shoots after biomass measurement were sent to Waypoint Analytical Laboratory, Wilson, NC, for nutrient analysis. Mycorrhizal plant growth response (MR) was calculate based on the difference between AM inoculated and non-inoculated plants (Sawers et al., 2017).

Percentage AM root colonization was quantified using the Gridline Intersection method (see section 2.2.7, from chapter 2). Total colonization is the sum of percentage root colonized by the presence of arbuscules, vesicles, and hyphae in the root. They are defined by the structure formed inside the host roots (see section 1.1 from chapter 1). If the roots that intersect the Gridline (see section 2.2.7 chapter 2) had only hyphae in the intersection point, then it was considered as percentage root colonized by presence of hyphae. Similarly, if the roots that intersect the Gridline had only arbuscules or arbuscules + hyphae or arbuscules + vesicles or all three, then it was considered as percentage root colonized by presence of arbuscules. If the roots that intersect the Gridline had only vesicles or vesicles and hyphae, then it was considered as percentage root colonized by presence of vesicles. For the field studies described above, we only assessed total

colonization, but for the greenhouse experiment we differentiated colonization associated with these three structures.

3.2.5 Field Experimental Design for *CASTOR* Mutant

The inbred lines B73 and W22 wildtype, and B73xW22 and W22xB73 hybrid together with corresponding lines homozygous for a mutation in the *CASTOR* gene were grown in both field locations described in section 3.2.1. The experimental design and samples collection were the same as mentioned in sections 3.2.1 and 3.2.3.

3.2.6 Statistical Analysis

Data analysis includes only the percentage root colonization in the field experiment. Also, data analysis includes the percentage root colonization, difference in plant height, chlorophyll content, shoot tissue nutrient content, shoot and root biomass between AM inoculated and non-inoculated plants in the greenhouse experiment. These data analyses were based on six plants per line per location for field experiment or six plants per line per treatment in the greenhouse. Also, the correlation was measured between replications i.e., average of three plants per replication per line. All the figures were made using ggplot2 from R software (version 2022.07.2 Build 576 ©2009-2022 RStudio, PBC). Differences among means were analyzed using a two-way ANOVA followed by Tukey HSD tests. Pearson correlation tests were also performed using R software.

3.3 Results

3.3.1 AM root colonization in different maize lines in two field locations

An experiment was conducted to assess AM root colonization of 31 maize lines and 26 hybrids in two field locations (Table 3.1). One field location (i.e., Clayton) has a high soil P (78.75 P-index) and we hypothesized that high P availability would inhibit AM root colonization (Mosse, 1973; Thomson, Robson & Abbott, 1986; Breuillin *et al.*, 2010). Therefore, we selected another field location (Goldsboro) low in P (16.25 P-index) that we hypothesized support the establishment of AM symbiosis. Preliminary sampling of 6-week-old plants in Clayton indicated that there was AM colonization at this site, so we evaluated colonization at both sites to study the influence of high P and low P fields on AM root colonization of maize inbred and hybrid lines.

Root colonization was measured seven weeks after planting (Table 3.1). Correlations across lines between replications within environment were not high. We observed a significant correlation between replication 1 and replication 2 in Clayton ($r=0.28$, $p<0.05$) but no significant correlation was observed between replications in Goldsboro. ANOVA analysis (Table 3.2) indicated that while there were significant line effects in each environment, the replication and line by replication effects were also extremely significant.

Looking at the average of the combined replications (six plants in most cases) we observed that, contrary to our initial hypothesis, the percentage AM root colonization was higher in the high P (Clayton) field compared to low P (Goldsboro) field in each of the 57 lines which were grown in both locations. We observed some significant differences in average root colonization among maize lines in each location (Table 3.1). In Clayton, H100 had significantly higher root colonization compared to Ky21xB73 and Oh43 whereas in Goldsboro, B97, CML228, IL14H, Ky21, and Mo17 were significantly higher than Ki3xB73.

Based on the overall averages, we observed significant correlation ($r=0.38$, $p<0.05$) between replications (rep 1 and rep 2) for AM colonization in inbred lines in Clayton and no significant correlation between replications in Goldsboro. There was a significant correlation ($r=0.43$, $p<0.05$) between two locations for AM colonization in inbred lines. Also, no significant correlation was observed between replications and between two locations for AM colonization in hybrid lines. Also, we observed no significant correlation between inbred lines and their corresponding hybrid lines (inbred parents crossed with B73) in both locations: Clayton and Goldsboro. Also, no significant correlation between root colonization was observed between two locations across the lines.

Table 3.1: Percentage root colonization at Clayton and Goldsboro field locations

Lines	Root Colonization % (mean \pm sd)	
	Clayton (high P)	Goldsboro (low P)
B73	37.22 \pm 10.03 ^{abcdefg}	22.29 \pm 4.31 ^{ab}
B97	43.36 \pm 13.13 ^{abcdefg}	38.53 \pm 8.25^a
B97xB73	39.74 \pm 15.14 ^{abcdefg}	19.01 \pm 10.71 ^{ab}
CML103	36.04 \pm 21.57 ^{bcdefg}	28.05 \pm 23.94 ^{ab}
CML103xB73	39.61 \pm 5.81 ^{abcdefg}	30.42 \pm 5.84 ^{ab}
CML228	38.91 \pm 11.41 ^{abcdefg}	36.33 \pm 11.82^a
CML228xB73	38.56 \pm 11.16 ^{abcdefg}	17.94 \pm 6.36 ^{ab}
CML247	30.19 \pm 6.85 ^{cdefg}	23.73 \pm 6.38 ^{ab}
CML247xB73	30.62 \pm 7.63 ^{bcdefg}	26.44 \pm 10.54 ^{ab}
CML277	40.20 \pm 13.50 ^{abcdefg}	20.28 \pm 5.38 ^{ab}
CML277xB73	48.71 \pm 9.70 ^{abcdef}	35.49 \pm 12.74 ^{ab}
CML322	30.72 \pm 12.94 ^{bcdefg}	21.78 \pm 8.22 ^{ab}
CML322xB73	39.82 \pm 19.32 ^{abcdefg}	27.35 \pm 13.38 ^{ab}
CML333	41.36 \pm 11.22 ^{abcdefg}	25.90 \pm 9.70 ^{ab}
CML333xB73	39.65 \pm 21.13 ^{abcdefg}	19.29 \pm 5.37 ^{ab}

Table 3.1 (continued):

CML52	23.41 ± 10.52 ^{cdefg}	14.85 ± 8.32 ^{ab}
CML52xB73	31.29 ± 3.97 ^{bcdefg}	23.33 ± 16.95 ^{ab}
CML69	22.58 ± 9.25 ^{efg}	16.99 ± 7.55 ^{ab}
CML69xB73	26.08 ± 8.34 ^{fg}	22.00 ± 10.52 ^{ab}
H100	64.20 ± 14.03^a	32.06 ± 7.53 ^{ab}
HP301	44.05 ± 14.89 ^{abcdefg}	27.63 ± 11.67 ^{ab}
HP301xB73	25.50 ± 10.06 ^{fg}	21.94 ± 15.66 ^{ab}
IL14H	37.02 ± 10.60 ^{bcdefg}	32.40 ± 10.61^a
IL14HxB73	33.50 ± 16.80 ^{bcdefg}	No plants
Ki11	29.34 ± 11.89 ^{bcdefg}	24.50 ± 10.59 ^{ab}
Ki11xB73	46.09 ± 3.14 ^{abcdef}	25.13 ± 11.54 ^{ab}
Ki3	43.65 ± 10.30 ^{abcdefg}	28.70 ± 7.68 ^{ab}
Ki3xB73	36.41 ± 15.69 ^{bcdefg}	11.73 ± 6.27^b
Ky21	37.78 ± 25.09 ^{bcdefg}	28.62 ± 6.17^a
Ky21xB73	26.62 ± 10.28^g	17.83 ± 15.70 ^{ab}
M162W	50.66 ± 8.04 ^{abcd}	35.77 ± 13.21 ^{ab}
M162WxB73	33.36 ± 7.29 ^{bcdefg}	26.68 ± 11.20 ^{ab}
M37W	35.01 ± 9.14 ^{bcdefg}	30.04 ± 10.45 ^{ab}
M37WxB73	40.96 ± 9.30 ^{abcdefg}	35.18 ± 9.60 ^{ab}
Mo17	56.06 ± 11.04 ^{ab}	36.84 ± 11.61^a
Mo17xB73	35.30 ± 13.26 ^{bcdefg}	28.08 ± 6.62 ^{ab}
Mo18W	45.63 ± 12.44 ^{abcdef}	18.59 ± 4.45 ^{ab}
Mo18WxB73	50.98 ± 10.59 ^{abc}	26.33 ± 9.22 ^{ab}
Ms71	42.74 ± 11.06 ^{abcdefg}	18.82 ± 6.63 ^{ab}
Ms71xB73	50.21 ± 14.07 ^{abcde}	23.58 ± 10.04 ^{ab}
NC262	46.98 ± 9.43 ^{abcdef}	29.15 ± 9.98 ^{ab}
NC304	26.65 ± 7.84 ^{bcdefg}	26.57 ± 2.98 ^{ab}
NC344	50.04 ± 10.03 ^{abcde}	25.07 ± 11.09 ^{ab}
NC350	42.81 ± 6.12 ^{abcdefg}	27.28 ± 7.89 ^{ab}

Table 3.1 (continued):

NC350xB73	26.62 ± 14.14 ^{cdefg}	24.67 ± 7.94 ^{ab}
NC358	38.52 ± 3.58 ^{abcdefg}	18.41 ± 8.25 ^{ab}
NC358xB73	49.96 ± 10.40 ^{abcde}	31.26 ± 8.09 ^{ab}
Oh43	22.00 ± 6.36^g	No plants
Oh43xB73	30.37 ± 8.10 ^{defg}	23.27 ± 15.36 ^{ab}
Oh7B	47.23 ± 9.03 ^{abcdef}	35.29 ± 6.26 ^{ab}
Oh7BxB73	34.33 ± 6.06 ^{bcdefg}	24.57 ± 9.70 ^{ab}
P39	47.87 ± 11.68 ^{abcdef}	29.08 ± 13.18 ^{ab}
P39xB73	45.55 ± 12.41 ^{abcdef}	30.51 ± 3.91 ^{ab}
Tx303	42.01 ± 8.28 ^{abcdefg}	23.85 ± 6.65 ^{ab}
Tx303xB73	34.56 ± 7.31 ^{abcdefg}	27.08 ± 5.96 ^{ab}
Tzi8	29.46 ± 5.79 ^{bcdefg}	17.07 ± 9.48 ^{ab}
Tzi8xB73	39.06 ± 10.15 ^{abcdefg}	30.53 ± 9.84 ^{ab}

Lines not followed by the same letters were significantly different. sd = standard deviation
Root colonization was measured as an average of six plants from two replicates (i.e., 3 plants per replication)

Table 3.2: ANOVA for AM root colonization between lines and replicates in Clayton and Goldsboro field locations

Source	df	Sum of Square	Mean Square	F value	Pr(>F)
Clayton					
Lines	55	30065	546.6	6.21	<2e-16***
Replication	1	1763	1763.5	20.01	1.23e-05***
Lines*Replication	54	16405	303.8	3.45	6.38e-11***
Residuals	219	19292	88.1		
Goldsboro					
Lines	55	12626	229.6	3.35	1.21e-10***
Replication	1	1797	1797.1	26.26	6.47e-07***
Lines*Replication	55	12060	219.3	3.20	6.19e-10***
Residuals	223	15264	68.4		

*** Significant at p= 0.001 level

3.3.2 Greenhouse trial of AM colonization and other traits across lines

An experiment was conducted to examine variation in AM root colonization among 30 maize lines in the greenhouse. In addition to AM colonization, we assessed response to AM by measuring plant height, chlorophyll content, shoot and root biomass, and shoot tissue nutrient content in AM inoculated and non-inoculated treatments. AM root colonization was not observed in non-inoculated plants (data not shown) but in the inoculated AM treatment, we observed a significant correlation in AM colonization across lines between replicates ($r=0.59$, $p<0.001$). ANOVA showed significant lines and replications effects but no significant line by replication interaction (Table 3.3).

Based on the averages of combined replicates, significant differences in total percentage root colonization were observed among maize lines. NC350 had significantly higher total colonization than Ki3 (Fig 3.1). The correlation between total colonization in the greenhouse and at both field sites were not significant.

A functional indication for AM symbioses is the formation of arbuscules with or without vesicles in the plant roots (Smith & Read, 2008). There was a significant positive correlation between percentage root colonized by presence of arbuscules (arbuscular colonization) and shoot biomass, root biomass, plant height (4 and 6 weeks after sowing), chlorophyll content (4 and 6 weeks after sowing) and shoot tissues potassium content, and a significant negative correlation between arbuscular colonization and plant height (2 weeks after sowing), shoot tissues nitrogen and phosphorus content (Table 3.3).

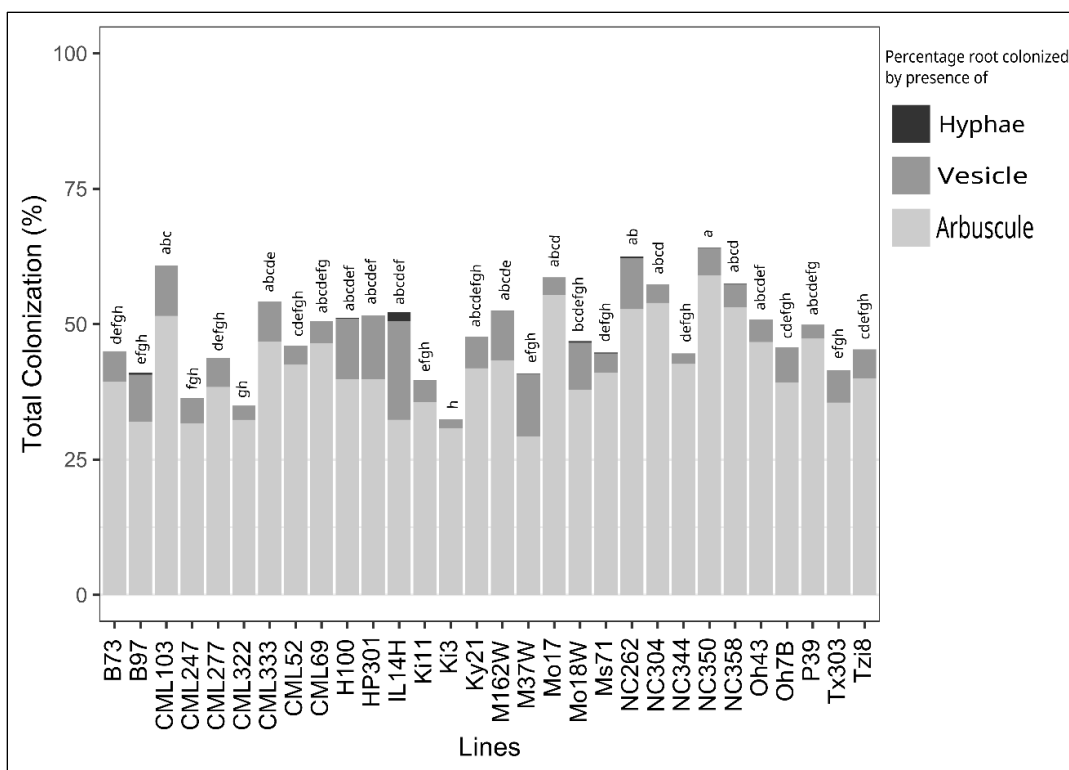


Figure 3.1: AM root colonization of 30 maize lines. Total colonization of maize lines inoculated with soil AM inoculum in greenhouse conditions. The bar graph shows the percentage total root colonization in y-axis and maize lines on the x-axis. The three different colors in the bar graph represents different types of colonization. Black, dark gray, and light gray color indicates percentage root colonization by presence of hyphae only, vesicles and hyphae, and arbuscules and hyphae (with or without vesicles), respectively. No colonization was observed in non-AM treatment (data not shown), but significant differences in AM root colonization were observed among maize genotypes in the AM inoculated treatment. Different letters represent significant differences ($p < 0.05$) in total colonization of maize lines.

Table 3.3: ANOVA for AM root colonization between maize lines and replicates in the greenhouse experiment

Source	df	Sum of Square	Mean Square	F value	Pr(>F)
Lines	29	11175	385.4	5.31	1.72e-08***
Replication	1	1565	1564.6	21.58	7.50e-06***
Lines*Replication	29	2889	99.6	1.37	0.173
Residuals	118	8557	72.5		

*** Significant at $p = 0.001$ level

Table 3.4: Correlation between percentage root colonization indicated by presence of arbuscules and shoot biomass, root biomass, plant height, chlorophyll content, and shoot tissue nutrient content in the greenhouse experiment

	Arbuscular colonization
Shoot biomass	0.45 ***
Root biomass	0.11 *
Plant height (2 WAS)	-0.25 ***
Plant height (4 WAS)	0.15 **
Plant height (6 WAS)	0.34 ***
Chlorophyll content (2 WAS)	ns
Chlorophyll content (4 WAS)	0.34 ***
Chlorophyll content (6 WAS)	0.42 ***
Nitrogen	-0.17 **
Phosphorus	-0.19 ***
Potassium	0.36 ***

WAS = Weeks after sowing

ns not significant

* Significant at p= 0.05 level

** Significant at p= 0.01 level

*** Significant at p= 0.001 level

Correlation between replications for shoot dry weight ($r=0.49$, $p<0.001$) and root dry weight ($r=0.54$, $p<0.001$) were significant. There was no significant differences in the response (difference between AM inoculated and non-inoculated plants) to mycorrhizal colonization in terms of shoot (Fig 3.2a) and root (Fig 3.2b) dry weight between maize lines. In general all maize lines responded positively to AM inoculation and there was a significant positive correlation between arbuscular colonization and shoot and root dry weight (Table 3.3).

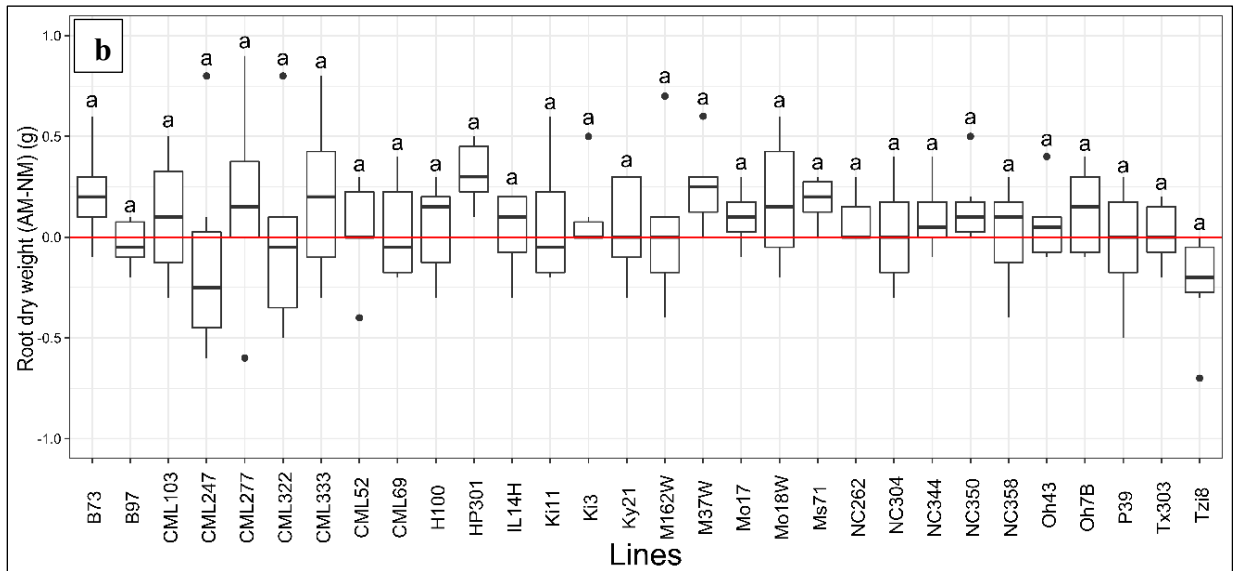
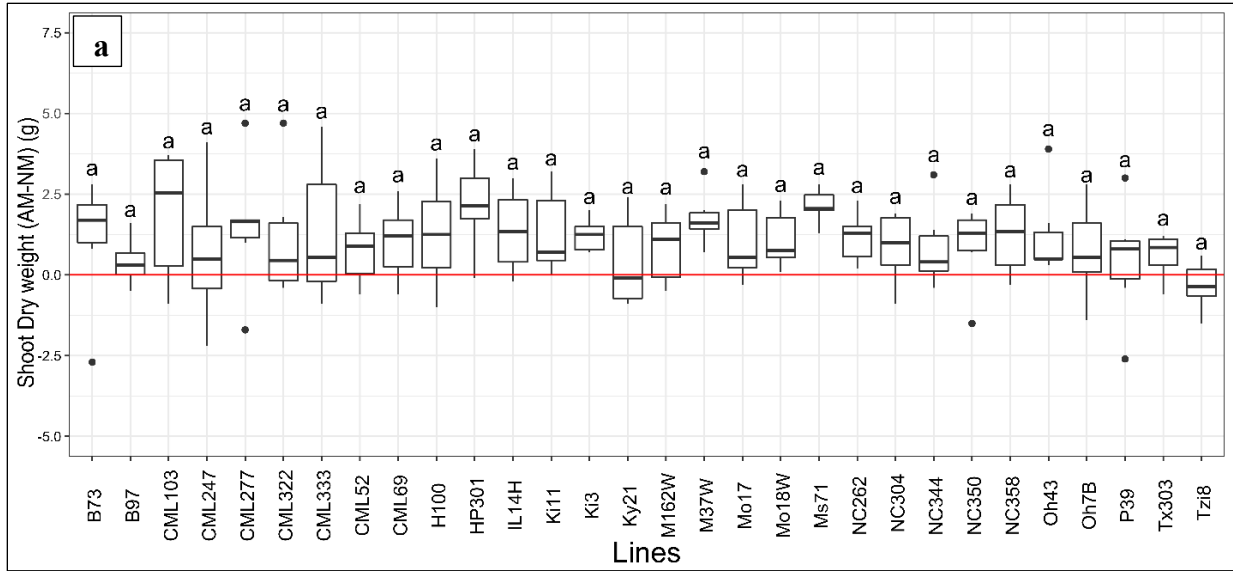
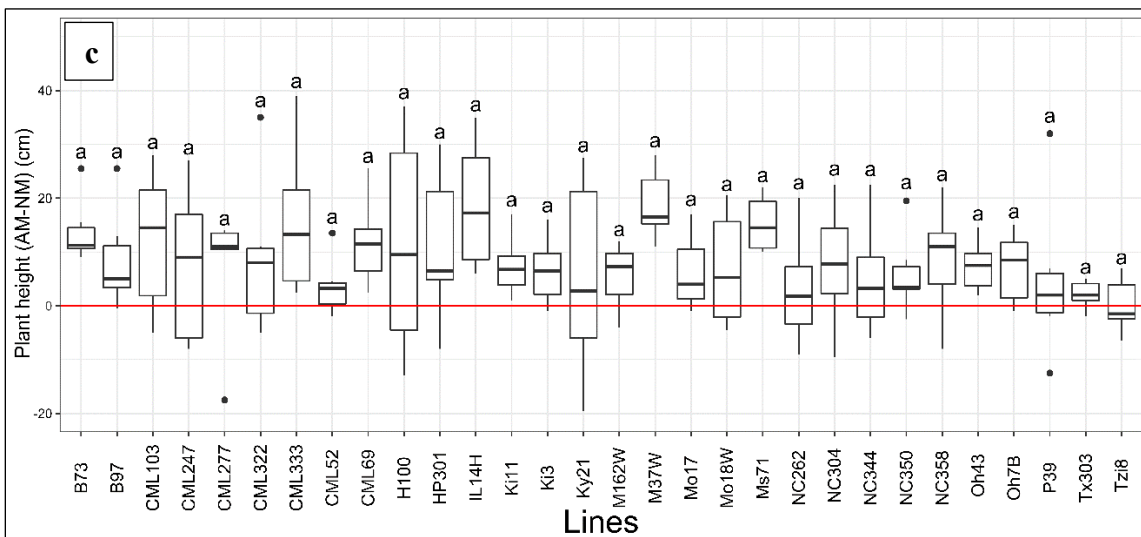
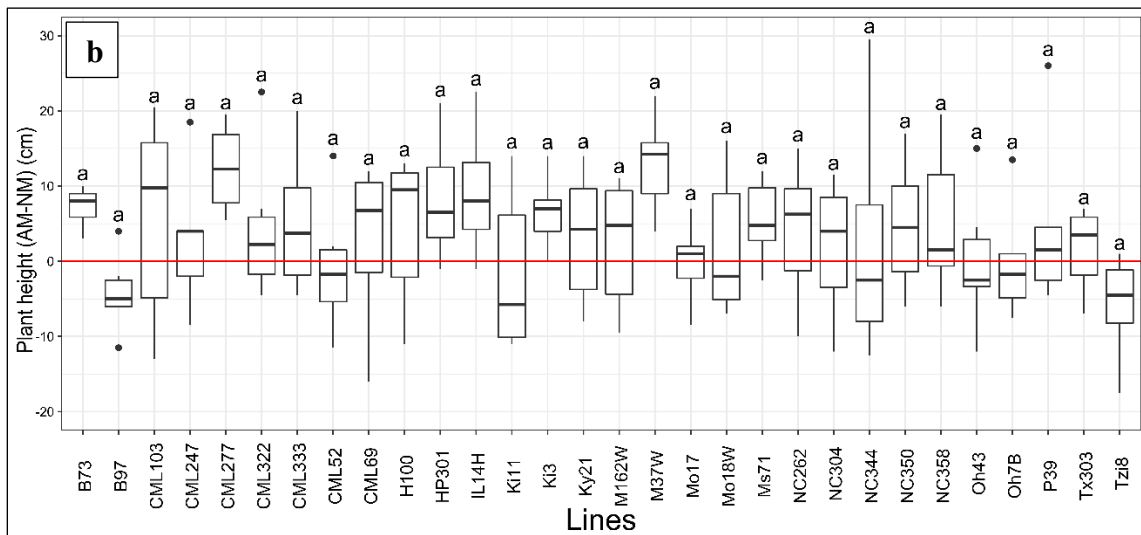
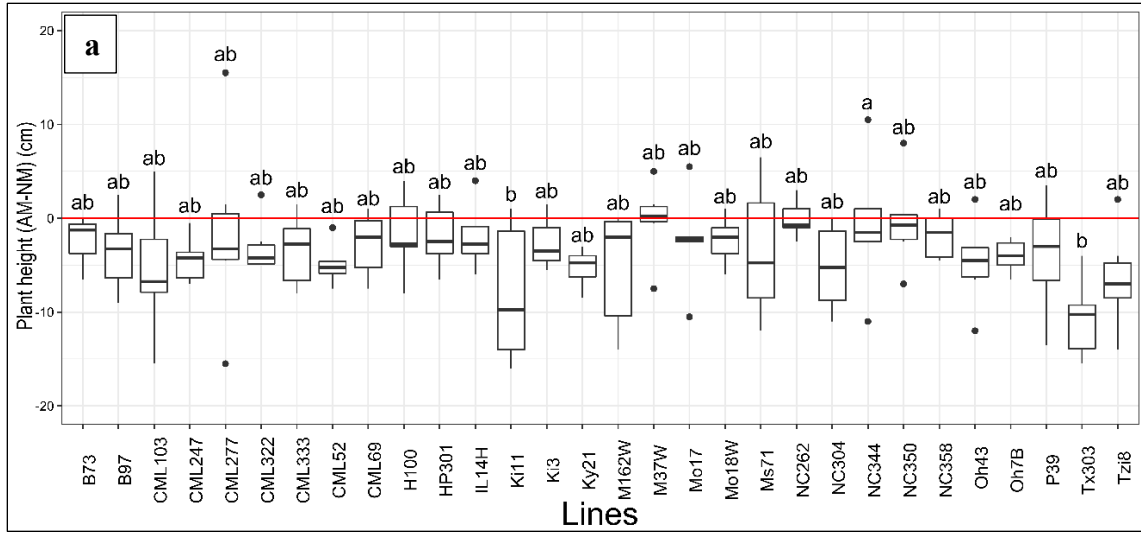


Figure 3.2: Effect of root colonization in shoot/root dry weight in 30 maize lines. Difference in (a) shoot dry weight (b) root dry weight between AM inoculated (AM) and non-inoculated (NM) maize lines. The reference line (red color line) indicates 0 or no difference between AM and NM plants. A negative value indicates NM plants were heavier and vice versa. Maize lines that do not overlap with zero show a significant effect of AM inoculation on dry weight. Lines not followed by the same letter were significantly different (Tukey HSD, $p > 0.05$).

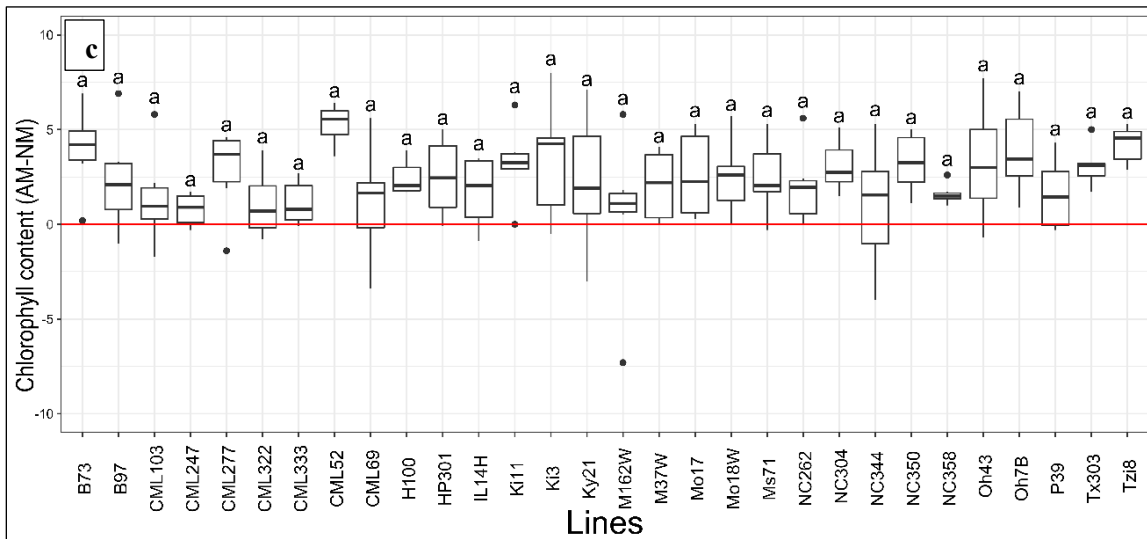
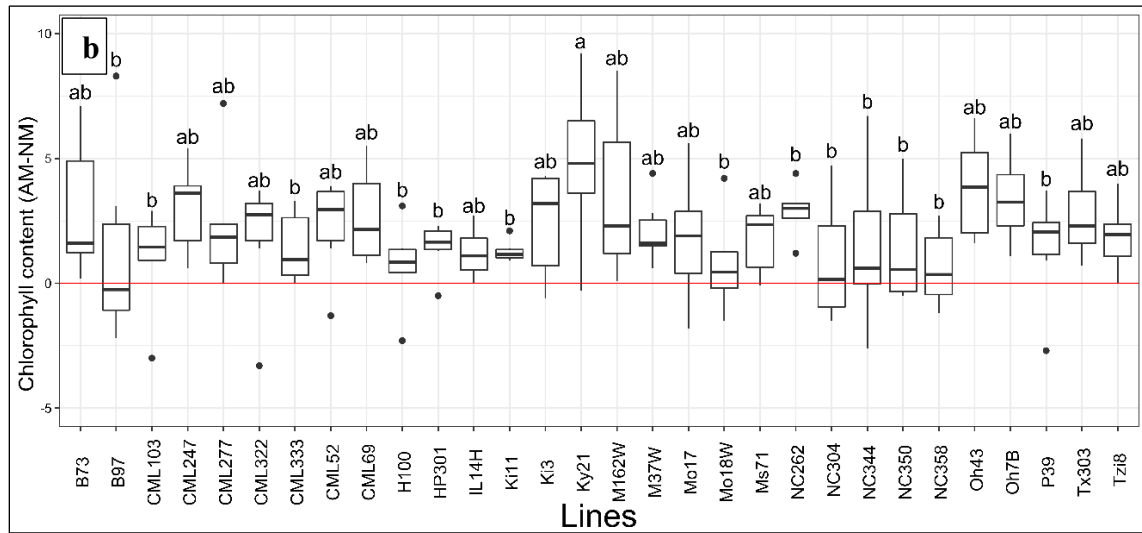
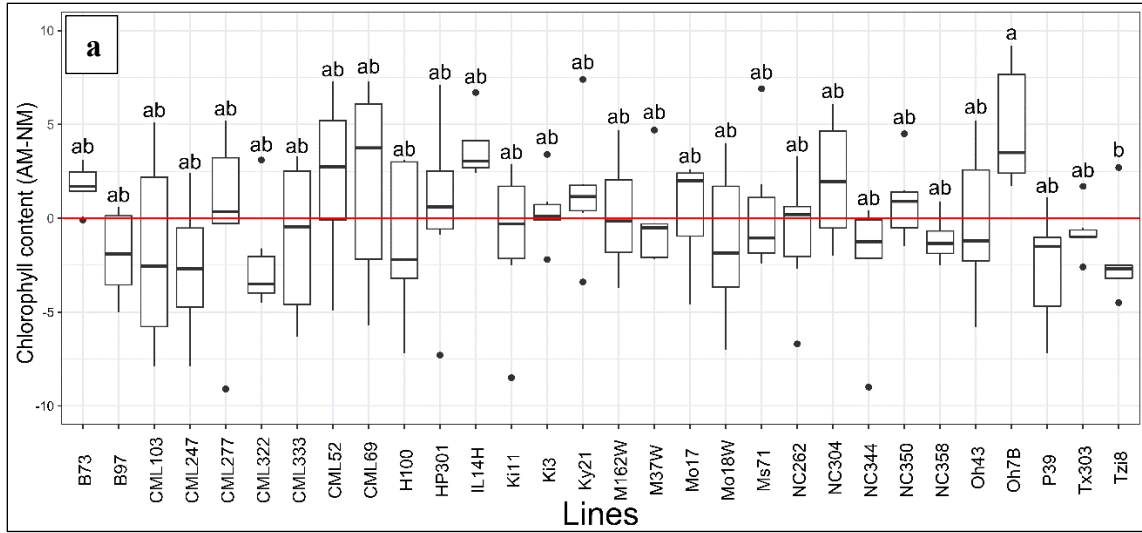
Correlation between replications for plant heights taken at different time period: 2 weeks ($r=0.77$, $p<0.001$), 4 weeks ($r=0.49$, $p<0.001$) and 6 weeks after sowing ($r=0.59$, $p<0.001$) were significant. After 2 weeks of seed sowing, the response to mycorrhizal colonization in terms of plant heights of NC344 were significantly higher than Ki11 and Tx303 (Fig 3.4). However, we did not observe a significant difference in the response to mycorrhizal colonization in terms of plant height between maize lines after 4 (Fig 3.5) and 6 (Fig 3.6) weeks of sowing.

Figure 3.3: Effect of root colonization in plant height in 30 maize lines. Difference in plant heights after (a) 2 weeks (b) 4 weeks (c) 6 weeks of sowing between AM inoculated (AM) and non-inoculated (NM) maize lines. The reference line (red color line) indicates 0 or no difference between AM and NM plants. A negative value indicates NM plants were taller and vice versa. Maize lines that do not overlap with zero show a significant effect of AM inoculation on plant heights. Lines not followed by the same letters were significantly different (Tukey HSD, $p > 0.05$).



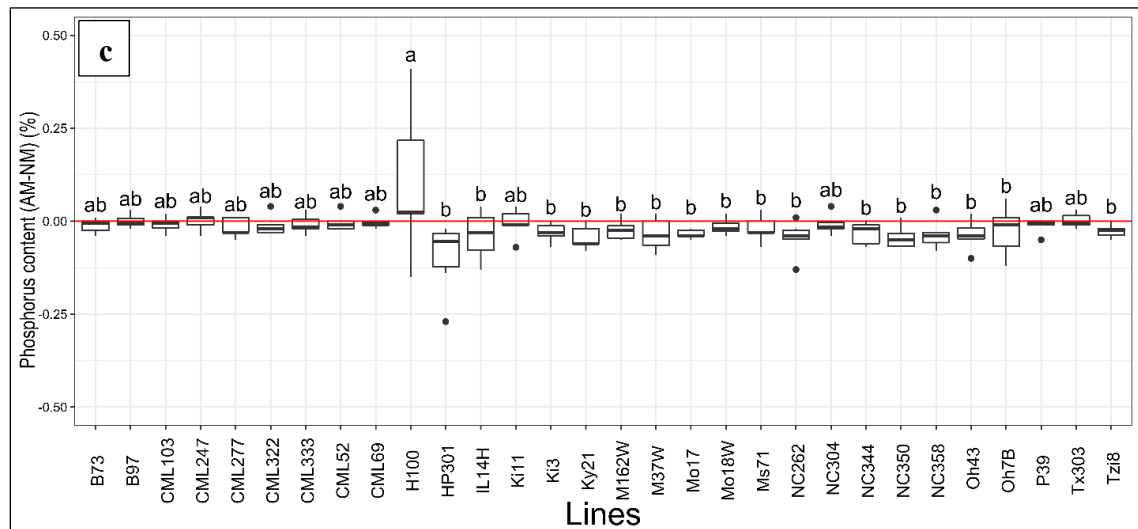
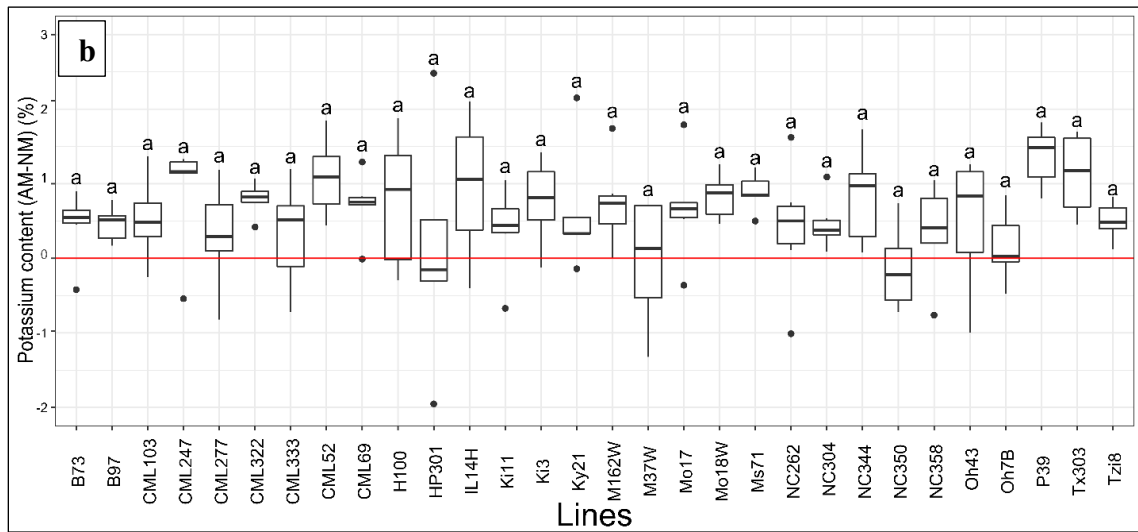
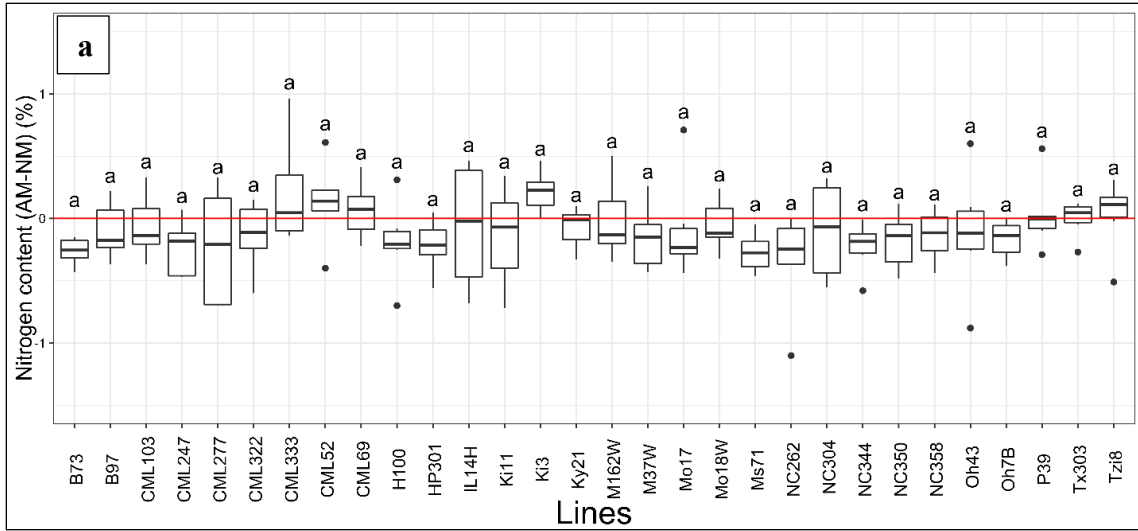
Similarly, correlation between replications for chlorophyll content measured at different time points: 2 weeks ($r=0.65$, $p<0.001$), 4 weeks ($r=0.62$, $p<0.001$), and 6 weeks after sowing ($r=0.73$, $p<0.001$) were significant. We observed that the response to mycorrhizal colonization for chlorophyll content after 2 weeks of sowing was significantly higher in Oh7B compared to Tzi8 (Fig 3.4a). Also, we observed that there was no significant correlation between colonization and chlorophyll content at 2 weeks (Table 3.3). However, there was significant positive correlation between AM root colonization and chlorophyll content at 4 and 6 weeks (Table 3.3). The response to mycorrhizal colonization in terms of chlorophyll content after 4 weeks were significantly higher in Ky21 compared to B97, CML103, CML333, H100, HP301, Ki11, Mo18W, NC262, NC304, NC344, NC350, NC358, and P39 (Tukey HSD, $p<0.05$) (Fig 3.4b) and no significant difference was observed among lines after 6 weeks (Fig 3.4c).

Figure 3.4: Effect of root colonization in chlorophyll content in 30 maize lines. Difference in chlorophyll content (a) 2 weeks (b) 4 weeks (c) 6 weeks after sowing between AM inoculated (AM) and non-inoculated (NM) maize lines. The reference line (red color line) indicates 0 or no difference between AM and NM plants. A negative value indicates NM plants had less chlorophyll content and vice versa. Maize lines that do not overlap with zero show a significant effect of AM inoculation on chlorophyll content. Lines not indicated by the same letters were significantly different (Tukey HSD, $p > 0.05$).



Correlation between replications across lines in leaf nitrogen content was not significant but correlations for leaf phosphorus ($r=0.37$, $p<0.05$) and potassium content ($r=0.70$, $p<0.001$) were significant. We observed that the response to mycorrhizal colonization in terms of shoot N and K content were not significantly different among maize lines (Fig 3.5a and 3.5b). However, we observed that the response to mycorrhizal colonization in terms of shoot phosphorus content was significantly higher in H100 compared to HP301, IL14H, Ki3, Ky21, M162W, M37W, Mo17, Mo18W, Ms71, NC262, NC344, NC350, NC358, Oh43, Oh7B, and Tzi8 (Fig 3.5c). There was a significant negative correlation between AM root colonization, and leaf N and P content and a significant positive correlation between colonization and leaf K content (Table 3.3).

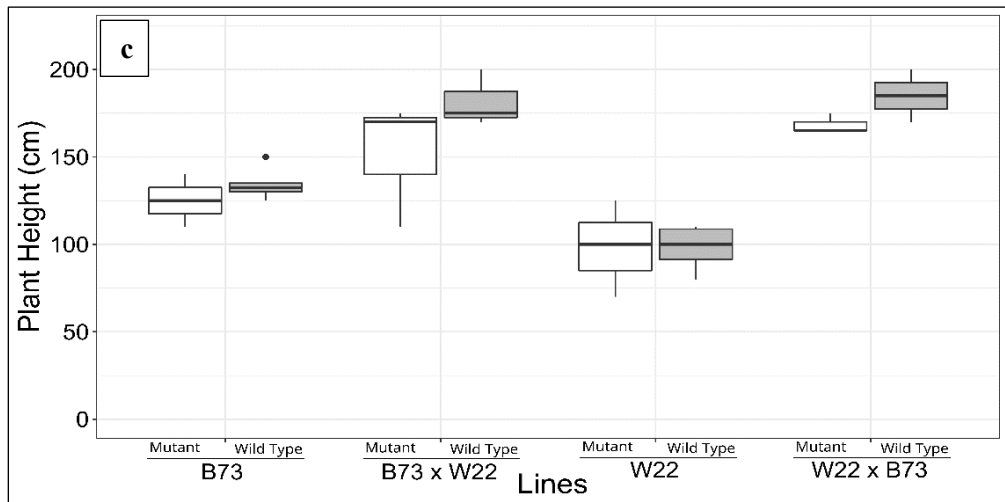
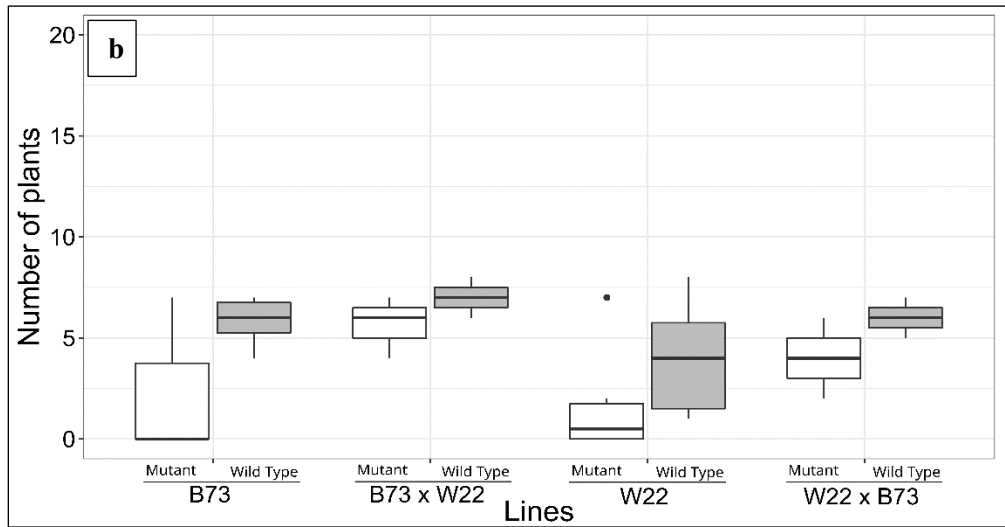
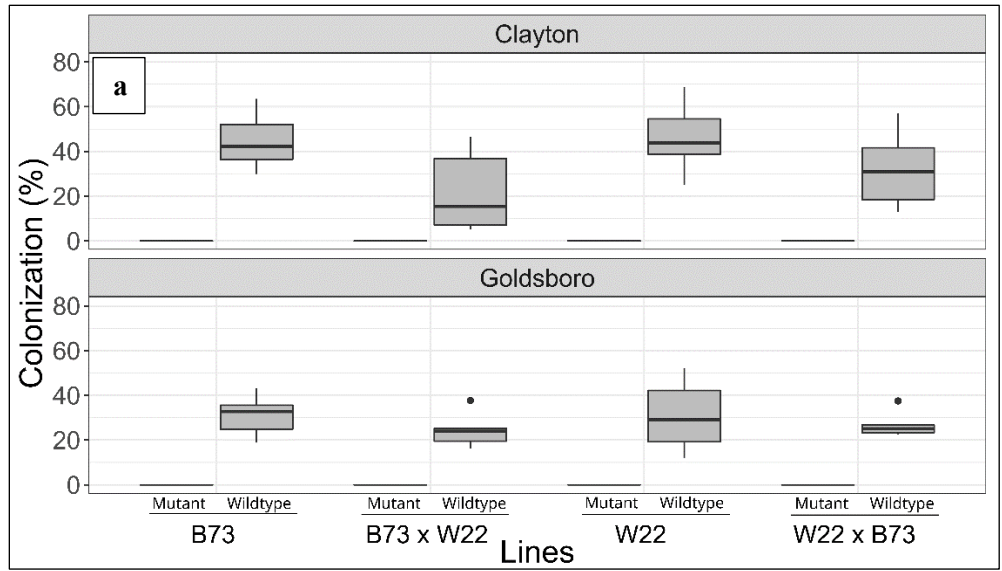
Figure 3.5: Effect of root colonization in shoot nutrients content in 30 maize lines. Difference in shoot tissue nutrient content (a) Nitrogen (N) (b) Potassium (K) (c) Phosphorus (P) between AM inoculated (AM) and non-inoculated (NM) maize lines. The reference line (red color line) indicates 0 or no difference between AM and NM plants. A negative value indicates NM plants had less shoot tissue nutrient content and vice versa. Maize lines that do not overlap with zero show a significant effect of AM inoculation on shoot tissue nutrient content. Lines not indicated by the same letters were significantly different (Tukey HSD, $p > 0.05$).



3.3.3 *CASTOR* mutant experiment

The *CASTOR* gene encodes a calcium channel protein that plays an essential role during AM fungal symbiosis. Maize mutant lines that do not have *CASTOR* gene are unable to form symbiotic relationships with AM fungi (Ramírez-Flores et al., 2020). Generally, in well fertilized soil conditions, it is thought that AM fungi are not beneficial to host plants. While soil at the field experiment in Clayton was well fertilized with a P and had a P-index of 78.75 whereas Goldsboro was deliberately chosen as a low P site and had a P-index of 16.25 (Appendix A: Table A1). Therefore, wildtype lines (able to form symbiotic associations with AM fungi) would be expected to derive marginal benefits from AM associations in high fertilized soil and *CASTOR* mutant lines when grown in well fertilized field soil would be similar to their wildtype equivalents. When we planted B73 and W22 lines that were homozygous for a *CASTOR* knockout mutation together with their wild type isolines, we observed that, as expected, the mutants did not support AM colonization (Figure 3.6a) but, somewhat unexpectedly the mutants had very poor plant standing (Figure 3.6b) and growth (Figure 3.6c) compared to their wild type isolines at both locations irrespective of soil P levels.

Figure 3.6: Colonization, plant stand, and growth of wild type and *CASTOR* mutant lines. Box plots showed 4 different maize lines in x-axis and (a) Percentage root colonization, (b) Plant stand, and (c) Plant height in y-axis between wild-type parents and their corresponding *CASTOR* mutants' lines. The whisker box plots represent the interquartile range. Data points that do not fall in whisker box ranges were considered as potential outlier data and represented by black dot (•).



3.4 Discussion

AM fungi are often beneficial to the host plants and low P condition has been shown to be necessary for AM fungi to form symbiotic association with host plants (Smith & Read, 2008). Field experiments were conducted at two locations in North Carolina: Clayton (high soil P) and Goldsboro (low soil P) to evaluate AM colonization of maize lines. We anticipated that we would observe lower colonization in the high P (78.75 P-index) field compared to the low P (16.25 P-index) field (Appendix A: Table A1).

We observed a significant difference in root colonization among maize lines in each location and comparatively higher root colonization in high soil P field (Clayton) compared to low soil P field (Goldsboro) (Table 3.1). Although, data from my previous chapter and previous studies showed that high soil P inhibits AM colonization in the host plants (Kaeppler *et al.*, 2000; Sawers *et al.* 2017; Liu *et al.*, 2018), we did not observe the same trend in our field experiment. A plausible explanation for these results is that P was not the only nutrient that varied between the two field sites. Several nutrients (K, Mn, and S) were present in higher amounts in Goldsboro compared to Clayton soil, while P and Zn were higher in soil at Clayton (Appendix A: Table A1). Also, the soil or environmental conditions, differing native AM fungi species present in the field might have affected the root colonization variation in these two locations. We have done one season study only with only two locations. Further field experiments over multiple locations are needed to definitively identify the environmental factors driving AM root colonization. Also, controlled experiments can be conducted in conjunction with field experiments to examine the relative contribution of soil nutrition to AM root colonization. For example, we observed that high fertilizer concentrations in the greenhouse can inhibit AM colonization in maize (Chapter 2 section 2.3.4).

We did not observe significant correlation between hybrids and their parental lines for root colonization. Also, we found that correlation is not consistent with other findings as well in our field experiment. It might be possible that we did not see any consistent correlation because of the variation in the soil or environmental conditions between the two locations.

In the field, it is challenging to evaluate the beneficial association of AM fungi, due to lack of suitable AM fungi-free controls. Therefore, we conducted a greenhouse experiment to study the colonization and response of maize lines response to AM and non-AM inoculation treatments. We observed significant difference in root colonization among maize lines (Fig 3.1). Several other studies also reported that root colonization varied among maize lines (Sangabriel-Conde *et al.* 2014; An *et al.* 2010; Kaepler *et al.* 2000; Sawers *et al.* 2017). In these studies, the number of lines evaluated for colonization were much lower compared to our study. We evaluated 30 maize lines and observed NC350 had significantly higher total colonization than Ki3 (Fig 3.1) whereas Sawers *et al.* (2017) evaluated six lines and reported that Mo18W had significantly higher total colonization compared to Oh43 and Mo17. When we look at our colonization data of the same six lines (B73, HP301, Mo17, Mo18W, Oh43, and P39) previously examined by Sawers *et al.* (2017). We observed that the total colonization was significantly higher in Mo17 compared to B73 and different from what Sawers *et al.* (2017) reported. The AM fungal genera/species or isolate used were different which may have contributed to difference in colonization in these lines. It might be possible that different AM fungi species colonize differently to same host. Therefore, control experiments, with different AM fungi species and more number of lines, would give insight regarding colonization variation with AM species and plant lines.

We observed that most maize lines responded positively to AM inoculation as assessed by shoot and root biomass (Fig 3.2 or Table 3.4). However, the response to mycorrhizal colonization

in terms of shoot and root biomass were not significantly different among lines (Fig 3.2). Several studies also reported that maize lines responded positively to AM inoculation for shoot dry weight and that this response were maize line-dependent (Kaeppeler *et al.*, 2000; Sawers *et al.*, 2017). Most maize lines evaluated in our study and Sawers *et al.* (2017) were similar but we did not observe the same trend. We observed that CML103 as a high response and Tzi8 as a low response line when comparing all 30 lines for shoot dry weight (Fig 3.2) whereas Sawers *et al.* (2017) reported that Oh43 as high response and Mo18W as low response line to AM inoculation for shoot dry weight. This variation to AM response may be due to different AM isolates used in our study.

Two weeks after sowing, response to mycorrhizal colonization in terms of plant heights of NC344 lines were significantly higher than Ki11 and Tx303 and we observed that the difference between AM inoculated and non-inoculated plants in most maize lines were below red reference lines (Fig 3.3a). This was also reflected in significantly negative correlation between colonization and plant height at 2 weeks (Table 3.3). It may be due to the fact that in the beginning of AM symbiosis, plants have to spend more resources to maintain symbiosis and AM fungi may not be functional in early maize growth stages to derive benefits from AM fungi by host plants. However, 4 and 6 weeks after seed sowing, we observed that AM inoculated plants were responding positively to AM symbiosis as reflected by a significant positive correlation between colonization and plant height at 4 and 6 weeks (Table 3.3). A similar trend was observed in chlorophyll content among 2, 4, and 6 weeks after sowing between AM inoculated and non-inoculated plants.

Shoot (leaf) tissue nitrogen and phosphorus content were negatively responded while shoot potassium content positively responded to AM inoculation (Table 3.4). However, we did not observe any significant difference between maize lines for the response to mycorrhizal colonization in terms of these traits (Fig 3.5a, b). It has been reported that, in a P limited conditions,

AM fungi facilitate P acquisition in host plants (Liu *et al.*, 2018; Sawers *et al.* 2017; Smith *et al.*, 2010). We observed that difference between shoot tissue P content between AM inoculated and non-inoculated plants was significantly higher in H100 compared to HP301, IL14H, Ki3, Ky21, M162W, M37W, Mo17, Mo18W, Ms71, NC262, NC344, NC350, NC358, Oh43, Oh7B, and Tzi8 (Fig 3.5c) while Sawers *et al.* (2017) reported that Oh43 and Pa36 had significantly higher shoot P content compared to Mo18W, Hp301, B73, and Mo17 in AM inoculated plants. We observed that most AM inoculated lines had lower P content compared to non-AM plants while Sawers *et al.* (2017) reported that the shoot P content was lower in AM plants compared to non-AM plants in high soil P conditions but not in low soil P conditions. It might be possible that different AM fungi species differentially provide P to the host plants and the AM species used in our and Sawers *et al.* (2017) study were different.

B73 and W22 lines homozygous for a *CASTOR* knockout mutation and their respective wild type isolines were planted in the same field locations in Clayton and Goldsboro and we observed that the mutants did not support AM colonization (Figure 3.6a) but, somewhat unexpectedly, the mutants had very poor plant stand (Figure 3.6b) and growth (Figure 3.6c) compared to their wild type isolines at both locations, and not just under low P conditions in Goldsboro. Generally, it has been thought that the only role for the *CASTOR* gene is in AM symbiosis. However, based on our observation, we hypothesize that the *CASTOR* gene may have other important roles.

In conclusion, AM root colonization of maize lines were observed between replications in field and greenhouse experiments, but the correlation was not high. Similarly, correlation between field and greenhouse was also not significant and we observed that colonization in high soil P field was comparatively higher than low soil P field. In the greenhouse experiment, we observed that

most maize lines responded positively to AM inoculation as determined by measurement of traits such as plant height, chlorophyll content, shoot and root biomass, and shoot (leaf) K content although they responded negatively with respect to shoot N and P content. Also, we evaluated wild type and *CASTOR* mutant lines and observed *CASTOR* mutants' growth were very poor (Figure 3.6b) compared to their wild type.

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APPENDICES

APPENDIX A

Table A1: Soil nutrient analysis from two Clayton and Goldsboro field locations:

	Central Crop Research Station, Clayton	Cherry Farm, Goldsboro
HM% (Percent Humic Matter)	0.20	0.22
W/V (weight per volume) (g/cm ³)	1.28	1.04
CEC (Cation Exchange Capacity)	2.58	6.08
BS% (% CEC occupied by basic cations)	67.25	77.5
Ac (Exchangeable acidity)	0.85	1.4
pH (Current Soil pH)	5.38	5.43
P-I (Phosphorus Index)	78.75	16.25
K-I (Potassium Index)	46.75	67.5
Ca% (% CEC occupied by Calcium)	43.75	48.75
Mg% (% CEC occupied by Magnesium)	14.5	23.25
S-I (Sulfur Index)	15	32.25
Mn-I (Manganese Index)	45.75	430.25
Mn-AI1 (Mn availability index for crop 1)	37.5	268.25
Zn-I (Zinc Index)	185	87.5
Zn-AI (Zinc availability Index)	185	87.5
Cu-I (Copper Index)	48.75	107.75
Na (meq/100 cm ³)	0.025	0.05
ESP (Exchangeable Sodium Percent)	1	1