IMPACT OF ARBUSCULAR MYCORRHIZAL FUNGI ON THE PHYSIOLOGY OF MAIZE GENOTYPES UNDER VARIABLE NITROGEN AND PHOSPHORUS LEVELS

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IMPACT OF ARBUSCULAR MYCORRHIZAL FUNGI ON THE PHYSIOLOGY OF MAIZE GENOTYPES
UNDER VARIABLE NITROGEN AND PHOSPHORUS LEVELS

by

Roberto Javier Crespo

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Increasing crop production to ensure future food security while reducing environmental pressure on agro-ecosystems requires improved water and nutrient use efficiency. The soil microbial community directly and/or indirectly has important consequences on food security since soil microbes participate in several soil processes. Thus, it is important to increase our understanding of AM fungal and maize genotype interactions, the impact of N and P fertilization and water condition on the symbiosis, and on the physiology and nutritional status of maize plants. In two greenhouse experiments AM inoculated plants exhibited root colonization values around 70% which was confirmed by the presence of the AM lipid biomarker (C16:1cis11). Nitrogen fertilization increased AM root colonization, but only compared to unfertilized plants. Root colonization and biomarker concentration in root and soil were similar among inoculated maize genotypes across conventional and drought tolerant hybrids. Mycorrhizal inoculation had a positive impact on maize plant P uptake, but neither increased N uptake nor chlorophyll content in leaves. Nitrogen fertilization increased P concentration in plant tissue under AM inoculation, but decreased P concentration under non-inoculated conditions. There were positive plant biomass and chlorophyll responses as N fertilization increased, but not for P fertilization. Except for increased P uptake, results
from both greenhouse studies are inconclusive about why most of the parameters evaluated were unresponsive or negatively affected by AM inoculation. In a field experiment, indigenous AM fungi effectively colonized maize roots to the same magnitude regardless of maize genotype and soil water condition. Increased soil extramatrical AM biomass, suggesting greater C allocation from plant to AM fungus, was observed under water-limited conditions and also among maize genotypes. In addition, while water limitation caused a shift in the overall soil microbial community, maize hybrids influenced specific microbial groups. Bacterial and actinomycete markers, and also total microbial biomass significantly increased under water stress. Interactions among AM fungi, plants and nutrients appear to be complex making plant responses to AM fungi difficult to predict and explain. Further studies on the mechanisms involved are needed to gain further insight into the complex relationships among AM fungi, maize and soil fertility management to maximize benefit from the AM fungi/plant symbiosis.
IMPACTO DE LAS MICORRIZAS ARBUSCULARES SOBRE LA FISIOLOGIA DE GENOTIPOS DE MAIZ BAJO NIVELES VARIABLES DE NITROGENO Y FOSFORO

Roberto Javier Crespo, Ph.D.

University of Nebraska, 2015

Asesor: Rhae Drijber

La comunidad microbiana del suelo tiene importantes consecuencias directas en la producción de los cultivos e indirectas en la seguridad alimentaria, dada su participación en los procesos de ciclado de nutrientes y agua, entre otros. De ahí, la importancia de mejorar el conocimiento relacionado a las interacciones entre hongos micorrícticos y genotipos de maíz, el impacto de la fertilización con nitrógeno (N) y fosforo (P), y el contenido de agua del suelo sobre la simbiosis hongo/planta, y el estatus fisiológico y nutricional de las plantas de maíz. En experimentos en invernadero las raíces de plantas de maíz inoculadas con micorrizas arbusculares (MA) exhibieron niveles de colonización de alrededor del 70%, lo cual fue confirmado con la presencia de un biomarcador lipídico (C16:1cis11). La fertilización nitrogenada incrementó la colonización de raíces por MA respecto al tratamiento sin fertilización, pero no hubo diferencia en colonización entre dosis de N aplicado. La colonización de raíces y la concentración del biomarcador en raíces de maíz y en el suelo fueron similares entre genotipos de maíz tolerantes y no tolerantes a sequía. La inoculación con MA tuvo un impacto positivo en la absorción de P de la plantas de maíz, pero no incremento la absorción de N ni la concentración de clorofila en hojas. La fertilización nitrogenada incrementó la concentración de P en
plantas inoculadas, pero disminuyó la concentración de P en plantas no inoculadas. La biomasa de la planta y el contenido de clorofila incrementaron con la dosis de fertilización nitrogenada, pero la respuesta no fue la misma con la fertilización fosforada. Excepto para la absorción de P, los resultados de ambos experimentos en el invernadero no permiten obtener conclusiones claras de porque varios de los parámetros evaluados no respondieron a los tratamientos o tuvieron un impacto negativo en plantas inoculadas con MA. En el estudio de campo, las micorrizas nativas del suelo colonizaron las raíces de maíz en la misma magnitud para todos los genotipos de maíz y niveles de agua del suelo. La biomasa micorrítica en el suelo varió entre genotipos de maíz, pero se incrementó bajo condiciones limitantes de agua en el suelo. Esto sugiere una mayor asignación de carbono desde la planta hacia el hongo micorrítico bajo condiciones de estrés hídrico. Mientras un bajo contenido de agua en el suelo causa un cambio en la toda la comunidad microbiana del suelo, los híbridos de maíz solo influenciaron a grupos microbianos específicos. Biomarcadores pertenecientes a bacterias y actinomicetes, y también, la biomasa microbiana total fueron significativamente incrementadas bajo condiciones de estrés hídrico. Las interacciones entre MA, la planta y los nutrientes parecen ser complejas, lo cual hace que la respuesta de la planta a la inoculación micorrítica sea difícil de predecir y explicar. Futuros estudios relacionados a los mecanismos involucrados son necesarios para obtener el conocimiento que permita explicar las complejas interrelaciones, y así, maximizar el beneficio de la simbiosis micorriza/planta.
To my Lord

To My Parents, Silvia and Leonardo

To my wife, Ana, and my children, Lucía and Emilio

To my Brother, Germán, and Marcela, Federico and Joaquín

To my godfather, Roberts “Coco” Bastida (RIP)

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CHAPTER 1. INTRODUCTION

The world population has dramatically grown during the second half of the 20th century, rising from 2.6 to 6.1 billion people, and it is expected to reach 9 billion by 2050 (U.S. Census Bureau, 2015). The Green Revolution has been successful in increasing food, feed and fiber production through greater crop yields since the mid-20th century. Greater crop yields have been mostly due to the larger use of inorganic fertilizers together with improved crop varieties that use fertilizers more efficiently than the old ones (Sayer and Cassman 2013; Payne and Ryan 2010). However, the Green Revolution contribution to protect the environment and preserve fragile ecosystems from cultivation (Payne and Ryan 2010), is questioned (Tittonell, 2014).

Despite the increase in food production in the last decades, it will demand even more production in the future to ensure food security, and so to overcome the main factors limiting production. In theory, there are large areas of land in the world that could be incorporated into cropping land. However, in practice, there are many impediments to do that (Lambin and Meyfroidt 2011), imposing a need to increase yields. Water availability constitutes another main factor limiting yield in crop production, and water for irrigation is competing with water for human and animal consumption. Nitrogen is involved in many different functions and is a major component of proteins, DNA/RNA and pigments such as chlorophyll (Marschner 1995) and represents one of the most commonly deficient nutrients in crop production (Tilman et al., 2002). Both crop irrigation and fertilization can substantially increase yields as well as costs (Boomsma and Vyn 2008). However, increasing crop yields at the expense of increasing fertilization
has been a concern mainly because the negative impact of nitrogen (N) and phosphorus (P) losses to the environment (Tilman et al. 2002; Cassman et al. 2003; Schröder et al. 2011). Nitrogen in the nitrate form can be easily leached under wet soil conditions necessitating appropriate fertilization management to reduce pollution of surface and ground water. Conversely, water deficits under arid and semi-arid conditions may limit the uptake and use of inorganic N by crops. In summary, it is imperative to increase productivity without expanding agricultural land or competing for water resources and at the same time reducing the environmental pressure on agro-ecosystems. Even more, there is a general consensus that intensification of agriculture need to focus on appropriate water and nutrient management to improve their use efficiency (Tilman et al. 2002; Cassman et al. 2003), and maximize crop production without a negative impact on the environment (Tittonell 2014).

Soil microbial community influences important ecosystem services such as plant productivity, carbon storage, nutrient retention and cycling, and water pollution among others (Verbruggen et al. 2011; Köhl et al. 2014). Therefore, soil microbial community directly and/or indirectly has important consequences on food security. Thus, for the implementation and development of intensified sustainable agriculture managements tending toward food security it is imperative to understand the agricultural systems and the relationships and interactions between below and above ground biodiversity. As these relationships are better understood, agricultural systems will become more efficient and therefore, greater agricultural production and food security will be possible to achieve.

**Soil microbial community’s role in plant nutrition**

Soil microbial community includes hundreds of species belonging mostly to
microbes (i.e. bacteria, actinomycetes and fungi) but also species belonging to the microfauna (i.e. protozoa and archezoa) and mesofauna (i.e. nematode) (Coyne 1999; Sylvia et al. 1999). Soil microorganisms have an important role in soil quality and functioning (Chowdhury et al. 2011) since they are involved in organic matter dynamic, nutrient cycling and several other soil processes (Acosta-Martinez et al. 2008). Some microorganisms in the soil are able to improve soil fertility and therefore help with crop nutrition and productivity. For example N-fixing bacteria are key groups in the soil microbial community due to their potential for improving N acquisition to the crop (Salvagiotti et al. 2008). Also ectomycorrhizal fungi can stimulate populations of the P-solubilizing bacteria fluorescent Pseudomonas in soils through fungal exudates, and also can be a carbon source for other microbial communities after fungal hyphae die (Siddiqui and Pichtel 2008). Arbuscular mycorrhizal (AM) fungi are soil fungi that develop symbiotic associations with most plant species (Johnson et al. 1997; Rodriguez and Sanders 2014). These fungi colonize the plant root and the soil around the root and can provide water to the host plant. In addition, AM fungi can uptake nutrients from the soil solution, transport them, and transfer to the plant. Thus, AM fungi help the plant to attenuate water stress effects and enhance plant growth and yield (Augé 2001; Ruiz-Lozano 2003; Busso et al. 2008; Sheng et al. 2008; Zhu et al. 2012).

**Relationships and interactions between arbuscular mycorrhiza fungi and plants**

**Impacts of AM fungi on plants**

Arbuscular mycorrhiza fungi obtain carbon compounds and other nutritional requirements from the symbiotic plant roots (Barea and Jeffries 1995; Smith and Read 2008; Alizadeh and Nadian 2010). Although the amount of carbon coming from the plant
to the AM fungi is difficult to measure, some studies have estimated that between 10 and 20% of the carbon compounds produced by the plant in the photosynthetic process are allocated in the AM fungi (Cardon and Whitbeck 2007) which contributes to the soil carbon cycle due to the rapid hyphae turn over in the soil (Zhu and Miller 2003). The C sink that represents AM fungi for the plant may be considered a penalty for plant production, but colonization of plant roots by AM fungi has several positive impacts on plant growth.

As a benefit, AM fungi could be directly involved in the host’s defense signaling against phytopathogens and/or indirectly contributing to the intensification of the plant defense responses including augmentation of plant nutrition and damage compensation (Smith and Read 2008; Haneef Khan et al. 2010). The mechanisms associated to reduced incidence of plant root disease and pathogens are not well understood. Enhanced nutritional status by AM fungi in inoculated plants has been attributed as the main cause affecting the plant defense process (St-Arnaud et al., 1995). Increased phenolic metabolism in plant roots has been suggested as a part of a mechanism involved in biocontrol (Morandi et al., 1984). Also, more than one mechanism, including phenolic production, has been proposed as a more complex mechanism involved in plant defense.

On AM fungi inoculated tomato (Solanum lycopersicum L.) plants, tolerance to Fusarium oxysporum f. sp. lycopersici was attributed to alterations in the plant physiology, improvement of the plant nutritional status, anatomical changes and/or production of phenolic compounds (Morandi et al. 1984; Ozgönen et al. 2001).

Plants can naturally respond to water stress at morphological and anatomical levels to avoid the stress or to increase its tolerance (Bray 1997). However, it has been
widely agreed that AM fungi may alleviate the response and performance of host plants under water limiting condition in the soil (Augé 2001; Ruiz-Lozano 2003). Although preexisting theories (Safir et al. 1971; 1972) supported the idea that water stress tolerance was related to increased P uptake level via AM fungi symbiosis by the plant, it now appears there is no definable relationship between these two factors (Augé 2001). Prior studies showed that AM fungi hyphae can penetrate soil pores inaccessible to root hairs, and thereby extract enough water to maintain a level sufficient for the plant (Allen 1982; Hardie 1985). Furthermore, Ruiz-Lozano (2003) suggested that the AM fungi symbiosis could contribute to water stress tolerance by a combination of physical, nutritional, physiological and cellular effects. Besides improved water uptake by the mycelium from the soil, AM fungi can alter hormonal levels in the host plant to change the opening of stomata and then affect their conductance (Ruiz-Lozano 2003). Additional mechanisms that have been proposed are increasing turgor by lowering leaf osmotic potential and improving plant recovery after drought by maintaining the soil-root continuum through the hyphae (Khosro et al. 2011). Ruiz-Lozano and Azcón (1995) experimentally proved the positive effects of the AM fungi on plant growth and water uptake by addition of water to a compartment where only AM fungi hyphae can reach. They demonstrated that, under lower water soil content, AM fungi colonized plant maintained stomata open longer than non-AM fungi plants and therefore had higher gas exchange rates (Ruiz-Lozano 2003) and higher biomass production under no limiting nutrient conditions compared to a non-AM fungi colonized plant.

In addition, many studies have demonstrated increased plant P uptake by AM fungi colonized plants compared to non-AM fungi colonized plants especially in soils
with low or medium P content (Liu et al. 2000; Javiad 2012). Based on previous studies, AM fungi can produce organic acids and, with or without synergetic interaction with P-solubilizing bacteria, can contribute to solubilize the insoluble P present in rocks (Cabello et al. 2005; Duponnois et al. 2005, Wu et al. 2005; Antunes et al. 2007). This constitutes an additional mechanism of some AM fungi species in term of enhancing plant P uptake.

The benefits of AM fungi have been shown to be greater for immobile nutrients (e.g. P and Zn) rather than mobile ones in the soil solution. The benefit of plant AM fungi colonization on plant N uptake is controversial. Inorganic [i.e nitrate (NO$_3^-$) and ammonium (NH$_4^+$)] and organic N (i.e. amino acids) sources from soil can be effectively taken by AM fungi and translocate to the host plant representing a significant route for N uptake by the plant (Jin et al. 2012). Although it varies with experimental conditions and host species, it has been shown that between 21 and 75% of the total N uptake in the roots come from AM fungi extraradical mycelium (Tian et al. 2010). Under humid climates, this may translate into significantly less leaching of NO$_3^-$ (Miransari and Mackenzie 2010) thus reducing groundwater and surface water contamination. However, mycorrhizal colonization enhances plant N uptake more effectively under dry soil than under humid soil conditions (Tobar et al. 1994). Furthermore, when the soil P content was high, there has been a negative interaction with plant N uptake, imposing a limit to the increasing AM fungi nutrient uptake and transfer to the plant (Valentine et al. 2001). In legumes, higher P uptake is correlated with higher N fixation. Symbioses of AM fungi, N fixing bacteria and legumes may be the best example where three different organisms improve productivity and become in a great significance for agriculture.

Arbuscular mycorrhiza fungi can also supply other nutrients such as potassium
(K), calcium (Ca), copper (Cu) and zinc (Zn) from the soil solution to the plant.

Subramanian and Charest (1997) showed higher K, Mg, Mn, and Zn contents in maize grain from AM fungi inoculated plants than non-inoculated plants. For legumes, higher Zn, Cu and Mn uptake from symbiosis with AM fungi has positively affected nodulation and therefore N uptake from symbiotic N fixation (Smith et al. 1979). Similar to N uptake, high P level in the soil could decrease other nutrients uptake (Liu et al. 2000), possibly due to the reduced AM fungi soil colonization under high soil P content. An exception could be P and Zn uptakes which were positively correlated on AM fungi colonized maize plants (Jansa et al. 2003). The higher accumulation of some nutrients in AM fungi colonized plants with respect to non-AM fungi colonized plants could be due to greater accumulation of plant biomass since nutrient concentration between both mycorrhizal and non-mycorrhizal plants are similar (Javaid 2009).

The enhanced water, P and other nutrients uptake of AM fungi colonized plants has the potential to improve plant growth and yield and has awakened the interest of using AM fungi as a biological fertilizer for many crops. According to Khosro et al. 2011, the height of an inoculated small tree, Sesbania grandiflora L. was twice that of non-inoculated plants when they were in symbiosis with Glomus fasciculatum. Larger biomass accumulation and greater yields were reported in AM fungi colonized groundnut (Arachis hypogaea L.) plants (Jackson and Mason 1984) and chickpea (Cicer arielinum L.) plants (Alloush et al. 2000). However, studies on the impacts of inoculating with AM fungi on plant yields are scarce as most studies concentrate on vegetative stages and do not harvest seeds/grains. Regarding maize, several studies have reported the benefits of AM fungi and maize symbiosis in term of nutrient uptake; however, there is not studies
reporting plant height or number of leaves in grasses as a function of AM fungi inoculation. Despite all the positive impacts of AM fungi on the host plant, mycorrhizal symbioses are part of a complex interaction among the plant, the fungus, and the environment which do not always result in a positive mycorrhizal response (Smith and Smith 2011; Johnson et al. 1997). Thus, plant colonization by AM fungi can form a continuum from mutualism to parasitism (Johnson et al. 1997).

**Interactions between AM fungi, host plant, and the environment**

Plant and fungal genotypes, and the soil environment have a key role in the outcome of the plant-AM fungi symbiosis (Hamel 2004; Daei et al. 2009; Smith and Smith 2011). For example, the level of mycorrhizal response varied with the kind of organic amendment added to cowpea [*Vigna unguiculata* (L.) Walp] which could be related to changes in AM fungi species under each amendment (Muthukumar and Udaian 2002). Also, several studies on mint (*Mentha* sp.) (Abdul and Janardhanan 1997), radicchio (*Cichorium intybus* L.) (Dalpé et al. 1996), maize (*Zea mays* L.) (Clark and Zeto 1996) and chrysanthemum (*Dendranthema grandiflora* Kitam) (Silveira and Lima 1996) have shown differences in plant growth or metabolism with different AM fungi -plant species combinations.

It has been agreed that the choice of fungal species is important in order to maximize the benefits of the AM fungi symbiosis and the outcome of mycorrhizal studies (Jakobsen et al. 1992; Johnson et al. 1997; Helgason et al. 2002; van der Heijden et al. 2003). Koch et al. (2006) reported that two genetically different AM isolates affected root length of carrot (*Daucus carota* L.) plants. When these two AM isolates grew under two different P concentrations not only the carrot plant was affected but also AM isolates
responded differently in term of hyphal length and spore density (Koch et al. 2006). Munkvold et al. (2004) showed a large difference in P uptake in cucumber (*Cucumis sativus* L.) plants inoculated with different AM isolates from different geographic origin. Also, mycelia from different AM fungi species can vary in terms of hyphal diameters, extent of growth away from the root, and ability to absorb and translocate nutrients to the root (Smith and Smith 2011). Drew et al. (2003) studied the effects of pore size on the growth of *Glomus intraradices* and *G. mosseae* and their ability to transport P from the bulk soil to the plant host. Inoculated plants had similar growth and total P uptake under different soil media conditions; however, it was observed that plants inoculated with *G. intraradices* had more P coming from AM fungi than plants inoculated with *G. mosseae* indicating that *G. intraradices* obtained a greater proportion of P at a distance from the host roots (Drew et al. 2003). In addition, sand pore size in the growing media affected growth of *G. intraradices* (but not *G. mosseae*) and hyphal diameter distributions of both fungi. Previous studies provided evidence that different AM species can be functionally complementary in term of soil exploration and nutrient uptake, transport and translocation to the host plant and they are have some phenotypic plasticity in response to the soil environment. Thus, the choice of AM fungi isolate within a species can also be important for optimal results.

As mentioned previously, it is known that AM fungi development varies among plant species and genotypes (Liu et al. 2000). In terms of P uptake, mycorrhizal response has often shown differences between modern and older cultivars or wild accessions of the same species. Zhu et al. (2001) found that the response to mycorrhizal inoculation in term of the improvement in plant P uptake of modern wheat cultivars was generally lower than
the older cultivars. This suggests that the trait responsible for establishing the AM fungi symbiosis in older and/or local varieties of wheat may have been weakened during modern breeding programs (Manske 1989; Zhu et al. 2001). Additionally, Hetrick et al. (1995) hypothesized that recent wheat cultivars developed in fully fertilized soils may have resulted in selection against genotypes that interact with, or respond to, mycorrhizal fungi. In maize, Khalil et al. (1994) found that, while some old and unimproved varieties did not show a positive response to mycorrhizal inoculation, others exhibited a 400% growth increase. Inbred maize lines in general have a poor rooting ability and decreased capacity to uptake nutrients; therefore, a high response to AM fungi inoculation is expected (Khalil et al. 1994). Research by Kaeppler et al. (2000) supports this finding showing that inbred lines varied substantially in vegetative growth and response to AM fungi colonization among genotypes.

Since the commercial introduction in 1996 of Bt technology in maize, limited research has been conducted on the effects of Bt maize hybrids on the AM fungi community, which could be considered a nontarget organism in the soil environment. Ambiguous results regarding the response of AM fungi inoculated Bt maize hybrids demonstrated a reduction in AM fungi colonization in Bt maize lines growing under greenhouse conditions when compared to non-Bt maize hybrids (Cheeke et al. 2012). However, future research should be conducted in the field to verify this finding as well as several other ecological aspects (Cheeke et al. 2012). At the beginning of the current century two root and shoot architecture contrasting maize genotypes were commercially available (Modarres et al. 1997). Liu et al. (2000; 2003) tested mycorrhizal formation in those different maize genotypes against a conventional maize hybrid, and reported that
the maize hybrid with leafy normal stature architecture had greater mycorrhizal colonization than both leafy reduced stature and conventional hybrids. These authors also showed the influence of soil N level on shoot N/P ratio, root colonization and extraradical hyphal production and their effects on the uptake of other nutrients (Liu et al. 2000).

Global climate change coupled with the expansion of maize production to marginal areas has led plant breeders and agronomists to focus on development of maize hybrids that use N and water more efficiently (Boomsma et al. 2009). Efficient use of water and N under water limitation has prompted several commercial seed companies to develop maize genotypes with enhanced drought tolerance. Programs to develop drought-tolerant maize varieties focused on identification of beneficial morpho-physiological traits (Campos et al. 2004). Although maize is an effective host to AM fungi, evaluation of AM fungi function has not been included at any maize breeding programs thus far for either conventional or drought-tolerant maize (Boomsma and Vyn 2008; Hogemeyer, T. personal communication).

Because of the nutritional and protective benefits that AM fungi confer to their hosts, there is considerable interest in understanding the ecology, physiology and molecular interactions involved in this symbiosis. Additionally, the enhanced adaptation to low soil water and/or nutrient content that AM fungi confers to the host plant could provide suitable criteria for the selection of inoculants. Considerable progress is being made in our understanding of the plant-AM fungi interaction by genetic and molecular analysis (Harrison 1997; Kaeppler et al. 2000), but less is known regarding environmental impacts on the symbiosis and functional traits of different AM fungi species. Furthermore, the genetic basis of variation among host plant species and
genotypes for the plant-mycorrhizal interaction is still controversial and not well understood. Given the complexity of interactions among plants, AM fungi and the environment only through understanding of underlying mechanisms appropriate management strategies can be selected to favor the benefits of the mycorrhizal-plant symbiosis and maximize resource use efficiency.

**Objective and hypotheses**

The general objective of this dissertation was to contribute to the understanding of AM fungal and maize genotype interactions under variable N and P fertilization and variable water availability. Specific questions of this dissertation are related to the impact of soil N and P availability on AM fungi colonization of maize plants, and on the growth, development and nutritional status of those plants and presented in Figure 1.1.

To address the specific questions regarding nutrient fertilization, two experiments were carried out in greenhouses located at University of Nebraska-Lincoln in Lincoln, NE between January and August 2013 under the following hypotheses:

A) Experiment 1
   i. Arbuscular mycorrhiza fungi enhance the ability of maize plant to take up both P and N.

B) Experiment 2
   i. Arbuscular mycorrhiza fungi enhance the ability of both drought sensitive and tolerant maize hybrids to take up of both P and N.
   ii. Arbuscular mycorrhiza fungi improve the physiological performance of both drought sensitive and tolerant maize hybrids.

In addition, a soil survey in a field experiment located near Brule, NE, was
conducted to address specific questions regarding water availability under the following hypotheses:

i. Reduction in soil water level increases root colonization by AM fungi and soil AM FAMEs biomarkers among all maize genotypes.

ii. Soil microbial community biomass, based on FAMEs biomarkers, decreases as soil water level decreases.

**Dissertation layout**

This dissertation contains five chapters. Chapter 1, is the general introduction where hypotheses are presented. Results from experiment 1 are presented in Chapter 2. Those results were useful to carry a second experiment and as a taken decision tool to determine the lab work. Chapter 3 presents the results from experiment 2 while Chapter 4 presents results from soil field sampling at Brule, NE. Lastly, in Chapter 5, a summary of all previous chapters is presented.

**References**


Alizadeh, O. and H.A. Nadian. 2010. Evaluation effect of water stress and nitrogen rates on amount of absorption some macro and micro elements in corn plant mycorrhizae


New York, NY.


• Do maize genotypes have different AMF colonization?
• Does environment modify maize AMF colonization?
  – Does N fertilization impact AM fungi colonization of maize roots?
  – Does P level modify the effect of N on AMF colonization?
  – Does P fertilization have an impact on AM fungi colonization of maize roots?
  – Does N level modify the effect of P on AMF colonization?
  – Does water availability have an impact on AMF colonization?
• Are the environment effects on AMF colonization different across genotypes?
• Do maize growth, development and nutritional status change with AMF inoculation?
• Do maize genotypes have a different growth, development and nutritional status response to AMF inoculation?
• Do environmental conditions modify the maize growth, development and nutritional status responses to AMF inoculation?
  – Does maize response to N levels on growth, development and nutritional status change with AMF inoculation?
  – Does P level modify the growth, development and nutritional responses of AMF colonized maize to N levels?
  – Does the maize response to P levels on growth, development and nutritional status change with AMF inoculation?
  – Does N level modify the growth, development and nutritional responses of AMF colonized maize to P levels?
• Do maize genotypes have a different environmental response on growth, development and nutritional status to AMF inoculation?
• Do environmental conditions impact AM fungi abundance and proportion in soils?
  – Does N fertilization impact AM fungi in soils?
  – Does plant growth modify the effect of N on soil AM fungi?
  – Does water availability impact AM fungi in soils?

Figure 1.1 Specific questions addressed in this dissertation.
CHAPTER 2. MAIZE RESPONSE TO NITROGEN FERTILIZATION RATE AND INOCULATION WITH ARBUSCULAR MYCORRHIZAL FUNGI: A PRELIMINARY GREENHOUSE STUDY

1. Introduction

Arbuscular mycorrhizal (AM) fungi are soil fungi that develop symbiotic associations with most plant species (Rai 2001) and may provide water to the host plant under conditions of water stress (Ruiz-Lozano 2003). Arbuscular mycorrhizal fungi also transport nutrients such as phosphorus (P) and nitrogen (N), as well as other nutrients, from the soil to the plant and in return obtain carbon from the plant host (Barea and Jeffries 1995; Smith and Read 2008; Alizadeh and Nadian 2010). Thus, AM fungi through colonization of the plant’s root system may help the host plant to attenuate water stress and improve nutrient uptake for enhanced plant growth and yield (Ruiz-Lozano 2003; Sheng et al. 2008; Zhu et al. 2012).

The environmental conditions set mainly by soil water and nutrient levels are important to determine the impact of AM fungi on the host plant (Jin et al. 2012). Research has shown that immobile nutrients such as P are more effectively taken up and translocated to the host plant by AM fungi than mobile ones such as N (Munkvold et al. 2004; Koch et al. 2006). For N, complex interactions between N and other nutrients such as P could make results specific to individual host species. There have been few studies demonstrating the influence of AM fungi inoculum and its interactions among N, P and other nutrients on plant response. Some reports indicate enhanced N uptake by plant colonized by AM fungi. Tobar et al. (1994) demonstrated enhanced P uptake, and also N
uptake, when AM fungi colonized lettuce plants were grown under sub-optimal water levels. In AM inoculated plants, George et al. (1995) attributed the enhanced N uptake to improved P uptake. However, AM fungi species differ in their capacity to uptake and transport P and therefore, N uptake can also differ among AM fungi species (George et al. 1995). Additionally, Azcón et al. (2003) stated that not only are the amounts of P and N in soil important, but also the balance between them is important to AM fungi root colonization and plant response.

Many studies have demonstrated the beneficial effect of the AM fungi symbiosis on plant P uptake and transfer (Smith and Smith 2011) and it is well known that AM fungi enhance plant growth under low input agriculture or in soils of poor fertility (Azcón et al. 2003; Brundrett et al. 1999). According to Subramanian and Charest (1997); however, AM fungi may not significantly improve plant nutritional status and growth in high fertility environments despite the prevalence of the symbiosis under these conditions. Thus, the significance of this relationship to high input cropping systems showing significant root and soil colonization by AM fungi warrants greater attention given the dominance of high-yielding maize cropping systems in the central Great Plains (Grigera et al. 2007; Tian et al. 2013). In the present study, we investigated the interaction of N and P fertilization on the physiological response of maize to AM fungal inoculation. The objectives of this study were: 1) to determine the impact of AM fungal inoculation and N fertilizer rate on the physiological response and P uptake of maize plants, and 2) to determine the impact of N fertilization on AM fungal colonization of maize roots.
2. Materials and methods

Production of AM inoculum

Four AM fungi species, *Glomus deserticola*, *Glomus intraradices*, *Glomus mosseae* and *Gigaspora gigantean*, were individually propagated on maize (*Zea mays* L.) plants (Hybrid P0621HR®, Pioneer Hi-Bred, Johnston, IA, US) in a greenhouse located at the University of Nebraska-Lincoln in Lincoln, NE. Plants grew for four months under a night/day temperature of 24/28 °C and a 15 h photoperiod with supplemental light (400 µmol m⁻² s⁻¹). A pasteurized mixture of 25% soil and 75% sand was used. Plants were irrigated as needed and fertilized weekly with standard Hoagland solution (Hershey 1994). At the end of the four-month period, soil and maize roots were harvested, kept separate by AM fungi species, and stored at 4 °C. The presence of each AM fungi species was checked by FAME biomarker techniques (Grigera et al. 2007) and cross contamination with species other than the added inoculate was checked by spore evaluation (Brundrett et al. 1996).

Plant growth conditions and treatments

Maize plants (Pioneer P1395XR-NSU9, Pioneer Hi-Bred International, Inc., Johnston, IA, US) were grown in a greenhouse experiment. Treated plants were co-inoculated with all four AM fungi species using 25 g from each AM inoculum for a total of 100 g soil and roots containing AM spores and mycelia. Black plastic 12.7 by 12.7 by 20.3 cm pots (Anderson Plant Bands™, Anderson Die and Manufacturing Inc. Portland, OR) were filled to ¾ volume with the pasteurized soil-sand mixture previously described. The 100 g of AM fungi mixed inoculum was then placed on top, and then topped with additional pasteurized soil-sand mixture. For the non-AM inoculated controls, 100 g of
the pasteurized soil/sand mixture was used instead of the AM inoculum.

Maize seed was pre-germinated for 3-4 days at room temperature on tap water wetted tissue paper by placing the seeds in rows and rolling up the tissue paper to encase the seeds. In the greenhouse, two germinated maize seeds were planted per pot, and thinned to one plant per pot after 4-5 d when maize plants were 4-5 cm tall. Plants were irrigated as needed by adding water/nutrient solution to a 20 cm diameter aluminum plate (Eco-Foil® Round Cake Pan, Handi-foil Corp., Wheeling, IL, US) placed under the pots.

Inoculated and non-inoculated maize plants were fertilized weekly after transplanting with 200 ml of fertilizer solution containing four levels of N (0, 59, 294 and 588 mg N pot$^{-1}$) in modified Hoagland solution. Nitrogen treatments were called N0, N1, N2 and N3 respectively. Maize plants grew for 7 weeks between January 18, 2013 and March 8, 2013 under greenhouse conditions with supplemental light (400 µmol m$^{-2}$ s$^{-1}$), a 14-15 h photoperiod and 24-28 ºC temperature. Plants were watered as needed.

**Plant harvest, sampling and measurements**

At 7 weeks after transplanting (WAT), whole maize plants were cut at the soil surface and aboveground fresh weight was recorded. Plants were placed in labeled paper bags, dried at 70 ºC for 72 h, and dry weight was recorded. Dry shoots were ground into a powder using a blender and total P and N contents (Plank 1992; Gavlak et al. 1996) were determined. The number of leaves per plant and plant height to the node of the last fully expanded leaf was recorded weekly between transplanting and the seventh WAT. A leaf was considered fully expanded when the leaf collar in the base of the leaf blade was visible and the ligule tips were not touching each other. Chlorophyll content was measured weekly on the last fully expanded leaf using a SPAD-520DL chlorophyll
content meter [Soil-Plant Analysis Development (SPAD) Section, Minolta Camera Co., Ltd., Osaka, Japan].

Immediately after removing plant shoots, the roots were gently removed from the soil, washed with tap water and dried on paper towels. Root fresh weight was recorded and a 3 to 4 g sample of the finest roots was removed using scissors. For each individual root system, the fine roots collected were placed into 50 ml sterile polypropylene tubes (Corning®, 4558, Corning Inc., Corning, NY, US), submerged in liquid nitrogen and stored at -20 ºC for fatty acid methyl ester (FAME) biomarker analysis. For FAME analysis, approximately 1 g of frozen roots was freeze-dried (Labconoc FreeZone 6L freeze dry system with a FreeZone Bulk Tray Dryer, Labconco Corp., Kansas City, MO, US) for 72 h at -50 ºC and -200 mbar. Freeze dried fine roots were ground and homogenized (Omni Bead Ruptor 24 Homogenizer, Omni International, Inc., Kennesaw, GA, US) in 2 ml polypropylene micro tubes (Sarstedt, Inc., Newton, NC, US) with five 2 mm zirconium ceramic beads per tube. The setting on the homogenizer was three cycles of 30 sec with intervals of 20 sec between cycles, at 25 ºC and 7.1 m s⁻¹. FAMEs were extracted from 30-50 mg freeze dried, ground roots and quantified using gas chromatography (Grigera et al. (2007). The fatty acids C16:1cis11 and C18:1cis11 were selected as biomarkers for AM fungi. The remaining washed and dried roots were placed into plastic storage bags (Ziploc®, S. C. Johnson & Son, Inc., Racine, WI, US) and stored at -20 ºC for determining root colonization by AM fungi. Percent root colonization was quantified in 1 cm long segments obtained from a 1.0 to 1.5 g fresh weight root sample (Brundrett et al. 1996; Giovannetti and Mosse 1980). Root segments were stained with 0.05 percent (w/v) black ink (Vierheilig et al. 1998) using the gridline intersect method (Brundrett et al.
Experimental design and statistical analysis

The experiment was 2x4 factorial arranged in a randomized complete design. The factorial arrangement included two arbuscular mycorrhizal fungal (AMF) treatments (with mycorrhizae, +M; without mycorrhizae, -M) and four N levels (0N, 1N, 2N and 3N). Four replications were used for each treatment combination; thus, the experiment had a total of 32 pots. PROC MIXED ANOVA in SAS (SAS, 9.3, SAS Institute, Cary, NC, USA) was used to analyze the plant shoot N and P content, shoot dry and fresh weight, and root fresh weight data. PROC GLIMMIX Repeated Measures ANOVA was used to analyze plant height and chlorophyll content measured over time, and a two-way ANOVA was used to compare effects of mycorrhizal and N treatments on plant growth. Mean values were separated using the LSD procedure ($P < 0.05$).

3. Results

Maize plant growth

Plants inoculated with AM fungi accumulated more biomass at 7 WAT than non-inoculated plants and plant biomass increased with N fertilization ($P < 0.0001$) compared to 0N (Table 2.1). However, there was a significant N*AMF interaction ($P = 0.0047$) for plant dry weight (Table 2.1). Without N fertilization, non-inoculated maize plants had greater biomass ($P = 0.0584$) than AM inoculated plants while with N fertilization, AM inoculated maize plants had greater biomass than non-inoculated plants (Table 2.2, Figure 2.1). Nitrogen fertilization increased dry weight accumulation only in AM inoculated maize plants with respect to non-fertilized plants (Table 2.2, Figure 2.1). Root fresh
weight was not affected by N fertilization \((P = 0.475)\) or AM fungi \((P = 0.138)\), and there was no N*AMF interaction \((P = 0.92)\) (Table 2.1).

Arbuscular mycorrhizal inoculation did not affect maize plant height \((P = 0.1041)\) at 7 WAT (Table 2.1). Nitrogen fertilization increased plant height when compared to non-fertilized controls \((P = 0.0196; \text{Table 2.1})\), and this was mainly due to a larger reduction in plant height for AM inoculated plants under non-fertilized conditions (Table 2.2). There was a significant AMF*time interaction \((P = 0.0002)\) for plant height (Figure 2.2). Plant height increased over time, and AM inoculated maize plants were taller at 4 and 5 WAT and shorter at 7 WAT compared to non-inoculated plants.

The number of plant leaves at 7 WAT was not affected by N fertilization level or inoculation with AMF \((P = 0.0894 \text{ and } 0.3786, \text{ respectively})\) (Table 2.1). There was also no significant AMF*time interaction for number of leaves \((P = 0.9291)\) (Table 2.1). The number of leaves per plant increased over time \((P < 0.0001)\) at a rate of approximately 0.15 leaf per day.

**Chlorophyll, P and N contents**

Chlorophyll content was not affected \((P = 0.0939)\) by AM fungal inoculation (Table 2.1), but increased with increasing level of N fertilization \((P < 0.0001)\) at 7 WAT for both AM inoculated and non-inoculated plants (Tables 2.1 and 2.3). There was no significant AMF*N interaction for chlorophyll content \((P = 0.1602)\) (Table 2.1). However, there were significant N*time and AMF*time (Figure 2.3) interactions for chlorophyll content \((P < 0.0001 \text{ for both interactions})\). Inoculated maize plants had higher chlorophyll content at early growth stages (4 and 5 WAT) than non-inoculated plants, but not by later stage (7 WAT) (Figure 2.3). At 7 WAT, chlorophyll content in leaves of both
AM inoculated and non-inoculated maize plants decreased at two lower N fertilization levels (N0 and N1) and increased at two higher N fertilization (N2 and N3) (Table 2.3).

Both P concentration and content in plant shoots were always higher in AM inoculated maize plants than non-inoculated plants regardless of N fertilization (Tables 2.1, 2.2 and 2.3; Figure 2.4). There was a significant N*AMF interaction for both P concentration and content ($P = 0.0027$ and 0.0004, respectively; Tables 2.1 and 2.2). Nitrogen fertilization had no effect on shoot P concentration or content in non-inoculated plants (Tables 2.3 and 2.4). In AM inoculated plants, additions of N fertilizer decreased shoot P concentration, but did not differ with level of N fertilizer added (Table 2.3; Figure 2.4) but increased shoot P content due to greater plant growth (Table 2.4). Shoot N concentration decreased with AM inoculation ($P < 0.0001$; Table 2.1), but there was no difference in N content between inoculated and non-inoculated plants ($P = 0.0577$; Table 2.2). However, there was a significant N*AMF interaction for both N concentration and content ($P = 0.0222$ and 0.0098, respectively; Tables 2.1 and 2.2). Although shoot N concentration and content increased with increasing levels of N fertilizer for both AM inoculated and non-inoculated maize plants (Tables 2.3 and 2.4; Figure 2.5), in the absence of N fertilizer, both shoot N concentration and content were not different between inoculated and non-inoculated plants ($P = 0.1154$) (Tables 2.1, 2.2, 2.3 and 2.4).

**Arbuscular mycorrhizal root colonization and biomarkers**

Root colonization was confirmed on AM inoculated plants and no cross contamination was found between inoculated and non-inoculated plants ($P < 0.0001$) (Table 2.5). From 1.0 to 1.5 g fresh weight fine roots, 500 to 1,000 line-root intercepts containing stained or unstained roots were counted (data not shown). Both AM
inoculation and N fertilization influenced \((P < 0.0001)\) percent root colonized by AM fungi, and there was a significant N*AMF interaction \((P < 0.0001)\) (Table 2.5). Root colonization of inoculated plants was significantly lower in non-fertilized plants (N0) than when nitrogen fertilizer was applied (N1, N2 and N3) (Table 2.5).

Inoculation of plants with AM fungi led to significantly higher concentrations of both AM fatty acid biomarkers C16:1cis11 and C18:1cis11 \((P < 0.0001)\) in roots compared to non-mycorrhizal roots (Table 2.5). The highest concentration of the AM biomarker C16:1cis11 was found in AM colonized roots at 1N fertilization level (7.46 nmol mg\(^{-1}\) of root; data not shown). Addition of N fertilizer decreased AM biomarker C16:1cis11 concentration in roots; however, there was no significant difference \((P = 0.2165)\) in AM biomarker concentration among N fertilization levels (Table 2.5). In contrast to C16:1cis11, the concentration of the AM biomarker C18:1cis11 did not show a clear trend, and N fertilization did not significantly influence the concentration of AM biomarkers C18:1cis11 in maize roots \((P = 0.8689)\) (Table 2.5).

4. Discussion

In the present study the impact of AM inoculation and N fertilization rate on the physiological response and nutrient uptake of maize plants was determined. Arbuscular mycorrhiza fungi increased plant dry biomass and the number of leaves when N fertilizer was added. In plants inoculated with AM fungi, the increase in plant biomass was greater as N fertilization level increased. Plant biomass of non-inoculated maize plants did not respond to increasing N rate. Zhu et al (2010) did not find differences in shoot dry weight or fresh weight between inoculated and non-inoculated maize plants. However, according
to Zhu et al. (2010) and Ortas (2012) there was positive effect of AM fungi on maize roots and root dry weight. In *Vicia unguiculata* L., Arumugan et al. (2010) reported 20% higher dry weight of shoots on AM inoculated plants than non-inoculated plants. Azcón et al. (2003) reported that dry biomass of shoots of inoculated and non-inoculated maize plants increased as N and P levels increased, but dry mass was not different at high N and P nutrient levels. Valentine et al. (2001) showed dry biomass was significantly influenced by nutrient supply, AM inoculation and their combination. In contrast, dry weight of AM inoculated cucumber (*Cucumis sativus* L.) decreased 19% compared to non-inoculated plants when N and P concentrations were high.

Although there are many studies that assess the impact of AM fungi on a large diversity of plant species, few of them use maize as the AM host plant. Moreover, few studies include plant height and/or number of leaves as a way to evaluate the impact of AM fungi on the plant. In one such study, Arumugan et al. (2010) reported inoculated *Vicia unguiculata* plants were 30% longer and 21% heavier than non-inoculated plants. In the present study, the height of AM inoculated maize plants was shorter than non-inoculated plants at 7 WAT. Although AM inoculated maize plants were shorter, they had more biomass and leaves than non-inoculated plants by the end of the experiment.

Plant P concentration and content (i.e. total P uptake in mg per plant) was significantly increased with AM inoculation. This agrees with results reported by Valentine et al. (2001) for cucumber plants; however, these authors showed reduced P uptake on inoculated plants compared to non-inoculated plants when both soil P and N concentrations are high. We also show an increase in P content with the addition of N fertilizer compared to the non-fertilized control, but a decline in P concentration with the
addition of N fertilizer. Phosphorus concentration was not different as N fertilization rate increased for either inoculated or non-inoculated plants. The reduction in plant tissue P concentration on AM inoculated plants with the addition of fertilizer N may be related to suppression of the external hyphal network (Azcón et al. 2003). In response to increased N fertilization, N concentration and content of both AM inoculated and non-inoculated maize plants increased. While the increase in N concentration was greater for non-inoculated than inoculated plants, N uptake was greater for inoculated compared to non-inoculated plants. This may indicate alternative pathways for N uptake in inoculated plants compared to non-inoculated, or result from differences in plant growth responses. In contrast, Valentine et al. (2001) found no effect of AM inoculation on plant N content. This shows the complexity of plant nutrient uptake in response to AM inoculation.

Chlorophyll content of AM inoculated and non-inoculated maize plants varied over time. At 7 WAT, inoculated plants showed lower chlorophyll content than non-inoculated plants. In contrast, Valentine et al. (2001) and Arumugan et al. (2010) reported increased amounts of chlorophyll on AM inoculated cucumber and *Vicia unguiculata* plants respectively, at similar harvest times as our experiment. According to Arumugan et al. (2010) this increase in chlorophyll content might be due to increased stomatal conductance, photosynthesis, transpiration and plant growth. Also, higher chlorophyll content could be due to larger and more numerous bundle sheath chloroplasts in inoculated plants (Arumugan et al. 2010). Nitrogen limits plant growth in most environments since this nutrient is an essential part of the chlorophyll molecule (Brady and Weil 2010). Any appreciable lack of N in the soil could result in a suppression of chlorophyll formation, and therefore decrease photosynthetic capacity of leaves and plant
growth. Thus, the greater chlorophyll content of non-inoculated plants at 7 WAT could be due to higher N concentration in those plants with respect to inoculated maize plants. However, the higher chlorophyll content of non-inoculated plants at 7 WAT did not translate into higher biomass, more leaves or greater root fresh weight.

Our results showed a positive benefit of AM fungi to maize through greater plant biomass in agreement with the results of other studies (Azcón et al. 2003; Arumugan et al. 2010; Ortas 2012). This enhanced biomass was greater with the addition of N fertilizer to the soil. The increased plant biomass was not due to taller plants or more leaves per plant, indicating no effect on plant size and/or structure. Although our experiment did not test plant water status, the greater dry weight biomass of inoculated plants could be partly due to improved water status as shown in many studies (Porcel and Ruiz-Lozano 2004; Seng et al. 2008; Zhu et al. 2010). Increased P uptake by inoculated plants resulted in greater N uptake, but lower N concentration in shoot and chlorophyll content. This suggests that the increase in plant biomass of AM inoculated plants was not related to enhanced photosynthesis since N concentration and chlorophyll content of inoculated plants were lower than non-inoculated plants.

This greenhouse study demonstrated a positive effect of AM inoculation on maize dry weight and P concentration regardless of N fertilization compared to non-inoculated plants. However, AM inoculation improved both P and N uptake as N fertilization increased. Although plant chlorophyll and tissue N concentration increased with increasing N fertilizer addition, it had little effect on dry matter accumulation suggesting other factors were limiting plant growth in this greenhouse experiment. The reduction in plant tissue P concentration on AM inoculated plants with the addition of fertilizer N may
be related to suppression of the external hyphal network. Further studies on the mechanism involved into the complex relationships among AM fungi, maize and soil fertility management is needed to maximize the benefit from this ubiquitous symbiosis.

**Acknowledgements**

We wish to acknowledge Ruth Miller, Parker Theisen, Oscar Perez-Hernandez, Lauren Segal and Liz Jeske for their technical assistance.

**References**


of maize (*Zea mays* L.) to arbuscular mycorrhizal inoculation during and after drought stress at tasselling. Mycorrhiza 7:25-32.


Plant Soil Environ. 58:186-191.
Table 2.1. Means and probability values from ANOVA of main effects and interactions on plant shoot dry weight (pDW, g), plant shoot fresh weight (rFW, g), root fresh weight (rFW, g), plant height (H, cm), number of leaves (L), chlorophyll content (Ch, mg m⁻²), nitrogen concentration (N, %) and phosphorus concentration (P, %) across mycorrhizal inoculation (AMF inoculated, +M; non-inoculated, -M), and nitrogen fertilization treatments (0N: without N, 1N: 59 mg N pot⁻¹, 2N: 294 mg N pot⁻¹ and 3N: 588 mg N pot⁻¹) at 7 weeks after transplanting.

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Letters indicate statistically different (P > 0.05) means for AMF inoculation and N treatments by LSD procedure. ns = not significant, * = P < 0.05, ** = P < 0.01
Table 2.2. Means and probability values from ANOVA of main effects and interactions on nitrogen content (N, mg plant\(^{-1}\)) and phosphorus content (P, mg plant\(^{-1}\)) across mycorrhizal inoculation (AMF inoculated, +M; non-inoculated, -M), and nitrogen fertilization treatments (0N: without N, 1N: 59 mg N pot\(^{-1}\), 2N: 294 mg N pot\(^{-1}\) and 3N: 588 mg N pot\(^{-1}\)) at 7 weeks after transplanting.

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Letters indicate statistically different ($P > 0.05$) means for AMF inoculation and N treatments by LSD procedure. ns = not significant, * = $P < 0.05$, ** = $P < 0.01$
Table 2.3. Mean values of plant shoot dry weight (pDW, g), plant shoot fresh weight (pFW, g), root fresh weight (rFW, g), plant height (H, cm), number of leaves (L), chlorophyll content (Ch, mg m⁻²), nitrogen concentration (N, %) and phosphorus concentration (P, %) across mycorrhizal inoculation and nitrogen fertilization treatments (0N: without N, 1N: 59 mg N pot⁻¹, 2N: 294 mg N pot⁻¹ and 3N: 588 mg N pot⁻¹) at 7 weeks after transplanting.

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Letters within each column indicate statistically different (P > 0.05) means by LSD procedure.
Table 2.4. Mean values of nitrogen content (N, mg plant\(^{-1}\)) and phosphorus content (P, mg plant\(^{-1}\)) across mycorrhizal inoculation and nitrogen fertilization treatments (0N: without N, 1N: 59 mg N pot\(^{-1}\), 2N: 294 mg N pot\(^{-1}\) and 3N: 588 mg N pot\(^{-1}\)) at 7 weeks after transplanting.

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Letters within each column indicate statistically different \((P > 0.05)\) means by LSD procedure.
Table 2.5. Means and probability values from ANOVA of main effects and interactions on root colonization by arbuscular mycorrhizal fungi (AMF) (Rc, %), and root AM FAME C16:1cis11 and C18:1cis11 (nmol mg\(^{-1}\) of root) biomarkers at 7 weeks after transplanting across mycorrhizal inoculation (AMF inoculated, +M; non-inoculated, -M), and nitrogen fertilization treatments (0N: without N, 1N: 59 mg N pot\(^{-1}\), 2N: 294 mg N pot\(^{-1}\) and 3N: 588 mg N pot\(^{-1}\)).

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Letters indicate statistically different ($P > 0.05$) means for AMF inoculation and N treatments by LSD procedure. ns = not significant, $* = P < 0.05$, $** = P < 0.01$
Figure 2.1. Plant dry weight for inoculated (black, +M) and non-inoculated (gray, -M) maize plants under different N fertilization levels (0N: without N, 1N: 59 mg N pot⁻¹, 2N: 294 mg N pot⁻¹ and 3N: 588 mg N pot⁻¹) at 7 weeks after transplanting. Letters indicate statistically different ($P > 0.05$) means for inoculated (small letter) and non-inoculated (capital letter) plants, respectively.
Figure 2.2. Plant height of inoculated (+M) and non-inoculated (-M) maize plants over time. ns = not significant, * = $P < 0.05$. 
Figure 2.3. Chlorophyll content of inoculated (+M) and non-inoculated (-M) maize plants over time. ns = not significant, * = $P < 0.05$. 
Figure 2.4. Phosphorus concentration of inoculated (black, +M) and non-inoculated (gray, -M) maize plants across N fertilization treatments (0N: without N, 1N: 59 mg N pot\(^{-1}\), 2N: 294 mg N pot\(^{-1}\) and 3N: 588 mg N pot\(^{-1}\)) at 7 weeks after transplanting. Letters indicate statistically different \(P > 0.05\) means for inoculated (small letter) and non-inoculated plants (capital letter) plants, respectively.
Figure 2.5. Nitrogen concentration of inoculated (black, +M) and non-inoculated (grey, -M) maize plants across N (0N: without N, 1N: 59 mg N pot\(^{-1}\), 2N: 294 mg N pot\(^{-1}\) and 3N: 588 mg N pot\(^{-1}\)) levels at 7 weeks after treatment. Letters indicate statistically different \((P > 0.05)\) means for inoculated (small letter) and non-inoculated plants (capital letter) plants, respectively.
CHAPTER 3. PHYSIOLOGICAL RESPONSE OF MAIZE HYBRIDS TO INOCULATION WITH ARBUSCULAR MYCORRHIZAL FUNGI UNDER VARIABLE NITROGEN AND PHOSPHORUS LEVELS

1. Introduction

Arbuscular mycorrhizal (AM) fungi form symbioses with most plant species including major crops such as wheat, corn, soybean and sorghum (Smith and Read 1997). By establishing associations with plant roots they provide more efficient uptake of nutrients via an extended soil mycelial network in exchange for carbon from the plant (Liu et al. 2000a, b; Liu et al. 2002). This extended hyphal network can also improve water uptake to attenuate plant water stress (Augé 2001; Ruiz-Lozano 2003). Thus, AM fungi can improve nutrient use efficiency from fertilizer and soil thereby positively influencing plant growth, reproduction and yield (Subramanian and Charest 1997; Miller 2000; Borowicz 2001).

In general, AM fungi are considered beneficial for plants. Johnson et al. (1997) concluded that AM fungi form a continuum from mutualism to parasitism. Where in this continuum a particular AM-plant symbiosis falls and the response of both plant and fungus is complex, and depends on both plant and fungal species/genotype as well as the environment (Smith and Smith 2011; Hamel 2004; van der Heijden et al. 2003; Helgason et al. 2002; Johnson et al. 1997). Most plant species can be successfully inoculated with different AM species under lab or greenhouse conditions and the choice of AM fungi isolate within a species can influence the outcome (Sanders 2003; Munkvold et al. 2004). In contrast, under field conditions, multiple AM species may colonize an individual
plant’s root system and the soil around it at one time. Furthermore, different degrees of AM colonization could be occurring over time and colonization could vary spatially within the root system for each AM species on plant host. Thus, it is difficult to conduct a greenhouse and field experiments out to assess AM fungi specificity. Therefore, field experiment results have being inconclusive and ecological specificity (i.e. a given AM fungi species can colonize a range of plant species and a given plant species can be colonized by several different AM fungi species) between fungus and plant species may be the main reason for that (Smith and Read 1997; Sanders 2003). Molecular approaches that allow AM diversity to be linked to functional properties could provide more accurate information on the composition of natural occurring AM fungi and their function in the community under study (Munkvold et al. 2004). Thus, improved management practices could be selected and the maximum benefit from AM fungi obtained.

Crop intensification practices such as fertilization substantially increase production costs (Boomsma and Vyn 2008). Soil nutrient supply, via fertilization or mineralization of soil organic matter, can influence the outcome of the mycorrhiza/plant symbiosis (Liu et al. 2000b; 2003). Among nutrients, N is more important to crop production mainly because N is needed in abundance for proteins and nucleic acids as well as many different plant functions (Marschner 1995). Additionally, N is highly soluble in water and therefore can be leached under humid soil conditions or be less available under arid and semi-arid conditions. The benefits of AM fungi have been shown to be greater for immobile nutrients (e.g. P and Zn) compared to mobile ones such as N in the soil solution (Jin et al. 2012).
In terms of P uptake, older theories hypothesized that increased P uptake by AM fungi was related to increased water uptake, but newer evidence shows no relationship between water uptake and P uptake (Augé 2001). Cooper and Tinker (1978) hypothesized that host plant demand could control P uptake by AM fungi since these researchers did not find correlation between P transport and length of external mycelia. More recently, in high yield maize (Zea mays L.) production, Grigera et al. (2007) showed that not only soil P availability may influence AM P uptake, but also the maize plant P requirement linked to crop physiology and growth stage can drive P uptake by AM fungi. Regarding N uptake, between 21 and 75% of the total N uptake in the roots can be attributed to AM fungi. Toussaint et al. (2004) reported at least 21% of the total N uptake in the AM roots came from the AM fungal extraradical mycelium in an in vitro culture of carrot roots (Daucus carota L.) colonized with Glomus intraradices Schenck & Smith. Under the same culture conditions and utilizing the same host plant and AM fungi species, Govindarajulu et al. (2005) and Jin et al. (2005) reported between 30 and 50% of N uptake coming from AM fungal extraradical mycelium. In a root and hyphae compartment system, Tanaka and Yano (2005) reported that up to 75% of the N in maize plants might come from symbiosis with AM Glomus aggregatum Schenck & Smith. Researchers showed that ammonium-N was delivered at a rate 10-fold higher than nitrate-N which indicated that ammonium-N was the preferred form of N transfer by AM fungus and this preference was not dependent on plant demand for N (Tanaka and Yano 2005). Tanaka and Yano (2005) highlighted the importance of reducing nitrification to avoid affecting the AM fungus capacity for N uptake. They speculated that uptake of a
more mobile form of N as nitrate by AM fungus is determined by other factors related to
the plant (i.e. transpiration rate) rather than by fungus (Tanaka and Yano 2005).

Plant response to AM fungi has often shown differences between modern and
older cultivars or wild accessions of the same species. Zhu et al. (2001) found that the
response to mycorrhizal inoculation of modern wheat cultivars was generally lower than
that of older cultivars. This suggests that the trait responsible for establishing the AM
symbiosis in older and/or local varieties of wheat may have been weakened during
modern breeding programs (Manske 1989; Zhu et al. 2001). Additionally, Hetrick et al.
(1995) hypothesized that recent wheat cultivars developed in fully fertilized soil may
have resulted in selection against genotypes that interact with, or respond to, mycorrhizal
fungi. In corn, Khalil et al. (1994) found that, while some old and unimproved varieties
did not respond to mycorrhizal inoculation, others exhibited a 400% growth increase.
Inbred maize lines in general have a poor rooting ability, and, therefore a decreased
capacity to uptake nutrients (Ali, L. personal communication). Research by Kaeppler et
al. (2000) supports this finding showing that inbred lines varied substantially among
genotypes in vegetative growth and response to AM root colonization.

At the beginning of the current century two shoot architecture contrasting maize
genotypes were commercially available (Modarres et al. 1997). According to an
unpublished study reported by Liu et al. (2000a), these two contrasting shoot architecture
maize hybrids also differ in root architecture. Leafy normal-stature hybrid has a more
branched but smaller root system than conventional maize hybrids while on the other
hand, leafy reduced-stature hybrid has a less branched and smaller root system than leafy
normal-stature hybrid (Liu et al. 2000a). Liu et al. (2000a; 2003) tested mycorrhizal
formation in those different maize genotypes against a conventional maize hybrid, and reported that the maize hybrid with leafy normal-stature architecture had greater mycorrhizal colonization than both leafy reduced-stature and conventional hybrids. Leafy normal-stature hybrid has higher shoot:root biomass ratio and higher photosynthetic potential (Modarres et al. 1997; Liu et al. 2000a). Thus, with smaller root system, leafy normal-stature hybrid is dependent on AM colonization to enhance nutrient and water uptake and therefore, support higher photosynthetic potential (Liu et al. 2000a). Liu et al. (2000a) highlighted that leafy normal-stature hybrid has high total leaf area which indicates a high capacity to produce and supply carbon compounds to AM fungi. These authors also showed differences in root colonization and extraradical hyphal production among maize hybrids in response to soil P and N levels. Both root colonization by AM fungi and extraradical hyphae length decreased for all hybrids as soil P level increased. On the other hand, both hybrids increased AM root colonization when N was applied; however, AM root colonization on leafy normal-stature hybrid decreased under the highest N dose while AM colonization on leafy reduced-stature did not differ among N doses (Liu et al. 2000a). Extraradical hyphae length increased with N fertilization, but was strongly repressed under the highest N rate for both maize hybrids (Liu et al. 2000a).

Breeding programs to develop drought-tolerant hybrids or varieties have focused on using transgenic technologies or identification of beneficial morpho-physiological traits (Campos et al. 2004). Regarding AM colonization of Bt maize, results have been contradictory. de Vaulx et al. (2007) reported that AM fungi colonization of roots did not differ among Bt maize varieties. In contrast, Cheeke et al. (2012) demonstrated a reduction in AM colonization of multiple Bt maize lines growing under controlled
greenhouse conditions. However, the authors indicated that future research should be conducted in the field to verify their findings as well as ecological drivers (Cheeke et al. 2012). Furthermore, to the best of the author’s knowledge, although maize is an effective host for AM fungi, currently there is no breeding program evaluating the impact of the AM symbiosis in conventional or drought-tolerant maize hybrids (Boomsma and Vyn 2008; Hogemeyer, T. personal communication).

Given the complexity of interactions among plants, AM fungi and the environment, greater understanding of the mechanisms underlying the symbiosis is needed to select appropriate hybrids and crop management strategies to foster the benefits of the mycorrhizal-plant symbiosis and maximize resource use efficiency. In this study, a greenhouse experiment was carried out to evaluate the influence of AM fungi on the physiological response and nutritional status of conventional and drought tolerant maize hybrids under variable nitrogen and phosphorus levels.

2. Materials and methods

Production of AM fungal inoculum

Four AM fungal species, *Glomus deserticola* (CA113), *Glomus intraradices* (IA506), *Glomus mosseae* (CA201) and *Gigaspora gigantean* (MN922A) were propagated on maize plants (Hybrid P0621HR®, Pioneer Hi-Bred, Johnston, IA, US) in a greenhouse located at the University of Nebraska-Lincoln in Lincoln, NE. Ten 1 L pots were filled with a pasteurized mixture of 25% soil and 75% sand and planted with 4-5 maize seedlings. Plants were grown for four months under a night/day temperature of 24/28 °C and a 15 h photoperiod with supplemental light (400 µmol m² s⁻¹). Plants were
irrigated as needed and fertilized weekly with 200 ml of standard Hoagland solution (Hershey 1994). At the end of the four month period soil and maize roots were harvested, and stored at 4 °C. The presence of AM fungi was checked by quantification of an AM specific fungal biomarker, C16:1cis11 (Grigera et al. 2007) and absence of cross contamination with other AM fungal species was checked by spore identification according to Brundrett et al. (1996).

**Plant material, treatments and growth conditions**

Two commercial drought sensitive (DS) and two commercial drought tolerant (DT) maize hybrids were selected for evaluation in a greenhouse experiment. Drought sensitive hybrids were Hybrid 2A555, Mycogen Seeds Inc., Indianapolis, IN (DS1) and Public Hybrid Nebraska N510D (DS2) and drought tolerant hybrids were Hybrid P0876HR NW01, Pioneer Hi-Bred Inc., Johnston, IA (DT1) and Hybrid X432297wp, Syngenta Group Company, Greensboro, NC (DT2). Two treatments (non-mycorrhizal and the co-inoculation of all four aforementioned AM fungi species) were set up for each maize hybrid. The AM fungi inoculum containing AM fungi spores and extra-radical hyphae was 100 g of soil and roots composed by the mixture of 25 g from each AM fungi species. Black plastic 12.7 by 12.7 by 20.3 cm pots (Anderson Plant Bands, Anderson Die and Manufacturing Inc. Portland, OR) were filled with similar sterilized soil-sand mixture previously described up to cover ¾ of its volume, and AM fungi inoculum was placed on the top. In the non-AM fungi inoculated treatment, 100 g of sterilized soil/sand mixture was placed on the top of the partially filled pot instead of the AM fungi inoculum. After placing the AM fungi treatment, the pot was filled with sterilized soil/sand mixture. To facilitate plant/root and soil harvesting/collection, a 25 by 40 cm
polyethylene bag (Icienceware®, Bel-Art Products, Wayne, NJ, US) was placed inside each pot. Bag corners were cut to facilitate water drainage.

Maize seed was sterilized and scarificated in 15% bleach (6.15% sodium hypochlorite, Clorox® Ultra, Clorox Professional Products Company, Oakland, CA) for 15 min followed by rinsing once with sterile distilled water for 5 min. After that, maize seed was treated with 70% ethanol for 1 min and rinsed once with sterile water for 5 min. To get uniform plant size, seed was pre-germinated by placing seed in lines and rolling on tap water wetted tissue paper during 3-4 d at room temperature. In the greenhouse, two germinated maize seeds were planted per cell in growing trays filled with a mixture of sand, vermiculite and perlite (1/3:1/3:1/3), and irrigated as needed in the sub-irrigation tray placed under the growing tray. After 4-5 d, one 4-5 cm tall maize plant per pot was transplanted to previously filled and inoculated pots. Plants were immediately irrigated after transplanting on the media surface, and kept irrigated as needed in a 20 cm circular plastic plate (Gardener’s Blue Ribbon® Vinyl Saucer, Woodstream Corp., Lititz, PA, US) placed under the pots.

Inoculated and non-inoculated maize plants were fertilized at the beginning (five days after transplanting) of the experiment with a starter fertilizer (20:20:20 Jack’s Professional Formula, J.R. Peters, Inc., Allentown, PA). Starter fertilization provided 80 mg pot⁻¹ of nitrogen, 35 mg pot⁻¹ of phosphorus and 66 mg pot⁻¹ of potassium. Two weeks after starter fertilizer was applied, maize plants were fertilized weekly with 200 ml of fertilizer solution combining one of three levels of nitrogen (0, 147 and 294 mg pot⁻¹) and one of two levels of phosphorus (43 and 86 mg pot⁻¹) applied via a modified Hoagland solution. The nitrogen and phosphorus combinations formed six nutrient
treatments that were named as following: T1: 43 mg P pot⁻¹ and 0 mg N pot⁻¹, T2: 43 mg P pot⁻¹ and 147 mg N pot⁻¹, T3: 43 mg P pot⁻¹ and 294 mg N pot⁻¹, T4: 86 mg P pot⁻¹ and 0 mg N pot⁻¹, T5: 86 mg P pot⁻¹ and 147 mg N pot⁻¹, and T6: 86 mg P pot⁻¹ and 294 mg N pot⁻¹. On pots, each nutrient treatment was identified with different colored plastic labels to facilitate their application. Maize plants grew for 10 weeks between June 12, 2013 and August 21, 2013 under greenhouse conditions with natural light (400-500 µmol m⁻² s⁻¹), a 14-15 h photoperiod and 24-30 °C temperature. Plants were watered as needed with tap water.

**Plant harvest, sampling and measurements.**

Number of leaves per plant and plant height to the node of the last fully expanded leaf was recorded weekly beginning the first week after transplanting. A leaf was considered fully expanded when the leaf collar, in the base of the leaf blade, was visible and the ligule tips are not touching each other (Ritchie et al., 1997). Also, the chlorophyll content was measured weekly on the last fully expanded leaf using a CCM-300 chlorophyll content meter (Opti-Sciences, Inc., Hudson, NH, US).

One third of the experiment (i.e. 192 plants) was harvested at 6 (July 24, 2013), 8 (August 7, 2013) and 10 (August 21, 2013) weeks after transplanting (WAT). At harvest time, maize shoots were cut off at the soil surface, weighed and fresh weight was recorded. Maize shoots were placed in paper bags, dried at 70 °C for 72 h, and dry weight recorded. Dry shoots were ground to 1 mm using a Wiley mill (Model 4 Wiley® Mill, Thomas Scientific, Swedesboro, NJ, US) and total P content was determined on ashed samples digested with hydrochloric and perchloric acids according to AOAC method 964.06 (AOAC, 1990) and developed colorimetrically using the molybdovanadate
method (AOAC, 1990; method 965.17) on a spectrophotometer (Molecular Devices SpectraMAX 250, Ramsey, MN; 400 nm) (Bremer et al. 2008). A subsample of ground dry shoot samples was roll milled and total N content was determined using a combustion method N analyzer (Leco FP 528, Leco Corp., St. Joseph, MI) (AOAC, 1990; method 968.06).

Root samples were taken at 6, 8 and 10 WAT when whole plants were harvested. Right after the plant was cut, roots and soil were divided in half. One half of the root and soil sample was washed with tap water to remove soil and dried out with paper towels. The 2-3 cm top portion of dried root system including the brace roots, was removed and fine roots were mixed and divided in two sub-samples which were placed into labelled 50 ml sterile polypropylene tubes (VWR® High-Performance Centrifuge Tubes, VWR International LLC., Radnor, PA, US) and submerged in liquid nitrogen. Root sub-samples were stored at -80 ºC for later analysis. The other half of the root and soil sample was placed in plastic storage bags (Ziploc®, S. C. Johnson & Son, Inc., Racine, WI, US) and stored briefly in 4 ºC refrigerator until subsampling. Subsampling of the refrigerated sample resulted in a 400 g soil sample that stored at -20 ºC for later determination of AM fungi fatty acid biomarker. The remainder of the refrigerated samples was washed with tap water to remove soil from fine roots. Roots were dried out with paper towel, placed in plastic bags, and stored at -20 ºC in freezer plastic bags for determination of root colonization by AM fungi and AM fungi fatty acid biomarker.

Percent root colonization by AM fungi was quantified by staining 1 cm long root segment with 0.05 percent (w/v) black ink (Brundrett et al. 1996; Vierheilig et al. 1998). Percent root colonization by AM fungi was calculated using the gridline intersect method.
(Brundrett et al. 1996; Giovannetti and Mosse 1980). To quantify AM fungi fatty acid biomarker, C16:1cis11 in soil samples, a 10 g ± 0.2 soil sub-sample was used. Thus, fatty acid was quantified by gas chromatography – mass spectrophotometry methodology as used by Grigera et al. (2007). Also AM fungi fatty acid biomarker, C16:1cis11 was quantified on root samples. To quantify C16:1cis11 fatty acid, a 1 g root sample was freeze dried (Labconoc FreeZone 6L freeze dry system with a FreeZone Bulk Tray Dryer, Labconco Corp., Kansas City, MO, US) for 72 h at -50 °C and -200 mbar. Then, freeze dried samples were ground and homogenized (Omni Bead Ruptor 24 Homogenizer, Omni International, Inc., Kennesaw, GA, US) in 2 ml polypropylene micro tubes (Sarstedt, Inc., Newton, NC, US) with five 2 mm zirconium ceramic beads per tube. On the homogenizer, three cycles of 30 sec with dwells of 20 sec between cycles, at 25 °C and 7.1 m s⁻¹ were used. Thus, FAMEs were quantified on 30-50 mg freeze dried and ground root samples by gas chromatography – mass spectrophotometry methodology as used by Grigera et al. (2007). The fatty acid C16:1cis11 was selected as biomarker for AM fungi.

**Experimental design and statistical analysis**

The experiment was 2x4x2x3x3 factorial arranged in a randomized complete block design. The factorial arrangement included two arbuscular mycorrhizal fungal (AMF) treatments (with mycorrhizae, +M; without mycorrhizae, -M) and four maize hybrids (abbreviated DS1, DS2, DT1, DT2) under two P (43 mg P pot⁻¹, +P; 86 mg P pot⁻¹, ++P) and three N levels (without N, 0N; 147 mg N pot⁻¹, 1N; 294 mg N pot⁻¹, 2N) at three harvest times (6, 8 and 10 WAT). Four replications were used for each treatment combination; thus, the experiment had a total of 576 pots. PROC MIXED ANOVA in
SAS 9.3 (SAS, 9.3, SAS Institute, Cary, NC, USA) was used to measure the response in N and P content in plant, and shoot dry and fresh weight. PROC GLIMMIX Repeated Measures ANOVA was applied to measure the effects of treatments over time, and a four-way ANOVA was used to compare effects of mycorrhisal, hybrid, N and P treatments on plants physiology response. Mean values were separated using LSD procedure ($P < 0.05$).

3. Results

Maize plant growth parameters

Maize plant growth parameters are shown in Table 3.1 while probabilities of repeated measures ANOVA are shown in Table 3.2. Maize plant height increased over time ($P < 0.0001$) between the 1st and the 10th WAT and also for all three harvest times ($P < 0.0001$). There was a significant ($P < 0.0001$) harvest time interaction with all of the other factors (i.e. AMF, hybrid and nutrient level) (Table 3.1). Non-inoculated plants were taller than AMF inoculated plants ($P < 0.0001$) (Table 3.1; Figure 3.1) while plant height increased as N and P fertilization increased ($P < 0.0001$) (Table 3.1). Plant height also varied among hybrids ($P < 0.0001$) (Table 3.1). Hybrid DT2 was the tallest while hybrid DS2 was the shortest at 10 WAT (Table 3.5). There were AMF*hybrid, AMF*nutrient level and hybrid*nutrient level interactions for plant height (Table 3.2).

Shoot dry and fresh weights increased over time for all three harvest times ($P < 0.0001$) (Table 3.1) and with increasing N and P fertilization ($P < 0.0001$) (Table 3.1). Time*hybrid interactions were significant for both dry ($P = 0.0027$ and 0.0140, respectively) and fresh weight while time*AMF and time*nutrient level interactions were
only significant \( (P = 0.0004 \text{ and } < 0.0001) \) for dry weight (Table 3.2). In addition, hybrids responded differently to AMF inoculation and therefore, AMF*hybrid interaction was significant \( (P < 0.0001) \) for both dry and fresh weights (Table 3.5). Non-inoculated maize plants weighed 27 and 26\% more than inoculated plants based in dry and fresh weight, respectively. Hybrid DT1 accumulated more biomass than all other hybrids based on both dry and fresh weights (Tables 3.1). Conversely, hybrid DS2 had the lowest dry weight while hybrid DT2 had the lowest fresh weight (Tables 3.1). Both drought tolerant maize hybrids, DT1 and DT2 had the highest dry weights. As a percentage of fresh weigh, hybrids DT1 and DT2 also had the highest values with 18.2 and 18.6\% of fresh weight, respectively (data not shown).

The number of leaves per plant increased over time \( (P < 0.0001) \) (Table 3.1) at an average rate of approximately 0.23 leaf day\(^{-1}\) and for all hybrids. Earlier harvest time (6 WAT) resulted in fewer leaves, fewer nodes (personal observation) and hence shorter plants. Mean leaf number per plant at the end of the experiment (10 WAT) was 15, 18, 16, and 16 for hybrids DS1, DS2, DT1 and DT2, respectively; however, they were not statistically different \( (P = 0.0534) \). At the end of the experiment, all hybrids had some plants silking and/or tasseling (personal observation). An increasing number of leaves were observed on non-inoculated maize plants compared to inoculated plants (16.3 vs 16.1, respectively) \( (P < 0.0001) \) at 10 WAT. Also, number of leaves increased with higher N fertilization, but not with increasing P fertilization (Table 3.1).

**Chlorophyll, P and N contents**

Chlorophyll content varied over time \( (P < 0.0001) \) with a peak at 4 WAT and then, decreased to the lowest value at 7 WAT (data not shown). After that, chlorophyll content
increased again up to the end of the experiment (10 WAT) (data not shown). The higher chlorophyll values at the beginning of the experiment could be due to the initial starter fertilizer, which included N. Chlorophyll content in the leaves of both AMF inoculated and non-inoculated plants increased as N level increased (Figure 3.2). At all N and P levels, AMF inoculated plants had lower chlorophyll content compared to non-mycorrhizal plants. Chlorophyll content also varied among hybrids ($P < 0.0001$) (Tables 3.1 and 3.5). A drought sensitive hybrid, DS1, had the highest chlorophyll content. It was 23% higher than the other drought sensitive hybrid, DS2, that had the lowest chlorophyll content (Table 3.1).

Nitrogen concentration of dry maize shoots decreased ($P < 0.0001$) while total N uptake per plant increased over time ($P < 0.0001$) (Table 3.3 and 3.4; Figure 3.3). Both N concentration and content increased as N fertilization increased ($P < 0.0001$) and were not different across hybrids ($P = 0.0682$ and $0.3493$, respectively) (Table 3.3, 3.4 and 3.6; Figure 3.3). Although non-inoculated plants showed higher N uptake than AMF inoculated plants ($P < 0.0001$), the difference in N concentration was not significant ($P = 0.5666$) (Table 3.3 and 3.4). In contrast, both P concentration and content of dry maize shoots were higher in inoculated plants than non-inoculated plants ($P < 0.0001$ and $P = 0.0020$, respectively) (Table 3.3 and 3.4) and increased with N fertilization (Figure 3.4). Phosphorus uptake differed across maize hybrids ($P = 0.0019$) (Table 3.6) and over time ($P < 0.0001$). Both C concentration and content of dry shoots increased as N fertilization increased ($P < 0.0001$) (Table 3.3 and 3.4) and varied over time ($P < 0.0001$) (Table 3.4); however, while C concentration was lower at 10 WAT compared to 6 WAT, C content doubled at 10 WAT compared to 6 WAT (Table 3.3). Also, there was a significant
hybrid*time interaction ($P = 0.0498$) and time*AMF*nutrient level interaction ($P = 0.0209$) for C concentration. Carbon content among maize hybrids also varied with AM inoculation ($P = 0.0083$). Symptoms of N and P deficiency were observed at the lowest N and P levels. When N was not applied (N0) plant leaves became yellow on the mid rib in young plants (Picture 3.1), and were brown and sapless necrotic by the end of the experiment. In all maize plants, the lower P treatment (+P) caused purple areas on the stems (Picture 3.2) and leaf tips that progressed to the leaf edges over time.

**AMF root colonization and biomarkers**

Root colonization data confirmed that AMF inoculation was effectively imposed on inoculated maize plants (Tables 3.5 and 3.7). None of the maize plants in the non-inoculated treatments were colonized by AMF (Tables 3.5 and 3.7). Plants inoculated with AMF had approximately 67% root colonization at 10 WAT average across hybrids and nutrient levels. Root colonization was significantly different among harvest times ($P < 0.0001$) and higher at the end of the experiment (10 WAT) (Tables 3.7 and 3.8). Root colonization by AMF increased as N fertilization increased ($P = 0.0013$) (Table 3.8).

The concentration of the AMF biomarker C16:1$\text{cis}11$ in both root and soil samples varied over time ($P < 0.0001$) and was significantly affected by AMF inoculation ($P < 0.0001$) (Tables 3.5 and 3.7). The low concentration of C16:1$\text{cis}11$ in the soil of non-inoculated plants reflects background levels from dead AMF in the pasteurized soil and bacteria that also contain this biomarker. Since the roots were not colonized by AMF in the non-inoculated plants, low levels of the biomarkers in roots would have come from other microorganisms containing C16:1$\text{cis}11$ (Table 3.5).
4. Discussion

Inoculation of maize hybrids having differential tolerance to drought with AM fungi had a positive impact on P but not N uptake during our 10 week greenhouse experiment. Furthermore, N fertilization of AM inoculated plants stimulated P uptake, while P uptake was repressed by N addition in non-inoculated maize plants. Under greenhouse conditions, Chu et al. (2013) and Tian et al. (2013) observed higher P uptake in AM inoculated maize than non-inoculated maize plants. Phosphorus concentration was not different among non-inoculated maize genotypes, but differed between old and modern maize genotypes for the inoculated plants (Chu et al. 2013). In addition to increased P uptake, Ruiz-Lozano et al. (1995) reported improved N uptake in lettuce (*Lactuca sativa* L.) when colonized by AM fungi. Conversely, Tobar et al. (1994) did not find differences in N uptake between mycorrhizal and non-mycorrhizal lettuce plants under optimal water conditions, but P and N uptake increased in mycorrhizal lettuce plants under sub-optimal water levels. These results partially agree with our results where maize plants colonized by AM fungi were more efficient in taking up soil P than non-inoculated plants, but not necessarily N uptake under greenhouse conditions.

Increased AM fungal biomass in soil and maize roots, and increased root colonization by AMF, led to decreased shoot plant biomass and number of leaves in AM inoculated compared to non-inoculated maize plants for all hybrids. This increased carbon allocation to AM biomass in roots and soil by the end the experiment indicates a carbon drain from the plant to AM fungi under greenhouse conditions. In contrast to the lower shoot dry biomass in inoculated maize hybrids reported in the present study, several other studies have observed higher dry and/or fresh weights on inoculated plants
(Chu et al. 2013; Tian et al. 2013; Zhu et al. 2010; Liu et al. 2000a; Ruiz-Lozano et al. 1995). In agreement with our results, Chu et al. (2013) found differences in shoot dry weight among inoculated, but not non-inoculated maize genotypes. The higher dry biomass with AM inoculation reported by Chu et al. (2013) was attributed to increased nutrient uptake. In our study, higher P uptake on inoculated plants did not translate into higher dry plant biomass.

Baylis (1975) and Liu et al. (2000a) equated higher dry biomass and/or higher total leaf area in maize hybrids to greater dependence on AMF for nutrient uptake. In our greenhouse study, although inoculated maize plants showed lower dry weights than non-inoculated plants, maize hybrids showed a differential response in shoot dry weight (also fresh weight and number of leaves) to AM inoculation. This was not expressed by differences in root colonization or AM biomarker concentration in roots or soil. These results contradict results from Liu et al. (2000a). Further experiments are needed to unravel the complex interactions among maize hybrids, AM species, cropping system management and the environment.

In our experiment, AM inoculation decreased chlorophyll content of maize leaves. Charest et al. (1993) also reported slightly reduced chlorophyll content in AM maize plants growing under greenhouse conditions similar to our experiment. In contrast, Zhu et al. (2010) and Sheng et al. (2008) reported contrasting results even when they carried out experiments under comparable greenhouse conditions. These researchers showed that inoculated maize plants increased the concentration of chlorophyll by 14 – 18% when compared to non-inoculated plants (Sheng et al. 2008; Zhu et al. 2010). In our study, lower chlorophyll content in AM inoculated plants could be attributed to lack of available
N for plant since AM fungi captured more N to balance C content in mycelium, and thus, less N was transferred to the plant.

Maize root colonization ranged from 65-69% and did not differ among maize hybrids. This was higher than the 30-45% reported by Chu et al. (2013) but lower than the 76 - 77% reported by Tian et al. (2013) for similar soil P contents in seven and nine week greenhouse experiments, respectively. Ortas and Akpinar (2011) reported a high variation (between 60 and 96%) in root colonization across six different maize hybrids in an eight week greenhouse study. Chu et al. (2013) also showed differences in AM root colonization between old and modern maize hybrids at 18 ppm soil P, but there was no difference in root colonization when soil P content increased to 38 ppm. Charest et al. (1993) reported comparable root colonization between two maize hybrids, but one hybrid produced greater biomass than the other one. Liu et al. (2000a) found that P application reduced AM root colonization on leafy normal and leafy reduced maize hybrids. When N was applied, root colonization ranged between 43 and 71% compared to the treatment without N application (34 – 53%) for both maize hybrids (Liu et al. 2000a). Additionally, Liu et al (2000a) reported a positive response in AM root colonization to N fertilization on the leafy reduced hybrid, but a negative response on the leafy normal hybrid only at high N fertilization level.

In the present study only AM inoculation and time affected the concentration of the C16:1cis11 biomarker in roots and soil with no differences among hybrids and N or P fertilization level. Although AM root colonization quantified by microscopy increased with N fertilization, this did not translate into higher biomarker concentration in the roots and may reflect the different components of the fungus being measured (Grigera et al. ...)
In a recent field experiment, increasing N fertilization rates did not change AM colonization or biomarker concentration in roots of maize (Tian et al. 2013), but significantly reduced AM biomarker concentration in soil (Jeske 2012). Thus, the impact of AM fungi on maize can differ greatly between field and greenhouse experiments, making drawing conclusions from greenhouse grown plants under confined conditions difficult.

Arbuscular mycorrhizal fungi are considered beneficial for plants. However, since host plant–AM fungi–environment interactions are difficult to assess both in the field and greenhouse, and are often inconclusive, there is little information to support or refute the existence of an AM fungal continuum from mutualism to parasitism (Johnson et al. 1997) in maize. In our study it is unclear why several plant physiological variables were unresponsive or negatively affected by AM inoculation. Our best guess is that the constrained growth conditions experienced by maize in the greenhouse swing the pendulum to the parasitism side of the continuum where more carbon is being allocated to the symbiont without a clear benefit to the plant.

In conclusion, under greenhouse conditions non-inoculated maize hybrids grew better than AM inoculated hybrids, despite higher P uptake in inoculated plants. Nitrogen fertilization stimulated P uptake in AM plants and repressed uptake in non-AM plants; however, this still did not compensate for lower plant biomass with AM inoculation. Maize hybrids responded differently to AM inoculation, particularly for dry and fresh weights, plant height and number of leaves. In the field, AM fungi have been shown to provide a nutritional benefit to corn (Liu et al. 2002), but under greenhouse conditions may act more as a carbon drain thereby depressing plant growth. Further investigation
into the complex relationships among AM fungi, maize and soil management is needed to maximize benefit from this ubiquitous symbiosis.

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References


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Table 3.1. Mean values of plant height (H, cm), shoot dry weight (DW, g), shoot fresh weight (FW, g), number of leaves (L), and leaf chlorophyll (Ch, mg m$^{-2}$) across harvest times, arbuscular mycorrhiza (AM) fungi inoculation (+M; -M), maize hybrid (DS=drought sensitive; DT=drought tolerant) and nutrient treatments (T1: +P0N: 43 mg P pot$^{-1}$ and 0 mg N pot$^{-1}$, T2: +P1N: 43 mg P pot$^{-1}$ and 147 mg N pot$^{-1}$, T3: +P2N: 43 mg P pot$^{-1}$ and 294 mg N pot$^{-1}$, T4: ++P0N: 86 mg P pot$^{-1}$ and 0 mg N pot$^{-1}$, T5: ++P1N: 86 mg P pot$^{-1}$ and 147 mg N pot$^{-1}$, and T6: ++P2N: 86 mg P pot$^{-1}$ and 294 mg N pot$^{-1}$) at 10 weeks after transplanting.

<table>
<thead>
<tr>
<th>Harvest Time</th>
<th>Mean H</th>
<th>DW</th>
<th>FW</th>
<th>L</th>
<th>Ch</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 WAT</td>
<td>152.6c</td>
<td>11.8c</td>
<td>104.7c</td>
<td>9.8c</td>
<td>296.3b</td>
</tr>
<tr>
<td>8 WAT</td>
<td>163.4b</td>
<td>20.3b</td>
<td>111.3b</td>
<td>15.7b</td>
<td>273.6c</td>
</tr>
<tr>
<td>10 WAT</td>
<td>167.0a</td>
<td>26.8a</td>
<td>117.1a</td>
<td>16.2a</td>
<td>318.9a</td>
</tr>
</tbody>
</table>

| AM fungi inoculation: | |
|---------------------|--------|----|----|---|----|
| -M                  | 175.2a | 22.7a | 127.6a | 14.1a | 303.9a |
| +M                  | 146.7b | 16.6b | 94.5b  | 13.7b | 288.7b |

| Maize hybrid:       | |
|---------------------|--------|----|----|---|----|
| DS1                 | 160.6b | 19.0bc | 110.5b | 13.4b | 330.3a |
| DS2                 | 150.4c | 18.5c  | 109.3b | 14.6a | 268.3c |
| DT1                 | 156.7b | 21.4a  | 117.8a | 13.6c | 289.3b |
| DT2                 | 176.1a | 19.8b  | 106.6b | 14.1b | 297.2b |

<p>| Nutrient treatments: | |
|---------------------|--------|----|----|---|----|
| T1: +P0N            | 142.1c | 16.1d | 93.2c  | 13.6c | 262.2d |</p>
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<td>109.6b</td>
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<td>22.5b</td>
<td>126.7a</td>
<td>14.2a</td>
<td>328.7a</td>
</tr>
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<td>T4: ++P0N</td>
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<td>15.7d</td>
<td>93.4c</td>
<td>13.5c</td>
<td>276.2c</td>
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<td>19.6c</td>
<td>111.0b</td>
<td>13.9b</td>
<td>292.9b</td>
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<td>24.1a</td>
<td>132.5a</td>
<td>14.3a</td>
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Table 3.2. Probability values from Repeated Measures ANOVA, four-way ANOVA of main effects and interactions on plant height (H, cm), shoot dry weight (DW, g), shoot fresh weight (FW, g), number of leaves (L), and leaf chlorophyll (Ch, mg m\(^{-2}\)) of shoots by harvest time (HT), arbuscular mycorrhiza fungi (AMF) inoculum, maize hybrid (Hy), and Nutrient treatment (Nut).

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<td>**</td>
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</tr>
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<td>AMF</td>
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</tr>
<tr>
<td>Hy</td>
<td>**</td>
<td>**</td>
<td>**</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>HT*Hy</td>
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</tr>
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</table>

ns: not significant, * = \(P<0.05\), ** = \(P<0.01\)
Table 3.3. Mean values of nitrogen (N), carbon (C), and phosphorus (P) concentrations (%) and contents (mg plant\(^{-1}\)) across harvest times, arbuscular mycorrhiza fungi (AMF) inoculation (+M; -M), maize hybrid (DS=drought sensitive; DT=drought tolerant) and nitrogen treatments (0N: without N, 1N: 147 mg N pot\(^{-1}\), and 2N: 294 mg N pot\(^{-1}\)) at 10 weeks after transplanting.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>C</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>(%)</td>
<td>(mg plant(^{-1}))</td>
<td>(%)</td>
</tr>
<tr>
<td>Harvest time:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>104.6b</td>
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</tr>
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<td>AMF inoculation:</td>
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Table 3.4. Probability values from Repeated Measures ANOVA, four-way ANOVA of main effects and interactions on nitrogen (N), carbon (C), and phosphorus (P) concentrations (%) and content (mg plant\(^{-1}\)) of shoots by harvest time (HT), arbuscular mycorrhiza fungi (AMF) inoculum, maize hybrid (Hy), and Nutrient treatment (Nut).

<table>
<thead>
<tr>
<th></th>
<th>N (%)</th>
<th>(mg plant(^{-1}))</th>
<th>C (%)</th>
<th>(mg plant(^{-1}))</th>
<th>P (%)</th>
<th>(mg plant(^{-1}))</th>
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</tr>
<tr>
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<td>ns</td>
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ns: not significant, * = \(P<0.05\), ** = \(P<0.01\)
Table 3.5. Mean values of plant height (H, cm), dry weight (DW, g), fresh weight (FW, g), number of leaves (L), chlorophyll (Ch, mg m\(^{-2}\), root colonization by AMF (Rc, %), and root (rF, nmol mg\(^{-1}\) of root) and soil biomarkers (sF, nmol g\(^{-1}\) of soil) across hybrids (DS=drought sensitive; DT=drought tolerant) and mycorrhizae inoculation at 10 weeks after transplanting.

<table>
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<th>H</th>
<th>DW</th>
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<th>rF</th>
<th>sF</th>
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</tr>
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<tr>
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<td>17.0de</td>
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<tr>
<td>DS1</td>
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<td>21.0bc</td>
<td>121.9b</td>
<td>13.5de</td>
<td>339.3a</td>
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<td>0.5b</td>
<td>2.0b</td>
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<td>13.8c</td>
<td>298.7a</td>
<td>0.0b</td>
<td>0.5b</td>
<td>5.2b</td>
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<tr>
<td>DT2</td>
<td>191.1a</td>
<td>23.5a</td>
<td>124.8ab</td>
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<td>306.3a</td>
<td>0.0b</td>
<td>0.7b</td>
<td>6.2b</td>
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Letters within each column indicate statistically different \((P > 0.05)\) means by LSD procedure.
Table 3.6. Mean values of nitrogen (N), carbon (C), and phosphorus (P) concentration (\%) and content (mg plant\(^{-1}\)) across hybrids (DS=drought sensitive; DT=drought tolerant) and mycorrhizae inoculation at 10 weeks after transplanting.

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>N (%)</th>
<th>C (mg plant(^{-1}))</th>
<th>P (mg plant(^{-1}))</th>
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<td></td>
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</tr>
<tr>
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<td>144.4a</td>
<td>42.2a 9073.3ab</td>
</tr>
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<td>0.67a</td>
<td>140.0ab</td>
<td>42.6a 9681.4a</td>
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<tr>
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<td>0.72a</td>
<td>150.5a</td>
<td>42.5a 9517.9ab</td>
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</table>

Letters within each column indicate statistically different (\(P > 0.05\)) means by LSD procedure.
Table 3.7. Percent of root colonization (Rc, %) by arbuscular mycorrhizal fungi (AMF) and soil AMF FAME (nmol g\(^{-1}\) of soil) and root AMF FAME (nmol mg\(^{-1}\) of root) biomarker C16:1cis11 at 6 and 10 weeks after treatment (WAT) for non-inoculated (-M) and inoculated (+M) plants.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Rc</th>
<th>Soil AMF FAME</th>
<th>Root AMF FAME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 WAT</td>
<td>10 WAT</td>
<td>6 WAT</td>
</tr>
<tr>
<td>-M</td>
<td>0.0b</td>
<td>0.0b</td>
<td>2.1b</td>
</tr>
<tr>
<td>+M</td>
<td>59.2a</td>
<td>75.1a</td>
<td>7.1a</td>
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</table>

Letters within each column indicate statistically different (\(P > 0.05\)) means by LSD procedure.
Table 3.8. Percent of root colonization by arbuscular mycorrhizal fungi (AMF) at 6 and 10 weeks after transplanting (WAT) and across N treatments (0N: without N; 1N: 147 mg N pot$^{-1}$; 2N: 294 mg N pot$^{-1}$) for inoculated plants.

<table>
<thead>
<tr>
<th>Harvest Time</th>
<th>N level</th>
<th>AMF root colonization</th>
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<tr>
<td></td>
<td>1N</td>
<td>62.9aB</td>
</tr>
<tr>
<td></td>
<td>2N</td>
<td>61.2aB</td>
</tr>
<tr>
<td>10 WAT</td>
<td>0N</td>
<td>70.4bA</td>
</tr>
<tr>
<td></td>
<td>1N</td>
<td>74.8bB</td>
</tr>
<tr>
<td></td>
<td>2N</td>
<td>79.9bB</td>
</tr>
</tbody>
</table>

Lower case letter = difference across harvest times ($P > 0.05$).

Upper case letter = difference across N level within harvest time ($P > 0.05$).
Figure 3.1. Plant height of non-inoculated (-M) and AM inoculated (+M) maize plants. Lower case letter = difference across AMF treatments ($P > 0.05$).
Figure 3.2. Chlorophyll content on leaves of maize plants under two phosphorus (+P: 43 mg P pot\(^{-1}\) and ++P: 86 mg P pot\(^{-1}\)) and three nitrogen levels (0N: without N, 1N: 147 mg N pot\(^{-1}\) and 2N: 294 mg N pot\(^{-1}\)). Lower case letter = difference across nutrient treatments ($P > 0.05$).
Figure 3.3. Nitrogen concentration of dry maize shoots at 6 and 10 weeks after transplanting (WAT) across three nitrogen fertilizations levels (0N: without N, 1N: 147 mg N pot\(^{-1}\) and 2N: 294 mg N pot\(^{-1}\)). Lower case letter = difference across harvest times within N level (\(P > 0.05\)). Upper case letter = difference across N level within harvest time (\(P > 0.05\)).
Figure 3.4. Phosphorus concentration of non-inoculated (-M) and AM inoculated (+M) maize plants across three nitrogen fertilization levels (0N: without N, 1N: 147 mg N pot\(^{-1}\) and 2N: 294 mg N pot\(^{-1}\)). Lower case letter = difference across AM inoculation within N level ($P > 0.05$). Upper case letter = difference across N level within harvest time ($P > 0.05$).
Picture 3.1. Nitrogen deficiency symptom on corn leaves for plant under 0N (without nitrogen fertilization) (A) and 1N (147 mg N pot$^{-1}$) (B) treatments at 8 weeks after transplanting.
Picture 3.2. Phosphorus deficiency symptom on corn stems for plant under the lower P level (+P: 43 mg P pot⁻¹) at 8 weeks after transplanting.
CHAPTER 4. SOIL MICROBIAL COMMUNITY RESPONSE TO WATER STRESS DIFFERS AMONG FIELD GROWN MAIZE HYBRIDS

1. Introduction

Soil microbial communities are key to soil quality and function (Chowdhury et al. 2011) since microorganisms are involved in organic matter dynamics, nutrient cycling and several other soil processes (Acosta-Martinez et al. 2008). As a key part of the soil microbial community, arbuscular mycorrhizal (AM) fungi are important to water and nutrient acquisition through symbioses with plant roots. It has been shown that AM fungi extend hyphae into the soil surrounding the root and help to uptake and transfer mainly non-mobile nutrients such as P, Zn, Ca and Cu to the plant (Barea and Jeffries 1995; Alizadeh and Nadian 2010). In exchange, the AM symbiont receives carbon compounds from the host plant (Smith and Read 2008) that ultimately contributes to the soil carbon cycle due to the rapid turnover of hyphae in the soil (Zhu and Miller 2003).

Soil microbial communities respond to changes in the soil environment by altering their activity level and metabolic processes, activating stress tolerance mechanisms, forming resistant resting structures or through death (Chowdhury et al. 2011). In a wheat-fallow rotation, Drijber et al. (2000) found that plant inputs and soil conditions during the wheat cycle influenced soil microbial biomass and community structure. However, during fallow, when fresh plant inputs would be limiting, microbial communities responded to physicochemical differences in the soil resulting from tillage (Drijber et al. 2000). Jansa et al. (2003) observed a reduction in AM fungi colonization of maize roots after intensive tillage; however, it varied with the AM species and genus.
Also, under field conditions, any change in one factor such as tillage can result in changes in soil nutrient content, microbial activity and also weed populations that could influence AM activity (Jansa et al. 2003). Although it has been shown that soil microbial communities and AM fungi play an important role in nutrient cycling, the factors affecting their population dynamics and the AM fungal/plant symbiosis are poorly understood.

Water stress is the most important abiotic stress for plants in arid and semiarid regions leading to reduced plant growth and yield. In general, fungi can better tolerate decreasing soil water contents compared to bacteria, and under drought conditions fungi often dominate bacteria in terms of overall biomass (Reichardt et al. 2001). However, soil fungal biomass has been reported to decline with water stress in some instances (Frey et al. 1999; Stromberger et al. 2007; Chowdhury et al. 2011). Plants can naturally respond to water stress to avoid the stress or to increase its tolerance (Bray 1997). Several studies have demonstrated that AM fungi may help alleviate drought stress in the host plant (Augé 2001; Ruiz-Lozano 2003). Arbuscular mycorrhiza fungi can provide water to the host plant through an extended mycelium that penetrates soil pores inaccessible to root hairs, and thereby extract enough water to maintain a level sufficient for the plant (Allen 1982; Hardie 1985). Furthermore, Ruiz-Lozano (2003) suggested that AM fungi could improve water stress tolerance through more complex processes involving a combination of physical, nutritional, physiological and cellular effects.

Mycorrhizal symbioses are part of a complex interaction among the plant, fungus, and the environment (Smith and Smith 2011; Johnson et al. 1997). According to Hamel (2004), plant and fungal genotypes have a key role in the AM symbiosis. For example,
Zhu et al. (2001) found that the response to mycorrhizal inoculation of modern wheat cultivars was generally lower than the older cultivars. This suggests that the trait responsible for establishing the AM symbiosis in older and/or local varieties of wheat may have been weakened during modern breeding programs (Manske 1989; Zhu et al. 2001). Additionally, Hetrick et al. (1995) hypothesized that recent wheat cultivars developed in fully fertilized soil may have resulted in selection against genotypes that interact with, or respond to, mycorrhizal fungi. In maize, response to AM fungi varied among old and unimproved varieties while inbred maize lines with poor root ing ability showed higher response to AM inoculation (Khalil et al. 1994). Research by Kaeppler et al. (2000) also showed a differential response among plant stages, and plant/AM genotypes. Most plants can be successfully colonized by AM isolates under greenhouse conditions, but the same response is not necessarily found under field conditions. This indicates that the soil environment plays a key role in the establishment and functioning of the AM/plant symbiosis where changes in environmental conditions, spatially or temporally, may result in specific fungi colonizing only certain plants or parts of the plant root system (Sanders 2003).

Currently there are few studies linking changes in soil microbial biomass and or community composition to water management or differential interactions of microorganisms with crop plant species/varieties. In a rainfed rice experiment, Reichardt et al. (2001) showed that anaerobic bacteria decrease and aerobic bacteria increase after transplanting until crop harvest in accordance with decreasing soil moisture. In a complementary greenhouse experiment, fungal biomass was predominant only under drought conditions or during fallow after rice was harvested (Reichardt et al. 2001).
According to these researchers, under flooding an oxygenated film in the root rhizosphere promotes bacterial growth and this positive effect disappears under water limiting conditions (Reichardt et al. 2001). Although several studies documented the beneficial effect of AM fungi on major crop species such as maize (Subramanian and Charest 1997), sorghum (Cho et al. 2006), soybean (Porcel and Ruiz-Lozano 2004), wheat (Beltrano and Ronco 2008) and sunflower (Gholamhoseini et al. 2013), we are aware of no field studies linking plant genotypes (i.e. hybrid and/or varieties for a species), AM fungi and soil microbial community composition to plant water stress. In the present study, the objective was to describe the fatty acids methyl ester (FAMEs) profile for soil microbial communities under selected maize hybrids grown to R2-R3 in the field in response to two contrasting water levels.

2. Materials and methods

Field experiment and sampling

An irrigated field experiment located near Brule, NE (41° 09’ 25.30 N, 102° 01’ 50.71” W) was carried out in summer 2012. Soil at experimental site is classified as a Kuma loam, fine-silty, mixed, mesic, Pachic Argiustolls. The field was planted to 98 maize hybrids representing a wide range of genotypes and managed under no-till. Starter phosphorus and nitrogen liquid fertilizer was uniformly applied across all experimental plots at planting at a rate of 56 L ha⁻¹ (ammonium polyphosphate solution, 10-34-00) while nitrogen fertilizer at a rate of 224 kg ha⁻¹ (urea, 46-0-0) was applied at V8 stage of maize. Pesticides were applied as recommended for western NE.

Plots for each hybrid had two rows separated by 76 cm and 6.1 m in length; final
plant density was 71,000 plants ha\(^{-1}\). Two water levels: well watered (WW) and water stressed (WS, 40% of well watered) were imposed on hybrids. Well watered treatment was the amount of water enough to raise maize in western NE.

Six maize hybrids (B87, GEMS-182, PHW52, Va99, LH60 and NC262) out of the 98 hybrids were selected from an eight groups’ cluster dendogram maximizing the genetic variation and minimizing the flowering time between selected hybrids. Results for the six selected hybrids were provided by Dr. A. Lorenz and Dr. L. Ali and are shown in Appendix A. On August 7-8, 2012 when maize plants were at the R1-R2 growth stage, ten 3.5 cm diameter x 20 cm deep soil samples per hybrid and water level were taken in plots belonging to the selected hybrids. Soil cores were taken between rows and within 10-12 cm from the maize stalks The ten soil samples were pooled to one sample and stored at 4 °C for no more than 10 days until processed in the lab.

**Measurements**

Roots present in soil samples were removed with tweezers, washed with tap water and dried with paper towels. Soil samples was divided in half. One half was stored to -20 °C until used for fatty acid methyl ester determination. The other half was air dried for one week, sieved and soil pH, electrical conductivity (EC) and Mehlich phosphorus content (P) were determined. Soil EC and pH analysis was done on a 1:1 soil water slurry according to the protocol described by Smith and Doran (1996). Mehlich P content in soil samples was determined according the protocol described by Frank et al. (2012). Root sub-samples were stored at -20 °C for measurement of percent root colonization (Re) by AM fungi. Percent root colonization was quantified in 1 cm long segments obtained from a 1.0 to 1.5 g fresh weight root sample (Brundrett et al. 1996; Giovannetti and Mosse
Root segments were stained with 0.05 percent (w/v) black ink (Brundrett et al. 1996; Vierheilig et al. 1998). Percent root colonization by AM fungi was calculated by the gridline intersect method (Brundrett et al. 1996; Giovannetti and Mosse 1980).

Fatty acid methyl esters (FAMEs), including the biomarker C16:1cis11, indicative of AM fungi, were extracted from soil by mild alkaline hydrolysis (Grigera et al. 2007). Released FAMEs were separated by gas chromatography and identified by retention time and confirmed by mass spectrophotometry. Fatty acid methyl esters were quantified using peak relative to the internal standard C19:0 and reported as concentration in nmol g⁻¹ of soil (Grigera et al. 2007). The FAME biomarker, C16:1cis11, although largely attributed to AM fungi is also found in low abundance in a few bacterial genera (Zelles 1999).

**Experimental design and statistical analysis**

The experiment was arranged as a randomized complete block design with six maize hybrids and blocked by water treatment, WW and WS. Four replications were sampled for each treatment combination. PROC MIXED ANOVA in SAS (SAS, 9.3, SAS Institute, Cary, NC, USA) was used to measure the microbial community response using broad taxonomic groups defined by 19 specific FAME biomarkers (Appendix B): bacteria, saprophytic fungi, cyclopropyl fatty acids, AM fungi, actinomycetes and microeukaryotes (i.e. soil fauna), to maize hybrid and water level. Mean values were separated using LSD procedure ($P < 0.05$).

Difference in microbial community structure (i.e. distribution FAME biomarkers representing taxonomic groups defined above) were determined using canonical correlation analysis (SAS 9.3, SAS Institute, Cary, NC, USA) on standardized nmol% FAME data. Squared Mahalonobis distances were used to identify significant differences
among microbial communities with hybrid and water level. Analyses were done using SAS software. Plots were made in Origin (Origin, 7.0, OriginLab Corp., Northampton, MA, USA).

3. Results

Soil P content, pH and EC from the experimental site at Brule was similar between water treatments and did not differ among maize hybrids (Table 4.1). Percent root colonization (Rc) by AM fungi ranged from 61.6 to 69.8% and was not significantly different among hybrids or with water level (Table 4.1). The concentration of the AM biomarker C16:1cis11 was significantly higher ($P < 0.0001$) under water stress than well watered conditions. The concentration of the AM biomarker in soil differed ($P = 0.0150$) among hybrids (Table 4.2). The highest concentration of the AM biomarker C16:1cis11 was found in soil planted to hybrid LH60, and was not different from hybrids NC262, Va99 and B87 (Table 4.2). The lowest concentrations were found in soil planted to hybrids GEMS and PHW52. Bacterial and actinomycete marker concentrations were significantly higher under water stress ($P = 0.0253$ and $P = 0.0026$, respectively) compared to well watered treatment (Table 4.2). There was no statistical difference ($P = 0.0884$ and $P = 0.5604$) in bacterial and actinomycete markers among hybrids (Table 4.2). Cyclopropyl, saprophytic fungal and micro-eukaryote FAME marker concentrations were neither affected by water treatment nor hybrids (Table 4.2). The hybrid*water level interaction was not significant for all microbial markers (Table 4.2).

Nineteen selected FAMEs were used for canonical correlation analysis after nmol% transformation (concentrations in nmol g$^{-1}$ of selected FAMEs are listed in
Appendix B). Two significant \( P < 0.0001 \) canonical functions were identified by canonical discriminant analysis. The first canonical function (DA1) accounted for 44.8\% of the total variability \( P < 0.0001 \), while the second canonical function (DA2) accounted for 29.2\% of the total variability \( P = 0.002 \) (Figure 4.1) for a total of 74\%. Although both canonical axes factored into treatment separation, soil microbial communities were separated more by water treatment on discriminant axis 1 (Can 1) and by hybrid on discriminant axis 2 (Can 2) (Figure 4.1). Mahalonobis distances (Table 4.3) showed a significant separation in microbial community structure between water treatments \( P < 0.05 \) and also between hybrids within water levels \( P < 0.05 \) (Figure 4.1). Under well watered conditions, hybrids Va99 and LH60 were significantly \( P < 0.05 \) separated from hybrids B87 and GEMS-182 (Figure 4.1). Similarly, under water stress, two groups of hybrids were clearly separated, hybrids B87 and GEMS-182 from hybrids Va99, LH60 and NC262 (Figure 4.1). Hybrid PHW52 was not significantly different from either group (Figure 4.1; Table 4.3).

The AM fungal marker, C16:1cis11, was strongly positively correlated with water stress on Can 1 (Figure 4.2). Similarly, actinomycete markers, 10MeC18:0 and 10MeC19:0 and some bacterial markers, iC14:0, iC15:0, aC15:0, iC16:0, also showed positive correlations with water stress on Can 1 (Figure 4.2). Other bacterial markers different from the aforementioned, and the cyclopropyl, saprophytic fungal and micro-eukaryote FAME markers were negatively correlated with water stress on Can 1 (Figure 4.2). The micro-eukaryotic markers, C20:2, C20:4 and C20:5, and some bacterial markers, were positively correlated with specific hybrids on Can 2 (Figure 4.2).
4. Discussion

The present study assesses the impact of soil water stress and maize genotype on AM fungi in soil and maize roots as well as changes in soil microbial biomass and community structure. Root colonization by AM fungi did not differ between water treatments or among maize hybrids (Table 4.1); however, AM community composition within the roots differed among hybrids but not water level (Masao Higo, personal communication). This is in contrast to AM biomass in the soil, where AM fungal biomass was higher under water stress compared to well watered treatment and there were significant differences in soil extraradical AM biomass among hybrids (Table 4.2). In a greenhouse experiment, Liu et al. (2000) determined AM root colonization and soil extraradical AM fungal biomass on three different maize hybrids. Their findings showed root colonization and extraradical hyphal length differed among maize genotypes. Conversely, Zhu et al. (2012) reported a reduction in AM root colonization of inoculated maize plants under drought stressed conditions when compared to plants under no water restriction. In a field experiment, Al-Karaki et al. (2004) reported a reduction in root colonization in two wheat genotypes under water stressed conditions compared to well watered plants.

It has been argued that the AM symbiosis with a host plant results in altered rates of water movement and nutrient flux into and throughout the host plant (Augé 2001). These exchanges have consequences on tissue hydration, and plant physiology and biochemistry that can lead to specific changes in carbon allocation to AM fungi. Most studies generalize the effects of AM fungi on the host plant; however, it is recognized that the plant and also the AM fungal response can vary depending on both host plant and
AM species (Ruiz-Lozano et al. 1995). Augé (2001) stated that the size of the plant, as determined by plant genotype, can affect plant water relations and drought response. The plant/AM symbiosis often affects nutrient acquisition. Consequently the symbiosis can also affect nutrient allocations, mainly carbon, and several other aspects of host/AM fungi physiology and biochemistry. Host plant and AM genotypes plus environmental conditions can influence plant and AM fungal responses, particularly when soil water becomes limiting. Thus, changes in plant biochemistry resulting from the AM-maize hybrid interaction could explain why different maize genotypes resulted in varying amounts of soil AM extraradical biomass when no differences in root colonization among hybrids was found. This could also be due to colonization of hybrids by different AM species (Masao Higo, personal communication).

Canonical analysis of FAMEs (Figures 4.1 and 4.2) confirmed the dominant influence of soil water status on both soil AM fungal biomass and microbial community structure. Nevertheless, soil water status did not affect all FAMEs similarly. Bacterial and actinomycete FAMEs significantly increased their concentration relative to the total amount under water stress when compared to well watered condition (Table 4.2). Although, the total biomass of microbial taxonomic/functional groups with the exception of AM fungi did not differ among maize hybrids (Table 4.2), there appeared to be subtle shifts in specific FAMEs with hybrid on both canonical axes. Andresen et al. (2014) did not find changes in soil bacterial and fungi FAMEs in sandy soil dominated by native species after summer drought treatment. In a multifactorial experiment, Andresen et al. (2014) found a diversified response pattern for bacteria, but not fungi, and suggested this response can be due to multiple factors such as substrate availability and soil temperature.
in addition to soil water content. Drijber et al. (2000) reported an increased amount of the AM FAME biomarker C16:1cis11 under a dryer sod plot versus plots under fallow or cropped to wheat.

Several studies have reported that soil physicochemical factors, plant community composition and environmental conditions affect soil microbial communities (Zul et al. 2007). Results from Zhang et al. (2014) suggest that water availability is the key factor structuring soil bacterial communities in semi-arid ecosystems. Uhlířová et al. (2005) reported lower microbial biomass and activity on dry soils due to water and nutrient deficiencies. These researchers showed that gram+ bacteria and actinomycetes dominated dry soils (Uhlířová et al. 2005). Wu et al. (2010) also recognized the importance of environmental conditions; however, they did not find changes in soil microbial biomass and community structure in soil samples incubated under different soil water contents.

In the present study, higher concentrations of bacterial FAMEs under water stress were somewhat surprising. However, this may signal increased plant root exudation under plant water stress favoring bacterial growth in the rhizosphere (Song et al. 2012). An alternative hypothesis could focus on preservation of bacterial habitat through improved stable aggregation under drought stress. This is supported by higher AM biomass under water stress and the known role of AM fungi in aggregate formation and stabilization (Wright et al. 1999; Rillig 2004; Boomsma and Vyn 2008). Soil aggregates promote formation of larger soil pores between aggregates, and also increase formation of capillary pores inside soil aggregates. Inside capillary pores water films surrounding adjacent particles merge, holding water to the particles; therefore, capillary pores are responsible for increasing water holding in all soils but they are particularly important
under drought and arid or semiarid conditions (Brady and Weil 2009). Bacteria within these capillary pores would be more physically protected and resistant to drought stress.

Cyclopropyl FAMEs have been considered as microbial stress markers in soil environments under extremes of pH, drought, contamination, etc. (Grogan and Cronan 1997; Chang and Cronan Jr. 1999). In the present study, the concentration of cyclopropyl FAMEs did not differ between water treatments (Table 4.2); however, the relative amount decreased under water stress (Figure 4.2). Thus, the water stress imposed in the field was either not severe enough to impact the stress response of bacteria in soil or there was a shift in species composition that had a greater influence on cyclopropyl abundance in the soil.

Although the concentration of the cyclopropyl markers, saprophytic fungi and micro-eukaryote FAMEs were neither affected by the soil water status nor maize hybrid (Table 4.2), the relative abundance of these markers were affected by soil water status. In soil, the fungal community has varying functional and metabolic capabilities. Thus, while AM fungi establish symbiotic relationships with host plant to obtain C, saprophytic fungi obtain C from through decomposition of soil organic matter, plant residues and/or root exudates (Butler et al. 2003). Thus, it is not surprising that AM and saprophytic fungi exhibit different responses to water stress and maize hybrid. No changes in the amount of saprophytic fungi at the time of sampling (R2-R3) could signal equal availability of carbon substrates within the field regardless of maize hybrid. Also, the similar amount of active fungal biomass in the soil suggests that the difference in water status did not impact the rate of decomposition of organic matter and thus fungal growth and turnover. Future research should examine the relationships among microbial biomass and
community composition, soil organic matter dynamics and nutrient supply to the maize crop as impacted by water stress and hybrid at this experimental site.

Our results confirmed that AM fungi effectively colonized roots to the same degree regardless of maize hybrid and under water limitation. Increased C allocation from plant to AM fungi was observed under water limitation as evidenced by greater AM biomass in the soil compared to well watered. While water limitation caused a greater shift in the overall soil microbial community, maize hybrid influenced specific microbial groups and/or FAME biomarkers. Further investigation into the complex relationships among the soil microbial community, maize genotype and soil water management is needed to maximize benefit from microbial-plant interactions.

**Acknowledgements**

We wish to acknowledge Liakat Ali, Masao Higo and Liz Jeske for their technical assistance.

**References**


Al-Karaki, G., B. McMichael and J. Zak. 2004. Field response of wheat to arbuscular


Table 4.1. Mean values and ANOVA table of soil phosphorus content (P), soil pH, soil electrical conductivity (EC), and root colonization (Rc) among water levels and maize hybrids.

<table>
<thead>
<tr>
<th></th>
<th>P (ppm)</th>
<th>pH</th>
<th>EC (µS cm⁻¹)</th>
<th>Rc (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Water level</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well watered</td>
<td>2.2a</td>
<td>7.2a</td>
<td>361a</td>
<td>65.5a</td>
</tr>
<tr>
<td>Water stressed</td>
<td>2.1a</td>
<td>7.3a</td>
<td>371a</td>
<td>63.8a</td>
</tr>
<tr>
<td><strong>Hybrid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B87</td>
<td>2.0a</td>
<td>7.4a</td>
<td>412a</td>
<td>69.8a</td>
</tr>
<tr>
<td>GEMS</td>
<td>2.0a</td>
<td>7.2a</td>
<td>378a</td>
<td>61.6a</td>
</tr>
<tr>
<td>PHW52</td>
<td>2.1a</td>
<td>7.2a</td>
<td>373a</td>
<td>65.0a</td>
</tr>
<tr>
<td>Va99</td>
<td>1.9a</td>
<td>7.4a</td>
<td>357a</td>
<td>65.4a</td>
</tr>
<tr>
<td>LH60</td>
<td>2.3a</td>
<td>7.2a</td>
<td>307a</td>
<td>62.9a</td>
</tr>
<tr>
<td>NC262</td>
<td>2.2a</td>
<td>7.2a</td>
<td>370a</td>
<td>63.0a</td>
</tr>
<tr>
<td><strong>Water level x Hybrid</strong></td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
</tbody>
</table>

**ns** = no significant differences.

Letters within each column for each factor indicate statistically different \( (P > 0.05) \) means by LSD procedure.
Table 4.2. Mean and ANOVA table of known FAME biomarker concentration (nmol g\(^{-1}\) of soil) and total biomass among

<table>
<thead>
<tr>
<th></th>
<th>Bacteria</th>
<th>AMF</th>
<th>Cyclopropyl</th>
<th>Actinomycete</th>
<th>Saprophytic fungi</th>
<th>Micro-eukaryote</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water level:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well watered</td>
<td>25.6b</td>
<td>4.4b</td>
<td>7.6a</td>
<td>3.9b</td>
<td>9.4a</td>
<td>3.2a</td>
<td>76.9b</td>
</tr>
<tr>
<td>Water stressed</td>
<td>29.1a</td>
<td>6.5a</td>
<td>8.2a</td>
<td>4.8a</td>
<td>10.5a</td>
<td>2.9a</td>
<td>87.6a</td>
</tr>
<tr>
<td>Hybrid:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B87</td>
<td>26.5a</td>
<td>5.9a</td>
<td>8.0a</td>
<td>4.5a</td>
<td>10.6a</td>
<td>3.1a</td>
<td>83.1a</td>
</tr>
<tr>
<td>GEMS</td>
<td>25.0a</td>
<td>4.5b</td>
<td>7.7a</td>
<td>4.1a</td>
<td>9.5a</td>
<td>2.8a</td>
<td>76.4a</td>
</tr>
<tr>
<td>PHW52</td>
<td>24.1a</td>
<td>4.6b</td>
<td>7.2a</td>
<td>4.0a</td>
<td>8.4a</td>
<td>2.8a</td>
<td>71.9a</td>
</tr>
<tr>
<td>Va99</td>
<td>30.2a</td>
<td>6.1a</td>
<td>8.2a</td>
<td>4.8a</td>
<td>11.0a</td>
<td>3.4a</td>
<td>89.7a</td>
</tr>
<tr>
<td>LH60</td>
<td>30.1a</td>
<td>6.2a</td>
<td>8.4a</td>
<td>4.6a</td>
<td>11.0a</td>
<td>3.3a</td>
<td>90.5a</td>
</tr>
<tr>
<td>NC262</td>
<td>28.3a</td>
<td>5.5ab</td>
<td>7.8a</td>
<td>4.4a</td>
<td>9.1a</td>
<td>3.0a</td>
<td>82.0a</td>
</tr>
</tbody>
</table>

|                  |          |     |             |               |                   |                 |        |
| Water level      | *        | *   | ns          | *             | ns                | ns              | *      |
| Hybrid           | ns       | *   | ns          | ns            | ns                | ns              | ns     |
| Water level x Hybrid | ns  | ns  | ns          | ns            | ns                | ns              | ns     |

AMF = Arbuscular mycorrhiza fungi; ns = not significant differences; * = \( P < 0.05 \)

Letters within each column and for each factor indicate statistically different \( (P > 0.05) \) means by LSD procedure.
Table 4.3. Probability table for dissimilarity Mahalanobis distances between relative concentrations (nmol%) of 19 FAMEs profile among water levels (well waterway, WW; water stressed, WS) and maize hybrids.

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Well watered</th>
<th>Water stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B87</td>
<td>GEMS</td>
</tr>
<tr>
<td>B87</td>
<td>1</td>
<td>0.5835</td>
</tr>
<tr>
<td>GEMS</td>
<td>0.5835</td>
<td>1</td>
</tr>
<tr>
<td>LH60</td>
<td>0.006</td>
<td>0.0111</td>
</tr>
<tr>
<td>NC262</td>
<td>0.0029</td>
<td>0.0018</td>
</tr>
<tr>
<td>PHW52</td>
<td>0.2946</td>
<td>0.8329</td>
</tr>
<tr>
<td>Va99</td>
<td>0.0015</td>
<td>0.0086</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B87</td>
<td>0.0051</td>
<td>0.0709</td>
</tr>
<tr>
<td>GEMS</td>
<td>0.0014</td>
<td>0.0133</td>
</tr>
<tr>
<td>LH60</td>
<td>0.0003</td>
<td>0.0036</td>
</tr>
<tr>
<td>NC262</td>
<td>0.0009</td>
<td>0.0105</td>
</tr>
<tr>
<td>PHW52</td>
<td>0.0066</td>
<td>0.0669</td>
</tr>
<tr>
<td>Va99</td>
<td>0.0003</td>
<td>0.0037</td>
</tr>
</tbody>
</table>
Figure 4.1. Canonical discriminant analysis of soil microbial communities by maize hybrid and water level. Maize hybrids: B87, GEMS, PHW, Va99, LH60, NC262. Letters W and S before maize hybrids mean ‘well watered” (filled symbols) and “water stressed” (open symbols), respectively.
Figure 4.2. Correlation of soil FAMEs with first two discriminant functions by maize hybrid and water level.
CHAPTER 5. SYNTHESIS

Current world population of 6.1 billion (with 11% undernourished and 10% with no access to safe water) is estimated to reach 9 billion by 2050 (U.S. Census Bureau 2015). This will necessitate increased food production to ensure future food security. Water availability and nutrients such as N and P are the main factors limiting crop yield (Tilman et al. 2002). Increasing crop yields by increasing fertilization has been a concern due to negative impacts of N and P losses on the environment (Tilman et al. 2002; Cassman et al. 2003; Schröder et al. 2011). Water shortages to crops may limit the uptake and use of nutrients, but increasing irrigation may lead to conflicts with human water needs. Thus, to increase crop productivity while reducing the environmental pressure on agro-ecosystems requires improved water and nutrient use efficiency (Tilman et al. 2002; Cassman et al. 2003) with a change in focus from input based agriculture to an agro-ecological process driven perspective (Tittonell, 2014).

The soil microbial community directly and/or indirectly has important consequences on food security, through microbial participation in energy and nutrient cycling, soil organic matter balance, aggregation and water retention by soils among other processes. Some microorganisms such as arbuscular mycorrhizal (AM) fungi develop symbiotic associations with most plant species (Johnson et al. 1997; Rodriguez and Sanders 2014) and can provide water and nutrients to the host plant helping to attenuate water stress and enhance plant growth and yield (Busso et al. 2008; Sheng et al. 2008). For example, immobile nutrients such as P could be more effectively taken up and translocated to the host plant when AM fungi colonize its roots (Munkvold et al. 2004;
Koch et al. 2006; Smith and Smith 2011) especially at low and medium soil P levels (Subramanian and Charest 1997).

Harnessing the benefits of the AM symbiosis on crop water and nutrient management is not a new concept (Safir et al. 1971), but it is a challenge for current input based food production systems. Most of this challenge resides in the fact that the outcome of the AM symbiosis (or the plant response) has been shown to vary depending on experimental conditions (the environment), the AM fungal partner and the plant genotype/species (Tian et al. 2010). For mobile nutrients like N, the benefits of the AM symbiosis have been inconclusive (Smith and Smith 2011). Additionally, interactions between soil N and P availability could result in outcomes more specific to individual host species, or genotypes within a species. Lastly, is the fact that the genetic improvement of maize hybrids has been evaluated under high input scenarios, which might have selected against traits that favor the AM symbiosis.

The general objective of this dissertation was to further the understanding of AM fungal and maize genotype interactions under variable environments. Specific questions of this dissertation were presented in Chapter 1 and reproduced in Figure 5.1. These questions are related to the impact of soil N and P availability on AM fungi colonization of maize plants, and on the growth, development and nutritional status of those plants.

These driving questions regarding the maize-AM fungi symbiosis were grouped into three main areas for discussion: i) impact of nutrient fertilization, plant host genotype, and their interaction on AM fungal colonization of maize roots and soil microbial communities, ii) impact of crop water availability, plant host genotype, and their interaction on AM fungal colonization of maize roots and soil microbial
communities, and iii) impact of nutrient fertilization, AM inoculation, plant host genotype, and their interaction on maize growth, development and nutritional status. The following discussion attempts to synthesize the most relevant results from this research. As it is not intended here to reiterate results presented in previous chapters, a reference to the corresponding chapter is given for the reader to consult.

Impact of nutrient fertilization, plant host genotype, and their interaction on AM fungi colonization of maize roots

The colonization of maize roots by AM fungi was evaluated in greenhouse experiments presented in Chapters 2 and 3 by two methods: microscopic observation of roots and FAME analyses of maize roots. The AM inoculated plants from both greenhouse experiments exhibited root colonization values around 70%, in contrast to the <1% root colonization by AM fungi for non-inoculated plants (see Chapters 2 and 3). FAME analyses also confirmed AM fungal colonization of maize roots by the presence of the AM lipid biomarker (C16:1cis11) in the roots of inoculated plants.

Nutrient fertilization effects on AM fungal colonization of maize roots included the evaluation of N fertilization (Chapters 2 and 3) and P fertilization (Chapter 3). Nitrogen fertilization increased AM root colonization as measured microscopically but not the concentration of the AM lipid biomarker (C16:1cis11) in the root (Chapter 3). This is not surprising as the two methods measure different aspects of the fungus. The concentration of the AM biomarker in soil, a measure of extramatrical hyphal biomass, was reduced with N fertilization. High N concentration in the soil may favor the C balance for establishment of AM symbiosis but limit AM growth and therefore, reduce
AM biomarker concentration in soil. Implications of these greenhouse results to crop responses at field scale are hypothesized in the next section.

The effects of plant host genotype and the soil environment by host genotype interaction on AM fungal colonization was evaluated with four maize hybrids in the greenhouse experiment presented in Chapter 3. Root colonization was similar among AM inoculated maize genotypes across harvest times suggesting no specificity for the AM symbiosis across conventional and drought tolerant hybrids tested. In addition, AM biomarker concentrations in root and soil samples were similar among inoculated maize hybrids. These results may be biased by the fact that the AM fungi were not indigenous to the soil but represent species common to agricultural soils that are commercially available.

**Impact of crop water availability, plant host genotype, and their interaction on AM fungi colonization of maize roots and soil microbial community abundance and structure (Chapter 4)**

The impact of crop water availability on AM fungal colonization of maize roots and soil microbial biomass and community structure was tested in the field for two water regimes: 100%, or well watered, and 40% of crop water requirements, on six maize hybrids. The colonization of maize roots by AM fungi in the field was evaluated by microscopic observation of roots harvested during maize R1-2 growth stages. Root colonization by AM fungi ranged from 61.6 to 69.8% indicating the presence of indigenous strains of AM fungi capable of colonizing maize roots. Root colonization by AM fungi neither differed between soil water treatments nor among maize hybrids. Thus,
all maize genotypes were equally colonized by indigenous AM fungi in the field. Although the extent of the symbiosis was similar, maize genotypes did show some specificity with respect to species colonizing the roots (Masao Higo, personal communication). Future studies should focus on linking AM diversity to functional properties of the AM-host symbiosis (Munkvold et al. 2004).

Soil microbial community abundance and structure was assessed by FAME analyses of soil from the root zone. The concentration of the AM biomarker in soil differed between water treatments and among maize genotypes. Thus, even when AM fungi equally colonized the roots of different maize genotypes, significant differences occurred in extramatrical AM biomass development among those genotypes regardless of water regime. Under water stress, the concentration of the AM biomarker in soil increased. It can be hypothesized that under water stress maize can promote the symbiosis leading to a higher extramatrical AM biomass available to access and transport water to the plant. This implies that specific changes in carbon allocation between the plant and AM fungus occur in response to crop water availability that in turn can alter water and nutrient uptake for the benefit of the host plant.

In addition to AM fungal responses to water stress, changes in the overall soil microbial community were observed. Bacterial and actinomycete markers, and also total microbial biomass significantly increased under water stress. This was contrary to the expectation that under well-watered conditions, plant growth should be optimal and be better positioned to stimulate microbial activity and growth. We speculated that the increase in bacterial biomass can be the result of several factors: i) greater availability of soluble carbon, possibly from root exudation, under water stress, and ii) increased
protection of bacteria inside micropores. The increase in AM fungal biomass in the soil could also lead improved aggregation and aggregate stability, providing greater habitat protection for soil bacteria inside of micropores. These micropores, capable of retaining capillary water, could also support a higher microbial bacterial and actinomycete biomass. Thus, increased soil bacterial biomass under water stress could be explained through the physical protection of bacteria inside AM fungal enhanced soil aggregates.

Saprophytic fungal and micro-eukaryote FAME biomarkers were not affected by soil water stress. Thus, either the imposed water stress was not sufficient to reduce the biomass of these microorganisms, or their biomass was more responsive to other factors. It is not surprising that AM and saprophytic fungi exhibited contrasting responses to soil water stress since one relies on C from the host plant while the other is a decomposer organism.

Our results demonstrated that AM fungi effectively colonized maize roots to the same magnitude regardless of maize genotype. Root colonization by AM fungi did not differ between water treatments; however, increased C allocation from plant to AM fungus was observed under water limited conditions. While water limitation caused a shift in the overall soil microbial community, maize hybrids influenced specific microbial groups. Further investigation into the complex relationships among the whole soil microbial community, maize and soil water management is needed to maximize benefit from microorganism – plant interactions.

Impact of nutrient fertilization, plant host genotype, and their interaction on maize growth, development and nutritional status responses to AM fungi inoculation.
In the first greenhouse experiment inoculated plants accumulated more biomass and had more leaves than non-inoculated plants when N fertilization was applied (Chapter 2). Non-inoculated maize plants were taller than inoculated ones, but their biomass, number of leaves and root fresh weight did not respond to increasing N rate. Previous studies have attributed greater plant biomass in inoculated plants to enhanced water status (Porcel and Ruiz-Lozano 2004; Zhu et al. 2010). However, plant fresh weight was significantly affected by N addition rather than by AM inoculation.

Regarding nutritional status, plant P uptake increased in AM inoculated plants, and was significantly higher when no N fertilizer was applied. Increased P uptake with AM inoculation supports other published research (Azcón et al. 2003; Arumugan et al. 2010; Ortas 2012). The reduction in P content with N fertilization can be explained by the fact that N fertilization of inoculated plants increased total biomass, which may have a dilution effect on plant P content. Nonetheless, N fertilization also reduced AM biomarker content (see previous subsection) suggesting N fertilization may cause a reduction in the P uptake capacity of AM symbiosis through reduction in extramatrical AM hyphae. Increased N uptake as N fertilization increased was observed for both AM inoculated and non-inoculated maize plants. On non-inoculated plants, increased N content was coupled with higher chlorophyll content in plant leaves; however, it was not enough to result in higher biomass, more leaves or greater root fresh weight. Lower N contents on AM inoculated plants compared to non-inoculated ones could be due to dilution effect of N by the increase in plant biomass with AM inoculation.

Overall, improved plant dry weight in AM inoculated plants was not related to improved photosynthesis since N and chlorophyll content were lower than on non-
inoculated plants. Thus, interactions among AM fungi, plants and nutrients appear to be complex making plant response to AM fungi difficult to predict and explain. Further studies on the mechanisms involved are needed to gain further insight into the complex relationships among AM fungi, maize and soil fertility management to maximize benefit from the AM fungi/plant symbiosis.

The second greenhouse experiment, presented in Chapter 3, was carried out to evaluate the influence of AM fungi on the physiological response and nutritional status of conventional and drought tolerant maize hybrids under variable N and P levels. Similar to the first greenhouse experiment, AM inoculation of maize plants had a positive impact on P uptake. There were positive biomass and chlorophyll responses as N fertilization increased, but not for P fertilization. However, AM inoculation neither increased N uptake nor chlorophyll content in leaves. In contrast to the previous greenhouse experiment, inoculated plants had lower dry and fresh biomass, less number of leaves and were shorter than non-inoculated plants for all maize hybrids. Greater dry biomass in non-inoculated plants was not related to increased P uptake as Chu et al. (2013) suggested. These findings contrast with previous studies where maize growth, development and nutritional N status benefitted from the AM symbiosis (Hamel and Smith, 1991).

Although it is clear that AM fungi provided a nutritional benefit to maize under greenhouse conditions (increase P content), results from this study are inconclusive about why most of the parameters evaluated were unresponsive or negatively affected by AM inoculation. Nitrogen fertilization increased P uptake under AM inoculation, but decreased P uptake under non-inoculated conditions. Nitrogen fertilization increased AM
root colonization which may have favored P uptake. Nitrogen fertilization increased plant biomass, which may have a dilution effect on the P content of non-inoculated plants.

It can be speculated from these greenhouse results that since higher N fertilization during the initial growth of maize led to good establishment of AM root colonization despite reduced hyphae growth into soil, changes in water and nutrient fertilization later in the growth cycle may not result in observable changes in crop response as the capacity of the AM symbiosis is met. Thus, the plant may provide C for later AM hyphal growth only if needed to improve water and nutrient uptake. Further investigation into the complex relationships among AM fungi, maize genotypes and soil nutrient and water management is needed to maximize benefit from maize host plant / AM symbiosis.

References


Current Opinion in Environmental Sustainability 8:53-61.


• Do maize genotypes have different AMF colonization?
• Does environment modify maize AMF colonization?
  – Does N fertilization impact AM fungi colonization of maize roots?
  – Does P level modify the effect of N on AMF colonization?
  – Does P fertilization have an impact on AM fungi colonization of maize roots?
  – Does N level modify the effect of P on AMF colonization?
  – Does water availability have an impact on AMF colonization?
• Are the environment effects on AMF colonization different across genotypes?
• Do maize growth, development and nutritional status change with AMF inoculation?
• Do maize genotypes have a different growth, development and nutritional status response to AMF inoculation?
• Do environmental conditions modify the maize growth, development and nutritional status responses to AMF inoculation?
  – Does maize response to N levels on growth, development and nutritional status change with AMF inoculation?
  – Does P level modify the growth, development and nutritional responses of AMF colonized maize to N levels?
  – Does the maize response to P levels on growth, development and nutritional status change with AMF inoculation?
  – Does N level modify the growth, development and nutritional responses of AMF colonized maize to P levels?
• Do maize genotypes have a different environmental response on growth, development and nutritional status to AMF inoculation?
• Do environmental conditions impact AM fungi abundance and proportion in soils?
  – Does N fertilization impact AM fungi in soils?
  – Does plant growth modify the effect of N on soil AM fungi?
  – Does water availability impact AM fungi in soils?

Figure 5.1. Specific questions addressed in this dissertation.
## APPENDIX A

Mean values of measured parameters on six selected hybrids for six selected hybrids among water levels (provided by A. Lorenz and L. Ali).

<table>
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<th>Water level</th>
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Concentration (nmol g\(^{-1}\)soil) of 19 fatty acid methyl esters by microbial group, hybrid and water level.

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Concentration (nmol g\(^{-1}\)soil) of 19 fatty acid methyl esters by microbial group, hybrid and water level. (CONTINUED)

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Concentration (nmol g\(^{-1}\)soil) of 19 fatty acid methyl esters by microbial group, hybrid and water level. (CONTINUED)

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