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Characterization of arbuscular mycorrhizal fungi and endophytic bacteria in mycorrhizal and non-mycorrhizal mutant maize by a metagenomics approach

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Dedication

First, thanks to my parents, for showing me unconditional love and support even when I was passing through rough times. I know I don't say I love you too much, but I want to let you know that I do. I own my curiosity and passion for research and my brains to you.

Thanks also to Aslan, my dog. I know he can't read this but he's always giving me serotonin and affection, which I appreciate as it was needed at times.

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Characterization of arbuscular mycorrhizal fungi and endophytic bacteria in mycorrhizal and non-mycorrhizal mutant maize by a metagenomics approach

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Abstract: Arbuscular mycorrhizal fungi (AMF) establish mutualistic symbiosis with almost 80% of all plants. AMF are widely used in agriculture because they offer a substitute to chemical biofertilizers as they can boost plant growth under non-ideal conditions. Maize is the most important commercial and cultural crop in Mexico. The common symbiosis pathway (CSP) is a well conserved molecular signaling pathway in all plants that can achieve mycorrhizal and rhizobia symbiosis, which starts with specific receptors that recognize each microorganism. Here, maize-associated AMF species and endophytic bacteria composition from two variants of maize, a mycorrhizal wild type and a non-mycorrhizal mutant type, were assessed by using high-throughput MiSeq-Illumina sequencing. Mutant maize did not possess the *HUN* gen, a *CASTOR* homologous gene, that is involved in the CSP by starting upstream Ca^{2+} oscillations and activating molecular signaling cascade. Composite root samples of wild type (n=9) and mutant maize (n=10) were used for microbial characterization. Mycorrhization was observed in all samples. A total of 22 AMF species were found and only 7 species were shared by both types of maize. Differences among AMF composition were observed, where mutant plants showed absence of the AMF generalist species *Rhizophagus*. However, Shannon and Chao1 diversity indexes were higher in mutant plants than wild type maize. Regarding endophytic bacteria, 137 and 135 amplicon sequence variants (AVS) were found in wild type and mutant maize, where 105 were shared by both treatments. *Streptomyces and Bulkholderia* were the most predominant among all samples. *Streptomyces* abundance suggest that this genus may play a main role in bacterial and AMF composition, as it is known for improving AMF germination and symbiosis due to its chitinase production. As mutation of *HUN* gene was not sufficient to block complete symbiosis between AMF and the plant, further characterization of downstream genes of the CSP in maize and field experimentation are required.

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1. Chapter 1

1.1 Introduction

1.1.1 Importance of agriculture and maize in Mexico

Agriculture has become, directly or indirectly, the main source of food worldwide (INEGI, 2018). Agriculture has a meaningful impact worldwide as in 2018 it represented 3.4% of the global GDP (The World Bank, 2019) and 42% of the human beings in the world depend on this activity.

Mexico is considered the place of origin and biodiversity of maize, where many varieties are conserved and continue to be produced, supporting agricultural development in the country (García & Bergvinson, 2006). In Mexico, agriculture is the main activity within the primary sector (which is conformed mainly by three subsections: agriculture, livestock and fishing), and in 2018 employed 5 million 678 thousands of workers, which contributed to 84.5% of the total activities from the primary sector and 3.7% of the total Gross Domestic Product (GDP) (CEDRSSA, 2019). Additionally, 15% of the total national territory is destined just for the development of agriculture.

Mexico's main agricultural products are white and yellow maize (57%), sorghum (15%), bean grain (13%) and wheat (4.3%) (INEGI, 2017).

In 2017, the production of maize was about 31 million tons, which represented almost 70% of the total agricultural production and gave Mexico the 8th place in maize production worldwide (ASERCA, 2018 & INEGI, 2017). From the 15% of Mexico's national territory destined to sown, approximately 51% was dedicated just to maize production and represented the 14.47% national agricultural GDP. Maize main producers in Mexico are the states of Sinaloa, Jalisco, Mexico City, Michoacan and Guanajuato (which had 22, 14, 7, 7, 6% of the total production respectively) (ASERCA, 2018).

1.1.2 Obstacles in maize production

The production in agriculture depends on the genetic potential of the plant to adequately respond to changing environmental conditions that are caused by the excess or lack of water and nutrients, extreme temperature conditions, presence of plagues (microorganism, insects, mice, weed, etc.) and soil characteristics and conditions. In maize production, these conditions affect the plant health and its yield in corn cob. If the growth conditions aren't

adequate, the corn cob will have a smaller size, a reduced number of grains and won't be able to qualify through a quality test approval (FAO, n.d.). One of the most important factors that are currently affecting the production of maize is drought, submitting plants to hydric stress and shortening their life cycle and production (FAO, n.d.).

1.1.3 Arbuscular mycorrhiza

Arbuscular mycorrhizal fungi (AMF) are obligate soilborne biotrophs that form a mutualistic symbiosis with almost 80% of the land plants, specifically vascular plants (Lanfranco, 2016). AMF facilitate water and inorganic elements and minerals to the plants, especially nitrogen (N), phosphorus (P), copper (Cu), zinc (Zn) and potassium (K) (Al-Karaki, et al., 2003). They strictly depend on the plant host to survive as almost 20% of the photosynthesis products are consumed by the AM fungi (Bago, 2000).

The term mycorrhiza is used to describe the whole symbiotic association of the roots of the plants and the fungus and is the most widespread mutualistic relationship (Lanfranco, 2016). Mycorrhizal fungi can be classified into two major groups depending on the host plant anatomical traits: ectomycorrhizae, where the fungus lays strictly in intercellular spaces of outer root tissues and colonizes trees and shrubs, and endomycorrhizae, where the fungi penetrate the living host cells of the plants and mostly colonize vascular plants (Lanfranco, 2016). AM fungi present a large taxonomic and morphological diversity, which have led to the study of the colonization of several plants, including plants with economic importance and agricultural relevance.

Several investigations have reported the influence of the AM symbiosis on the plant responses to both biotic and abiotic stresses such as heavy metal contaminants, drought and salinity (Cramer, 2011). Nowadays, the increased interest in this association is related to AM fungi potential to support sustainable agriculture as it can support the reduction of chemical fertilizers and water and to impact sustainable crop production that needs to feed the global increasing human population (Gianinazzi, 2010).

AM fungi have existed for more than 400 million years (Honrubia, 2009). Because of their major importance during nutrient transfer to the plant, they are thought to have played an essential role during the land colonization by the plants. Schüßler and colleagues grouped the AM fungi within one monophyletic phylum in 2001, called Glomeromycota. This phylum is represented by more than 250 AM fungi species, four orders and 19 genera (Souza, et al., 2005; Walker, et al., 2007; Bonfante and Genre, 2016).

Huang et al. (2018) found that some AM fungi have special compatibility to certain plants and environments, as well as Campos et al. (2018) that demonstrated preferential host-symbiont associations, relatable to fungal colonization strategies that have implications on the delivery of certain characteristics required by the plant. The outcome of the plant-AMF symbiosis also relies on the AM fungi - plant combinations, the individuals *per se*, the climatic conditions, environment, and soil minerals' composition (Cardoso, 2017).

1.1.4 Arbuscular mycorrhizal fungi and development of symbiosis

To achieve this process several successive and well-characterized steps need to be followed by the plant and the fungus. The first step is the pre symbolic stage where the plant and the fungus have their first contact. During this step, germination of fungal spores induces the appearance of mycelium. The plant roots exudate diffusible molecules which are recognized by the soil spore mycelium promoting the hyphal branching and the production of fungal exudates that activate the symbiotic signaling pathway. Then the fungus contacts the root epidermis and starts to adhere, forming a structure called hyphopodia which precedes the root entry and initiates the symbiotic phase (Bonfante & Genre, 2010).

1.1.5 Arbuscular mycorrhizal fungi life cycle

Arbuscular mycorrhiza originates from the hyphae that proceed the fungal propagules from the soil (fungal spores, mycorrhizal fragmented roots or mycorrhizae from plants the proximity). When the hyphae contact the surface of a root epidermal cell, it changes its form to a colonizer hypha that will penetrate the epidermal cell and cross the intercellular space. In the external area of the roots, the hyphae will form intracellular structures called appressoria; in the intermediate zone hyphae will grow longitudinally and in the internal zone the hyphae will penetrate the epidermal cell and form arbuscules by dichotomic ramifications, where the interchange of nutrients will be carried out (INVAM, 2006). In the cortex of the roots, hyphae also form vesicles which function as storage of lipid reserves. Once the colonization is being executed, the hyphae produce ramifications, and the external mycelium starts the absorbance of nutrients and water. These ramifications also work as new colonizers for plants roots or propagules for the roots of plants in proximity and they also form spores, that, once they've matured, they complete the fungi life span (INVAM, 2006; Bonfante & Genre, 2010).

1.1.6 Pre-symbiotic signaling

AM fungi sense the proximity of a host via molecules that result from root exudates. These molecules induce the spore germination of the fungi, hyphal growth, increased physiological

activity and promote hyphal branching. The most well-known exudated molecules are called strigolactones (SLs) and are carotenoid-derived phytohormones. Strigolactones are volatile molecules with a short lifetime in the rhizosphere owing to a labile ether bond that can be easily hydrolyzed in water. This compound is volatile and transitory, and tends to form an abrupt concentration gradient, so its perception has been suggested to express the closeness of a host plant root (Parniske, 2008). AM fungi perceive strigolactones from root exudates even at low concentrations (10 nM) (Gutjahr et al., 2015).

Plant mutants that are unable to produce SLs can be useful to study and analyze the impact of these signalling molecules in AM development and further consequences in rhizosphere and endophytic bacterial composition and plant physiology and phenotype (Parniske, 2008). In recent studies, a group of scientists identified the loss of AM fungi exudates response in a rice mutant, as well as loss of physical contact and transcriptional responses to the signals between the two individuals. Researchers identified the gene that was responsible for the loss of the symbiosis, D14L. This gene is required to support the initial colonization processes (Kagiyama et al., 2018; Gutjahr et al., 2015).

Arbuscular mycorrhizal fungi also release molecules, called Myc factors, that start plant symbiotic responses which include transcriptional regulation, starch accumulation in roots, lateral root formation, among others. Most of these molecules are N-acetylglucosamine oligosaccharides and penta-chito-oligosaccharides (Genre et al., 2013). These factors have the capacity to induce calcium spikes and oscillations in root epidermal cells to activate plant symbiosis-related genes. Molecules known as Nod factors work in the same way as Myc factors and are release by rhizobia and endophytic bacteria in response to the flavonoids diffuses by host plant with nitrogen deficiency (Geurts & Bisseling, 2002; D'Haeze & Holsters, 2002).

There are at least seven genes identified that are needed for the AM symbiosis and the root-nodule symbiosis with the rhizobia in legumes. These genes (Annex 1) encode proteins that are involved in a signal transduction chain that is needed for the development of the intracellular structures of the plant required for the fitting and adaptation of the symbiotic fungi and bacteria by the host plant cell. Plants that are defective in one or more of these genes are characterized by a lack of capacity to form mycorrhizal symbiosis (Zhu et al., 2006; Oldroyd & Downie, 2008; Maekawa & Murooka, 2009; Gutjahr et al., 2008).

1.1.7 Spore germination

Spore germination forms the first step in the life cycle of AMF, because it represents the initiation of the vegetative growth process. Spore germination characteristics are important because they can be used to distinguish between the AMF genres. The dormancy of the spores is different for every species, and it can vary from two weeks to several months (Gazey et al., 1992). Spore germination is influenced by many factors like pH, humidity, host plant exudates, soil bacteria community, etc. (Siqueira, et al., 1985; Fitter & Garbaye, 1994).

1.1.8 Host cell colonization

Besides of arbuscules and vesicles, AM fungi can also produce other type of structures like the appressoria and hyphopodia (hyphal organs that facilitate the penetration of cells or tissues of other organisms), which are the first fungal structures after the first contact with the plant is made; the auxiliary cells which are structures produced by extraradical hyphae, the intraradical mycelium that transports the substances which have been absorbed by extraradical hyphae and the extraradical mycelium which performs the absorption and translocation of nutrients, propagation, spore production of new roots, etc. (Sharma et al., 2015).

At the end of the hyphal branch (hyphopodia) formation on the plant root surface, the nucleus of the epidermal cells beneath the zone moves to the opposite side of the cell of the fungal contact site. At the same time a cytoplasmic aggregation its form between the nucleus and the fungal contact site and it is rich in organelles such as endoplasmic reticulum, cytoskeleton, Golgi stacks, secretory membrane and it constitutes the pre-penetration apparatus (PPA) (Genre, Bonfante, et al., 2005; Genre, Chabaud, et al., 2008; Genre, Ivano, et al., 2012). After the PPA is developed completely, a penetrating hypha is formed from the hyphopodia and it passes over the epidermal cell wall and infiltrates the cell lumen following the PPA route. The fungus is now in the perifungal membrane where it will be hosted (Genre, Bonfante, et al., 2005, 2016). The formation of the PPA is not only carried out in epidermal cells, but also inside and outside of cortical cells that are preparing to retain the arbuscule formation. In these cells, the hyphae is not encapsulated in the cell, but the hyphae expands progressively to other cells in fine branches so it can fill up most of the cell lumen (Bonfante, 1984; Genre, et al., 2008).

The process and the rate of colonization determines the effectiveness of the mycorrhizal association (Smith, 1997). Giovannetti and colleagues (1993) demonstrated that using *Funneliformis mosseae* (as *Glomus mosseae*) as a fungal model in common sunflowers the

first appressoria was formed at 36 h from the first interaction of the fungus and the plant. 42-48 h after inoculation the first arbuscules were showing and 4 days later, 60% of the plant root was associated with hyphae and arbuscules.

Arbuscules result from the development of the symbiosis between the host plant cell and the AM fungus. Arbuscule design varies depending on both the fungal and plant genotypes (Parniske, 2008). The perifungal membrane (PAM) is the zone around the arbuscules and outlines the symbiotic interface, the region where the fungus is hosted and where the nutrients and signal exchanges are believed to be carried out (Bonfante, 2001; Bonfante and Balestrini, 2014).

1.1.9 Mycorrhizal and non-mycorrhizal maize as the main study model for this research

As mentioned in the section 1.1.6, AMF symbiosis starts with a molecular signal exchange by both plant and fungi. The plant roots diffuse strigolactones, that are recognized by the AMF, which then released Myc-factors. Plant perceives released Myc-factors that are attached to specific receptors MFRs, and a signaling cascade is started within the plant. This signaling cascade is regulated by the Common Symbiosis Pathway (CSP), which is also known to be used for the symbiosis of the plant with rhizobia and endophytic bacteria in a similar way (Kosuta et al., 2008; Ercolin & Reinhardt, 2011; Debellé, 2019).

To start the symbiosis between the plant and rhizobia, plant roots exudate flavonoids in response to a nitrogen deficiency. These molecules are recognized by the bacteria, which respond by releasing Nod factor molecules that are perceived by specific receptors called NFRs, that contain Lysing motifs (LysM) in the extracellular regions. Once the NFRs sense the Nod factors, a downstream signaling cascade is activated in the CSP to complete the symbiosis (Kumar, Vijayakumar & Choudhury, 2021).

CASTOR and *POLLUX* genes encode putative ion channels that are components of the CSP in *Lotus japonicus*. The two genes perform integral and complementary functions (Charpentier et al., 2008). *CASTOR* and *POLLUX* are omnipresent in all plant taxa for which sequences are known, including important economic plants like sorghum, soybean, grapevine, rice and maize (Chen et al., 2009).

Studies on *Lotus japonicus* and *Oryza sativa* have shown that mutants deficient in *CASTOR* show a phenotype where the roots of the plants are resistant to the colonization of the AMF (Oldroyd & Downie, 2008; Maekawa & Murooka, 2009; Gutjahr et al., 2008).

In maize, *CASTOR* orthologous gene was characterized by Ramírez-Flores et al. (2020) and given the name *HUN*. In order to create a *castor*-deficient maize genotype for this study, Dr. Ruairidh Sawers and Dr. Ramirez-Flores et al. (2019) performed a mutagenesis using the *Mutator* transposon system for the study model (Candela and Hake, 2008; Bennetzen, 1996). To achieve the mutagenesis, two corn individuals carry the mutant alleles of *HUN*. The first individual is a *hun* 1-1, which is in the 5'UTR promoter region, while *hun* 1-2, the second mutant allele, is found in the first exon (Figure 1).

Each mutant allele has its wild line because the distribution of Mu insertions of the wild genotype is almost equal to the mutant (except for the insertion in *HUN*), but the distribution between wild lines is different. In the study model, we expected the deficient *hun* 1-2 maize to not be able to show the symbiosis of the AMF.

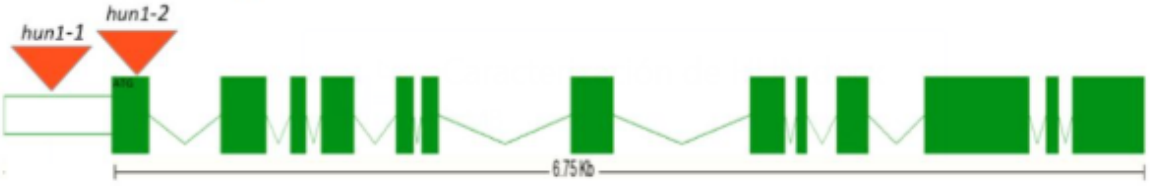


Figure 1. Localization of the insertions of *Mu* in *HUN gene.*

The green boxes represent the exons, the green line the intros, the white box represents the promoter region. *HUN* has a length of 6.75 kb and ATG located in the first exon. The insertion of the element *Mu* corresponds to the mutant allele *hun* 1-1 and it is located 36 bp upstream the ATG, while the second insertion is represented by *hun* 1-2, located 40 bp downstream the ATG.

1.1.10 Transfer of nutrients

AM fungi are responsible for transferring nutrients to the host plant in the mutualistic symbiosis. Fungal hyphae explore the soil substrate, where they act as an extension of the plant's roots for nutrients and water uptake. Some of the most documented minerals are phosphate (P), nitrogen (N), carbon (C), potassium (K), sodium (Na), among others. This process is completed in the periarbuscular membrane of the plant and fungi and it is found in the arbuscules, where the symbiotic relationship is being carried out (Al-Karaki et al., 2003; Lanfranco and Bonante, 2016).

1.1.11 Carbon metabolism

AM fungi consume 10-30% of the plant products from photosynthesis (Drigo, et al., 2010). On the fungal side, a high affinity monosaccharide transporter (MST) is responsible for carbon uptake from the interface compartment, and it is expressed in arbuscules and intracellular and extracellular hyphae. In extracellular hyphae, AM fungi can take glucose and xylose as well. For the mutualistic relation to happen, the exchanges of P and C need to be under strict control by both parts of the symbiosis (Lafranco, et al., 2016). Factors such as changes in soil moisture and temperature due to drought can negatively affect the flux of compounds exchanged by the two symbionts (Wang, et al., 2016).

Plants can control the fluctuation of sucrose directed to the roots, including the fungus. The sucrose deposited to the AM root is cleaved by symbiosis-induced sucrose synthases or invertases (Parniske, 2008).

1.1.12 Phosphate uptake

Phosphorus is one of the most important mineral macronutrients needed for the vital function of the plants, including photosynthesis, protein and nucleic acid synthesis, nitrogen fixation, among others (Awasthi et al., 2011).

Improved phosphate uptake is one of the main benefits of the AM symbiosis. Inorganic phosphate (Pi) is obtained by two main pathways in mycorrhizal plants, the first pathway is the direct one, obtained by the root epidermal cells and the other is the mycorrhizal pathway by the AM fungi (Smith and Smith, 2012).

Pi is characterized by a low mobility and availability in the soil. A study to know the percentage of Pi provided by the fungus performed by Facelli and colleagues, found that most of the Pi was obtained by the AM fungi, with a final percentage of 20-100% depending on the AM fungi species (Facelli et al., 2010).

The mycorrhizal Pi uptake pathway is mediated by Pi/H^+ transporters and the extraradical mycelium, where the Pi can be supplied by active Pi used for the synthesis of phospholipids, nucleic acids or proteins, or accumulate in vacuoles in the form of long-chain or short-chain polyphosphates (polyP) through the action of the polyP polymerase/vacuolar transporter chaperone complex (Tisserant et al., 2012). PolyP is the most common form of storage of Pi in hyphae as well as the main form of Pi translocation over one place to another within hyphae

(Kikuchi et al., 2014). PolyP translocation to the host plant is mediated by fungal aquaglyceroporin which is located in the intraradical mycelium and is the complex in charge of water transportation across the plasma membrane (Kikuchi et al., 2016). For the Pi transportation, PolyP is degraded in the intraradical mycelium by vacuolar endopolyphosphatase and exopolyphosphatase activities and then transports to the apoplastic interface compartment in the plant. In the plant the Pi/H^+ transporters are downregulated in root epidermal cells and the mycorrhiza-inducible Pi transporters are upregulated (Ahanger et al., 2014).

1.1.13 Nitrogen uptake

Nitrogen is found in soil in organic and inorganic forms and plants use both types of compounds. The organic forms are compounds like urea, amino acids, amines, peptides, proteins, etc. while the inorganic form is found in the way of nitrates and ammonium. In soils where N is poorly available (mainly because of drought or acidity), the contribution of the AM fungi to plant nutrition can be considerable (Casieri et al., 2013) with a percentage of 24-42% of the total N content in the plants (Facelli et al., 2010).

For the Nitrogen AM fungi pathway, the extraradical hyphae takes up (preferentially) ammonium (NH^{4+}), which is energetically less costly than alternative Nitrogen sources (NO^{3-}) and amino acids (Lafranco et al., 2016). López-Pedrosa, et al., showed that a NH4+ transporter gene is expressed in the extraradical mycelium of AM fungi (López-Pedrosa, et al., 2006; Pérez-Tienda, et al., 2011). Inside the extraradical mycelium, the Nitrogen compounds are transformed into amino acids and then are translocated to the intraradical hyphae within vacuoles to finally reconverted again into inorganic Nitrogen compounds by enzymatic activity of urease and proteases. Ammonium is the most commonly N form transported from the fungus to the plant (Dreyer, et al., 2019). The later transport is carried out by NH^{4+} transporters in the PAM (Calebrese et al., 2017).

1.1.14 Ecological importance of arbuscular mycorrhizal symbiosis

More than 80% of all vascular plants interact in a mutualistic symbiosis with AM fungi, an ancient fungal group present on earth for more than 200 million years. There is evidence that says that AM fungi have accompanied plants since the beginning of the land colonization by plants and AM fungi may have been the reason why it was so effective (Redecker et al., 2000). Almost all soils around every region of the world are the habitat of an elevated diversity of AM fungi. The AM fungi spores are propagative structures that are present in the soils and they

can remain latent quiescence for long periods, even years, especially if no host plants are available nearby. AM fungi spores need to be activated again to resume their biological functions as by root exudates of host plants, even though the germination of the spores does not depend only on the roots of the host plant (Nogueira, Zangaro, Nogueira, 2017). There exist different kinds of associations between fungi and plants and every combination can demonstrate different effects during plant growth. Some arbuscular mycorrhizal fungi are characterized by having a wide range of hosts while other fungi may be more specific. In the same way, some fungi can show a greater benefit to a plant than others and some plants can exploit the association better than other plants (Agrios, 2002).

Arbuscular mycorrhizae can stimulate the growth, development and nutrition of plants, especially if they are growing in soil with low-medium fertility and studies have shown that these effects are due to the impact of the mycorrhizae in the absorption of nutrients (mainly phosphorus) and water (Barea et al., 2002).

AM fungi are affected by many environmental and non-environmental factors, such as chemical, physical and biological soil characteristics, climate and seasonal weather changes, plant and microbial communities, etc. To improve the effectiveness of the AM fungi - plant symbiosis, good agricultural practices need to be carried out. Practices as monocultures (that generate soil infertility), lack of organic matter in soil, very acid or alkaline soils, excess of fertilization (specially phosphates), heavy metals, salinity, pesticides, etc. can cause negative effects on the AM fungi and the formation and function of the mycorrhizae. Nonetheless, AM fungi have shown tolerance to these abiotic stress conditions and help improve the plant resistance as well (Nogueira, Zangaro, Nogueira, 2017).

Arbuscular mycorrhizal interaction has shown effect supporting ecological services like the increase of soil quality and the biodiversity that is related to the plant communities and rhizosphere (Riling, 2015).

Because of the negative environmental impact and high increasing costs of chemical fertilizers, the study of microorganisms that enhance soil and plant health and nutrition and improve soil fertility is gaining importance nowadays and AM fungi are the main proposed individuals to replace chemical fertilizers. As the hyphae of the AM fungi explores the soil with its roots and helps the plants to exploit the soil minerals (absorbs nutrients selectively and solubilizes minerals that are normally insoluble) and water in a more efficient manner and it also exudates molecules that help to fight and reduce the presence of harmful fungi, bacteria and nematodes on the roots (Smith & Read, 2007).

1.1.15 Arbuscular mycorrhizal fungi and abiotic stress

AM symbiosis can alter both the physiology and the metabolism of the plants and it can induce early flowering, increased yield in biomass, flowers and grains (Al-Karaki, 2003). The symbiotic relationship between the plant and the fungi can improve plant growth and tolerance against several abiotic stresses such as greater tolerance to drought, heavy metals, salinity, extremely alkaline or acid soil and biotic stresses like pests or lack of nutrients in the soil (Nogueira, et al., 2017). Some of the attributes that this association provides are the improvement of the soil and rhizosphere properties, the enhanced development of a root system which results in an increased root area of exploration which improves the water absorption, enhanced absorption of nutrients (especially Phosphorus) which also boost the plant health, accelerates the activation of the host plant defense system, improves mitigation of oxidative damage, among others (Ahanger, 2014).

1.1.16 AMF and their relationship with the rhizosphere

It is well known that AMF, soil bacteria and plant roots do not work as individuals but as a team. The interaction of the soil microbiome, AM fungi and the plants are the key to understanding the processes that characterize this symbiosis. According to Meyer, Linderman and Paulitz (1986 & 1991) AM fungi affect the composition and number of the bacterial communities in the soil and the integration of these is responsible for giving the plant enhanced responses to stress and diseases (Andrade, 1997). It is thought that different bacterial communities may establish themselves according to the AM plant- fungus combinations and plant needs.

1.1.17 Bacterial endophytes and plant growth promoting bacteria

The term endophyte has been revised multiple times by different authors. At first, endophytes were defined as the microorganisms that could be isolated from surface-sterilized plant tissues or extracted from inside plant and have no negative influence on the plant growth (Hallmann, et al., 1997). Pathogens were not considered endophyte bacteria. This definition included microorganisms that had a symbiotic, commensalistic or mutualistic relation with the plant. Due to molecular advances and the fact that several microorganisms have been sequenced but not isolated, this definition had to be adjusted (Hurek et al., 2002; Conn and Franco, 2004; Pereira et al., 2011; Gaiero et al., 2013). Some bacteria and fungi can also change their endophytic phase because they can shift between mutualistic and parasitic states (Schulz et al, 2006; Gaiero et al., 2013). Consequently, a more adequate definition of endophyte is the

set of microbial genomes found inside the plant organs (Gaiero et al., 2013; Bulgarelli et al., 2013).

Several endophytic bacteria, also known as plant growth-promoting bacteria (PGPB) have shown to have beneficial effects on the plants. Some of the benefits include plant growth promotion, induction of increased resistance to pathogens, supply of fixed nitrogen to the host and other activities that mitigate the effect of several biotic and abiotic stresses (Mano et al., 2008; Etesami & Maheshwari, 2018; Gianpiero et al., 2018). Endophyte bacteria form a great reservoir of bacterial diversity with potential use in the biotechnology industry (Ryan et al., 2008; Abedinzadeh et al., 2019). Recent studies have shown that, in the plant-bacteria interactions, the plants under different environmental conditions are able to build their rhizosphere and endophytic microbiome and recruit environmental stress-resistant bacteria that contain specific characteristics required by the plant (Berendsen et al., 2012; Abedinzadeh et al., 2019) which is a crucial factor to grant stress tolerance.

1.1.18 Arbuscular mycorrhizal fungi molecular characterization approaches

The genomic and molecular analysis of samples of soil and roots can bring information about the presence and identity of the microorganisms (Gauna, 2013) that compose the microbiome of the roots and the endophyte microbiome of the plants and that affect positively and negatively the yield and health of the plants. With this knowledge, it is possible to compare the composition of the bacterial and fungal communities that live in symbiosis with mycorrhizal and non-mycorrhizal maize to study the real impact that the AMF has over the microbiome composition in roots and endophyte microbiome.

AMF are usually identified by the morphology of the spores that they produce. In cases where the spores are absent, the intraradical structures of the AMF can allow the identification to the family level. Due to several complications and discrepancies, as the different conditions that must meet for an AMF to sporulate and that some may not sporulate at all, molecular techniques are required to identify AMF in any given root sample without the need of spores. For the first approaches, researchers tried to identify the order of AMF due to its colonization patterns within the roots as well as using isoenzymes and antibodies (Sanders, 1992; Fitter, 1998). Nonetheless, these techniques are no longer used because of the inefficiency due to the similarities between the fungal species and the effects of the host plant to the fungal morphology (Smith & Smith, 1996).

AMF identification systems are based on ribosomal DNA. The genes that are used for the identification in this genome region are accessible at a high copy number and possess highly well conserved sectors that are variable, which make possible the identification and distinction of taxa at different levels (Redecker et al., 2003).

The great majority of molecular approaches for the identification and detection of AMF are PCR and sequencing based techniques, which characterize the nucleic acids of the fungi from small quantities of fungal DNA (Sanders, 2016). The first PCR attempts for AMF required amplifying a fragment of the 18S rRNA gene (Simon, et al., 1992). DNA was amplified using fungal spores and universal primers. Using the primer VANS1 (*Glomerales* specific), Simón et al., (1993) could amplify and sequence an approx. 545-550 bp fragment of the 18S rRNA gene of different AMF species of the Glomeromycetes class. This led to the design of new primers with taxonomic specificity according to the different genera and they have been used to detect AMF in colonized roots (Clapp, et al., 1995; Sulistyowati, 1995). In 1995, Bonito et al..used the primers VANS1 and NS21 to detect *Glomus intraradices* (most likely *Rhizophagus intraradices*) in different plant roots but they encountered a lot of problems during the characterization, more likely due to the VANS1 priming site being not well conserved in all groups of the AMF, therefore, the primer pair was not taxon specific and would amplify non-mycorrhizal DNA (Bonito, 1995).

Lanfranco et al., (1995) used another molecular technique called RAPD-PCR to generate a specific primer for the identification of *Glomus mosseae* (currently *Funneliformis mosseae*), correctly identifying the AMF not only in the spore form but also during the root colonization process, the fragment that was obtained from the PCR product was about 550 bp.

Using RFLP-PCR and the universal primers ITS1 and ITS4, Sanders et al., (1995) obtained PCR fragments of different length of spores from the order Glomerales collected from a natural community. With this approach, they could identify and sequence the 5.8S rRNA gene which led them to reveal differences among species from the same order.

As AMF are not able to grow without a plant host it is necessary to identify the vegetative stage of the fungi using DNA technology and molecular techniques to identify and quantify the fungus in colonized roots. Inefficient identification has led to keep improving the molecular characterization techniques (Senés-Guerrero & Schüßler, 2016). Nowadays, ribosomal genes and its spacers are common target regions for the identification of AMF. Nuclear ribosomal DNA that encodes for the highly conserved rDNA 5.8S with the two internal transcribed

spacers (ITS) is used to study the phylogenetic relationship between AMF groups, and ITS regions are used to design primers for the PCRs (Velandia, 2006).

It has been demonstrated that the ITS regions in the rDNA are highly conserved at a species level, but they are variable for superior taxons (Bruns et al., 1991). The ITS sequences of DNA are variable among fungi (Levesque et al., 1994). Nonetheless, the ITS sequences must have the right percentage of variation for the right identification of the species.

PCR techniques based on the ITS sequences can be used for the detection and identification of AMF at species levels and even to differentiate between samples isolated from the same species. The PCR primers design from the ITS can allow the investigation of the diversity of fungi among the plant roots, the rhizosphere and the quantification of all the presented fungi.

Small Subunit (SSU) of the ribosomal RNA were the first genes to be sequenced from AMF and the ITS were the specific targets for the phylogenetic analysis. The data of the SSU and the ITS allow the design of specific primers that are used for PCR amplification and therefore, used for the identification of AMF of both spores and fungi placed in plant roots.
ITS regions are highly variable. A single spore can contain more than one ITS region and these regions can also change between spores from the same species (Velandia, 2006).

In 2009, Krüger and colleagues improved a PCR primer set to characterize AMF rDNA from the group of the *Glomeromycota* at a species level. To achieve this, nuclear rDNA fragments were sequenced and analyzed to design primer mixtures, using one binding site in the small subunit (SSU), whole internal transcribed spacer (ITS) region and a partial large subunit (LSU). These primers were successful to amplify two amplicons, with primers SSUmAf-LSUmAr an amplicon of 1800 bp, and with primers SSUmCf-LSUmBr an amplicon of 1500 bp.

In 2020, Senés-Guerrero and colleagues developed a new molecular technique to identify AMF at a species level characterization (Senés-Guerrero et al., 2020). In this study, researchers used a strategy using the Miseq-Illumina platform to sequence an approx. 450 bp fragment of the LSU-D2 rDNA region.

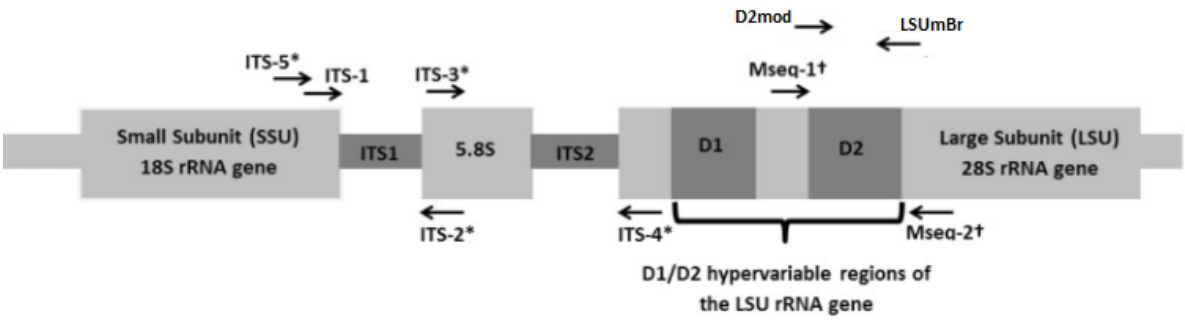


Figure 2. Diagram of the ribosomal gene complex.

It includes the sequence coding for the 18S rRNA gene, and internal transcribed region 1 (ITS1), the 5.8S rRNA gene coding region, internal transcribed region 2 (ITS2), the sequence coding for the 28S rRNA gene with the D1 and D2 variable regions.

Senés-Guerrero and colleagues found out that the selection of the DNA region is essential to obtain phylogenetic resolution to characterize sequences to the species level. Evolutionary Placement Algorithm was developed as an alternative to taxonomic assignment based on sequence identity or homology and was shown to be useful for the placement of the short sequences of AMF reads with high species-resolving accuracy (Senés-Guerrero & Schüßler, 2016). The 450 bp LSU-D2 rDNA region proved to be a good fragment used for the identification of the newly discovered AMF.

1.1.19 Endophyte bacteria and its molecular characterization approaches

To overcome the inability to grow on culture to identify most of the microorganisms that reside and colonize the inside of the roots, molecular-based techniques are used (Rathod et al., 2012). Many endophyte bacteria have been identified through molecular-based techniques that are culture-independent. These approaches include the extraction of DNA from the plant, rRNA cloning, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP) (Smalla et al., 2007).

To prevent the loss of information and deprivation of identification of many genera, considering the limited distribution and diversity of many endophytes, 16S rDNA PCR has gain popularity over the years, usually with the help of universal standard primers that target hypervariable regions of the 16S rRNA gene to perform 16S rRNA gene sequencing (Giangacomo et al., 2021).

1.2 Hypothesis

Mycorrhizal and mutant non-mycorrhizal maize plants will have different compositions of arbuscular mycorrhizal fungi (AMF) and bacteria root endophytes. Where, non-mycorrhizal maize will lack AMF symbiosis and compensate with endophyte bacteria, and mycorrhizal maize will present a diverse microbial community with higher corn yields.

1.3 Study objectives

1.3.1 General Objectives

To characterize the endophyte bacterial and arbuscular mycorrhizal fungi composition of a wild type maize variety (*B73)* and a genetically modified non-mycorrhizal maize (*hun1-2)* grown in Ameca, Jalisco, by a metagenomic approach.

1.3.2 Specific Objectives

To characterize the effect of the genetic modification on non-mycorrhizal maize (*hun1-2*) by determining the composition of the AMF community involved in maize productivity.

To characterize and compare the endophyte bacterial composition of wild type and mutant non-mycorrhizal maize and assess the diversity of plant growth promoting bacteria.

2. Chapter 2

2.1 Materials and methods

2.1.1 Confirmation of correct mutagenesis

To confirm mutations, genotypes *B73, huh 1-1* and *hun 1-2* were studied by our collaborators Dr. Ruairidh Sawers and Dr. Ramirez-Flores. DNA extraction was performed and genotypification done by PCR with two sets of primers of the left and right fragments at both sides of the transposon used for mutagenesis (Table 1). Results showed the expected fragments for *hun 1-1* and *hun 1-2* only in the homozygote mutant plants confirming the correct mutagenesis in the *HUN* promotor region and first exon.

Table 1. Primers for genotypification of *B73, hun 1-1* and *hun 1-2* gene mutations. Two sets of primers were used of the left and right fragments at both sides of the transposon used for mutagenesis.

Reaction	Forward Primer	Reverse Primer	Fragment length
Wild type (B73)	HUNF01-CGCGAAGAAACGCAGACATTCC	HUNR04-TAACCTGGAGCGAACAGAATCCAC	606 bp
Hun 1-1 (left fragment)	KP016- GTCGCAAGACACGGACAAC	RS185-CGCCTCCATTTCGTCGAATCACCTC	143 bp
Hun 1-1 (right fragment)	KP017- GACCACCGTGGAGCGATC	RS185-CGCCTCCATTTCGTCGAATCACCTC	438 bp
Hun 1-2 (left fragment)	HUNF03-CTTGGGCGCATTGGAAATTCATCG	RS183-CGCCTCCATTTCGTCGAATCCSCTT	839 bp
Hun 1-2 (right fragment)	HUNR04-TAACCTGGAGCGAACAGAATCCAC	RS183-CGCCTCCATTTCGTCGAATCCSCTT	433 bp

Maize phenotypic characteristics were also studied by Dr. Sawers and Dr. Ramírez-Flores. Seeds of *B73, hun 1-1* and *hun 1-2* were used to established two treatments: i) *Rhizophagus irregularis* MYKE ® PRO inoculation and ii) a negative control without fungi. One seed per pot was sown. Once the seeds started to germinate, water was applied twice a week and Hoagland nutritive solution was applied once a week. To set a phosphate deficiency in the plants, phosphorus was applied at 160 µM of KH_2PO_4 instead of 1 M in the Hoagland nutritive solution. All genotypes were starved 8 weeks after seedtime. To observe root colonization, lateral roots of the three genotypes were stained with trypan blue in acetoglyceride 0.005%. Colonization rate was calculated of root fragments using a modified gridline intersect method. Results showed colonization of roots with *Rhizophagus irregularis* in plants of *B73* and *hun 1-1* but was absent in *hun 1-2.* Therefore, plants of varieties *B73* and *hun 1-2* were used in field experimentation.

2.1.2 Field experiment

The study was carried out in the UNISEM experimental field at Ameca, Jalisco, in the summer of 2019 by Dr. Ruairidh Sawers and Dr. Ramírez-Flores at Langebio, Cinvestav. Plant families of mycorrhizal B73 (n=73) and non-mycorrhizal hun 1-2 maize (n=64) were distributed in a randomized complete block design with three blocks as replicates (Figure 3). A total of 45 seeds per family were sown in configurations of three rows with five plants each on 2 x 2.25 m squares. Fertilization doses were used as recommended for maize cultivation by FAO (2002). The first fertilizer dose was performed as maize seeds were sown, using 250 kg/Ha of diammonium phosphate (DAP) as nitrogen and phosphorus source (18-46-00, N-P-K). For the second dose only 250 kg/Ha of urea was used as nitrogen source and fertilizer (46-00-00, N-P-K) 40 days after seedtime. During maize cultivation development, UNISEM staff oversaw removing plagues, sick plants and weeds from the plants.

The study was carried out in the UNISEM experimental field at Ameca, Jalisco, in the summer of 2019 in collaboration with Dr. Ruairidh Sawers at Langebio, Cinvestav. Plant families of mycorrhizal (n=73) and non-mycorrhizal maize (n=64) were distributed in a randomized complete block design with three blocks as replicates (Figure 3). A total of 45 seeds per family were sown in configurations of three rows with five plants each on 2 x 2.25 m squares. After two months of being sown, the maize plants were in the flowering phase and roots were collected for endophyte bacteria and arbuscular mycorrhizal fungi (AMF) molecular characterization. Row 2 and plant 3 in the center of each family square was selected for analysis (Figure 4).

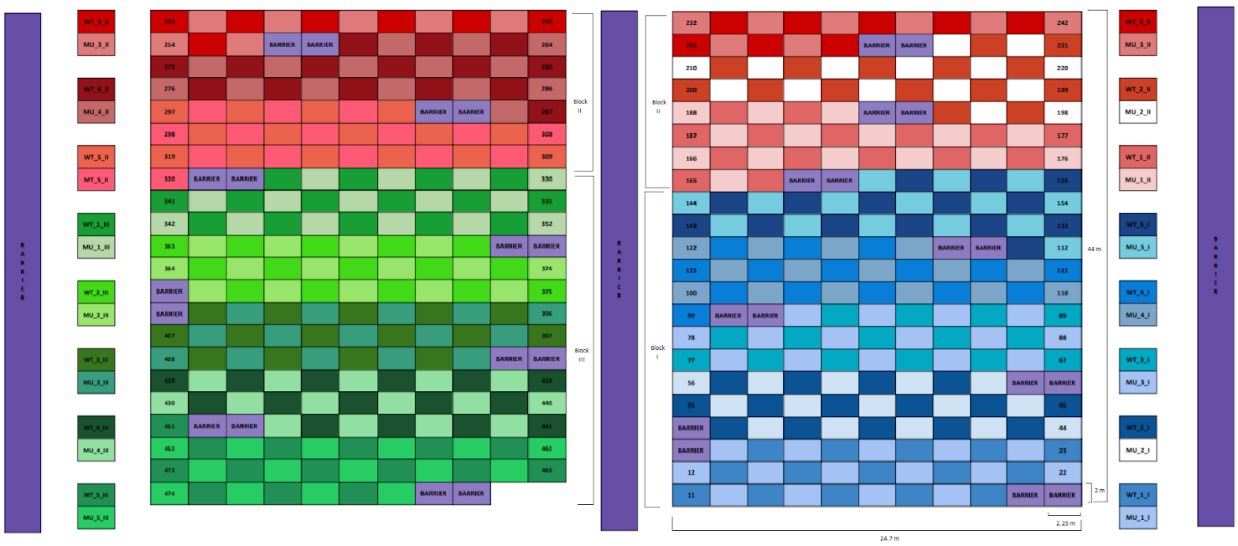


Figure 3. Experimental field sketch located in UNISEM field, Ameca, Jalisco.

Each square (2.25 m length x 2.00 m wide) represents a different maize family sown in 3 rows. Families were randomized and alternated between wild type and mutant maize. Blues, reds, and greens represent block I, II and

III, respectively. Each block was formed by 10 families (5 wild type and 5 mutant). Barriers represent a commercial UNISEM maize hybrid that serve as a physical control and was not included in the study.

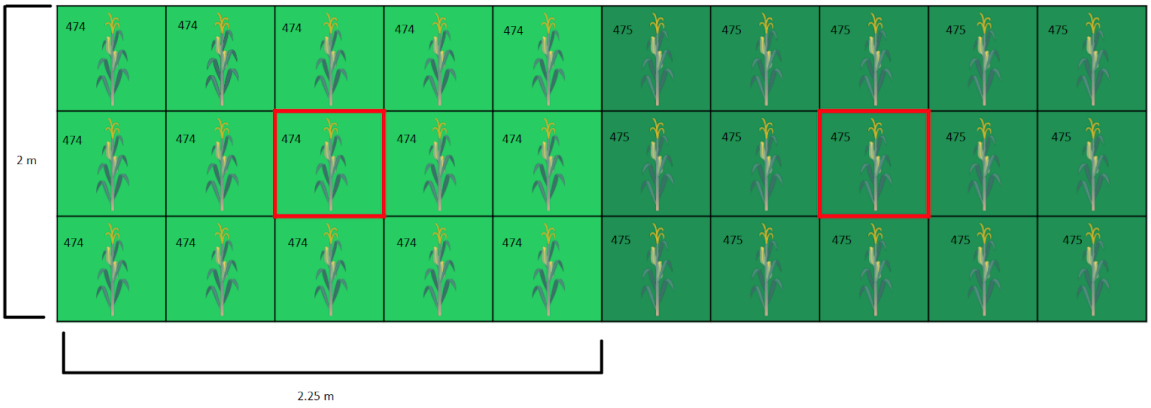


Figure 4. Representation of individual squares in each block.

Each square (2.25 m length x 2.00 m wide) represents a different maize family sown in 3 rows. Red square marks the plant in the second row and third position considered for molecular analysis. Light and dark green represents wild type and mutant maize families.

2.1.3 Root Sampling

For each sample, complete plants were dug with a sterile shovel in a radius of approximately 20 cm of the center of the plant. Once the soil was dug, the plant was removed and stones were detached from the roots. Scissors that were previously cleaned with 96° ethanol before each sampling were used to cut the roots and radical hairs of the plants. The roots and radical hair were stored in ziploc bags. Once the 137 samples were taken (73 mutant families and 64 wild type families), 30 sets of pool samples were clustered with 15 families of the same type of maize each pool as they were considered replicates. A total of 15 samples each of wild type mycorrhizal maize and genetically modified non-mycorrhizal maize was obtained. In the field, samples were stored in ziploc bags per pool, on ice. Once in the laboratory, samples were stored in an ULT freezer at -80°C until the moment of the DNA extraction. For the microbial molecular analysis, only nine pooled samples of block II and ten of block III were analyzed: i) nine mycorrhizal maize, one sample was lost during laboratory manipulation, and ii) 10 non-mycorrhizal maize.

2.1.4 DNA extraction

Root samples for microbial characterization (n=19) were processed as written by Senés-Guerrero and Schüßler (2015). Roots were washed with 80% ethanol, cut into fragments of 1 cm, transferred to a 2 mL tube and dried at 60 °C for 5 minutes to eliminate any traces of

ethanol. Before DNA extraction, 100 µL of water was added to the dried roots for 1 minute and the water removed before taking a subsample. 20 fragments of 1 cm of processed roots were added to a FastPrep lysing matrix A (MP Biomedicals, Santa Ana, CA, USA) with an additional ¼ inch sterile ceramic sphere. The FastDNA Spin Kit for soil (MP Biomedicals, Santa Ana, CA, USA) was used for DNA extraction and the kit protocol followed as recommended with the following modifications: samples were homogenized two times in the FastPrep instrument (MP Biomedicals, Santa Ana, CA, USA) for 40 seconds at a speed of 6.0 m/s and, after the first run, samples were put on ice for 5 minutes and homogenized again. Also, to precipitate proteins the PPS solution was added twice with an incubation on ice for 5 minutes between the treatments. Supernatants were transferred to clean 15 mL tubes to add the Binding Matrix silica suspension and mixed by inversion. Then, 750 µL of the mix was transferred to a SPIN filter and centrifuged at 14,000 x g for 1 minute. The catch tube was emptied and 500 µL of the remaining mixture was added to the SPIN filter and centrifuged again at 14,000 x g for another minute. Supernatant was discarded and 500 µL of SEWS-M with ethanol were added and resuspended with a wide bore tip pipet. Another centrifugation at 14,000 x g for 1 minute was carried out. The catch tube was emptied and replaced. Without any addition of another liquid, a second centrifugation was carried out at 14,000 x g for 2 minutes to dry the matrix of any residual wash solution, then it was air dried for 5 minutes at room temperature. Later, the Binding Matrix was resuspended in 80 µL of DNase/Pyrogen-Free Water and incubated for 5 minutes at 55 °C in a heat block. Finally, the sample was centrifuged at 14,000 x g for 1 minute to bring the eluted DNA into a clean tube. DNA yield and quality parameters were assessed by using 1 µL of DNA in a Nanodrop1000 (Thermofisher Scientific, Waltham, MA, USA). Also, 5 µL of DNA were loaded on a 1% agarose gel in 0.5X TBE electrophoresis buffer to analyze integrity of the DNA. Because of low 260/230 ratios (approximately 0.16) of all the samples, an extra purification step with AMPure XP Beads (Qiagen, Hilden, Germany) was performed using 30 µL of DNA and a 1.8 AMPure beads/DNA ratio. The mixtures of DNA and beads were incubated for 10 minutes in PCR tubes. Then, the tubes were placed on a magnet for 5 minutes. The supernatant was carefully removed to undisturbed the pellet. Two washes with ethanol 70% were performed while the tubes were still on the magnet with an incubation time of 30 seconds within washes. Remanent ethanol was removed with a 10 µL pipette and the pellet was incubated at room temperature for 5 minutes. Then the tubes were removed from the magnet and 15 µL Nuclease-Free Water (Thermofisher Scientific, Waltham, MA, USA) was used to resuspend the pellets. PCR tubes were put again on the magnet for 5 minutes. Nuclease-Free Water with the DNA sample was eluted and transferred to a new PCR tube. DNA quality parameters were assessed again using the Nanodrop1000 and quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) in a Qubit 2.0

Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). These purified and concentrated samples were used for 28S and 16S amplification.

2.1.5 Arbuscular mycorrhizal fungi PCR amplification

AMF amplification was performed using a nested PCR approach. The first PCR reaction was performed using the forward and reverse primer mixtures described by Krüger et al., (2009) (Table 2). Each individual PCR reaction consisted of 15 µL: 7.5 µL of 2X Phusion ® High-Fidelity DNA polymerase Master Mix HF (NEB, Frankfurt, Germany), 0.75 µL of 10 mM forward primer mixture, 0.75 µL of 10 mM reverse primer mixture, 1 µL of DMSO 45%, 1 µL of purified and concentrated DNA sample and 4 µL of MilliQ water.

Table 2. Primers and primer mixture used during the first PCR (Krüger, et al., 2009).

Step	Primer	Nucleotide sequence (5'-3')	Primer mixture
First PCR	SSUmAf1	TGGGTAATCTTTTGAAACTTYA	SSUmAf: forward primer mix SSUmAf1-2 (equimolar)
	SSUmAf2	TGGGTAATCTTRTGAAACTTCA	
	LSUmAr1	GCTCACACTCAAATCTATCAAA	LSUmAr: reverse primer mix LSUmAr1-4 (equimolar)
	LSUmAr2	GCTCTAACTCAATTCTATCGAT	
	LSUmAr3	TGCTCTTACTCAAATCTATCAAA	
	LSUmAr4	GCTCTTACTCAAACCTATCGA	

PCR tubes were maintained cold using a frozen metal block to prevent degradation and activity of the polymerase. PCR conditions were programmed as follows: initial denaturation step for 5 min at 99 °C followed by 35 cycles of 10 s denaturation step at 99 °C, 30 s of annealing step at 55 °C and 1 min of elongation step at 72 °C. A final elongation step was conducted at 72 °C for 10 min. First PCR products were stored at 4 °C until use. The expected result for the first PCR is a fragment of 1.8 kb (Krüger, 2009).

Second PCR reaction was performed using a reverse primer mixture of primers described by Krüger et al. (2009) and a forward primer described by Giménez-Bru et al. (2018) (Table 3). Each individual reaction consisted in 20 µL: 10 µL of 2X Phusion ® Master Mix, 1 µL each of forward and reverse primers at a stock concentration of 10 mM, 1.3 µL of 45% DMSO, 5.7 µL of milliq water and 1 µL of template from the first PCR were used.The conditions to perform

the nested PCR were 5 min of initial denaturation step at 99 °C followed by 35 cycles of 10 s of denaturation step at 99 °C, 30 s of annealing step at 55 °C and 1 min of elongation step at 72 °C. A final elongation step was performed at 72 °C for 10 min and the templates were stored in the thermocycler at 4 °C. A 1% agarose gel in the 1X TAE buffer was used to perform electrophoresis to confirm correct amplification of a 450 bp fragment (Gimérez-Bru et al., 2018).

Table 3. Primers and primer mixture used during nested PCR (Krüger, et al., 2009; Giménez-Bru, et al., 2018).

Step	Primer	Nucleotide sequence (5'-3')	Primer mixture
Nested PCR	LSU D2mod	TGAAATTGTTRAWARGGAAACG	LSU D2mod: forward primer
	LSUmBr1	DAACACTCGCATATATGTTAGA	LSUmBr: reverse primer mix LSUmBr1-5 (equimolar)
	LSUmBr2	AACACTCGCACACATGTTAGA	
	LSUmBr3	AACACTCGCATACATGTTAGA	
	LSUmBr4	AAACACTCGCACATATGTTAGA	
	LSUmBr5	AACACTCGCATATATGCTAGA	

PCR amplification of AMF samples was performed in triplicate, hence, 20 µL of each product were pooled together. 25 µL of the pooled product was used to preparate the sequencing libraries.

2.1.6 Endophytic bacteria PCR amplification

Root sample bacteria amplification was performed in 25 µL reactions following step by step the Illumina 16S Metagenomic Sequencing Library preparation manual (Illumina, CA, USA). The primers used for PCR target the 16S rDNA V3-V4 multivariable region as described by the Illumina protocol (Illumina, CA, USA). For each reaction, 12.5 µL of 2X Phusion ® was added, 5 µL of each forward and reverse primer 10 µM, and 2.5 µL of microbial DNA with a concentration of approximately 5 ng/µL. The conditions to perform the PCR were 3 min of initial denaturation step at 95°C, 25 cycles of 30 s of denaturation step at 95 °C, 30 s of annealing step at 55 °C and 30 s of elongation step at 72 °C. A final elongation step was performed at 72 °C for 5 min and the templates stored in the thermocycler at 4 °C. An 1%

agarose gel in 1X TAE buffer was used to perform electrophoresis to check the correct amplification of an approximate 550 bp fragment.

2.1.7 AMF sequencing libraries preparation

AMF libraries were generated by following the Illumina 16S Metagenomics Sequencing Library Preparation protocol ((Illumina, CA, USA). Products were purified using AMPure XP beads (Qiagen, Hilden, Germany) and they were visualized in a 1% agarose gel to validate the correct amplicon length. Indexes were added as described above and samples were purified using AMPure XP beads at a 1.2 ratio of beads/PCR product and visualized in a 1% agarose gel. The expected amplicon size was 630 bp.

For sequencing library preparation, AMF PCR products were purified using AMPure XP beads (Qiagen, Hilden, Germany) and visualized in a 1% agarose gel to validate the correct amplicon length. Nextera XT indexes (Illumina, CA, USA) were added by PCR at the edges of the amplicon to tag every sample in a unique way. PCR conditions for indexing AMF samples were the same as the nested PCR conditions except for the annealing temperature that was set at 63 °C and only 8 cycles of amplification were used. After, samples were cleaned by using two times AMPure XP beads at a ratio of 0.7 AMPure XP Beads/PCR product and indexed PCR products were observed in a 1% agarose gel. The expected amplicon size was 530 bp.

2.1.8 16S sequencing libraries preparation

Bacterial libraries were generated by following the Illumina 16S Metagenomics Sequencing Library Preparation protocol ((Illumina, CA, USA). Products were purified using AMPure XP beads (Qiagen, Hilden, Germany) and they were visualized in a 1% agarose gel to validate the correct amplicon length. Indexes were added as described above and samples were purified using AMPure XP beads at a 1.2 ratio of beads/PCR product and visualized in a 1% agarose gel. The expected amplicon size was 630 bp.

2.1.9 MiSeq sequencing

AMF and bacterial sample libraries were quantified using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) in a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Libraries average fragment length was calculated with the Qsep400 (BiOptic Inc., Taiwan). Sequencing libraries were prepared according to the 16S Metagenomic Sequencing Library Preparation manual by Illumina (Illumina, CA, USA) using

2 µL of PCR product and diluting in 10 mM Tris-HCl pH 8.0 to achieve concentrations of 4 nM per library. Five microliters of each library were aliquoted and mixed to generate the pool library. Pooled libraries were denatured with 0.2 N NaOH and diluted with a hybridization buffer to obtain a final concentration of 6.5 pM. PhiX was used as sequencing control and processed as described in the 16S Metagenomic Sequencing Library Preparation manual by Illumina (Illumina, CA, USA), diluted in hybridization buffer to accomplish the same concentration as the DNA libraries (6.5 pM). A volume of 520 µL of DNA libraries and 180 µL of PhiX control library at 6.5 pM each were combined to achieve a 30 % sequencing control concentration. Combined DNA library and PhiX control were incubated at 96 °C for 2 min and incubated on ice water for 5 min right before loading the library into the MiSeq reagent cartridge.
Sequencing was performed in a paired-end configuration (2 x 300 bp) with a MiSeq Reagent Kit v3 at a final concentration of 6.5 pM in a MiSeq sequencer System (Illumina, CA, USA).

2.1.10 16S Sequencing data analysis for bacteria

Raw sequences were used as input for QIIME2 version 2021.8 and a standard bioinformatic pipeline for microbial 16S rDNA amplicon sequencing was used to analyze the reads. Reads were filtered according to Q score preliminary to denoising with DADA2. An Amplicon Sequence Variants (ASVs) table was obtained. To designate the taxonomy, a pre-trained and trusted qiime2 classifier was used using 99% similarity and SILVA 138 database. Taxa bar plots were created to assign the matching taxonomy to the ASV table. Phyle and genera tables were downloaded from view.qiime2.org is CSV format to continue with the analysis.

Results were used as input in RStudio (version 2021.09.0). Shannon and Chao1 indexes boxplots were computed. Data was normalized with the DESeq2 package by square root. Genera that were under 10% presence were removed from the raw reads and analyzed to generate multivariate ANOVA and principal coordinate analysis (PCoA) with the Vegan package functions. Additionally, a PCoA with the block data was performed. BiodiversityR and ggplot2 packages were used to perform the statistical analysis and plot the results. For relative read abundance, taxa bar plots were generated. Genera with less than 2% presence were classified as “others”. Genera known to be beneficial to the plant were selected as plant growth promoting bacteria (PGPB) genera. An additional bar plot was computed using only the PGPB genera to compare the compositions between treatments.

2.1.11 28S Sequencing data analysis for AMF

28S raw reads were processed with the same protocol as the 16S raw sequences in section 2.1.9 to obtain Amplicon Sequence Variants (ASVs) table and representative sequences (RS).

2.1.12 Phylogenetic tree and AMF species delimitation

A reference phylogenetic tree was constructed with reference sequences (Krüger et al., 2012) and 1.5 kb sequences from recently described AMF available in public domains. This first phylogenetic tree was used as a backbone for the placement of the obtained RS. Sequences used as reference were aligned with MAFFT v7.490 (Katoh et al., 2002) and checked in CLC Genomics Workbench (Qiagen, Hilden, Germany). A maximum-likelihood phylogenetic tree was calculated with RAxML-HPC v.8.2.X on XSEDE at the CIPRES Science Gateway (Miller, Pfeiffer & Schwartz, 2012) using the GTRGAMMA model and 1,000 bootstraps (Stamakis, 2015).

Every RS was aligned to the previous sequence alignment with MAFFT. The resulting alignment was formed by 1.5 kb sequences from the described AMF phylogenetic backbone and the RS. This alignment file was used as input in the CIPRES Science Gateway using RAxML-HPC v.8.2.X on XSEDE. Evolutionary Placement Algorithm (EPA) function was used with the GTRGAMMA model (Berger et al., 2011; Berger and Stamatakis, 2011). For each RS, EPA assigned a place in the phylogenetic tree used as backbone. Sequences that had a maximum placement likelihood weight of > 0.5 were discarded (Zhang et al., 2013; Berger et al., 2011). The resulting phylogenetic tree with the placement of the RS was visualized in Archaeopteryx Treeviewer (Han and Zmasek 2009). RS were manually annotated to a taxonomic level corresponding to the branch where they were affiliated in the phylogenetic backbone tree (Giménez-Bru et al., 2018).

Results were used as input in RStudio (version 2021.09.0). Shannon and Chao1 indexes boxplots were generated. Data was normalized with the DESeq2 package by square root. Species that were under 10% presence were removed from the raw reads and analyzed to generate multivariate ANOVA and principal coordinate analysis (PCoA) with the Vegan package functions. Additionally, a PCoA with the block data was performed. BiodiversityR and ggplot2 packages were used to perform the statistical analysis and plot the results. For relative read abundance, taxa bar plots were generated.

3. Chapter 3

3.1 Results

3.1.1 Library generation and bioinformatic analysis

A total of 38 samples were processed for library generation (19 samples each for endophyte bacterial and AMF characterization). From the 19 samples, 10 were from mutant maize and 9 from wild type maize. All AMF PCR products resulted in the right size amplicons of 1.5 kb for the first PCR and 450 bp for nested PCR. Bacterial PCR products resulted in 550 bp amplicons. AMF and bacterial correct length amplicons were confirmed by agarose gel electrophoresis. Paired-end sequencing (2X300) resulted in a total 6,852,607 reads with a Q30 of 43.84%. For bacteria, 3,232,841 raw reads were obtained and for AMF a total of 3,619,766.

After sequencing, FASTAQ data of each library were downloaded from the Illumina Base Space platform and uploaded as raw data into QIIME 2.0 version 2021.8. Sequences were filtered by quality, discarding reads with a quality score <Q20, and denoised with DADA2 to obtain a table with amplicon sequence variants (ASVs). For bacteria, 1,580,976 ASVs resulted after chimera and singletons filtering (of the total raw reads, 48.9% were identified), and 758,450 ASVs for AMF (20.9%).

Rarefaction curves were computed to observe if sequencing depth was sufficient to represent the identity of AMF and endophytic bacterial samples (Figure 5). Asymptotes in all samples show that the sequencing depth was sufficient to describe the identity of species and genera composition of both AMF and endophytic bacteria.

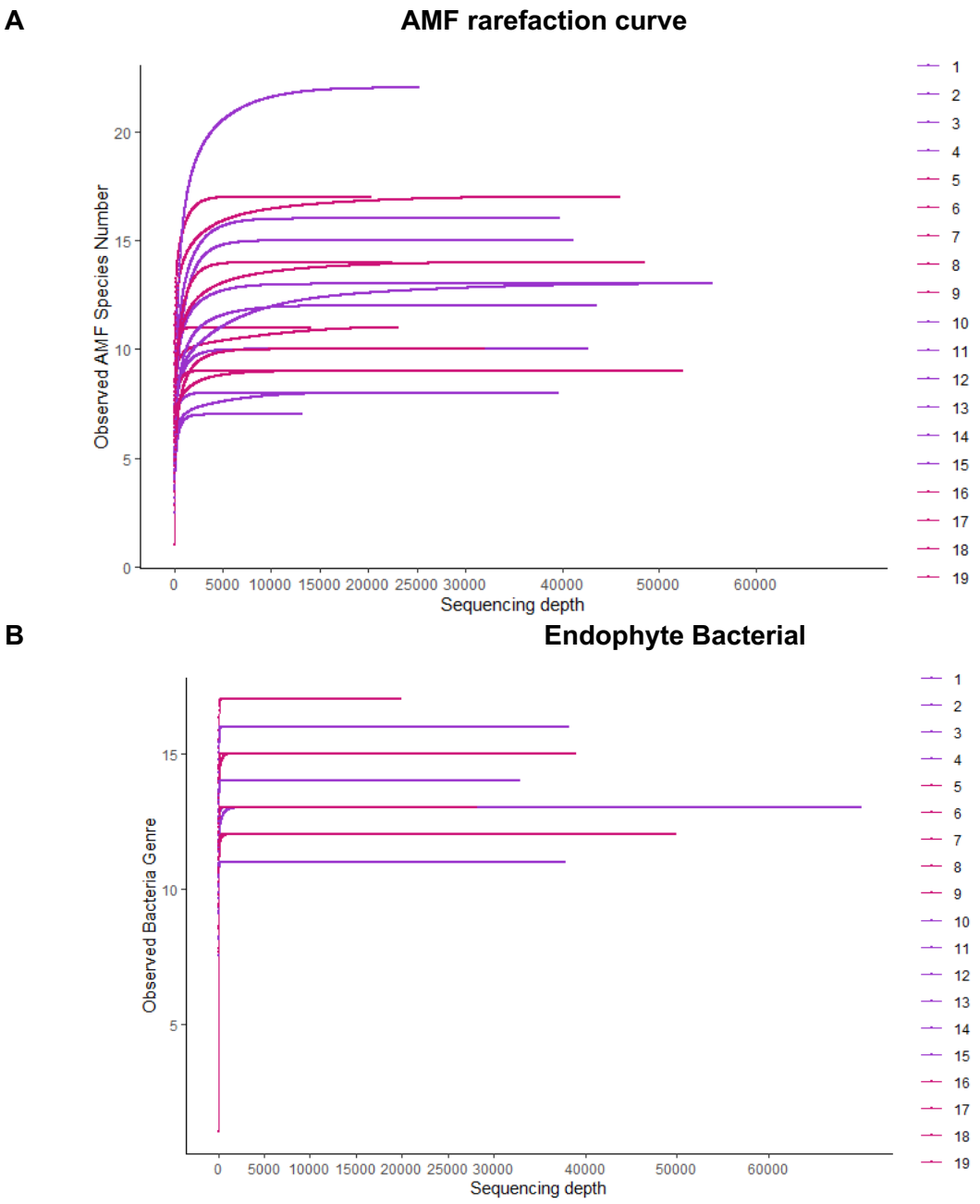


Figure 5. Rarefaction curves for AMF and endophyte bacteria.

Purple curves belong to WT samples while pink curves are MU samples. All curves appear to reach asymptotes, which indicates sufficiency in sequencing depth for a correct characterization of A) the species for AMF and B) genera for bacterial endophyte community.

3.1.2 AMF species diversity in wild type and mutant maize

The diversity of AMF was analyzed using the relative read abundance of the species present per individual sample (Figure 6), block and type of maize (Figure 7). A total of 22 species were observed, where seven were shared by both treatments. Twenty AMF species were found in wild type maize while only eight species were identified in mutant type maize.

Pervetustus simplex was found in 100% of the samples and was shared by both types of maize with a relative abundance ranging from 2.7-47.7% followed by *Dentiscutata savannicola,* present in 90% of the samples with a percentage of presence ranging from 5.2-75.9% (Figure 6).

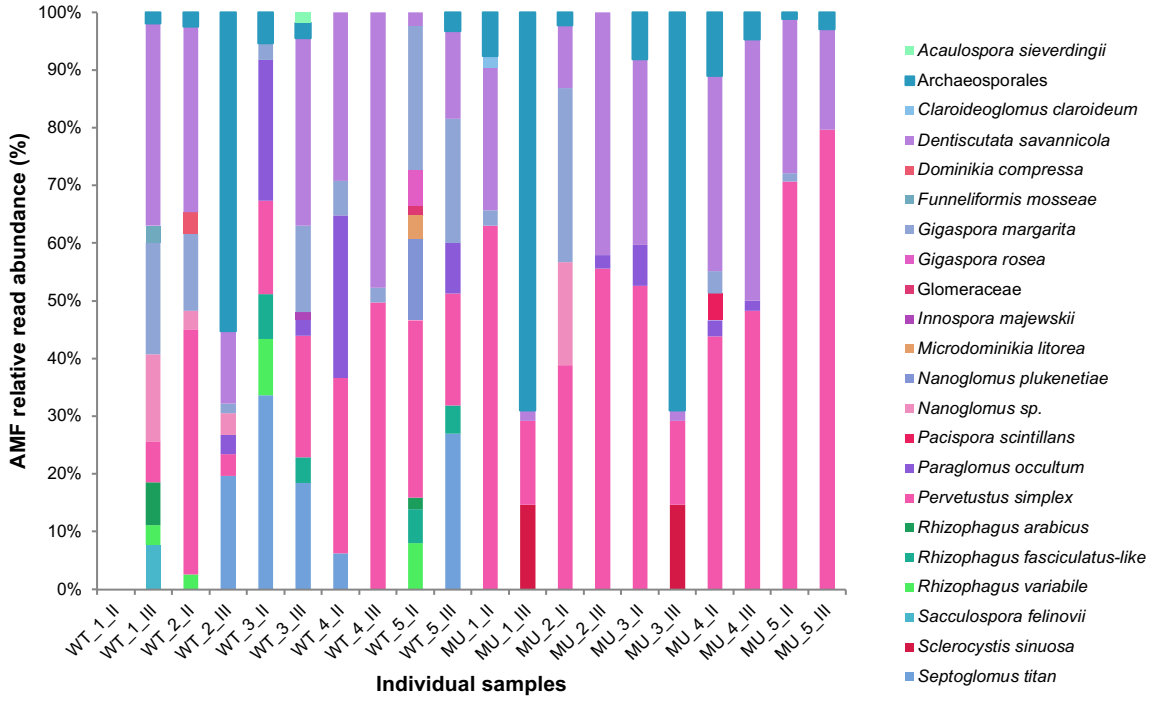


Figure 6. AMF species composition of individual samples.

WT and MU correspond to the type of maize: wild type or mutant type of maize. Arabic numerals correspond to a different family of wild type of mutant type family. Romanic numerals correspond to the number of block: 2 or 3. Sample WT_1_II was not included in this study because it was lost.

When grouped by maize type (Figure 7.B)*, Pervetus simplex* showed 47% abundance in mutant plants and was the most predominant AMF among the mutant treatment, while it showed 22.3% abundance in wild type maize, followed by *Dentiscutata Savannicola* with 28.9% and 25.2% abundance, respectively. Thirteen species of AMF were unique to wild type maize. Among the unique species observed in wild type maize grouped by blocks, *Septoglomus titan* (5.9 % - 12.4%)*, Rhizophagus variable* (2.9 %), *Rhizophagus fasciculatus*-like *(2.3%)* , *Rhizophagus arabicus* (1.9%) and *Rhizophagus clarus* (1.4 %)*,* were in higher abundance (Figure 7A). *Sclerocystis sinuosa* and *Pasispora scintillans* were present only in mutant plants (3.9% and 1% relative abundance, respectively) (Figure 7A).

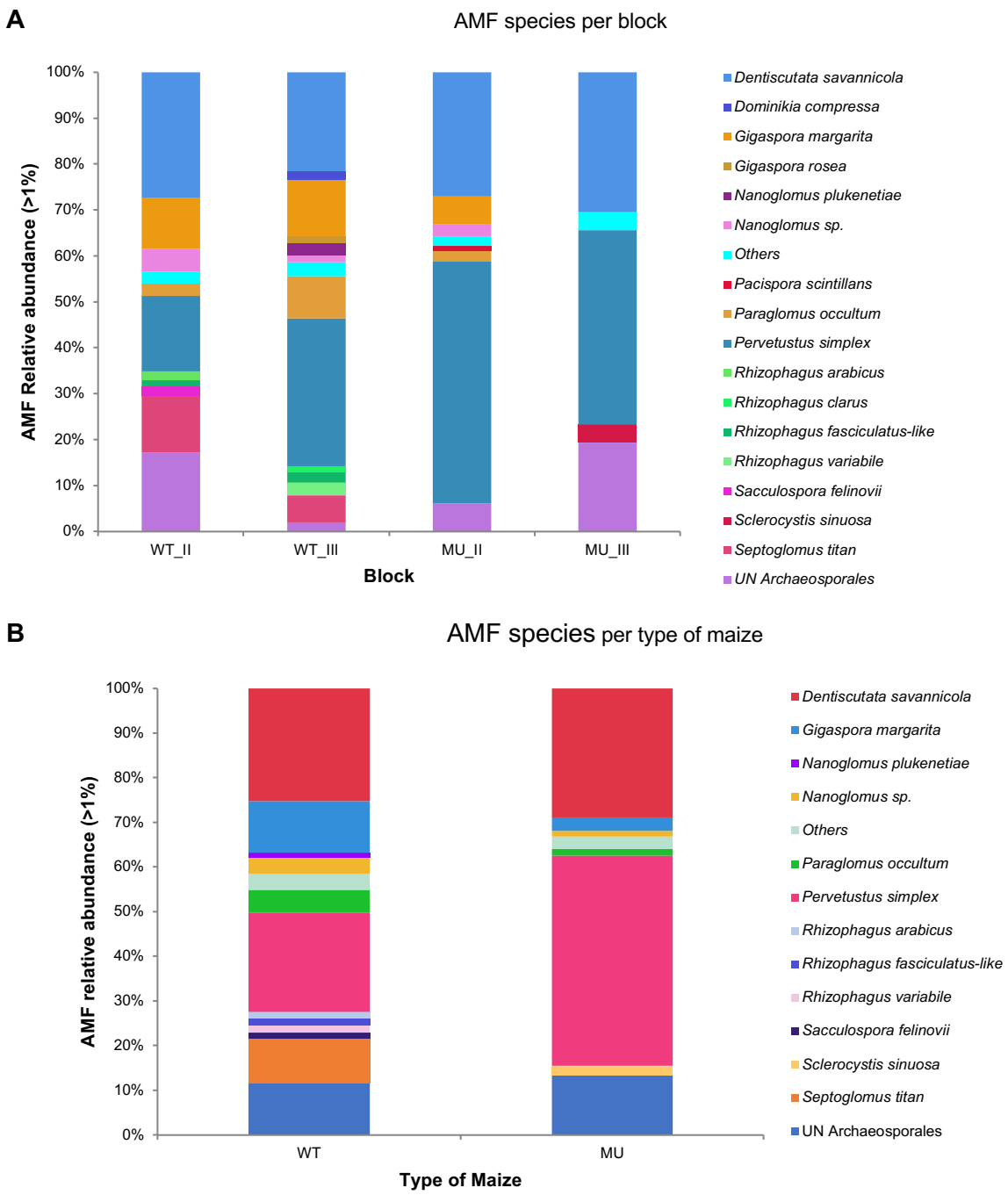


Figure 7. AMF species composition by block and type of maize.

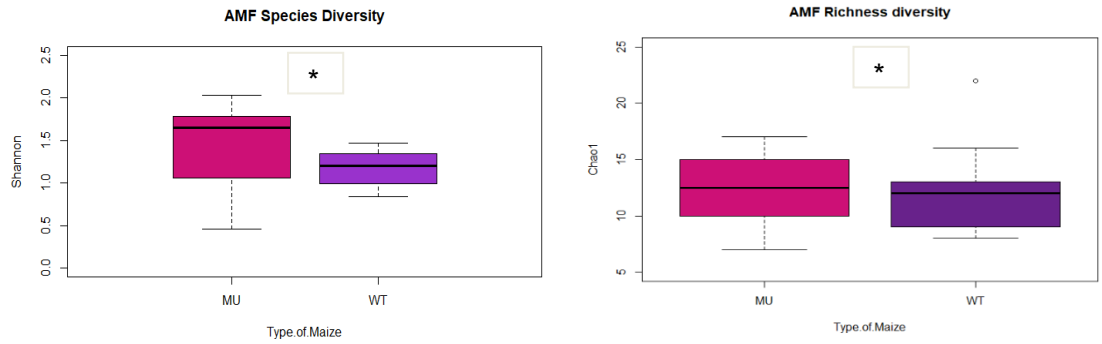
A) Species distribution by block. Each type of maize was evaluated in two blocks (II and III). B) Species distribution by type of maize. Species with presence >1% is shown, while species with a relative abundance <1% are grouped in others.

Differences in richness, evenness and dominance were computed using Shannon and Chao1 indexes (Figure 8.A). Significant differences between diversity and richness were found ($P<0.05$) between mutant and wild type maize. Shannon diversity index showed differences in the abundance of different species in AMF community. Shannon index was higher in mutant

plants than in wild type plants, resulting from higher evenness and richness. Chao1 index, showed significant difference (P<0.05) at species richness between wild and mutant maize, being higher in mutant plants. The Chao1 index also suggests a greater number of rare species in mutant plants than those observed in wild type maize.

A PCoA was also computed for the AMF communities between the two types of maize (Figure 8.B), results showed no differences among the treatments regarding the overall AMF species composition. Adding the variation of both PCoA axes, only 42.3% of the differences and variation from the species diversity could be described with type of maize and number of species variables.

A



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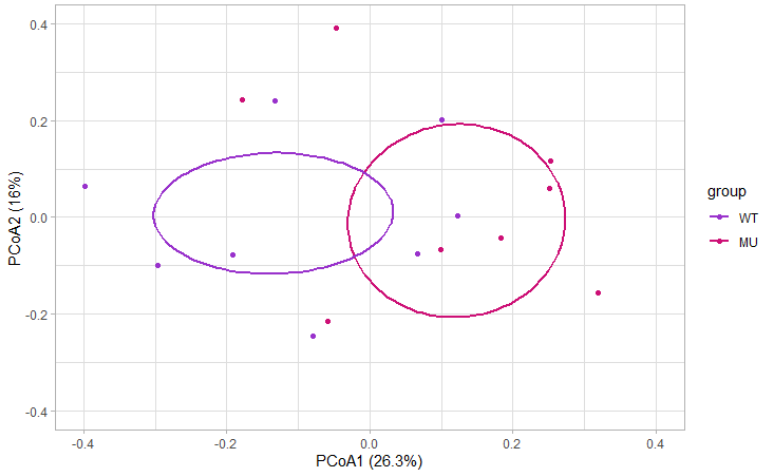


Figure 8. Diversity indexes and principal coordinate analysis of AMF species composition.

A) Shannon and Chao1 index and B) Principal coordinate analysis (PCoA) for AMF species composition.

A) Composition and abundance were compared between wild and mutant type maize using Shannon and Chao1 index where significant differences were found B) plots show no significant differences in AMF community composition between types of maize.

PCoA of the AMF communities between blocks was also computed and suggested that only block WT_II and MU_III showed significant differences in their AMF species diversity composition (Figure 9).

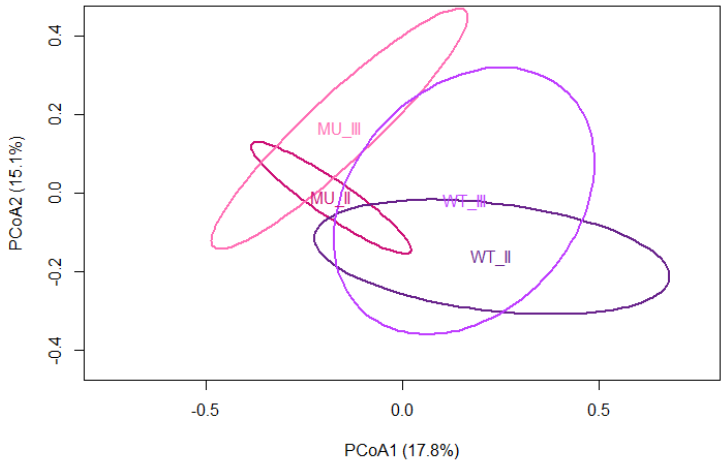


Figure 9. PCoA for differences in AMF community between blocks.

Principal coordinate analysis (PCoA) plots showed only significant differences in AMF community composition between block WT_II and MU_III.

3.1.3 Endophyte bacterial composition of wild type and mutant maize

All bacteria 16S PCR products resulted in the correct expected length of 550 bp. After sequencing, ASVs were clustered using a 99% similarity threshold and SILVA 138 database. Taxonomic classification was assigned at phylum (Figure 10) and genus (Figure 11) level by individual plant, block, and type of maize (Figure 12). For endophyte bacteria, sequences were annotated into 17 phyla, where 12 were shared by both treatments. Bdellovibrionota was observed only in wild type maize, while Fibrobacterota, Armatimonadota and Abditibacteriota were found only in mutant type maize.

Endophyte bacterial composition was highly similar between wild type and mutant treatments. Regarding phyla, the most abundant was Proteobacteria ranging from 32.04% to 65.69% relative abundance, followed by Actinobacteria from 17.83% to 50.60%. A total of 137 and 135 genera were found in wild type and mutant maize, respectively, where 105 of them were shared between treatments.

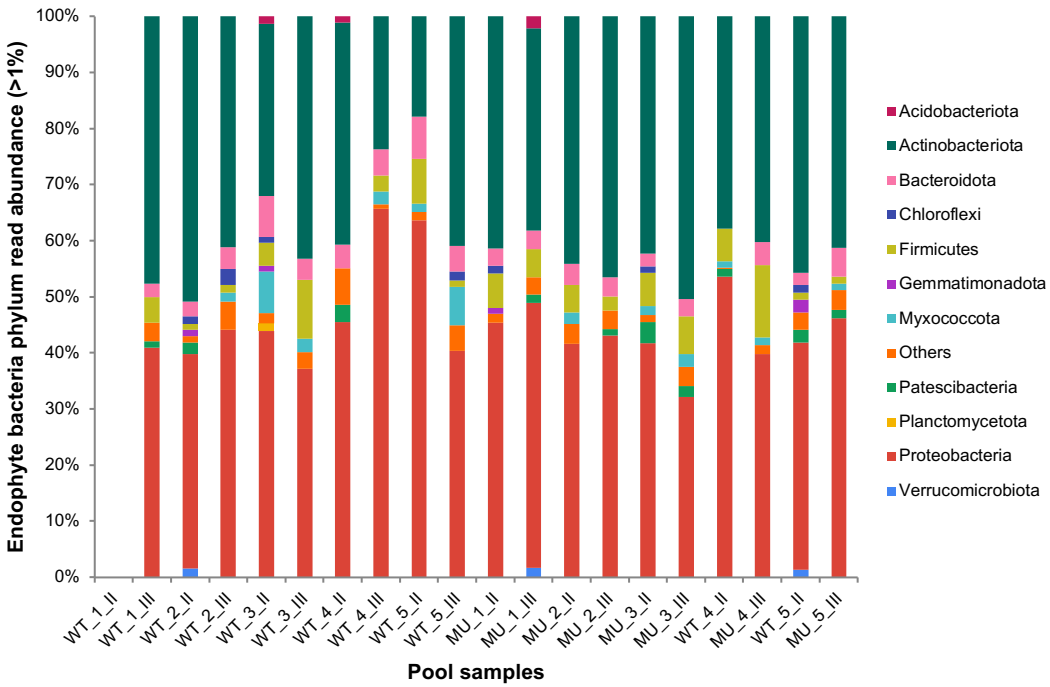


Figure 10. Endophyte bacterial phyla composition.

Phyla composition of samples with a relative abundance >1%, while phyla with a relative abundance <1% are grouped in others.

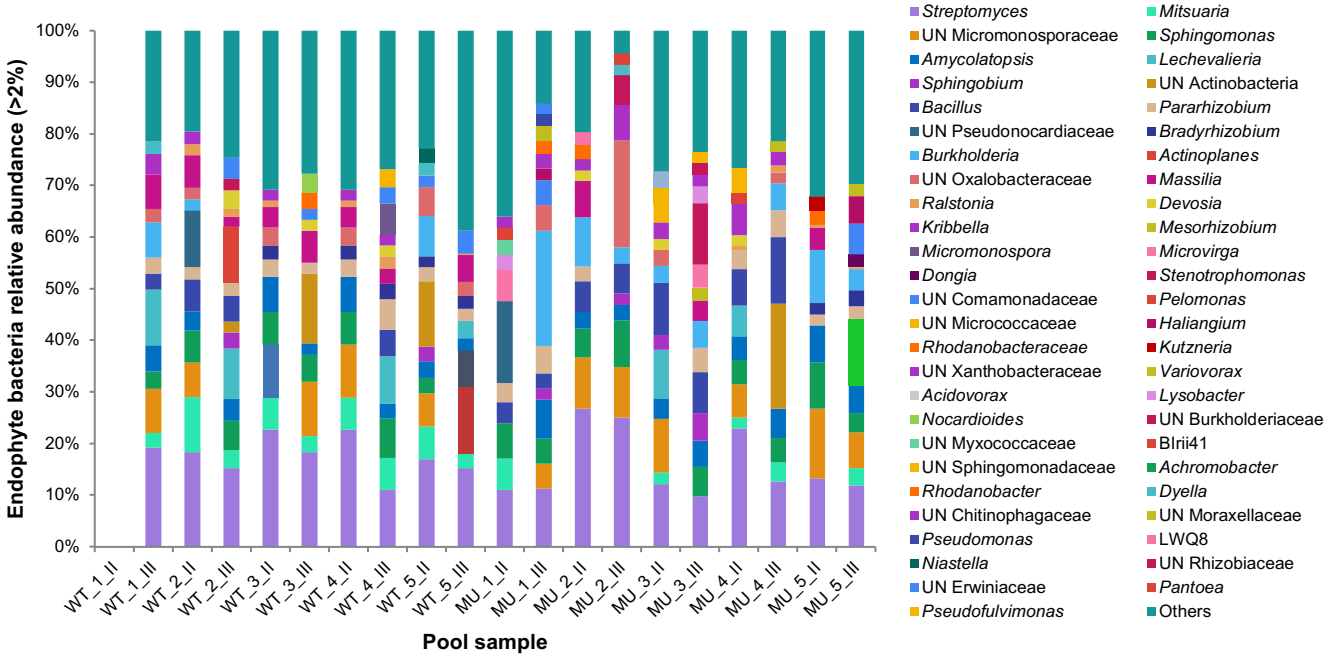


Figure 11. Endophyte bacterial genera composition.

Genera composition of samples with a relative abundance >2%, while genera with a relative abundance <2% are grouped in others.

Regarding genera, *Streptomyces* with 17.3% and 15.3% read relative abundance was observed in all wild type and mutant type maize, followed by UN Micromonosporaceae (7.8% and 6.6%), *Sphingomonas* (5.8% and 5.5%) and *Amycolaptosis* (3.9% and 4.6%) (Figure 12 B). *Micromonospora, Pelomonas, Nocardioides* and *Flavobacterium* were found only in wild type maize samples, while *Haliangium, Kutzneria* and *Achromobacter* only in mutant type maize samples (Figure 11).

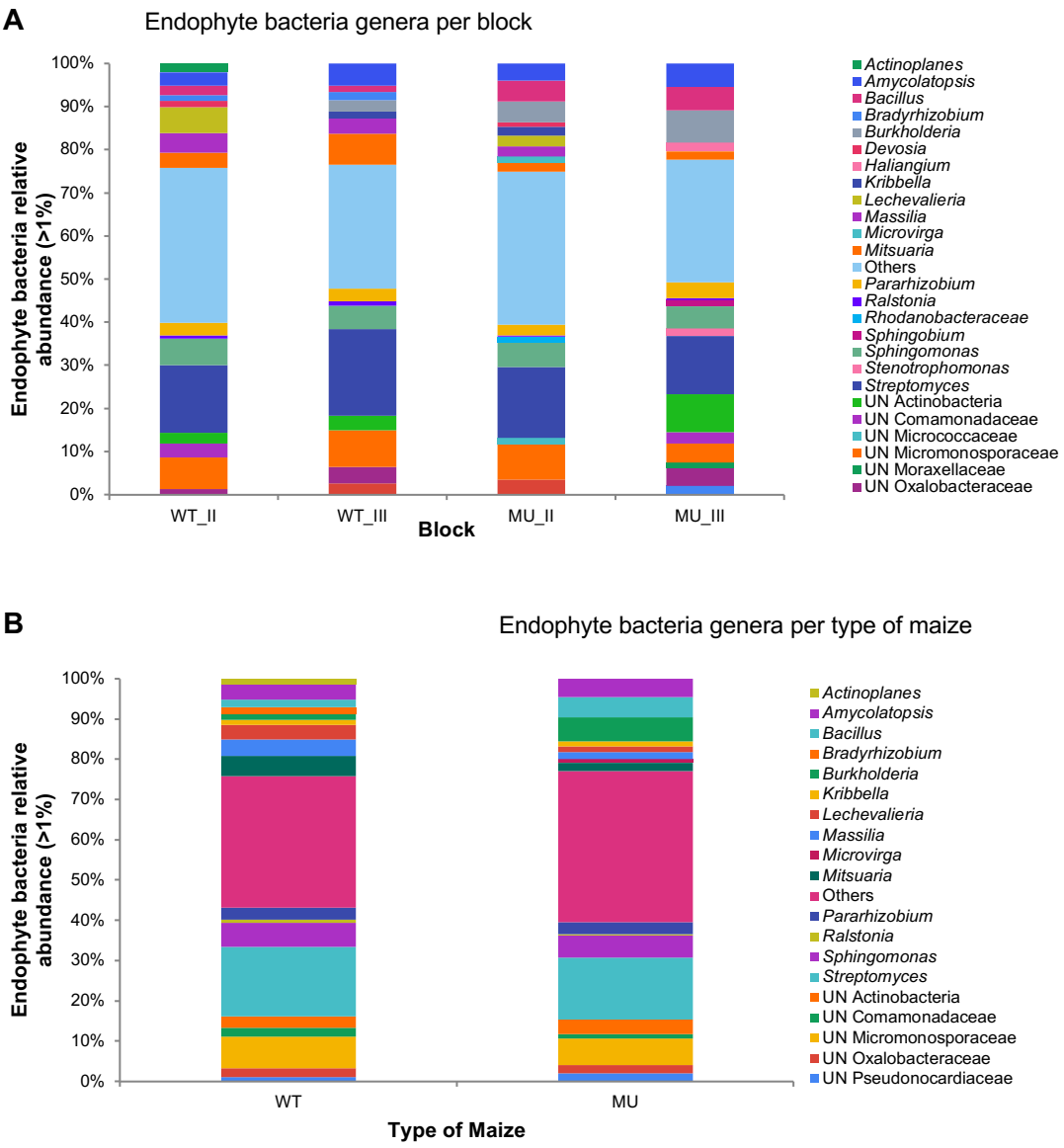


Figure 12. Endophyte bacteria genera composition by block and type of maize.

A) Genera distribution by block. Each type of maize was evaluated in two blocks (II and III) on a randomized complete block design. B) Genera distribution by type of maize. Genera with presence >1% is shown, while genera with a relative abundance <1% are grouped in others.

From all the bacteria genera that were found, 30.62% and 29.64% of them were grouped as unclassified in wild type and mutant maize, respectively. Mutant maize plants showed 18.23% abundance of unclassified genera, while wild type maize showed an abundance of 16.17%, with main unclassified genera belonging to *Micromonosporaceae* family.

Among the genera found in wild type and mutant type maize, 35.9% and 34.2% of them were found to be classified as plant-growth promoting bacteria (PGPB), respectively (Figure 13). Of these, 12 genera were shared by the two types of maize. The most abundant genera shared between the treatments was *Streptomyces* with a relative presence of 41.2% and 30.8% in wild type and mutant type maize, respectively, followed by *Amycolaptosis (*9.4% and 11.3%) and *Burkholderia* (6.8% and 14.5%). PGPB genera *Actinoplanes* and *Flavobacterium* were found only in wild type maize endobacterial community, while *Variovorax* and *Haliangium* were only present in mutant plants.

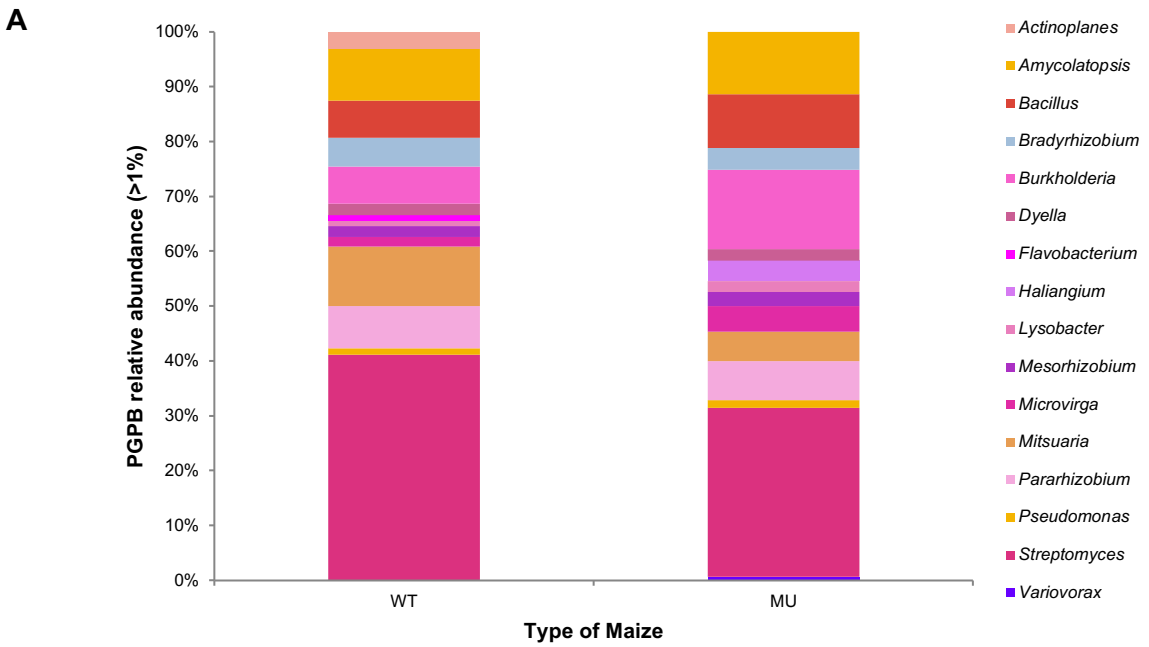
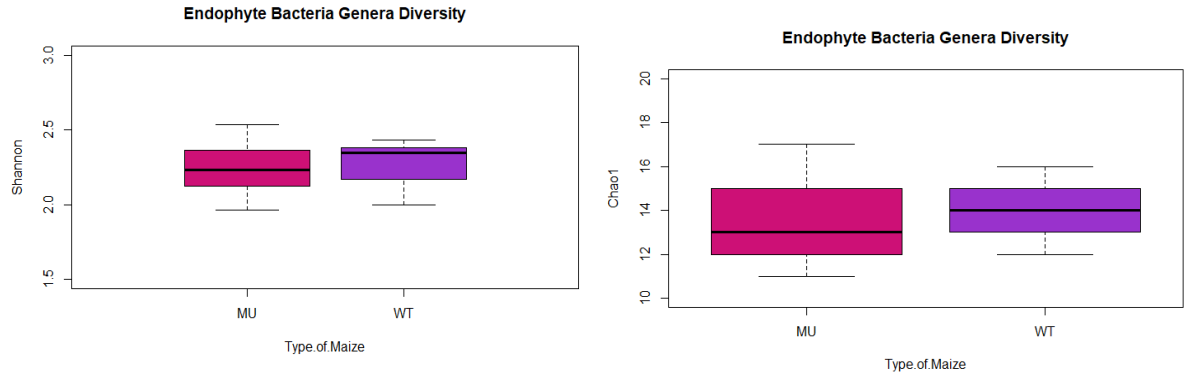


Figure 13. Relative read abundance (>1%) of PGPB composition in wild type and mutant type maize.

Shannon diversity index and Chao1 richness index were calculated to compare the number, abundance and influence of total endophyte bacteria and PGPB genera (Figure 14). No differences were found between the two treatments (P>0.05). Shannon diversity index for endophyte bacteria are above 2 for both treatments, suggesting high diversity composition in both type of plants.

A



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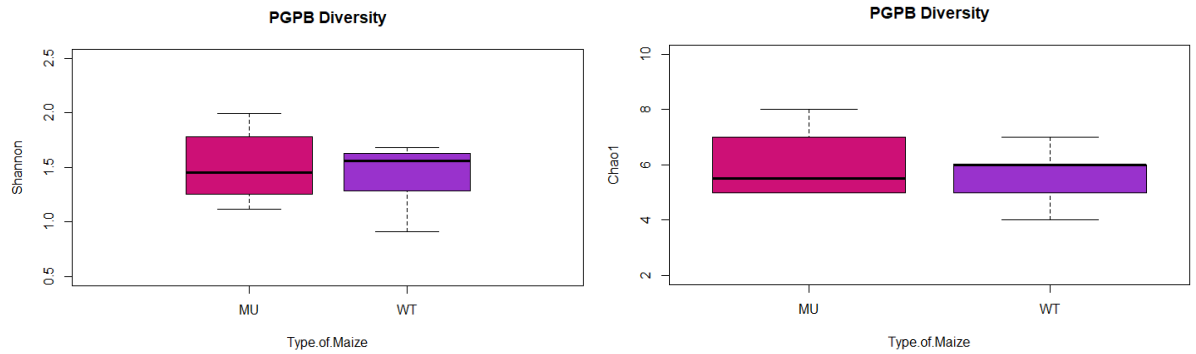

Figure 14. Shannon diversity index and Chao1 richness index for endophytic bacterial and PGPB genera composition of wild type and mutant type maize.

A) Diversity indexes of endophyte bacteria and B) PGPB genera composition.

A principal coordinate analysis (PCoA) was computed to visualize if endophyte and PGPB bacterial communities were significantly different among wild type and mutant maize. PCoA showed no significant differences between the bacterial community composition (Figure 15).

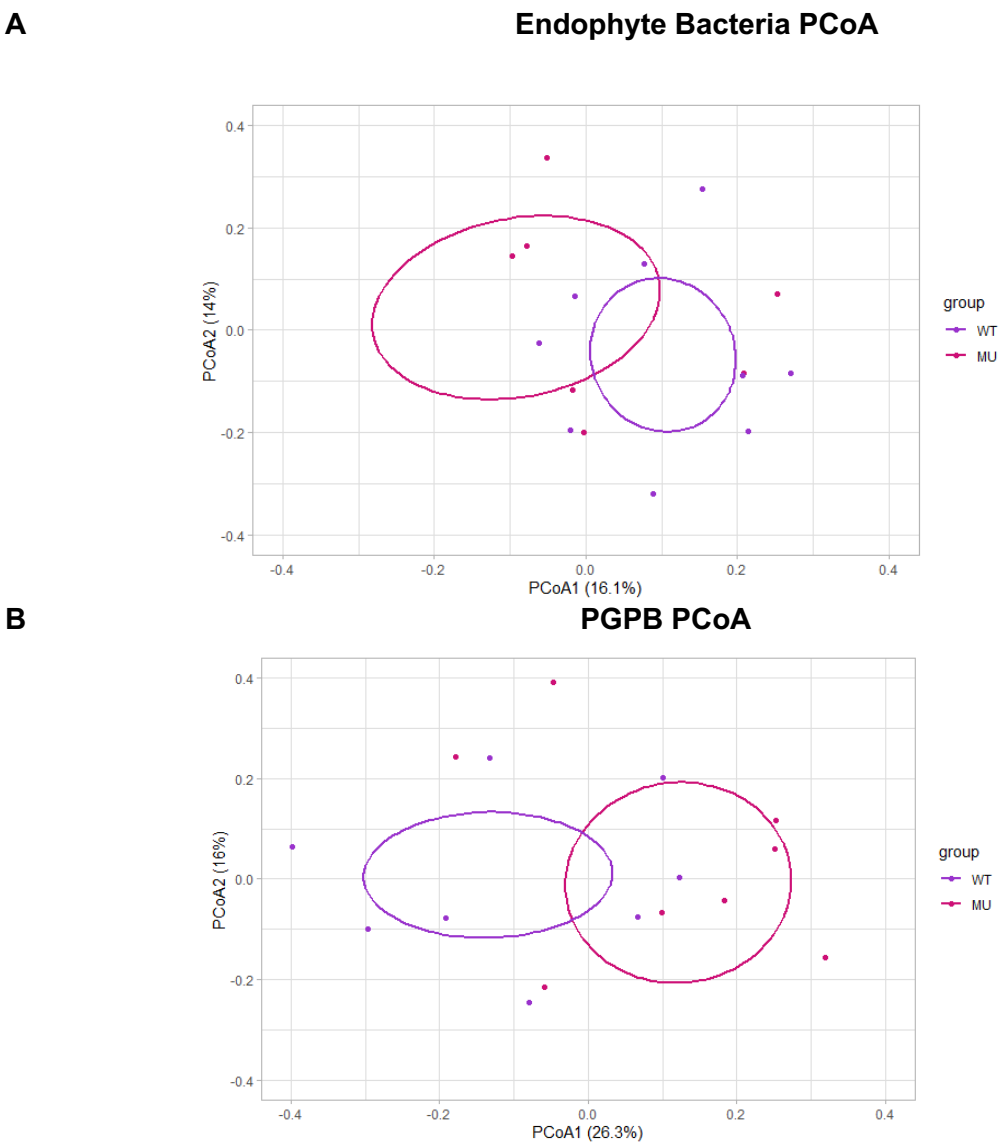

Figure 15. PCoA plots for endophytic bacterial and PGPB genera composition.

Principal coordinate analysis (PCoA) plots show no significant differences between types of maize A) endophyte bacteria and B) PGPB genera composition. The percentages of the axes represent the variation and dissimilarity between data.

PCoA of bacterial community to see differences among blocks showed no differences regarding endophyte bacterial community (Figure 16).

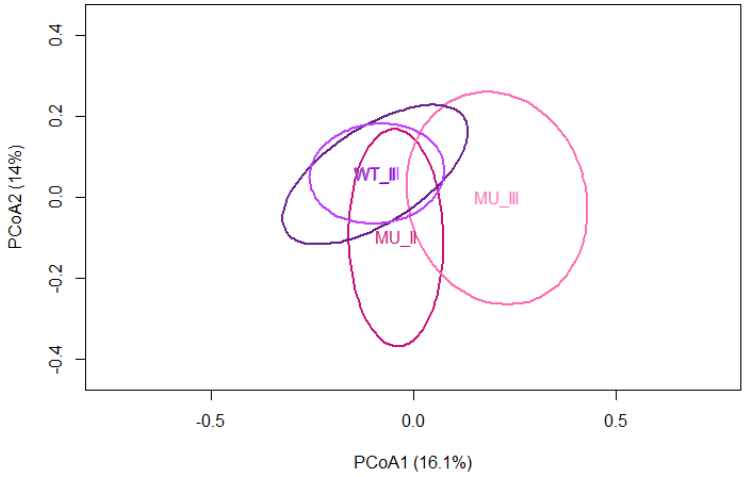

Figure 16. Principal coordinate analysis for endophytic bacterial composition between blocks.

4. Chapter 4

4.1 Discussion

4.1.1 Mutated study model

Symbiosis between plant roots and germinating AMF spores requires for both organisms to exchange signals that trigger the recognition interaction (Singh et al., 2019). In the plant, this process is led by the common symbiotic pathway (CSP), which is built by different components that include genes encoding for different proteins and biosynthesized molecules. Among these, *CASTOR*, a gene that encodes a K^+ channel protein and starts a Ca^{2+} signaling cascade is involved (Parniske, 2008). Different genomic studies have shown that *CASTOR* has several homolog genes in different plants, including species such as *Medicago truncatula, Lotus japonicus, Arabidopsis thaliana, Orizaba sativa* and *Sorghum bicolor* (Chen et al., 2009). However, *CASTOR* homologs in *Zea mays* are poorly characterized (Ramírez-Flores et al., 2020).

Genomic and proteomic studies have shown that *CASTOR* has a direct relation with Ca^{2+} spiking by permitting K^+ efflux to counterbalance Ca^{2+} influx (Bamba et al., 2008), which is one of the upstream steps in the calcium spiking response to the Nod and Myc factors (Miwa et al., 2006), diffuse molecules synthetized by bacteria and AMF, respectively, in response to release flavonoids and strigolactones by the plants.

Castor mutant varieties of *L. japonicus* have shown deficiency in calcium spiking (Oldroyd and Downie, 2008), which blocks the signaling cascade in the symbiosis common pathway. Another study on *L. japonicus* mutant varieties performed by Maekawa and Murooka (2009) showed absence of symbiosis with AMF and resistance to nitrogen-fixing bacteria symbiosis. Studies on *O. sativa castor-deficient* mutants, also displayed impaired AMF interaction, lacked cortex colonization, arbuscules and vesicles (Gutjahr et al., 2008). However, *Castor mutant* varieties of *M. truncatula,* on the other hand, showed a similar nodule number, intracellular hyphae and arbuscules as the wild type maize counterpart (Venkateshwaran et al., 2012), which indicated that *CASTOR* genes were not required in this plant to establish both AMF and nitrogen-fixing bacteria symbiosis.

HUN, the homologous gene of *CASTOR* in maize, used in this study was mutated by a mutagenesis transposon in both alleles by collaborator Dr. Ruairidh Sawers and its research

group. Allele *hun* 1-1 was mutated in a somatic event at the *HUN* promoter region, while *hun* 1-2 was mutated with a mutant insertion in the first exon.

4.1.2 AMF species diversity on wild type and mutant type maize

According to the results in this study, AMF were present in all mutant treatments. Diversity, evenness, and richness of the AMF species in the mutant maize were significatively higher than the observed in wild type maize counterparts. These results suggests that both mutations are not sufficient to block a complete AMF symbiosis. Hayashi et al. (2010) examined the function of a Ca^{2+} spiking downstream gene of the CSP, calcium and calmodulin-dependent kinase (CCaMK), in *L. japonicus* mutants that were defective in common symbiosis genes, including *CASTOR*. In the research, CCaMK was activated by the inoculation of *Mesorhizobium loti,* a nitrogen-fixing bacteria that establish symbiosis with plant roots, resulting in cytosolic Ca^{2+} spiking from an independent CSP input from signaling cascades, derived by the recognition of the bacterial Nod factors by lysin motif (LysM) receptor kinases. The results showed that the upstream gene mutants were suppressed of the defects in AM symbiosis and bacterial infection, demonstrating that mutations in the Ca^{2+} spiking upstream genes do not affect the oscillation of the Ca^{2+} concentration in response to Nod factors (Hayashi et al., 2010). Also, frequent natural mutations gain-of-function in CCaMK of a single amino-acid show to be sufficient to surpass the requirement of the Myc-factor perception and the resulting Ca^{2+} oscillations to activate the transcriptional response necessary to complete the AMF symbiosis (Tirichine et al., 2006; Miller et al., 2013).

AMF was assigned taxonomy at species level because the primers used in this study and designed by Krüger et al. (2009) and Gimenez-Bru (2018) targeted the LSU-D2 fragment that allowed phylogenetic analysis with species resolution.

Regarding AMF diversity, it has been demonstrated that AMF show a broad range of host plants without specificity for any plant species (Singh et al., 2019). However, it is known that AMF can have a certain preference for a plant based on the plant needs (Campos et al., 2018). Lee and Eom (2015) described that host plants show existence of certain genotypes preferences even within a single AMF species in the same environment. Up to this date, different studies have shown that AMF genetic differences are correlated to diverse functions (Munkvold et al., 2004; de Novais et al., 2014). Diversity in AMF communities are due to the diverse functional variation and adaptability that the AMF grants to the host plant.

In this study, AMF were assigned taxonomy at species level because the primers used in this study and designed by Krüger et al. (2009) and Gimenez-Bru (2018) targeted the LSU-D2 fragment that allowed phylogenetic analysis with species resolution. Species from the family *Rhizophagus,* considered a generalist symbiont, were not observed in none of the *HUN-mutant* treatments. During gene *B73, hun 1-1* and *hun 1-2* mutagenesis a confirmation of lack of symbiosis was performed by our collaborators. For this, *Rhizophagus irregularis* was inoculated and observed with trypan blue. *R. irregularis* was not observed in the *hun 1-2* mutant plants. Therefore, only this mutation was further studied in the field. In the results of this study, *R. irregularis* was not observed in any of the mutant plants, which coincide with Dr. Sawers and Dr. Ramírez-Flores results.

Other AMF from the class Glomeromycetes and order Paraglomerales (*Pervetustus simplex, Paraglomus* spp.) that have low phylogenetic relationship to the order Glomerales (e.g., *Rhizophagus* spp*., Sclerocystis* spp*.)* and Diversisporales (eg., *Dentiscutata* spp*., Gigaspora* spp.) were observed in all mutant and wild varieties (Oehl et al., 2011). *Pervetustus simplex* was the most observed AMF species and it is known to form extra-radical mycelia and mycorrhizal structures without vesicles. Other genera that lack intraradical vesicle formation are *Gigaspora* and *Dentiscutata* (Oehl et al., 2011) and were also observed in mutant and wild type maize. Absence of generalist *Rhizophagus* spp*.* in mutant plants suggests that the functional characteristics of the *Rhizophagus* species were not required or incompatible by the mutant maize variety. Most of the AMF observed in mutant and wild type maize lacked vesicles formation, which also suggest that these fungi were preferred by the plants. When observing the phenotype of mutant maize plants, a correlation between AMF without vesicles and lower chlorophyll content in the plant could be suggested. Further studies are required to confirm the effect of AMF without vesicles versus AMF with extraradical vesicles formation.

Contrary to our results, lower AMF community diversity of a mutant variety maize was demonstrated by Londoño et al. (2020), where three types of maize were studied: a domesticated species, a genetically modified hybrid and a non-modified hybrid. The genetically modified hybrid maize exhibited generally less AMF species than the landrace maize.

Species from *Dentiscutata, Rhizophagus, Gigaspora* and *Glomus* genera were observed to be predominant among wild type maize (Serralde & Ramiréz, 2004; Liu et al, 2016; Londoño et al., 2020). In this study, *Pervetus simplex,* a relative new molecularly characterized AMF (Błaszkowski et al., 2017) was found to be the first and second AMF in higher abundance of the mutant type and wild type maize, respectively.

4.1.3 Endophyte bacteria and PGPB community composition of wild type and mutant type maize

For bacteria, taxonomy was assigned at genus and phyla levels. Regions v1-v3 from the 16S rRNA subunit provide an approximation of 16S diversity but do not capture enough variation to discern between closely related taxa at species resolution. However, it is sufficient for the identification of taxa at genus level or above (Johnson et al., 2019).

Regarding endophytic bacterial communities, it has been shown that Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria were found to be the most predominant phyla in maize samples (Correa-Galeote, 2018; Liu et al., 2012; Liu et al., 2020), in agreement with the results in this study. Different genera had been found among maize seeds, i.e., *Burkholderia, Pseudomonas* and *Bradyrhizobium* being the most abundant. Other reported genera associated with maize are *Enterobacter, Erwinia, Herbaspirillum, Shigella* and *Serratia* (Pereira et al., 2011; Correa-Galeote, 2018; Liu et al., 2012; Liu et al., 2020), which were not found in this study. Contrary to other reports, in this study, *Streptomyces* was found to be the most predominant genus in both treatments.

Streptomyces are abundant and ubiquitously distributed in soil (Olanwewaju, 2019). Nozari et al. (2021) found that *Streptomyces* improved vegetative growth of maize under saline stress, while Warrad et al. (2020) found that *Streptomyces* boosted maize plant growth during drought stress. Nonetheless, none of these abiotic stresses were experimented during the field experiment. *Streptomyces* genera is a great source of bioactive molecules like antibiotics, phytohormones, antifungal compounds, cellulases, chitinases, etc. (Olanrewaju, 2019). Studies suggest that *Streptomyces* are often described to increase AMF spore germination and mycorrhizal symbiosis establishment (Agnolucci et al., 2015; Giovannini et al., 2020). In another study performed by Qi et al. (2019), *Streptomyces* spp. were found to have antifungal activity against thirteen phytopathogenic fungi, including *Fusarium oxysporum*, which suggests that *Streptomyces* spp. could be related to the AMF and overall fungal composition.

Bacterial endophytes regulate plant growth (plant growth promoting bacteria, PGPB) through different mechanisms such as N_2 fixation, production of siderophores, phytohormones, antibiotics, antifungal compounds, inducing systemic resistance, etc. (Ahemad & Kibret, 2014; Vejan et al., 2016). From the PGPB characterized in this study, three genera are well known to be nitrogen-fixing bacteria: *Bradyrhizobium, Mesorhizobium* and *Pararhizobium.* As mentioned in section 1.1.9 and 4.1.2, nitrogen-fixing bacteria or rhizobia, produce Nod factors that are recognized by specific receptors of the plant to start a calcium spiking response to

initiate the symbiosis process (Lerouge et al., 1990) and confirm the idea of CCaMK activating AMF and endophytic bacteria symbiosis ignoring the mutations of *CASTOR* gene.

Other genera found in this study also exhibited fixation of atmospheric nitrogen (*Pseudomonas, Burkholderia, Bacillus, Microvirga*); solubilize inorganic phosphorus (*Pseudomonas, Sphingomonas, Burkholderia, Variovorax, Bacillus*); synthesized indole acetic acid (*Flavobacterium, Pseudomonas, Sphingomonas, Burkholderia, Dyella, , Variovorax, Bacillus*) and are involved in biocontrol (*Streptomyces, Haliagium, Amycolaptosis, Actinoplanes, Lysobacter, Burkholderia, Dyella, Variovorax, Bacillus, Microvirga*) (Ahemad & Kibret, 2014; Vejan et al., 2016; Jiao et al., 2019; Cabrera et al., 2020).

Reports suggest that there are qualitative and quantitative differences in bacteria composition according to plant genotypes in maize (Garcia de Salamone, 1996). However, Ikeda et al. (2013) described no significative differences in the bacterial endophytic community isolated from six different maize genotypes. In this study, Shannon and Chao1 indexes were statistically non-significant different between the diversity and richness of the wild type and mutant type maize, which confirms no significant variation between the endophytic bacterial and PGPB composition in the two studied genotypes.

Approximately 30% of bacterial abundance was grouped as unclassified, which suggest the presence of uncultured bacterial groups. In addition, genera found by other authors (PGPB genera such as *Enterobacter, Erwinia, Herbaspirillum, Shigella* and *Serratia*) that were not detected in this study may be due to maize varieties, soil, location and environment differences in the maize field (Ikeda et al., 2013).

5. Chapter 5

5.1 Conclusions

In this study, we characterized the effect in the endophyte root microbial community of a mutation in the gene *CASTOR* involved in the common symbiosis pathway (CSP) in the most important crop in Mexico, maize. By mutating the *CASTOR* gene (known as *HUN* in maize), we expected to block the arbuscular mycorrhizal fungi (AMF) symbiosis with the plant to compare the effect of the absence of AMF in endophytic bacterial community and corn yield. The mutation in the *HUN* gene showed to be non-efficient to prevent AMF symbiosis from occurring. Several reasons could lead to this result such a poor mutagenesis, action of a CCaMK activated protein (Hayashi et al., 2010) or, as shown in *M. truncatula,* this gene could be unnecessary for the symbiosis to occur or compensated with functional downstream common symbiosis pathway genes (Tirichine et al., 2006; Venkateshwaran et al., 2012; Miller et al., 2013). AMF diversity in the mutant plant was significantly higher than the diversity observed in wild type maize. Richness index Chao1 was also higher in mutant plants, which suggest a higher diversity in rare AMF species observed in mutagenic maize. Endophytic bacterial composition and plant growth-promoting bacteria (PGPB) diversity, on the other hand, showed no significant differences between treatments. However, an infrequent abundance of *Streptomyces* species was found, which is known to be an important source in biosynthesizing antibiotics, cellulases, chitinases and phytohormone molecules (Olanrewaju, 2019). As plant recruit endophytic bacteria and reshape its bacterial composition to cover its needs (Berendsen et al., 2012; Abedinzadeh et al., 2019), results suggest that *Streptomyces* was recruited to improve AMF germination and the symbiosis process. To confirm these results, further studies with AMF absence are required to reassess the endophytic bacterial composition, mainly *Streptomyces* role, as well as plant and endorhiza behavior. In addition, plant phenotypic characterization (plant height, corn weight, total number of grains per cob and per line, 1000-grains weight, total grain weight, chlorophyll optical density, etc.) are also required to correlate observed characteristics in the plants with the microbial composition studies.

6. References

Abbaspour, H., Saeidi-Sar, S., Afshari, H. & Abdel-Wahhab, H. (2012). Tolerance of mycorrhiza infected Pistachio (Pistacia vera L.) seedling to drought stress under glasshouse conditions. Journal of Plant Physiology, 169 (7): 704-709.

Abbendizadeh, M., Etesami, H., & Alikhani, H. (2019). Characterization of rhizosphere and endophytic bacteria from roots of maize (Zea mays L.) plant irrigated with wastewater with biotechnological potential in agriculture. Biotechnology Reports, 21.

Agricultural Sustainability Institute ASI. (N.d.). What is sustainable agriculture? Retrieved from https://asi.ucdavis.edu/programs/ucsarep/about/what-is-sustainable-agriculture

Agnolucci, M., Battini, F., Cristani, C. & Giovannetti, M. (2015). Diverse bacterial communities are recruited on spores of different arbuscular mycorrhizal fungal isolates. Biology and Fertility of Soils, 51(3): 379-389.

Ahanger, M., Hashem, A., Abd-Allah, E. & Ahmad, P. (2014). Arbuscular mycorrhiza in crop improvement under environmental stress. Emerging Technologies and Management of Crop Stress Tolerance, 2: 69-95.

Ahemad, M., & Kibret, M. (2014). Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. Journal of King Saud University Science, 26(1): 1–20.

Ahmad, P., Ashraf, M., Azooz, M.M., Rasool, S. & Akram, S. Potassium startation in induced oxidative stress and antioxidant defende responses in Brassica juncea. Journal of Plant Interaction, 9(1): 460-467.

Al-Karaki, G. & Al-Raddad, A. (1997). Effects of arbuscular mycorrhizal fungi and drought stress on growth and nutrient uptake of two wheat genotypes differing in drought resistance. Mycorrhiza, 7: 83-88.

Al-Karaki, G., McMichael, B. & Zak, J. (2003). Field response of wheat to arbuscular mycorrhizal fungi and drought stress. Mycorrhiza, 14: 263–269.

Aliasgharzadeh, N., Saleh Rastin, N., Towfighi, H., Alizadeh, A. (2001). Occurrence of arbuscular mycorrhizal fungi in saline soils of the Tabriz Plain of Iran in relation to some physical and chemical properties of soil. Mycorrhiza, 11: 119–122.

Amtmann, A., Trouillard, S. & Armengaud, P. (2008) The effect of potassium nutrition on pest and disease resistance in plants. Physiolia Plantarum, 133 (4): 682-691.

Aroca, R., Vernieri, P., & Ruiz Lozano, J. M. (2008). Mycorrhizal and non-mycorrhizal Lactuca sativa plants exhibit contrasting responses to exogenous ABA during drought stress and recovery. Journal of Experimental Botany, 59: 2029-2041.

Atul-Nayyar, A., Hamel, C., Hanson, K. & Germida, J. (2009). The arbuscular mycorrhizal symbiosis links N mineralization to plant demand. Mycorrhiza, 19: 239–246.

Bago, B., Pfeffer, P. E., & Shachar-Hill, Y. (2000). Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiology, 124: 949–958.

Balestrini, R., Bonfante, P. (2014). Cell wall remodeling in mycorrhizal symbiosis: a way towards biotrophism. Frontier in Plant Science, 5: 237.

Banba, M., Gutjahr, C., Miyao, A., Hirochika, H., Paszkowski, U., Kouchi, H., & Imaizumi-Anraku, H. (2008). Divergence of evolutionary ways among common sym genes: CASTOR and CCaMK show functional conservation between two symbiosis systems and constitute the root of a common signaling pathway. Plant Cell Physiology 49 (11): 1659–1671.

Besserer, A., Puech, V., Kiefer, P., Gomez, V., Jauneau, A., Roy, S., Portais, J., Roux, C., Bécard, G., & Séjalon-Delmas, N. (2006). Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. PLoS Biology 4 (7): e226.

Berendsen, R., Pieterse, C. & Bakker, P. (2012). The rhizosphere microbiome and plant health. Trends in Plant Science, 17 (8): 478-486.

Błaszkowski, J., Kozłowska, A., Crossay, T. & Symanczik, S. (2017). A new family, Pervetustaceae with a new genus, *Pervetustus* , and *P. simplex sp*. nov. (Paraglomerales), and a new genus, Innospora with *I. majewskii* comb. nov. (Paraglomeraceae) in the Glomeromycotina. Nova Hedwigia, 105 (4): 397-410.

Bonfante, P. & Genre, A. (2008). Plants and arbuscular mycorrhizal fungi: an evolutionary-developmental perspective. Trends in Plant Science, 13 (9): 492-498.

Bonfante, P. (1984). Anatomy and morphology of VA mycorrhizae. VA Mycorrhiza (1): 5–33.

Bonfante, P. (2001). At the interface between mycorrhizal fungi and plants: the structural organization of cell wall, plasma membrane and cytoskeleton. Hock B. (Ed) Fungal Associations (9): 45–61.

Brodt, S., Six, J., Feenstra, G., Ingels, C. & Campbell, D.. (2011). Sustainable Agriculture. Retrieved from https://www.nature.com/scitable/knowledge/library/sustainable-agriculture-23562787/

Brundrett, M. (1991). Mycorrhizas in natural ecosystem. Advances in Ecological Research (21): 300–313.

Cabrera, R., García, H., Aguirre, E. & Orozco, J. (2020). *Amycolatopsis* BX17: An actinobacterial strain isolated from soil of a traditional milpa agroecosystem with potential biocontrol against *Fusarium graminearum*. Biological Control 147(1): 104285.

Calabresem S., Kohler, A., Niehl, A., Veneault-Fourrey, C., Boller, T. & Courty, P. (2017). Transcriptome analysis of the Populus trichocarpa–*Rhizophagus irregularis* Mycorrhizal Symbiosis: Regulation of Plant and Fungal Transportomes under Nitrogen Starvation. Plant and Cell Physiology 58 (6): 1003–1017.

Campos, C., Carvalho, M., Brigido, C., Goss, M. & Nobre, T. (2018). Symbiosis specificity of the preceding host plant can dominate but not obliterate the association between wheat and its arbuscular mycorrhizal fungal partners. Frontiers in Microbiology, 9: 2920.

Cantrell, I.C., Linderman, R.G. (2001). Pre Inoculation of lettuce and onion with VA mycorrhizal fungi reduces deleterious effects of soil salinity. Plant and Soil, 233: 269–28.

Casieri, L., Ait Lahmidi, N., Doidy, J., Veneault-Fourrey, C., Migeon, A., Bonneau, L., Courty, P.E., Garcia, K., Charbonnier, M., Delteil, A., Brun, A., Zimmermann, S., Plassard, C. & Wipd, D. (2013). Biotrophic transportome in mutualistic plant-fungal interactions. Mycorrhiza, 23: 597–625.

Charpentier, M., Bredemeier, R., Wanner, G, Takeda, N., Schleiff, E. & Parniske, M. (2008). Lotus japonicus CASTOR and POLLUX Are Ion Channels Essential for Perinuclear Calcium Spiking in Legume Root Endosymbiosis. The Plant Cell, 20(12): 3467–3479.

Chen, C., Fan, C., Gao, M & Zhu, H. (2009). Antiquity and Function of CASTOR and POLLUX, the Twin Ion Channel-Encoding Genes Key to the Evolution of Root Symbioses in Plants. Plant Physiology, 149(1): 306-17.

Chen, M., Arato, M., Borghi, L., Nouri, E. & Reinhardt, D. (2018). Beneficial Services of Arbuscular Mycorrhizal Fungi – From Ecology to Application. Frontiers in Plant Science, 9: 1270.

Clark, R. & Zeto, S. (1996). Mineral acquisition by mycorrhizal maize grown on acid and alkaline soils. Soil Biology and Biochemistry, 28 (11): 1495-1503.

Colla, G., Rouphael, Y., Cardarelli, M., Tullio, M., Rivera, C. & Rea, E. (2008). Alleviation of the salt stress by arbuscular mycorrhizal and zucchini plants grown at low and high phosphorus concentration. Biology and Fertility Soils, 44: 501-509.

Colombo, R., Benavidez, M., Bidondo, L., Silvani, V., Bompadre, M., Statello, M., Scorza, M., Scotti, A. & Godeas, A. (2019). Arbuscular mycorrhizal fungi in heavy metal highly polluted soil in the Richuelo river basin. Revista Argentina de Microbiología, 52(2): 145-149.

Correa-Galeote, D., Bedmar, E. & Arone, G. (2018). Maize endophytic bacterial diversity as affected by soill cultivation history. Frontiers in Microbiology, 9(484): 1-9.

Cramer, G., Urano, K., Delrot, S., Pezzotti, M. & Shinozaki, K. (2011). Effects of abiotic stress on plants: a system biology perspective. BMC Plant Biology, 11: 163.

D'Haeze, W. & Holsters, M. (2002) Nod factor structures, responses, and perception during initiation of nodule development. Glycobiology 12(6): 79–105.

Debellé, F. (2019). The model Legume *Medicago tuncatula* (1st Ed). John Wiley & Sons, Inc. p: 523-528.

De novais, C., Lustrino, W., Conceicao, E., Saggin, O. & Siquiera, J. (2014). Inter- and intraspecific functional variability of tropical arbuscular mycorrhizal fungi isolates colonizing corn plants. Applied Soil Ecology, 76: 78–86.

Dreyer, I., Spitz, O., Kanonberg, K., Montag, K., Handrich, M., Ahmad, S., Schott-Verdugo, S., Navarro-Retamal, C., Rubio-Meléndez, M., Gomez-Parras, J., Riedelberger, J., Molina-Montenegro, M., Sucurro, A., Zucarro, A., Gould, S., Bauer, P., Schmitt, L. & Gohlke, H. (2019). Nutrient exchange in arbuscular mycorrhizal symbiosis from a thermodynamic point of view. New Phytologist 222(2): 1043–1053.

Drigo, B., Pijl, A.S., Duyts, H., Kielak, A.M., Gamper, H.A., Houtekamer, M.J., Boschker, H.T., Bodelier, P.L., Whiteley, A.S., van Veen, J.A., Kowalchuk, G.A. (2010). Shifting carbon flow from roots into associated microbial communities in response to elevated atmospheric CO2. PNAS, 107:10938–10942.

Egerton-Warburton, L. M., Querejeta, J. I., & Allen, M. F. (2007). Common mycorrhizal networks provide a potential pathway for the transfer of hydraulically lifted water between plants. Journal of Experimental Biology, 58: 1473-1483.

Etesami, H. & Maheshwari, D. (2018). Use of plant growth promoting rhizobacteria (PGPRs) with multiple plant growth promoting traits in stress agriculture: action mechanisms and future prospects. Ecotoxicology and Environmental Safety, 156: 225-246.

Ercolin, F. & Reinhardt, D. (2011). Successful joint ventures of plants: arbuscular mycorrhiza and beyond. Trends in Plant Science, 16(7): 356 - 362.

Evelin, H., Kapoor, R & Giri, B. (2009). Arbuscular mycorrhizal fungi in alleviation of salt stress. Annals of Botany, 104(7): 1263–1280.

Facelli, E., Smith, S.E., Facelli, J.M., Christophersen, H.M. & Smith, F. (2010). Underground friends or enemies: model plants help to unravel direct and indirect effects of arbuscular mycorrhizal fungi on plant competition. New Phytologist, 185: 1050–1061.

FAO. (2017). Global information and early warning system on Food and Agriculture. Retrieved from https://www.fao.org/giews/en/

FAO. (2009). Cambio Climático: El impacto en la agricultura y los costos de adaptación. Retrieved from http://www.fao.org/fileadmin/user_upload/AGRO_Noticias/docs/costo%20adaptacion.pdf

Ferrol, N., Azcón-Aguilar, C., Bago, B., Franken, P., Gollote, A., González, M., Harrier, L., Lanfranco, L., Tuinen, D. & Gianinazzi, V. (2004). Genomics of Arbuscular Mycorrhizal Fungi. Applied Mycology & Biotechnology, 4: 379-403.

Frechill, S., Lasa, B., Ibarretxe, L., Lamsfus, C., Aparicio Trejo, P. (2001). Pea response to saline stress is affected by the source of nitrogen nutrition (ammonium or nitrate). Plant Growth Regulation, 35: 171-179.

Gaiero, J., McCall, C., Thompson, K., Day, N., Best, A. & Dunfield, K. (2013). Inside the root microbiome: bacterial root endophytes and plant growth promotion. American Journal of Botany, 100(9): 1738-1750.

Galarza, J., Piña, U., Muñoz, D. & Rivera, G. (2004). Situación actual y perspectiva del maíz en México 1990-2004. Retrieved from https://www.gob.mx/cms/uploads/attachment/file/97933/maiz90-04.pdf

Gamalero, E., Lingua, G., Berta, G. & Glick, B. (2009). Beneficial role of plant growth promoting bacteria and arbuscular mycorrhizal fungi on plant responses to heavy metal stress. Canadian Journal of Microbiology, 55: 501–514.

Garcia de Salamone, I., Döbereiner, J., Urquiaga, S., Boddey, R. (1996) Biological nitrogen fixation in Azospirillum strain-maize genotype associations as evaluated by 15N isotope dilution technique. Biology and Fertility Soils, 23: 249–256.

Gaur, A. & Adholeya, A. (2004). Prospects of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils. Current Science, 86(4): 528-534.

Genre, A., Chabaud, M., Belzergue, C., Puech, V., Novero, M., Rey, T., Fournier, J., Rochange, S., Bécard, G., Bonfante, P. & Barker, D. (2013). Short Chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca2+ spiking in Medicago truncatula roots and their production is enhanced by strigolactone. New Phytologist, 198: 190–202.

Genre, A., Chabaud, M., Timmers, T., Bonfante, P., Barker, D.G. (2005). Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in Medicago truncatula root epidermal cells before infection. Plant Cell, 17: 3489–3499.

Genre, A., Chabaud, M., Faccio, A., Barker, D.G., Bonfante, P. (2008). Prepenetration apparatus assembly precedes and predicts the colonization patterns of arbuscular mycorrhizal fungi within the root cortex of both Medicago truncatula and Daucus carota. Plant Cell, 20: 1407–1420.

Genre, A., Ivanov, S., Fendrych, M., Faccio, A., Zársky, V., Bisseling, T., Bonfante, P. (2012). Multiple exocytotic markers accumulate at the sites of perifungal membrane biogenesis in arbuscular mycorrhizas. Plant Cell Physiology, 53: 244–255.

Geurts, R. & Bisseling, T. (2002). Rhizobium Nod Factor Perception and Signalling. Plant Cell, 14: 239–249.

Giangacomo, C., Mohseni, M., Kovar, L. & Wallace, J. (2021). Comparing DNA Extraction and 16S rRNA Gene Amplification Methods for Plant-Associated Bacterial Communities. Phytobiomes Journal, 5: 190 - 201.

Gianinazzi, S., Gollotte, A., Binet, M. N., van Tuinen, D., Redecker, D. & Wipf, D. (2010). Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. Mycorrhiza, 20: 519–530

Giovannini, L., Palla, M., Agnolucci, M., Avio, L., Sbrana, C., Turrini, A. & Giovanetti, M. (2020). Arbuscular Mycorrhizal Fungi and Associated Microbiota as Plant Biostimulants: Research Strategies for the Selection of the Best Performing Inocula. Agronomy 10(1): 106.

Govindarajulu, M., Pfeffer, P.E., Jin, H., Abubaker, J., Douds, D.D., Allen, J.W., Bücking, H., Lammers, P.J., Shachar-Hill, Y. (2005). Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature, 435: 819–823.

Gutjahr, C., Banba, M., Croset, V., An, K., Miyao, A., An, G., Hirochika, H., Imaizumi, H. & Paszkowski, U. (2008). Arbuscular Mycorrhiza–Specific Signaling in Rice Transcends the Common Symbiosis Signaling Pathway. The Plant Cell, 20: 2989–3005.

Gutjahr, C., Gobatto, E., Choi, J., Riemann, M., Johnston, M., Summers, W., Carbonell, S., Mansfield, C., Yang, S., Nadal, M., Acosta, I., Takano, M., Jiao, W., Schneeberger, K., Kelly, K. & Paszkowski, U. (2015). Rice perception of symbiotic arbuscular mycorrhizal fungi requires the karrikin receptor complex. Science, 350: 1521–1524.

Guether, M., Neuhäuser, B., Balestrini, R., Dynowski, M., Ludewig, U., & Bonfante, P. (2009). A mycorrhizal-specific ammonium transporter from Lotus japonicus acquires nitrogen released by arbuscular mycorrhizal fungi. Plant Physiology, 150: 73–83.

Hayashi, T., Banba, M., Shimoda, Y., Kouchi, H., Hayashi, M., & Imaizumi-Anraku, H. (2010). A dominant function of CCaMK in intracellular accommodation of bacterial and fungal endosymbionts. Plant Journal, 63: 141–154.

Honrubia, M. (2009). The Mycorrhizae: a plant-fungus relation that has existed for more than 400 million years. Anales del Jardín Botánico de Madrid, 66 (1): 334.

Huang, H., Zi, X., Lin, H. & Gao, J. (2018). Host-specificity of symbiotic mycorrhizal fungi for enhancing seed germination, protocorm formation and seedling development of over-collected medicinal orchid, Dendrobium devonianum. Journal of Microbiology, 56(1): 42-48.

Ikeda, A., Stringari, D., Adamoski, D. & Kava, V. (2013). Morphological and genetic characterization of endophytic bacteria isolated from roots of different maize genotypes. Microbial Ecology, 65: 154–160.

Jiao, X., Zhang, X., Lu, X. & Qin, R. (2019). Effects of maize rotation on the physicochemical properties and microbial communities of American ginseng cultivated soil. Scientific Reports, 9: 8615.

Johnson, J., Spakowicz, D., Hong, B., Petersen, L., Demkowicz, P., Chen, L., Leopold, S., Hanson, B., Agresta, H., Gerstein, M., Sodergren, E. & Weinstock, G. (2019). Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nature Communications, 10: 5029.

Karimi, A., Khodaverdiloo, H., Sepehri, M. & Rasouli-Sadaghiani, M. (2011). Arbuscular mycorrhizal fungi and heavy metal contaminated soils. Journal of Hazardous Materials, 402: 123919.

Kikuchi, Y., Hijikata, N., Ohtomo, R., Handa, Y., Kawaguchi, M., Saito, K., Masuta, C. & Ezawa, T. (2016). Aquaporin-mediated long-distance polyphosphate translocation directed towards the host in arbuscular mycorrhizal symbiosis: application of virus-induced gene silencing. New Phytologist, 211(4): 1202-1208.

Kobae, Y., Tamura, Y., Takai, S., Banba, M., Hata, S. (2010). Localized expression of arbuscular mycorrhiza-inducible ammonium transporters in soybean. Plant Cell Physiology, 51: 1411–1415.

Koegel, S., Ait Lahmidi, N., Arnould, C., Chatagnier, O., Walder, F., Ineichen, K., Boller, T., Wipf, D., Wiemken, A., Courty, P.E. (2013). The family of ammonium transporters (AMT) in Sorghum bicolor: two AMT members are induced locally, but not systemically in roots colonized by arbuscular mycorrhizal fungi. New Phytologist, 198: 853–865.

Kosuta, S., Hazledine, S., Sun, J., Miwa, H., Morris, R., Downie, A. & Oldroyd, G. (2008). Differential and chaotic calcium signatures in the symbiosis signaling pathway of legumes. PNAS, 105(28): 9823–9828.

Krüger, M., Stockinger, H., Schüßler, A. (2009). DNA-based species level detection of Glomeromycota: one PCR primer set for all arbuscular mycorrhizal fungi. New Phytologist, 183: 212–223.

Kumar, B., Vijayakumar, S. & Choudhyru, S. (2021). Exploring nod factor receptors activation process in chickpea by bridging modelling, docking and molecular dynamics simulations. International Journal of Biological Macromolecules, 189: 965-979.

Kumar, R., Kumawat, N. & Sahu, Y. (2018). Role of Biofertilizers in Agriculture. Popular Kheti, 5(4): 63-66.

Laffite, H. (N.d.). Estreses abióticos que afectan al maíz. Retrieved from http://www.fao.org/3/x7650s12.htm

Lanfranco, L., Wyss, P., Marzachi, C., Bonfante, P. (1995). Generation of RAPD-PCR primers for the identification of isolates of Glomus mosseae, an arbuscular mycorrhizal fungus. Molecular Ecology 4(1): 61-68.

Lanfranco, L., Bonfante, P., & Genre, A. (2016). The mutualistic interaction between plants and arbuscular mycorrhizal fungi. Microbiology Spectrum 4(6): 1-20.

Lee, H. & Eom, A. (2015). Growth characteristics of rhizophagus clarus strains and their effects on the growth of host plants. Mycobiology, 43(4): 444–449.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.C., & Dénarié, J. (1990). Symbiotic host-specificity of Rhizobium meliloti is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature, 344: 781–784.

Lingua, G., Bona, E., Todeschini, V., Cattaneo, C., Marsano, F., Berta, G. & Cavaletto, M. (2012). Effects of Heavy Metals and Arbuscular Mycorrhiza on the Leaf Proteome of a Selected Poplar Clone: A Time Course Analysis. PLoS ONE, 7(6): e38662.

Liu, Y., Zuo, S., Zou, Y., Wang, J. & Song, W. (2012). Investigation on diversity and population succession dynamics of endophytic bacteria from seeds of maize (Zea mays L., Nongda108) at different growth stages. Annals of Microbiology, 63: 71–79.

Liu, W., Zhang, Y., Jiang, S., Christie, P, Deng, Y., Murray, P., Li, X. & Zhang, J. (2016). Arbuscular mycorrhizal fungi in soil and roots respond differently to phosphorus inputs in an intensively managed calcareous agricultural soil. Scientific Reports, 6: 24902.

Liu, Y., et (2020). Investigating the endophytic bacterial diversity and community structures in seeds of genetically related maize (Zea mays L.) genotypes. Biotech, 10: 27.

Londoño, D., Meyer, E., da Silva, K., González, A., Dutra, R., Macedo, L., Luiz, S., Onofre, R., Fonseca, R. & Lovato, P. (2020). Root colonization and arbuscular mycorrhizal fungal community composition in a genetically modified maize, its non-modified isoline, and a landrace. Mycorrhiza, 30: 611–621.

López-Pedrosa, A., González-Guerrero, M., Valderas, A., Azcón-Aguilar, C., Ferrol, N. (2006). GintAMT1 encodes a functional high-affinity ammonium transporter that is expressed in the extraradical mycelium of Glomus intraradices. Fungal Genetics and Biology, 43: 102–110.

Maekawa, M. & Murooka, Y. (2009). Root Hair Deformation of Symbiosis-Deficient Mutants of Lotus japonicus by Application of Nod Factor from *Mesorhizobium loti*. Microbes and Environments, 24(2): 128–134.

Mano, H. & Morisaki, H. (2008). Endophytic Bacteria in the Rice Plant. Microbes and Environments, 23(2): 109-117.

Manter, D., Delgado, J., Holm, D. & Stong, R. (2010). Pyrosequencing Reveals a Highly Diverse and Cultivar-Specific Bacterial Endophyte Community in Potato Roots. Microbial Ecology, 60: 157–166.

Miller, M.A., Pfeiffer, W., & Schwartz, T. (2010). Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Gateway Computing Environments Workshop (GCE), 2010: 1-8.

Miller, J.B., Pratap, A., Miyahara, A., Zhou, L., Bornemann, S., Morris, R.J., & Oldroyd, G.E.D. (2013). Calcium/calmodulin dependent protein kinase is negatively and positively regulated by calcium, providing a mechanism for decoding calcium responses during symbiosis signaling. Plant Cell, 25: 5053–5066.

Miwa, H., Sun, J., Oldroyd, G. E. & Downie, J. A. (2006). Analysis of Nod-factor-induced calcium signaling in root hairs of symbiotically defective mutants of Lotus japonicus. Mol. Plant Microbe Interaction, 19: 914–923.

Munkvold, L., Kjøller, R., Vestberg, M., Rosendahl, S. & Jakobsen, I. (2004). High functional diversity within species of arbuscular mycorrhizal fungi. New Phytologist, 164: 357–364.

Nogueira, E., Zangaro, W., & Nogueira, M. (2017). Diversity and Benefits of Microorganism from the Tropics (1st Ed). Springer, 245-267.

Nozari, R., Ortolan, F., Astarita, L. & Romanato, E. (2021). *Streptomyces spp*. Enhance vegetative growth of maize plants under saline stress. Brazilian Journal of Microbiology, 52: 1371-1383.

Oehl, F., Da-Silva, G., Goto, B., Costa, L. & Sieverding, E. (2011). Glomeromycota: two new classes and a new order. Mycotaxon, 116: 365-379.

Olanrewaju, O.S., Babalola, O.O. (2019) Streptomyces: implications and interactions in plant growth promotion. Applied Microbiology and Biotechnology, 103: 1179–1188.

Oldroyd, G. & Downie, J. (2008). Coordinating nodule morphogenesis with rhizobial infection in legumes. Annual Review of Plant Biology, 59: 519-546.

Parida, S.K. & Das, A. B. (2009). Salt tolerance and salinity stress effect on plants. Ecotoxicology and Environmental Safety, 65: 553-469.

Parniske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nature Reviews Microbiology, 6: 763-775.

Pawlowska, T. & Charvat, I. (2004). Heavy-metal stress and developmental pattern of arbuscular mycorrhizal fungi. Applied and Environmental Microbiology, 70(11): 6643-6649.

Pereira, P., Ibáñez, F., Rosenblueth, M., Etcheverry, M. & Martínez, E. (2011). Analysis of the Bacterial Diversity Associated with the Roots of Maize (*Zea mays L.*) through Culture-Dependent and Culture-Independent Methods. International Scholarly Research Network, 11: 1-10.

Pérez-Tienda, J., Testillano, P.S., Balestrini, R., Fiorilli, V., Azcón-Aguilar, C., Ferrol, N. (2011). GintAMT2, a new member of the ammonium transporter family in the arbuscular mycorrhizal fungus Glomus intraradices. Fungal Genetics Biology 48: 1044–1055.

Porcel, R., Aroca, R. & Ruiz-Lozano, J. (2011). Salinity stress alleviation using arbuscular mycorrhizal fungi: A review. Agronomy and Sustainable Development, 32: 181–200.

Porcel, R., Aroca, R., Azcon, R., & Ruiz-Lozano, J. M. (2006). PIP aquaporin gene expression in arbuscular mycorrhizal Glycine max and Lactuca sativa plants in relation to drought stress tolerance. Plant Molecular Biology, 60: 389-404.

Porcel, R., Azcon, R., & Ruiz-Lozano, J. M. (2004). Evaluation of the role of genes encoding for D1-pyrroline-5-carboxlyate synthetase (P5CS) during drought stress in arbuscular mycorrhizal Glycine max and Lactuca sativa plants. Physiological and Molecular Plant Pathology, 65: 211-221.

Pulford, I., Watson, C. (2003) Phytoremediation of heavy-metal contaminated land by trees: a review. Environmental International, 29(4): 529-540.

Qi, D., Zou, L., Zhou, D., Chen, Y., Gao, Z., Feng, R., Zhang, M., Li, K., Xie, J. & Wang, W. (2019). Taxonomy and broad-spectrum antifungal activity of *Streptomyces sp.* SCA3-4 isolated from rhizosphere soil of *Opuntia stricta*. Frontiers in Microbiology, 10: 1390.

Ramírez-Flores, R., Pérez-Limón, S., Li, M., Berrales-Gamez, B., Albinsky, D., Paszkowski, U., Olalde-Portugal, V. & Sawers, R. (2020). The genetic architecture of host response reveals the importance of arbuscular mycorrhizae to maize cultivation. eLife 19(9): e61701.

Rathod, S., Ahmad, M., Gade, A. & Shrivastav, R. (2012). Microbial Endophyte: Progress and Challenges. Biotechnology for Medicinal Plants, 4(1): 101 – 121.

Redecker, D., Hijri, M., Dulieu, H. & Sanders, I. (1999). Phylogenetic Analysis of a Dataset of Fungal 5.8S rDNA Sequences Shows That Highly Divergent Copies of Internal Transcribed Spacers Reported from *Scutellospora castanea* Are of Ascomycete Origin. Fungal Genetics and Biology, 28: 238–244.

Redecker, D., Hijri, I, Wiemken, A. (2003). Molecular identification of Arbuscular Mycorrhizal Fungi in roots: perspectives and problems. Folia Geobotanica, 38: 113-124.

Rillig, M.C., Aguilar-Trigueros, C.A., Bergmann, J., Verbruggen, E., Veresoglou, S. D. & Lehmann, A. (2015). Plant root and mycorrhizal fungal traits for understanding soil aggregation. New Phytologist, 205: 1385–1388.

Rohyadi, A., Smith, F., Murray, R. & Smith, S. (2003). Effects of pH on mycorrhizal colonisation and nutrient uptake in cowpea under conditions that minimise confounding effects of elevated available aluminium. Plant and Soil, 260: 283–290.

Ruiz-Lozano, J. M., & Aroca, R. (2010). Host response to osmotic stresses: stomatal behaviour and water use efficiency of arbuscular mycorrhizal plants. H. Koltai & Y. Kapulnik (Eds.), Arbuscular mycorrhizas: Physiology and Function (pp. 239–256). Berlin: Springer.

Ruiz-Lozano, J. M., Mar Alguacil, M., Barzana, G.,Vernieri, P., & Aroca, R. (2009). Exogenous ABZ accentuates the differences in root hydraulic properties between mycorrhizal and non mycorrhizal maize plants through regulation of PIP aquaporins. Plant Molecular Biology, 70: 565-579.

Ryan, R., Germaine, K., Franks, A., Ryan, D. & Dowling, D. (2008). Bacterial endophytes: recent developments and applications. FEMS Microbiology Letters, 278: 1-9.

Sadhana, B. (2014). Arbuscular Mycorrhizal Fungi (AMF) as a Biofertilizer- a Review. International Journal of Current Microbiology and Applied Sciences, 3(4): 384-400.

Sanders, I., Alt, M., Groppe, K, Boller, T., Wiemken, A. (1995). Identification of ribosomal DNA polymorphisms among and within spores of the Glomales: application to studies on the genetic diversity of arbuscular mycorrhizal fungal communities. New Phytologist, 130(3): 419-427.

Senés-Guerrero, C., & Schüßler, A. (2016). A conserved arbuscular mycorrhizal fungal core-species community colonizes potato roots in the Andes. Fungal Diversity, 77: 317–333.

Senés-Guerrero, C., Giménez, S., Pacheco, A., Gradilla-Hernández, S., & Schüßler, A. (2020). New MiSeq based strategy exposed plant-preferential arbuscular mycorrhizal fungal communities in arid soils of Mexico. Symbiosis, 81: 235–246.

Serralde A.M. & Ramírez M.M. (2004) Análisis de poblaciones de micorrizas en maíz (Zea mays) cultivado en suelos ácidos bajo diferentes tratamientos agronómicos. Ciencia y Tecnología Agropeccuaria, 5: 31– 40.

Sharma, N., Yadav, K., Cheema, J., Badda, & Aggarwal, A. (2015). Arbuscular Mycorrhizal Symbiosis and Water Stress. Pertanika Journal of Tropical Agricultural Science, 38: 427 - 453.

Simón, L., Lévesque, R., Lalonde, M. (1993). Identification of Endomycorrhizal Fungi Colonizing Roots by Fluorescent Single-Strand Conformation Polymorphism-Polymerase Chain Reaction. Applied and Environmental Microbiology, 59(12): 4211-4215

Smith, S., Smith, F.(2012). Fresh perspectives on the roles of arbuscular mycorrhizal fungi in plant nutrition and growth. Mycologia, 104: 1–13.

Singh, P., Srivastava, D., Jaiswar, A. & Adholeya, A. (2019). Effector proteins of Rhizophagus proliferus: conserved protein domains may play a role in host-specific interaction with different plant species. Brazilian Journal of Microbiology, 50(3): 593-601.

Tisserant, E., Kohler, A., Dozolme, P., Balestrini, R., Benabdellah, K., Colard, A., Croll, D., Da Silva, C., Gomez, S., Koul, R., Ferrol, N., Fiorilli, V., Formey, D.., Franken, P., Helber, N., Hijri, M., Lanfranco, L., Linquist, E., Liu, Y., Malbreil, M., Morin, E., Poulain, J., Shapiro, H., Tuinen, D., Waschke, A., Azcón, C., Bécard, G., Bonfante, P., Harrison, M., Küster, H., Lammers, P., Paszkowski, U., Requena, N., Rensing, S., Roux, C., Sanders, I., Shachar, Y., Tuskan, G., Young, J., Gianinazzi, V. & Martin, F. (2012). The transcriptome of the arbuscular mycorrhizal fungus Glomus intraradices (DAOM 197198) reveals functional tradeoffs in an obligate symbiont. New Phytologist, 193: 755–769.

Tirichine, L., Imaizumi, H., Yoshida, S., Murakami, Y., Madsen, L., Miwa, H., Nakagawa, T., Sandal, N., Albrektsen, A., Kawaguchi, M., Downie, A., Sato, S., Tabata, S., Kouchi, H., Parniske, M., Kawasaki, S. & Stougaard, J. (2006). Deregulation of a Ca2+/calmodulindependent kinase leads to spontaneous nodule development. Nature, 441: 1153–1156.

Union of Concerned Scientist UCSUSA. (2017). What is sustainable agriculture? Retrieved from https://www.ucsusa.org/resources/what-sustainable-agriculture.

Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., & Nasrulhaq Boyce, A. (2016). Role of plant growth promoting rhizobacteria in agricultural sustainability-a review. Molecules, 21: 573.

Venkateshwaran, M., Cosme, A., Han, L., Banba, M., Satyshur, K., Schleiff, E., Parniske, M., Imaizumi, H. & Ané, J. (2012). The Recent Evolution of a Symbiotic Ion Channel in the Legume Family Altered Ion Conductance and Improved Functionality in Calcium Signaling. The Plant Cell, 24: 2528–2545.

Vigani, G., Rolli, E., Marasco, R., Dell'Orto, M., Michoud, G., Soussi, A., Raddadi, N., Borin, S., Sorlini, C., Zocchi, G. & Daffonchio, D. (2018) Root bacterial endophytes confer drought resistance and enhance expression and activity of a vacuolar H+ -pumping pyrophosphatase in pepper plants. Environmental Microbiology, 21(9): 1-90.

Wang, Z., Bi, Y., Jian, B., Zhakypbek, Y., Peng, S., Liu, W. & Liu, H. (2016). Arbuscular mycorrhizal fungi enhance soil carbon sequestration in the coalfields, northwest China. Microorganisms, 8(2): 230.

Warrad, M., Hassan, Y., Mohamed, M., Hagagy, N., A-Maghrabi, A., Selim, S., Saleh, A. & AbdElgawad, H. (2020). A bioactive fraction from Streptomyces sp. Enhances maize tolerance against drought stress. Journal of Microbiology and Biotechnology, 30(8): 1156–1168.

Weissenhorn, I., Leyval, C., Belgy, G. & Berthelin, J. (1995). Arbuscular mycorrhizal contribution to heavy metal uptake by maize (Zea mays L.) in pot culture with contaminated soil. Mycorrhiza, 5: 245-251.

Willis, A., Rodrigues, B. & Harris, P. (2013). The ecology of arbuscular mycorrhizal fungi. Critical Reviews in Plant Science, 32: 1–20.

Wu, Q. & Ying-Ning, Z. (2017). Arbuscular mycorrhizal fungi and drought stress in plants. Arbuscular mycorrhizas and stress tolerance of plants, 1: 25-41.

Zhang, J., Jia, W., Yang, J., & Ismail, A. M. (2006). Role of ABA in integrating plant responses to drought and salt stresses. Field Crop Research, 97, 111-119. Auge, R. M. (2004). Arbuscular mycorrhizae and soil/plant water relations. Canadian Journal of Soil Science, 84: 373-381.

Zhu, H., Riely, B., Burns, N., Ane, J. (2006). Tracing nonlegume orthologs of legume genes required for nodulation and arbuscular mycorrhizal symbioses. Genetics, 172(4): 2491-2499.

Annex 1.

Common symbiosis pathway components. Plant genes required for the symbiosis of AMF, gene function and corresponding phenotypes (Parniske, 2008).

Gene	Mutants	Function	Ca^{2+} spiking	Mutant AM phenotype	Root-nodule phenotype
SYMRK	*Lotus japonicus: symrk, sym2* *Medicago truncatula: dmi2,* *Medicago sativa nork* *Pisum sativum: sym19*	Common entry of the point into the symbiotic signalling pathway. Receptor-like kinase. Perceives the extracellular signals from microbial symbionts and transduce this perception event through its intracellular kinase domain.	No	Impaired intracellular passage through the outer cell layers.	Non-nodulating
CASTOR	*Lotus japonicus: sym4, sym7* *Zea mays: hun1-1, hun1-2*	Cation channel. Compensates for the rapid charge imbalance that is produced during calcium spiking.	No	Impaired intracellular passage through the outer cell layers and impaired arbuscule formation.	Non-nodulating
POLLUX	*Lotus japonicus: sym23, sym73, sym85* *Medicago truncatula: dmi1* *Pisum sativum: sym8*	Cation channel. Compensates for the rapid charge imbalance that is produced during calcium spiking.	No	Impaired intracellular passage through the outer cell layers.	Non-nodulating
NUP85	*Lotus japonicus: sym24, sym73, sym85*	Putative nuclear pore component.	No	Impaired intracellular passage through the outer cell layers. Temperature sensitive.	Temperature sensitive.
NUP133	*Lotus japonicus: sym133, sym45*	Putative nuclear pore component.	No	Impaired intracellular passage through the outer cell layers.	Temperature sensitive.

				Temperature sensitive.	
CCaMK	*Lotus japonicus: sym15, sym72* *Medicago truncatula: dmi3* *Pisum sativum: sym9*	Calcium and calmodulin-dependent protein kinase. Response to calcium spikes.	Yes	Impaired epidermal opening, impaired intracellular passage through the outer cell layers and impaired arbuscule formation.	Non-nodulating.
CYCLOPS	*Lotus japonicus: sym6, sym30, sym82* *Medicago truncatula: ipd3*	Acts in an infection-specific branch of the symbiotic signalling network.	Yes	Impaired intracellular passage through the outer cell layers and impaired arbuscule formation.	Small, non-infected nodules.