MYCORRHIZA IN TROPICAL AND NEOTROPICAL ECOSYSTEMS

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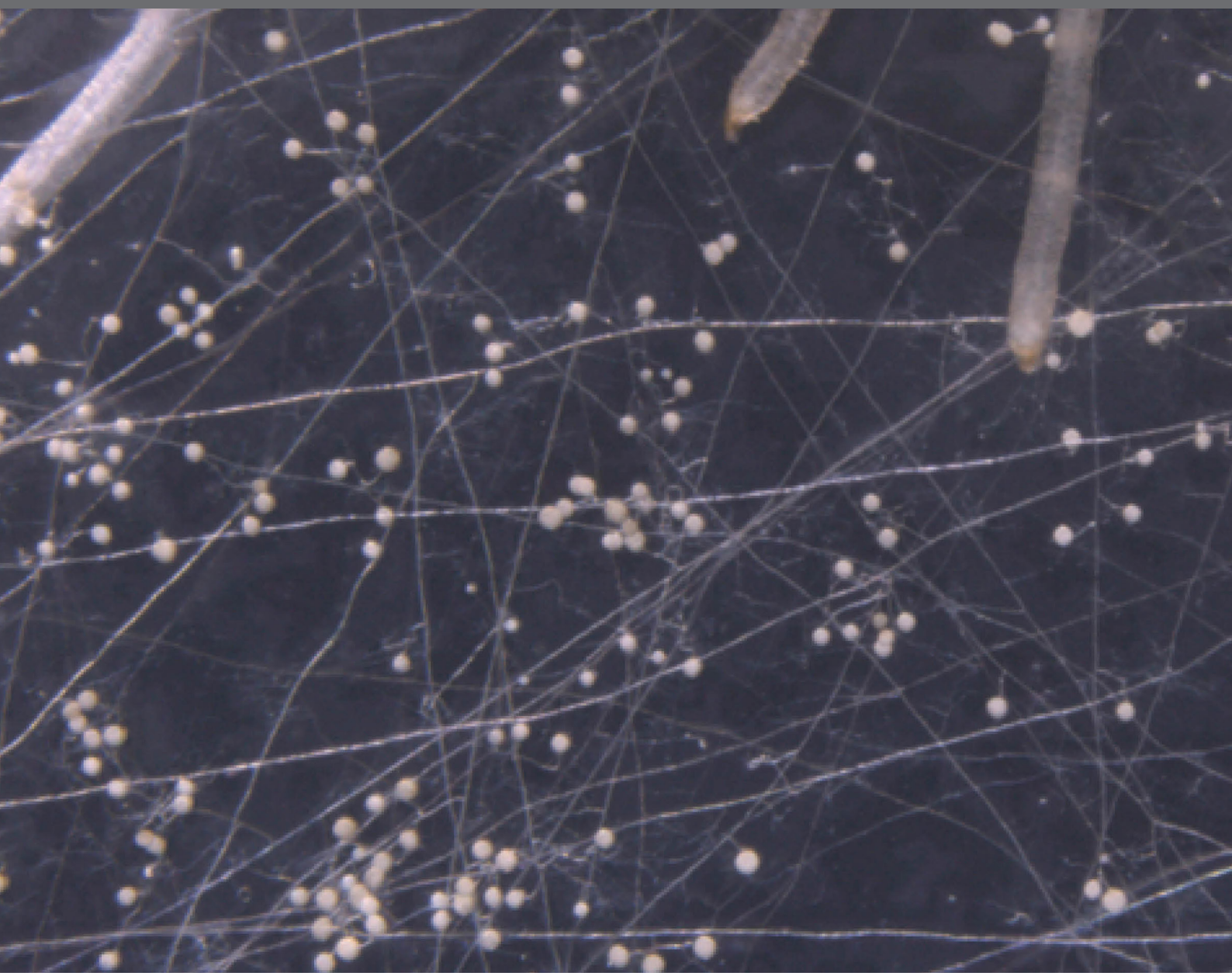

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MYCORRHIZA IN TROPICAL AND NEOTROPICAL ECOSYSTEMS

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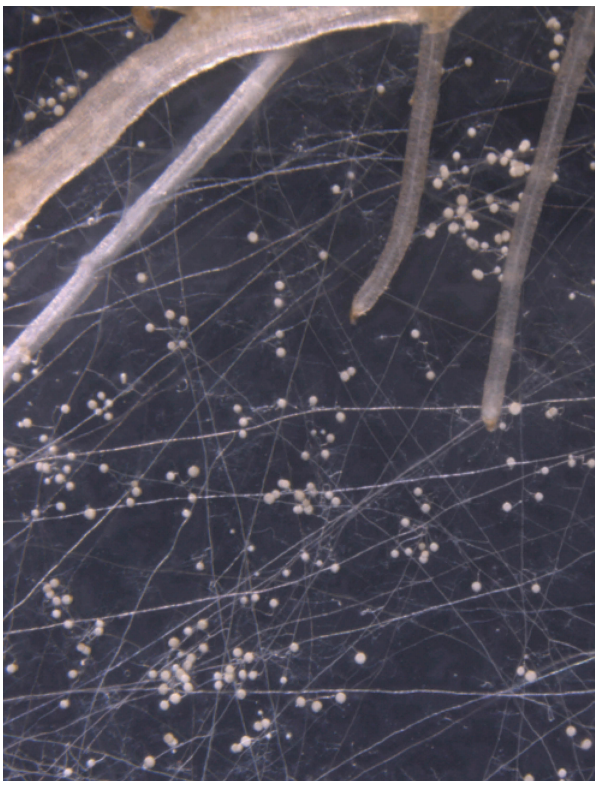

In vitro co-culture of Rhizophagus irregularis with carrot roots showing extraradical mycelium acting as root extension and asexual spores.
Image credit: M. Hijri.

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EDITORIAL
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Editorial: Mycorrhiza in Tropical and Neotropical Ecosystems

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Editorial on the Research Topic

Mycorrhiza in Tropical and Neotropical Ecosystems

Mycorrhizal symbiosis is a mutualistic plant-fungus association that plays a major role in the function, maintenance and evolution of biodiversity and agroecosystems stability and productivity. The fungus provides mineral nutrients, water, protection against pathogens, alleviation of abiotic stresses such as salinity, drought and pollution, to the plant which, in return, provides carbon as an energy source to fungus. Threats to biodiversity destruction in tropical and neotropical agroecosystems should encourage fast inventory of the diversity and function of mycorrhizae in tropical latitudes which are biodiversity hotspots. Mycorrhizal fungi and Plant Growth Promoting Rhizobacteria (PGPR) are important components in forestry and agriculture which have encouraged their utilization over the past decades (Diédhiou et al., 2005; Hijri, 2016). Mycorrhizal- and PGPR-based commercial inoculants are sold world-wide as biofertilizers in a variety of formulations in agriculture, horticulture and even in forestry. However, the success of these products is variable because of limited data on large-scale field applications particularly in tropical and sub-tropical agrosystems (Hijri, 2016).

Ectomycorrhizal (EM) associations can influence plant community assembly and facilitate plant coexistence in boreal and temperate regions (van der Heijden et al., 1998), but little is known in tropical and neotropical forests (Bâ et al., 2012; Ebenye et al., 2017). To this end, Essene et al. compared assembly patterns of EM fungi in bulk soil to EM root tips collected from three ecologically distinct species of dipterocarp in a Bornean lowland tropical rain forest. They found that soil type had a stronger role than Dipterocarp host species in shaping the EM fungal community.

Asmelash et al. presented a review on the potential role of arbuscular mycorrhizal fungi (AMF) to significantly improve successful restoration success of degraded lands where levels of infective AM abundance and diversity are often low. They concluded that successful restoration of infective propagules can potentially improve the restoration success of degraded lands.

Symanczik et al. compared the performance of three non-native AMF strains (*Rhizoglomus irregulare, Claroideoglomus claroideum*, and *Cetraspora helvetica*) with native communities contained in three soils (conventional, organic, and permaculture) from naranjilla plantations in Ecuador. The growth response experiment has shown that two of the three non-native AMF, a mixture of the three and soil from a permaculture site led to significantly better acquisition of phosphorus (up to 104%) compared to non-inoculated controls. These results suggest that the use of non-native AMF and local soils as inoculants represent a valid approach to improve nutrient uptake efficiency of naranjilla. In another study, Cely et al. compared the

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effect *Rhizophagus clarus* (syn. *Rhizoglomus clarus*) and a supply of conventional fertilizer on growth and yield of two crops, soybean (*Glycine max* L.) and cotton (*Gossypium hirsutum* L.), under field conditions in Brazil. They showed that mycorrhizal inoculation significantly increased root colonization (∼20%), P and N content as well as crop yield in both inoculated soybean and cotton. The authors conclude that *R. clarus* inoculation increased the effectiveness of fertilizer application in soybean and reduce the fertilizer dosage in cotton. Séry et al. used different approaches based on the selection of native AMF (*Acaulospora colombiana* and *Ambispora appendicular*) and their application in cassava crop production in greenhouse trials and under field conditions in Ivory Coast. Greenhouse trials showed that *A. colombiana* significantly improved the growth of cassava and enhanced tolerance to water stress. Furthermore, combined inoculation of *A. colombiana* and *A. appendicula* enhanced plant resistance against nematode attacks. In field conditions, the *A. colombiana* single inoculation and the dual inoculation significantly improved cassava yield compared to the control. However, no significant difference was observed between native and commercial inoculants which shows that interactions of introduced mycorrhizal inoculants and native communities can provide contrasting results.

The fundamental importance of mycorrhizal associations in tropical agrosystems is not restricted to crops, but extends to forestry as Cely et al. tested two native AMF *Claroideoglomus etunicatum* and *Acaulospora* sp., two native strains of *Rhizobium* sp., and one non-native PGPR strain of *Burkholderia* sp. on wood production of a fast-growing tree *Schizolobium parahyba* var. *amazonicum* (Huber ex Ducke) in Brazil under field conditions. Different combinations of microbial inoculants were complemented with two doses of conventional fertilizers in two planting methods, seed sowing and seedling planting. Among all combinations, two of them have shown a significant increase of wood yield or seedling growth in each planting method. The authors concluded that inoculation of *S. parahyba* with AMF and PGPR increased wood yield by approximately 20% compared to the application of fertilizer alone.

Arbuscular mycorrhizal fungi have been largely used for alleviating stress effects on host-plants by increasing the nutrient availability and enhancing the productivity in these plants (Dodd and Pérez-Alfocea, 2012). In extreme polluted environments by petroleum hydrocarbon and trace elements (de la Providencia et al., 2015), we do not yet have any clear evidence that AMF directly degrade petroleum hydrocarbon, however, they might stimulate soil metabolic activity of microorganisms, particularly bacteria and fungi, resulting to an acceleration of the immobilization and translocation of trace elements and the degradation of organic pollutants.

Nath et al. presented a mini review on reactive oxygen species (ROS) balance between its generation and scavenging, which is an essential indicator of adaptive defense response of plants under biotic and abiotic stresses. They showed that AMF and *Piriformospora indica* are well-known to colonize plant root, to enhance ROS-metabolism and to maintain ROS-homeostasis.

Chandrasekaran et al. conducted a comprehensive meta-analysis on AM efficiency on C3 and C4 plants under salt stress across 60 published studies. The authors compared the response of some parameters such as AMF and plants identities, soil textures, experiment conditions (greenhouse *versus* field), and the results clearly showed a positive effect on plants upon AM inoculation under salinity stress, regardless of the photosynthetic pathway. However, the authors found that C3 plants showed significantly more mycorrhizal inoculation than C4 plants. Interestingly, the meta-analysis showed that single inoculation with *R. irregularis* had a positive effect on C3 plants while inoculation with *Funneliformis mosseae* had a positive effect on C4 plants.

Using a greenhouse trial, Huang et al. tested the effect of the AMF *F. mosseae* on the subcellular compartmentalization and chemical forms of lead (Pb) in black locust (*Robinia pseudoacacia* L.), a plant species that is tolerant to Pb in polluted soils. Inoculation with *F. mosseae* significantly increased the proportion of Pb in the cell wall and soluble fractions, while it decreased the proportion in the organelle fraction of roots, stems, and leaves. Interestingly, AM inoculation increased the proportion of inactive Pb and reduced the proportion of water-soluble Pb in the roots, stems, and leaves.

CONCLUSION

Overall, the studies presented in the issue of "Mycorrhiza in Tropical and Neotropical Ecosystems" have documented the effectiveness of commercial and native mycorrhizal inoculants in tropical agrosystems and forestry. The results demonstrate that inoculation with either native or non-native AMF inoculants improved crop yield under field conditions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Potential Role of Arbuscular Mycorrhizal Fungi in the Restoration of Degraded Lands

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Experiences worldwide reveal that degraded lands restoration projects achieve little success or fail. Hence, understanding the underlying causes and accordingly, devising appropriate restoration mechanisms is crucial. In doing so, the ever-increasing aspiration and global commitments in degraded lands restoration could be realized. Here we explain that arbuscular mycorrhizal fungi (AMF) biotechnology is a potential mechanism to significantly improve the restoration success of degraded lands. There are abundant scientific evidences to demonstrate that AMF significantly improve soil attributes, increase above and belowground biodiversity, significantly improve tree/shrub seedlings survival, growth and establishment on moisture and nutrient stressed soils. AMF have also been shown to drive plant succession and may prevent invasion by alien species. The very few conditions where infective AMF are low in abundance and diversity is when the soil erodes, is disturbed and is devoid of vegetation cover. These are all common features of degraded lands. Meanwhile, degraded lands harbor low levels of infective AMF abundance and diversity. Therefore, the successful restoration of infective AMF can potentially improve the restoration success of degraded lands. Better AMF inoculation effects result when inocula are composed of native fungi instead of exotics, early seral instead of late seral fungi, and are consortia instead of few or single species. Future research efforts should focus on AMF effect on plant community primary productivity and plant competition. Further investigation focusing on forest ecosystems, and carried out at the field condition is highly recommended. Devising cheap and ethically widely accepted inocula production methods and better ways of AMF *in situ* management for effective restoration of degraded lands will also remain to be important research areas.

Keywords: AMF, ecological restoration, facilitation, inoculation, land degradation, mycorrhiza, monoxenic culture, succession

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INTRODUCTION

Ecological restoration has emerged to be the central theme of global environmental policies (Aradottir and Hagen, 2013; Jacobs et al., 2015). Restoration of at least 15% of the world's degraded ecosystems is one of the 20 2011–2020 targets of the UN Convention on Biological Diversity (CBD, 2010[1]). In 2011, world leaders endorsed the "Bonn challenge" which is a global commitment to

[1] https://www.cbd.int/sp/targets/

restore 150 million hectares of deforested and degraded lands by 2020 (Aradottir and Hagen, 2013). In 2014, the New York Declaration on Forests put forward even a bigger global commitment of restoring 350 million hectares of deforested and degraded lands until 2030 (Jacobs et al., 2015). Most importantly, in 2015, the UN concretized these global commitments by adopting the 2030 Sustainable Development Goals which has one of the 17 targets (Target 15) dealing on ecological restoration (UN, 2015). However, restoration experiences so far show that many restoration projects achieve limited success or fail completely (Thomas et al., 2014) and therefore, extra effort is needed to achieve the huge global restoration commitments put on the table. Here we propose AMF inoculation and *in situ* management for better restoration outcome of degraded lands.

About 93% of flowering plant families (Brundrett, 2009) and 92% of land plant families (Wang and Qiu, 2006) are estimated to have mycorrhizal associations. These associations, based on their structure and physiological relationship with symbionts, are categorized in to seven; of which arbuscular mycorrhiza is one (Brundrett et al., 1996; Barea et al., 2011). Arbuscular mycorrhiza is the most predominant and evolutionarily the ancestor of all the association types (Wang and Qiu, 2006). AMF produce arbuscules, hyphae, and vesicles within host plants' root cortical cells (Brundrett and Abbott, 2002). However, some species within the family Gigasporaceae do not form vesicles but instead, form auxiliary cells of unknown function (Redecker and Raab, 2006). In few other cases as well, arbuscules develop poorly or may be absent (Koide and Mosse, 2004). AMF are; (1) obligate biotrophs completely depending on host plants for organic carbon (File et al., 2012), (2) evolutionarily intimately associated with plants (Taylor et al., 1995), (3) multiple nucleated, and (4) asexually reproducing eukaryotes (Schüßler et al., 2007).

Arbuscular mycorrhizal fungi are keystone organisms with myriads of ecosystem roles. The external hyphae network (extraradical mycelium) of the fungi permeate in to the microsites of rocks and soils surrounding the plant roots (Finlay, 2008; Barea et al., 2011) increasing the root absorbing surface area 100 or even 1000 fold (Larcher, 1995). Therefore, AMF increase plants' nutrient and water relation (e.g., Birhane et al., 2012, 2015; Banerjee et al., 2013), and can improve plants' field survival and establishment (e.g., Pouyu-Rojas and Siqueira, 2000; Habte et al., 2001; Ouahmane et al., 2006; Dag et al., 2009; Kapulnik et al., 2010; Karthikeyan and Krishnakumar, 2012; Manaut et al., 2015). AMF improve soil structure, soil water relation, plants' tolerance to biotic and abiotic stresses, increase plants' nutrient supply, plants' growth, yield and reproductive success and reduce fertilizer requirement (Finlay, 2008; Gianinazzi et al., 2010; Simard and Austin, 2010; Barea et al., 2011; Al-Karaki, 2013; Soka and Ritchie, 2014). AMF influence plant community structure (Van der Heijden et al., 1998; Hartnett and Wilson, 1999; Renker et al., 2004; Heneghan et al., 2008; Lin et al., 2015) and are considered to have a pivotal role in plant community assembly and succession (Janos, 1980; Renker et al., 2004; Kikvidze et al., 2010). Therefore, AMF have significant role in ecological restoration.

The potential role of AMF in ecological restoration has been well recognized even before restoration ecology emerged as a scientific field of study (see Janos, 1980 and the references there). However, as of yet, there is no report available to confirm that AMF inoculation has grown to be a biotechnological tool that is widely applicable in ecological restoration. Review articles dealing on the subject are also very few. To our knowledge, those review articles that dealt on the subject are Skujins and Allen (1986), Brundrett and Abbott (2002), Jeffries et al. (2002) and Renker et al. (2004). Other reviews (e.g., Perry et al., 1987; Quoreshi, 2008; Sanon et al., 2010; Al-Karaki, 2013) did not deal on AMF specifically and the one by Koide and Mosse (2004) allocated some paragraphs for the topic. Therefore, although there is a large number of articles and sufficient knowledge on the ecosystem role of AMF, their role in the restoration of degraded lands is relatively little reviewed. Hence, the purpose of this review article is to gather data from published articles and assess the effects AMF have on measurable ecological restoration attributes and ecological processes.

FEATURES OF DEGRADED LANDS

There is no single internationally approved definition of land degradation (World Resource Institute, 2015[2]). However, land degradation is often defined as a long-term loss of ecosystem function and productivity caused by disturbances from which the land cannot recover unaided (e.g., Bai et al., 2008). Meanwhile, reduction in net primary productivity has commonly been used to measure the level of land degradation and restoration (Bai et al., 2008). The Society for Ecological Restoration (SER), however, recommends nine attributes to measure restoration success (SER, 2004). Earlier, Aronson et al. (1993) adapted Odum (1969) succession traits to formulate their own restoration attributes. The SER restoration attributes are excellent parameters (Ruiz-Jaen and Aide, 2005), however, it is the Aronson et al. (1993) restoration attributes that have commonly been used by restoration ecologists (Choi, 2004; Ruiz-Jaen and Aide, 2005). Therefore, in this article, the attributes listed by Aronson et al. (1993) are adapted and used to safely characterize degraded lands (**Table 1**).

Degraded lands are characterized by low levels of AMF abundance and diversity. An experiment carried out in Brazil (Cardozo-Junior et al., 2012) compared the abundance and diversity of AMF on lands of differing degradation levels and also determined the same on a young restoring site. The result of the observation clearly showed that as the scale of degradation increases, the abundance and diversity of AMF reduces and when restoration presumes both AMF abundance and diversity increase (Cardozo-Junior et al., 2012). Elsewhere also, it was reported that an effective exclosure increased AMF abundance (Birhane et al., 2010). These are in agreement to the remarks by Abbott and Robson (1991) and Schnoor et al. (2011) which indicated that although AMF are ubiquitous, the very few conditions where a natural ecosystem can be devoid of AMF is in areas that are severely eroded or disturbed.

[2]http://www.wri.org/faq/what-degraded-land

TABLE 1 | Features of degraded lands compared to reference climax ecosystems (Based on Aronson et al., 1993).

Structural indicators of degraded lands	Functional indicators of degraded lands	
Low total plant cover	Low biomass productivity	
Low perennial and annual plant species richness	Low soil organic matter	
Low aboveground phytomass	Poor soil water relation	Lowered soil water reserves
Low beta diversity (species turnover along environmental gradient)		Low coefficient of rainfall efficiency (the amount of water infiltrating to middle and deep soil layers)
Decreased life form spectrum (Decreased number of species with different modes of adaptation)		Reduced length of water availability period
Reduced number of keystone species	Low rain use efficiency (RUE)	
Low soil microbial biomass	Poor nutrient cycling index (the ratio of the amount of nutrients mainly N&P recycled to the amount leaching or lost)	
Low soil microbial diversity	Low nitrogen use efficiency (NUE)	

A greenhouse experiment with simulated erosion was able to demonstrate that erosion of soil beyond 7.5 cm could make soil loose AMF completely (Habte, 1989). Likewise, Jasper et al. (1989) were able to experimentally determine that, while AMF maintained their infective potential in extremely dry soil conditions, their infective potential was significantly lowered when the soil was disturbed. Hyphae are important source of inoculum but are highly susceptible to disturbance and hence, disturbance leads to lowered infective potential of AMF (Brundrett and Abbott, 2002).

Furthermore, several studies conducted in agricultural fields have shown that disturbance not only reduces AMF abundance, diversity and infectivity but also results in drastic shift in the AMF community (Schnoor et al., 2011). Most species of the most common AMF families (Glomeraceae, Acaulosporaceae, and Gigasporaceae) have distinctive biomass allocation strategies whereby species of the Glomeraceae allocate most of their biomass in the intraradical hyphae while species of the Gigasporaceae allocate most of their biomass in the extraradical hyphae and species of the Acaulosporaceae produce low biomass both intra and extraradically (Maherali and Klironomos, 2007). Similarly, these distinctive fungal groups have distinctive life history with most species of the Glomeraceae being ruderals while that of Gigasporaceae and Acaulosporaceae are competitors and stress tolerators respectively (Chagnon et al., 2013). Ruderal AMF species are disturbance tolerant since they have shorter extraradiacal mycelium and have the following life history strategy viz., grow faster, have short life cycle and invest earlier and more abundantly in spore formation, fuse fragmented hyphae more readily, and form cross-walls that enable infected root pieces and severed hyphal fragments to heal and re-colonize host roots (Chagnon et al., 2013). Meanwhile, AMF communities of disturbed sites are characteristically dominated by disturbance tolerant species of the family Glomeraceae and more specifically the genus Glomus (Chagnon et al., 2013).

The number of surviving propagules of AMF in soils also declines with time in the absence of host plants (Brundrett and Abbott, 2002). Alexander et al. (1992) have reported that heavy logging in a Malaysian forest significantly reduced (75% reductions) the abundance and infectivity of AMF propagules. Therefore, considering the fact that land degradation significantly reduces plant cover, increases soil disturbance and erosion, low levels of AMF abundance, diversity and infective potential can be considered as a peculiar feature of degraded lands. Degraded lands are also prone to invasion by exotic alien species. This is because, the low level of native plants diversity can potentially provide vacant niche for invasives (Mack et al., 2000).

WHAT IS ECOLOGICAL RESTORATION?

Ecological restoration, according to Hobbs et al. (2007) is a process of assembly and succession mediated by disturbance. Succession refers to the more or less regular and predictable replacement of seral communities while community assembly refers to the species dynamics of each seral community. Community assembly is typically viewed as a hierarchical process with local species assemblages representing subsets of a larger species pool (Kikvidze et al., 2015). There are three level filters that result in a particular seral community assemblages viz. (1) speciation, extinction and migration, (2) dispersal, and (3) habitat filters (abiotic factors) and biotic filters like competition and facilitation (Gotzenberger et al., 2011). A typical community assembly was observed by Gleason (1927) whereby ponds at similar locality with similar environmental condition formed different kinds of wetland communities.

Restoration strategies of degraded terrestrial systems usually center on manipulation of species order of arrival and modification of filters to accelerate succession and/or jump start succession (Young et al., 2001; Hobbs et al., 2007; Gómez-Aparicio, 2009). The seral community assemblage is very important in ecological succession since it can determine the latter seral community assemblage and hence, succession trajectory. Egler (1954), argued that ecosystem development

can be accelerated by controlling initial species composition and succession to achieve the desired end point. Some field observations (e.g., Cortines and Valcarcel, 2009) have shown that this has practical application in ecological restoration. This phenomenon is known as the priority effect (Young et al., 2005; Zedler, 2005). Hence, designing initial species composition and ensuring their survival and establishment is an important step in ecological restoration (Angelini et al., 2011). With the growing appreciation to plant–plant facilitation interaction and due to better adaptation to resource limited conditions, early/mid successional shrubs are gaining preference to late successional tree/shrubs to startup restoration process of degraded lands (Gómez-Aparicio, 2009; Padilla et al., 2009).

Restoration ecologists not only provide appropriate conditions for desired species to establish but they also, in the meantime, devise ways of preventing the establishment by invasives (Zedler, 2005). Disturbance is also an important factor in ecological restoration since it can modify filters and community assemblage (Choi, 2004). Restoration ecologists have observed that some low level of natural disturbance (e.g., logging, fire, flooding, etc.) can enhance biological diversity and hence, ecological restoration (Palmer et al., 1997).

Based on the scope and complexity of intervention, ecological restoration ranges from species reintroduction to population restoration to community restoration (Young et al., 2001). Based on the restoration goal, it ranges from reclamation to rehabilitation to true restoration. An ecosystem that is slightly disturbed can restore back to its pre-disturbance status (true restoration). As the magnitude of disturbance increases the return to pre-disturbance status may be impossible and hence, return to an intermediate successional status of the given community (an alternative steady state) may be achieved (rehabilitation). When the disturbance is severe, the threshold of irreversibility is passed and the return to pre-disturbance community status or intermediate successional status will be completely impossible and hence, restoration can only result in a novel community stature (reclamation) (Aronson et al., 1993). In the advent of climate change, to have reclamation as a restoration goal is considered to be relevant since novel climatic conditions are anticipated in the future (Choi, 2004).

Considering the wide range of concepts embedded in ecological restoration, as shown above, a comprehensive definition is crucial. Hence, the SER defined ecological restoration as, the process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed (SER, 2004). This is the most widely accepted definition of ecological restoration (Harris et al., 2006; Higgs et al., 2014). Likewise, for this article, this definition is adopted.

In tropical lands ecological restoration, tree planting (Lamb et al., 2005; Holl et al., 2010; Aerts and Honnay, 2011) and re-vegetation/reforestation (Cortines and Valcarcel, 2009; Al-Karaki, 2013) are known to be the most effective and widely used biological measures. Accordingly, in this article ecological restoration is considered to be the re-vegetation of degraded sites mainly through tree/shrub planting.

AMF AND THE MYCORRHIZOSPHERE ECOLOGY

The rhizosphere is a narrow zone of soil affected by the presence of plant roots (Hrynkiewicz and Baum, 2011). It is extremely important and active area for root activity and metabolism (Saharan and Nehra, 2011). Roots release a multitude of organic compounds (e.g., exudates and mucilage) derived from photosynthesis and other plant processes making the rhizosphere a hot spot of microbial activities mainly that of fungi and bacteria (Hrynkiewicz and Baum, 2011). The physical, chemical and biological environment of the rhizosphere is hence, clearly distinct from the bulk soil (Barea et al., 2002).

Similarly, the rhizosphere of the mycorrhizal plant can be referred to as the mycorrhizosphere (Barea et al., 2002). Mycorrhizosphere comprises both the root and hyphae influence zones or the rhizosphere and hyphosphere (Timonen and Marschner, 2006). Mycorrhizal hyphal growth in soils is extensive, with mycelial lengths reaching 111 m cm^{-3} or 0.5 mg g^{-1} or 900 kg ha^{-1} of soil (Simard and Austin, 2010). Hence, the mycorrhizosphere provide a critical link between plants, other microorganisms and the soil (Hrynkiewicz and Baum, 2011).

Intricate interactions take place within the mycorrhizosphere. The most important ones could be interactions between; AMF and the plant, AMF and bacteria, AMF and other fungi, and among AMF themselves. These interactions commence when plant roots exude strigolactones (SLs) (Parniske, 2008; Gutjahr, 2014). Under phosphate or nitrogen limiting conditions plants exude elevated amounts of SLs into the rhizosphere (Gutjahr, 2014). SLs are carotenoid-derived plant hormones (Gutjahr, 2014) that induce AMF spore germination and hyphal branching (Parniske, 2008). They are also known to induce seed germination in parasitic plants, such as *Striga* (Parniske, 2008) and are also involved in suppression of shoot branching and shaping root architecture (Gutjahr, 2014).

The AMF on their part, produce mycorrhiza (Myc) factors. Myc-factors induce calcium oscillations in root epidermal cells and also activate plant symbiosis-related genes (Parniske, 2008). Then the AMF form special type of appressoria called hyphopodia which develops from mature hyphae (Parniske, 2008). As a consequence of sequential chemical and mechanical stimulation, plant epidermal cells produce a pre-penetration apparatus (PPA) (Parniske, 2008). Subsequently, a fungal hypha that extends from the hyphopodium enters the PPA, which guides the fungus through root cells toward the cortex. The fungus then leaves the plant cell and enters the apoplast, where it branches and grows laterally along the root axis (Parniske, 2008; Gutjahr, 2014). These hyphae induce the development of PPA-like structures in inner cortical cells, subsequently enter these cells, and branch to form arbuscules (Parniske, 2008). Upon getting nourished via the arbuscules the fungi will develop extraradical mycelium whose leading tips form new spores to continue the lifecycle of the fungi (Parniske, 2008). Vesicles, which are proposed to function as storage organs of the fungus, when applicable, are formed in the apoplast (Parniske, 2008).

Arbuscular mycorrhizal fungi have a significant role in plants' P nutrition and sometimes, 100% of the P may be

provided by the AMF (Smith et al., 2011). In return, plants allocate, according to most authors, 4–20% of the photosynthate to the AMF (Lerat et al., 2003). It has been shown that plants preferentially allocate more carbon in favor of the more beneficial fungi (e.g., Bever et al., 2009). Moreover, plants allow the arbuscules to live in their cells as long as the AMF is delivering phosphorous and maybe other nutrients efficiently (Parniske, 2008). The observation that mutation of the arbuscule specific phosphate transporter PT4 results in premature degradation of arbuscules suggests that the lifetime of arbuscules is influenced by their ability to deliver phosphate and probably other nutrients (Parniske, 2008). This provides the plant with a means to maintain efficient arbuscules and penalize inefficient ones with early degradation. Conceptually, this mechanism allows the plant not only to discriminate between efficient and inefficient fungal species but also allows to remove potentially 'good' fungal symbionts that are attached to a poor phosphate source. This concept allows fungal clones and species to compete for arbuscule formation, which allows succession in an established root system (Parniske, 2008). Meanwhile, the formation of fungal colonization structures and the extent of root colonization are largely under plant control (Gutjahr, 2014).

Arbuscular mycorrhizal fungi are known to play role in plant nutrition as long as they collaborate with other soil microbes. It was experimentally proven that mechanisms underlying the increased P-uptake in arbuscular mycorrhizal plants were solely due to AMF synergistic interactions with P-solubilizing microorganisms and/or greater soil volume explored by the AMF hyphae (Antunes et al., 2007). Otherwise, the AMF unlike ectomycorrhiza are not able to neither solubilize phosphate nor decompose organic matter (Simard and Austin, 2010). The well-known activities of nitrogen-fixing bacteria and P-solubilizing microorganisms improving the bioavailability of the major plant nutrients N and P are very much enhanced in the mycorrhizosphere where synergistic interactions of such microorganisms with mycorrhizal fungi have been demonstrated (Barea et al., 2002). In particular, mycorrhizal inoculation improved the establishment of both inoculated and indigenous P-solubilizing rhizobacteria and, again P-solubilizing rhizobacteria usually behave as mycorrhiza-helper-bacteria, promoting mycorrhiza establishment by both the indigenous and the inoculated mycorrhizal fungi (Barea et al., 2002).

Arbuscular mycorrhizal fungi also interact with decomposer fungi (Soka and Ritchie, 2014) and phosphate solubilizing fungi (PSF) synergistically (Osoria and Habte, 2001). Accordingly, presence of mycorrhizal fungi is known to alter the rates of above and below ground litter decomposition due to chemical changes in the roots and interactions with the decomposer fungi (Soka and Ritchie, 2014). PSF were also observed to have lesser effect in plant nutrition when applied alone and maximum effect took place when both AMF and PSF were inoculated showing the synergistic interaction between the AMF and PSF (Osoria and Habte, 2001). In the meantime, AMF are also known to have antagonistic relationship with root pathogens (Soka and Ritchie, 2014) and even leaf pathogens (Parniske, 2008).

Arbuscular mycorrhizal fungi may also interact with each other synergistically. It was experimentally found out that AMF effects are greater when AMF consortia inoculums are applied than single AMF (Banerjee et al., 2013). After long years of observation, Barea et al. (2011) concluded that the use of native AMF consortia has the maximum effect. A meta-analysis on 306 studies also indicated that plant response was substantially lower when plants were inoculated with single AMF species, compared with inoculations with multiple AMF species (Hoeksema et al., 2010). This could be due to synergistic interaction between the various AMF species. Different species of AMF have different hyphal growth patterns, anastomoses and branching frequencies (Parniske, 2008). These differences probably reflect different strategies and the occupation of different niches within the soil (Parniske, 2008).

AMF AND MEASURABLE RESTORATION ATTRIBUTES

Improved plant fitness (survival, growth and reproduction), nutrient uptake and accumulation, tolerance of adverse conditions (biotic and abiotic stresses) and altering plant community structure [competition/facilitation, diversity (richness and evenness) and succession] and that of animal communities (Direct effects on organisms which feed on fungi and indirect effects due to changes in plant fitness) were identified to be the pivotal role AMF play in ecological restoration (Brundrett and Abbott, 2002).

Based on Aronson et al. (1993), the functional and structural attributes to measure ecological restoration include; soil organic matter, soil water relation, nutrient cycling index, plant diversity and soil microbial diversity and abundance, and plant productivity. Therefore, the role of AMF in soil organic matter content, soil water relation, nutrient cycling index, plant stress tolerance, plants survival, establishment and growth on degraded soils, plant diversity, soil microbial diversity and abundance, and plant succession, competition/facilitation and productivity is highlighted below.

AMF Improve Soil Aggregation; Hence Increase Soil Organic Matter and Soil Water Relation

Fungi and most importantly AMF may be the most effective soil organisms in stabilizing soil structure (Augé, 2004). AMF hyphae grow into the soil matrix to create the skeletal structure that holds primary soil particles together to form soil aggregates (Augé, 2004; Al-Karaki, 2013). AMF also improve soil aggregation by influencing bacterial communities that can improve soil aggregate formation (Rilling, 2004). Furthermore, the dead AMF hyphae produce glomalin which is hydrophobic stable aggregate former (Barea et al., 2002; Simard and Austin, 2010). Hence, AMF increase both soil aggregation and stability. AMF may stabilize soils up to 5 months after their host's death (Soka and Ritchie, 2014).

Meanwhile, as a result of the significant amount of mycorrhiza derived soil carbon (Rilling, 2004) and improved soil aggregation and stability, AMF increase soil organic matter content and stability (Rilling, 2004; Leifheit et al., 2014). Improved soil aggregation also increases soil water relation. It was observed that a naturally non-mycorrhizal plant planted in mycorrhizal soils tolerated drought more than the ones planted in a non-mycorrhizal soils indicating that AMF hyphae improves water holding capacity of soils (Marschner, 1995).

AMF Improve Plant Nutrition and Nutrients Cycling Index

The most important role of AMF is their role in phosphorous nutrition (Skujins and Allen, 1986). There are also data indicating that AMF can transfer nitrogen from one plant to another (e.g., Requena et al., 2001), increase the utilization of different forms of nitrogen by plants and can also take up nitrogen directly and transfer it to host roots (Govindarajulu et al., 2005). However, there is considerable doubt as to the cost-benefit of AMF in plant N nutrition (Smith and Smith, 2011). Although few data exist, AMF were observed to improve potassium nutrition in plants (Dag et al., 2009; Garcia and Zimmermann, 2014). AMF can also increase the uptake of other macro and micro nutrients by plants (Birhane et al., 2012). Generally, the external mycelium of AMF establishes an underground network that links the different plants and hence sequester carbon, nitrogen, and phosphorous and also allow the transfer of these nutrients among plants (Rodriguez-Echeverria et al., 2007). These important roles of AMF therefore play great role in nutrient cycling where the need for further nutrient inputs is significantly reduced (Gianinazzi et al., 2010; Al-Karaki, 2013).

Arbuscular mycorrhizal fungi not only improve nutrient cycling but also reduce nutrient leaching from the soil (Rodriguez-Echeverria et al., 2007). In a comprehensive assessment done by Bender et al. (2015), it was possible to determine the role AMF have in nutrient cycling and leaching. Accordingly, it was determined that while AMF inoculation increased nutrient uptake by plants it also reduced leaching of dissolved organic N and un-reactive P (Bender et al., 2015).

AMF Increase Plants' Abiotic Stress Tolerance

It was, several times, demonstrated that AMF can increase plants' tolerance to drought and salinity (Al-Karaki, 2013). AMF are also known to alleviate heavy metal stress in plants (Leyval et al., 1997; Hildebrandt et al., 2007; Soares and Siqueira, 2008; Amir et al., 2013). By inoculating plants with drought tolerant AMF, up to 42% reduction in plants' water requirement could be achieved (Gianinazzi et al., 2010). Also, Navarro et al. (2013) found out that, *Citrus* rootstocks inoculated with AMF showed significantly increased growth than non-inoculated individuals despite the fact that inoculated individuals were irrigated with saline water and the non-inoculated ones got irrigated with non-saline water.

The mechanism by which AMF increase plants' tolerance to drought, salinity and heavy metal stresses is mainly nutritional (Marschner, 1995; Soares and Siqueira, 2008; Birhane et al., 2012; Al-Karaki, 2013; Navarro et al., 2013). Soares and Siqueira (2008) demonstrated that both P fertilization and AMF inoculation of plants significantly improved plants' growth on heavy metal polluted soils. Hence, they concluded, AMF increase plants' heavy metal stress tolerance mainly through P nutrition.

The non-nutritional mechanisms by which AMF increase plants' tolerance to drought include; hormonal changes, hyphal soil improvement (delayed soil drying), hyphal ability to scavenge water from micro-pores, increased plants' photosynthetic rate, and accumulation of compatible osmolites (Marschner, 1995; Birhane et al., 2012; Al-Karaki, 2013). Likewise, immobilizing heavy metals in their biomass mainly cell wall, vesicles and in the glomaline is the non-nutritional mechanism by which AMF improve plants' tolerance to heavy metals stress (Hildebrandt et al., 2007).

The positive AMF effects on plants' drought tolerance can improve plants' salinity tolerance as well. Better water intake by plants can effectively dilute salts within the plants' cells (Larcher, 1995). Other non-nutritional mechanisms by which AMF improve plants' salinity tolerance include; exclusion of salt from plant cells by accumulating the salt within the fungal hyphae, production of enzymes involved in antioxidant defense, and change in cell wall elasticity and membrane stability (Al-Karaki, 2013).

AMF Increases Plants' Resistance and Tolerance to Pathogens and Herbivores

There are several published articles showing the role of AMF in increasing plant tolerance against biotic stressors. The meta-analysis of 144 published papers clearly reveals that (Yang et al., 2014). Considering the role AMF have in bioprotection, Gianinazzi et al. (2010) described AMF as 'health insurance' of plants. One mechanism by which AMF increase plants' pathogen tolerance could be the synergistic interaction of AMF have with plant growth promoting rhizobacteria (PGPR). PGPR have a very well documented role in plant pathogen inhibition (Figueiredo et al., 2010). The fact that AMF stimulate the synthesis of plant secondary metabolites (Gianinazzi et al., 2010) may also explain why AMF inhibit herbivory. Plants' secondary metabolites are known to have role in plants' defense against herbivores (Larcher, 1995). The other reason by which AMF increase plants' herbivory tolerance is compensatory growth. A microcosm investigation revealed that mycorrhizal plants did not show a reduction in total above ground biomass despite their leaves being fed by grasshoppers indicating that mycorrhiza helped the plant to compensate in growth after herbivory (Kula et al., 2005).

AMF Increase Tree/Shrub Seedlings Growth, Productivity, Field Survival and Establishment on Degraded Lands

Lekberg and Koide (2005), carried out a meta-analysis based on 290 published experiments to determine the role of AMF on plant growth and productivity. The analysis also determined the effects of three common AMF management methods; inoculation, short fallow, and reduced soil disturbance. The result of the meta-analysis revealed that AMF generally increase individual plant's

growth and productivity. Inoculation and short fallow resulted in significantly positive effects on plants' growth and productivity (Lekberg and Koide, 2005). A recent meta-analysis on 304 papers also concluded that AMF inoculation increases the growth and productivity of plants grown alone (Lin et al., 2015). A similar result was also reported by Birhane et al. (2014). Huante et al. (2012) also did experiment on six tree species and found out that AMF inoculation has significant effect on seedlings growth and most significantly slow growing tree species. **Figure 1** below shows how AMF inoculation can significantly increase tree seedlings growth.

Tree survival and field establishment is an important factor in the restoration of degraded lands. Hence, AMF are important since they can significantly improve tree seedlings field survival and establishment. Pouyu-Rojas and Siqueira (2000), Habte et al. (2001), Ouahmane et al. (2006), Dag et al. (2009), Kapulnik et al. (2010), Karthikeyan and Krishnakumar (2012), and Manaut et al. (2015) have demonstrated the positive effect AMF have in these regards. Pouyu-Rojas and Siqueira (2000) investigated the AMF effect on seven tree species seedlings survival and establishment on degraded pot soils. They found out that AMF inoculation in the nursery or during transplanting have equally significantly positive effect on trees survival and establishment. Later on Habte et al. (2001) determined the effect AMF nursery inoculation has on field establishment of *Acacia koa* and accordingly, AMF was shown to improve transplanted tree seedlings growth and establishment by increasing seedlings P nutrition. The role of native AMF inoculation was also demonstrated to have a significant positive effect on the field survival and establishment of *Cupressus atlantica* Gaussen seedlings on a degraded Moroccan field site (Ouahmane et al., 2006).

Similarly, Kapulnik et al. (2010) determined AMF nursery inoculation effect on seedlings field establishment and growth of *Olea europaea* L. Meanwhile, they were able to observe that AMF inoculation improved seedlings field performance significantly and most importantly for the first 2.5 years from transplanting. They also observed that AMF effect decreased with increasing seedlings age. Karthikeyan and Krishnakumar (2012) also determined AMF effect on survival and establishment of *Eucalyptus tereticornis* Sm. on pot soil of highly degraded origin (mine spoils). Meanwhile, they were able to observe that AMF inoculation almost doubled seedling survival and significantly increased establishment. Recently, Manaut et al. (2015) demonstrated that native AMF consortia inoculation of *Ceratonia siliqua* L. seedlings more than doubled seedlings' survival and significantly improved seedlings' height and collar diameter.

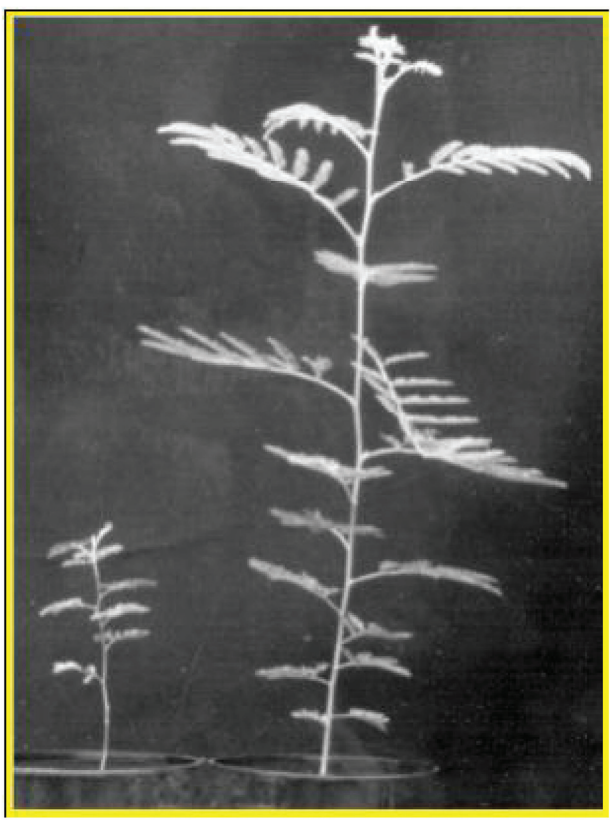

FIGURE 1 | *Acacia koa* A. Gray grew significantly tall in a low-P soil when inoculated with AM fungus (adopted from Miyasaka et al., 2003).

AMF Drive Succession and Influence Plant Community Structure

According to Janos (1980), the mycorrhizal fungus status and the fertility of soil influence the occurrence of plant species. It is also hypothesized that AMF are drivers and as well, passengers of plant community succession (Zobel and Öpik, 2014). Meanwhile, the AMF status of a site determines the composition of a seral plant community, and the composition of that particular seral plant community determines the composition of infective AMF communities which will further influence the composition of the next seral plant community (Janos, 1980; Renker et al., 2004). Thus, if specific compatible relationships between certain AMF and plant taxa are required for mutual symbiont survival, the loss of compatible AMF species or individuals may limit the distribution of a particular plant species (Renker et al., 2004). Plant-soil feedback (plant-AMF feedback) is also an important concept explaining the role AMF have in succession (Kikvidze et al., 2010). Positive feedbacks promote the development of early successional communities and negative feedbacks promote plant species replacement to drive succession (Kikvidze et al., 2010).

Arbuscular mycorrhizal fungi could also potentially influence plant community structure by affecting richness or evenness of coexisting plants (Brundrett and Abbott, 2002). Only some 240 AMF morphospecies have been described forming associations with 80% of terrestrial plants (Lee et al., 2013). This indicates that AMF have no host specificness. Meanwhile, a single mycorrhizal fungus can link different plants together, thus forming mycorrhizal networks (Simard and Austin, 2010; Song et al., 2014). These networks have been shown to facilitate regeneration of new seedlings, alter species interactions, and change the dynamics of plant communities therefore, increasing plant diversity (Simard and Austin, 2010). Sowing seeds of

plant species in microcosms that resembled the grassland community of the temperate zone, on soils of AMF inoculated and non-inoculated, Van der Heijden et al. (1998), Vogelsang et al. (2006), and Schnitzer et al. (2011) were able to observe that AMF inoculation improved plant community diversity by mainly increasing plants' fitness and evenness. AMF may also be important organisms to inhibit invasion by alien species. This could be indirectly by reducing the vacant niche through increased native plants survival, establishment and diversity or can be by direct inhibition of invasives. Janos et al. (2013) reported that the presence of established extraradical mycelium prevented the survival and establishment of seedlings migrating from another ecosystem.

AMF Increase Soil Microbial Diversity and Abundance

Arbuscular mycorrhizal fungi hyphae and root litter are the most abundant carbon source in the soil (Brundrett and Abbott, 2002). Therefore, AMF provide increased supply of energy for soil microbes to flourish. The fact that AMF influence plant communities is also considered to be one of the potential mechanisms by which AMF influence soil microbial communities (Rilling, 2004). Furthermore, AMF hyphal exudates may also stimulate microorganisms present in the mycorrhizal hyphosphere. However, the effect is variable: AMF hyphal exudates may stimulate some microorganisms but still inhibit others (Herman et al., 2012). Hence, AMF may increase the diversity and abundance of microorganisms that are beneficial to plants' growth and health.

AMF Effects on Plant Community Primary Productivity and Plant Competition

Arbuscular mycorrhizal fungi inoculation increases plant productivity at community level and the effect increases with increase in plant species richness following the common ascending but asymptotic diversity-productivity pattern (Schnitzer et al., 2011). At low plant diversity soil microbes suppress plant productivity since their pathogenic effect increases and as the plant diversity increases, plant productivity can increase up to fivefold (**Figure 2**; circle and triangle). In the absence of soil microbes plant productivity has a weak positive linear relationship with plant diversity (**Figure 2**; square).

Sowing seeds of plant species that resembled the grassland community of the temperate zone on AMF inoculated and non-inoculated soils, Van der Heijden et al. (1998) and Vogelsang et al. (2006) were able to determine AMF effect on plant productivity at community level. Accordingly, AMF improved plant community productivity (Van der Heijden et al., 1998; Vogelsang et al., 2006). Furthermore, Van der Heijden et al. (1998) observed that increasing AMF richness resulted in increased productivity while Vogelsang et al. (2006) observed although AMF species richness increased productivity, the effect was not significant compared to single AMF inoculums effect. So, the former observation is Van der Heijden et al. (1999) argued, due to niche complementarity while the latter is Vogelsang et al. (2006) argued, due to sampling effect. The "niche complementarity" theory argues that the presence of many species and functional types results in more complete utilization of resources because different species specialize on different resources, resulting in higher overall productivity while the "sampling effect" theory argues species identity is more important than diversity and asserts that productivity increases with diversity solely due to an increased probability that communities with more species contain a few very productive species that disproportionately contribute to community-wide productivity (Schnitzer et al., 2011).

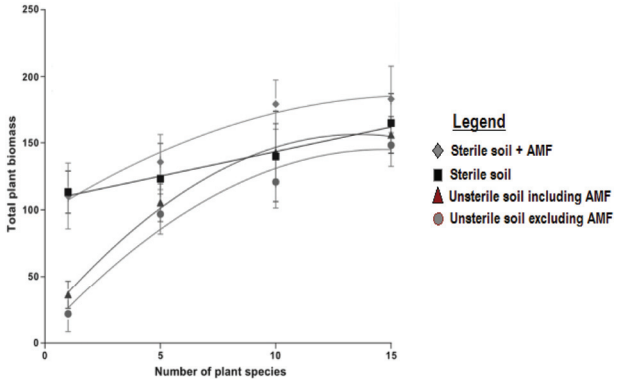

FIGURE 2 | The role of microbes and AMF in plant diversity-productivity relationship (adopted from Schnitzer et al., 2011).

Despite the fact that the above research observations reveal that AMF increase plant productivity at community level, the recent meta-analysis based on 304 study results, which also cited the above research observation, found out that, at community level, AMF inoculation either has no effect on plant productivity or even has a negative effect (Lin et al., 2015). AMF inoculation increases plant productivity at community level only when experiments were conducted in the green house (Lin et al., 2015). Klironomos et al. (2000) were, in a greenhouse setting, able to determine the AMF effect on plant diversity and that of productivity. Accordingly, it was observed that AMF inoculation increased plant productivity but not for all AMF species. Inoculating *Glomus intraradices* N. C. Schenck and G. S. Sm. even lowered productivity compared to the non-inoculated plant community (Klironomos et al., 2000).

Similarly, variable AMF effect is observed in plant competition. Plant species competitive ability response to AMF inoculation depends on plants' functional group, mycorrhizal status, plants' life history (Scheublin et al., 2007; Lin et al., 2015), and also maybe below ground functional traits of the plant species (Birhane et al., 2014). The meta-analysis conducted by Lin et al. (2015) also concludes, AMF inoculation significantly increases N-fixing forbs, decreases C3 grasses and non-N-fixing forbs and woody plants, and has no effect on C4 grasses competitive ability whether these functional groups compete intra or inter-specifically (Lin et al., 2015). According to Birhane et al. (2014), in a pot experiment, AMF inoculation did not have

positive effect on the competitive ability of both *Acacia etbaica* Schweinf. and *Boswellia papyrifera* Hochst. seedlings grown together. In other instances, mycorrhizal networks may result in asymmetric competition by favoring strong carbon-donor roots (Weremijewicz and Janos, 2013) or vice-versa (Walder et al., 2012).

AMF BIOTECHNOLOGY FOR THE RESTORATION OF DEGRADED LANDS

Degraded lands have low level of infective AMF and nursery seedlings around degraded sites may less likely be infected with sufficient AMF (e.g., Michelsen, 1992). Therefore, these sites can support the growth of late successional tree species when appropriate AMF inocula are reintroduced. Late successional tree species are obligately mycotrophic and may necessarily require AMF for their survival and fitness (Janos, 1980). More importantly, at the early stages of seedlings growth, mycorrhizal early/mid successional tree/shrub species can be even more AMF dependent than the late successional ones (Kiers et al., 2000). Therefore, AMF inoculation could potentially be considered as an important biotechnological tool in degraded lands restoration.

Arbuscular mycorrhizal fungi show no host specificity to forge symbiotic relationship with plants and are very ubiquitous, found almost in every soil (Abbott and Robson, 1991; Brundrett and Abbott, 2002; Barea et al., 2011; Al-Karaki, 2013). Hence, many researchers argue that AMF inoculation is likely to be valuable in only few conditions such as mine fields where indigenous AMF inoculum is surely little or none available (Brundrett and Abbott, 2002). Koide and Mosse (2004) suggested instead of going for AMF inoculation it would be quite economical and appropriate to focus on managing the indigenous AMF population of a site. According to Renker et al. (2004), inoculation is an important but the last option. However, contrary to having several dispersal agents such as; wind, water, rodents, birds, worms, and ants (Brundrett and Abbott, 2002), AMF were observed to have poor dispersal. Accordingly, Hailemariam et al. (2013) were able to observe that within a single piece of farm land, soil AMF status and infectiveness can vary in short distances indicating poor dispersal. Similarly, Friese and Allen (1991), also indicated that AMF have poor dispersal. Therefore, to overcome the dispersal limitation of AMF, inoculation may be a worthily intervention.

Meanwhile, AMF inoculation has proved to be effective under wide range of soil conditions (Janos, 1980; Brundrett and Abbott, 2002) including on soils with good AMF abundance (e.g., Banerjee et al., 2013). Positive AMF effect is not ensured by the presence of abundant indigenous AMF but by both abundance (quantity) and efficiency (quality) of indigenous fungal populations (Onguene and Kuyper, 2005). Veiga et al. (2011) also demonstrated that AMF inoculation suppressed weeds and, interestingly enough, hypothesized that AMF inoculation could suppress ruderal plants which are known to invade degraded sites (Veiga et al., 2011). This is particularly important in ecological restoration since ruderal plants could invade degraded lands and compete with tree/shrub seedlings planted.

If the importance of AMF inoculation in the restoration of degraded lands is agreed, the next question to ask will be; what kind of inocula should be prepared? AMF show wide range of functional diversity (Johnson et al., 1997; Klironomos, 2003; Smith et al., 2011) and their effect is within the mutualism-parasitism continuum (Johnson et al., 1997). Likewise, Hoeksema et al. (2010) summarized that certain plants functional groups viz. non-N-fixing forbs and woody plants and C4 grasses show more positive responses to AMF inoculation. Klironomos (2003) also demonstrated that exotic-native AMF strain-host or vice-versa combination results in highly parasitic interaction. Therefore, deciding on the type of inoculum to prepare is a very important step. Based on the currently available data, the use of native inocula should be preferred to the use of exotic inocula. Early seral AMF should be used when seedlings are inoculated for restoration, even for late seral tree species (Allen et al., 2003). Late seral AMF have big spores and demand much carbon and hence, seedlings may not benefit from them. Instead, seedlings benefit from early successional AMF which are usually having small spores and smaller carbon demand (Allen et al., 2003). Likewise, the use of inocula from grasslands is promoted. AMF abundance in grasslands can be more than tenfold than that of in the forestlands and AMF from grasslands do have significantly high inoculation effect (Fischer et al., 1994). That was why Onguene and Kuyper (2005) applied fresh grassland whole-soil inoculum on various soils and three tree species seedlings. According to the result Onguene and Kuyper (2005) obtained, although early successional grassland inoculum had positive effect for most of the cases (80%), the fact that it is an inoculum from grassland resulted in significantly negative effect on *Terminalia superba* Engl. and Diels seedlings grown on agricultural and early successional forest soils. Hence, Onguene and Kuyper (2005) concluded; allochthonous AM inocula may not be always effective. Hence, the use of planting site adapted AM inocula may be recommended. The other reason for the observed negative effect may also be related to host plant's fungi preference (Onguene and Kuyper, 2005). There are data to demonstrate that inocula from conspecific source show better affinity to the host plants' root (e.g., Kiers et al., 2000). Similarly, there are data to show that plant species even that do co-occur may prefer to associate with distinct AM fungi communities (e.g., Wubet et al., 2006; Davison et al., 2011). There are also data to show that distinctively different AMF communities colonize seedlings' and adults' roots of a single tree species (e.g., Wubet et al., 2009). Therefore, one has to ask; does inoculating seedlings with AM inocula from seedlings' rhizosphere or adults' deliver better positive effect? Kiers et al. (2000) have found out that although conspecific inocula from adults had better affinity to inoculated seedlings, the effect on their growth was mostly relatively small showing that, inocula even from conspecific adults, may not be suited for seedlings inoculation.

Selecting few of the dominant planting site adapted AMF species, multiplying them and applying as inocula may not be also a very good idea specially when there are established AMF in the planting site. Increasing the density of few of the dominant AMF species and applying as inocula had resulted in negative effects on plant growth by disrupting indigenous AMF

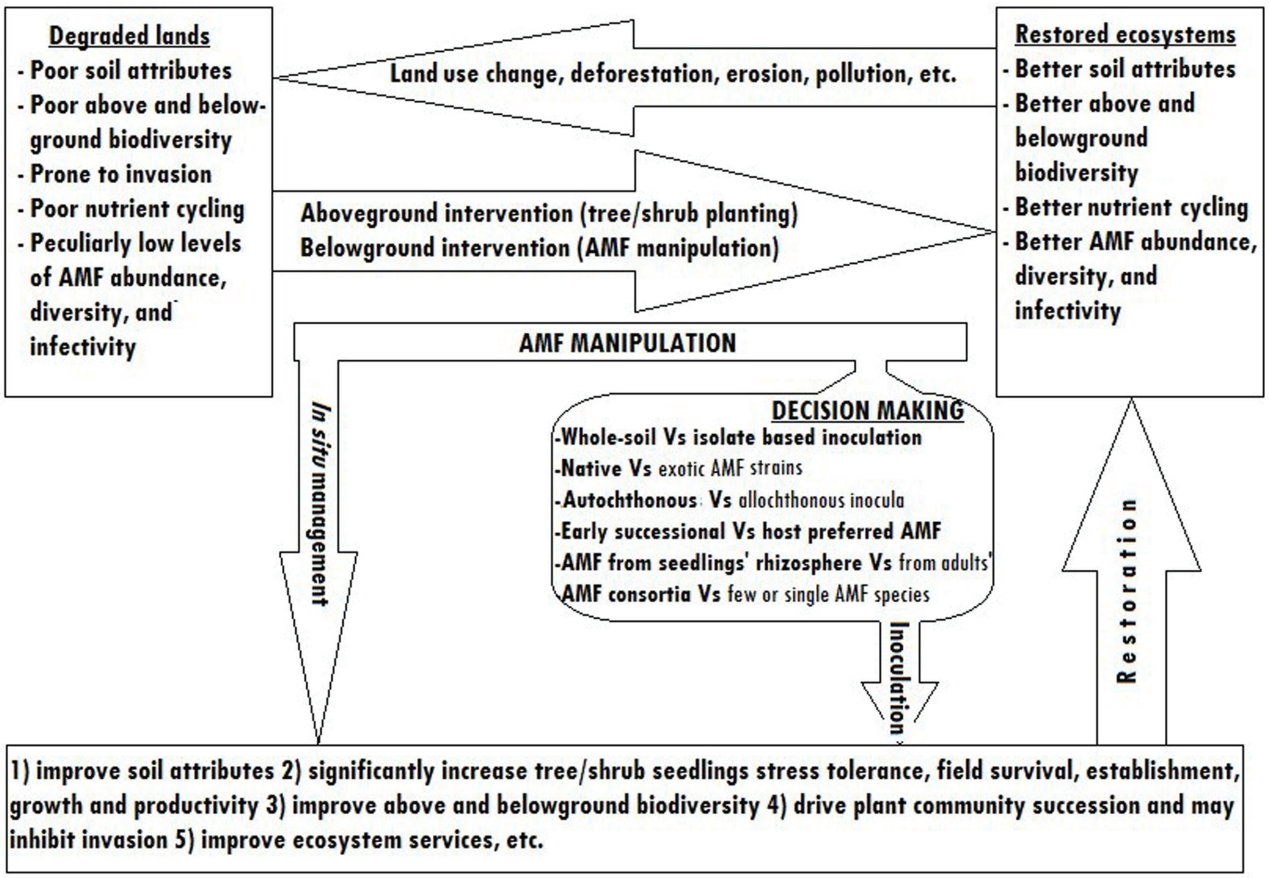

FIGURE 3 | Simplified schematic model showing degraded lands restoration through AMF manipulation.

community structure and thereby creating competition among AMF to ultimately result in inoculum failure (Janoušková et al., 2013). Therefore, in areas with low levels of indigenous AMF abundance, multiplying all not only the dominant AMF species and applying all may be the best option.

The AMF richness in AM inocula is considered to improve inocula effectiveness. Plant response is substantially lower when inoculated with single AMF species and the response keeps increasing from multiple fungal species to whole-soil inoculums (Hoeksema et al., 2010). Likewise, Barea et al. (2011) compiling long years of experience in AMF research recommend the use of autochthonous foundation shrub inoculated with autochthonous AMF consortia inoculums to best restore degraded lands of the Mediterranean. The shrub not only acts as a foundation species but also serves as a resource island for AMF (Barea et al., 2011). However, not all ecologists agree by the application of AMF species rich inocula; some argue that better results due to inocula with better AMF species richness is due to sampling effect and selecting single effective AMF species should get the attention of restoration ecologists. Sampling effect is discussed earlier.

The other challenge associated with AMF biotechnology is related with inocula production for large-scale application. This is due mainly to the obligate nature of AMF. Meanwhile, AMF cannot be cultured axenically (Azcón-Aguilar et al., 1999; Fortin et al., 2005) and host plant based AMF multiplication is mandatory. These host plant based conventional inocula production methods (substrate based pot culturing and substrate free methods of hydroponics and aeroponics techniques) are costly and large scale production of AMF inocula may hardly be possible. Effective monexenic *in vitro* culturing of AMF has been made possible few decades ago (Bécard and Fortin, 1988) and in India, using this method, large-scale industrial production of biologically clean AMF inocula was possible (Adholeya et al., 2005). Readers are directed to read Adholeya et al. (2005) and Cranenbrouck et al. (2005) to grasp the potential and the technique of monoxenic *in vitro* AMF culture production for large-scale application. Readers are also directed to read Azcón-Aguilar et al. (1999) to get proper definitions of axenic and monoxenic cultures.

However, until now, monexenic *in vitro* culturing is not widely practiced. This is due mainly to the fact that; (1) undesired contamination is hardly avoidable and the technique is technology and skill demanding (Bago and Cano, 2005), (2) there are ethical and legal concerns, and (3) it is rather very hard to identify each genotype (even morphotype) hence, most

if not all, AMF are not readily culturable (Fortin et al., 2005). AMF momoxenic *in vitro* culturing uses transformed [using *Rhizobium rhizogenes* (Riker et al.) Young et al.] hairy roots as host owing to the fact that these hairy roots are better suited than the non-transformed hairy roots since they grow on hormone free media and without developing shoots and leaves (Puri and Adholeya, 2013). Meanwhile, AMF monoxenic culture as it is practiced now could potentially be challenged with biosafety related issues.

Due to the lack of cheap and easy AMF inocula production for large scale application, managing the *in situ* AMF is sometimes considered to be an effective AMF biotechnology for the restoration of degraded lands. The meta-analysis by Lekberg and Koide (2005) showed that short fallow could be as good as inoculation to improve plants growth and productivity. It was shown that an obligately arbuscular mycorrhizal pioneer nurse shrub *Lavandula stoechas* L. improved the field survival and establishment of *Cupressus atlantica* Gaussen seedlings by increasing, among others, *in situ* infective AMF abundance (Duponnois et al., 2011). Kumar et al. (2010) also compared different plant composition effects on *in situ* management of AMF on a degraded coal mine spoil. Accordingly, they demonstrated that using cover crops mainly grasses and N-fixing shrubs in the plant composition, significantly enhanced AMF abundance, diversity and infectiveness. Hence, AMF can be manipulated by fallowing or/and by designing the plant species composition to ultimately result in increased AMF abundance which intern facilitates restoration. However, some investigations indicated that grass cover can significantly suppress individual tree/shrub seedlings-saplings growth (Riginos, 2009) or may have variable seasonal effects (Good et al., 2014). Therefore, investigation on cover plant management options to effectively manage AMF and facilitate tree/shrub seedlings growth can be an important research topic.

Nowadays, substrate free inocula preparation methods and *in vitro* production on excised plant roots are being intensively researched to make AMF inoculation less costly (Ijdo et al., 2011). The pot culture inocula preparation method, although it is labor intensive and costly, can be a source of employment especially in developing countries. Therefore, pot culture based AMF biotechnology will remain to be a feasible way of degraded lands restoration in most parts of the world. **Figure 3** shows the simplified schematic model of degraded lands restoration using AMF.

CONCLUSION

This review paper has compiled facts regarding the AMF role in the above and belowground ecosystem processes relevant to ecological restoration. Accordingly, it is possible to conclude that AMF; have a well documented positive role in nutrient cycling and improved soil attributes. AMF also improve plants' tolerance to biotic and abiotic stresses, and significantly increase tree/shrub seedlings survival, establishment and growth. AMF play pivotal role in plant community succession and may directly or indirectly prevent invasion by alien plant species. At plant community level, AMF increase both above and below ground biodiversity but their effect on primary productivity maybe low. The AMF effect on plant competition is also variable and mostly negative. Available data as of yet, indicate that there are very few outfield experiments done on AMF effects on tree/shrubs seedlings survival and establishment. This review was not also able to clearly trace a research result showing the AMF effect on the competitive ability of tree seedlings planted with annual and perennial grass and/or herbaceous weeds. Based on the currently available data, however, it can be concluded that AMF inoculation can significantly increase the success of degraded lands restoration and for better results reducing competitors and seedlings density (increased seedling spacing) is recommended.

Based on the data reviewed in this article, we recommend for future AMF effect researches to give emphasis to outfield experiments. The AMF effect on the competitive ability of tree seedlings compared with annual and perennial herbaceous weeds should be investigated. Data reviewed here showed that almost all research observations conducted on AMF effect at community level are on microcosms of grasslands; and mainly temperate grasslands. Future researches should focus on forest communities of both the temperate and tropics. For an effective large scale application of AMF inocula biotechnology, pot based inocula multiplication will remain to be significantly cost ineffective. Therefore, investigating and researching on cost effective multiplication methods of substrate free and *in vitro* culture and/or optimization of the effects of low-cost fresh AMF inoculation techniques like using grassland top soil or managing AMF *in situ* using several cover crops including grasses need further attention in the future. Optimization of monoxenic *in vitro* AMF culture products and using non-transformed hairy root organ could also be an important research area until axenic *in vitro* AMF culturing is ultimately made possible.

AUTHOR CONTRIBUTIONS

FA did all the data gathering and write-up. TB and EB considerably contributed intellectually by providing comments and guidance at every milestone of the manuscript development. All authors approved publication of the article.

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Petroleum Contamination and Plant Identity Influence Soil and Root Microbial Communities While AMF Spores Retrieved from the Same Plants Possess Markedly Different Communities

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Phytoremediation is a promising *in situ* green technology based on the use of plants to cleanup soils from organic and inorganic pollutants. Microbes, particularly bacteria and fungi, that closely interact with plant roots play key roles in phytoremediation processes. In polluted soils, the root-associated microbes contribute to alleviation of plant stress, improve nutrient uptake and may either degrade or sequester a large range of soil pollutants. Therefore, improving the efficiency of phytoremediation requires a thorough knowledge of the microbial diversity living in the rhizosphere and in close association with plant roots in both the surface and the endosphere. This study aims to assess fungal ITS and bacterial 16S rRNA gene diversity using high-throughput sequencing in rhizospheric soils and roots of three plant species (*Solidago canadensis, Populus balsamifera,* and *Lycopus europaeus*) growing spontaneously in three petroleum hydrocarbon polluted sedimentation basins. Microbial community structures of rhizospheric soils and roots were compared with those of microbes associated with arbuscular mycorrhizal fungal (AMF) spores to determine the links between the root and rhizosphere communities and those associated with AMF. Our results showed a difference in OTU richness and community structure composition between soils and roots for both bacteria and fungi. We found that petroleum hydrocarbon pollutant (PHP) concentrations have a significant effect on fungal and bacterial community structures in both soils and roots, whereas plant species identity showed a significant effect only on the roots for bacteria and fungi. Our results also showed that the community composition of bacteria and fungi in soil and roots varied from those associated with AMF spores harvested from the same plants. This let us to speculate that in petroleum hydrocarbon contaminated soils, AMF may release chemical compounds by which they recruit beneficial microbes to tolerate or degrade the PHPs present in the soil.

Keywords: petroleum hydrocarbon pollutants, 454 high-throughput sequencing, phytoremediation, arbuscular mycorrhizal fungi, bacteria, microbial ecology, plant–microbe interaction

INTRODUCTION

During the past century, industrial production, urbanization, energy consumption, transportation and human population have expanded exponentially, resulting in increased soil, water and air pollution, which in turn has placed the environment under substantial pressure (Samanta et al., 2002; Chen and Kan, 2008). Together, these factors produced a large number of highly polluted sites all over the planet, usually containing complex mixtures of toxic and carcinogenic, organic and inorganic compounds. Organic contaminants such as polycyclic aromatic hydrocarbons (PAH) are known mutagens and carcinogens that enter the food chain together with lipophilic compounds (Boffetta et al., 1997; Henner et al., 1997; Poirier, 2004). Inorganic contaminants mainly consist of metalloids and trace metals with soil retention times of up to thousands of years. Like organic compounds, they reduce plant growth, negatively impact the soil microbiota (McGrath et al., 2001), and decrease the quality of the environment (Gremion et al., 2004) to such an extent that they pose serious health risks to humans and animals.

Polluted sites may be cleaned by physico-chemical strategies including excavation and storage, washing, and chemical treatments. Yet, most *ex situ* treatments only contain contamination without eliminating it. They damage or even destroy soil microbial communities, and are unfit for application over large areas because they are prohibitively expensive. An alternative, most promising *in situ* approach for multi-contaminated sites is phytoremediation (Salt et al., 1995, 1998; Peuke and Rennenberg, 2005), which uses plants and their associated soil microbial communities (fungi and bacteria) to accumulate pollutants within the plants, and/or degrade them in the soil (Peuke and Rennenberg, 2005; Pilon-Smits, 2005; Bieby Voijant et al., 2011).

In the past few years, phytoremediation has become increasingly popular owing to its efficiency, cost effectiveness and respect for the integrity of the soil structure and biology. However, in most cases, phytoremediation is achieved through a complex interaction between plants and the myriad of bacteria and fungi living in the rhizospheric soil and in association with plant roots (endophytic and epiphytic microorganisms) (Pilon-Smits, 2005; Jing et al., 2007; Bourdel et al., 2016; Rohrbacher and St-Arnaud, 2016; Thijs et al., 2016). Therefore, improving the efficiency of phytoremediation requires a thorough knowledge of the microbial diversity living in the rhizospheric soil and in association with plant roots (either in the rhizosphere or the endosphere), and of their interactions with plants. Several studies demonstrated that, in contaminated soils, the root exudates released in the plant rhizosphere promote the selection of microorganisms able to degrade pollutants and stimulate the expression of several genes involved in xenobiotic compound degradation (Bell et al., 2014; Yergeau et al., 2014; Pagé et al., 2015; Rohrbacher and St-Arnaud, 2016; Thijs et al., 2016). For example, Pagé et al. (2015) used a metatranscriptomic approach to compare the gene expression of 10 oxygenases related to petroleum hydrocarbon degradation between the bulk and rhizospheric soils of *Salix purpurea.* They found that among the 10 genes examined, four of them were significantly over-expressed in rhizospheric soil compared with bulk soil. Bell et al. (2014) studied the fungal and bacterial diversity from the rhizospheric soils of eleven cultivars of willow planted in three hydrocarbon contaminated sites, and they found that the abundance of some petroleum hydrocarbon-degrading microorganisms, such as some classes of *Proteobacteria* (*Alpha-*, *Beta-*, and *Gamma-Proteobacteria*) and *Dothideomycetes* (fungi), were significantly enhanced in highly contaminated (HC) plots compared with the low and non-contaminated plots.

Arbuscular mycorrhizal fungi (AMF) are well known as soil fungi able to establish a mutualistic symbiosis with most of land plants (Smith and Read, 2008). In exchange for carbon resources, AMF provide the host plants with nutrients and protect them against soil-borne pathogens (St-Arnaud and Vujanovic, 2007; Smith and Read, 2008; Ismail et al., 2011). In addition, many reports have shown that AMF may play an important role in soil phytoremediation processes (Liu and Dalpé, 2009; Wu et al., 2009; Gao et al., 2011; Hassan et al., 2013). In the soil surrounding plant roots, AMF share the same micro-environment with other rhizospheric microorganisms and several studies have suggested that AMF species collaborate with some of these microorganisms in phytoremediation process (Alarcón et al., 2008; Liu and Dalpé, 2009; Teng et al., 2010). However, AMF also harbor their own hyphosphere microorganisms on the surface of their spores and mycelia (Hijri et al., 2002; Bonfante and Anca, 2009; Scheublin et al., 2010; Lecomte et al., 2011), and the role of these microbes in phytoremediation processes is unknown.

Using 454 sequencing, Iffis et al. (2016) have conducted a study of bacteria and fungi associated to AMF spores harvested from soil collected from the rooting zone of three plant species (*Solidago canadensis, Populus balsamifera,* and *Lycopus europaeus*) growing spontaneously in waste decantation basins of a former petrochemical plant. They have found a large diversity of bacteria and fungi in association with the AMF spores. They also found that the AMF-associated fungal and bacterial communities were significantly affected by both petroleum hydrocarbon pollutant (PHP) concentrations and plant species identity. Furthermore, Iffis et al. (2016) observed that some AMF taxa were either positively or negatively correlated with some fungal and bacterial groups, suggesting that AMF may also play a role in shaping the microbial communities associated with their spores. Similarly, Iffis et al. (2014) showed that the intraradical propagules (vesicules and spores inside plant roots) of AMF extracted from *S. rugosa* roots, sampled in a PHP contaminated site, also harbored a large diversity of bacteria and fungi.

Based on these studies, we inferred that plant species, AMF community and PHP concentrations are among the major driving forces that shape microbial communities living in the rhizosphere and in association with the roots of plants growing in petroleum hydrocarbon polluted sites. The current study aims to understand the contribution of each of these factors on bacterial and fungal community structures in rhizospheric soils and roots sampled in polluted sedimentation basins. Our objectives were: (i) to assess the bacterial and fungal diversity associated with roots and their surrounding soil from mycorrhizal plants spontaneously growing in waste decantation

basins of a former petrochemical plant; (ii) to test the effects of PHP and plant species identity on the microbial community structure in the soil and plant roots; and (iii) to compare the microbial community structure of soil and roots with the microbial community structure associated with the AMF spores in order to verify the hypothesis that AMF are able to recruit specific microbial communities on the surface of their spores and mycelia.

To do so, soil and root samples of three plant species (*S. canadensis, P. balsamifera,* and *L. europaeus*) growing spontaneously in three petroleum hydrocarbon polluted sedimentation basins that were used in Iffis et al. (2016) to assess the AMF-spore associated microbes were subjected to DNA extraction, PCR amplifications targeting the ITS regions for fungi and 16S rRNA gene for bacteria, then the PCR products were sequenced using the 454 FLX+ high throughput sequencing platform to profile the microbial communities structure.

Overall, our results show a difference in OTU richness and community structure composition between soil and roots for both bacteria and fungi. We also found that PHP concentrations have a significant effect on the fungal and bacterial community structures in both soil and roots while, plant species identity had a significant effect only on the root bacteria and fungi. Furthermore, the comparison between the results of this study and Iffis et al. (2016) study showed that the microbial community structures found in soil and root differed from those found in association with the AMF spores harvested from the same samples. These results support the hypothesis that AMF can recruit specific microbial communities on the surface of their spores and mycelia. To our knowledge, this is the first research work devoted to compare the microbial communities of soil, roots and those associated with AMF spores in petroleum hydrocarbon contaminated sites.

MATERIALS AND METHODS

Experimental Design and Sampling

Field sampling and experimental design were previously described in Iffis et al. (2016). Briefly, *S. canadensis, P. balsamifera,* and *L. europaeus* are three plant species naturally growing in three petroleum contaminated basins of a former petrochemical plant located on the south shore of the St-Lawrence River near Montreal, QC, Canada (45.70 N, 73.43 W) (Desjardins et al., 2014) (Supplementary Figure S1). *P. balsamifera* trees were between 0.5 and 1 m height and they were approximately one or 2 years old. The root system of three individual plants with their surrounding soils were collected for each plant species and from each basin, totalizing 27 samples (three basins × three plant species × three individual plants per species). Here, we define rhizosphere soil as soil surrounding roots and under influence of plant roots obtained by shaking the roots in a non-sterile plastic bag (Ziploc Freezer bags, dimensions: 26.8 cm × 27.3 cm) and collecting the remaining soil. Samples were immediately put and transported in a cooler filled with ice packs. Soils were homogenized by shaking bags. Soil subsamples were collected and stored at −20°C until use.

The root system from each plant was washed several times in tap water until elimination of all soils debris attached, and then cut into 1 cm long pieces followed by another round of wash by sterilized water. Since a part of the root samples was already used for microscopic examination, the remaining amounts were insufficient for individual DNA extractions. Therefore, the root replicates of each plant species per basin were pooled in 10 ml tubes, and ground in liquid nitrogen with a mortar and pestle. Then, 500 mg aliquots of the ground root material was collected in 1.5 ml tubes, totaling nine samples for plant roots. As the remaining soil amount was sufficient for individual DNA extraction, the soil samples were kept separate for each replicate per plant species from each basin, so there was a total of 27 soil samples. For each replicate, the soil surrounding the roots was collected in plastic bags, homogenized by shaking, then 500 mg aliquots of this soil were collected in 1.5 ml tubes for DNA extraction. Root and soil tubes were conserved at −20°C until use.

The analysis of PAH and total alkane (C10–C50) concentrations from the soil of the three contaminated basins was carried out using a commercial laboratory service (Maxxam Analytical Laboratories, Montreal, QC, Canada), and the results are available in Supplementary Table S10 of Iffis et al. (2016). Basin 1 was the most highly contaminated with mean concentration of alkanes (C10–C50) equal to 2200 mg/kg of soil (termed as HC), basin 2 was the least contaminated with 153 mg/kg of soil (termed as LC), while the basin 3 was moderately contaminated with 800 mg/kg of soil (termed as MC) (Iffis et al., 2016).

DNA Extraction and Polymerase Chain Reactions

DNA extraction was performed in both soil and roots samples using the NucleoSpin Soil Kit (MACHEREY-NAGEL, United States) following the manufacturer's instructions. DNA was eluted in a 50 μl of elution buffer, and was stored at −20°C until use.

To identify the bacterial taxa, soil and root DNA samples were subjected to PCR amplification targeting the V1–V4 hypervariable region of 16S rRNA gene with primer pair UnivBactF 9 (5′GAGTTTGATYMTGGCTC-3′) and BSR534/18 (5′-ATTACCGCGGCTGCTGGC-3′). We choose these primers because they were successfully used and tested in 454 pyrosequencing by Bell et al. (2011, 2014) and Yergeau et al. (2012). To identify the fungal taxa, the ITS regions were targeted using primer pair ITS1F and ITS4 (Bell et al., 2014). For soil DNA samples, PCR amplifications were performed once on each DNA sample, totaling 27 PCR tubes of soil samples. For root DNA, since plant root replicates were pooled within each plant species per basin, PCR amplifications were performed in triplicate on each DNA sample, also totaling 27 PCRs tubes.

Both 16S and ITS primers were tagged with adaptors and unique multiplex identifier (MID) tags from the extended MID set recommended by Roche Diagnostics (Roche, 2009). PCRs were performed in 50 μl volumes containing 5 μl of 10× PCR buffer, 0.2 mM of dNTP mix, 1 μl of BSA (100 mg/ml),

1 µl $MgCl_2$ (25mM), 0.4 mM of each primer, 2 µl of DNA template and 1 U of *Taq* DNA polymerase (QIAGEN, Toronto, ON, Canada). PCRs were run on a thermal cycler Pro S thermocycler (Eppendorf, Mississauga, ON, Canada) using an initial denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final elongation step at 72°C for 10 min. After electrophoresis separation and UV light visualization, the PCRs products were purified with the QIAquick Gel Extraction Kit (QIAGEN, Toronto, ON, Canada) following the manufacturer's instructions. Then, DNA concentrations of the purified products were measured using the Qubit 2.0 fluorometer (Life Technologies, Burlington, ON, Canada). Four pools were prepared by mixing equimolar volumes of PCR products. The four pools were 16S rRNA gene amplicons of soil samples, 16S rRNA amplicons of root samples, ITS amplicons of soil samples and ITS amplicons of root samples. These pools were sent for sequencing to the McGill University and Genome Québec Innovation Centre using the Roche 454 FLX+ pyrosequencing platform (Roche, Branford, CT, United States), with one-eighth of sequencing plate per pool.

The sequences generated in this study were deposited in the NCBI Sequence Read Archive and are available under the project number SRP100801.

Bioinformatic Processing

Sequence processing was performed in Mothur (v.1.34.4) (Schloss et al., 2009) as described in the Mothur wiki[1], with some minor modifications (Bell et al., 2015). Briefly, for both soil and root 16S rRNA gene sequences, the ".sff" files of the different samples were first merged in one ".sff" file, then ".qual," and ".fasta" files were obtained from the ".sff" file using "merge.files" and "sffinfo" commands, respectively. Low quality and short sequences were removed using the "trim.seqs" command, with the following parameters: maxambig = 0, maxhomop = 8, bdiffs = 1, pdiffs = 2, qwindowaverage = 30, qwindowsize = 50, minlength = 300 and then, we reduced the dataset to only unique sequences using the "unique.seqs" command. After sequence alignments against the Mothur interpreted Silva bacterial database using "align.seqs," the non-aligned sequences were removed using the "screen.seqs" command with the following criteria: start = 1044, optimize = end, criteria = 95. Prior to sequence classifications, the datasets were first subjected to a second simplification using the "unique.seq" command, followed by the commands "pre.cluster" and "chimera.uchime" to reduce the sequencing errors. Sequence classifications were carried out with the Mothur implementation of the RDP database using the "classify.seqs" command. Sequences identified as "Mitochondria," "Chloroplast," "Archaea," "Eukaryota," or "unknown" were removed using the "remove.lineage" command. Distance matrices were generated with "dist.seqs" command and OTUs were obtained using the "cluster.split" command (method = average, processors = 2, splitmethod = classify, large = T). Removal of the singleton reads was carried out using the "split.abund" command and then, to have an equal number of reads per sample, datasets were standardized by a random sub-sampling using the "sub.sample" command. OTU tables at 97% similarity were generated following the steps for "create.database."

Overall, the steps for soil and root ITS datasets processing were similar to those for 16S rRNA gene datasets processing. Most of the modifications were introduced owing to the absence of reference database for ITS sequence alignments. After elimination of low quality and short sequences by the "trim.seqs" command (maxambig = 0, maxhomop = 8, bdiffs = 1, pdiffs = 2, qwindowaverage = 30, qwindowsize = 50, minlength = 250), we ran "chop.seqs" and "chimera.uchime" commands to standardize sequence length (numbases = 249) and reduce the sequencing errors, respectively. Then, the ".fasta" files were clustered into OTUs at 97% identity using the CD-HIT software (Li and Godzik, 2006) and reformatted to ".list" file in order to continue Mothur processing. The remaining steps of the ITS datasets processing were the same as 16S rRNA gene datasets processing, except sequence classification was performed using the UNITE_97_v6 reference database, and the "remove.lineage" command was not used in ITS datasets processing.

Statistical Analysis

All statistical analyses were performed in R (version 3.1.1). To compare the microbial diversity and OTU richness between soil and root datasets, Student's *t*-tests or Wilcoxon tests (depending on the normality of the residuals) were carried out on Shannon diversity indices and the Chao richness estimator using the "Rcmdr" package. Student's *t*-tests were performed on the fungal Chao values (normally distributed), while Wilcoxon tests were performed on the bacterial Chao and Shannon values, and on the fungal Shannon values (non-normally distributed residuals). Student's *t*-tests or Wilcoxon tests were also performed to compare the most abundant fungal and bacterial classes relative abundances between soil and roots. To verify the efficiency of our sampling efforts and sequencing depth, rarefaction curves were drawn for each individual sample using the "rarefy" function in the "vegan" package. Depending on the normality of the residuals, the effect of contamination levels and plant species identity on Shannon diversity indices of fungi and bacteria were tested by ANOVA or Kruskal–Wallis tests using the "Rcmdr" package in R (ANOVA tests were carried out on soil fungi, root fungi and soil bacteria data, while Kruskal–Wallis tests were performed on root bacteria data.

To test the effect of contamination levels, plant species and habitats (soil, roots, and AMF spores) on the bacterial and fungal community structures, PERMANOVA analyses were performed using the "adonis" function in the "vegan" package on Bray–Curtis values obtained from the community structure matrices previously normalized by the Hellinger transformation (Legendre and Legendre, 2012). For the PERMANOVA carried out across habitats, we did two PERMANOVA tests. The first was performed to compare between the microbial (bacterial and fungal) communities present in soil and roots, while the second was performed to compare between the microbial communities present in the three habitats (soil, roots, and AMF spores). As the AMF spore-associated fungi dataset did not contain OTU matching with Glomeromycota, all the taxa matching with

[1]http://www.mothur.org/

Glomeromycota in soil and root matrices were removed, prior to the PERMANOVA test preformed to compare the fungal communities between the three habitats. Since the microbial datasets of soil, roots and AMF spores were obtained from different sequencing pools with different sequencing depth, the matrices were first summed at the genera level and then expressed as percentages, prior to the merging of matrices, rarefaction of data and PERMANOVA analyses. The percentages were performed by calculating the report of abundance of each OTU per site on the sum of OTUs abundances per site. To test the homogeneity of dispersion of the different communities against PHP concentration and plant species identity, beta-dispersion analyses were performed on the Bray–Curtis matrices using the "betadisper" function in the "vegan" package. To reveal which fungal or bacterial taxa were affected by the contamination levels or plant species, Kruskal–Wallis tests or ANOVA were performed on the abundances at class level and on the most abundant thirty OTUs of fungi and bacteria in both soil and root datasets. Non-metric multidimensional scalings (NMDS) were performed to visualize the effects of contamination levels, plant species and biotopes on community composition. NMDS ordinations were calculated from the Bray–Curtis matrices using the "metaMDS" function from the vegan package. Krona charts were prepared using the KronaTools[2] (Ondov et al., 2011) to compare the AMF-associated microbial communities (Iffis et al., 2016) with the soil and roots microbial communities.

RESULTS

Soil Microbial Diversity versus Root Microbial Diversity

After quality filtering and standardizing the number of sequences in the different datasets, the soil 16S rRNA gene dataset allowed us to retrieve a total of 23085 reads (855 per sample) which were assigned to 4083 OTUs, while a total of 25839 reads (957 per sample) were obtained from the soil ITS dataset and were assigned to 215 OTUs. For the root 16S rRNA gene dataset, we retrieved a total of 2403 reads (89 per samples) which were assigned to 820 OTUs, while a total of 13716 reads (508 per samples) were obtained from the root ITS dataset and assigned to 188 OTUs.

Rarefaction curves showed that the sampling effort for fungi was close to saturation for all samples with Good's coverage values ranging between 0.97 and 0.99 for soil fungi, and 0.94 and 0.98 for root fungi. Contrary to fungi coverage, the sampling efforts were relatively low yielded for bacteria, in particular for the samples related to *L. europaeus* and *P. balsamifera*. The Good's coverage values of bacteria ranged between: 0.68 and 0.81 for soil bacteria, and between 0.22 and 0.70 for root bacteria (**Figure 1** and Supplementary Table S1).

The Shannon diversity indices and Chao richness estimators (**Figures 2A,B**) of bacterial OTU were significantly higher in soils than in roots (Wilcoxon test, $P < 0.0001$). For fungi, Wilcoxon test on Shannon diversity indices showed no significant difference in fungal diversity between soil and root datasets ($P = 0.15$). However, a significant difference in OTU richness was observed between the soil and root samples for fungi (Student's *t*-test, $P = 0.05$), with Chao values higher in the soil samples (mean Chao value = 68.66) compared to root samples (mean Chao value = 59.58) (**Figures 2A,B**).

PERMANOVA analyses showed that the bacterial and fungal community structures present in soil were significantly different from the communities found in association with plant roots ($P < 0.001$). For both bacteria and fungi, NMDS ordinations showed a clear change in community structures across the two habitats (stress value = 0.14 and 0.22, respectively) (**Figures 3A**, **4A**).

BLAST searches of the bacterial 16S rRNA gene sequences showed that, at class or phylum level, the bacterial profiles were similar in soil and root datasets. The most abundant bacterial taxa identified in the two datasets belong to classes *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria*, and phyla *Actinobacteria* and *Acidobacteria*. However, except for *Alphaproteobacteria* and *Betaproteobacteria*, which were found approximately in similar proportions in soil and root datasets (36 and 33% for *Alphaproteobacteria,* and 14 and 12% for *Betaproteobacteria*), the proportions of the other most abundant bacterial groups were different between the soil and root datasets. *Acidobacteria* and *Deltaproteobacteria* had higher proportions in the soil dataset (11 and 5.25%, respectively) compared to the root dataset (4 and 3.41%, respectively) (Student's *t*-test, $P < 0.05$) while, the percentages of *Actinobacteria* (represented mainly by *Streptomyces*) and *Gammaproteobacteria* were higher in the root dataset (19 and 14%, respectively) than in the soil dataset (6 and 9%, respectively) (Wilcoxon test, $P < 0.0001$ for *Actinobacteria* and Student's *t*-test = 0.15 for *Gammaproteobacteria*) (Supplementary Figures S2A,B). In addition, within each bacterial group, abundances and taxonomic affiliations of the most OTUs found in the root dataset were different from those found in the soil dataset (Supplementary Figures S3A,B). For instance, in the root dataset, the OTUs related to *Alphaproteobacteria* were represented mainly by *Bradyrhizobium*, *Skermanella*, *Sphingobium,* and *Hoeflea*, while in the soil dataset, *Alphaproteobacteria* were dominated by *Sphingomonas*, *Skermanella,* and *Dongia*. Similarly, *Betaproteobacteria* were represented mainly by *Ideonella*, *Duganella,* and *Limnobacter* in the root dataset, while they were dominated by *Caenimonas*, *Burkholderiales,* and *Ferrovum* in the soil dataset.

For the ITS sequences, BLAST searches showed that almost all fungal taxa identified in the soil dataset were also identified in the root dataset. Most fungal taxa found in the two datasets belong to the classes *Dothideomycetes*, *Sordariomycetes*, *Agaricomycetes*, *Chytridiomycetes,* and *Glomeromycetes*. *Dothideomycetes*, *Agaricomycetes,* and *Chytridiomycetes* were found in similar proportions in the root and soil datasets. *Dothideomycetes* represented 23 and 24% of the OTUs in soil and root datasets, respectively. *Agaricomycetes* represented 10 and 7% of OTUs in the soil and root datasets, respectively. Similarly, *Chytridiomycetes* represented 5 and 4% of OTUs in the root and soil datasets, respectively. However, the proportions of

[2]https://github.com

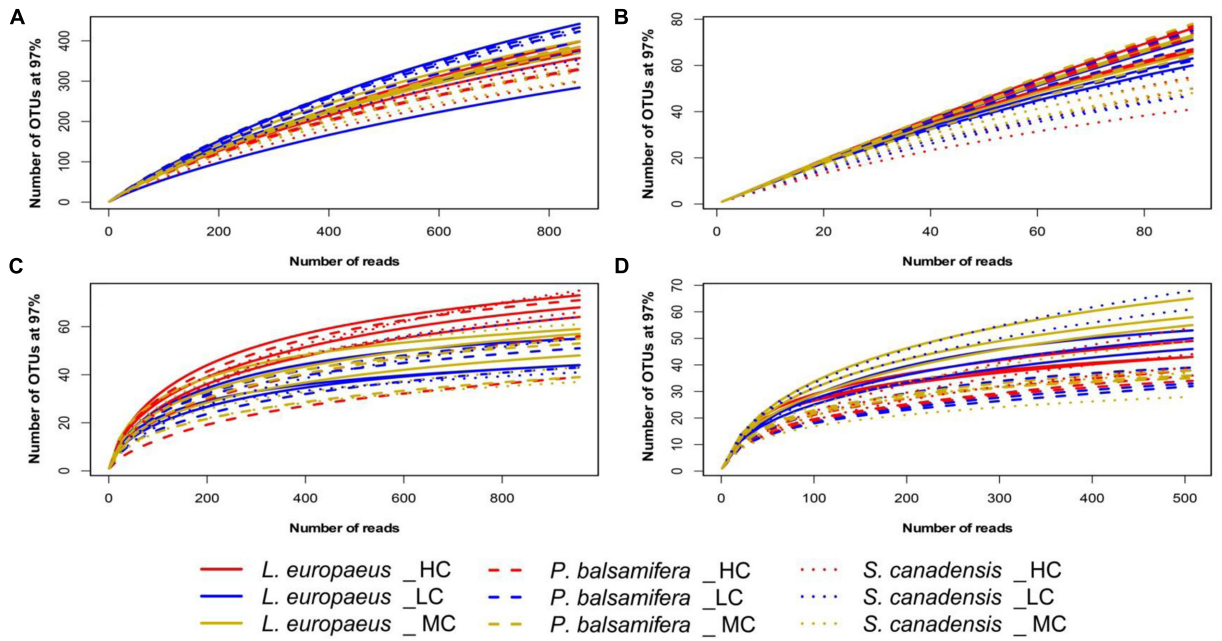

FIGURE 1 | Rarefaction curves of OTUs for individual samples across the different datasets: **(A)** soil bacteria, **(B)** root bacteria, **(C)** soil fungi, **(D)** root fungi.

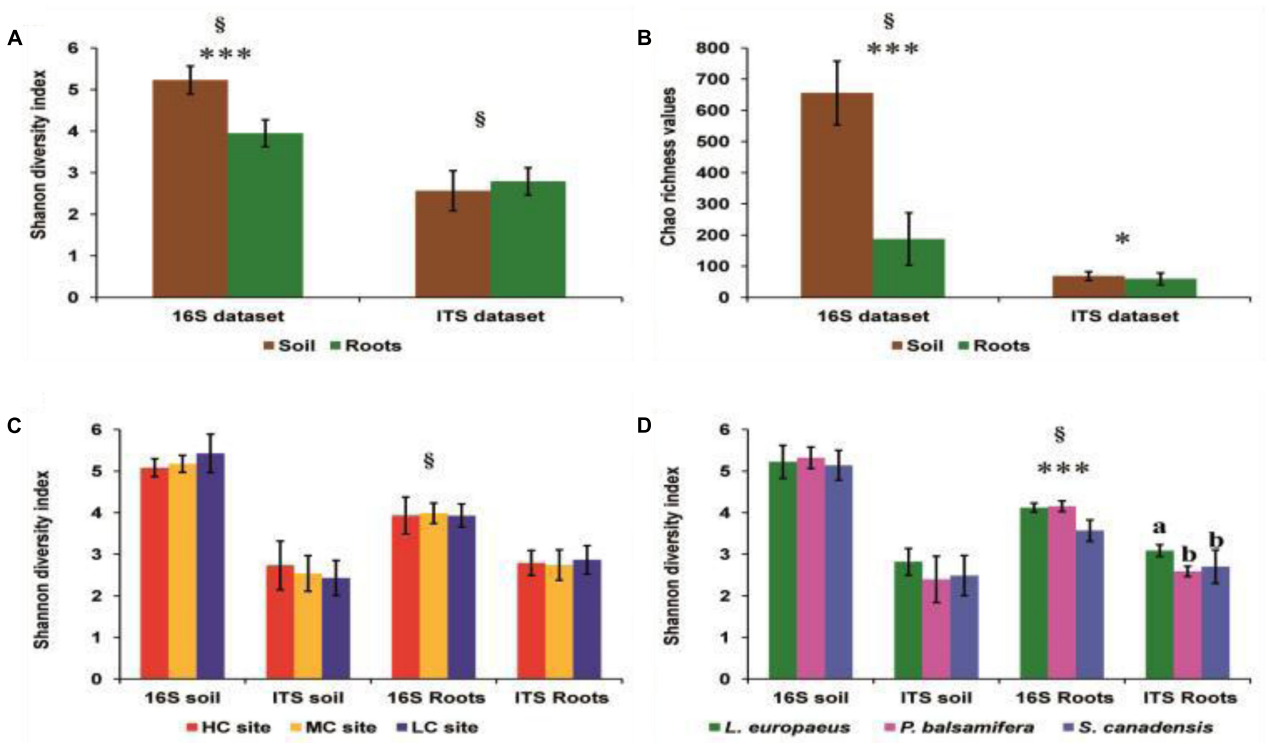

FIGURE 2 | (A) Comparison of Shannon diversity indices of bacteria and fungi across rhizosphere soil and roots. **(B)** Comparison of Chao estimator values of bacteria and fungi across rhizosphere soil and roots. **(C)** Comparison of Shannon diversity indices of the different pyrosequencing datasets across contamination concentrations. **(D)** Comparison of Shannon diversity indices of the different pyrosequencing datasets across plant species. HC, high contamination; CM, moderate contamination, and LC, low contamination. *: significant at 5%; ***: significant at 0.1%; §*P*-values calculated using Wilcoxon or Kruskal–Wallis tests. Different letters over columns "a and b" indicate significant differences according to Tukey's range tests.

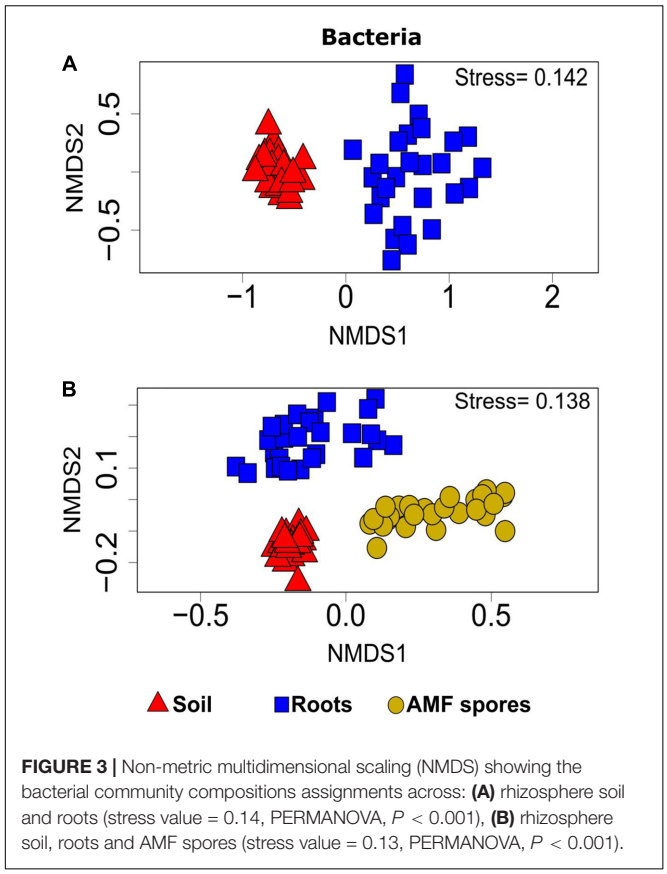

FIGURE 3 | Non-metric multidimensional scaling (NMDS) showing the bacterial community compositions assignments across: **(A)** rhizosphere soil and roots (stress value = 0.14, PERMANOVA, $P < 0.001$), **(B)** rhizosphere soil, roots and AMF spores (stress value = 0.13, PERMANOVA, $P < 0.001$).

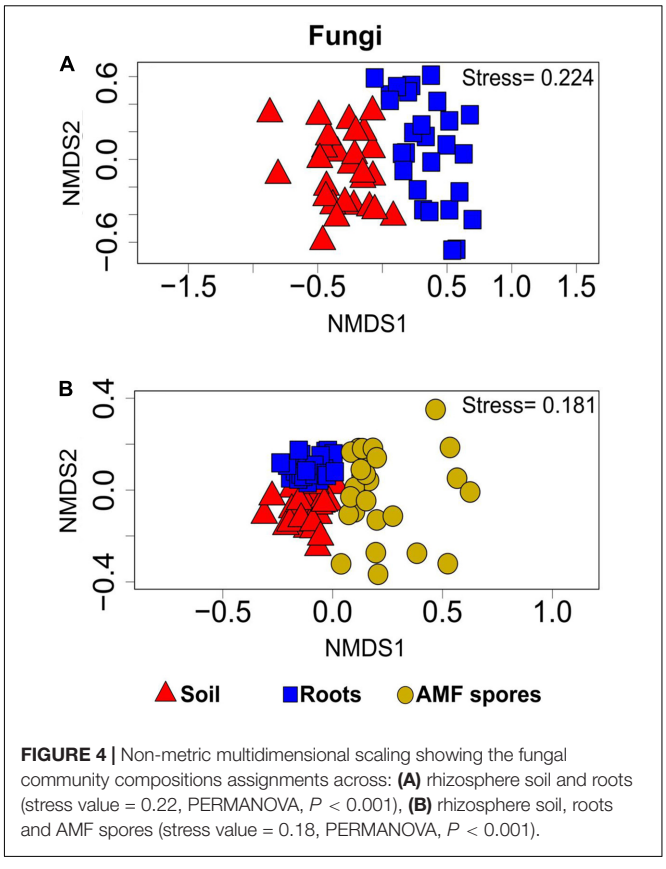

FIGURE 4 | Non-metric multidimensional scaling showing the fungal community compositions assignments across: **(A)** rhizosphere soil and roots (stress value = 0.22, PERMANOVA, $P < 0.001$), **(B)** rhizosphere soil, roots and AMF spores (stress value = 0.18, PERMANOVA, $P < 0.001$).

Sordariomycetes and *Glomeromycetes* were clearly different between soil and root samples (Wilcoxon test, $P \leq 0.0001$). *Sordariomycetes* were found as the most dominant taxa in the soil dataset, with 37% of OTUs, whereas they represented only 5% of the root dataset. *Glomeromycetes* represented 20% of the root dataset, but only 1% of the fungal sequences in the soil dataset (Supplementary Figures S2C,D). At the genera level, abundances and taxonomic affiliation of the fungal OTU found in roots were also different from those found in soil. For example, *Dothideomycetes* were found at similar proportions in both soil and roots habitats (23% in soil and 24% in roots), however, the *Dothideomycetes* genera found in soil and roots were different. *Leptosphaeria* was the most dominant genus of *Dothideomycetes* found in roots (37%), while unclassified *Pleosporales* was the most dominant *Dothideomycetes* found in soil (42%) and *Leptosphaeria* represent only 10% (Supplementary Figures S4A,B).

PHP and Plant Species Identity Effects on Soil and Root Bacterial Diversity

ANOVA tests revealed a nearly significant effect of the contamination concentration on Shannon diversity indices of bacteria in the soil dataset ($P = 0.077$), with a slightly higher diversity in the LC site than in HC, and MC sites. On the other hand, there was clearly no effect of contamination on the bacterial diversity indices in the root dataset (**Table 1** and **Figure 2C**). However, when comparing the shifts in Shannon diversity indices by plant species, a highly significant effect of plants species identity on bacterial diversity was observed in the root dataset ($P < 0.001$), with a higher diversity of bacteria in *L. europaeus* and *P. balsamifera* than in *S. canadensis*. In contrast, there was no effect of plant species identity on the bacterial diversity in the soil dataset (**Table 1** and **Figure 2D**).

PERMANOVA analysis revealed that the community structure of bacteria was significantly affected by the contamination level for both soil and root datasets ($P = 0.01$ and 0.034, respectively). However, a significant effect of plant species identity on the bacterial community structure was observed only on the root dataset ($P = 0.001$), while no effect of plant species was observed on bacteria in the soil dataset ($P = 0.42$) (**Table 1**).

The variations in diversity and community composition of bacteria were corroborated by the non-metric multidimensional scaling (NMDS) plots in both soil and root datasets. In NMDS plots performed across contamination concentrations, a clear separation of the bacterial communities was observed between the LC and HC sites in both soil and root datasets (stress value = 0.15 and 0.22, respectively) (**Figures 5A,B**). However, the NMDS plot performed across plant species showed that the bacterial communities were determined by plant species identity only in the root dataset, where bacteria from *S. canadensis* roots grouped apart those from the other plant species (stress value = 0.15 and 0.22, respectively) (**Figure 6A,B**).

TABLE 1 | *P*-values of ANOVA and PERMANOVA analyses of the effects of contamination level and plant species identity on the Shannon diversity indices and community structures of bacterial and fungal communities present in soils and roots of *Solidago canadensis*, *Populus balsamifera,* and *Lycopus europaeus* growing spontaneously in three petroleum hydrocarbon polluted sedimentation basins.

	Contamination level			Plant species		
	ANOVA on Shannon index	**PERMANOVA on the community structure**	**Beta-dispersion analysis**	**ANOVA on Shannon index**	**PERMANOVA on the community structure**	**Beta-dispersion analysis**
Soil bacteria	0.0777	**0.011**	0.25	0.544	0.428	0.49
Root bacteria	§0.7242	**0.034**	0.46	§ **0.0001**	**0.001**	**0.031**
Soil fungi	0.424	**0.002**	0.72	0.151	0.37	0.10
Root fungi	0.714	**0.006**	0.51	**0.000965**	**0.002**	0.06

The bolded values are significant at P < 0.05. §P-values calculated using Kruskal–Wallis tests. For each group, n = 9.

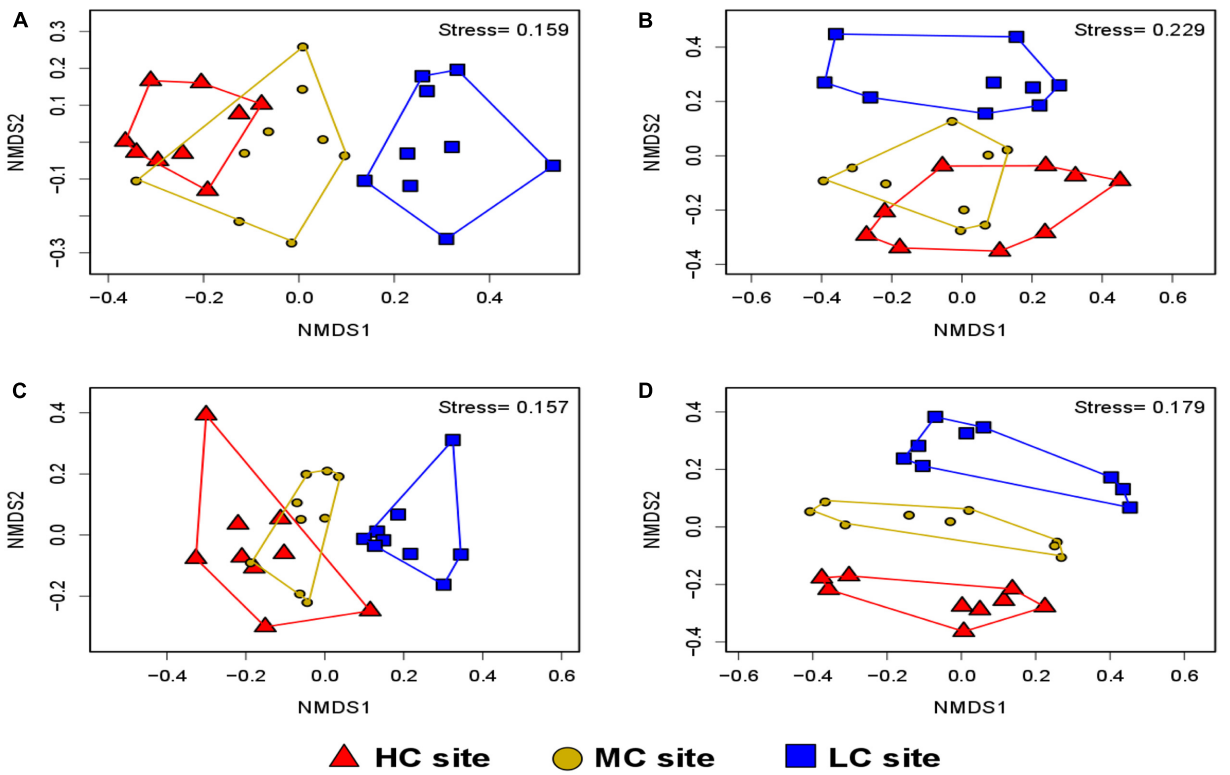

FIGURE 5 | Non-metric multidimensional scaling showing the community composition assignments of: **(A)** soil bacteria (stress value = 0.15, *P* = 0.011), **(B)** root bacteria (stress value = 0.22, *P* = 0.034), **(C)** soil fungi (stress value = 0.15, *P* = 0.002), and **(D)** root fungi across contamination concentrations (stress value = 0.17, *P* = 0.006). PERMANOVA analysis showed significant effects of contamination levels on the community composition of soil bacteria, root bacteria, soil fungi and root fungi (*n* = 9).

Kruskal–Wallis tests performed on the soil bacterial OTUs showed clearly that the contamination level had a stronger effect on the bacterial composition than plant species (Supplementary Table S2). Among the most abundant 30 OTUs, 17 were significantly affected by the contamination concentrations, two by plant species, and two by both factors, while no effect was found on the remaining nine OTUs. Most of the OTUs affected by contamination belong to *Alphaproteobacteria* (*Sphingomonas*, *Skermanella*, *Dongia*, *Rhizobiales*), *Betaproteobacteria* (*Caenimonas*, *Burkholderiales*, *Ferrovum*, *Comamonadaceae*), *Gammaproteobacteria* (*Xanthomonadales*, *Thermomonas*, *Steroidobacter*) and *Acidobacteria* groups (Supplementary Table S2).

At class level, the proportions of *Alphaproteobacteria* and *Acidobacteria* groups were significantly increased in the HC (40.8 and 11.6%) and MC (37.7 and 12.9%) sites than in the LC site (29.8 and 8.7%) (ANOVA, $P = 0.012$ and 0.002). *Betaproteobacteria* showed also a slightly higher proportion in

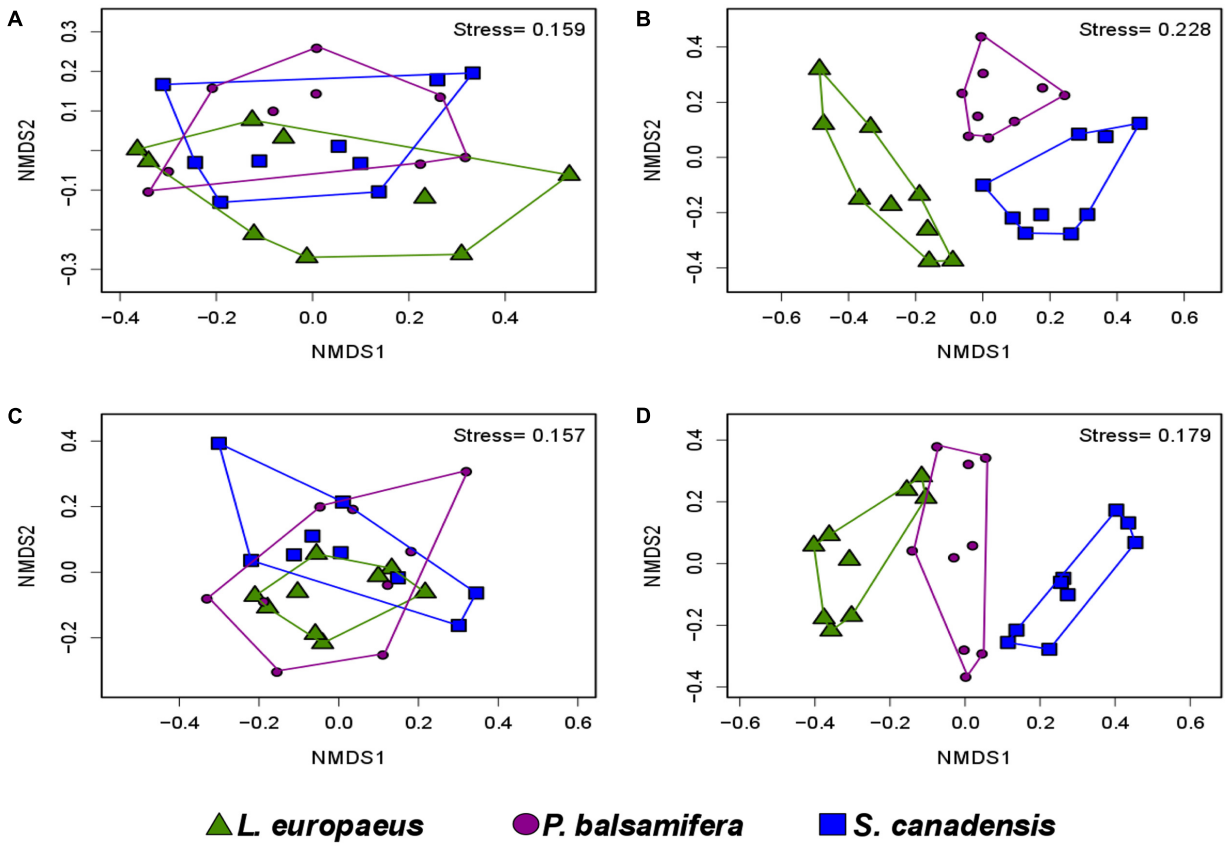

FIGURE 6 | Non-metric multidimensional scaling showing the community compositions assignments of: **(A)** soil bacteria (stress value = 0.15), **(B)** root bacteria (stress value = 0.22), **(C)** soil fungi (stress value = 0.15), and **(D)** root fungi per plant species identity (stress value = 0.17). PERMANOVA analysis showed significant effects of plant species identity only on the community composition of root bacteria and root fungi (n = 9, P = 0.001 and 0.002, respectively).

the HC and MC sites (13.3 and 15%) than in the LC site (12.8%), though ANOVA test did not show a significant difference. On the other hand, the abundance of *Gammaproteobacteria* was slightly higher in the LC and HC sites (10.8 and 10.4%) than in the MC site (7.2%), though the difference was also not significant (**Figure 7A**).

Kruskal–Wallis tests performed on the root bacterial OTUs showed that the most abundant OTUs were affected by both contamination concentrations and plant species identity. Among the 30 most abundant OTUs, 10 were significantly affected by the contamination concentration, 13 by plant species and two by both contamination and plant species. Bacterial OTU related to *Alphaproteobacteria* (*Bradyrhizobium*, *Skermanella*, *Sphingobium*, *Hoeflea*, *Hyphomicrobium,* and *Altererythrobacter*), *Actinobacteria* (*Streptomyces*, *Actinoplanes*, *Streptomyces,* and *Lentzea*), *Betaproteobacteria* (*Ideonella*, *Duganella* and *Limnobacter*) and *Gammaproteobacteria* (*Rhizobacter*, *Steroidobacter,* and *Pseudomonas*), were the most affected by contamination levels or plant species (Supplementary Table S2).

When comparing the relative abundances of the root bacteria at class or phyla level across the contaminated sites, we observed that *Actinobacteria* were in higher proportions in the HC (20.4%) and MC sites (22.5%) than in the LC site (15.6%) (ANOVA, $P = 0.041$). On the other hand, *Betaproteobacteria* class was in higher abundance in the LC (13.9%) and MC sites (14.1%) than in the HC site (6.9%) (ANOVA, $P = 0.003$).

Across plant species, *Alphaproteobacteria* were clearly more abundant in *P. balsamifera* (38.3%) and *L. europaeus* roots (33.8%) than in *S. canadensis* (25.8%) samples (ANOVA, $P < 0.001$). By contrast, the abundance of *Gammaproteobacteria* was higher in *S. canadensis* (24.2%) than in *P. balsamifera* (10.1%) and *L. europaeus* (8.4%) roots (Kruskal–Wallis, $P = 0.004$). For *Betaproteobacteria*, their abundance was slightly higher in *S. canadensis* and *L. europaeus* roots (12.6% in both plant species), compared to *P. balsamifera* (9.6%), but the *P*-value was not significant (**Figure 7B**).

PHP and Plant Species Identity Effects on Soil and Root Fungal Diversity

ANOVA tests showed that there was no effect of contamination on the Shannon diversity indices of fungi in either soil ($P = 0.424$) or root ($P = 0.714$) datasets (**Table 1** and **Figure 2C**). However, there was a highly significant effect of plant species identity on the fungal diversity in roots ($P < 0.001$). Tukey's range test showed that the divergence in root fungal diversity has occurred

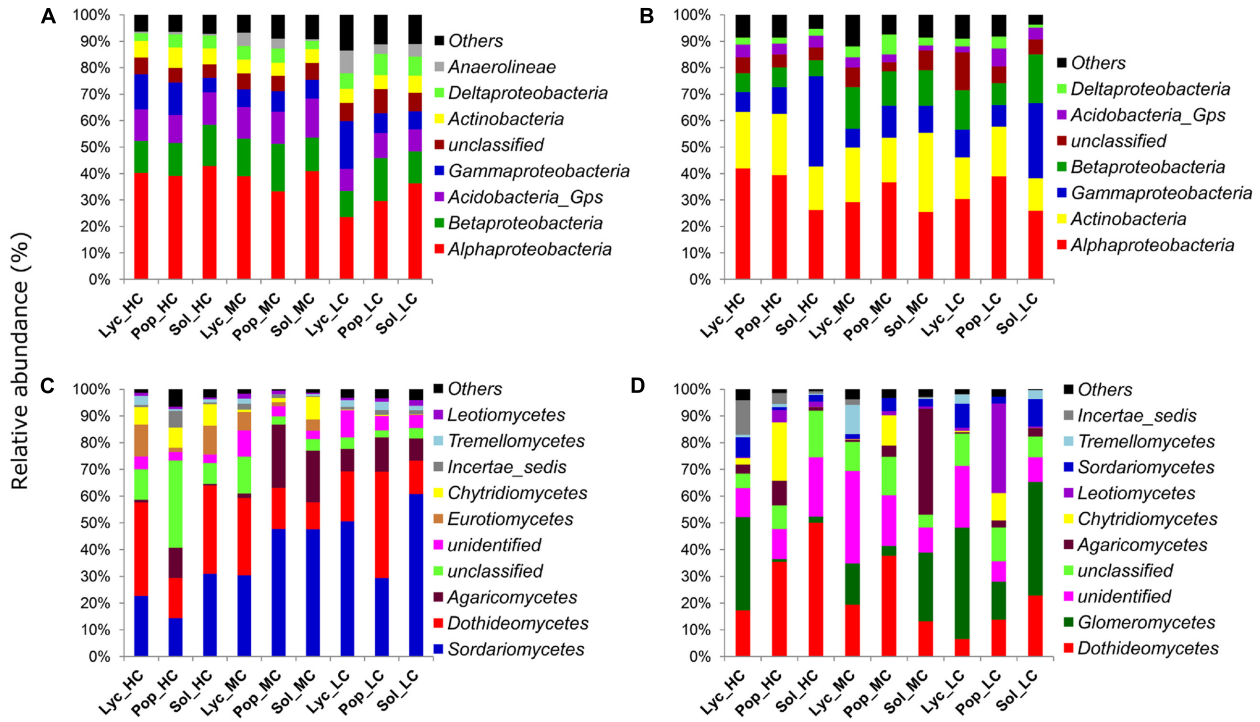

FIGURE 7 | Relative abundances of major: **(A)** soil bacteria classes, **(B)** root bacteria classes, **(C)** soil fungi classes and **(D)** root fungi classes. Sol_HC, *Solidago canadensis* in the highly contaminated site; Pop_HC, *Populus balsamifera* in the highly contaminated site; Lyc_HC, *Lycopus europaeus* in the highly contaminated site; Sol_MC, *S. canadensis* in the moderately contaminated site; Pop_MC, *P. balsamifera* in the moderately contaminated site; Lyc_MC, *L. europaeus* in the moderately contaminated site; Sol_LC, *S. canadensis* in the least contaminated site; Pop_LC, *P. balsamifera* in the least contaminated site; Lyc_LC, *L. europaeus* in the least contaminated site.

in *L. europaeus*, which showed the highest diversity compared with *P. balsamifera* and *S. canadensis*. No effect of plant species identity on the fungal diversity was found in soil (**Table 1** and **Figure 2D**).

PERMANOVA analysis showed that contamination had a significant effect on the community structure of fungi in both soil and root samples ($P = 0.002$ and $P = 0.006$, respectively), while plant species identity had a significant effect in roots only ($P = 0.002$) (**Table 1**). NMDS plots showed a clear separation between the community composition of the HC and LC sites, while the community of the MC site was intermediary in both soil and root datasets (**Figures 5C,D**). NMDS plots across plant species showed differences in community structure in roots only, where a distinct grouping of the fungal communities was found between *L. europaeus* and *P. balsamifera*, with the community of *S. canadensis* being intermediate (**Figures 6C,D**).

The Kruskal–Wallis tests confirmed that soil fungi were more affected by the contamination levels than by plants species identity, while a similar amount of root fungi were affected by either contamination and plants species (Supplementary Table S2). Among the 30 most abundant soil fungal OTUs, 13 were significantly affected by contamination, four by plants species, one by both, while the 12 remaining OTUs were not affected. The soil fungal OTUs significantly affected by contamination concentration or plants species identity belong mainly to the fungal classes *Sordariomycetes* (*Emericellopsis* sp. [OTU 5], *Lasiosphaeriaceae* [OTU 13] and *Fusarium* sp. [OTU 19]), *Agaricomycetes* (*Thelephoraceae* [OTU 13]), *Dothideomycetes* (*Leptosphaeria* sp. [OTU 11] and *Pycnidiophora* sp. [OTU 27]), *Eurotiomycetes* (*Penicillium* sp. [OTU 6]), and *Chytridiomycetes* (*Spizellomyces plurigibbosus* [OTU 8]). On the other hand, among the 30 most abundant root fungal OTUs, nine were affected by contamination, 10 by plants species, eight by both, while three OTUs were not affected. Most of the root fungal OTUs significantly affected by contamination levels and/or plant species identity belong to the classes *Dothideomycetes* (*Leptosphaeria* sp. [OTU 1], *Pleosporales* sp. [OTU 15] and *Phoma herbarum* [OTU 7]), *Chytridiomycetes* (*Olpidium brassicae* [OTU 6] and *S. plurigibbosus* [OTU 2]), *Glomeromycetes* (*Claroideoglomus* [OTU 10] and *Entrophospora infrequens* [OTU 4]), *Leotiomycetes* (*Helotiales* [OTU 9]) and *Sordariomycetes* (*Fusarium sacchari* [OTU 22] and *Myrothecium* sp. [OTU 29]) and *Agaricomycetes* (*Sebacinaceae* [OTU 5]).

In soil, at class level, *Sordariomycetes* showed more abundance in the LC (46.9%) and MC sites (41.9) than in the HC site (22.6%) (Kruskal–Wallis, $P = 0.02$). Contrarily, *Eurotiomycetes* and *Chytridiomycetes* were more abundant in the HC site (8.1 and 7.3%) than in MC (4.1 and 3.7%) and LC sites (0.1 and 0.3%) (Kruskal–Wallis, $P \leq 0.001$) (**Figure 7C**). *Dothideomycetes* were also slightly more abundant in the HC

(27.7%) than MC and LC sites (18.1 and 23.7%), though the difference was not significant by Kruskal–Wallis rank test. In roots, *Dothideomycetes* and *Chytridiomycetes* were more abundant in the HC site (34.3 and 8.1%) than in MC (23.4 and 3.9%) and LC sites (14.4 and 3.6%) (Kruskal–Wallis, $P \leq 0.05$). Contrarily, *Glomeromycetes* and *Sordariomycetes* were more abundant in the LC site (32.8 and 7.3%) compared to MC (14.9 and 3.2%) and HC sites (12.7 and 3.7%) (Kruskal–Wallis, $P = 0.02$ and 0.04). When the abundances of root fungi were compared across plant species, we observed that the proportions of OTUs belonging to different fungal classes also varied between plant species identity. *Dothideomycetes* were slightly more abundant in *P. balsamifera* (29%) and *S. canadensis* (28.7%) than in *L. europaeus* (14.4%) (Kruskal–Wallis, $P = 0.09$), while *Glomeromycetes* were more abundant in *L. europaeus* (30.7%) and *S. canadensis* (23.5) than in *P. balsamifera* (6.3%) (Kruskal–Wallis, $P = 0.004$). *Agaricomycetes* were in higher proportions in *S. canadensis* (14.7%) compared to *L. europaeus* (1.6%) and *P. balsamifera* (5.3%) (Kruskal–Wallis, $P = 0.018$). *Chytridiomycetes* and *Leotiomycetes* were more abundant in *P. balsamifera* (14.5 and 13.2%, respectively) than in *L. europaeus* (1 and 0.6%, respectively) and *S. canadensis* (0.04 and 1.2%, respectively) (Kruskal–Wallis, $P < 0.001$) (**Figure 7D**).

Soil and Root Microbial Diversity versus AMF Spore-Associated Microbial Diversity

The comparison of the soil and root microbial communities with those identified in association with AMF spores harvested from the same plant rhizospheres (Iffis et al., 2016) revealed that the community structure of AMF-associated microorganisms significantly differed from the communities identified in the rhizospheric soil and plant roots (PERMANOVA, $P < 0.001$ for both bacteria and fungi). The NMDS plots showed a distinct grouping of soil and root microbial communities compared with AMF spore-associated microbial communities (**Figures 3B**, **4B**). We previously found that *Gammaproteobacteria* and *Betaproteobacteria* were the most dominant classes associated within AMF spores (their abundances were 49 and 23%, respectively), while the most dominant fungi belonged to the unclassified fungi (55%), *Pezizomycetes* (13%) and *Dothideomycetes* (13%) (Iffis et al., 2016). In the present study, *Alphaproteobacteria* was the most dominant bacterial class in both soil and root datasets (36% in soil and 33% in roots), while *Sordariomycetes* was the most dominant fungal group in soil (37%) and *Dothideomycetes* was the most dominant in roots (24%). Here, *Gammaproteobacteria* represented only 9% of OTUs in soil and 14% in roots, while *Pezizomycetes* formed only 1.3% of OTUs in roots and 0.8% in soil (Supplementary Figure S5). The pezizomycete OTU was represented by *Sphaerosporella brunnea,* an ectomycorrhizal species that we previously found abundant in willow roots grown in the same contaminated site (Bell et al., 2014). However, here we did not find any significant effect of plant species on this fungal OTU (Supplementary Table S2). Differences in community structures between the AMF spore-associated microbiomes and the rhizospheric and root microbiome were also found at the genus level (Iffis et al., 2016). Indeed, *Alphaproteobacteria* OTU were represented mainly by the genera *Sphingomonas* in soils and *Bradyrhizobium* in roots, while this group was represented mainly by the genus *Caulobacter* in the AMF spore-associated microbiome (Iffis et al., 2016). Similarly, *Betaproteobacteria* OTU were represented mainly by the genera *Duganella* and *Janthinobacterium* in the spore microbiome, while they were formed mainly by unclassified *Betaproteobacteria* and *Caenimonas* in soil, and by *Duganella* in roots (Supplementary Figure S3). For fungi, *Septoria* was the most representative genus of *Dothideomycetes* associated with the AMF spores, while in soil and roots, the *Dothideomycetes* were represented mainly by *Pleosporales* (Supplementary Figure S4).

DISCUSSION

In rhizospheric soil, plant roots, bacteria and fungi form tripartite associations ranging from beneficial to harmful interactions based on exchange of complex signaling dialogs and nutrient compounds by which each partner influences the other to avoid the different biotic and/or abiotic stresses able to disrupt their life cycle. Therefore, the microbial communities living in soil or in association with roots are intimately linked to the different exudates released in the rhizosphere (root and microbial exudates), to soil composition and to climatic conditions. In this study, we assessed the variation in bacterial and fungal diversity across PHP concentrations, plant species identity and habitats (soil versus roots). Furthermore, taking advantage of the fact that soil and root samples used in this study were the same as those used previously in Iffis et al. (2016) study, we compared the bacterial and fungal diversity found in the current study to that found associated with the AMF spores in the Iffis et al. (2016) study to test the hypothesis that microbial communities living in association with AMF spores are selected by the AMF and not only randomly recruited from those found in the rhizosphere and roots of the host plants.

In our study, rarefaction curved of bacterial diversity were not saturated because of the limited number of sequences per sample, thus only dominating bacterial taxa are discussed. The comparison of microbial communities across PHP concentrations revealed that *Alphaproteobacteria* were favored in the high contaminated HC site, both in soil and in roots. *Actinobacteria* were also among the most dominant groups in the plant roots of the HC site. The high abundance of *Alphaproteobacteria* and *Actinobacteria* in the HC site may be related to their PHP tolerance and/or their ability to degrade PHP compounds. Several studies carried out on the microbial communities in PHP contaminated sites showed that *Alphaproteobacteria* and *Actinobacteria* were often found in higher abundances in soils containing high amounts of organic contaminants (Greer et al., 2010; Bell et al., 2014; Yergeau et al., 2014; Pagé et al., 2015). Furthermore, *Sphingomonas* (the most dominant *Alphaproteobacteria* in the soil dataset), *Bradyrhizobium* (the most dominant *Alphaproteobacteria* in the root dataset), and *Streptomyces* (the most dominant *Actinobacteria* in soil and roots) were shown to degrade a

range of recalcitrant PAH compounds, such as phenanthrene, pyrene, and naphthalene (Rentz et al., 2008; Qu and Spain, 2011; Balachandran et al., 2012; Bourguignon et al., 2014). The presence of *Gammaproteobacteria* in similar abundances in the LC and HC sites, both in soil and root datasets, may be related to the large spectrum of activities of the species belonging to this class. For example, in PHP contaminated soils, *Pseudomonas* (the most dominant genus of *Gammaproteobacteria* in roots) was shown to degrade a range of PAH compounds such as phenanthrene, alkane, and naphthalene (Ma et al., 2006; Ní Chadhain and Zylstra, 2010; Sun et al., 2014). On the other hand, in agricultural soils, *Pseudomonas* taxa are known as potential plant growth-promoting bacteria able to establish a symbiotic association with plant roots and to play an important role in plant growth, nitrogen fixation and phosphate solubilization (Rodrìguez and Fraga, 1999; Desnoues et al., 2003; Sharma et al., 2013). In the case of fungi, *Dothideomycetes* and *Chytridiomycetes* were the fungal classes found in higher abundance in the HC site. To our knowledge, PHP tolerance or biodegradation abilities of *Chytridiomycetes* was never studied. Surprisingly, except the study of Iffis et al. (2016), none of the published studies carried out nearby the basins of our study have found *Chytridiomycetes*, either in rhizospheric soils and sediments or in association with plant roots (Bell et al., 2014; Stefani et al., 2015; Bourdel et al., 2016). However, these studies found that *Dothideomycetes* were among the most dominant fungal classes in the PHP contaminated sites. Furthermore, several studies reported that some species belonging to *Dothideomycetes* are able to tolerate or break down a range PHP compounds (Junghanns et al., 2008; Ferrari et al., 2011; Harms et al., 2011; Stefani et al., 2015). For example, *Alternaria* and *Cladosporium*, which were detected here both in soil and root datasets, have been shown to degrade crude oil and a variety of its derivative products such as phenanthrene, benzo[a]pyrene, fluoranthene, and anthracene (Giraud et al., 2001; Potin et al., 2004; Mohsenzadeh et al., 2012; Ameen et al., 2016).

Our results also showed that the OTU richness of bacteria and fungi were significantly decreased in root samples in comparison to the soil samples. Generally, the microbial diversity was shown to increase in rhizospheric soils compared to the different plant compartments (roots, stem, or leaves) (Xu et al., 2012; Turner et al., 2013a; Edwards et al., 2015). The increase of microbial richness in soils compared to roots may be related to the difference in environmental conditions and nutrient bioavailability in the two ecological niches (soil versus roots). Indeed, plant roots have a selective effect on both rhizospheric and endophytic microorganisms, however, the selective effect is much higher in the endosphere (inside roots) owing to the complexity and specificity of plant–microbe interactions and plant immune system responses (Bais et al., 2006; Hardoim et al., 2008; Oldroyd, 2013). Generally, before root colonization, plants and microorganisms engage in a complex chemical dialog, and only the bacteria or fungi recognizing the signaling pathways are allowed to penetrate and colonize plant roots (Bertin et al., 2003; Bais et al., 2006; Oldroyd, 2013). Furthermore, once inside roots, the endophytes are subjected to stress caused by the new conditions and consequently, only the microbes able to adapt to the intraradical conditions can proliferate inside root compartments (Jones et al., 2007; Parniske, 2008; Gaiero et al., 2013; Brader et al., 2014). For example, plant root infection by nitrogen fixing bacteria (e.g., *Bradyrhizobium*) and arbuscular mycorrhizal fungi, which are found in higher proportion in root samples than in soil samples, is achieved through an exchange of complex chemical signaling between the plant roots and microbes (Bonfante and Anca, 2009; Philippot et al., 2013). In the nitrogen-fixing bacterial symbiosis, plant roots release in the rhizosphere specific signaling compounds, composed mainly of flavonoids, which stimulate these bacteria to produce a series of lipochitooligosaccharide compounds (nodulation factors) that are required to activate the rest of the symbiosis signaling pathway (Fisher and Long, 1992; Jones et al., 2007). An analog strategy to nitrogen-fixing bacterial infection was also described between plant roots and AMF. The signaling begins with root exudation of strigolactones in the rhizosphere (Parniske, 2008). Perception of strigolactones by AMF stimulate the spores to answer by releasing other signaling compounds, so called "Myc factors," which trigger the symbiosis pathway (Parniske, 2008; Maillet et al., 2011; Oldroyd, 2013). Unlike the conditions faced by microorganisms living in the roots, in the rhizosphere, the soil surrounding the roots is rich in nutrients. A large range of soil organic matter, as well as root exudates composed mainly of carbohydrates, amino acids and organic acids are present at the soil-root interface and stimulate the proliferation of the rhizosphere-living fungi and bacteria (Bertin et al., 2003; Somers et al., 2004; Bais et al., 2006; Philippot et al., 2013; Quiza et al., 2015).

The comparison of microbial communities between soil and roots showed that the proportions of OTUs belonging to some groups of fungi (e.g., *Sordariomycetes* and *Glomeromycetes*) and bacteria (e.g., *Gammaproteobacteria*, *Actinobacteria,* and *Acidobacteria*) were different between rhizospheric soils and plant roots. In addition, at the genus rank, we found that the community structures of fungal and bacterial genera identified in rhizospheric soils were different from those identified in plant roots. Even if the root microbiome was considered as a community derived from the rhizospheric soil (Compant et al., 2010; Gaiero et al., 2013; Turner et al., 2013a), several studies demonstrated that the microbial community composition (fungi and bacteria) in rhizospheric and bulk soils are different from those of plant roots (Smalla et al., 2001; Xu et al., 2012; Shakya et al., 2013; Edwards et al., 2015; Quiza et al., 2015). Usually, the same phyla or classes of microorganisms were found in soils and roots, but their abundances varied between the two habitats. Moreover, differences in the taxonomic affiliations were often reported when the comparisons were carried out at genus or species rank (Gottel et al., 2011; Turner et al., 2013a,b; Edwards et al., 2015). In our study, most of the bacteria and fungi identified in high proportions in roots were already known to be endophytic, mycorrhizal or obligatory biotrophic microorganisms, establishing a symbiotic or pathogenic associations with plants. For example, *Bradyrhizobium* and *Pseudomonas* (the most dominant *Alphaproteobacteria* and *Gammaproteobacteria* found in the root dataset) are plant growth-promoting bacteria able to

establish endosymbiotic associations with the roots of several plant species (Fisher and Long, 1992; Kneip et al., 2007; Sharma et al., 2013). Similarly, *Glomeromycetes* are known as obligate biotrophic fungi that require a host plants for their growth and reproduction (Simon et al., 1993; St-Arnaud and Vujanovic, 2007; Smith and Read, 2008).

While there is a growing need in the world to decontaminate polluted soils, microbe-assisted phytoremdiation can be an alternative biotechnology for remediation and revegetation of contaminated soils. The outcome of our results could improve photoremediation technology, particularly in highly contaminated sites by organic pollutants, by combining endophytic and mycorrhizal inoculants with appropriate plants.

The shifts in the community structures of AMF-associated bacteria and fungi across soils and roots observed in this study support the hypothesis that AMF select the microbial communities living in association with their spores and mycelia. As with plant roots, AMF may release carbon resources and other signaling molecules that make the surface of spores and mycelia favorable and then selective for the growth of specific microorganisms, as proposed previously (Roesti et al., 2005; Bharadwaj et al., 2011; Lecomte et al., 2011; Agnolucci et al., 2015; Iffis et al., 2016). For example, Bharadwaj et al. (2011) conducted *in vitro* cultures of 10 AMF-associated bacteria isolates and observed that the growth rates of the isolates were significantly increased by the addition to the culture medium of a broth medium in which AMF have been already cultured (they considered the broth medium as being rich in AMF exudates). In another study, Roesti et al. (2005) performed a cross-inoculation experiment with two AMF species (*Glomus geosporum* and *G. constrictum*) and two host plant species (*Plantago lanceolata* and *Hieracium pilosella*), and observed the AMF spore-associated bacterial communities were more determined by AMF-identity than plant species identity. However, little is known about AMF exudates composition and their effects on soil microorganisms. Therefore, further investigations on this topic will be required to fully understand the mechanisms by which AMF spores recruit their associated microorganisms.

CONCLUSION

The high throughput amplicon sequencing approach used in our study allowed us to characterize the variations in bacterial and fungal communities in soils and roots across petroleum hydrocarbon concentrations and plant species. Overall, we found that bacterial and fungal communities associated to plant roots varied significantly across both PHP concentrations and plant species identity, while they were affected only by PHP concentrations in soil. Our results also showed that the bacterial and fungal OTU richness and community structures differed significantly between soil and roots. Furthermore, comparisons between the AMF spore-associated microbiome described previously in Iffis et al. (2016) and the results of the present study showed that the microbial communities living in association with AMF spores significantly differed from those found in the surrounding soil and roots.

AUTHOR CONTRIBUTIONS

BI: Performed the experiments, done bioinformatics and statistics and wrote the paper. MS-A: Helped to design the experiments and wrote the paper. MH: Supervised, conceived, designed the experiments and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01381/full#supplementary-material

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Molecular Characterization of Arbuscular Mycorrhizal Fungi in an Agroforestry System Reveals the Predominance of *Funneliformis* spp. Associated with *Colocasia esculenta* and *Pterocarpus officinalis* Adult Trees and Seedlings

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Pterocarpus officinalis (Jacq.) is a leguminous forestry tree species endemic to Caribbean swamp forests. In Guadeloupe, smallholder farmers traditionally cultivate flooded taro (*Colocasia esculenta*) cultures under the canopy of *P. officinalis* stands. The role of arbuscular mycorrhizal (AM) fungi in the sustainability of this traditional agroforestry system has been suggested but the composition and distribution of AM fungi colonizing the leguminous tree and/or taro are poorly characterized. An in-depth characterization of root-associated AM fungal communities from *P. officinalis* adult trees and seedlings and taro cultures, sampled in two localities of Guadeloupe, was performed by pyrosequencing (GS FLX+) of partial 18S rRNA gene. The AM fungal community was composed of 215 operational taxonomic units (OTUs), belonging to eight fungal families dominated by Glomeraceae, Acaulosporaceae, and Gigasporaceae. Results revealed a low AM fungal community membership between *P. officinalis* and *C. esculenta*. However, certain AM fungal community taxa (10% of total community) overlapped between *P. officinalis* and *C. esculenta*, notably predominant *Funneliformis* OTUs. These findings provide new perspectives in deciphering the significance of *Funneliformis* in nutrient exchange between *P. officinalis* and *C. esculenta* by forming a potential mycorrhizal network.

Keywords: arbuscular mycorrhizal community, *Colocasia esculenta*, *Funneliformis*, Guadeloupe, *Pterocarpus officinalis*, pyrosequencing, tropical agroforestry

INTRODUCTION

Pterocarpus officinalis L. is one of the dominant wetland tree species of the seasonally flooded swamp forests in the Caribbean and the Guiana regions (Eusse and Aide, 1999; Bâ and Rivera-Ocasio, 2015). It covers large areas of the coastal floodplain as individual trees and small patches adjacent to mangroves, and along rivers and in mountains (Eusse and Aide, 1999). In the

Caribbean, this unique *P. officinalis* swamp forest provides a habitat for many species of plants and animals and reduces soil erosion along the margins and riverbanks in coastal and mountain areas (Saur and Imbert, 2003; Bâ and Rivera-Ocasio, 2015). Despite its ecological interest, most of the populations of *P. officinalis* in the Caribbean islands are restricted to a small area due to wetland drainage and urban development (Bâ and Rivera-Ocasio, 2015). Furthermore, the low genetic diversity found within and between populations of *P. officinalis* is exacerbated by a strong inbreeding depression (Muller et al., 2009). As a consequence, management and conservation measures must be implemented to preserve the remaining *P. officinalis* populations.

In some Caribbean islands, a dominant management strategy for *P. officinalis* conservation is to plant agricultural crops under these stands. The Guadeloupean smallholder farmers notably conduct taro (*Colocasia esculenta* L. Schott) monocultures under the *P. officinalis* stands in freshwater flooding swamp forests because of higher crop yields compared to other agricultural practices (Saur and Imbert, 2003). No fertilizer or pesticide are used, and the *P. officinalis* trees are not impacted (no cutting), preserving the tree genetic diversity, and the ecosystem processes (e.g., nutrient cycling, local biodiversity) (Rivera-Ocasio et al., 2006; Bâ and Rivera-Ocasio, 2015). The ecological mechanisms sustaining the functioning of this traditional agroforestry system remains poorly investigated. Indeed heavy leaching of soils brought by seasonal flooding contributes to the shortage of available P and N and should be detrimental for taro monocultures (Bâ and Rivera-Ocasio, 2015). *Pterocarpus* stands might be beneficial to understory taro cultures by (i) maintaining humidity and temperature at certain levels to prevent water stress (Sanou et al., 2012), and (ii) improving the N input on soil and non-legume plants through biological nitrogen fixation process (Koponen et al., 2003; Saur and Imbert, 2003). The transfer of N from legume to the non-legume can occurred through root exudation, root and nodule decomposition and mineralization, as well as mediated by plant-associated arbuscular mycorrhizal (AM) fungi (Kaiser et al., 2015). Both *P. officinalis* and *C. esculenta* establish a symbiosis with AM fungi (Saint-Etienne et al., 2006; Wang and Qiu, 2006; Fougnies et al., 2007), notably improving plant phosphorus (P) nutrition (Smith and Read, 2008), but also potentially plant N nutrition (Hodge et al., 2001; Walder et al., 2012). In addition, *P. officinalis* requires P from AM fungi not only for their nutrition but also for efficient nodule formation and nitrogen fixation (Fougnies et al., 2007; Le Roux et al., 2014). Whereas, the diversity of nitrogen-fixing bacteria associated with *P. officinalis* in Caribbean swamp forests has been described (Le Roux et al., 2014), AM fungal diversity has been poorly investigated.

Arbuscular mycorrhizal fungi are the most common and widespread symbiosis involving 86% of land plants including many important crops (Davison et al., 2012). The extraradical phase of AM fungi acts as an extension of the root system for the uptake of nutrients in exchange for plant-synthesized carbon. AM fungi are assumed to exhibit non-specific symbiosis but a given AM taxa could have different effects depending on plant species (Thioye et al., 2017). The relatively low host specificity of AM fungi, increases the possibility that extraradical fungal hyphae links multiple plant species to form common mycorrhizal networks (CMNs) in a plant community (Cheng and Baumgartner, 2004; Ingleby et al., 2007). Mycorrhizal networks are known to drive nutrient transfers (mainly C and N) between adult trees and seedlings for one plant species, and among different plants species (e.g., legume and non-legume) (Cheng and Baumgartner, 2004; He et al., 2004; Selosse et al., 2006; Wahbi et al., 2016). However, mycorrhizal networks have been mainly assessed at the fungal strain level in controlled conditions (Walder et al., 2012) and rarely at the community level (Montesinos-Navarro et al., 2012). Thanks to the development of high-throughput sequencing approaches such as pyrosequencing, the complexity of AM fungal community among plant roots can be deeply assessed (Hart et al., 2015), and a wide range of ecological studies based on the diversity of AM fungal taxonomic markers such as the SSU rRNA gene has been performed (Öpik et al., 2006, 2014).

The current study aims the molecular characterization of AM fungal community composition and distribution between *P. officinalis* and *C. esculenta* crops in a traditional Guadeloupean agroforestry system (swamp forests) in order to evaluate if the sustainability of the system might be explained by a high similarity or a high dissimilarity of AM fungal community between *Pterocarpus* and taro. In addition, the comparison of AM fungal community of *P. officinalis* adult trees and seedlings with the ones of taro were investigated because of the potential importance of seedlings in the sustainability of the agroecosystems since they are conserved inside the taro cultures. Consequently, two main questions were assessed, (i) What are the predominant mycorrhizal taxa in the traditional agroforestry system, and (ii) What is the degree of similarity of AM fungal members among *P. officinalis* adult trees, seedlings and taro?

MATERIALS AND METHODS

Study Sites and Sampling

We conducted the sampling during 2012 in two representative *Pterocarpus* swamp forests located in the Grande-Terre island of Guadeloupe: Grande Ravine (GR) (16°13′N, 61°28′W) and Belle Plaine (BP) (16°17′N, 61°31′W) (**Supplementary Figure S1**). The *P. officinalis* stands, which comprise 45 and 52 adult trees for GR and BP sites, respectively, are fairly forested and contain dense populations of understory regenerating seedlings. The GR forest site (approximately 0.3 ha) is located along the GR river and taro plants (*C. esculenta*) were cultivated by smallholders farmers under adult trees and between naturally regenerating seedlings. Some individuals of understory plant species such as *Ficus* sp.1, *Commelina* sp.1, and *Mimosa pudica* were naturally associated with *P. officinalis*. The BP forest site (approximately 0.4-ha), is located around the bay of the Grand Cul-de-sac Marin, in the near mangrove area and taro plants were also cultivated between *Pterocarpus* trees and regenerating seedlings. Understory species like *Musa* sp. and *Ficus* sp. are represented by a few individuals and are widely

spaced from one another. In each forest site, soil cores (200 g of fresh soil) were randomly collected near three adult trees (more than 25 m high), three seedlings (1 < height < 2 m) and three taro plants. Overall, 18 soil cores were stored at 4°C before being processed. Roots were separated from soil, gently washed with tap water and dried with Silica-gel until molecular analyses. Soil physico-chemical parameters were measured at the Celesta-lab (Mauguio, France) (Supplementary Table S1).

Molecular Analyses

For each root sample, the three replicates were pooled and subjected to liquid nitrogen grinding for homogenization. Total DNA was extracted from a sub-sample (100 mg of dried root) using a FastPrep-24 homogenizer (MP Biomedicals Europe, Illkirch, France) and the FastDNA® SPIN kit (MP Biomedicals Europe) according to manufacturer's instructions. The quality of DNA extracts was improved by adding 20–30 mg Polyvinylpolypyrrolidon (PVPP) during the first step of DNA extraction. Two DNA extractions were done per root sample.

Glomeromycota (AM fungi) sequences were amplified using the nuclear 18S rRNA gene primers NS31 [5′- (10-bp MID) TTGGAGGGCAAGTCTGGTGCC -3′] (Simon et al., 1992) and AML2 [5′- (10-bp MID) GAACCCAAACACTTTGGTTTCC -3′] (Lee et al., 2008). MIDs (multiplex identifier) were designed by Eurofins Genomics (Eurofins Genomics GmbH[1]). PCR conditions were performed according to Sanguin et al. (2016). Two PCR products per root sample were pooled before purification using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, Velizy-Villacoublay, France) following manufacturer's guidelines. Overall 18 PCR products were subjected to bi-directional 454-sequencing (1/4th plate Roche GS FLX+ run using the GS FLX Titanium sequencing kit XL+) by Eurofins Genomics.

Data Processing and Taxonomic Assignment of AM Fungal Sequences

Four hundred and fifty-four-sequencing data were analyzed using Mothur software according the standard operating procedure[2] proposed (Schloss et al., 2011), except for the quality cutoffs, for which it has been set up at Q30 (*trim.seqs* command). All sequence reads were then depleted of barcodes and primers (final length 230 bp), and sequences < 100 bp or with ambiguous base calls or with homopolymer runs exceeding 8 bp were also removed. A pre-clustering step (Huse et al., 2010) was also performed to remove sequences still likely due to pyrosequencing errors. Chimeric sequences were checked by using UCHIME (Edgar et al., 2011) and removed. Finally, sequences were identified using the Glomeromycota-based alignment database (Krüger et al., 2012) and sequence similarity ≥60% at the family level.

Clustering of sequences in operational taxonomic unit (OTUs) was performed using *dist.seqs* and cluster commands in Mothur. Then, the number of sequences from each sample was normalized with *sub.sample* command. This sub-sampling step allows reducing the number of spurious OTUs and is crucial to obtain robust estimation of alpha and beta diversity (Gihring et al., 2012). Finally, OTUs were defined at 97% similarity level for taxonomic affiliation.

Statistical Analyses

Diversity (Shannon, inverse Simpson [1/D]), richness (number of OTUs, Chao1) and evenness (Pielou) indexes were estimated using R version 3.3.2 (R Core Team, 2016) and the R package vegan (Oksanen et al., 2016). The sequencing effort was evaluated using the coverage calculator and Boneh estimator (Boneh et al., 1998) implemented in Mothur. We assessed the effects of forest site, plant species, *Pterocarpus* age categories and their interactions on the AM fungal community composition by non-parametric permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) implemented in the *perm.anova*() function from the R package RVAideMemoire (Hervé, 2016). The differences in AM fungal community structure among forest sites and plant species were assessed using PERMANOVA in *adonis*() function (McArdle and Anderson, 2001), both from the R package vegan. The AM fungal community structure was based on the Bray–Curtis dissimilarity index as defined in *vegdist*() function from the R package vegan. Multivariate dispersion was estimated using the *betadisper*() and *permutest*() functions (999 permutations; alpha = 0.05) from the R package vegan because it can affect PERMANOVA results. Differences in the relative abundances of AM fungal OTUs among forest sites or plant species were estimated using Kruskal–Wallis' test implemented in *kruskal.test*() function from the R package stats. The frequency of AM fungal OTUs was determined using the *strassoc*() function from the R package indicspecies (De Cáceres and Legendre, 2009).

The determination of AM fungal OTUs preferentially associated with a given forest site was performed using the corrected Pearson's phi coefficient of association ("r.g") implemented in the *multipatt*() function (De Cáceres et al., 2010) from the R package indicspecies. AM fungal OTUs preferentially associated with a given plant species was assessed using the corrected indicator value index ("IndVal.g"species) from the R package indicspecies. A procedure based on determination of species (i.e., OTU) and group (i.e., plant type) combinations was applied using successively *combinespecies*() and *multipatt*() functions. This procedure was demonstrated to bear more ecological informations and to determine more robust predictive indicator value than by considering species or group independently (De Cáceres et al., 2012). Two different probabilities were calculated, i.e., A (specificity), representing the probability of a sample to be defined by a group (i.e., plant type), given that the species or the species combinations have been detected, and B (sensitivity) representing the probability of finding the species or the species combinations in different samples characterized by a given group (i.e., plant type). Only AM fungal OTUs present in two samples among three groups defined (i.e., plant type) were subjected to analysis. We considered

[1] www.eurofinsgenomics.eu

[2] http://www.mothur.org/wiki/454_SOP

as valid indicators the OTUs showing both A (specificity) and B (sensitivity) superior to 0.8 and 0.6, respectively, as recommended in Suz et al. (2014).

The AM fungal community membership among *C. esculenta* and *P. officinalis* adult trees and seedlings was assessed using venn diagram analysis with the R package VennDiagram (Chen, 2016), and the relative abundance of AM fungal taxa shared among plants was characterized using bipartite network analysis with the *plotweb*() function from the R package bipartite (Dormann et al., 2008).

RESULTS

AM Fungal Community Composition among Forest Sites

The global 454-pyrosequencing data were composed of 210,676 reads, and 155,544 reads (74%) passed the quality control steps. The average read length was 230 bp. After trimming, pre-clustering and chimera detection steps, 70,949 sequences were classified using a Glomeromycota-based alignment database. A total of 31,803 non-Glomeromycota sequences were removed from the dataset (70,949), as well as singletons (1515 sequences) that are mostly considered as artifacts and can lead to overestimations of AM fungal diversity. The AM fungal sequences (37,631 reads) were assigned to a total of 215 OTUs based on a sequence similarity threshold $\geq$ 97%. The sequence number between samples was rarefied to 440 sequences per sample (threshold based on the sample with the lower number of sequences) to improve statistical robustness. A high diversity coverage (94–98%) was reached for all samples, with less than eight potential OTUs that were not retrieved (Boneh estimation, Supplementary Table S2). The Boneh estimation showed that the sequencing depth was sufficient to estimate and compare the microbial diversity of the samples (Supplementary Table S2).

The taxonomy assignment of OTUs (Supplementary Table S3) revealed a main affiliation to Glomeraceae (83.5% of sequences), but also to Acaulosporaceae (9.7%), Gigasporaceae (5.3%), Archaesporaceae (0.9%), Geosiphonaceae (0.3%), Diversisporaceae (0.1%), Paraglomeraceae (0.05%), and Pacisporaceae (0.03%). In Glomeraceae, *Funneliformis* represents the most abundant genus (62% of sequences), followed by fungi of uncertain position in *Glomus* sensus lato (20%), *Rhizophagus* (2%), and *Sclerocystis*/*Glomus* (<1%) (Supplementary Table S3). The low evenness estimated for all AM fungal communities (<0.3) revealed the strong predominance of few OTUs and numerous rare OTUs (Supplementary Table S2). Three OTUs, i.e., OTU1 and OTU2, belonging to Glomeraceae and OTU3 to Acaulosporaceae families, represented more than 80% of sequences (Supplementary Table S3). The most predominant OTU (OTU1, 58% of sequences) was affiliated to *Funneliformis*.

Richness, diversity and evenness were calculated for the different AM fungal communities. No significant difference was observed at both forest sites (Supplementary Table S2). AM fungal community structure analysis, based on Bray–Curtis index, also showed no significant difference between both forest sites (**Table 1**). However, four OTUs affiliated to *Acaulospora, Gigaspora*, and *Incertae sedis Glomus* showed significantly higher abundance in BP, and three OTUs affiliated to *Rhizophagus* and *Funneliformis* in GR (**Figure 1**, for all comparisons see Supplementary Table S4). Among the 215 AM fungal OTUs, only four were determined as preferentially associated at a given forest site (Supplementary Table S5), among which two of them, i.e., *Gigaspora* (OTU28) and *Rhizophagus* (OTU16) were exclusively found in BP and GR sites, respectively.

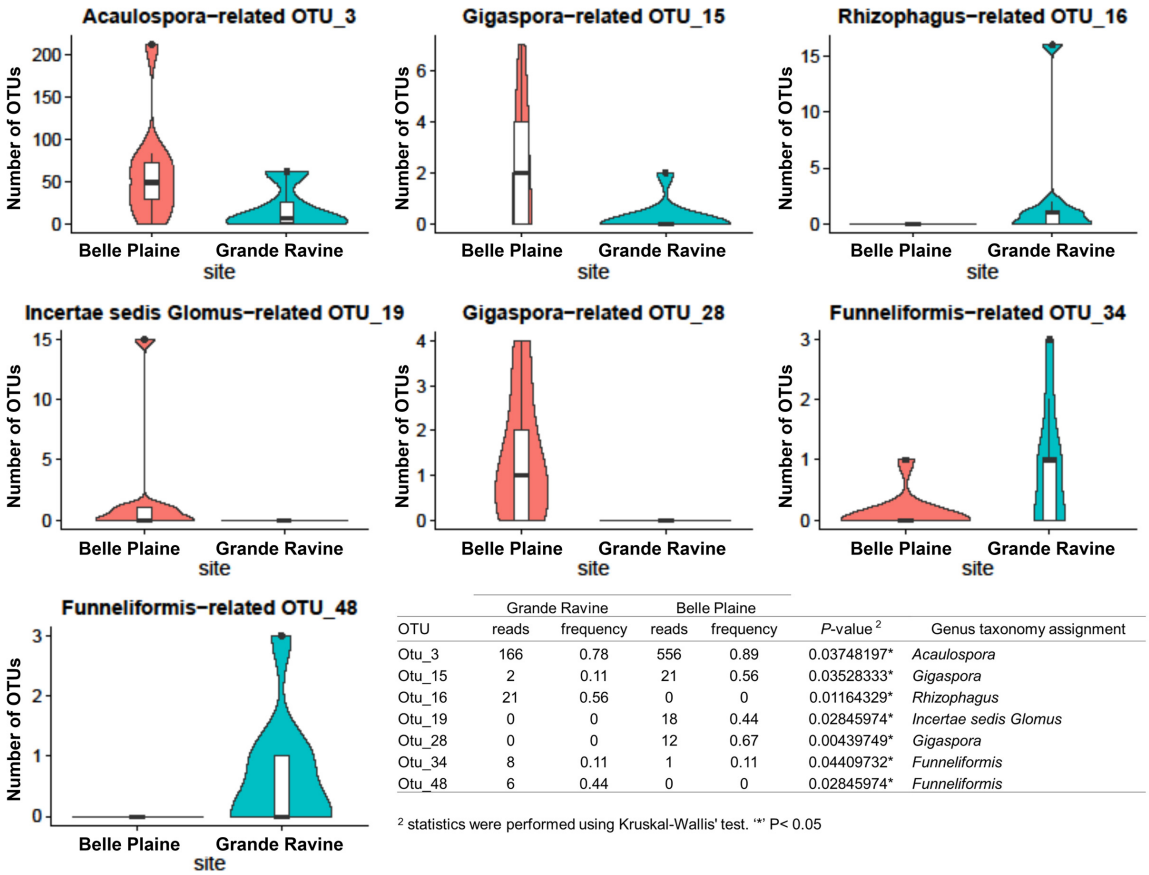
AM Fungal Community Composition among Plant Types

Arbuscular mycorrhizal fungal community richness (for the two indices Chao1 and OTUs number) was significantly different between taro and *Pterocarpus* ($P = 0.033$; Supplementary Table S2) whereas only a locality-dependent plant type effect was observed on AM fungal community structures ($P = 0.011$; **Table 1**). The analysis of AM fungal community structure

TABLE 1 | Impact of locality and plant type on arbuscular mycorrhizal (AM) fungal community structures in agroforestry systems.

Locality	Factors	Df	SS	MS	F. Model	R^2/N perm	*P*-value[1]
All	Locality	1	0.11671	0.11671	1.298	0.05730	0.242^{ns}
	Plant	2	0.26401	0.132006	1.4610	0.12962	0.173^{ns}
	Locality × Plant	2	0.57188	0.285939	3.1647	0.28077	**0.011***
	Residuals	12	1.08422	0.090352		0.53231	
	Total	17	2.03682			1	
Belle Plaine	Plant	2	0.38777	0.19389	1.4766	0.32984	0.079^{ns}
	Residuals	6	0.78786	0.13131		0.67016	
	Total	8	1.17563			1.00000	
Grande Ravine	Plant	2	0.44812	0.224058	4.5362	0.60192	**0.036***
	Residuals	6	0.29636	0.049393		0.39808	
	Total	8	0.74448			1.00000	

[1]*The significance of multivariate analysis of variance was assessed using PERMANOVA with adonis() function (iterations = 999 permutations). *$P < 0.05$, $^{ns}P > 0.05$. Multivariate dispersion was tested using the betadisper() and permutest() functions (iterations = 999 permutations; alpha = 0.05) revealing a significant homogeneity of group dispersions.*



OTU	Grande Ravine reads	Grande Ravine frequency	Belle Plaine reads	Belle Plaine frequency	*P*-value [2]	Genus taxonomy assignment
Otu_3	166	0.78	556	0.89	0.03748197*	*Acaulospora*
Otu_15	2	0.11	21	0.56	0.03528333*	*Gigaspora*
Otu_16	21	0.56	0	0	0.01164329*	*Rhizophagus*
Otu_19	0	0	18	0.44	0.02845974*	*Incertae sedis Glomus*
Otu_28	0	0	12	0.67	0.00439749*	*Gigaspora*
Otu_34	8	0.11	1	0.11	0.04409732*	*Funneliformis*
Otu_48	6	0.44	0	0	0.02845974*	*Funneliformis*

[2] statistics were performed using Kruskal-Wallis' test. '*' P< 0.05

FIGURE 1 | Abundance of arbuscular mycorrhizal (AM) fungal operational taxonomic units (OTUs) between Grande Ravine and Belle Plaine forest sites. Only OTUs with significant ($P < 0.05$) differences in their relative abundance between the both forest sites are shown. Table represented comparison of AM fungal OTU abundances between localities (Belle Plaine and Grande Ravine) with statistics were performed using Kruskal–Wallis' test. $*P < 0.05$.

for each forest site confirmed the plant type effect for the GR forest site ($P = 0.036$) (**Table 1**). The NMDS analysis (**Supplementary Figure S2**), taking into account all OTUs, highlighted the dissimilarity among AM fungal communities of *Pterocarpus* adult trees compared to both taro and *Pterocarpus* seedlings in the GR site. Out of a total of 7750 and 9730 AM fungal OTUs and OTU pair combinations for the BP and GR sites, 8 and 27 AM fungal indicators were determined, respectively (**Table 2** and Supplementary Table S6), confirming the significant and preferential association of *Gigaspora* (OTU22 and OTU33) with taro. In the GR forest site, six AM fungal OTUs (OTU1, OTU2, OTU4, OTU5, OTU6, and OTU48) belonging to the Glomeraceae were significantly associated with both taro and *Pterocarpus* seedlings, and two AM fungal OTUs (OTU18 and OTU3) with *Pterocarpus* adult trees.

TABLE 2 | Single of AM fungal operational taxonomic units (OTUs) associated with a plant type in *Pterocarpus*-taro in Guadeloupean agroforestry systems.

Locality	Plant	Indicator taxa Taxonomy (OTU)	Frequency[1] (P.a/P.s/T)	*A*[2] (specificity)	*B* (sensibility)	IndVal.g	*P*-value[3]
Belle Plaine	T.	*Gigaspora (33)*	0.0/0.0/1.0	1.00	1.00	1.00	0.027*
Grande Ravine	T.	*Gigaspora (22)*	0.0/0.0/1.0	1.00	1.00	1.00	0.035*
	P.a	*Geosiphon (18)*	0.0/0.0/1.0	1.00	1.00	1.00	0.032*
		Archaeospora (10)	1.0/0.0/0.3	0.96	1.00	0.98	0.032*
	T. +P.s	*Incertae sedis Glomus (4)*	0.0/1.0/1.0	1.00	1.00	1.00	0.032*
		Rhizophagus (23)	0.0/1.0/1.0	1.00	1.00	1.00	0.032*

[1]*Frequency indicates the presence of an OTU in samples of a given plant type. P.a, Pterocarpus adult tree; P.s, Pterocarpus seedling; T., taro.* [2]*The corrected Indval coefficient of association ("IndVal.g") was used as model to determine indicator OTUs.* [3]$*P < 0.05$, $^{ns}P > 0.05$.

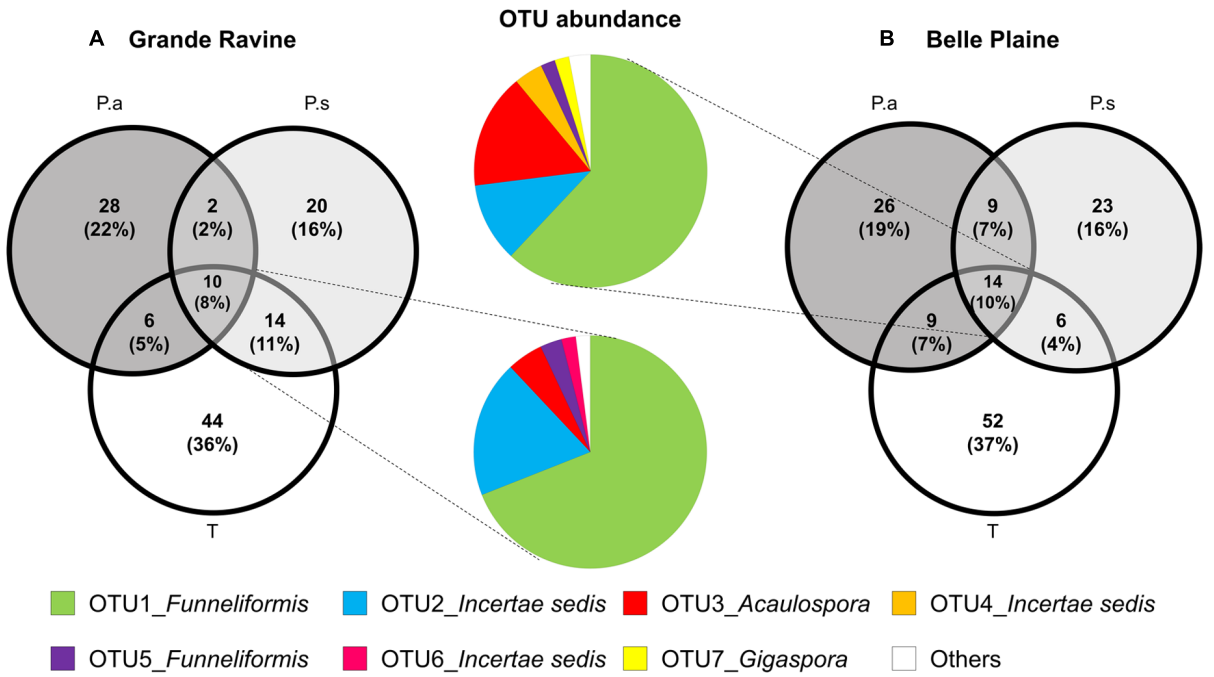


FIGURE 2 | Distribution of AM fungal OTUs shared among taro (T), *Pterocarpus* adult trees (P.a) and seedlings (P.s) in **(A)** Grande Ravine and **(B)** Belle Plaine forest sites. All sequences were clustered in OTUs (97% similarity). Data were rarefied to 440 sequences per sample. Venn diagrams represent the percentage of AM fungal OTUs specific to a given plant or overlapping among plants. Color pie charts represents the abundance of OTUs shared among the three plants for both forest sites. The category "Others" combines OTUs representing less than 1% (8 OTUs for BP and 5 for GR).

Venn diagram analysis of OTU-based AM fungal community revealed a low membership characterized by a high number of OTUs specific to one plant and few overlapping OTUs among taro and the two age categories of *Pterocarpus*, i.e., 10 and 8% of common OTUs in BP and GR sites, respectively (**Figure 2A**). Taro showed the highest number of specific OTUs (>35%) compared to *Pterocarpus* (<25%) in both forest sites. Bipartite network analysis showed that the overlapping AM fungi between plants species was mainly composed of *Funneliformis* OTUs, with 53 and 61% of sequences in BP and GR forest sites, respectively (**Figure 2B**). Rare OTUs mainly constituted the plant-specific OTUs, which fit with the low number of indicator taxa associated with the different types of plants.

DISCUSSION

Arbuscular mycorrhizal fungi might play a major role in the functioning of the traditional Guadeloupean agroforestry system associating *P. officinalis* trees, their naturally regenerating seedlings, and an understory taro monoculture, and the degree AM fungal community similarity among plants has been hypothesized as one of the main factors. Significant differences in AM fungal community richness and structure were observed among the different plant types, but not in terms of diversity. Some AM fungal OTUs were preferentially associated with a given plant type, but a highly predominant AM fungal OTU affiliated to *Funneliformis* was detected among all plants.

Characteristics of AM Fungal Community

A relatively high AM fungal richness (>120 OTUs) was observed compared to other AM fungal surveys using high-throughput methods from a wide range of in tropical forest ecosystems (22–207 OTUs; De Beenhouwer et al., 2015; Holste et al., 2016; Rodríguez-Echeverría et al., 2016). As shown by Bainard et al. (2011), tree-based cropping systems, combining different tree species (white ash, hybrid poplar and Norway spruce) with annual crops (corn, soybean, and winter wheat), can present a highly diverse AM fungal community. However, the robustness of comparisons between different tropical agro-ecosystems remains questionable due to the scarcity of studies in tropical regions. Furthermore, OTU-based fungal richness is highly dependent on the bioinformatics treatment applied (mainly sequence quality filtering and clustering methods) (Bálint et al., 2016); the biological material analyzed (roots, spores, and extraradical mycelium) (Varela-Cervero et al., 2015) or the methodology used (spore identification, PCR-cloning, pyrosequencing) (Davison et al., 2015). The predominance of Glomeraceae, which is the most widespread family in natural and managed ecosystems (Oehl et al., 2010; Brearley et al., 2016) and Acaulosporaceae was in agreement with several surveys carried out in tropical environments (Leal et al., 2013; De Beenhouwer et al., 2015; Holste et al., 2016). Only 4% of all detected AM fungal OTUs were poorly affiliated to a reference taxa in databases, which

contrasted with previous data in dry afromontane forests (Wubet et al., 2003) and dry tropical regions (Rodríguez-Echeverría et al., 2016), where up to 18 and 15% of OTUs were considered as new taxa, respectively.

Characteristics of AM fungal community (diversity, richness, and structure) were relatively comparable in this cropping system between the two forest sites (separated of 15 km). However, some AM fungal were associated to a given forest site, notably for BP site. The soil characteristics and soil hypoxia are known as major drivers of AM fungal community (Helgason and Fitter, 2009; Oehl et al., 2010; Alguacil et al., 2016) and differences observed between the both forest sites in soil nutrient contents (N, Ca, and Na; Supplementary Table S1) and flooding duration might have favored specific taxa. The low number of indicator species observed in our work was consistent with the study by Moora et al. (2014), which showed that forest plantations or cultivated lands have very few AM indicator species compared to primary forests or permanent grasslands.

Degree of AM Fungal Community Similarity between Tree and Culture

The analysis of AM fungal community composition and structure demonstrated a site-dependent host plant effect due to low abundant OTUs notably belonging to *Gigaspora* for taro, *Geosiphon* and *Archaeospora* for *Pterocarpus* adult trees. In addition, the significant differences observed between *Pterocarpus* adult trees and seedlings confirmed the modification of AM fungal communities according to the plant age (Wubet et al., 2009). Our data corroborate previous molecular studies conducted in tropical forests where divergent AM fungal communities of co-occurring plant species were reported (Uhlmann et al., 2004; Wubet et al., 2009; Mangan et al., 2010). However, three highly abundant AM fungal taxa (80% of sequences) were associated to the three plant types, notably *Funneliformis* (OTU1) that is considered as a generalist AM fungus (Öpik et al., 2006). *Funneliformis* was shown to form CMNs for transport of N, particularly in tropical environments where N is poorly available (Walder et al., 2012; Munroe and Isaac, 2014). CMNs might play a major role in the sustainability of *Pterocarpus*-taro agroforestry systems and high crop yields. First, the CMNs maintained by *Pterocarpus* could provide an AM fungal mycelium reservoir enabling a faster colonization of short-lived crops under swamp forests (Kuyper et al., 2004) compared to an AM spore reservoir (Brundrett and Abbott, 1994). Secondly, the CMNs could be involved in the N transfers from *Pterocarpus* trees or seedlings to taro in N-deficient soils of swamp forests, as observed between other legume and non-legume associations (Thilakarathna et al., 2016).

Funneliformis has been, however, described as a ruderal and stress tolerator taxa mainly involved in plant protection against biotic and abiotic stress rather than in plant nutrition (competitor) (Chagnon et al., 2013). Indeed, *Funneliformis* was shown to protect tropical plants against certain pathogens (Cardoso and Kuyper, 2006). Several strategies could be set up to experimentally test the significance of *Funneliformis* in nutrient and/or plant protection in systems associating *Pterocarpus*-taro. Compartmented microcosms with inoculated *Funneliformis* strains and the use of a ^{15}N-labeled growth substrate as designed in Walder et al. (2012) could be used to demonstrate the N transfers through *Funneliformis* CMNs between the two studied plants (**Figure 3A**). Moreover the benefits of *Pterocarpus*-taro associations compared to taro monoculture could be estimated experimentally in pot

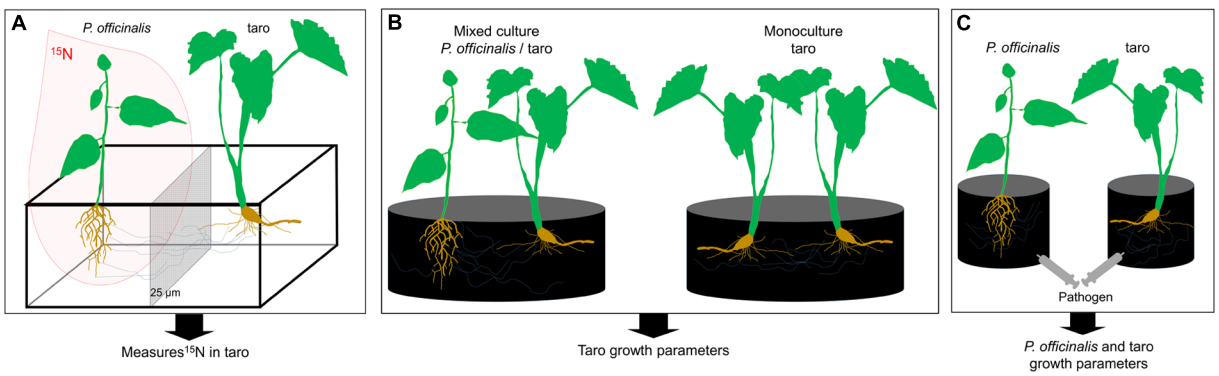


FIGURE 3 | Conceptual experimental design to evaluate role of *Funneliformis* in the nitrogen transfer from *Pterocarpus* to taro through CMNs **(A)**, in the potential benefit of *Pterocarpus-taro* associations **(B)**, and in plant pathogen defense **(C)**. For the three experimental design, all compartments are filled with sterile growth substrate and amended with nutritive solution [without N for **(A)**]. Inoculated (*Funneliformis* strain) and non-inoculated (control), are compared. **(A)** Microcosms, consisting of two plant individuals, set up in compartmented containers subdivided by nylon mesh screens (25 µm). This type of screen is pervious for fungal hyphae but not for roots and allows the separation between two plants. Three months after planting, an isotopic labeling experiment is conducted utilizing ^{15}N. Plants are harvested 20 days after labeling. Percentage of root colonized by the inoculated *Funneliformis* strain and ^{15}N abundance of taro plants are determined. **(B)** Pot culture experiments, consisting either to a model of monoculture (taro/taro) or a culture association (*P. officinalis*/taro). Plants are harvested after 12 weeks of growth and taro growth parameters are measured. The experiment aims the determination of the "competitor" status of *Funneliformis*. **(C)** Pot culture experiments, consisting in taro monoculture, to study the potential role of *Funneliformis* in plant pathogen defense. Plants are grown for 3 months to allow the establishment of *Funneliformis* and then are inoculated with a pathogen. Plants are harvested after 4 weeks of growth and growth parameters of both plants are measured. The experiment aims the determination the "ruderal" status of *Funneliformis*.

cultures (**Figure 3B**), allowing the evaluation of *Funneliformis* contribution as competitor taxa (Chagnon et al., 2013). Finally, the introduction of a pathogen in inoculated or not inoculated pot cultures could be used to evaluate the significance of *Funneliformis* regarding plant protection (**Figure 3C**), but also the contribution of *Funneliformis* as ruderal taxa (Chagnon et al., 2013).

CONCLUSION

Our study highlights the high AM fungal diversity and richness associated with roots of *Pterocarpus* (adult trees and seedlings) and taro. Although the AM fungal community is significantly different in terms of membership and structure between the types of plants, *Pterocarpus* and taro had few but predominant overlapping AM fungi, notably *Funneliformis* spp. (OTU1). From an agricultural point of view, in addition to the good tolerance of taro to waterlogging and shade under *Pterocarpus* swamp forests (Saur and Imbert, 2003), the preservation of *Pterocarpus* adult trees and their seedlings could be one of the main factors leading to high taro crop yields by maintaining N input on soil and a source of AM fungal inoculums that might form potential CMNs crucial for the establishment of taro.

DATA

Raw data are available under the BioProject ID PRJNA384862 (https://www.ncbi.nlm.nih.gov/bioproject).

AUTHOR CONTRIBUTIONS

AB and AnG designed the research and collected the samples. AlG and HS developed the methodology and performed statistical analyses. AlG and HS generated data. AB, AlG, and HS wrote the initial manuscript. AB, AlG, AnG, and HS contributed to the final manuscript. All the authors shared, edited and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01426/full#supplementary-material

FIGURE S1 | Map of Guadeloupe with the two sampling forest sites.

FIGURE S2 | Non-metric multidimensional scaling (NMDS) ordination of *Pterocarpus* and taro root-associated AM fungal communities in Grande Ravine and Belle Plaine forest sites. Different colors represented the three types of plants.

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Soil Type Has a Stronger Role than Dipterocarp Host Species in Shaping the Ectomycorrhizal Fungal Community in a Bornean Lowland Tropical Rain Forest

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The role that mycorrhizal fungal associations play in the assembly of long-lived tree communities is poorly understood, especially in tropical forests, which have the highest tree diversity of any ecosystem. The lowland tropical rain forests of Southeast Asia are characterized by high levels of species richness within the family Dipterocarpaceae, the entirety of which has been shown to form obligate ectomycorrhizal (ECM) fungal associations. Differences in ECM assembly between co-occurring species of dipterocarp have been suggested, but never tested in adult trees, as a mechanism for maintaining the coexistence of closely related tree species in this family. Testing this hypothesis has proven difficult because the assembly of both dipterocarps and their ECM associates co-varies with the same edaphic variables. In this study, we used high-throughput DNA sequencing of soils and Sanger sequencing of root tips to evaluate how ECM fungi were structured within and across a clay–sand soil nutrient ecotone in a mixed-dipterocarp rain forest in Malaysian Borneo. We compared assembly patterns of ECM fungi in bulk soil to ECM root tips collected from three ecologically distinct species of dipterocarp. This design allowed us to test whether ECM fungi are more strongly structured by soil type or host specificity. As with previous studies of ECM fungi on this plot, we observed that clay vs. sand soil type strongly structured both the bulk soil and root tip ECM fungal communities. However, we also observed significantly different ECM communities associated with two of the three dipterocarp species evaluated on this plot. These results suggest that ECM fungal assembly on these species is shaped by a combination of biotic and abiotic factors, and that the soil edaphic niche occupied by different dipterocarp species may be mediated by distinct ECM fungal assemblages.

Keywords: tropical rain forest, mycorrhizal associations, ectomycorrhizal fungi, Dipterocarpaceae, host specificity

INTRODUCTION

Mycorrhizal fungi provide the physiological link between soil nutrients and at least 80% of all terrestrial plant species (Wang and Qiu, 2006). A growing body of research demonstrates that mycorrhizal associations can influence plant community assembly and facilitate plant coexistence (van der Heijden et al., 1998; Bever, 2002; McKane et al., 2002; Reynolds et al., 2003). However, the majority of this research has taken place in temperate, herbaceous communities, which are easy to manipulate experimentally and grow over multiple generations. The role that mycorrhizal associations play in the assembly of long-lived tree communities is much less understood, especially in tropical forests, which have the highest tree diversity of any ecosystem (Leigh et al., 2004). In Neotropical rain forests, mycorrhizal fungal communities are mostly comprised of arbuscular mycorrhizal (AM) fungi (Janos, 1985), the earliest-evolved and widespread mycorrhizal associations across all terrestrial ecosystems (Smith and Read, 2010; Lewis, 2016). Ectomycorrhizal (ECM) fungal associations are less common, formed by ∼4.5% of tree species worldwide (Brundrett, 2009), and are generally associated with monodominant or co-dominant tree stands in Neotropical and African tropical rain forests (Torti et al., 2001; McGuire et al., 2008).

The mycorrhizal ecology in the lowland tropical rain forests of Southeast Asia is distinct from that of their Neotropical and African counterparts; the canopy in this region is characterized by high levels of species richness within the family Dipterocarpaceae (Ashton, 2009), and all 500+ species surveyed to date, including fossilized specimens, have been shown to form ECM associations (Lee et al., 2002; Ducousso et al., 2004; Brearley, 2012). These ECM associations have been proposed as one of the factors that originally facilitated and continued to maintain the familial dominance of Dipterocarpaceae (Torti et al., 2001; Brearley, 2012). ECM associations have also been suggested, but never tested, as a mechanism for maintaining the coexistence of so many closely related tree species in this family (Smits, 1994). This assumption is based on the fact that individual ECM trees can associate with dozens of different ECM fungal species (Lewis et al., 2008; Avolio et al., 2009), and each of these species can exhibit varying enzymatic capabilities and foraging strategies (Agerer, 2001), and segregate and partition resources between different soil horizons (Dickie et al., 2002; Baier et al., 2006; McGuire et al., 2013). The composite ECM fungal community on the roots of a single tree may be functionally distinct (McGuire et al., 2010), providing one tree access to a different pool of soil resources than a neighboring tree with different ECM fungal associates.

Many studies have investigated other biotic and abiotic factors besides ECM associations that influence the distribution and coexistence of dipterocarp species, in particular on the island of Borneo, which contains the highest dipterocarp richness recorded anywhere on earth: 276 species in 13 different genera (Maury-Lechon and Curtet, 1998). One striking ecological pattern in these lowland rain forests is the affinity many species of dipterocarp exhibit for specific soil types (Baillie et al., 1987; Paoli et al., 2006; Sukri et al., 2012). This specialization influences dipterocarp assembly at local (Davies et al., 2005), mesoscale (Paoli et al., 2006), and regional (Potts et al., 2002) scales, and dipterocarp species associated with different soil types have different physiological (Baltzer et al., 2005) and demographic (Russo et al., 2005) traits.

The extent and scale at which edaphic variables influence the biodiversity and distribution of trees has long been a contentious issue in Neotropical rain forests (Pitman et al., 1999; Hubbell, 2001; Condit et al., 2002; Phillips et al., 2003). The consistency with which this pattern has been observed in this region suggests that dipterocarps are strongly influenced by differences in nutrient availability (Bungard et al., 2002; Russo et al., 2005, 2008; Sukri et al., 2012). As ECM associations are the primary interface of dipterocarp nutrient acquisition, these associations could be important mediators of different dipterocarp species' habitat preference observed across soil edaphic gradients.

Despite the significant role that ECM fungi may play in structuring dipterocarp composition, few studies have been conducted on mixed-dipterocarp ECM communities using DNA sequences to identify either the symbiont or host. The only intensive molecular survey of ECM diversity in a mixed-dipterocarp forest (Peay et al., 2010) found that changes in ECM community structure, similarly to above-ground dipterocarp community structure (Davies et al., 2005), correlated with differences in soil nutrient content. As distributions of ECM host taxa can also influence ECM fungal assembly (Bruns et al., 2002; Ishida et al., 2007; Morris et al., 2008), the influence of the soil abiotic environment can be confounded when ECM host taxa co-vary with the same edaphic variables as their ECM symbionts. In order to determine whether ECM fungi can mediate the habitat specialization of their dipterocarp hosts, the relative influence of the soil abiotic environment needs to be decoupled from host tree identity. A recent study conducted a reciprocal transplant of dipterocarp seedlings across soil types, and found no evidence for ECM-fungal host specificity (Peay et al., 2015). However, the seedling stage is a small portion of the host lifespan, and since ECM communities associated with a tree may change over time (Twieg et al., 2007), it is also important to look at these patterns with adult dipterocarps.

In this study, we used a high-throughput DNA sequencing approach to evaluate how ECM fungi are structured across a clay–sand soil nutrient ecotone in a mixed-dipterocarp rain forest in Malaysian Borneo. We first evaluated patterns of ECM fungal assembly in bulk soil on each side of the ecotone. Then, we collected ECM root tips from three ecologically distinct adult species of dipterocarp in the genus *Shorea*: *S. acuta* (Ashton), a sand specialist, *S. inappendiculata* (Burck), a clay specialist, and *S. almon* (Foxw.), a soil generalist found on both sides of the ecotone. This design allowed us to decouple the relative influence of host tree and soil type on ECM assembly, and enabled us to test whether ECM fungi were more strongly structured by soil or host specificity in adult trees.

MATERIALS AND METHODS

Site Description

Lambir Hills National Park (hereafter, Lambir) is located about 30 km inland from the northern coast of Borneo, in the state of Sarawak, Malaysia (4°10′51″ N, 114°01′12.6″ E). Lambir is an aseasonal lowland tropical rain forest, with a daily temperature range of 24–36°C and an average annual rainfall of 3000 mm (Lee et al., 2002). This study was conducted on a 52-ha Forest Dynamics Plot established in 1992 by the Smithsonian Center for Tropical Forest Science (CTFS), the Sarawak Forestry Department and the Plant Ecology Laboratory of Osaka City University, Japan. The forest on the plot has never been commercially logged (Lee et al., 2002). The plot is divided into 1300 permanent 20 m^2 quadrats (Yamakura et al., 1995). All living woody plants on the plot with DBH $\geq$ 1 cm are identified and mapped every 5 years. As of the 2012 census, there were more than 1000 identified tree species on the plot, which makes this one of the richest plots in terms of trees species in the CTFS network (Condit et al., 2002).

The Lambir Forest Dynamics Plot is roughly split 1:3 by an east–west escarpment that separates two distinct sedimentary formations, which give rise to very different soil chemistries and associated plant communities. Detailed descriptions of Lambir's geomorphology are available elsewhere (Baillie et al., 1987; Lee et al., 2002). In brief, the parent substrate that forms the cuestas found on the more elevated northwestern side of the plot is sandstone, which yields sandy loams. This sandstone overlies an older layer of shale sediments, which are exposed at the southern and southeastern edges of the plot and give rise to more clayey soils (hereby referred to as "clay"). The sandy soils are relatively nutrient-poor, well-drained, and accumulate a thick humic layer and dense root mat at the soil surface, while the clay soils are nutrient rich, have a high water-retention capacity, and a very thin (>1 cm) organic surface layer with no root mat. Cluster analyses of tree species distributions relative to each soil type on the plot have shown that the majority of trees (86.6%) exhibited soil habitat specificity (Davies et al., 2005).

Experimental Design

To estimate the local ECM fungal composition found on each soil type at Lambir, soil cores were collected from six 20 m × 20 m plots on the clay and sand sides of the ecotone in July 2010 (**Figure 1A**). In each plot, one 20 cm^2 core was taken with an open-ended soil corer of 2.86 cm in diameter from each corner and the middle of the plot. All five soil cores were composited over a sterile 2 mm sieve to remove roots, stones, and organic detritus, and then stored at −20°C until analysis.

To evaluate the influence of different host species within and across the soil ecotone, we collected ECM root tips from three species of dipterocarp in the genus *Shorea*: a clay specialist, *S. inappendiculata*, a sand specialist, *S. acuta*, and a soil generalist, *S. almon* (Davies et al., 2005). We chose to focus on this genus because it contains the most species (55), has the highest basal area (487.8 m^2), and largest number of the stems (23,813) in the plot (Lee et al., 2002). Individuals were randomly selected from Lambir's 2012 tree census database with the following criteria: they had to be living, reproductively mature (Itoh et al., 2004), and more than 5 m from another tree. We used a partially crossed sampling design: five individuals of *S. inappendiculata*

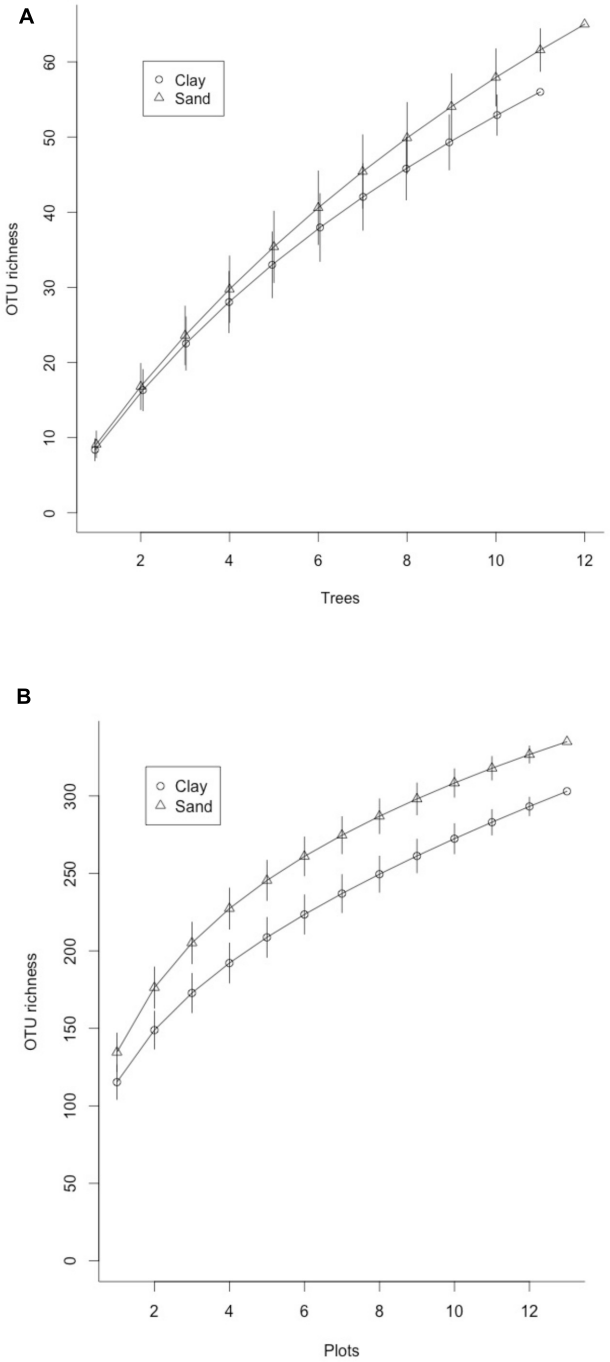

FIGURE 1 | Rarefaction curves for ectomycorrhizal (ECM) fungal OTUs found in **(A)** bulk soil samples pooled from 12 plots on sand and clay soils in Lambir Hills National Park and **(B)** ECM fungal OTUs found on root tips sampled from three species of dipterocarp trees on sand and clay soils in Lambir Hills National Park. Error bars represent 95% confidence intervals for each iteration.

and six individuals of *S. acuta* were sampled from their respective soil habitat preference. Five and six individuals of *S. almon* were sampled on the sand and clay sides of the soil ecotone, respectively.

At each tree sampled for ECM root tips, four 10 cm^3 cores of soil and roots were excavated with an EtOH-sterilized knife 0.5 m from the stem at each cardinal direction. The tree species of the roots within the cores were not confirmed, but sampling was conducted near the base of target trees to increase the likelihood of that species' root capture. Root cores were then either gently rinsed with water to remove soil and examined on the day of collection, or soaked in water to prevent roots from drying out for no more than 24 h prior to examination. After most of the soil was rinsed off of each core, fine roots were removed and examined under a dissection microscope. ECM root clusters were identified visually, and a representative root tip was taken from each cluster in the order they were encountered. Although the target sample size was 20 root tips per tree, four of the trees had only 10, 19, 13, and 18, respectively (**Table 1**). ECM root tips were individually stored at room temperature in CTAB buffer until DNA extraction.

TABLE 1 | Genera of ECM fungi observed in bulk soil samples using next-generation sequencing of the ITS1 regions using Illumina MiSeq.

Genus	Sand plots	Clay plots	Total
Amanita	5.97	1.09	3.57
Amphinema	0	0.11	0.05
Apodus	0	0.02	0.01
Austropaxillus	0.12	0.06	0.1
Barssia	0.06	0	0.03
Boletus	0.02	0.01	0.01
Cantharellus	0.1	0	0.05
Clavulina	0	0.04	0.02
Cortinarius	30.38	1.39	16.1
Craterellus	0.32	1.88	1.09
Elaphomyces	1.3	0.03	0.67
Entoloma	0.13	0	0.07
Fimetariella	0	0.08	0.04
Gelasinospora	0.25	0.03	0.14
Helvella	0.07	0	0.04
Hydnellum	0.08	0.02	0.05
Hydnum	0.02	0	0.01
Hygrophorus	0.11	0	0.06
Inocybe	4.99	5.33	5.16
Lactarius	1.62	25.96	13.61
Lactifluus	0	0.02	0.01
Lasiosphaeria	0.01	0.02	0.01
Lyophyllum	5.19	0	2.64
Peziza	0.35	0.4	0.37
Phellodon	0	0.02	0.01
Piloderma	1.18	0	0.6
Podospora	0.02	0.01	0.01
Ramaria	0	1.22	0.6
Russula	21.45	25.94	23.66
Sarcodon	0.85	0	0.43
Scleroderma	0	0.07	0.03
Sebacina	0.37	0.31	0.34
Sphaerosporella	23.34	27.83	25.55
Thelephora	0.37	0.02	0.2
Tomentella	1.27	7.93	4.55
Tremellodendron	0	0.06	0.03
Tuber	0	0.09	0.04

The percent of sequences assigned to each genus is shown within each soil type.

Molecular Analyses

We identified the ECM fungal community in the soil core samples using a barcoded, high-throughput Illumina sequencing method previously described (McGuire et al., 2013). MoBio PowerSoil Extraction Kits (MO BIO Laboratories Inc., Carlsbad, CA, United States) were used to extract DNA from each soil sample. Extractions were done in triplicate to ensure a more complete characterization of microbial DNA from each sample (Feinstein et al., 2009). We targeted the internal transcribed spacer region 1 (ITS1) of ribosomal DNA using a modification of the fungal-specific primer pair ITS1F and ITS2 (White et al., 1990) adapted for the Illumina platform (McGuire et al., 2013). Both primers included Illumina adapter sequences, a 2-bp linker sequence and primer pad, with the 3′-primer (ITS2) also incorporating a 12-bp sample barcode sequence. PCR was conducted in 25 μl reactions containing 10 μM of each primer. PCR cycles were performed as follows: 94°C for 3 min, then 35 cycles of 94°C for 45 s, 50°C for 60 s, 72°C for 90 s, then 10 min at 72°C. PCR products (amplicon length ∼250 bp) were visualized using gel electrophoresis and successful reactions were quantified using the Quant-iT PicoGreen dsDNA assay (Thermo Fisher Scientific Inc., Waltham, MA, United States) with a spectrofluorometer. Sequencing was done at the New York Medical College (Valhalla, NY, United States) using an Illumina Miseq (Illumina Inc., San Diego, CA, United States). Sequences were deposited into GenBank under accession numbers MG018027-MG018198.

Raw sequences that Illumina generated from the bulk soil samples were demultiplexed using an in-house (University of Colorado) Python script and then processed following the UPARSE pipeline (Edgar, 2010). Demultiplexed reads were filtered by removing sequences with quality scores <23, dereplicating them, and then removing singletons and sequences <75% similar to any sequence in the UNITE database (Koljalg et al., 2005). A *de novo* database was constructed by clustering the remaining sequences into operational taxonomic units (OTUs) using a 3% sequence radius. Raw, demultiplexed sequences, rarified to 20,700 sequences per sample, were then mapped to this filtered database at a 97% similarity threshold to calculate sequence counts per OTU per soil sample. Taxonomy was assigned to each OTU using BLAST, and ECM fungi were identified from matches to known ECM taxa based on recent phylogenetic and stable isotope data (Tedersoo et al., 2010). Raw sequences were deposited in NCBI's Sequence Read Archive (SRA) database under the accession number PRJNA413552.

To identify the ECM fungi associating with the root tip samples, we used a Sanger sequencing approach. We isolated DNA from each root tip using the Qiagen DNEasy Plant Mini Kit (Qiagen, Venlo, Netherlands) with a slightly modified protocol

for tough plant tissue. Each root tip and its storage CTAB buffer were transferred to a 2 mL screw cap tube with 0.5 mm metal beads, and Qiagen-supplied lysis buffer was added to bring the total volume to 1 ml. Root tips were pulverized using a Mini Bead Beater 16 (Biospec Products Inc., Bartlesville, OK, United States) at max speed for 30 s increments until the root tissue was completely homogenized, and then lysate was incubated at 65°C for an hour. All subsequent steps adhered to the manufacturer's guidelines. We amplified the ITS1 and ITS2 region of ribosomal RNA using the fungal-specific primer pair ITS1f and ITS4 (Gardes and Bruns, 1993). PCR was conducted in 20 μl reactions with 10 μM of each primer using the following cycle parameters: 95°C for 3 min, then 14 cycles at 95°C for 35 s, 55°C for 55 s, 72°C for 60 s, then 14 cycles at 95°C for 35 s, 55°C for 55 s, 72°C for 120 s, then 8 cycles at 95°C for 35 s, 55°C for 55 s, 72°C for 180 s, then 72°C for 10 min. PCR products (amplicon lengths ∼700 bp) were visualized using gel electrophoresis. Where multiple bands were visible, amplicons were isolated from the gel using either the GelElute Extraction Kit (5 Prime Inc., Gaithersburg, MD, United States) or the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, United States). If multiple amplicons were within the target size range for the ITS region (500–700 bp), then each of the amplicons was gel purified. All successful reactions were sequenced in a single direction by the Beckman Coulter Genomics facility in Danvers, MA, United States, using the primer ITS1f.

Sequences from the root tip samples were manually edited with Geneious v. 7.3 (Biomatters, Auckland, New Zealand) to remove priming sites and low quality bases from the 5′ and 3′-ends of the reads. Sequences with quality scores <23 or reads shorter than 100 bp were excluded from further analysis. Remaining sequences were clustered into OTUs using a 3% sequence radius in USEARCH v. 8.0.1517 (Edgar, 2010). Chimeras were removed using both *de novo* and open-reference-based detection in USEARCH using the UNITE database as a reference (Koljalg et al., 2005). All root tip sequences were mapped to this filtered database with a 97% similarity cutoff to calculate sequence counts per OTU per tree. Taxonomy was assigned to each OTU using UBLAST with a database of ITS sequences curated from a previous study on the plot (Peay et al., 2010), sporocarps collected on the plot and identified by the Peay Lab (Stanford University, Stanford, CA, United States), and vouchered sporocarp specimens from the Forest Research Institute of Malaysia (FRIM) herbarium. To ensure accuracy, these taxonomic assignments were compared to the top BLAST hit on GenBank (Benson et al., 2009). The OTU sequences were then filtered to include only sequences that matched to known ECM lineages following Tedersoo et al. (2010).

Statistical Analyses

Rarefaction curves for bulk soil and root tip ECM OTUs were constructed using the package BiodiversityR (Kindt and Kindt, 2015) in R version 3.20 (R Core Team, 2014). The Chao1 estimator was run for both root tips and bulk soil ECM fungi using the estimateR function of the Vegan package in R. To examine the relative influence of soil type and host species on ECM assembly, we used permutational analysis of variance (permANOVA, 10,000 permutations) (Anderson, 2001) and visualized results using non-metric multidimensional scaling (NMDS) ordination using Primer v. 6.1.13 with permANOVA+ v. 1.0.3 (Clarke and Gorley, 2006). Analysis of both bulk soil and root tip ECM fungal community structure was conducted using Bray–Curtis dissimilarity of square root-transformed OTU counts for each sample. For pairwise comparisons of the root tip ECM fungal communities found on each host species nested within the factor *Soil Type*, we excluded the one *S. inappendiculata* that was sampled on sandy soil because insufficient permutations (3) were possible to conduct a pairwise test with either *S. almon* or *S. acuta*.

RESULTS

Of the 2853 fungal OTUs recovered from the bulk soil cores, 197 were from established ECM lineages, assigned to 38 genera (**Table 1**). The most abundant genera were *Russula* (Russulaceae), *Cortinarius* (Cortinariaceae), *Lactarius* (Russulaceae), *Inocybe* (Inocybaceae), *Tomentella* (Thelephoraceae), and *Amanita* (Amanitaceae). The majority of the taxa recovered were present in only one soil type; 130 (65%) of the 197 taxa were only found on either sand or clay. Mean OTU richness was not significantly different in sand soils compared to clay (38.7 vs. 42.8). While rarefaction curves indicated that Illumina sequencing did not fully capture the total OTUs found in each soil type (**Figure 1**), the Chao1 estimator also did not predict significantly higher OTU richness in sand vs. clay soils (69.3 vs. 69.0, respectively).

Of the 193 OTUs recovered from the root tip samples, 112 were from established ECM lineages, assigned to 20 genera (**Figure 2A**) and 16 families (**Figure 2B**). The most common genera were *Russula* (Russulaceae), *Tomentella* (Thelephoraceae), *Boletus* (Boletaceae), *Lactarius* (Russulaceae) *Amanita* (Amanitaceae) and *Craterellus* (Cantharellaceae). The majority of the taxa detected in root tip samples had restricted distributions relative to soil type. Of the 112 taxa identified, 101 (90%) were found in root tips collected in only one soil type. The same was true for different host species; 98 (87.5%) of the taxa were found associating with only one species of *Shorea*, while 13 (11.5%) were found in multiple species, only two of which (1.7%) were found in all three. Ten of the 11 taxa found in only two species were shared between *S. almon* and *S. acuta*, and 9 of the 13 taxa found in multiple tree species were among the most common found in the plot. Observed OTU richness was not significantly higher for root tips collected from trees on sand soils than clay soils, although rarefaction curves did not show significant separation and indicate that we did not completely capture the diversity found in either side of the ecotone (**Figure 1B**). The Chao1 estimate also did not predict significantly higher ECM richness in sand soils (9.7) compared to clay soils (8.3).

When we qualitatively compared the ECM taxa found in bulk soil versus on root tips, we found that 30% of the ECM genera were shared in the sand and 35% were shared in the clay (**Figure 3**). However, due to the difference in sequencing methodologies a formal statistical test could not be done.

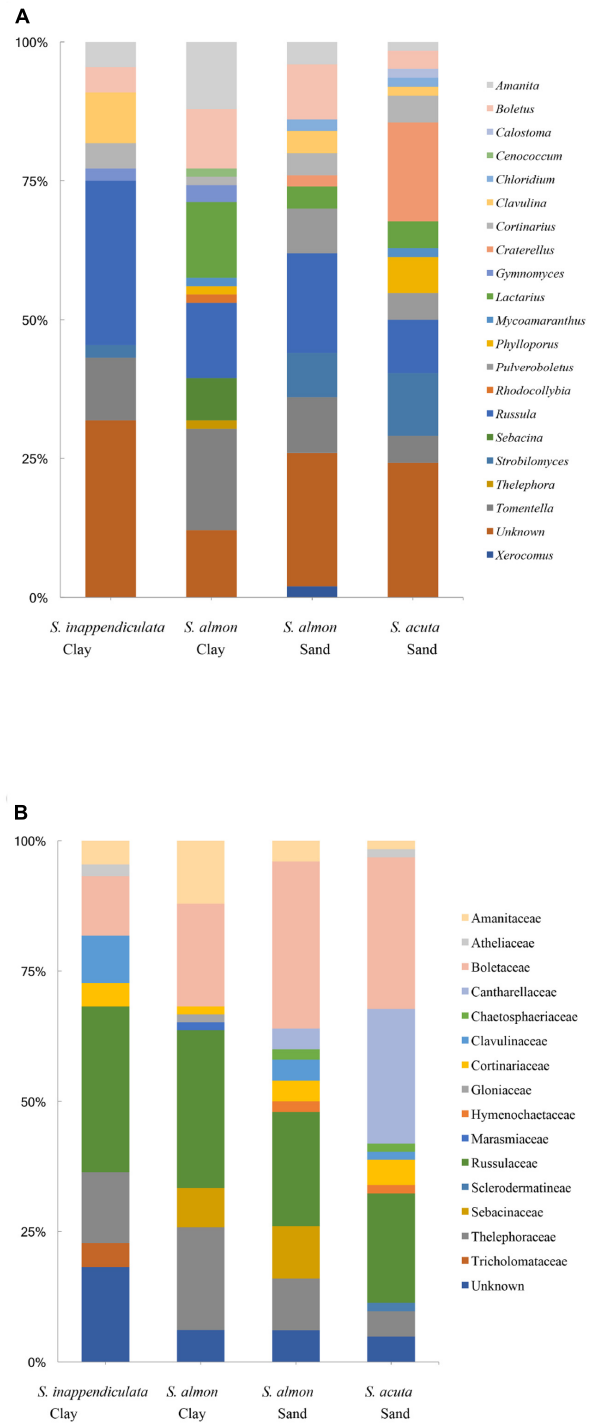

FIGURE 2 | Genera **(A)** and families **(B)** of ECM fungi observed on root tip samples using Sanger sequencing of the ITS1 and ITS2 regions. Percent of sequences assigned to each genus is shown with respect to each host species and soil type.

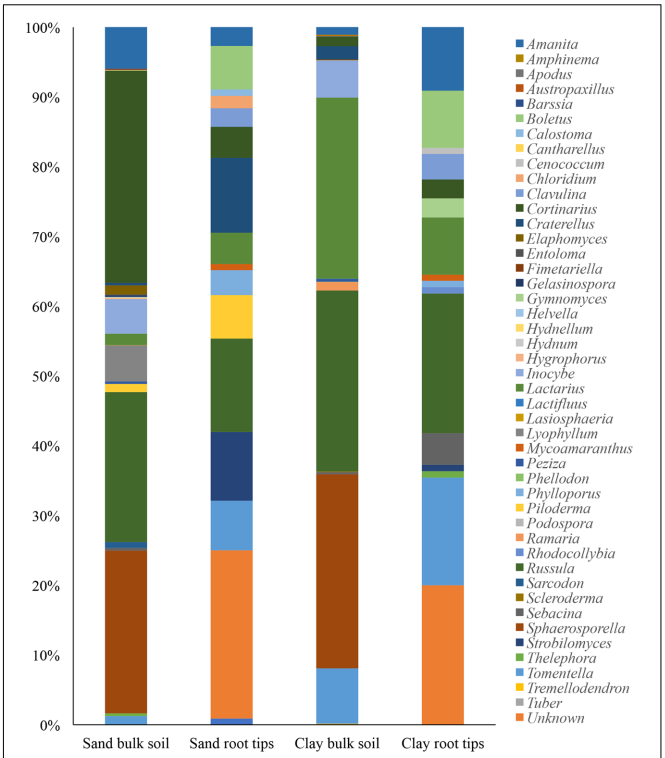

FIGURE 3 | Genera of ECM fungal taxa detected in each soil type, with bulk soil taxa (from Illumina sequencing) and root tip taxa (from Sanger sequencing) aligned to see qualitative differences.

Analysis by permANOVA indicated that ECM fungal communities in both the bulk soil and root tip samples were significantly structured by soil type (**Figures 4A,B**). The global test of the effect of host species nested within soil type on the ECM root tip community was marginally insignificant ($p = 0.054$), yet pairwise comparisons revealed significant clustering was due to differences between *S. almon* and *S. inappendiculata* on the clay side of the ecotone ($p = 0.03$), but not between *S. almon* and *S. acuta* on the sand side of the ectone ($p = 0.67$). This host effect is illustrated by the two different clusters of root tip samples from the clay side of the plot in the NMDS ordination (**Figure 4B**).

DISCUSSION

We found strong edaphic specialization across the soil ecotone for both the root tip and bulk soil components of ECM fungal communities, supporting the hypothesis that soil type is the primary determinants of ECM fungal community assembly. The strong edaphic segregation of ECM fungal communities in this study is consistent with previous work at Lambir Hills finding that ECM fungi exhibit sharp compositional shifts across clay and sand soil types (Peay et al., 2010, 2015). Nonetheless, differences in ECM community composition were detected between two dipterocarp species within the same soil type, suggesting that host tree identity also plays a role in structuring ECM fungal communities, albeit a much weaker role than soil edaphic properties.

Our detection of ECM fungal host specificity in this study is in contrast with previous results from this dipterocarp forest

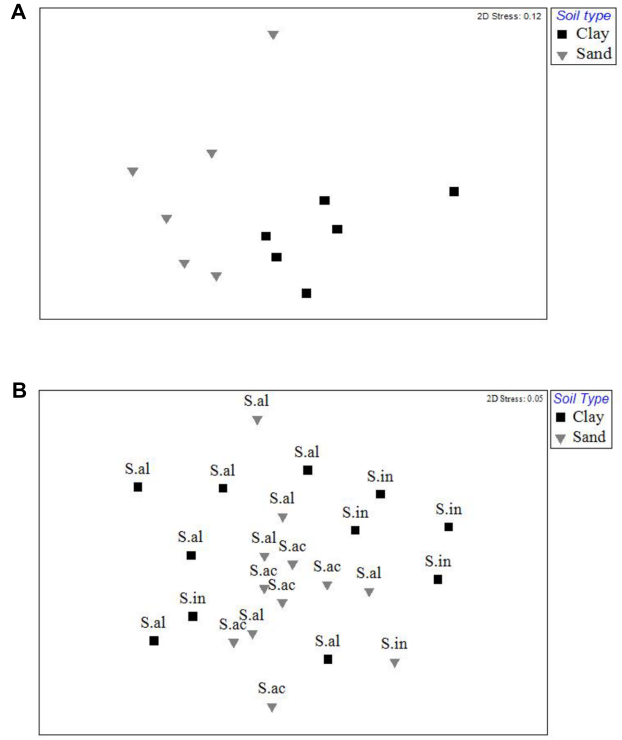

FIGURE 4 | (A) Non-metric multidimensional scaling ordination of the ECM fungal communities found on two different soil types at Lambir Hills National Park. Each point represents pooled OTU counts from six different 400 m^2 plots on each soil type. Distance between points represents rank dissimilarity using a Bray–Curtis index. **(B)** Non-metric multidimensional scaling ordination of ECM fungal communities found on root tips collected from three species of dipterocarps at Lambir Hills National Park. Each point represents the pooled OTU counts from an individual tree. Distance between points represents rank dissimilarity using a Bray–Curtis index. Letters denote samples from individual tree species (S.al = *Shorea almon*, S.in = *Shorea inappendiculata*, S.ac = *Shorea acuta*).

(Peay et al., 2015). Specifically, a recently published reciprocal seedling transplant study conducted at Lambir Hills found no effect of host species identity on ECM fungal composition in 13 different dipterocarp seedling roots, but found significant structuring of ECM fungal composition across clay and sand soil types (Peay et al., 2015). The conflicting results from the current study may be due to the fact that adult ECM fungal communities are not reflective of seedling ECM fungal communities, as ECM fungal assembly in an individual tree may change as the host ages (Nara et al., 2003). In temperate forests, ECM fungi fall along a continuum of host specialization (Horton and Bruns, 1998), and there is some evidence that ECM fungal composition diverges with greater phylogenetic distance between host species (Ishida et al., 2007). While we only sampled three *Shorea* species, ECM fungal composition was only distinct in the two *Shorea* species that were less phylogenetically related (Ashton, 2003). More experimentation will be necessary to determine if the differences we observed in the ECM assembly of *S. almon* and *S. inappendiculata* is an example of strict host specificity, i.e., related to host–fungus genetic compatibility (Bruns et al., 2002), the result of indirect host effects via the nutrient content of leaf litter input (Cullings et al., 2003; Uriarte et al., 2015), or other host-related modifications to the local abiotic environment (Dickie et al., 2002).

Some ECM lineages exhibited consistent segregation across soil types at the level of both root tip and bulk soil, suggesting there are underlying physiological differences in these taxa that may drive their community assembly across the ecotone. In temperate and boreal ecosystems, differences in ECM assembly are often correlated with soil characteristics such as nitrogen content, water retention, pH, and cation exchange capacity (Lilleskov et al., 2002; Toljander et al., 2006; Piculell et al., 2008; Avolio et al., 2009; Polme et al., 2013) and these patterns may reflect a variety of physiological optima for different fungal taxa (Smith and Read, 2010). Different lineages of ECM fungi have also been shown to exhibit varying enzymatic capabilities and foraging strategies, or so-called "exploration types" (Agerer, 2001, 2006), and taxa with similar exploration types have been observed to respond similarly to environmental gradients of nitrogen (Lilleskov et al., 2002) and carbon (Markkola et al., 2004). Given the general taxonomic similarity between the ECM fungal communities found at Lambir and those observed in temperate and boreal forests (Peay et al., 2010), the mechanisms responsible for driving these differences are likely similar to those found elsewhere.

The greater relative abundance of Thelephoraceae in clay soils and *Cortinarius* in sandy soils is consistent with a previous study at this site (Peay et al., 2015), implying that these patterns are robust and not simply due to sampling bias or seasonal variation. While the specific mechanisms driving the assembly of taxa within each soil type require further study, one potential explanation is related to the different hyphal exploration types associated with each of these lineages. There is evidence that some species of *Cortinarius* form medium-range rhizomorphs specialized for the acquisition of organic N from leaf litter and humus, and are sensitive to increased availability of mineral N (Lilleskov et al., 2011). These taxa would be expected to provide a competitive advantage to hosts growing in oligotrophic soils rich in organic matter, such as the sandy soils found at Lambir Hills. Likewise, some taxa in the family Thelephoraceae form short, hydrophilic hyphae that may be favored by hosts in soils with greater labile nutrient availability, such as clay.

While edaphic filtering appeared to be the strongest community assembly mechanism for ECM fungi both in bulk soil and in root tips, the compositions of these two fractions were distinct, most notably with respect to the paucity of Boletaceae detected in the bulk soil samples. This finding contrasts with both the root tip samples and previous ECM surveys conducted in the plot, where taxa in this family were among the most abundant. This discrepancy may be due to the tendency of this lineage to form long-distance exploratory rhizomorphs (Agerer, 2001), which may have been excluded during the removal of roots that were sieved from the bulk soil sampling. Similarly, there is general evidence that taxa can differ in their investment into different structures such as root mantles, fruiting bodies, or extraradical hyphae (Gardes and Bruns, 1996;

Koide et al., 2005). Another notable difference between the root tip and bulk samples is the greater relative abundance of ECM Ascomycota in the bulk soil samples, which may reflect a greater proportion of Ascomycota hyphal or propagule biomass in the soil rather than in mycorrhizal association with roots. Differential biomass allocation between fruiting bodies, ectomycorrhizae, extraradical hyphae, and propagules has been observed previously in both primary forests (Gardes and Bruns, 1996; Taylor and Bruns, 1999) and pine plantations (Koide et al., 2005). It is also possible that these results are simply due to differences in sequencing depth, as the root tip samples were analyzed by Sanger sequencing and the bulk soil samples were sequenced on the Illumina platform.

The results of this study suggest that ECM fungal assembly at Lambir is shaped by a combination of biotic and abiotic factors. Like previous ECM fungal studies conducted in the plot, both the bulk soil and root tip fractions of ECM communities were strongly structured by the soil differences between the clay and sand sides of the soil ecotone. Given the fact that differences in ECM community composition have been previously observed at Lambir (Peay et al., 2010), it appears that this result is robust and likely reflects some functional or physiological differences between the ECM taxa found in either side of the soil ecotone. Although the mechanisms remain unclear, the differences that we observed between the ECM fungal assemblies found in two of the three species of *Shorea* suggest that the soil edaphic niche occupied by some dipterocarp species may be mediated by distinct ECM fungal assemblages. Additional studies that incorporate a broader phylogenetic range of dipterocarp host species and include measurements of host-associated soil physiochemical modifications would enable a more mechanistic evaluation of this hypothesis.

AUTHOR CONTRIBUTIONS

Sampling design was conceived by AE, JL, KP, and KM. Laboratory and data analyses were done by AE, KS, and KM, and manuscript writing was completed by all authors.

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Subcellular Compartmentalization and Chemical Forms of Lead Participate in Lead Tolerance of *Robinia pseudoacacia* L. with *Funneliformis mosseae*

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The effect of arbuscular mycorrhizal fungus on the subcellular compartmentalization and chemical forms of lead (Pb) in Pb tolerance plants was assessed in a pot experiment in greenhouse conditions. We measured root colonization, plant growth, photosynthesis, subcellular compartmentalization and chemical forms of Pb in black locust (*Robinia pseudoacacia* L.) seedlings inoculated with *Funneliformis mosseae* isolate (BGC XJ01A) under a range of Pb treatments (0, 90, 900, and 3000 mg Pb kg^{-1} soil). The majority of Pb was retained in the roots of *R. pseudoacacia* under Pb stress, with a significantly higher retention in the inoculated seedlings. *F. mosseae* inoculation significantly increased the proportion of Pb in the cell wall and soluble fractions and decreased the proportion of Pb in the organelle fraction of roots, stems, and leaves, with the largest proportion of Pb segregated in the cell wall fraction. *F. mosseae* inoculation increased the proportion of inactive Pb (especially pectate- and protein-integrated Pb and Pb phosphate) and reduced the proportion of water-soluble Pb in the roots, stems, and leaves. The subcellular compartmentalization of Pb in different chemical forms was highly correlated with improved plant biomass, height, and photosynthesis in the inoculated seedlings. This study indicates that *F. mosseae* could improve Pb tolerance in *R. pseudoacacia* seedlings growing in Pb polluted soils.

Keywords: arbuscular mycorrhizal fungus, symbiosis, bioremediation, Pb, plant tolerance

INTRODUCTION

Lead (Pb) is one of the most widespread toxic metals (Fahr et al., 2013) and tends to remain in soil for long periods of time (LaBelle et al., 1987). Pb pollution may be naturally occurring or result from anthropogenic activities (Yadav, 2010), creating potential hazards to ecosystems (Lantzy and Mackenzie, 1979). Pb pollution has also been a serious threat to human health and food safety (Cuypers et al., 2016), especially harmful to young children (Datko-Williams et al., 2014). Over the last decade, evidence has been shown that Pb exposure-related health injury occur already at

lower blood Pb levels than previously documented (Henry et al., 2015). Recently, the Centers for Disease Control and Prevention has lowered the reference value of blood Pb level to 5 $\mu g\ dL^{-1}$ (Burns and Gerstenberger, 2014). To adhere the Pb reference value the diminishment of Pb level in soils is of high interest at present. As the demand for conversion of post-industrial lands continues to raise the remediation of Pb polluted soils represents one principal task to fulfill this requirement (Yousaf et al., 2016).

The established traditional techniques (e.g., washing, electrochemical methods, thermal processes, physical separation, stabilization/solidification, and burial) for clean-up of metal contaminated soils are generally expensive and harmful to soil microbial diversity (Rajkumar et al., 2012). Bioremediation has received increasing attention as it represents an eco-friendly and low-cost technology (Kotrba et al., 2009) using living plants (Janoušková and Pavlíková, 2010) or microorganisms (Brito et al., 2014) to remove toxic heavy metals (HM) in contaminated areas. Various plants growing in HM contaminated soils have developed specialized mechanisms for tolerating intracellular HM. Subcellular compartmentalization is an important way to eliminate the toxicity of HM in plants (Cobbett, 2003). Once taken up by plants, HM can exist in different chemical forms, including inorganic, water-soluble, pectate- and protein-integrated, undissolved phosphate and oxalate forms (Wu et al., 2005). For example, conversion of cadmium (Cd) into insoluble phosphate precipitates and pectate- or protein-bound forms is the primary means for reducing Cd mobility and toxicity in *Nasturtium officinale* (Wang J.B. et al., 2015). Copper (Cu) bound to the cell walls in fibrous roots of *Malus sieversii* mainly exists in phosphate and oxalate forms, which can explain some of the variation in Cu sensitivity in *M. sieversii* (Wang et al., 2016). Recently, Li et al. (2016) reported that the cell walls and intercellular spaces are the main location of Pb accumulation in the roots of *Conyza canadensis*. The cell walls restrict Pb uptake into plant roots and act as an important barrier to protect root cells (Wang Y. et al., 2015). Pb fixation by pectates and proteins in the cell walls and sequestration in the vacuoles were found to be responsible for Pb detoxification in *Lolium perenne* (He et al., 2015).

Arbuscular mycorrhizal fungi (AMF) are ubiquitous in terrestrial ecosystems (Sędzielewska-Toro and Delaux, 2016), forming symbiotic interactions with 80% of land plants. AMF can improve plant nutrient acquisition (Orrell and Bennett, 2013), enhance plant photosynthesis, and influence the fate of HM in both plants and the soil (Brito et al., 2014; Saia et al., 2015). Inoculation with *Funneliformis mosseae* resulted in a higher Pb tolerance of *Eucalyptus grandis* × *urophylla* and has been related to the retention of Pb in the roots, the binding of Pb to the cell walls, vacuolar compartmentalization of Pb in the soluble fraction, and increase in the proportion of less bioactive Pb (Liao et al., 2014). Thus, AMF may be used to advance plant-based environmental remediation by altering subcellular compartmentalization and chemical forms of HM in plants. Hence, it is necessary to select an appropriate HM-tolerant plants species that could form a symbiotic relationship with AMF.

Black locust (*Robinia pseudoacacia* L.) is a leguminous tree species widely planted on the Loess Plateau, China (Zhang et al., 2016). *R. pseudoacacia* is frequently found in HM contaminated areas and it may serve as an indicator of Pb pollution (Serbula et al., 2012). *R. pseudoacacia* plants are well grown and commonly colonized by AMF such as *F. mosseae* in the Qiandongshan lead–zinc polluted area (Yang et al., 2015b,c). Recent studies have shown that photosynthesis and antioxidant enzymes (e.g., superoxide dismutase and ascorbate peroxidases) in the leaves of *R. pseudoacacia* are enhanced by *F. mosseae* under Pb stress (Yang et al., 2015a). Additionally, *F. mosseae* is effective at accumulating Pb in plant root systems (Yang et al., 2015a). However, detailed information on the impact of *F. mosseae* on the subcellular compartmentalization and chemical forms of Pb in *R. pseudoacacia* under Pb stress are rare. To understand the efficiency of bioremediation of Pb in soils, there is a need to study the molecular mechanism how AMF colonization enhances Pb tolerance in woody legumes (Leguminosae) such as *R. pseudoacacia*.

One objective of our study was aimed to clarify whether *F. mosseae* is of significance for Pb tolerance in seedlings of *R. pseudoacacia*. For this purpose, inoculated as well as non-inoculated seedlings with *F. mosseae* were exposed to different Pb levels (0, 90, 900, and 3000 mg Pb kg^{-1} soil). The impact of Pb was studied on the basis of plant growth, gas exchange (CO_2 assimilation, stomatal conductance for water vapor) and chlorophyll fluorescence. A second objective of this study was to give detailed insights in the detoxification mechanism of Pb from the molecular perspective, such as subcellular compartmentalization and conversion of Pb into inactive forms, and their alteration due to inoculation with *F. mosseae*. In this respect, the impact of *F. mosseae* on Pb tolerance was studied on the basis of Pb uptake in relation to the proportion of Pb located in different plant tissue, subcellular fractions and chemical forms.

MATERIALS AND METHODS

Fungal Inoculum and Plant

Funneliformis mosseae (BGC XJ01A) spores were purchased from the Institute of Plant Nutrition and Resource, Beijing Academy of Agriculture and Forestry Sciences (Beijing, China). The fungus was propagated with fine sand for 3 months using *Zea mays* under greenhouse condition. The average colonization was 91.7%. The fungal inoculum consisted of sand, infected root fragments, external hyphae, and spores (∼26 spores g^{-1}).

Robinia pseudoacacia L. is a Pb-tolerant tree species (Yang et al., 2015c). The seeds were collected from Northwest A&F University (Yangling, Shaanxi Province, China) in 2013. Seeds were surface sterilized with 10% H_2O_2 for 10 min, washed with distilled water for several times, and then soaked for 24 h before germinating on sterile filter paper in a Petri dish in an incubator at 28°C.

Growth Substrate

Soil used in this study was collected from the surface horizon (0–30 cm) on the campus of Northwest A&F University. The soil was

air-dried, homogenized, and ground in a ceramic mill and passed through a 2 mm sieve before performing chemical analyses. After being mixed with washed fine sand (<2 mm), the substrate (sand/soil, 1:2 v/v) was autoclaved at 121°C for 2 h.

The properties of the soil were as follows (per kilogram of dry soil) after autoclaving: pH 7.66 (soil/water = 1:2.5, w/v), organic matter 14.85 g, ammonium-nitrogen 7.37 mg, nitrate-nitrogen 25.77 mg, available phosphorus 11.48 mg, and available potassium 128.96 mg, total Pb 6.58 mg. Measurements were performed according to the method described by Bao (2000).

The autoclaved substrate was divided into four subsamples. To three of the subsamples, $Pb(NO_3)_2$(aq) was added to produce substrates with different Pb levels, i.e., 90, 900, and 3000 mg kg^{-1} (mass of Pb/mass of dry soil) based on our pre-experiment (Yang et al., 2015c). An equivalent amount of distilled water was added to the control (0 mg Pb kg^{-1} soil). The four subsamples were supplied with an appropriate amount of NH_4NO_3 to compensate for the quantity of nitrate added as $Pb(NO_3)_2$. After addition of Pb solution, the growth substrate was allowed to stabilize for 1 month before used.

Experimental Design and Plant Culture

The experiment was setup as a 4 × 2 factorial design consisting of four Pb levels and one AMF inoculum and non-inoculated control which were arranged in a completely randomized design with 30 replicates per treatment combination. The experiment was performed from March to July 2014 in a greenhouse located at Northwest A&F University. Plants were kept at average room temperature (35/20°C, day/night) under a natural light regime during the period of plant growth. Soil moisture was determined by a soil moisture meter (Field Scout TDR 100, Spectrum Technologies Inc., Plainfield, IL, USA) and maintained at approximately 60% of field capacity by adding the amount of lost water to each pot daily.

Four uniform pre-germinated seeds were sown in each plastic pot (10 cm × 8 cm) with approximately 450 g growth substrate; each pot received 20 g fresh inoculum for mycorrhizal treatment or 20 g sterilized inoculum with 10 mL AMF-free filtrate (10 μm pore size) of unsterilized inoculum (soil:water = 1:10 w/v) as the non-mycorrhizal treatment (Sheng et al., 2008). The seedlings were thinned to one plant per pot 10 days after emergence. Plants were watered daily with tap water (35 mL) and supplemented with 0.25 × fresh Hoagland's nutrient solution (35 mL) (Hoagland and Arnon, 1950) once a week throughout the growth stage.

After 4 months growth, the photosynthetic parameters and growth of six randomly selected plants per treatment were measured. The whole plants were washed with tap water to remove soil or dust deposits, and the roots were immersed in 20 mM Na_2EDTA for 15 min to remove metal ions adhering to root surface (Chen et al., 2014). The plants were then washed with deionized water and dried with paper towels. Thereafter, the plants were separated into roots, stems, and leaves. The fresh roots (except for fine roots used for determination of AMF colonization), stems, and leaves were immediately frozen in liquid nitrogen (−196°C) and stored at −70°C for further analysis.

Physiological Measurements

Mycorrhizal Colonization and Growth Parameters

Mycorrhizal colonization (MC) was determined for fresh roots using the method described by Phillips and Hayman (1970). Then the MC was calculated according to Ban et al. (2015). Plant height and stem diameter (at 1 cm above the soil surface) were measured by precision straight edge (Deli 8200, Ningbo, China) and vernier caliper (Yifante ECV150C, Wuxi, China), respectively. Root dry weight, stem dry weight, and leaf dry weight were recorded after oven-drying to constant weight at 70°C. Total biomass was calculated as: root dry weight + stem dry weight + leaf dry weight.

Gas Exchange and Chlorophyll Fluorescence Parameters

Six healthy and functional leaves (youngest fully expanded from six different plants) were taken from each treatment and each leaf was measured five times for every photosynthetic parameter (Huang et al., 2011). Net CO_2 assimilation rate (A), stomatal conductance to water vapor (gsw), intercellular CO_2 concentration (C_i), and transpiration (E) were measured with a portable open-flow gas exchange system LI-6400 (LI-COR, USA) on a cloudless day from 9:00 to 11:30 a.m. in the glasshouse at 25°C. Automatic measurements were made under optimal conditions: photosynthetically active radiation 1000 ± 12 μmol m^{-2} s^{-1}, CO_2 concentration 350 ± 2 cm^3 m^{-3}, leaf temperature 28.0 ± 0.8°C, relative humidity 60%, ambient water vapor pressure 1.35 kPa, and flow rate of atmosphere 0.5 dm^3 min^{-1}.

Fluorescence assays were addressed to the same leaves as used for the photosynthetic measurements. The fluorescence parameters were measured at room temperature between 9:00 and 11:30 a.m. by a MINI-Imaging-PAM system (Imaging-PAM, Heinz Walz GmbH, Germany) as described by Gong et al. (2013). The seedlings were placed in darkness for 30 min and the minimal fluorescence in the dark-adapted state (F_o) was recorded. A saturating pulse of irradiation (2000 μmol m^{-2} s^{-1}, 3 s) was applied to determine the maximal fluorescence (F_m) in the dark-adapted state. Then, the leaves were illuminated with actinic light (300 μmol m^{-2} s^{-1}, 10 min) for evaluating the minimal fluorescence (F_o') and maximal fluorescence (F_m'). The incident photosynthetically active irradiance (EPAR), effective photochemical efficiency of PSII (ΦPSII), and steady-state value of fluorescence (F_s) under actinic light were recorded. Using both light and dark fluorescence parameters, we calculated: (1) the maximal quantum yield of PSII in the dark-adapted state, $F_v/F_m = (F_m - F_o)/F_m$, (2) the effective photochemical efficiency of PSII, $\Phi PSII = (F_m' - F_s)/F_m'$, (3) the potential activity of PSII, $F_v/F_o = (F_m - F_o)/F_o$, (4) the photosynthetic electron transport rate, $ETR = (F_m' - F_s)/F_m' \times EPAR$, (5) the photochemical quenching coefficient, $qP = (F_m' - F_s)/(F_m' - F_o')$, and (6) the non-photochemical quenching coefficient, $qN = (F_m - F_m')/F_m'$.

Pb Analysis

To determine the fractions of Pb present in *R. pseudoacacia*, a fractionation procedure was adapted from a published protocol with slight modifications (Weigel and Jäger, 1980). Cells were separated by gradient centrifugation at 4°C into three different fractions: cell wall fraction (FI), organelle fraction (FII), and soluble fraction (FIII). In brief, 0.5 g of frozen tissues was homogenized in a pre-cold (4°C) extraction buffer [containing 50 mM Tris–HCl (pH 7.5), 250 mM sucrose, and 1.0 mM dithiothreitol ($C_4H_{10}O_2S_2$)] at the ratio of 1:10 (w/v) with a chilled mortar and a pestle. The homogenate was passed through a nylon cloth (80 μm mesh size) and liquid was squeezed from the residue. The residue on the cloth was washed twice with a homogenization buffer. The pooled washes, together with the first filtrate, were centrifuged at 300 × *g* for 30 s. The resulting pellet combined with the residue of nylon cloth filtration was designated as FI, containing mainly of cell walls and cell wall debris. The resulting supernatant solution was further centrifuged at 12,000 × *g* for 45 min. The pellet and supernatant solution were referred to as FII and FIII, respectively. The different fractions were dried at 70°C on an electric heating plate to a volume of approximately 1–2 mL and then wet-digested with concentrated acid HNO_3/$HClO_4$ (4:1, v/v). The recovery rate of Pb was calculated as: (cell wall fraction + organelle fraction + soluble fraction) Pb/total Pb × 100%. The proportion of Pb of the fraction was calculated with the following formula: Pb content in each fraction/total Pb content in the respective tissue × 100%.

To detect different chemical forms of Pb in plant tissues, we performed five extraction processes using for each a different extraction solvent (Wang et al., 2008), i.e., 80% ethanol ($F_{Ethanol}$; inorganic, soluble Pb), distilled water (F_{d-H_2O}; organic, soluble Pb), 1 M sodium chloride (F_{NaCl}; pectate and protein- Pb), 2% acetic acid (F_{HOAc}; Pb phosphate), 0.6 M hydrochloric acid (F_{HCl}; Pb oxalate), and Pb in residues ($F_{Residue}$). Briefly, 0.5 g of frozen tissues were homogenized with a mortar and a pestle in liquid nitrogen (–196°C), diluted at a ratio of 1:50 (w/v) with the extraction solution, and then shaken for 22 h at 25°C. The resulting extracts were centrifuged at 5000 × *g* for 10 min. The precipitates were washed twice by re-suspending in the respective extraction medium, shaking at 25°C for 2 h, and centrifuging at 5000 × *g* for 10 min. The supernatants from each of the three repetitions were then pooled for each of the five extraction solutions. The resulting supernatant solvent from extraction solutions were evaporated on an electric heating plate to a volume of approximately 1–2 mL, followed by digestion with HNO_3:$HClO_4$ (4:1, v/v). To measure the Pb content in residues, plant materials were digested with HNO_3–$HClO_4$ (4:1, v/v) at the end of the sequential extraction. The recovery rate of Pb was calculated as: ($F_{Ethanol} + F_{H_2O} + F_{NaCl} + F_{HOAc} + F_{HCl} + F_{Residue}$) Pb/total Pb × 100%. The proportion of Pb of the chemical form was calculated as: Pb content of the chemical form/total Pb content in the respective tissue × 100%.

To determine the total Pb content in different tissues, oven-dried subsamples were ground into powder and sieved through a nylon mesh (100 μm). Then, 0.5 g aliquots of the samples were wet-digested in HNO_3:$HClO_4$ (4:1, v:v). Total Pb content as well as Pb content in different subcellular fractions and chemical forms was determined by atomic absorption spectrophotometry (Shimadzu AA-6300C, Kyoto, Japan). The flame composition was acetylene (flow rate 2 L min^{-1}) and air (flow rate 15 L min^{-1}). The working conditions of the instrument were as follows: current 7.5 mA, wavelength 217 nm, slit width 1.3 nm, burner height 7.5 mm, negative high voltage of photomultiplier tube 576 V, and auxiliary gas pressure 160 kPa.

Quality assurance and quality control for Pb in *R. pseudoacacia* was conducted using the standard reference material bush leaves (GBW07602), which was treated in the same way as the plant samples. The recovery for standard was approximately 95.3–108.5%. The standard solutions with different concentrations in the range of 1–10 μg mL^{-1} were aspirated in turn into flame and their absorbance values were recorded at 217 nm wavelength. The calibration curve was constructed by plotting on a linear graph paper using the absorbance of standards versus their concentrations. The correlation equations were defined as $y = 0.019x + 0.004$, $R^2 = 0.999$. Reagent blanks and analytical duplicates were also used where appropriate, to ensure the accuracy and precision in the analysis. Pb content was determined three times for each sample and the relative standard deviation (SD) of Pb content was calculated (<4.0%).

Statistical Analysis

All values were expressed as mean ± SD ($n = 6$). The Kolmogorov–Smirnov test was applied to assess data normality and the Levene test for homogeneity of variance in SPSS 22.0 (SPSS Inc., Chicago, IL, USA). All the original datasets followed a normal distribution in this study. Potential differences among different Pb treatments were analyzed using two-way and three-way analysis of variance (ANOVA) followed by Duncan's multiple comparison at $P < 0.05$. An independent *t*-test was performed to detect significant differences in plant growth and physiological parameters between the inoculated and non-inoculated controls within one Pb level. A two-factorial ANOVA and Pearson correlation analysis were performed to examine the influence of Pb exposure as well as the mycorrhizal treatment on physiological parameters as well as on the proportions of Pb in subcellular fractions and chemical forms and to detect possible correlations between these traits ($n = 48$). Graphs were drawn using SigmaPlot 10.0 (Systat Software, San Jose, CA, USA).

RESULTS

Mycorrhizal Colonization and Plant Growth

None of the plants from non-inoculated treatments were colonized by *F. mosseae*. The symbiotic relationship between *F. mosseae* and *R. pseudoacacia* was well established in inoculated treatments irrespective of Pb treatment. Inoculated *R. pseudoacacia* showed 88.16% MC under control condition (0 mg Pb kg^{-1} soil), while low Pb treatment (90 mg Pb kg^{-1} soil)

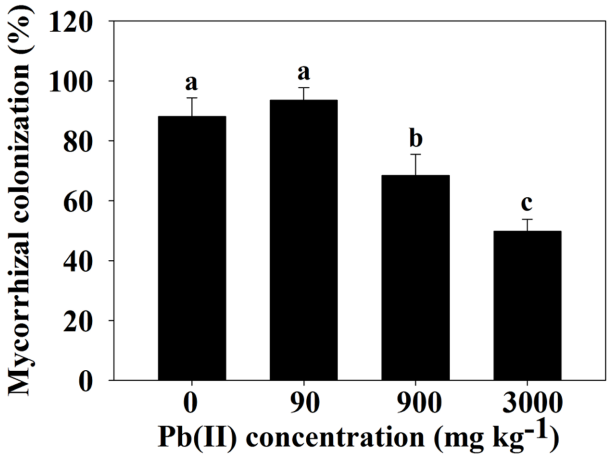

FIGURE 1 | Colonization of *Robinia pseudoacacia* roots by *Funneliformis mosseae* in response to 0, 90, 900, and 3000 mg Pb kg^{-1} soil for 4 months. Shown are means ± SD (n = 6). Different lower case letters indicate significant differences among different Pb levels within inoculated treatment ($P < 0.05$; ANOVA with *post hoc* Duncan).

TABLE 1 | Plant growth and physiological parameters of *Robinia pseudoacacia* seedlings with (+M) or without (−M) *Funneliformis mosseae* at 0 mg Pb kg^{-1} soil for 4 months.

Parameter	−M	+M
MC (%)	0	88.16 ± 6.18***
Root dry weight (g plant^{-1})	1.99 ± 0.03	2.27 ± 0.06**
Stem dry weight (g plant^{-1})	0.58 ± 0.02	0.66 ± 0.02**
Leaf dry weight (g plant^{-1})	2.16 ± 0.03	2.33 ± 0.02***
Total biomass (g plant^{-1})	4.73 ± 0.08	5.26 ± 0.06***
Plant height (cm)	25.03 ± 0.86	42.20 ± 0.56***
Stem diameter (mm)	2.95 ± 0.03	3.35 ± 0.03***
A (μmol CO_2 m^{-2} s^{-1})	6.53 ± 0.45	8.39 ± 1.12**
gsw (mmol H_2O m^{-2} s^{-1})	0.11 ± 0.00	0.14 ± 0.00***
C_i (μmol CO_2 mol^{-1})	150.67 ± 5.25	144.73 ± 4.99 NS
E (mmol H_2O m^{-2} s^{-1})	2.61 ± 0.15	2.90 ± 0.09*
F_v/F_m	0.74 ± 0.01	0.83 ± 0.01***
ΦPSII	0.72 ± 0.01	0.80 ± 0.01***
F_v/F_o	2.89 ± 0.19	4.91 ± 0.28***
ETR	31.52 ± 1.99	39.19 ± 0.52**
qP	0.79 ± 0.01	0.85 ± 0.01***
qN	0.68 ± 0.01	0.58 ± 0.01***

*Shown are means ± SD (n = 6). Asterisks indicate significant differences between inoculated and non-inoculated seedlings at 0 mg Pb kg^{-1} soil (*P < 0.05, **P < 0.01, ***P < 0.001; t-test). NS, not significant; MC, mycorrhizal colonization; A, net CO_2 assimilation rate; gsw, stomatal conductance to water vapor; C_i, intercellular CO_2 concentration; E, transpiration rate; F_v/F_m, maximum quantum yield in the dark-adapted state of PSII; ΦPSII, actual quantum yield of PSII in light-adapted steady state; F_v/F_o, potential activity of PSII; ETR, electron transport rate; qP, photochemical quenching coefficient; qN, non-photochemical quenching values.*

resulted in a slight increase in MC (93.56%). With increasing Pb level, MC of *F. mosseae* was significantly decreased and the lowest MC (49.77%) appeared at 3000 mg Pb kg^{-1} soil (**Figure 1**).

Under control conditions (0 mg Pb kg^{-1} soil), the growth parameters of *R. pseudoacacia* seedlings showed significant differences between non-inoculated and inoculated treatments. Inoculated seedlings showed significantly higher plant biomass (roots, stems, leaves, and total), plant height, and stem diameter than non-inoculated seedlings (**Table 1**). Compared to the controls, seedlings exposed to the lowest Pb level (90 mg Pb kg^{-1} soil) showed a significant improvement in plant growth, which was greater for inoculated seedlings than for non-inoculated seedlings (e.g., by 21% versus 16% in root dry weight). In contrast, exposure to higher Pb levels (900 and 3000 mg Pb kg^{-1} soil) resulted in a significant reduction in plant growth, which was greater for non-inoculated seedlings than for inoculated seedlings (e.g., by 41% versus 26% in root dry weight at 3000 mg Pb kg^{-1} soil; **Figure 2**).

Gas Exchange and Chlorophyll Fluorescence

In the leaves of control seedlings at 0 mg Pb kg^{-1} soil, gas exchange parameters (except for C_i) significantly differed between non-inoculated and inoculated treatments. Inoculated seedlings had significantly higher A, gsw, and E values compared to non-inoculated seedlings (**Table 1**). Compared to control data, A, gsw, and E were significantly increased at 90 mg Pb kg^{-1} soil and significantly reduced at 900 and 3000 mg Pb kg^{-1} soil in both inoculated and non-inoculated seedlings. However, the C_i values of both inoculated and non-inoculated seedlings were higher at 900 and 3000 mg Pb kg^{-1} soil compared to the respective seedlings at lower Pb level. Moreover, inoculated seedlings had significantly higher A, gsw, and E and lower C_i compared to non-inoculated seedlings at the highest Pb level (e.g., by 58, 22, 44, and 39% versus 86, 47, 71, and 72%, respectively, at 3000 mg Pb kg^{-1} soil; **Figure 3**).

Under control conditions, we observed significantly higher F_v/F_m, ΦPSII, F_v/F_o, ETR, and qP, and significantly lower qN in the leaves of inoculated seedlings compared to non-inoculated seedlings (**Table 1**). Compared to control levels, F_v/F_m, ΦPSII, F_v/F_o, ETR, and qP of both inoculated and non-inoculated seedlings increased at 90 mg Pb kg^{-1} soil, followed by a significant reduction at 900 and 3000 mg Pb kg^{-1} soil. On the contrary, the qN showed a decrease at 90 mg Pb kg^{-1} soil, while an increase was found at 900 and 3000 mg Pb kg^{-1} soil. Inoculated seedlings showed significantly higher F_v/F_m, ΦPSII, F_v/F_o, ETR, and qP and significantly lower qN compared to non-inoculated seedlings at the highest Pb level (e.g., by 33, 28, 43, 31, 32, and 14% versus 39, 37, 75, 41, 39, and 28%, respectively, at 3000 mg Pb kg^{-1} soil; **Figure 4** and Supplementary Figure S1).

Pb Uptake and Translocation

Under control conditions, no significant difference in Pb content between non-inoculated and inoculated treatments was detectable. Compared to the control conditions, exposure to Pb resulted in an increase in Pb content in all tissues for inoculated and non-inoculated seedlings in a concentration-dependent manner, with the highest Pb content found in the roots under all Pb treatments. Inoculated seedlings showed

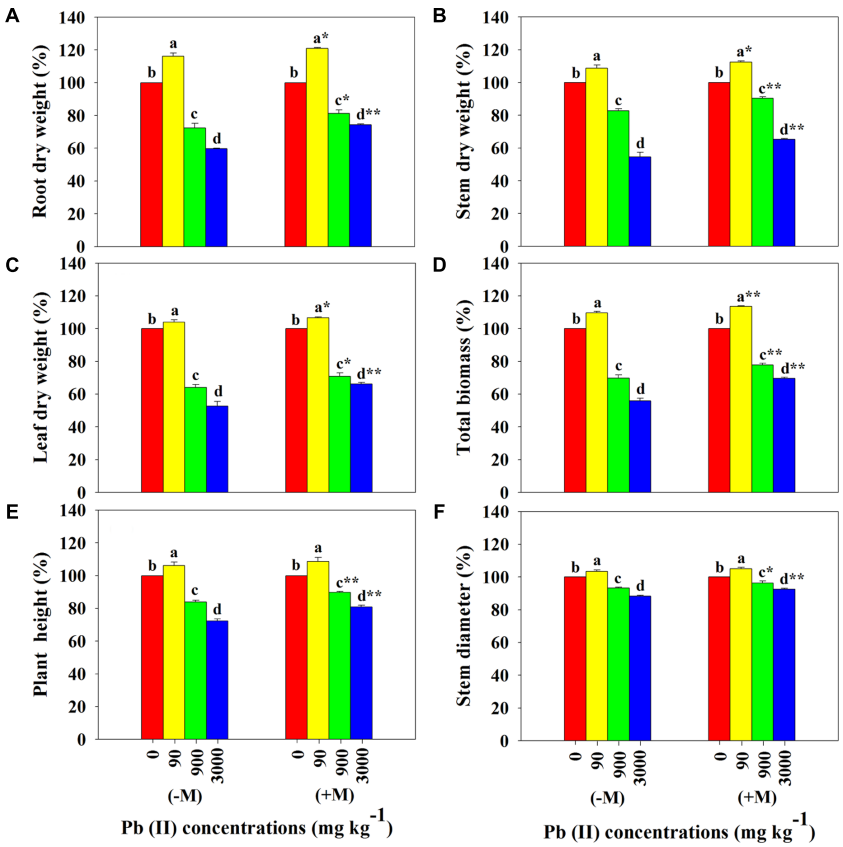

FIGURE 2 | Relative differences in plant growth of *Robinia pseudoacacia* seedlings with (+M) or without (–M) *Funneliformis mosseae* in response to 0, 90, 900, and 3000 mg Pb kg^{-1} soil for 4 months. (A) Root dry weight, **(B)** stem dry weight, **(C)** leaf dry weight, **(D)** total biomass, **(E)** plant height, and **(F)** stem diameter. Shown are means ± SD (n = 6). Asterisks indicate significant differences between inoculated and non-inoculated seedlings within one Pb level (*$P < 0.05$, **$P < 0.01$; t-test). Different lower case letters indicate significant differences among different Pb levels within inoculated or non-inoculated treatment ($P < 0.05$; ANOVA with *post hoc* Duncan).

significantly higher Pb content in the roots and stems at 90, 900, and 3000 mg Pb kg^{-1} soil and significantly lower Pb content in the leaves at 3000 mg Pb kg^{-1} soil compared to non-inoculated seedlings under Pb exposure (**Table 2**).

Subcellular Compartmentalization of Pb

The recovery rate of Pb was higher than 90% for most treatments. The proportion of Pb content in each subcellular fraction was related to the total Pb content accumulated in the respective tissue. Irrespective of Pb treatment or AMF inoculation, the proportion of Pb was highest in the FI fraction, ranging from 49 to 84% (**Figure 5**). Under control conditions, the proportions of Pb in the subcellular fractions of each tissue significantly differed between non-inoculated and inoculated seedlings. Both the FI and FIII were significantly higher and the FII was lower for inoculated seedlings compared to non-inoculated seedlings. Compared to control conditions, exposure to Pb (90, 900, and 3000 mg Pb kg^{-1} soil) resulted in a significant increase in the proportions of FI and FIII in all analyzed tissues in a concentration-dependent manner, and both fractions were significantly higher for inoculated seedlings than for non-inoculated seedlings (e.g., 62% versus 55% for FI in roots of seedlings at 90 mg Pb kg^{-1} soil). In contrast, the proportion of FII was significantly decreased in the tissues of seedlings under Pb exposure, and this fraction was significantly lower for inoculated seedlings compared to non-inoculated seedlings (e.g., 26% versus 39% in roots of seedlings at 90 mg Pb kg^{-1} soil; **Figure 5**).

Chemical Forms of Pb

The proportion of Pb content in each of chemical form was also related to the total Pb content accumulated in the respective tissue (**Figure 6**). For control seedlings (0 mg Pb kg^{-1} soil), the proportions of Pb in various chemical forms significantly differed between non-inoculated and inoculated seedlings. For example, inoculated seedlings showed significantly lower proportions (13–55%) of water-soluble Pb (including F_{d-H_2O} and $F_{Ethanol}$) and significantly higher proportions (16–38%) of HOAc-exactable Pb (F_{HOAc}) in the roots compared to non-inoculated seedlings.

Compared to controls, the lowest Pb level (90 mg Pb kg^{-1} soil) resulted in a significant increase in the proportion of Pb in F_{d-H_2O} fraction in all analyzed tissues of the non-inoculated seedlings, whereas that of inoculated seedlings were

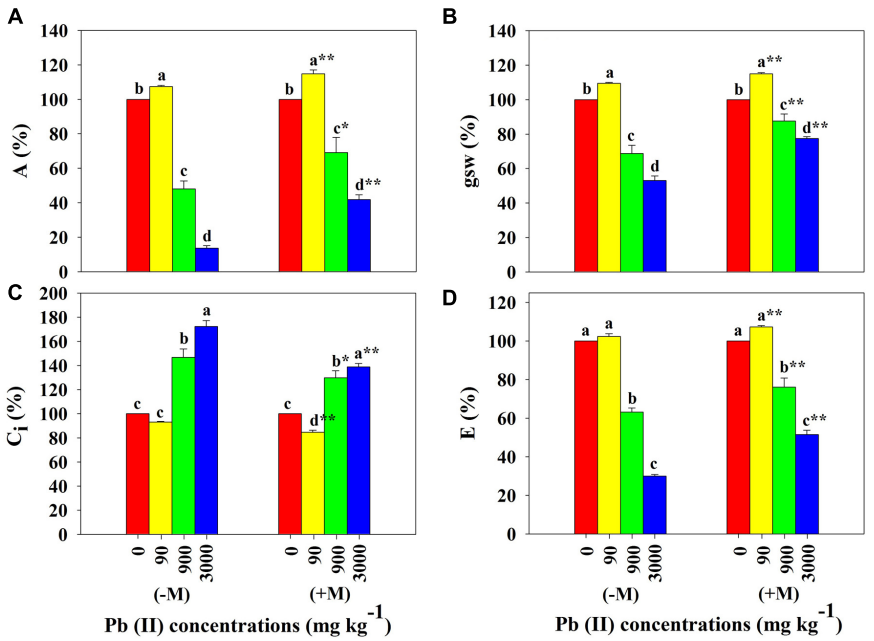

FIGURE 3 | Relative differences in gas exchange in leaves of *Robinia pseudoacacia* seedlings with (+M) or without (–M) *Funneliformis mosseae* in response to 0, 90, 900, and 3000 mg Pb kg^{-1} soil for 4 months. (A) Net CO_2 assimilation rate, A; **(B)** stomatal conductance to water vapor, gsw; **(C)** intercellular CO_2 concentration, C_i; and **(D)** transpiration rate, E. Shown are means ± SD (n = 6). Asterisks indicate significant differences between inoculated and non-inoculated seedlings within one Pb level (*P < 0.05, **P < 0.01; t-test). Different lower case letters indicate significant differences among different Pb levels within inoculated or non-inoculated treatment (P < 0.05; ANOVA with *post hoc* Duncan).

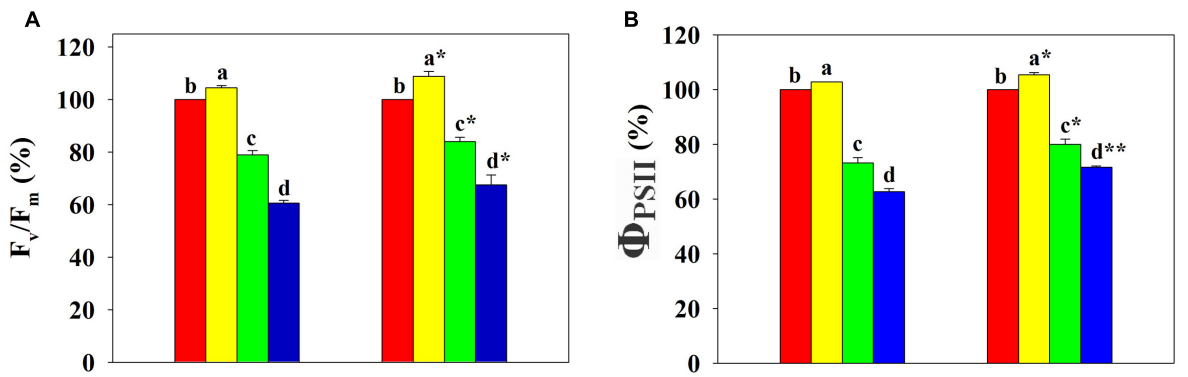

FIGURE 4 | Relative differences in chlorophyll fluorescence in leaves of *Robinia pseudoacacia* seedlings with (+M) or without (–M) *Funneliformis mosseae* inoculation in response to 0, 90, 900, and 3000 mg Pb kg^{-1} soil for 4 months. (A) The maximum quantum yield in the dark-adapted state of PSII, F_v/F_m; and **(B)** the actual quantum yield of PSII in light-adapted steady state, ΦPSII. Shown are means ± SD (n = 6). Asterisks indicate significant differences between inoculated and non-inoculated seedlings within one Pb level (*P < 0.05, **P < 0.01; t-test). Different lower case letters indicate significant differences among different Pb levels within inoculated or non-inoculated treatment (P < 0.05; ANOVA with *post hoc* Duncan).

significantly decreased. At higher Pb levels, the seedlings showed a significant reduction in the proportion of water-soluble Pb and a significant increase in the proportion of inactive Pb forms (including F_{NaCl}, F_{HOAc}, F_{HCl}, and $F_{Residue}$) in all tissues tested, irrespective of AMF inoculation. Additionally, inoculated seedlings retained significantly lower proportions of water-soluble Pb along with significantly higher proportions of inactive Pb forms compared to non-inoculated seedlings at 900 and 3000 mg Pb kg^{-1} soil (e.g., 21 and 79% versus 28 and 72%, respectively, at 900 mg Pb kg^{-1} soil, respectively; **Figure 6**).

Two-Factorial ANOVA and Correlation Analysis

The proportions of Pb content in different subcellular fractions and chemical forms were generally correlated with the relative differences in plant growth and physiological parameters

TABLE 2 | Pb content in roots, stems, and leaves of *Robinia pseudoacacia* seedlings with (+M) or without (−M) *Funneliformis mosseae* exposed to 0, 90, 900, and 3000 mg Pb kg^{-1} soil for 4 months.

Pb treatment (mg Pb kg^{-1} soil)	Pb content in plant tissues (μg plant^{-1})		
	Root	Stem	Leaf
0			
−M	1.17 ± 0.05g	0.14 ± 0.00g	0.31 ± 0.01d
+M	1.23 ± 0.02g	0.11 ± 0.00g	0.26 ± 0.01d
90			
−M	178.66 ± 4.88f	16.78 ± 0.49f	38.07 ± 0.68b
+M	270.17 ± 6.88e	20.32 ± 0.56e	34.58 ± 2.22b
900			
−M	335.75 ± 52.45d	36.70 ± 2.19d	65.95 ± 2.91c
+M	882.26 ± 27.25b	67.62 ± 2.77b	63.08 ± 2.96c
3000			
−M	758.06 ± 35.04c	47.16 ± 2.23c	112.77 ± 7.77a
+M	1342.12 ± 60.19a	73.88 ± 2.85a	94.71 ± 1.17b

Shown are means ± SD (n = 6). Different lower case letters in a column indicate significant differences between inoculated and non-inoculated seedlings within the same tissue at different Pb levels (P < 0.05; ANOVA with post hoc Duncan).

of *R. pseudoacacia* seedlings (Supplementary Tables S1–S4). Additionally, Pb, AMF, and their interactions showed significant effects on plant growth and photosynthetic parameters (Supplementary Table S5).

DISCUSSION

Pb has become ubiquitous in the soil due to natural deposits and intensive human activities (Fahr et al., 2013). AMF could offer an attractive system to facilitate plant-based environmental clean-up and strengthen plant tolerance to HM (Göhre and Paszkowski, 2006). In this study, we cultivated Pb-resistant *R. pseudoacacia* with *F. mosseae* exposed to different Pb levels in a pot experiment, in order to investigate the growth performance of *R. pseudoacacia* plants and Pb accumulation patterns in different tissues of *R. pseudoacacia*. The results indicate the potential of *R. pseudoacacia* for accumulation of Pb in phytoremediation and highlights the binding of inactive Pb forms to cell walls as a mechanism of Pb tolerance in plants with AMF.

Effects of AMF Symbiosis under Pb Stress

It is well documented that a symbiosis with AMF can improve photosynthesis, water use efficiency, and growth of plants (Chen et al., 2005; Zhu et al., 2011). In the current study, a symbiosis with *F. mosseae* showed significant positive effects on the gas exchange parameters, efficiency of PSII photochemistry, and plant growth in *R. pseudoacacia* seedlings under control conditions (0 mg Pb kg^{-1} soil; **Figures 3**, **4**). This may be attributed to AMF regulation of the availability and uptake of water and nutrients by decreasing stomatal resistance, and increasing CO_2 assimilation, and accelerating transpiration fluxes (Zhu et al., 2011). Low levels of HM (e.g., 50 μg L^{-1} Cu and Cd) have been shown to exert a stimulatory effect on plant growth of mycorrhizal (*Acaulospora laevis* and *Glomus caledonium*) and non-mycorrhizal *Z. mays* (Liao et al., 2003). Similar to these findings, across all Pb levels investigated in the present study, the lowest Pb level (90 mg Pb kg^{-1} soil) resulted in an increase in plant growth (e.g., higher total dry weight, plant height, and stem diameter; **Figure 2**) coinciding with an improvement of photosynthesis (e.g., higher A, gsw, E, F_v/F_m, and ΦPSII; **Figures 3**, **4**) in *R. pseudoacacia* seedlings, regardless of mycorrhization with *F. mosseae*. This growth promotion effect of low Pb may be associated with stimulated metabolism (e.g., photosynthesis) and enzyme activities (e.g., superoxide dismutase and peroxidase) under low Pb stress (Schützendübel and Polle, 2002). Additionally, we found that seedlings inoculated with *F. mosseae* gained a higher performance in growth and photosynthesis than non-inoculated seedlings, consistent with previous findings in other plants. Chen et al. (2007) highlighted the importance of *F. mosseae* in accelerating growth of *Coreopsis drummondii*, *Pteris vittata*, and *Trifolium repens* in Cu mine tailings.

In this study, the application of higher Pb levels (900 and 3000 mg Pb kg^{-1} soil) resulted in an inhibition of photosynthesis, along with a decline in plant growth. The photosynthetic parameters of inoculated and non-inoculated seedlings exhibited similar patterns in response to Pb application; but the effect of AMF inoculation on photosynthetic parameters was strengthened at relatively low Pb level. In stressful conditions with HM, component disruption of the photosynthetic apparatus can occur and photosynthetic processes would be negatively affected in plants (Powles, 1984). Because of a significant reduction in ETR and ΦPSII in the leaves of *R. pseudoacacia* (**Figure 4** and Supplementary Figure S1), it is assumed that higher Pb could destroy the PSII reaction center or disrupt electron transport in the photosynthetic apparatus. Nonetheless, we found that the photosynthetic parameters of inoculated seedlings were less impaired during exposure to higher Pb levels compared to non-inoculated seedlings. For instance, the relative difference in F_v/F_m was significantly lower for leaves of the inoculated seedlings compared to non-inoculated controls for the same Pb treatments (900 or 3000 mg Pb kg^{-1} soil). The higher performance of inoculated plants exposed to high Pb can be related to their improved availability of water and nutrients due to the AMF symbiosis. As we have normalized the physiological parameters of plants grown under Pb exposure to those under control conditions, the higher performance of inoculated plants under high Pb stress may also be attributed to other mechanisms that resist Pb uptake from soil and/or tolerate Pb within the cell (Wang Y. et al., 2015). AMF could facilitate Pb retention within the roots and stems, while reducing Pb accumulation in the leaves (**Table 2**). Thus, although significant Pb accumulation resulted in photosynthesis stress, it was seemed to be diminished for inoculated plants.

In this study, Pb accumulated in the roots, stems and leaves of *R. pseudoacacia* in a concentration-dependent manner, and Pb retention in the roots was significantly higher for inoculated

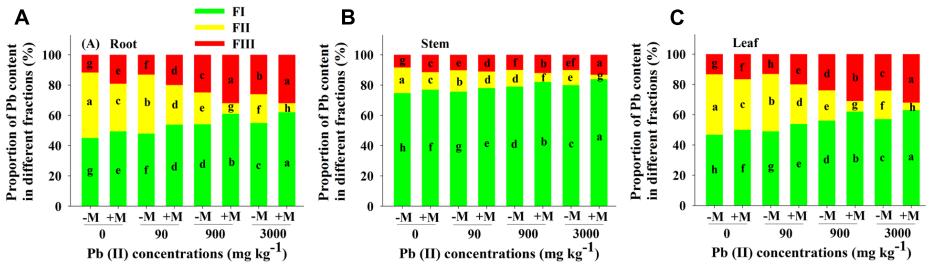

FIGURE 5 | Proportion of Pb in different subcellular fractions in roots (A), stems **(B)**, and leaves **(C)** of *Robinia pseudoacacia* seedlings with (+M) or without (–M) *Funneliformis mosseae* in response to 0, 90, 900, and 3000 mg Pb kg^{-1} soil for 4 months. Shown are means ± SD (n = 6). Different lower case letters within a column indicate significant differences between inoculated and non-inoculated seedlings within the same tissue among different Pb levels ($P < 0.05$; ANOVA with *post hoc* Duncan). Cells were separated by gradient centrifugation at 4°C into cell wall (FI), organelle (FII), and soluble (FIII) fractions.

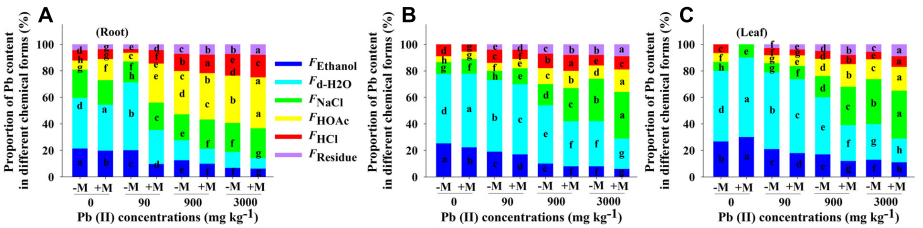

FIGURE 6 | Proportion of Pb in different chemical forms in roots (A), stems **(B)**, and leaves **(C)** of *Robinia pseudoacacia* seedlings with (+M) or without (–M) *Funneliformis mosseae* in response to 0, 90, 900, and 3000 mg Pb kg^{-1} soil for 4 months. Shown are means ± SD (n = 6). Different lower case letters within a column indicate significant differences between inoculated and non-inoculated seedlings within the same tissue among different Pb levels ($P < 0.05$; ANOVA with *post hoc* Duncan). Different chemical forms of Pb were sequentially extracted by 80% ethanol ($F_{Ethanol}$), deionized H2O F_{d-H_2O}), 1 M NaCl (F_{NaCl}), 2% HOAc (F_{HOAc}), and 0.6 M HCl (F_{HCl}). $F_{Residue}$, Pb in residues.

seedlings compared to non-inoculated plants over the entire range of Pb levels applied (**Table 2**). AMF shows high tolerance to HM (Xu et al., 2012) and AMF symbiosis could create a more balanced environment for the roots by enriching HM at or in the mycorrhizal structure, decreasing free ion activity, and reducing toxicity (Chen et al., 2007). During symbiotic interaction between AMF and plants, hyphal network may functionally extend the root system of their hosts to take up HM from an enlarged soil volume (Göhre and Paszkowski, 2006). This explains the twofold higher Pb content in the roots of inoculated seedlings compared to non-inoculated seedlings of *R. pseudoacacia* (**Table 2**). The mitigation of negative impacts induced by HM could vary to a large extent, depending on HM species, soil HM level, fungal symbiotic partner, and/or plant environment (Hildebrandt et al., 2007). In the present study, the colonization of *F. mosseae* was markedly reduced in the roots of *R. pseudoacacia* at 900 and 3000 mg Pb kg^{-1} soil (**Figure 1**), indicating the negative effect of high Pb on the root colonization of AMF. A similar phenomenon was found by Weissenhorn and Leyval (1995) in the roots of *Z. mays* under Cd stress (up to 10 mg L^{-1}), maybe due to limited hyphal extension and restricted spore germination at high HM levels (Weissenhorn et al., 1994). It is noteworthy that the root colonization rate by *F. mosseae* was not completely eliminated at 3000 mg Pb kg^{-1} soil in the current study. Thus, we assume that *F. mosseae* possesses a high tolerance toward Pb and it maybe a candidate for applications in reclamation of Pb polluted soils. This assumption needs to be tested by further long-term experiments.

Molecular Mechanism of Pb Tolerance

Under Pb stress, some molecular mechanisms are rapidly activated to minimize the potential toxicity of HM in plants (Wang Y. et al., 2015). Compartmentalization of HM is an important mechanism of detoxification/adaptation in plant cells (Zhao et al., 2015). After entering the cells, HM are bound to various subcellular compartments and exhibit different ecotoxicological significances. The cell walls, as the first protecting barrier, are mainly composed of polyoses (including cellulose, hemicellulose, and pectin) and proteins (Wang Y. et al., 2015). The negatively charged sites provided by functional groups, such as hydroxyl, carboxyl, amino, and aldehyde groups (Hu et al., 2012), can bind HM ions and limit their transport across the cell membrane (Bhatia et al., 2005; Arias et al., 2010). In the present study, a large proportion of Pb was found in the cell wall fraction in the roots, stems, and leaves of *R. pseudoacacia* seedlings across all Pb treatments, irrespective of AMF inoculation (**Figure 5**). This observation leads to the conclusion that the cell walls function as the primary barrier to Pb entry into the cytoplasm and limit organelles from suffering Pb toxicity in *R. pseudoacacia*. Further research is needed to evaluate the contribution of Pb sequestration in the cell walls of *R. pseudoacacia* roots.

The cell matrix between the cell walls and organelles can be regarded as an intracellular buffer (Arias et al., 2010). Soluble cellular components store HM, while they contain organo-ligands, mainly sulfur-rich peptides, organic alkali, and organic acids. Complexing metals with organo-ligands in these storage sites can decrease free ion activity and reduce HM toxicity (Bhatia et al., 2005). In the current study, the proportion of Pb in the soluble fraction was significantly increased in different tissues of *R. pseudoacacia* seedlings with increasing Pb level (**Figure 5**). This supports the hypothesis that complex formation of metals with organo-ligands is a molecular mechanism reducing HM toxicity in *R. pseudoacacia* seedlings. Meanwhile, the proportion of Pb in different subcellular fractions was significantly affected by AMF inoculation (Supplementary Table S5). Compared to the non-inoculated seedlings, *R. pseudoacacia* seedlings with *F. mosseae* showed significantly higher proportions of Pb in the cell wall and soluble fractions in the roots, stems, and leaves of *R. pseudoacacia* seedlings over the entire range of Pb level applied (**Figure 5**). This indicates that AMF facilitated the immobilization of Pb in the cell wall and soluble fractions of plant tissues, similar to the finding in *Medicago sativa* with *Glomus intraradices* under Cd stress (Wang et al., 2011). Moreover, the proportion of Pb in the subcellular compartments was highly associated with photosynthesis and plant growth of *R. pseudoacacia* seedlings (Supplementary Tables S1, S2). Thus, we conclude that the selective distribution of Pb in the cell wall and soluble fractions is a strategy for Pb tolerance and detoxification during the growth of *R. pseudoacacia* seedlings with AMF.

A high level of HM in plant tissue does not necessarily mean a high toxicity for a plant, since the HM may exist in chemical forms with low or no phytotoxicity (Fu et al., 2011). In the present study, we found that inoculation with *F. mosseae* promoted the conversion of Pb into inactivate forms (F_{NaCl}, F_{HOAc}, F_{HCl}, and $F_{Residue}$; **Figure 6**). The undissolved phosphate (F_{HOAc}) and oxalate (F_{HCl}) fractions of Pb have been already described as effective means in Pb detoxification (Zhang et al., 2013; Wang Y. et al., 2015) as they are less harmful than soluble Pb to plant cells. Wang et al. (2016) have indicated that the fibrous roots of apple tree showed largest proportions in the HOAc and HCl extractable Cu forms. Similarly, Zhao et al. (2015) showed that NaCl- and HOAc-extractable HM may be responsible for the adaptation of *Porphyra yezoensis* to Cd stress.

According to the high abundance of inactive forms of Pb in different tissues of inoculated plants, we therefore assume that (i) AMF may have a significant impact on the detoxification of Pb in plants through the transformation of Pb into the inactive forms, and (ii) transformation of Pb in inoculated seedlings may base on chelation of Pb by specific polar materials (e.g., hydroxyl or carboxyl groups) to form a non-toxic complex (Andrade et al., 2010), thereby contributing to improved plant growth and physiological performance. During exposure to high Pb, better photosynthesis and performance of PSII in the leaves of inoculated plants (e.g., higher gsw and F_v/F_m) may be attributed to the retention of Pb in different subcellular fractions and chemical forms in the roots, which could prevent the disruption of photosynthesis apparatus and membrane integrity. AMF symbiosis could improve the capacity of gas exchange, the efficiency of photochemistry and non-photochemistry of PSII, and regulate the energy bifurcation between photochemical and non-photochemical events in the leaves of seedlings.

Given its high stress tolerance and fast growth, *R. pseudoacacia* is considered suitable for soil and vegetation restoration in HM contaminated areas (Yang et al., 2015b,c). In this study, the majority of Pb was found to be retained in the roots of *R. pseudoacacia* under Pb stress, with a significantly higher accumulation in seedlings with *F. mosseae*. Compared with non-inoculated seedlings, the improved physiological parameters were highly associated with Pb compartmentalization in different chemical forms, including (1) increased proportion of Pb in the cell wall and soluble fractions, with the highest proportion of Pb in the cell wall fraction; and (2) increased proportion of inactive Pb, especially F_{NaCl} and F_{HOAc}, in plant tissues. These provide new insights into the role of AMF on Pb tolerance in woody legumes from a molecular perspective. From an ecological point of view, *R. pseudoacacia* inoculated with *F. mosseae* may be used for remediating Pb polluted soils.

AUTHOR CONTRIBUTIONS

All authors participated in the conception of the topic after critically revising it for important intellectual content. LH and HZ wrote the manuscript. YS, YY, and HC assisted with data analysis, manuscript preparation, and revision. MT served as the primary investigator, conceived the project, and finalized the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Mycorrhizal Symbiotic Efficiency on C_3 and C_4 Plants under Salinity Stress – A Meta-Analysis

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A wide range of C_3 and C_4 plant species could acclimatize and grow under the impact of salinity stress. Symbiotic relationship between plant roots and arbuscular mycorrhizal fungi (AMF) are widespread and are well known to ameliorate the influence of salinity stress on agro-ecosystem. In the present study, we sought to understand the phenomenon of variability on AMF symbiotic relationship on saline stress amelioration in C_3 and C_4 plants. Thus, the objective was to compare varied mycorrhizal symbiotic relationship between C_3 and C_4 plants in saline conditions. To accomplish the above mentioned objective, we conducted a random effects models meta-analysis across 60 published studies. An effect size was calculated as the difference in mycorrhizal responses between the AMF inoculated plants and its corresponding control under saline conditions. Responses were compared between (i) identity of AMF species and AMF inoculation, (ii) identity of host plants (C_3 vs. C_4) and plant functional groups, (iii) soil texture and level of salinity and (iv) experimental condition (greenhouse vs. field). Results indicate that both C_3 and C_4 plants under saline condition responded positively to AMF inoculation, thereby overcoming the predicted effects of symbiotic efficiency. Although C_3 and C_4 plants showed positive effects under low (EC $<$ 4 ds/m) and high ($>$8 ds/m) saline conditions, C_3 plants showed significant effects for mycorrhizal inoculation over C_4 plants. Among the plant types, C_4 annual and perennial plants, C_4 herbs and C_4 dicot had a significant effect over other counterparts. Between single and mixed AMF inoculants, single inoculants *Rhizophagus irregularis* had a positive effect on C_3 plants whereas *Funneliformis mosseae* had a positive effect on C_4 plants than other species. In all of the observed studies, mycorrhizal inoculation showed positive effects on shoot, root and total biomass, and in nitrogen, phosphorous and potassium (K) uptake. However, it showed negative effects in sodium (Na) uptake in both C_3 and C_4 plants. This influence, owing to mycorrhizal inoculation, was significantly higher in K uptake in C_4 plants. For our analysis, we concluded that AMF-inoculated C_4 plants showed more competitive K^+ ions uptake than C_3 plants. Therefore, maintenance of high cytosolic K^+/Na^+ ratio is a key feature of plant salt tolerance. Studies on the detailed mechanism for the selective transport of K in C_3 and C_4 mycorrhizal plants under salt stress is lacking, and this needs to be explored.

Keywords: arbuscular mycorrhizal fungi, salinity stress, meta-analysis, C_3 and C_4 plants, nutrient uptake, plant biomass

INTRODUCTION

Approximately 70% of the farm soils on earth are either salt-affected or subjected to salinity (Munns and Tester, 2008). Furthermore, it has been predicted that 30% of the cultivable soils will become unusable by 2050 due to salinity, and this warrants appropriate salt alleviating technologies for sustaining food production (Wang et al., 2003). Plants can be divided in to three different types in terms of the way in which they use photosynthesis, C_3, C_4, and CAM. The difference between these types is how plant uses carbon dioxide in the growth process. Although C_4 plants are relatively few, i.e., 3% of plant species compared with the much more numerous C_3 plants (∼7500 C_4 species to nearly ∼250000 C_3 species), they account for approximately 30% of primary productivity (Sage and Zhu, 2011). As described previously in detail, Zhu et al. (2008) and de Bossoreille de Ribou et al. (2013) reported that the conversion efficiency (from solar energy to biomass) during photosynthesis is 4.6% for C_3 and 6% for C_4 plants. Thus, global productivity has to better exploit the superior engine of C_4 photosynthesis, both by using it on a greater scale (i.e., engineer the C_4 photosynthetic pathway into C_3 plants) and by improving the ability of C_4 plants to resist environmental stress (Hibberd et al., 2008; Kajala et al., 2011; de Bossoreille de Ribou et al., 2013). Among the major food crops, C_3 and C_4 plants are widely studied for their response to salt stress (Stepien and Klobus, 2005; Chaves et al., 2009; Ivlev et al., 2013). Mane et al. (2011) reported a higher shoot/root ratio at 300 mM NaCl in the C_4 plant Bajra, in which plant biomass production was increased by 29.17%. In C_4 maize plants, shoot and root growth was not impacted up to 1.5 g NaCl/Kg dry substrate (Sheng et al., 2008). Conversely, shoot and root growth of C_3 plants wheat (Mohammad et al., 1998) and tomato (Datta et al., 2009) were decreased proportionately with the increase in NaCl concentration up to 125 mM. Earlier researchers (Omoto et al., 2012; Ivlev et al., 2013) have attributed this successful salt-tolerance effect in C_4 plants to the CO_2 concentrating mechanism of these plants under stress conditions.

Symbiotically associating microorganisms have been widely used for modulating stress factors by increasing the nutrient availability and sustaining productivity in these plants (Dodd and Perez-Alfocea, 2012; Pellegrino et al., 2015). Symbiotically associated and soil inhabiting arbuscular mycorrhizal fungi (AMF) have been widely studied for their effective scavenging of soil nutrients, owing to their larger surface root volume (Parniske, 2008). The symbiosis between plant roots and AM fungi is one of the best plant's strategies for growing under salt stress (Evelin et al., 2009; Porcel et al., 2012). AM fungi have been frequently reported to improve host plants' tolerance to salt stress. Improved salt tolerance following mycorrhizal colonization may be the result of a more efficient nutrient uptake (Porras-Soriano et al., 2009; Hajiboland et al., 2010; Evelin et al., 2012), and ion balance (Giri et al., 2007; Evelin et al., 2012; Wu et al., 2013). For example, plants colonized with *Rhizophagus irregularis* increased the concentrations of nitrogen (N), phosphorous (P), and potassium (K) whereas mycorrhizal plants have decreased (Na) sodium concentrations than the non-mycorrhizal plants at all levels of salinity in tomato and fenugreek plants (Hajiboland et al., 2010; Evelin et al., 2012). Also, plants colonized with *Funneliformis mosseae* maintained higher concentrations of N, P, and K and decreased the Na concentrations in cotton and citrus plants (Wu et al., 2013). Several researchers suggest the role of AM fungi in influencing the ionic balance of the cytoplasm or Na^+ efflux from plants thereby increasing K^+:Na^+ ratios under salt stress (Giri et al., 2007; Hajiboland et al., 2010; Evelin et al., 2012; Wu et al., 2013). Moreover, proline content has been reported to change during stress among mycorrhizal plants, and thus, it may serve as a parameter to evaluate the effects of AMF and salinity on plants (Sannazzaro et al., 2007; Echeverria et al., 2013). Several studies have also indicated that AMF symbiosis can increase stomatal conductance, transpiration and photosynthetic rate and water use efficiency in plants exposed to salinity stress than non-mycorrhizal plants (Sheng et al., 2008; Evelin et al., 2012; Ruiz-Lozano et al., 2012; Elhindi et al., 2016; Shamshiri and Fattahi, 2016).

At the same time, variations in mycorrhizal symbiotic efficiency among different plant species have been reported. The mycorrhizal symbiotic efficiency in C_3 and C_4 plants varied depending on the plant species involved. Mycorrhizal colonization in C3 plants such as tomato (Abdel Latef and Chaoxing, 2011) and menthol mint (Bharti et al., 2013) were 55 and 64%, respectively. Whereas in C_4 plants such as maize (Estrada et al., 2013) and *Acacia auriculiformis* (Giri et al., 2003), the mycorrhizal colonization was 64 and 68%, respectively. Moreover, efficiency also varied among isolates of AM fungi irrespective of individual host plant or location of origin. For example, C_3 plants, *Olea europaea* colonized with *F. mosseae*, *R. irregularis,* and *R. claroideum*, increased salt tolerance in terms of plant growth and nutrient acquisition particularly N, P, and K uptake. Among AMF species, *F. mosseae* was the most efficient fungus in reducing the detrimental effects of salinity, and this effect was due to increased K uptake (Porras-Soriano et al., 2009). In addition, *Glomus deserticola* exhibited a higher symbiotic efficiency in C_3 plants, *Lactuca sativa* compared to *Glomus* sp. under saline condition. According to Zou and Wu (2011), the C_4 plant *Poncirus trifoliate,* trifoliate orange seedlings inoculated with five AMF species (*Diversispora spurca*, *Claroideoglomus etunicatum*, *F. mosseae, G. versiforme,* and *Paraglomus occultum*) exhibited different symbiotic efficiency. They proposed that *G. versiforme* showed the best efficiency in alleviating salt stress of trifoliate orange, and *C. etunicatum* exhibited the lowest efficiency. Hence, mycorrhizal development would mostly depend on the compatibility of both AMF and host plants. Enhanced mycorrhizal symbiosis with C_4 plants, when compared with generally grown C_3 plants has been documented (Hetrick et al., 1988a; Hetrick, 1991; Wilson and Harnett, 1997). Among C_4 plants, grasses are reported to be obligate for mycorrhizal symbiosis (Hetrick, 1991). C_4 grasses showed 98 to 99% mycorrhizal dependency, with positive effects for inoculation resulting up to 63 to 215% greater plant dry weight, than the non-mycorrhizal plants, whereas the C_3 grasses showed 15 to 75% mycorrhizal dependence and recorded 0.12 to 4.1 times larger plant dry weight (Hetrick et al., 1988b).

The variations in the root morphology of the two plant types play a crucial role in mycorrhizal colonization. C_3 grasses with

fibrous, highly branched root systems are believed to function more independently to support mycorrhizal symbiosis than the C_4 grass with a coarser and less-branched root system (Hetrick et al., 1990). For woody plants, plant species with thick roots and few root hairs were not much responsive to AMF, whereas woody species with fine roots and abundant root hairs are highly responsive one (Siqueira and Saggin-Junior, 2001). Duponnois and Plenchette (2001) reported a positive correlation on the leguminous mycorrhizal response, the root-hair density and length. It is apparent that the mycorrhizal infection of the root confers less susceptibility to the deleterious effects of salinity and can enable the plants to compensate for root growth and other functions.

Quantitative analytical studies have been widely conducted on AMF colonization and nutrient uptake in normal soils and have also been reported in AMF plant symbiotic relationships under abiotic stress conditions, including drought and metal stresses (Veresoglou et al., 2012; Jayne and Quigley, 2014; Bunn et al., 2015; Pellegrino et al., 2015; Andrade-Linares et al., 2016). Quantifiable validation showed that plants colonized by mycorrhizal fungi have better growth and better reproductive responses under water deficit conditions (Jayne and Quigley, 2014). Despite this fact and despite the in-depth reviews on the AMF mediated salt stress alleviation where data are available (Evelin et al., 2009; Porcel et al., 2012), quantitative analytical studies on these aspects are few. Recently, we conducted meta-analysis report on the symbiotically efficient AMF species and reported that salt-stress alleviation in different plants is due to enhanced nutrient uptake and antioxidant enzyme activity (Chandrasekaran et al., 2014). Additionally, our previous meta-analytical study has confirmed the general overall positive effect in mycorrhizal plants based on plant biomass and nutrient uptake under saline conditions.

Therefore, the present study sought to answer the following questions:

(i) Are there any characteristic differences in plant growth response among C_3 and C_4 plants upon mycorrhizal inoculation under salt stress?
(ii) Are there any uptake of specific nutrient (s) essential for salt stresses alleviation in C_3 and C_4 mycorrhizal plants?
(iii) Which soil type and salinity level favorably influence the mycorrhizal inoculation in C_3 and C_4 plants?
(iv) What are the most effective mycorrhizal inoculation between AMF species and C_3 and C_4 plants species under salt stress?

MATERIALS AND METHODS

Literature Search and Data Collection

To build a database, searches were conducted in Web of Science, and the references cited in publications were retrieved from 1998 to 2013. We performed our literature survey using these key words: *C_3/C_4 plants**, '*mycorrhiza**/*arbuscular mycorrhiza**** and *salt stress/salinity stress**. Using the Boolean truncation ('*') character ensured the presence of words required for a literature survey.

These searches resulted in 700 published and unpublished online references. To meet this set of criteria, 250 publications that were likely to contain relevant information were considered (Appendix S1). The selected publications were checked for the following inclusion criteria (i) plant biomass and nutrient uptake, (ii) crop plants (annual and/or perennial), (iii) influence on AMF inoculation compared with uninoculated control and (iv) the experiments that were performed in saline conditions, or at least an EC $\leq$ 4 dS/m and/or >40 mM NaCl levels. The number of studies selected at various stages is shown in the flow diagram in **Figure 1**. Finally, 60 studies fulfilling all of the required criteria were screened, and 582 trials (bias-corrected) that potentially met the selection criteria of presenting information on the effects of AMF inoculation under salinity stress were selected (see Supplementary Information Dataset). If a particular research paper reported results from more than one study system that had independencies (e.g., AMF inoculum, plant species, and level of salinity), each system was considered as a trial. Based on the criteria mentioned above, our meta-analysis included 229 observations from 25 studies for C_3 photosynthetic type and 352 observations from 35 studies for C_4 photosynthetic types.

Data Acquisition

For each study, we ensured that the data included mean, standard deviation (SD) and replicate number/sample size (n) for a control as well as the AMF inoculation under salt stress. If standard errors (SEs) were reported, data were transformed to include SD with the equation $SE = \text{SD}\ (n^{-1/2})$ using MetaWin 2.1 statistical calculator. In publications where the means and errors were presented in the graph, the image was digitized, and Dexter (GAVO data center) software was used to estimate the values[1].

Effect Size and Publication Bias

Effect sizes were calculated as the natural log of response ratio (LRR) as a metric for the response of AMF inoculation under salinity stress, due to its statistical properties (approximate normal distribution) and biological interpretation (Hedges et al., 1999; Rosenberg et al., 2000). These calculations were conducted by the Meta-Win v2.1 software (Rosenberg et al., 2000).

$$LRR = ln\left(\frac{\overline{X}^E}{\overline{X}^C}\right) = ln\left(\overline{X}^E\right) - ln\left(\overline{X}^C\right)$$

$$V_{\ln R} = \frac{(S^E)^2}{N^E(\bar{X}^E)^2} + \frac{(S^C)^2}{N^C(\bar{X}^C)^2}$$

where R is the response ratio, X^C is the control mean (without AMF inoculation under salt stress), X^E is the treatment mean (with AMF inoculation under salt stress), S^C is the control SD, S^E is the treatment SD, N^C is the control replication number, and N^E is the treatment replication number.

[1] http://dc.zah.uni-heidelberg.de/sdexter/

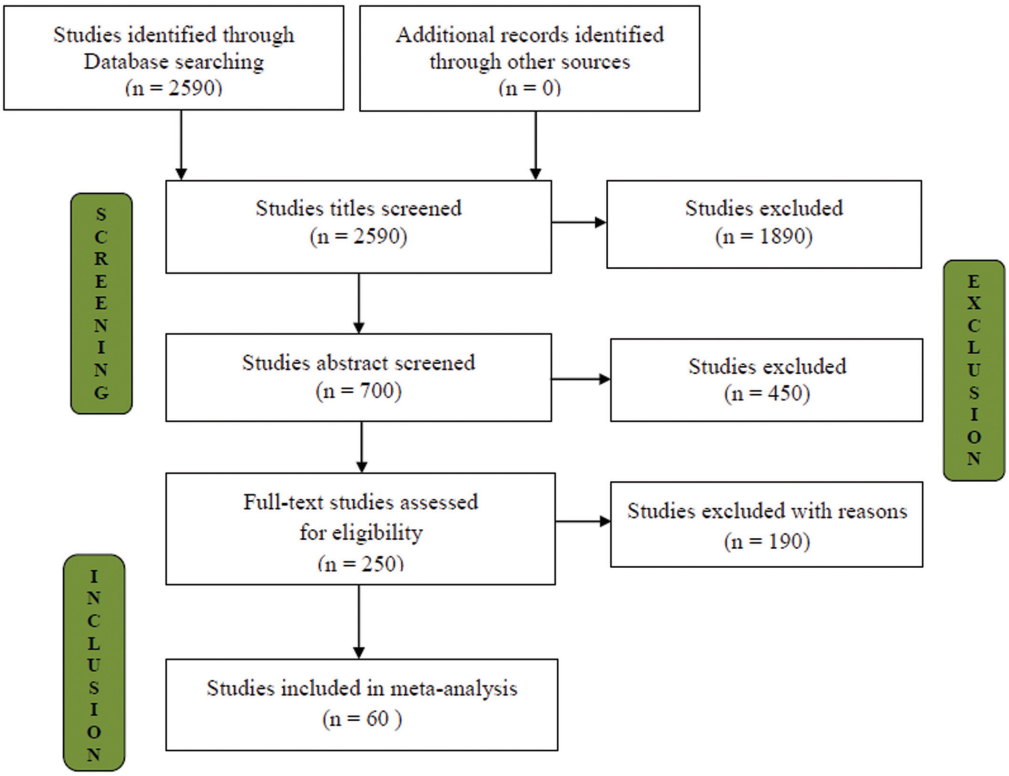

FIGURE 1 | Selection of studies for inclusion in the meta-analysis.

In the framework of our meta-analysis, under salinity stress, each response parameter observed after AMF inoculation was assessed by Spearman's rho rank correlating effect size against sample size. If larger effect sizes were more likely to be published than smaller effect sizes, a significant correlation would indicate publication bias. We utilized the fail-safe number of the Rosenthal method to determine the number of non-significant, unpublished or missing studies, which would have to be added to our meta-analysis to nullify its overall effect size (Rosenberg, 2005). If this number was larger than $5n + 10$ (n = number of studies), then publication bias was safely ignored. We also checked the existence of publication bias via scatter-plots and/or funnel plots of effect size vs. sample size or variance, respectively, according to Nakagawa and Santos (2012; Supplementary Figures S1 and S2).

Categorical Independent Variables

Categorical analyses were made on the data to determine the comparative responses of C_3 and C_4 plants to AMF inoculation under soil salinity by considering the different fixed factors mentioned below.

Photosynthetic type had two levels: C_3 and C_4 plants corresponding to the two data sets, which allowed testing for any significant differences on the AMF-mediated plant growth and nutrient uptake under salt stress among photosynthetic types.

Plant group had two levels: *monocot* and *dicot*. The plants were classified using the PLANTS database of the USDA, Natural Resources Conservation Service[2].

Plant types: Studies were coded to include the following variables: *annual herb*, *perennial herb*, *woody,* and *grass.*

Plant growth habit had seven levels: *forb/herb*, *shrub*, *herb*, *grass*, *tree*, *sub shrub,* or *shrub/tree.*

Soil type had five levels: *sandy*, *sandy loamy*, *loamy*, *clayey loamy,* and *silty* soil. Soil types were classified using the soil database of the USDA Natural Resources Conservation Service[2].

Soil salinity had three levels: *low*, *moderate,* and *high* salinity. The levels of salinity data reported were classified, referring to the USDA Natural Resources Conservation Service. Accordingly, *Low level salinity* with EC $\leq$ 4 dS/m, *moderate level salinity* with 4–8 dS/m and *high salinity* with more than 8 dS/m were considered.

AMF inocula had two levels: *single* and *mixed*. Single species inoculants were considered using one of the AMF species, whereas the mixed species inoculants had more than one AMF species. The AMF inoculants used were either isolated from field soil or obtained from commercial suppliers. The AM fungal species were classified and named according to the nomenclature of Redecker et al. (2013).

Plant species and plant family: Forty plant species belonging to the families of Anacardiaceae, Asteraceae, Caryophyllaceae,

[2]http://plants.usda.gov/java/

Chenopodiaceae, Euphorbiaceae, Fabaceae, Lamiaceae, Liliaceae, Malvaceae, Moraceae, Poaceae, Rutaceae, Solanaceae, and Verbenaceae were included in our analysis.

Experimental duration had three levels: short studies lasting up to 2 months; intermediate studies of 2–4 months and long-time studies of more than 4 months were considered.

Experimental condition had two levels: *greenhouse experiments* conducted indoors under controlled environmental conditions and *field experiments* conducted in uncontrolled environmental conditions were considered.

Statistics

Random-effects model meta-analyses were conducted for each of the categorical independent variables to test their influence on the impact of AMF inoculation and the alleviation of salt stress effects in C_3 and C_4 plants using MetaWin 2.1 software. All models were weighted by the inverse of variance in LRR (Rosenberg et al., 2000). A permutation procedure with 4,999 iterations was used because the number of their effects violated the criterion of normality (Adams et al., 1997). Confidence intervals were then estimated through a bootstrap procedure that implemented bias-correction (Rosenberg et al., 2000).

Most of the studies included in the datasets contained more than one trial in experimental setup. To handle this common issue in ecological meta-analyses and the independence violation of these studies (Nakagawa and Santos, 2012), the following two modifications were applied: (i) if multiple trials shared the same control, they were modified using the methodology of Lajeunesse (2011), and (ii) multiple trials originating from the same study were brought to a single effect size through fixed-effects using the meta-analytical procedure (Lajeunesse, 2011; Lehmann et al., 2014). These approaches ensured that the random-effects component of the meta-analysis was restricted to trials that belonged to different studies. Trials of one study were not reduced if the effect sizes that originated from different experimental systems were represented by independent variables (Rosenberg et al., 2000).

Potentially meaningful statistical inferences are presented for those variables for which data for at least two categories were available for either C_3 or C_4 datasets containing reasonable sample sizes. This was done to allow at least a rudimentary comparison between C_3 and C_4 species. For each categorical analysis, the total heterogeneity was calculated among studies (Q_T) within group heterogeneity (Q_W), or between group heterogeneity (Q_B). Studies were considered significant when Q_B was significant ($P < 0.05$) and described at least 10% of the total variation ($Q_B/Q_T \geq 0.1$; Rosenberg et al., 2000). Zero (0) effect sizes signify no difference in effects between the experimental and control groups, negative values represent effects where the control group attains a greater significance than the experimental group, and positive values represent effects where the experimental group attains a greater significance than the control group. AMF inoculation effects were estimated as a percentage change, relative to the control (%), using the equation $[\exp(\text{LRR}) - 1] \times 100$. A sensitivity analysis was conducted to test for any disproportional impact of single studies and their reproducibility (Hedges et al., 1999). In this study, we tested significant results, and only robust or bias-corrected results are presented in the results section (Supplementary Figures S3–S5).

RESULTS

Overall AMF Inoculation Effects on Plant Growth and Nutrient Uptake under Salt Stress

The results of meta-analysis of 60 studies conducted on different host plants under salt stress show that mycorrhizae are a significant factor affecting plant growth and nutrient uptake in both C_3 and C_4 plants irrespective of plant photosynthetic type (Supplementary Figure S6; Supplementary Table S1). Shoot, root and total biomass of AMF-inoculated plants increased by 67.1, 57.8, and 71.1%, respectively. Also, the AMF-inoculated plants under saline conditions significantly increased N, P, and K uptake by 93.2, 86.8, and 42.7%, respectively. In addition, Na uptake and proline accumulation decreased significantly by 22.2 and 6.5%, respectively, under saline condition in AMF inoculated plants (Supplementary Figure S6; Supplementary Table S1).

Under normal condition our analysis provided strong, quantitative evidence that AM fungi positively influenced plant biomass (shoot, root and total biomass by 32.3, 24.8, and 39.3%, respectively) and nutrient uptake (N, P, and K uptake by 17.1, 37.5, and 21.5%, respectively) in both C_3 and C_4 plants irrespective of photosynthetic types. In addition, overall AMF inoculation responses increased positively in both C_3 and C_4 plants by 23.7 and 25.3%, respectively. Furthermore, both C_3 and C_4 plants increased total biomass by 31.8 and 46.1%, respectively, relative to uninoculated controls. C_3 plants showed greater AMF mediated increase in P uptake (35.6%) and N uptake (19.9%) compared to C_4 plants P uptake (33.5%) and N uptake (16.7%), however, no statistical differences were observed between C_3 and C_4 plants. We also found that AMF inoculation increased K uptake by 21.7 and 22.8% for C_3 and C_4 plants, respectively. On the other hand, Na uptake decreased by 2.1% in C_3 plants and increased by 23.6% in C_4 plants. Mycorrhizal inoculation increased proline accumulation in C_4 plants by 19.1% and C_3 plants by 5.6% compared to noninoculated plants (Supplementary Figure S7).

C_3 vs. C_4 Plant Response to AMF Inoculation in Saline Soils

Results illustrated that overall AMF inoculation responses increased in both C_3 and C_4 plants by 42.9 and 44.5%, respectively (**Figure 2**; Supplementary Table S2). Both C_3 and C_4 plants increased total biomass under saline conditions by 69.5 and 70.8%, respectively, relative to uninoculated controls. Mycorrhizal inoculation was also found to increase shoot biomass and root biomass in C_3 and C_4 plants under saline conditions. In addition, AMF inoculation mediated increase in P uptake was observed in both C_3 (73.1%) and C_4 plants (101.4%). In C_3 and C_4 plants, N uptake increased by 77.6 and 112.3%, respectively, but due to low sample size this result needs

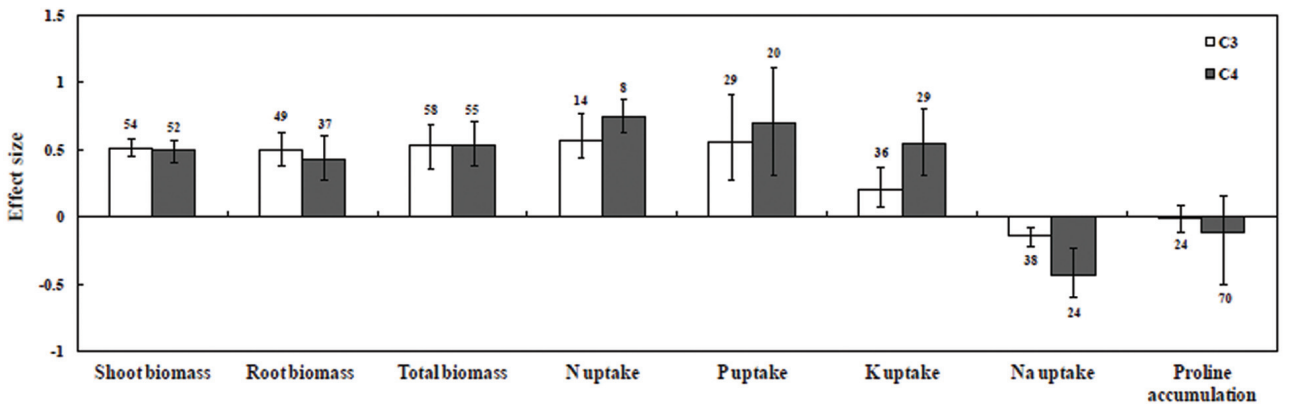

FIGURE 2 | Comparative photosynthetic growth and nutrient uptake responses of arbuscular mycorrhizal fungi (AMF)-inoculated plants under salt stress. Error bars are means ± bias CIs. Where the CIs do not overlap the horizontal dashed lines, the effect size for a parameter is significant at $P < 0.05$. A number of studies were included for the meta-analysis mentioned above the bar.

to be treated with caution. K uptake in C_4 plants increased significantly ($P \leq 0.05$) compared to C_3 plants. Na uptake decreased significantly by 13.4% in C_3 plants and 35.2% in C_4 plants. On the other hand, C_4 plants showed a 10.9% decrease in proline accumulation and C_3 plants showed a 1.6% decrease.

C_3 and C_4 Plant Response to Plant Types and Fungal Symbionts

The categorical analysis of plant types, plant groups and growth habitat had a significant effect on effect sizes (**Figure 3**). Among C_3 mycorrhizal plants, *Cicer arietinum* followed by *L. sativa*, *Lotus glaber*, *Cajanus cajan,* and *Solanum lycopersicum* had significant positive effect size. C_4 species *A. nilotica*, *Cyamopsis tetragonoloba*, *Gmelina arborea*, *A. auriculiformis,* and *Allium sativum* were found to be effective under salt stress. Moreover, C_3 plants such as *L. tenuis* had significant negative effect size on plant growth. Among C_4 plants *P. trifoliate and Spartina alterniflora* and some trials with *Zea mays* had negative responses under saline conditions. The significant positive effect size on plant types under salt stress was recorded on both monocotyledonous and dicotyledonous plant types (**Table 1**). In the analyses of data indicated in C_3 plants, monocotyledonous plants showed increased effect size whereas in C_4 plants, dicotyledonous plants showed increased effect size under salt stress conditions compared to those of monocotyledonous type (**Figure 3**). Plant type (annual vs. perennial) had a significant ($P < 0.05$) effect size as evidenced by the result that C_4 perennial plants have higher effect size, than C_3 plants. In our study we have also observed that C_4 annual plants performed better than C_3 plants under mycorrhizal condition. No significant effect size was observed in C_4 grass, in which the confidence interval overlapped with zero. Among plant functional groups, mycorrhizal C_4 herbs were found to have more effective amelioration of salt stress compared with other functional groups (**Figure 3**).

Different AM fungal inoculation types increased the growth of both plant types under saline conditions. Single and mixed AMF species inoculations differed significantly. C_3 plants showed increased effect size in single species inoculation compared with C_4 plants. On the other hand, C_4 plants showed a greater increase in effect size with mixed AMF inoculation than the C_3 plants

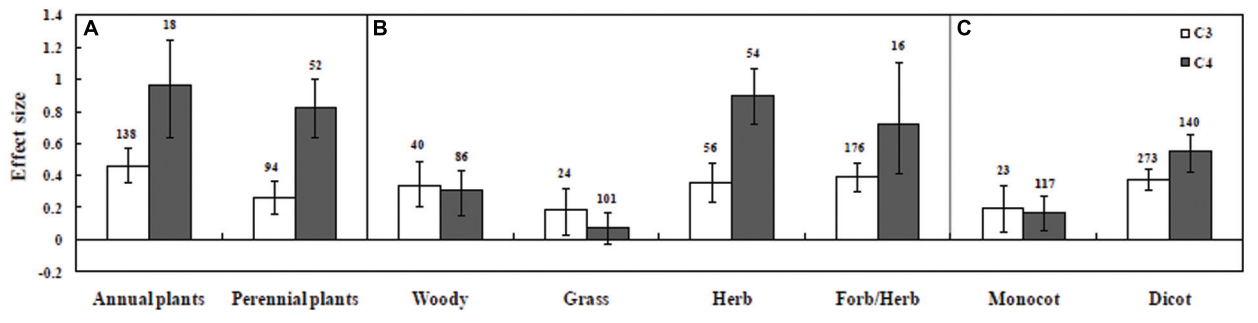

FIGURE 3 | Arbuscular mycorrhizal fungi inoculation responses of plants under salt stress. Categorical analysis of **(A)** plant duration, **(B)** plant functional groups, and **(C)** plant types. Error bars are means ± bias CIs. Where the bias CIs do not overlap the horizontal, dashed lines, the effect size for a parameter is significant at $P < 0.05$. All effect sizes differed significantly from zero. The number of studies is shown above the bar.

TABLE 1 | Comparative photosynthetic types significance analyzed in the categorical analyses based on the significance of the variation among categories (Q_B) and the amount of the total variation (Q_T) described by Q_B/Q_T under salt stress.

Categorical independent variable		Q_B	Q_B/Q_T	P_{random}
Response variable	C_3	229.8693	0.2172	0.0002
	C_4	116.8027	0.2625	0.0002
AMF species	C_3	16.7240	0.0238	0.2232
	C_4	96.9317	0.1881	0.0004
AMF inoculation	C_3	12.7338	0.0173	0.0278
	C_4	0.7907	0.003	0.4666
Plant species	C_3	222.275	0.2735	0.0002
	C_4	217.6239	0.3431	0.0002
Plant group	C_3	57.8540	0.173	0.0036
	C_4	137.3704	0.2376	0.0002
Plant family	C_3	41.2789	0.1340	0.0420
	C_4	104.0544	0.1858	0.0002
Plant types	C_3	56.0280	0.0937	0.0002
	C_4	88.2995	0.1595	0.0002
Plant growth habit	C_3	24.5270	0.0351	0.0174
	C_4	135.757	0.2359	0.0002
Experimental condition	C_3	1.0427	0.0014	0.5044
	C_4	0.1821	0.00001	0.7876
Level of salinity	C_3	61.6394	0.1190	0.0716
	C_4	155.1245	0.2393	0.0002
Degree of salinity	C_3	1.5496	0.0021	0.7272
	C_4	16.0778	0.0374	0.0050
Experimental duration	C_3	24.0317	0.0239	0.0084
	C_4	33.999	0.1604	0.0006
Soil type	C_3	32.1172	0.1552	0.0420
	C_4	25.4710	0.10043	0.0070

(**Figure 4**). Categorical analysis indicated significant ($P = 0.0002$) differences among AMF species (**Table 1**). Among the AMF species, *R. irregularis* and *F. mosseae* showed increase in effect size of C_3 species by 52.3 and 52.3%, respectively, compared with C_4 plants with a 19.2 and a 99.1% increase, respectively. Nevertheless, *R. irregularis* alone had a significant difference for C_3 and C_4 plants under salinity stress.

C_3 and C_4 Plant Response to Soil Texture, Salinity and Experimental Settings

Soil type significantly influenced the effect sizes of C_3 and C_4 datasets (**Figure 5A**). In the C_3 dataset, plants grown in sandy soil yielded higher effect size than plants grown in silty or clay loamy soil. On the other hand, in the C_4 dataset, silty soil yielded higher effect size than sandy soil. In addition, the effect size of C_3 plants was significantly increased due to mycorrhizal inoculation than it was in C_4 plants grown under salinity stress. The effect of soil salinity on the relative response to AMF inoculation is presented in **Figure 5B**. Soil salinity significantly influenced the effect size in plants ($P < 0.05$). AMF-inoculated plants grown in moderate salinity showed a significant difference between C_3 and C_4 plants, whereas effect in size variation between C_3 and C_4 plants under low and high salinity levels were not significant. AMF-inoculated plants grown in moderate salinity yielded higher effect size for C_3 plants compared with those of C_4 plants (**Figure 5B**).

In our dataset, experimental duration significantly influenced the effect size in both C_3 and C_4 plants. AMF inoculation in short-duration C_4 plants had a significant effect on plant growth compared with that of C_3 plants. On the other hand, C_3 species showed a greater increase in effect size of intermediate duration (**Table 1**). Experimental conditions had a significant effect on the C_3 dataset where plants grown under controlled-greenhouse conditions had higher effect size values compared to that of the C_4 dataset (**Table 1**). However, C_4 plants showed a greater increase in effect size under field conditions when compared to the C_3 plants.

C_3 and C_4 Plant Response to Biomass

Among plant biomass studies, AMF inoculation had a significant positive effect (67.11%, $P < 0.05$) on shoot biomass whereas no significant ($P > 0.05$) effects were detected in root and total biomass under saline condition (**Figure 2**; Supplementary Table S2). Categorical analysis of shoot biomass showed that among AMF species, *R. irregularis* followed by *F. mosseae* were found to be effective with C_3 plants. On the other hand *F. mosseae*, followed by *R. fasciculatus* and *R. irregularis* were effective with C_4 plants.

C_3 and C_4 Plant Response to P, N, and K Uptake

Among all nutrient studies included for meta-analysis, P uptake (86.8%) showed a significant positive response for all types of categorically fixed factors. A significant effect on P uptake was found in this study (Supplementary Figure S6; Supplementary Table S2). Although Kendall's tau and Spearman rank correlation test for publication bias was found to be significant, this was ignored based on Rosenthal Fail-safe calculation (Supplementary Table S2). Categorical analysis of P uptake among AMF species was found to have a significant positive effect under salt stress ($P = 0.02$). Among AMF species, C_4 plants inoculated with *R. fasciculatus* had a more significant positive effect than *R. irregularis* and *F. mosseae,* whereas an opposite trend was observed in C_3 species. Across the study, single inoculants recorded higher P uptake than mixed inoculants in both C_3 and C_4 species ($P > 0.05$). In addition, there were significant differences among plant species for P uptake under salt stress ($P = 0.01$). Among C_4 plant species, *A. nilotica* and *C. tetragonoloba* were found to be efficient for P uptake, whereas among C_3 plant species, *Trifolium subterraneum*, *C. arietinum,* and *S. lycopersicum* had larger effect size. The significant positive effects of AMF inoculation on P uptake under salt stress were recorded, and there were higher levels with dicotyledonous plants than with monocotyledonous plants. Among the plant photosynthetic types, though, P uptake had a positive effect on size; their effect on mycorrhizal inoculation did not differ between C_3 and C_4 plants (**Figure 2**; Supplementary Table S2). Furthermore, categorical analyses indicated that there was no significant difference between the effect sizes for P uptake in plants with response to varied degrees of salinity. However,

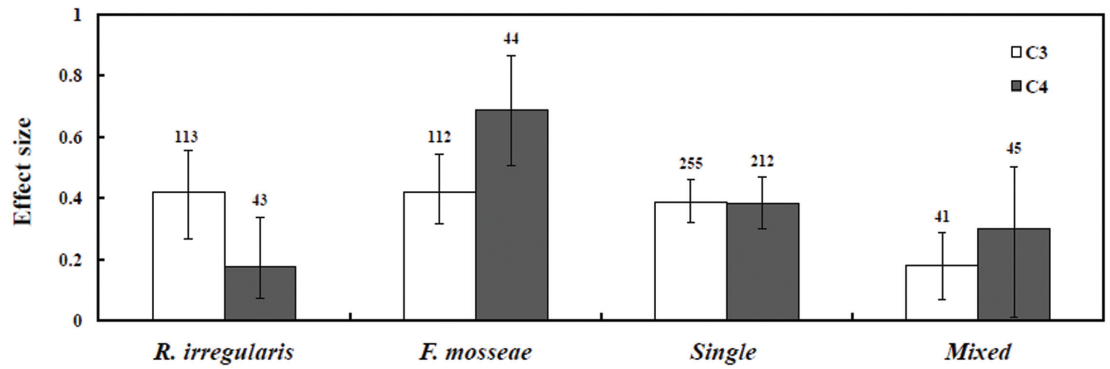

FIGURE 4 | Effect sizes of AMF species-categorical analysis. Error bars are means bias CIs. Where the CIs do not overlap the horizontal dashed lines, the effect size for the parameter is significant at $P < 0.05$. A number of studies included for meta-analysis are mentioned above the bar.

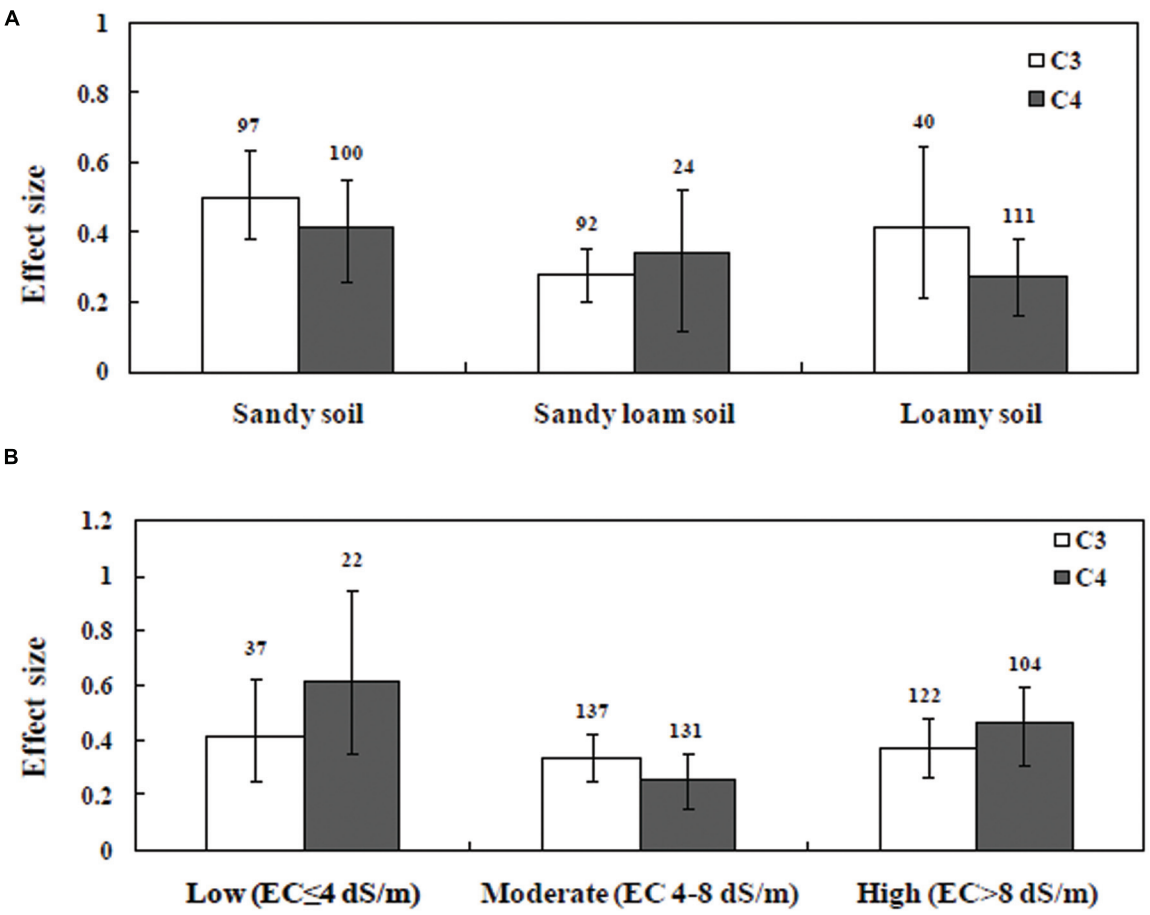

FIGURE 5 | Arbuscular mycorrhizal fungi inoculation responses of plant under salt stress. Categorical analysis of **(A)** soil types **(B)** degree of salinity. Error bars are means ± bias CIs. Where the bias CIs do not overlap the horizontal dashed lines, the effect size for a parameter is significant at $P < 0.05$. The number of studies included for the meta-analysis is mentioned above the bar.

at a moderate degree of salinity, P uptake was higher when compared with those at high and low levels of salinity. There was no significant difference observed between experimental duration trials. Short-duration studies showed a higher effect size in P uptake compared with moderate- and long-duration studies under salt stress.

In this study, it was observed that the N uptake (93.2%) had a positive effect size for AMF inoculation under salt stress

conditions (Supplementary Figure S6; Supplementary Table S2). Significant publication bias observed in Kendall's tau and the Spearman rank correlation was safely ignored (Supplementary Table S2). Categorical analysis among groups was not significant, except for plant functional groups and plant species, indicating inconsistencies and low sample sizes in the mycorrhizal plants in response to N uptake under salt stress. Among plant photosynthetic types, N uptake increased positively in C_3 and C_4 plants, but C_4 plants had increased effect size (**Figure 2**).

K uptake showed variation among studies, but it was consistently significant in AMF inoculated C_3 and C_4 plants (Supplementary Figures S6 and S8; Supplementary Table S2). Categorical analyses showed significant differences between AMF species. In C_4 plants, *R. fasciculatus* showed a greater increase in K uptake than *F. mosseae* and *R. irregularis*. However, in C_3 species, *F. mosseae* followed by *R. irregularis* was found to be effective. Categorical analysis of plant species also showed a significant positive effect ($P = 0.0002$) on K uptake under saline conditions. K uptake in C_4 plants was found to be significantly greater than those of C_3 plants (**Figure 2**). Among C_4 species, increased K uptake was observed in *A. nilotica* (due to low sample size, treat the results with caution) followed by *Z. mays,* while in C_3 species, *S. lycopersicum* was found to be more effective for K uptake. When compared among all plant functional groups ($P = 0.0002$), K uptake in herbs was significantly greater than those of tree and grass types. In addition, the K uptake in a low and high degree of salinity was significantly higher than moderate salinity across studies.

C_3 and C_4 Plant Response to Na Uptake and Proline Accumulation

In contrast to N, P, and K uptake, mycorrhizal plants had a consistent negative response to Na uptake by an average of 22.2% across all studies. Furthermore, variations among studies were significant ($P < 0.05$, Supplementary Figure S6; Supplementary Table S2), indicating consistency among groups with mycorrhizal responses to salt stress. Among AMF species, *R. irregularis* was found to have a negative impact on C_4 plants. On the other hand, *F. mosseae* showed a negative effect size in C_3 plants under salt stress. Categorical analysis indicates that plant species ($P = 0.001$) and plant duration ($P = 0.02$) contributed significantly to Na uptake. Among the plant types, woody plants were found to have more negative impact for Na uptake, but bias CI overlapped with zero. Grasses were significantly negative for Na uptake in AMF inoculated plants. Among plant photosynthetic type, C_4 plants were found to be more negative for Na uptake than those of C_3 plants. Among plant species, Na uptake for *S. lycopersicum* was found to be more negative in C_3 plants, whereas *Z. mays* had a more negative size in C_4 plants. Moreover, a negative effect for Na uptake was comparatively greater in monocotyledonous plants than in dicotyledonous plants.

Both increases and decreases in proline accumulation under salt stress have been ascribed to AM symbiosis. The present study showed that proline accumulation has been found to decrease when the plant is colonized by AMF (6.5%), but this effect was not significant since the confidence intervals overlapped with zero (Supplementary Figure S6; Supplementary Table S2). However, categorical analysis of AMF species showed that in both C_3 and C_4 plants, *R. irregularis* and *F. mosseae* inoculated plants had more reduced level of proline accumulation but, *R. fasciculatus* inoculated plants had increased proline accumulation under salt stress. Within the categorical analysis of C_3 mycorrhizal plants, increased proline content compared to non-mycorrhizal plants under saline condition was observed in *M. arvensis*, *C. arietinum,* and *L. glaber* plants. However, within C_4 mycorrhizal plants under saline condition, significant positive effect on proline accumulation was seen in *G. arborea*, *Jatropha curcas,* and *A. sativum* plants, but *Z. mays* and *Pennisetum glaucum* showed significant negative effects on proline accumulation. Among plant functional group, C_4 monocotyledonous plants had reduced level of proline accumulation, whereas C_3 dicotyledonous plants had significant increase in proline accumulation than those of non-mycorrhizal plants. All of the other plant functional groups and experimental conditions showed no significant differences in proline accumulation under salt stress.

DISCUSSION

AMF Symbiotic Efficiency of C_3 and C_4 Plants in Saline Soils

Plant growth diminishes under salt stress due to (a) spending of more energy to avoid the toxic effects of NaCl and (b) deficiency in nutrients (Munns and Tester, 2008). On the other hand, mycorrhizal inoculation was found to enhance the efficiency of the host plants by increasing their growth and photosynthetic efficiency (Chandrasekaran et al., 2014; Elhindi et al., 2016; Shamshiri and Fattahi, 2016). In the present study, we have observed that the overall AMF inoculation response increased in both C_3 and C_4 plants, and the total plant biomass was found to be enhanced in both C_3 and C_4 plants. Similar results were also observed for both C_3 and C_4 plants under normal condition. Previous meta-analyses have also documented enhanced plant growth due to AMF inoculation (Hoeksema et al., 2010; Treseder, 2013; Pellegrino et al., 2015). Resolute evidence was also provided for AMF species causing a significant impact on many ecological predictor variables on C_3 and C_4 photosynthetic plant types under salinity stress. Thus far, studies on salt-stress tolerance in mycorrhizal plants have suggested that AMF inoculated plants grew better due to improved mineral nutrition and physiological processes such as photosynthesis or water use efficiency, production of osmoregulators, higher K^+/Na^+ ratios and compartmentalization of sodium within some plant tissues (Giri et al., 2007; Elhindi et al., 2016). The saline-stress-alleviation effect on C_3 and C_4 plants may be attributed to enhanced mycorrhizal growth response. In a saline environment, root growth is delayed with the effect of salt on cell toxicity and due to low soil water potential (Psarras et al., 2008). An increase in salt concentrations proportionately increases the mycorrhizal response in *Sesbania* and *Gmelina* plants (Giri and Mukerji, 2004; Dudhane et al., 2011). We also observed salinity-level variations in the mycorrhizal symbiosis of C_3 and C_4 plants.

AMF-inoculated plants grown in moderate salinity yielded a higher effect size for C_3 plants than those of C_4 plants.

Nutrient (s) Mediated Salt Stress Tolerance in C_3 and C_4 Mycorrhizal Plants

Overall meta-analysis showed a significant ($P < 0.05$) increase in mean-effect sizes in mycorrhizal plants in all salinity levels, indicating the fact that AMF inoculation increased P uptake. The ability of AM fungal to acquire P is known to differ among isolates, and a similar result is expected for other functions as well (Hoeksema et al., 2010; Treseder, 2013). C_4 plants inoculated with *R. fasciculatus* showed a more positive effect than *R. irregularis*, and *F. mosseae,* whereas the opposite trend was observed in C_3 species. Therefore, our results suggest that environment (i.e., the level of salinity) restricted an association to a single species. A higher response to P uptake was observed in the Fabaceae family for both C_3 and C_4 plants, indicating many trends common to both photosynthetic types. In ways similar to our study, Treseder (2013) provided meta-analytical evidence for a positive, significant relationship between per cent root colonization and plant biomass due to high P uptake.

In our analysis, we found that among plant photosynthetic types, mycorrhizal C_4 plants had a higher N uptake than C_3 plants, but due to low sample size this result needs to be treated with caution. This could be explained based on the C_4 mode of photosynthesis that had indirect consequences on the use of water by these plants (Ehleringer and Monson, 1993). Improved efficiency in nitrogen use during photosynthesis has been suggested to allow C_4 plants to develop a higher leaf area for effective carboxylation and the translocation of photosynthates toward roots under nitrogen-limited conditions, and this favored the increase in mycorrhizal colonization for nutrient acquisition. However, to the best of our knowledge, the exact mechanism for why AMF enhances N nutrition under salt-stress conditions is yet to be understood.

K plays a number of essential roles in plants, including enzyme activation, protein synthesis, photosynthesis, osmoregulation, stomatal regulation, energy transfer, phloem transport, cation-anion balance, and stress resistance (Wang et al., 2013). Salinity stress decreases the level of K^+ as a competitor of Na^+. Because, Na^+ and K^+ have similar physiological properties, cytoplasmic Na^+ fights for similar binding sites and hence limits the metabolic processes that depend on K^+ (Shabala and Cuin, 2008). Mycorrhizal inoculation can enhance the K absorption under saline conditions and prevent the translocation of Na. Thus, increasing the K^+/Na^+ ratio by the AMF inoculation under high salinity levels could be one of the reasons for enhancing plant tolerance to the highest salinity level through Na exclusion (Tang et al., 2006; Giri et al., 2007; Elhindi et al., 2016). In the current study, we have seen that K uptake by C_3 and C_4 plants were positively influenced by the AMF inoculation, and K uptake was significantly higher in C_4 plants. Salt-stressed root growth is restricted by the osmotic and toxic effects of ions, which result in lower nutrient uptake and inhibition of the translocation of mineral nutrients, especially K^+ (Wang et al., 2013). Therefore, we suggest that the inoculation enhancing exploration of the external mycorrhizal hyphae beyond the nutrient-depleted zones can increase P and K uptake in both plant types.

C_3 and C_4 Plant Species Responding to AMF Symbiosis

The present study showed that C_4 plants had more significant increase in P uptake efficiency compared with C_3 plants under salt stress. This result may be attributed to the fact that C_4 plants seem to be highly benefited from mycorrhizal inoculation than the selected C_3 plants due to the preferential P allocation for photosynthesis in those C_4 plants (Tang et al., 2006). Under high-salinity stress, enhanced *de novo* biosynthesis of CO_2 concentrating phosphoenolpyruvate carboxylase (PEPC) enzyme in C_4 plants requires more P for their additional ATP requirement (Doubnerova and Ryslava, 2011). AMF inoculation can help plants meet out their P demand for improving their survival with salt stress (Doubnerova and Ryslava, 2011). The retention of P within tissues of C_4 plants could elicit higher total P content within the plant (Kishor et al., 2005). Therefore, even a little increase in nutrient uptake via AMF may contribute to a larger increase in plant P content (Maherali and Klironomos, 2012).

The present analysis reveals that C_4 plant species showed significantly better response to mycorrhizal inoculation particularly for K uptake. Among C_4 plants, *Z. mays* was found to be more competitive for K^+ uptake. Automatic exclusion of Na^+ ions in the soil could be achieved by C_4 plants in saline soils (Omoto et al., 2012). This was again reflected by a decrease in proline accumulation in C_4 compared to C_3 plants under salinity stress (Omoto et al., 2012). AMF inoculated plants showed a significantly higher K and lower Na uptake than the uninoculated ones under salinity stress, which suggests a preferential intake of K^+ rather than of Na^+ into the root xylem. Therefore, the enhancement of growth in mycorrhizal plants under saline conditions could be attributed to decreased Na^+ uptake, with enhanced K uptake resulting in a higher K^+/Na^+ ratio (Elhindi et al., 2016).

The investigations carried out so far on proline accumulation in AMF symbiosis are limited, and contradictory (Kishor et al., 2005). Proline accumulation was found to increase when the plant was colonized with AMF (Maiale et al., 2004; Echeverria et al., 2013). Some studies demonstrated that AMF inoculation significantly decreased proline accumulation (Jahromi et al., 2008; Borde et al., 2011), and several other studies also showed little or no effect on proline accumulation (Hajiboland et al., 2010). Across the studies, we could find variations in proline accumulation among *Glomus* spp. The inoculation of *R. fasciculatus* and *Glomus* sp. increased proline accumulation, whereas a decrease in proline accumulation was observed in *R. irregularis* and *F. mosseae* inoculated C_3 and C_4 plants. In addition, C_3 plant species (*M. arvensis* and *C. arietinum*) and some of the C_4 plant species (*G. arborea, J. curca,* and *A. sativum*) showed a significant increase in proline accumulation, whereas some of the C_4 plant species (*Z. mays* and *P. glaucum* and) and C_3 plant species (*Trigonella foenum* and *S. lycopersicum*)

had a significant decrease in proline accumulation. The low accumulation of proline in AMF-inoculated C_4 plants may be attributed to their inherent higher salinity stress tolerance than the high-proline accumulation in stressed C_3 plants. An increase or a decrease in plant proline was also found to be directly proportional to Na uptake; as decreased Na uptake in C_4 plants decreased proline accumulation. In addition to this, mycorrhizal inoculation had no effect on proline accumulation in C_3 plants. Earlier research studies also support the present quantitative analytical finding that the proline accumulation response to salt stress is a good indicator of stress perception among plant species with regards to their saline tolerance ability (Evelin et al., 2009; Garg and Chandel, 2012).

CONCLUSION

With quantitative analytical evidence, we suggest that AMF inoculation positively influences plant growth and nutrient uptake in both C_3 and C_4 plant types in saline soils. Among AMF species, *F. mosseae* was found to be well suited for C_3 plants, whereas *G. fasciculatum* showed a high growth response with the C_4 plants under salinity stress. Mycorrhizal inoculation in both C_3 and C_4 plants under salinity stress exhibited an intriguing pattern of responses, where higher effectiveness of AMF inoculation was observed under low and high salinity levels than under moderate levels. But, significantly high symbiotic efficiency was observed in C_3 mycorrhizal plants under moderate levels of salinity. Our study also described that under mycorrhizal conditions, C_3 annual plants performed significantly higher than C_4 plants, with an exception in perennial plants. Future research on mycorrhizal symbioses with C_3 perennial plants and C_4 annual plants can answer several questions that arose from the present analysis and may lead to progress in symbiotic efficiency of mycorrhizal fungi under salinity stress. Among the two plant photosynthetic types, C_4 plants showed more competitive K^+ ion uptake than C_3 plants. Salt-stressed, AMF-inoculated C_4 plants exhibited higher K^+/Na^+ ratio than those of salt stressed C_3 plants and non-mycorrhizal plants. Studies on the detailed mechanism for the selective transport of K in C_3 and C_4 mycorrhizal plants under salt stress are lacking, and this needs to be explored. Our study concludes that AMF inoculation had a positive effect on plant growth and nutrient uptake in both C_3 and C_4 plants grown under salinity stress contrary to current perceptions.

AUTHOR CONTRIBUTIONS

MC and TS: Conception and design of the work. MC: Performed the work. MC, KK, RK, and DW: acquisition of data. MC, SS, SH, S-HO, and TS: analyzed the data. MC, KK, RK, SS, SH, MJ, S-HO, GS, and TS: critical revision of manuscript. MC, MJ, GS, and TS: wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01246

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Reactive Oxygen Species Generation-Scavenging and Signaling during Plant-Arbuscular Mycorrhizal and *Piriformospora indica* Interaction under Stress Condition

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A defined balance between the generation and scavenging of reactive oxygen species (ROS) is essential to utilize ROS as an adaptive defense response of plants under biotic and abiotic stress conditions. Moreover, ROS are not only a major determinant of stress response but also act as signaling molecule that regulates various cellular processes including plant-microbe interaction. In particular, rhizosphere constitutes the biologically dynamic zone for plant–microbe interactions which forms a mutual link leading to reciprocal signaling in both the partners. Among plant–microbe interactions, symbiotic associations of arbuscular mycorrhizal fungi (AMF) and arbuscular mycorrhizal-like fungus especially *Piriformospora indica* with plants are well known to improve plant growth by alleviating the stress-impacts and consequently enhance the plant fitness. AMF and *P. indica* colonization mainly enhances ROS-metabolism, maintains ROS-homeostasis, and thereby averts higher ROS-level accrued inhibition in plant cellular processes and plant growth and survival under stressful environments. This article summarizes the major outcomes of the recent reports on the ROS-generation, scavenging and signaling in biotic-abiotic stressed plants with AMF and *P. indica* colonization. Overall, a detailed exploration of ROS-signature kinetics during plant-AMF/*P. indica* interaction can help in designing innovative strategies for improving plant health and productivity under stress conditions.

Keywords: plant root, ROS-metabolism, ROS-signaling, stress, arbuscular mycorrhizal fungi

INTRODUCTION

Plant–microbe interactions cover a broad range of relationships between plant and microbial community in which either of the partners participate by imposing a beneficial, negative or neutral effect on its counterpart. Moreover, plant roots are continuously exposed to a large number of microbes present in the rhizosphere that influence plant life cycle and overall fitness

(Sanders, 2011; Mine et al., 2014). Plant–microbe symbiotic interactions have been the focus of recent plant stress research, where the outcomes of these interactions were credibly evidenced to alleviate biotic and abiotic stress-impacts and consequently enhance the plant fitness (Goh et al., 2013; Schouteden et al., 2015; Doty, 2016). In the present scenario, a relatively small number of beneficial plant-microbe interactions are well characterized and utilized (Farrar et al., 2014). Microbial counterpart- arbuscular mycorrhizal fungi (AMF)-mediated stress tolerance and growth enhancements have been extensively reported in colonized host plants during symbiotic interaction studies (Muthukumar and Udaiyan, 2010; Porcel et al., 2012; Tahat and Sijam, 2012). Notably, a number of recent works have discussed the significance of *Piriformospora indica,* a arbuscular mycorrhizal-like fungi which is able to grow in pure culture and without the presence of the plant. *P. indica*, a multifunctional and versatile root endophytic fungus belongs to Sebacinales (order-Basidiomycota) and is involved in the improvement of growth, yield, and plant tolerance to major biotic and abiotic stresses (Sherameti et al., 2008; Vadassery et al., 2009a,b; Cruz et al., 2013; Jogawat et al., 2013; Prasad et al., 2013; Bakshi et al., 2014; Johnson et al., 2014; Gill et al., 2016; Trivedi et al., 2016). Both fungal counterparts viz., AMF and *P. indica* are capable of improving plant fitness via changing mainly the chemical plasticity through altering reactive oxygen species (ROS) generation-scavenging under biotic and abiotic stresses (Beneventi et al., 2013; Goh et al., 2013; Hashem et al., 2016; Mo et al., 2016). ROS can be both radical and non-radical forms and generated in normal metabolic processes e.g., as a result of electron transport chains in chloroplast and mitochondria. However, adverse conditions including abiotic and biotic stresses can significantly accelerate the generation of ROS at cellular level (Apel and Hirt, 2004; Gill and Tuteja, 2010; Rasool et al., 2013). Radical forms of ROS majorly include superoxide radicals ($O_2^{\bullet -}$), perhydroxy radical ($HO_2^{\bullet}$) and alkoxy radicals (RO); whereas, hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) are included in non-radical molecular form. Compared with non-radicals, radical forms of ROS are more toxic due to their highly reactive nature (Gill and Tuteja, 2010; Sewelam et al., 2016). In plants, enzymatic and non-enzymatic systems are the two major components of ROS-scavenging system. The list of major enzymatic components includes superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), guaiacol peroxidase (GPX), glutathione reductase (GR), peroxidase (POD), and catalase (CAT). Major antioxidant metabolites namely glutathione (GSH) and ascorbic acid (AsA) belong to the list of non-enzymatic component (Apel and Hirt, 2004; Gill and Tuteja, 2010; Rasool et al., 2013). Notably, NADPH oxidases and respiratory burst oxidase homologs are the key components of ROS generation system in plants (Suzuki et al., 2013; Kadota et al., 2015).

In order to alert the plants for stress-adaptation, initial generation of ROS was reported to act as long distance signals in response to stress (Mittler et al., 2011; Sewelam et al., 2016). Furthermore, ROS are also thought to be generated during early stages of symbiotic interactions of mycorrhizal fungi associated with plant roots (Fester and Hause, 2005; Tanaka et al., 2006; Puppo et al., 2013; Espinosa et al., 2014; Kiirika et al., 2014). Though, to efficiently utilize ROS as signaling molecule, plants must sustain a precise balance between ROS generation and ROS-scavenging pathways in order to finally mitigate the potential toxic effects of ROS (Mittler et al., 2004; Baxter et al., 2014). In plants, stress signals include redox homeostasis, antioxidants signaling and continuous production/scavenging of ROS at cellular level (Bose et al., 2014; Jajic et al., 2015). However, severity or prolonged duration of biotic and abiotic stresses can reduce the capability of plant to neutralize excess ROS production that alternatively cause oxidative stress and finally affect cellular essential metabolic activities and viability (Gill and Tuteja, 2010; Barna et al., 2012; Nath et al., 2016).

Despite the previous facts, literature is scanty on how the generation, signaling and metabolism of ROS can be modulated in plants with AMF/*P. indica* association under stress conditions. Hence, this paper aims to briefly appraise ROS accumulation, homeostasis, and signaling during plant-AMF and *P. indica* interaction in response to major stress conditions.

ROS GENERATION AND SCAVENGING DURING PLANT-ARBUSCULAR MYCORRHIZAL INTERACTION UNDER STRESS CONDITIONS

Reactive oxygen species profiling in AMF-inoculated roots of several plants including *Medicago truncatula, Zea mays,* and *Nicotiana tabacum* has evidenced important role of mycorrhizal colonization/arbuscules in the scavenging of major ROS such as H_2O_2 (Fester and Hause, 2005). AMF-colonization improved drought tolerance in olive plants, where compared to non-colonized olive plants, AMF-colonized plants exhibited lesser accumulation of ROS (H_2O_2) and malondialdehyde (MDA), a lipid peroxidation product (Fouad et al., 2014). Similar results were also reported in other test plants including date palm (Benhiba et al., 2015) and *Citrus reticulata* (Sarkar et al., 2016), where improved drought tolerance was dedicated to AMF-mediated improvements in the antioxidant defense of host plants and alleviate drought stress-effects. A recent report also confirmed the role of AMF (*Glomus versiforme*) colonization in the enhancement of ROS-metabolism via its modulatory role in the activities of antioxidant enzymes including SOD, CAT, APX, GR, and MDHAR in drought stressed water melon plants (Mo et al., 2016). AMFs colonization-mediated strengthening of antioxidants defense systems was advocated to control ROS-metabolism and eventually alleviate oxidative stress in host plants under stress conditions (Peterson et al., 2004; Vos et al., 2013; Wu et al., 2014; Hashem et al., 2016). Involvement of ROS generation was also suggested in providing resistance in soybean against nematode (*Meloidogyne javanica*) infection (Beneventi et al., 2013). In mycorrhizal tomato roots, reduction of infection caused by root-knot nematode (*M. incognita)* was linked with ROS

metabolism (Vos et al., 2013). Enhanced activities of major antioxidant enzymes including SOD, CAT, POD, GR, and APX were argued to improve cadmium (Cd)-tolerance in tomato via AMF-mediated ROS-scavenging (Hashem et al., 2016). **Table 1** summarizes representative studies highlighting plant-AMF/AMF-like (*P. indica*) interaction and its link with ROS metabolism in response to various biotic and abiotic stress conditions.

LINK OF ROS SIGNALING WITH STRESS TOLERANCE DURING PLANT-ARBUSCULAR MYCORRHIZAL ASSOCIATION

In order to adapt with various biotic and abiotic stresses, plants are endowed with a highly complicated and elaborated signaling cascade. In response to stress conditions, plants utilize ROS as one of the key signaling players which also activate other defense related signaling pathways (Baxter et al., 2014; Xu and Brosche, 2014; Sewelam et al., 2016). Transcriptome analysis of *Glomus mosseae/Medicago sativa* during herbicide (atrazine) stress revealed higher stress tolerance via increased expression of electron transport related genes, ROS-scavenging antioxidants such as thioredoxin, glutaredoxin, and GPX. Additionally, a higher degradation of atrazine was also observed in mycorrhizal (*G. mosseae*)-treated *M. sativa* plants (versus non-treated plants), further corroborated its link with stress mitigation (Song et al., 2016).

Increasing evidences revealed that ROS-generation is one of the most frequent responses triggered in plants that represent a general point for different signaling cascades under stress (Sewelam et al., 2016). ROS generation is also one of the characteristics of the early host-defense system during initial microbial invasion with host plants and can also lead to the hypersensitive reaction and cell death at the site of interaction (Puppo et al., 2013). However, detailed reports on ROS signature kinetics are still very limited during initial stages of microbial interaction with plant. A transient increase of ROS was observed

TABLE 1 | Representative studies highlighting AMF/*P. indica* mediated-stress tolerance associated with the metabolism of reactive oxygen species (ROS) in different plants.

Name of the interacting fungi	Plant	Stress tolerance	ROS metabolism in colonized plants	*Potential stress tolerance-mechanism	Reference
Glomus mosseae	*Solanum lycopersicum* (Tomato)	Cadmium (Cd) stress	Increased level of SOD, CAT, POD, GR, and APX	AMF-mediated ROS scavenging	Hashem et al., 2016
G. mosseae	*Medicago sativa* (alfalfa)	Atrazine (Herbicide) stress	High level of thioredoxin, glutaredoxin and GPX	High GPX activity may link with alleviation of atrazine stress	Song et al., 2016
Piriformospora indica	*Hordeum vulgare* (Barley)	Salt stress	High antioxidant activities and glutathione-ascorbate cycle activation	Stress tolerance link with increase in antioxidants	Waller et al., 2005; Baltruschat et al., 2008
		Biotic stress (*Fusarium culmorum*)	Increased antioxidants	Stress tolerance link with increase in ROS metabolism	Waller et al., 2005
	Zea mays (Maize)	Biotic stress (*Fusarium verticillioides)*	High antioxidant enzymatic activities	High antioxidants proposed to link with stress tolerance	Kumar et al., 2009
P. indica and *Azotobacter chroococcum* (Co-inoculation)	*Triticum aestivum* (Wheat)	Zinc stress	High APX and peroxidase activity	Induced antioxidant activities	Abadi and Sepehri, 2016
Rhizophagus manihotis and Funneliformis mosseae	*Olea europaea* (Olive)	Drought stress	Low H_2O_2 in AMF-colonized plants	Low H_2O_2 level correlated with drought tolerance	Fouad et al., 2014
R. intraradices and F. mosseae	*Phoenix dactylifera* (Date Palm)	Drought stress	High antioxidant-enzymatic activities	Antioxidant defense system alleviates long term drought stress.	Benhiba et al., 2015
Glomus sps.	*Citrus reticulate* (Mandarin orange)	Drought stress	High antioxidant-enzymatic activities	Increased antioxidant defense system link with oxidative stress tolerance	Sarkar et al., 2016
G. mosseae	*S. lycopersicum* (Tomato)	biotic stress (*Meloidogyne incognita*)	Reduction of root-knot nematode infection	Involvement of ROS metabolism with reduction of the nematode infection	Vos et al., 2013

**ROS may be one of the associated mechanisms or it may likely to have link with stress tolerance.*

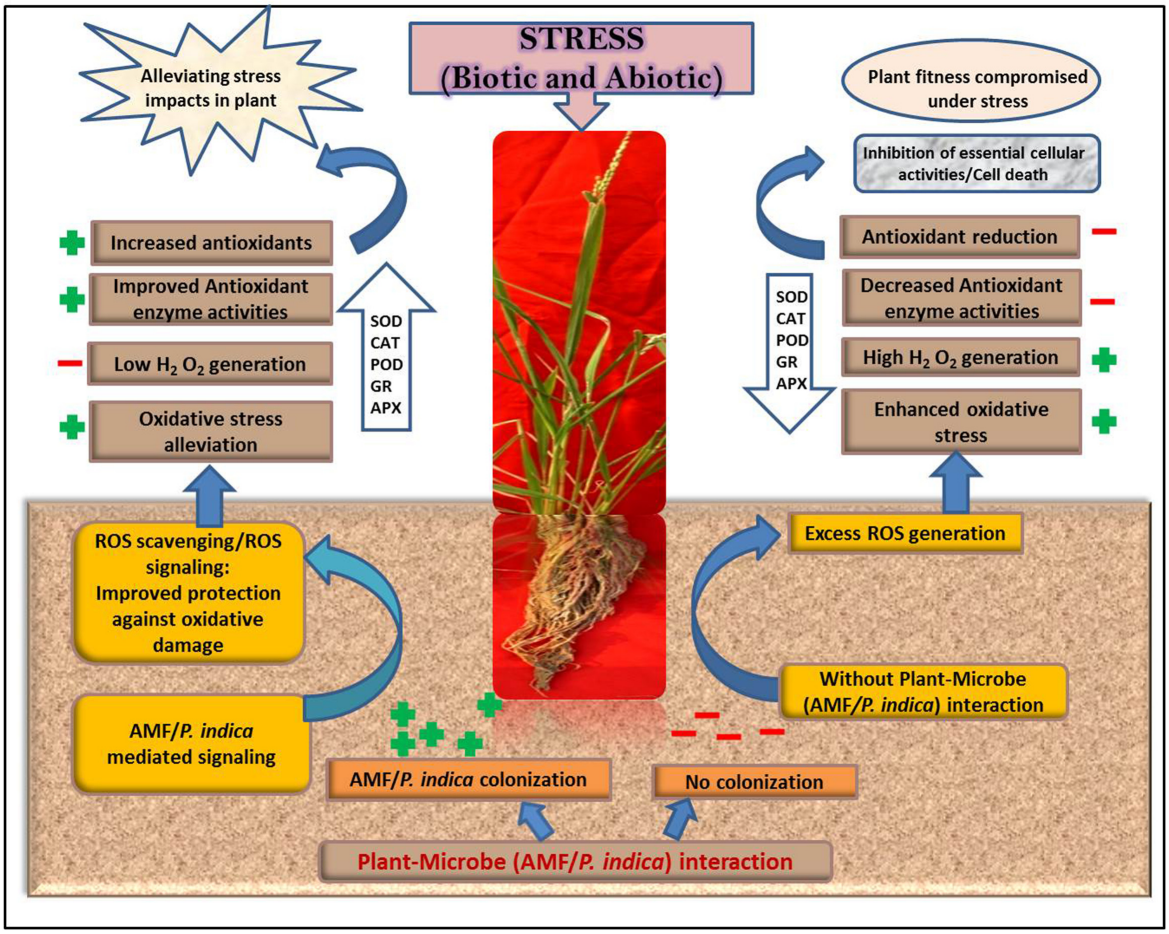

FIGURE 1 | Schematic representation of reactive oxygen species (ROS) generation and signaling during plant- arbuscular mycorrhizal fungi (AMF)/*Piriformospora indica* interaction in response to stress. The left panel of the figure indicates the ROS generation and signaling in presence of AMF/*P. indica* interaction, while the right panel demonstrates high ROS in absence of mycorrhizal interaction. AMF/*P. indica* colonization in plant roots can help the plant to cop the detrimental effect of stress, directly or indirectly, on plant functionality and metabolism. Altered ROS signaling/metabolism, in response to biotic and abiotic stress, link with stress tolerance in mycorrhizal colonized plants consequently provides stress tolerance; while, the scenario is just reverse in case of non-colonized plants i.e., high ROS production followed by the inhibition of plant cellular activities thus affecting the plant fitness. AMF/*P. indica* colonized plants were able to withstand stress induced damage by increasing the production of various antioxidant compounds, which helps to scavenge ROS and thus in turn enhance the activities of various antioxidant enzymes as listed inside the arrow. The positive (+) and negative (−) sign in figure denotes an increased and decreased levels, respectively. Superoxide dismutase (SOD), Catalase (CAT). Peroxidase (POD), Glutathione reductase (GR), and Ascorbate peroxidase (APX).

within seconds in root hairs of *Phaseolus vulgaris* after treatment with Nod factors (NFs), where specific role of ROS response during symbiotic association was proposed (Cardenas and Quinto, 2008). Moreover, among ROS, H_2O_2 is membrane-permeable and plays an important role in signaling cascade as well as in defense response under stressful environments (Xia et al., 2009; Saxena et al., 2016). Thus, H_2O_2 has emerged as an active signaling player which is also involved in regulation of specific biological reactions/cellular metabolism and stress tolerance (Neill et al., 2002; Yan et al., 2007; Saxena et al., 2016). In *M. truncatula–Sinorhizobium meliloti,* exogenously supplied H_2O_2 was associated with induced *MtSpk1* gene (encoding a putative protein kinase) and also its conformed functional role was argued in the control of genes linked to rhizobia symbiosis (Andrio et al., 2013).

ROS MODULATION DURING INTERACTION OF PLANTS AND *P. indica* UNDER STRESS CONDITION

Piriformospora indica mediated stress tolerance has been credibly reported in various crops including barley (Waller et al., 2005; Deshmukh and Kogel, 2007), wheat (Serfling et al., 2007), maize (Kumar et al., 2009), tomato (Sarma et al., 2011), and lentil (Dolatabadi et al., 2012). In rhizosphere, *P. indica* was reported to enhance the levels of alkaline phosphatase and acid phosphatase enzymes that in turn contributes for higher phosphate uptake in plants (Das et al., 2014). However, information is still meager on the relation of *P. indica* with the status of ROS in plants with mycorrhizal association. Nevertheless, the control of

ROS generation and the modulation of major components of antioxidant defense pathway were argued as a key mechanism underlying *P. indica* mediated improved stress tolerance in wheat, barley and maize (Waller et al., 2005; Serfling et al., 2007; Kumar et al., 2009). In plant roots, ROS generation and activation of defense related responses was reported during initial mycorrhizal associations (Pozo and Azcón-Aguilar, 2007). Notably, the generation of ROS was initially observed before physical contact of *P. indica* with plant roots and no H_2O_2 was reported after establishment of symbiotic relationship between *P. indica* and plant root (Vadassery et al., 2009a; Camehl et al., 2011; Vahabi et al., 2015).

H_2O_2 was found to induce *OXI1* (*Oxidative Signal Inducible1*) gene which consequently triggers defense response during pathogen infection (Rentel et al., 2004; Anthony et al., 2006; Petersen et al., 2009). In *Arabidopsis* roots, *OXI1* (a serine/threonine kinase) was shown to be required for oxidative burst/ROS-mediated responses including root hair elongation and disease tolerance against biotrophic pathogens (Rentel et al., 2004; Petersen et al., 2009). Though, under favorable co-cultivation conditions, H_2O_2 generation was repressed in *P. indica*-colonized *Arabidopsis* roots while stimulation of growth response via *P. indica* involved PLD-PDK1-OXI1 cascade in *Arabidopsis* (Camehl et al., 2011). Activation of the GSH-AsA cycle followed by increased antioxidant capacity was reported in *P. indica* colonized barley root (Waller et al., 2005). *P. indica*-mediated enhancement of antioxidants was reported to link with salt stress tolerance in the colonized barley plants (Baltruschat et al., 2008). Microbe derived effectors delivered during plant-mycorrhizal association can enhance the microbial infections and also manipulate the host metabolism. Recently, a study demonstrated that the expression of candidate effector (PIIN_08944) of *P. indica* was found to decrease the ROS burst activated by flg22 and chitin in barley (Akum et al., 2015). Co-inoculation of *P. indica* and *Azotobacter chroococcum* in wheat enhanced APX and peroxidase-antioxidant enzyme activities under zinc-deprived environment (Abadi and Sepehri, 2016).

Recently, the exudates released via *P. indica* interaction were reported to initially lead to ROS generation, accumulation of stress-responsive phytohormone, stomatal closure and induce the defense responsive genes in root and/or shoot of *Arabidopsis*. Moreover, after the establishment of physical contact of plant with *P. indica,* defense responsive genes expression/number, phytohormone and ROS levels turned down; whereas, the stomata re-opened (Vahabi et al., 2015). **Figure 1** schematically highlights the link of ROS generation, scavenging and signaling with plant-mycorrhizal association and their cumulative effect on the enhanced plant fitness under stress.

CONCLUSIONS AND PERSPECTIVES

Symbiotic microbial association can enhance the ROS-antioxidant defense system and ultimately improve the plant fitness under stress. Further, in future, exploration of ROS signatures kinetics during initial plant-arbuscular mycorrhizal association can enhance the basic understanding of mycorrhizal link with ROS generation. Additionally, molecular insights into the detailed kinetics of ROS metabolism in plant-mycorrhizal especially *P. indica* signaling are advocated to design innovative strategies via modulating the ROS metabolism and ultimately will help to improve plant productivity under stress conditions.

AUTHOR CONTRIBUTIONS

MN and NT developed the idea and wrote/finalized the MS. DB, RP, SG, and NA made the figures and developed table and helped in writing. All authors read and approved the final manuscript.

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ORIGINAL RESEARCH
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Application of Mycorrhiza and Soil from a Permaculture System Improved Phosphorus Acquisition in Naranjilla

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Naranjilla (*Solanum quitoense*) is a perennial shrub plant mainly cultivated in Ecuador, Colombia, and Central America where it represents an important cash crop. Current cultivation practices not only cause deforestation and large-scale soil degradation but also make plants highly susceptible to pests and diseases. The use of arbuscular mycorrhizal fungi (AMF) can offer a possibility to overcome these problems. AMF can act beneficially in various ways, for example by improving plant nutrition and growth, water relations, soil structure and stability and protection against biotic and abiotic stresses. In this study, the impact of AMF inoculation on growth and nutrition parameters of naranjilla has been assessed. For inoculation three European reference AMF strains (*Rhizoglomus irregulare*, *Claroideoglomus claroideum,* and *Cetraspora helvetica*) and soils originating from three differently managed naranjilla plantations in Ecuador (conventional, organic, and permaculture) have been used. This allowed for a comparison of the performance of exotic AMF strains (reference strains) versus native consortia contained in the three soils used as inocula. To study fungal communities present in the three soils, trap cultures have been established using naranjilla as host plant. The community structures of AMF and other fungi inhabiting the roots of trap cultured naranjilla were assessed using next generation sequencing (NGS) methods. The growth response experiment has shown that two of the three reference AMF strains, a mixture of the three and soil from a permaculture site led to significantly better acquisition of phosphorus (up to 104%) compared to uninoculated controls. These results suggest that the use of AMF strains and local soils as inoculants represent a valid approach to improve nutrient uptake efficiency of naranjilla and consequently to reduce inputs of mineral fertilizers in the cultivation process. Improved phosphorus acquisition after inoculation with permaculture soil might have been caused by a higher abundance of AMF and the presence of *Piriformospora indica* as revealed by NGS. A higher frequency of AMF and enhanced root colonization rates in the trap cultures supplemented with permaculture soil highlight the importance of diverse agricultural systems for soil quality and crop production.

Keywords: naranjilla, arbuscular mycorrhizal fungi, fungal communities, *Piriformospora indica*, farming practices, permaculture, next generation sequencing

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INTRODUCTION

Naranjilla (*Solanum quitoense*, Solanaceae) is a perennial shrub and an important cash crop in Ecuador, Colombia, and Central America where its fruits are used for a very popular beverage (Acosta et al., 2009). However, the cultivation of naranjilla causes severe ecological problems. Often located at steep sites and cultivated in monoculture, plantations are fully exposed to erosion and soil degradation (Revelo et al., 2010). In the course of this study, highly different measurements were obtained when comparing the soil quality of a conventional 2 years old naranjilla plantation in La Esperanza (province Carchi, Ecuador) and native forest soil directly adjacent to the plantation. Indeed, an enormous loss of soil organic matter (SOM, −66%) and available nutrients such as phosphorus (P, −85%), nitrogen (N, −52%), magnesium (Mg, −70%), potassium (K, −30%), and calcium (Ca, −55%) was detected after only 2 years of cultivation (Supplementary Table S1). Similarly, Mainville et al. (2006) have shown that deforestation dramatically reduced soil fertility in the Andean Amazon. Due to the rapid loss in soil nutrients, high inputs of mineral fertilizers are regularly applied to these plantations. Furthermore, naranjilla plants suffer from several diseases and pests, the most important ones being fusarium wilt, late blight, nematode (*Meloidogyne incognita*), and fruit borer (*Neoleucinodes elegantalis*) infestations (Revelo et al., 2010). In order to fight these pests an array of different pesticides is used by the farmers. However, after 2–3 years the productivity of the plantation decreases due to erosion, lack of nutrients and the high prevalence of resistant pathogens in the soil (Revelo et al., 2010). As a consequence, the land gets abandoned or converted into pasture and a new piece of primary or secondary forest is chosen for a new naranjilla plantation (Heiser, 1985; Revelo et al., 2010). Thus, the cultivation of naranjilla poses serious ecological problems relating to deforestation and forest soil degradation (Sowell and Shively, 2012) within some of the world's richest biodiversity hotspots as the Northern Ecuadorian mountain cloud forests (Brummitt and Lughadha, 2003).

Breeding of resistant varieties and grafting on resistant root stocks are current practices to improve pest resistance of naranjilla (Sowell and Shively, 2012). However, the problem of soil erosion and soil degradation remains unsolved. To counteract the negative impacts of current cultivation practices resilient and sustainable alternatives are crucially needed. Organic farming and permaculture systems pose valuable options. Both systems are known to widely exclude chemical fertilizers and pesticides and to apply the principle of diversification in terms of crop rotation, inter or mixed cropping to promote and maintain soil fertility (Mäder et al., 2000).

It has been shown that arbuscular mycorrhizal fungi (AMF) which form associations with 80–90% of all terrestrial plants (Parniske, 2008) can contribute to alleviate problems related to intensive cultivation practices. AMF were shown to fulfill a broad spectrum of beneficial functions for their host plants as well as for their environments like improved nutrient and water uptake, enhanced tolerance against biotic and abiotic stresses and improved soil structure to counteract soil erosion (Azcón-Aguilar and Barea, 1997; Augé, 2001; Hildebrandt et al., 2007; Pozo et al., 2010; Smith and Read, 2010; Porcel et al., 2012; Leifheit et al., 2014). At the same time it has been observed that intensive agricultural practices like the use of large amounts of mineral fertilizers and pesticides can have severe effects on AMF and other fungal symbionts (Bünemann et al., 2006; Kalia and Gosal, 2011) and consequently can negatively impact plant nutrition and growth (Verbruggen et al., 2010). Indeed it has been shown that the loss of fungal diversity disrupts major ecosystem services such as ecosystem variability and productivity and might decrease plant biodiversity (Van der Heijden et al., 1998; Wagg et al., 2014). On the other hand, there are several agricultural practices that can stimulate the presence of beneficial soil biota, such as crop rotation, diversification or reduction of tillage intensity (Köhl et al., 2014). However, there are no data available comparing the impact of different agricultural management practices such as conventional versus organic and permaculture systems on fungal communities in tropical rain forest ecosystems.

Beside the wide range of beneficial properties, AMF can be used as inoculant and act as a biological fertilizer and disease control agent in naranjilla problematics. Up to date, there are only few studies investigating the association of naranjilla and AMF. Collazos et al. (1986) analyzed the combined effect of inoculation of naranjilla with 10 different AMF species and P fertilization comparing three P levels (0, 50, and 100 kg P/ha). The authors observed increased leaf areas and highest P uptake after inoculation with *Acaulospora* sp., *Glomus* sp., and *Entrophospora colombiana* when grown without P and with the addition of 50 kg P/ha. Also Gonzalez and Osorio (2015) observed that inoculation with *Glomus aggregatum* increased biomass of naranjilla especially when grown under low P levels. Similarly Casierra-Posada et al. (2013) observed greatest biomass production after inoculation with *Scutellospora heterogama* and a mix of *Glomus* spp.

The current study aimed at testing a novel approach by using local soil from three different types of naranjilla plantations for inoculation in addition to three European reference AMF strains commonly used in inoculation experiments. A growth response experiment tested the impact of inoculation on biomass production and nutrient uptake of naranjilla at two different sampling times. In a second experiment, the same local soils were used to establish trap cultures enabling the study and description of native fungal communities in particular AMF inhabiting the roots of naranjilla using a next generation sequencing (NGS) approach. This description had the objective to reveal potential differences in the AMF community structure as affected by agricultural management practices of naranjilla plantations.

MATERIALS AND METHODS

Biological Material

Plant Material

Seeds of naranjilla (*Solanum quitoense*, cv. INIAP QUITOENSE 2009) have been obtained from Palora, a canton situated in the

province of Moròna Santiago in the eastern part of Ecuador (lat: −1,745709°, long: −77,91837°) and were germinated in AMF-free potting substrate (Ökohum Anzuchterde, Herrenhof, Switzerland) within seed trays at 20°C, 80% relative humidity and 8 h light regime in a glasshouse in the Botanical Garden of the University of Basel, Switzerland. After 21 days seedlings were transplanted and grown for another 21 days in compartmented seed trays with the same substrate. Uniform naranjilla seedlings at five leaf stage were used to start the growth response experiment and to initiate the trap cultures.

Inoculum Preparation

For inoculation, three well-studied, "reference AMF species" and a mix of the same three species (AMF mix) were used: *Rhizoglomus irregulare* (BEG 75, 15 spores/g inocula), previously referred to as *Rhizophagus irregularis* and earlier as *Glomus intraradices*, *Claroideoglomus claroideum* (BEG 155, 200 spores/g inocula), and *Cetraspora helvetica* (BEG 153, 13 spores/g inocula), previously referred to as *Scutellospora pellucida*. *R. irregulare* was produced in pot cultures with *Hieracium pilosella, Plantago lanceolata,* and *Allium porrum* as host plants in the greenhouse of the Botanical Institute, Basel, Switzerland, whereas *Cl. claroideum* and *Ce. helvetica* were produced in pot cultures with *P. lanceolata* and *A. porrum* as host plants in the greenhouse of the research station of ETH Zürich, Lindau-Eschikon, Switzerland.

The second type of inoculum were local soils (classified as andosols) coming from three directly adjacent but differently managed naranjilla plantations in Guamaní, in the canton Archidona in the province of Napo in Eastern Ecuador. The first sample was taken from a conventionally managed naranjilla plantation (Conv soil), the second from an ecologically managed plantation (Org soil) and the third from a permaculture site (Perm soil). Details about management practices and soil parameters of the three soils are given in **Table 1** and Supplementary Table S1. Soil parameters were analyzed by lbu (Labor für Boden- und Umweltanalytik, Eric Schweizer AG, Thun, Switzerland). For each site, an area of 25 m × 25 m was chosen from which soil samples of four random subplots of 5 m × 5 m were collected. Each subplot sample consisted of 10 subsamples of individually collected, pooled and sieved (5 mm) naranjilla rhizosphere soil at a depth of 5–20 cm. For the growth response experiment, all four subplot soil samples were pooled to obtain one local soil inoculum per site. For trap culture establishment subplot samples were used independently (an overview scheme is given in Supplementary Figure S1).

Experimental Design

Growth Response Experiment

Uniform naranjilla seedlings were individually transplanted into 440 ml plastic pots (gvz-rossat, Otelfingen, Switzerland) filled with 320 g experimental substrate consisting of heat-sterilized (160°C, 3 h) sand (quartz sand, 0.125–0.25 mm; Kaltenhouse, Alsace, France), Vermiculite [Vermiculit Typ "SF" (0.4–1.2 mm), gvz-rossat, Otelfingen, Switzerland] and autoclaved (120°C, 20 min) topsoil (from a local site in Buus, Switzerland) in the ratio 1:1:2 (v:v:v). After 14 weeks, half of the plants including the substrate were transferred into 1 L pots (gvz-rossat, Otelfingen, Switzerland) filled with additional 541 g of the experimental substrate. Nine treatments were applied: four reference AMF

TABLE 1 | Overview of the three local soils collected from differentially managed naranjilla plantation in Guamani (province Napo, Ecuador) used as local soil inoculants.

	Conventional soil	**Organic soil**	**Permaculture soil**
Location	lat: −0.706462° long: −77,610998°	lat: −0.721087° long: −77,608609°	lat: −0.720108° long: −77,613457°
System	Conventional	Organic	Organic
Fertilization	NPK fertilizer (ratio 10:30:10)	Worm and green waste compost, molasses	Worm and green waste compost, chicken manure
Pesticides	Ridomil, Cypermethrin, 2,4-Dichlorophenoxy-acetic acid	–	–
Age of plantation	1 year	1½ years	8 years
Cultivated plants	Naranjilla in monoculture	Naranjilla in monoculture (with sparsely scattered plants as banana, yucca, guavas, maize, dragon fruit, pineapple, peanuts)	Naranjilla amongst wild plants and banana, yucca, guavas, maize, dragon fruit, pineapple, peanuts
Soil parameters			
pH (H_20)	5.4	5.4	6
P_{Olsen} (mg/kg)	38.1	45.7	47
P* (mg/kg)	3.6	3.2	4.7
K* (mg/kg)	43.7	115	195.2
Mg*(mg/kg)	17.4	52.6	192.3
Ca* (mg/kg)	227	383	1626
N_{tot} (g/kg)	6.9	9.6	12.3
N_{min} (mg/kg)	72.4	82.3	176.8
C_{org} (%)	9.7	15.6	20.3

**1:10 EDTA extraction.*

Conv soil, conventionally managed soil; Org soil, organically managed soil; Perm soil, soil from a permaculture system; NPK fertilizer, nitrogen-phosphorus-potassium fertilizer. Soil parameters were analyzed by lbu (Labor für Boden- und Umweltanalytik, Eric Schweizer AG, Thun). For complete report see Supplementary Table S1.

treatments with *R. irregulare*, *Cl. claroideum*, *Ce. helvetica* and a mix of the three AMF species (AMF mix); three local soil treatments, namely Conv soil, Org soil, and Perm soil; and two control treatments comprising an AMF and a soil control produced from sterilized (120°C, 20 min) AMF mix inocula and a mix of the three local soils, respectively. Two hundred spores of each AMF species were used for the single AMF treatments, while a total of 100 spores of each AMF species were used to prepare the AMF mix. Due to high spore densities of *Cl. claroideum*, inocula were diluted with sterilized AMF mix inocula to add same inocula amounts as for *R. irregulare* and *Ce. helvetica*. The local soil treatments were supplemented with 15 g of pooled subplot soil per pot as described above. Since different inocula sources were added to the pots, their respective nutrient inputs were calculated but no relevant amounts were provided by the AMF or local soil inocula (nutrient analysis of all substrates can be found in Supplementary Table S1). All inocula were added to the planting hole before inserting the seedling. Each pot received 5 mL of a microbial wash to correct for possible differences in microbial communities (Koide and Elliott, 1989). The wash was prepared by wet sieving 100 g of each inoculum mix (either AMF- or local soil inocula mix for the respective treatments) through a 32 μm sieve and a paper filter (pore size 5–7 μm, FS 14 1/2; Schleicher & Schuell BioScience GmbH), yielding a final volume of 1 L. Plants were watered with distilled water according to their needs. Initially, plants were fertilized once after experimental start with 5 mL of a modified Hoagland solution containing 50% of the original P content (Gamborg and Wetter, 1975). After week 10, pots were fertilized weekly with 8 mL of the same Hoagland solution. Plants were grown in climate chambers (A. Schleiss AG, Magden, Switzerland) under controlled conditions with a 14 h light regime of 30000 lux, a temperature of 21–23°C and a relative humidity of 60–80%. To improve plant performance, growing conditions were modified after 14 weeks after transplantation into 1 L pots as follows: 14 h light regime of 60000 lux, a temperature of 23–25°C and a relative humidity of 80%. The experiment was carried out in a fully randomized design including 11 replicates per treatment.

Trap Culture Experiment

For trap culture establishment, the same plantlets and experimental substrate were used as described above. As inocula, soil of the four subplots per system was used directly without pooling to initiate trap cultures (Supplementary Figure S1). Naranjilla seedlings were grown for 6 month in 1 L plastic pots filled with 640 g of the experimental substrate. For inoculation, 50 g local soil inoculum of each subplot was spread in the planting hole before inserting the seedling. Irrigation and fertilization was performed in the same manner as the growth response experiment. Trap cultures were grown in the greenhouse with temperatures between 20 and 30°C and a relative humidity of 40–80%. The experiment was carried out in a fully randomized design including three replicates for each of the four subplot soil sample leading to a total of 12 replicates per naranjilla site (Supplementary Figure S1).

Harvest and Measurements

Growth Response Experiment

After 14 weeks the first five pots of each treatment were harvested by cutting the plant shoot just above the soil surface. Before drying the shoot at 55°C for 48 h, maximum leaf length (length of the biggest leaf), shoot height (height of the uppermost node) and shoot fresh weight were measured. Dried shoots were weighed for dry weight estimation and subsequently milled in a coffee mill (Siemens MC23200, Typ KM13, BSH Hausgeräte GmbH, München, Germany). Shoot P concentration was measured using the molybdate blue method (Murphy and Riley, 1962) on a Segmented Flow Analyzer (Skalar Analytical B.V., San++ Automated Wet Chemistry Analyzer, Breda, Netherlands) after incineration and acid extraction of the plant powder. Nitrogen concentration was measured at the University of Basel using a Thermo Flash 2000 elemental analyzer (Carlo Erba, Thermo Fisher Scientific) where samples were combusted and C and N quantified using a thermal conductivity detector. The root system was carefully removed from the substrate, washed, weighed, cut into small pieces and split in two parts. The main part was dried at 55°C for 48 h to determine root dry weight while 1 g was kept to determine AMF root length colonization (RLC).

The same harvesting procedures were applied at the second harvest 22 weeks after experimental start with the remaining plants (six replicates).

Trap Culture Experiment

Six months after trap culture establishment four root and soil samples were taken from the periphery of each pot using a soil borer. After pooling, roots were removed from the soil and carefully washed. Three subsamples of about 200 mg and one subsample of about 1 g of fresh roots were stored at −20°C for later DNA extraction and RLC assessments, respectively. Remaining soil was mixed and also stored at −20°C for later DNA extraction.

Determination of AMF Root Length Colonization (RLC) from Growth Response and Trap Culture Experiments

Clean frozen root samples were stained to identify and count AMF structures inside the root by a method modified from Phillips and Hayman (1970). Roots were bleached in 10% KOH (10% in water, w:v) overnight at room temperature, rinsed with deionized water, neutralized with 1% HCl (1% in water, w:v) for 30 min and transferred to a ink-vinegar solution [57 mL Black Parker Quink in 1000 mL household vinegar (5% acetic acid), Migros, Switzerland] and stained overnight. Finally, roots were rinsed with deionized water and kept in 50% glycerol for destaining. The percentage of root length colonized by AMF was estimated by a modified line intersection method (McGonigle et al., 1990) at 200× magnification using Leitz Laborlux S microscope (Ernst Leitz Wetzlar GmbH, Germany). One hundred line-intersections per root sample were scored for AMF structures.

DNA extraction and AMF Community Profiling from Trap Culture Experiment

DNA extraction was performed as described by Green et al. (1999). In short, 2 mL of CTAB (cetyltrimethyl ammonium bromide) extraction buffer [100 mM Tris, 1.4 M NaCl, 50 mM Na_2 EDTA, 2% (w/v) CTAB, 1% (w/v) PVP (K25 Roth, 4606)] were added to 200 mg of frozen trap culture roots and homogenized with the Homex 6 homogenizer (Bioreba AG, Reinach, Switzerland) in Bioreba extraction bags (Universal U-Unit, Art. Nr. 480100, Bioreba, Reinach, CH). Total genomic DNA was extracted using DNAeasy Plant Mini Kit (Qiagen, Hilden, Germany), following manufacturer's recommendation. DNA concentration was measured with Qubit^{TM} 3.0 Fluorometer (ThermoFisher Scientific).

Root fungal community profiling was performed as described recently by Schlaeppi et al. (2016) with the exception that we could employ 1300 bp sequence information to infer operational taxonomic units (OTUs). We sequenced one SMRT® Cells on a Sequel instrument at the Functional Genomic Centre Zurich[1] (Zurich, Switzerland). The raw sequencing data is stored at the European Nucleotide Archive database (accession no. PRJEB20759). **Supplementary Data S1** documents the bioinformatic analysis (barcode-to-sample assignments and the command line script). The sequencing run produced a total of 171,673 raw reads with at least five passes (filtering by accuracy was not selected). After quality filtering, demultiplexing and the exclusion of samples that did not reach at least 400 quality sequences, we delineated OTUs at a level of 97% sequence similarity using usearch v8.0.1517 (Edgar, 2013). Taxonomic identities were assigned to the OTU representative sequences using the UNITE database (Kõljalg et al., 2013) with BLAST in the QIIME environment (Caporaso et al., 2010). We did not filter for AMF sequences [original approach by Schlaeppi et al. (2016)], because the mixed soil inocula for the trap cultures also contain fungi other than AMF. The final OTU table consisted of 31 samples (Conv: $n = 11$; Org: $n = 9$; Perm: $n = 11$), together with a total of 105,321 quality sequences (range 459–8,442, median 3,512 sequences per sample).

Statistical Analyses

Root length colonization data of both experiments and plant data of the growth response experiment were analyzed using one-way ANOVA (with inoculation as the main factor with nine and three levels in the growth response and trap culture experiment, respectively) followed by Tukey's honest difference test with a significance level of $\alpha = 0.05$. Normality of residuals was tested using Shapiro–Wilk test. RLC data were arcsin-square root transformed to fit the assumption of normal distribution. Correlations were calculated using Pearson's correlation. Analyses were performed using JMP software version 11 (SAS, Cary, NC, United States).

We provide the statistical data analysis for fungal community profiling in R (v.3.2.2; R Core Team, 2014) including input files (OTU and taxonomy tables, OTU representative sequences and r-markdown script) as **Supplementary Data S2**. The OTU table was rarefied to the lowest sampling depth for ordination and richness analysis or, for the expression of relative abundance in %, it was normalized by sampling depth. β-diversity was assessed with Principal Coordinate Analysis (PCoA) and Permutational Multivariate Analysis of Variance (PERMANOVA), both analyses utilizing Bray–Curtis dissimilarity with packages include vegan (Oksanen et al., 2015) and phyloseq (McMurdie and Holmes, 2013).

[1] www.fgcz.ch

RESULTS

Response of Naranjilla to Inoculation with AMF and Local Soil Inocula

While maximum leaf length did not differ significantly between treatments at both harvests, shoot height was significantly affected by inoculation and differed between the first ($F = 3.16$, $p = 0.0078$) and second ($F = 3.68$, $p = 0.0026$) harvest (**Table 2**). At the first harvest, soil control plants were significantly taller than plants supplemented with conventional and permaculture soil, *R. irregulare* and the AMF mix. In contrast plants supplemented with Conv and Perm soil grew taller as compared to *R. irregulare*-inoculated plants. Similarly, shoot and root dry weights did not differ significantly between treatments, except for shoot dry weight at the first harvest ($F = 2.5$, $p = 0.0282$). Soil control plants had significantly higher shoot dry weights than *Cl. claroideum*-inoculated plants.

Inoculation of naranjilla significantly affected shoot P content at the first and second harvest ($F = 14.06$ and $F = 11.84$, $p = 0.023$ and $p < 0.0001$, respectively). After 14 weeks plants inoculated with *R. irregulare* contained more P in shoots than plants inoculated with *Ce. helvetica,* Conv soil and the two control treatments. After 22 weeks, differences between treatments increased and all reference AMF treatments (except of *Ce. helvetica*) and Perm soil treatment revealed a significantly higher P content than the control treatments (**Figure 1A**). Inoculation with *R. irregulare* and the AMF mix significantly increased shoot P concentrations ($F = 14.6$, $p < 0.0001$) at the first harvest while inoculation with *Cl. claroideum*, *R. irregulare*, the AMF mix, and Perm soil significantly increased shoot P concentration ($F = 15.4$, $p < 0.0001$) at the second harvest (Supplementary Table S2).

Also shoot N content was significantly affected by inoculation at the first and second harvest ($F = 3.81$ and $F = 4.31$, $p = 0.024$ and $p = 0.0009$, respectively). Highest shoot N contents were measured in soil control plants at the first harvest and plants supplemented with Conv soil at second harvest (**Figure 1B**). However, no significant differences were found between AMF treatments and control treatments. Shoot N concentrations were similar in all treatments at both harvests (Supplementary Table S2).

Root length colonization by AMF significantly correlated with shoot P content after 14 weeks ($r = 0.6783$, $p > 0.001$) and 22 weeks ($r = 0.8475$, $p < 0.0001$) and was significantly affected by inoculation at both harvests ($p < 0.0001$). Plants inoculated with *R. irregulare* and the AMF mix showed the highest RLC rates

TABLE 2 | Maximum leaf length, shoot height, shoot and root dry weights (DW) of naranjilla plants inoculated with different species of arbuscular mycorrhizal fungi (AMF), local soil inoculants from differentially managed Ecuadorian naranjilla plantations or two control treatments and grown for 14 weeks (WE 14) and 22 weeks (WE 22) in a sterilized potting substrate.

	Leaf length (cm)		Shoot height (cm)		Shoot DW (g)		Root DW (g)	
Treatment	**WE 14**	**WE 22**	**WE 14**	**WE 22**	**WE 14**	**WE 22**	**WE 14**	**WE 22**
Rhizoglomus irregulare	8.0	16.3	6.7 b	14.3 b	1.3 ab	5.44	0.57	1.89
Claroideoglomus claroideum	8.8	15.8	7.3 ab	15.3 ab	1.19 b	5.69	0.63	1.92
Cetraspora helvetica	8.3	16.7	7.4 ab	16.3 ab	1.55 ab	5.74	0.78	2.09
AMF mix	8.7	15.1	6.5 b	15.2 ab	1.23 ab	5.08	0.51	1.59
AMF control	7.8	15.5	7.3 ab	14.7 ab	1.45 ab	5.49	0.70	1.62
Conventional soil	8.0	16.1	6.6 b	16.8 a	1.69 ab	5.72	0.69	1.88
Organic soil	8.8	16.1	7.3 ab	16.3 ab	1.61 ab	6.17	0.64	1.74
Permaculture soil	8.5	15.2	6.8 b	16.9 a	1.53 ab	5.70	0.64	1.83
Soil control	8.5	15.1	8.8 a	15.0 ab	1.83 a	6.20	0.61	2.05
F_{ANOVA}	0.82	0.69	3.16	3.68	2.5	1.05	1.38	2.26
p-Value	ns	ns	0.0078	0.0026	0.0282	ns	ns	ns

Ns, not significant; AMF mix, mix of the three AMF species R. irregulare, Cl. claroideum, and Ce. helvetica; AMF control, sterilized inocula of the three AMF species; soil control, sterilized soil from all three naranjilla plantations. Different letters in the same column indicate significant different means [ANOVA, Tukey's honest significant difference (HSD) test, α = 0.05]. Data represent means (n = 5 at WE 14 and 6 at WE 22).

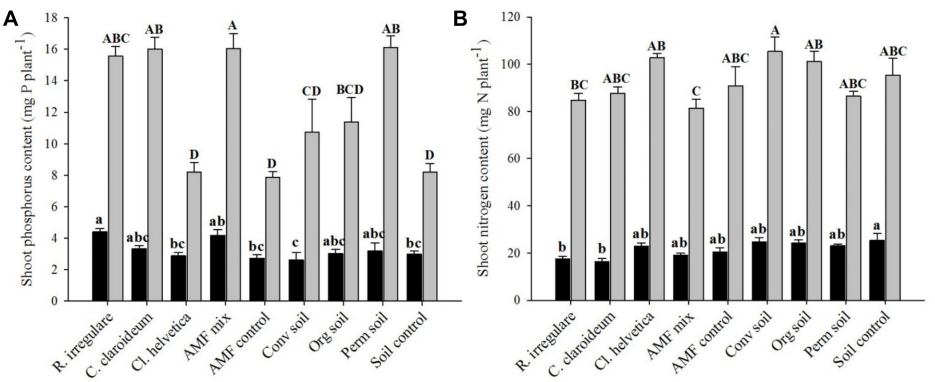

FIGURE 1 | Phosphorus **(A)** and nitrogen **(B)** content of naranjilla shoots inoculated with different species of arbuscular mycorrhizal fungi (AMF): *Rhizoglomus irregulare*, *Claroideoglomus claroideum*, *Cetraspora helvetica*, a mix of the three AMF species and an AMF control consisting of a sterilized AMF inocula mix; or local soil inoculants derived from: conventional (Conv soil), organic (Org soil), and permaculture (Perm soil) naranjilla plantations in Ecuador and a sterilized mix of the three soils as soil control and grown for 14 weeks (WE 14) and 22 weeks (WE 22) in a sterilized potting substrate. Different lower and upper case letters above bars indicate significant differences at WE 14 and WE 22, respectively [ANOVA, Tukey's honest significant difference (HSD) test, α = 0.05]. Data represent means + SE [*n* = 4 (for AMF control treatment due to exclusion of one contaminated sample), 5 (at WE 14), and 6 at (WE 22)].

while roots of *Ce. helvetica*-inoculated plants remained weakly colonized at both harvest time points (**Figure 2**). For most of the AMF treatments, RLC rates did not change between both harvests, only for the two local soil inocula, Org and Perm soil, RLC rates highly increased from the first to the second harvest. Roots of the control treatments remained in most cases free of mycorrhizal structures. Nevertheless one plant of the soil control (first harvest) and one plant of the AMF control treatment (second harvest) had roots highly colonized, most likely due to cross-contaminations and were consequently excluded from graphical representation and all statistical analyses. Roots of another AMF control plant contained at the second harvest only few hyphae but no arbuscules nor vesicles and hence this replicate was kept for further analyses.

AMF Communities in Differentially Managed Naranjilla Plantations

We conducted a trap culture experiment to characterize the AMF communities in the differentially managed soils from the naranjilla plantation site in Guamaní. After 6 month of growth in the greenhouse, RLC of trap cultured naranjilla plants significantly differed between the three local soil inocula ($F = 10.3$, $p = 0.0003$) and was the highest for plants inoculated with Perm soil (44%) compared to plants inoculated with Org soil (21%) and Conv soil (9%).

We determined the fungal taxa inside the roots of the trap culture plants based on amplicon sequencing. Initially, we compared the between-sample similarities based on the β-diversity index Bray–Curtis. PERMANOVA revealed that

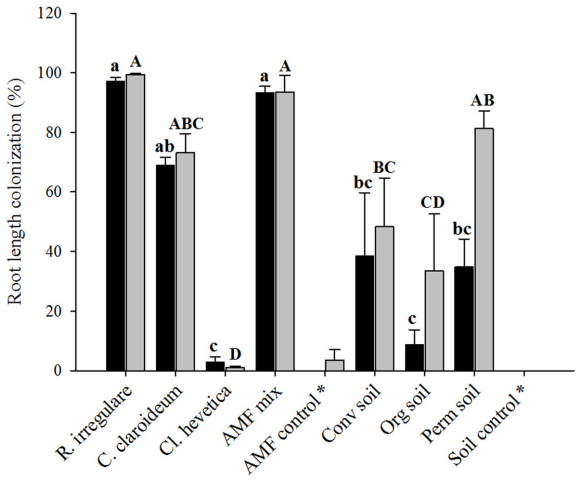

FIGURE 2 | Root length colonization of naranjilla roots after 14 (black bars) and 22 (gray bars) weeks of growth as affected by inoculation with different species of AMF: *Rhizoglomus irregulare*, *Claroideoglomus claroideum*, *Cetraspora helvetica*, a mix of the three AMF species and an AMF control consisting of a sterilized AMF inocula mix; or local soil inoculants derived from: conventional (Conv soil), organic (Org soil), and permaculture (Perm soil) naranjilla plantations in Ecuador and a sterilized mix of the three soils as soil control. Different lower and upper case letters above bars indicate significant differences at WE 14 and WE 22, respectively (ANOVA, Tukey's HSD test, $\alpha = 0.05$). Treatments with an asterisk were excluded from statistical analysis. Data represent means + SE [$n = 4$ (for AMF control treatment due to exclusion of one contaminated sample), 5 (at WE 14), and 6 at (WE 22)].

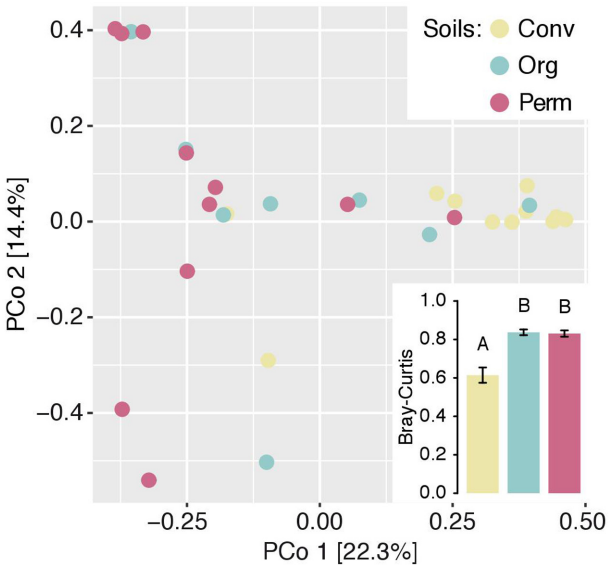

FIGURE 3 | Variation between naranjilla root communities from trap cultures. Differences between communities were assessed with principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity. Points in the unconstrained ordination represent individual samples and are colored by the type of soil used for the trap cultures. Data was subsampled to the sequence number of the sample with the lowest sampling depth. Percentage of variation given on each axis refers to the explained fraction of total variation in all samples. Inset: Average dissimilarity within groups. Each sample was compared with all other samples within its group and these Bray–Curtis dissimilarity were averaged for each sample and plotted by soil groups.

the fungal communities differed significantly between the different soil types (Fmodel $= 2.484$, $p = 0.002$; Supplementary Table S3). Unconstrained ordination supports this finding with separating the samples of the three different soil groups along Axis 1 (**Figure 3**, explaining 22.3% of the variation). We noted that the root fungal communities trapped from organic and permaculture soils were significantly more heterogeneous compared to those trapped from conventional soil (**Figure 3**, inset).

Closer inspection of the root fungi profiles revealed that the communities trapped from the differently managed soils differed in their composition (**Figure 4**). Of note, the high variation between replicate samples, especially the ones derived from organic and permaculture soils, precluded statistical support for these differences. Basidiomycetes and Ascomycetes were more abundant in root fungal communities trapped from conventional and organic soils, respectively. Consistent with the AMF RLC analysis, we found increasing abundances of AMF in roots if trapped from conventional, organic to permaculture soils (**Figure 4A**). This observation was also reflected in the number of detected AMF sequence groups (aka OTUs, operational taxonomic units): while the communities did not differ in the overall number of detected OTUs, we noted increasing numbers of AMF OTUs in roots if trapped from conventional, organic to permaculture soils (**Figure 4B**).

We defined 'abundant OTUs' when OTUs were reaching a minimal mean abundance of 1% in the dataset and we examined their distribution pattern in the root fungi profiles from the trap cultures (**Figure 4C** and Supplementary Figure S2). While numerous communities from conventional or permaculture soils tended to cluster with their group (indicative for similar community composition), we did not find a clear pattern for the root fungi profiles from the trap cultures with organic soil. We found mainly in roots of organic soil trap cultures abundant *Malassezia* spp. (Phylum Basidiomycota) and this, often in combination with the AMF fungus *R. irregulare*. In contrast, we noted predominantly in root samples of permaculture soil diverse AMF taxa such as *Acaulospora* or *Paraglomus* spp.. Interestingly, the beneficial Basidiomycete *Piriformospora indica* was primarily abundant and almost exclusively found in roots of Permaculture soil trap cultures.

DISCUSSION

The application of AMF in horticulture became more prominent within the last decades as the number of studies demonstrating improved plant growth after inoculation has steadily increased (Requena et al., 2001; Gianinazzi et al., 2012). The application of AMF in nurseries which involves growing plants in the presence of AMF prior to planting them in the field is especially beneficial since (i) growing substrates regularly lack natural soil microorganisms, (ii) the plants possess an already established

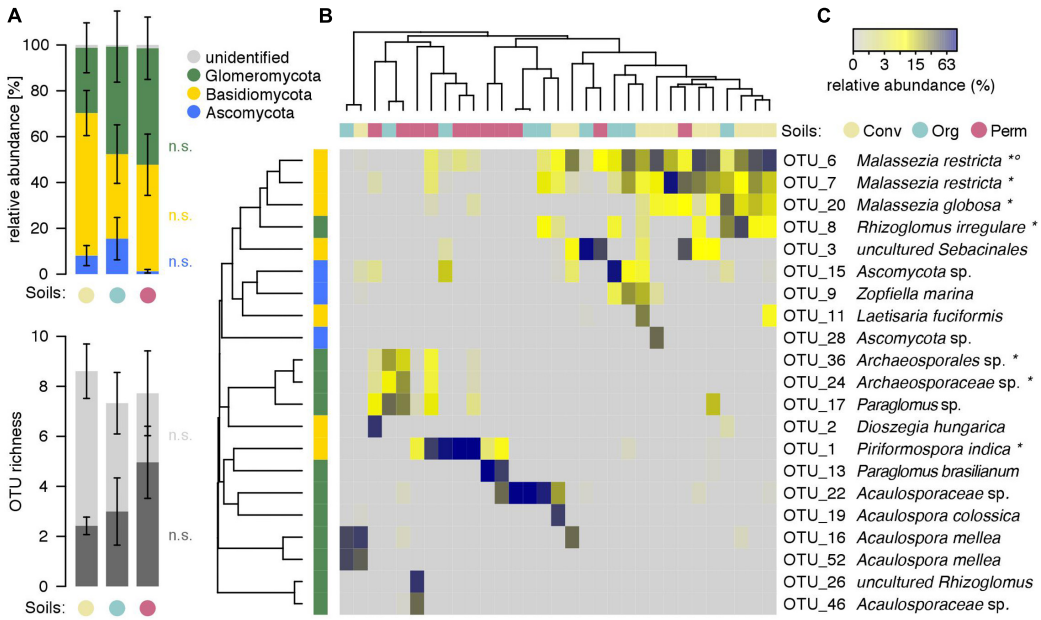

FIGURE 4 | Root fungi profiles from trap cultures. **(A)** Mean abundances of fungal phyla plotted by soil groups. Data were normalized by sampling depth and expressed as percentage relative abundance. **(B)** Mean fungal (light gray) and Glomeromycota (dark gray) OTU richness, reported by soil groups. The dataset was independently subsampled 100 times, OTU richness determined and averaged for each sample. **(C)** The heatmap displays all abundant (defined as >1% mean relative abundance) OTUs in percentage relative abundance in all samples. Dendrograms summarize similarities between samples (in columns) and between OTUs (in rows) based on Bray–Curtis. Non-parametric Kruskal–Wallis test was used to examine differences between soil groups (n.s., not significant, $^{*}P < 0.05$, $^{\circ}P < 0.05$ false discovery rate corrected).

mycorrhizal symbiosis for improving the plants' tolerance against biotic and abiotic stresses when transplanted to the field, and (iii) hyphal colonization of the soil and hyphal nutrient assimilation can start immediately after field transplantation. Thus, the application of AMF harbors a huge potential especially during the early stages of crop cultivation. At later stages, soil fertility becomes crucial for the crops' performance and many studies report that this fertility is more often associated with less intensive practices involving abundant and diverse microbial communities (Van der Heijden et al., 2008; Wagg et al., 2014). In the present study we aimed at investigating both aspects, the potential of AMF to improve plant growth and nutrition and the impact of different farming practices on soil fungal communities, in relation to the cultivation of naranjilla, an important cash crop in Ecuador, Colombia, and Central America.

Response of Naranjilla to Inoculation with AMF and Local Soil Inocula

We investigated the impact of inoculation of naranjilla with reference AMF strains and compared this with a novel inoculation method using natural soils originating from three differentially managed agricultural systems in Ecuador. Growth parameters, biomass production, and shoot nutrient content were measured twice, after 14 and 22 weeks, and revealed that successfully colonized plants were able to acquire more P in their shoots. The strong positive correlation of shoot P content and mycorrhizal RLC raised evidence for naranjillas' dependency on the association with AMF for nutrient acquisition which confirms findings of previous studies. Gonzalez and Osorio (2015) observed that *Glomus aggregatum* increased P uptake and biomass production of inoculated plants compared to non-mycorrhizal controls when grown under greenhouse conditions. Also, the commercial inoculum Mycobiol®, consisting of *Glomus* spp., *Entrophospora colombiana,* and *Acaulospora mellea* enhanced P acquisition and plant growth in a pot experiment (Casierra-Posada et al., 2013). In the present study inoculation with the reference AMF species *R. irregulare*, *Cl. claroideum,* and the AMF mix improved P uptake by the plants. Though not only pure AMF inocula also inoculation with natural soil from a naranjilla permaculture system enhanced P uptake to a similar degree. The approach of using local soils as inoculants has not yet been used for crop plant cultivation before. Up to date experience has been gained using this type of inoculant with few examples related to the cultivation of tropical tree seedling. In a study performed by Urgiles et al. (2014) inoculation with local forest soil was tested for its efficiency to promote the growth of two pioneer trees native to the tropical mountain rain forest in the Andes of Ecuador. The study revealed that application of local forest soil increased seedlings growth to a greater extent than application of a pot-produced inoculant consisting of native AMF species. The authors suggested that beside native AMF populations present in the soil potentially also other beneficial soil microorganisms accounted for improved seedlings performance. Evidence for a similar effect has been made in our study. Also, Rowe et al. (2007) observed improved

performance of late successional tree seedlings after inoculation with field soil as compared to inoculation with two different commercial inoculants. Beside enhanced seedlings growth also the colonization success in terms of RLC was significantly higher when field soil was used.

In accordance to the above mentioned studies we could also demonstrate that the application of local soils as inoculant represents a valid alternative to the use of commercial AMF products especially for ecosystems harboring healthy and microbial active soils. The approach of using local soils offers multiple advantages compared to the use of commercial inoculants. Firstly, the application of local soils helps to conserves the local below-ground biodiversity. Nowadays, the use of commercial AMF inoculants is growing and certain AMF species (mainly *R. irregulare*) are traded globally and used in agriculture and revegetation programs (Gianinazzi et al., 2012) even if they are not native in the applied soils. How introduced AMF species would alter existing AMF communities is still poorly understood as only few greenhouse studies have addressed this question. While some studies detected a decrease in diversity (Mummey et al., 2009; Koch et al., 2011) or functionality (Symanczik et al., 2015) of native AMF communities by introduced AMF inoculants, other studies observed no effect (Antunes et al., 2009). Without more precise knowledge about potential changes of native communities due to current application practices, there remains a risk of future unwanted consequences (Schwartz et al., 2006). Secondly it has been suggested that native AMF strains are better adapted to the local environmental conditions and thus better promote plant growth. A meta-study analyzing the influence of inoculum source revealed that inoculants consisting of local AMF strains yielded higher increases in mycorrhizal colonization and plant growth responses than inoculants from commercial sources (Maltz and Treseder, 2015). Moreover, the use of local soils from reference ecosystems as inocula is economically advantageous especially for smallholder farmers who otherwise might not be able to purchase expensive commercial inocula. Furthermore, the availability of high quality inoculants has to be considered in this context. A study of Corkidi et al. (2004) investigated the infectivity of 10 commercially available mycorrhizal inoculants by inoculating maize plants with recommended rates of each inoculant type. The observed percentages of root colonization ranged from highly insufficient (0%) to satisfactory (50%) and was explained by the presence or absence of viable propagules, the content and type of infective propagules, the amount of recommended application rates as well as unbeneficial soil-microbial interactions. This study indicates possible restrictions which might be associated with the use of commercial mycorrhizal inoculants.

Results from the growth response experiment highlight the potential of using local soils as inoculants especially when other sources of native AMF strains are unavailable. However, we also need to point out the importance of testing a soil before its application to guarantee the absence of pathogens and other growth inhibiting substances. Additional experiments using this source of inoculant are needed to verify our observations and to make more general predictions about the potential.

Root Fungal Communities Trapped from Differentially Managed Naranjilla Plantations

In a second step, we analyzed the root fungal communities trapped from the three differentially managed soils with a focus on AMF. Root analysis revealed that fungal communities significantly differ among the sites with Org and Perm soils harboring more heterogeneous communities than Conv soil. Since separate and non-homogenized soil samples were used as input for the trap cultures, we probably observe in the roots the local soil heterogeneities from the plots from which soil samples had been collected. These observations likely indicate that Org and Perm plots were more heterogeneous and thus more diverse than Conv plots. The higher heterogeneity of the fungal communities might be explained by the higher above ground diversity within these two types of management systems. The influence of plant community composition on belowground microorganisms has already been shown in the case of AMF. Several studies found a positive correlation between plant diversity and AMFs' species richness and abundance (Johnson et al., 2004; Lovelock and Ewel, 2005; Krüger et al., 2017). Similarly we have observed that AMF OTUs and mycorrhizal RLC steadily increased in roots when trap cultures were supplemented with soils from conventional to organic to those from permaculture systems. Also Oehl et al. (2003) observed that management systems with a high plant species richness were correlated with an increase in AMF species richness and abundance. Compared were three management systems: low-input, species-rich grassland; low- to moderate-input farming with a 7-year crop rotation and high-input, continuous maize mono-cropping. Results have shown that some AMF almost exclusively species from the genus of *Glomeraceae* occurred within all management systems, but that the majority of AMF was either restricted to grasslands or less intensively farmed systems with crop rotation especially species of the genera *Acaulosporaceae* and *Scutellosporaceae*. Similarly we have detected that *Acaulospora* spp. were almost exclusively present in roots supplemented with Perm soil, the management system with the highest above ground biodiversity.

It is well established that agricultural management practices highly impact AMF but also other fungal communities and that less intense farming systems favor a higher functionality, abundance, and diversity (Mäder et al., 2000; Oehl et al., 2003, 2004; Gosling et al., 2006; Verbruggen et al., 2010; Kalia and Gosal, 2011; Köhl et al., 2014). One important type of soil disturbance which has been shown to strongly affect soil fungal communities is the application of pesticides as reviewed by Kalia and Gosal (2011). Fungicides directly impact soil fungi with AMF being the most vulnerable candidates. Several studies have reported about fungicides' negative impacts like a decrease in hyphal growth and sporulation of AMF (Sukarno et al., 1993; Kurle and Pfleger, 1994; Chiocchio et al., 2010) a reduction in mycorrhizal root colonization rates (Abd-Alla et al., 2000) as well a decrease in overall fungal biomass of 85% (Bossuyt et al., 2001). Similarly extensive applications of P fertilizers has been shown to affect mycorrhizal performance in a similar manner as reviewed

by Jansa et al. (2006). The conventional naranjilla plantation from which the soil of this study was collected, regularly received high levels of external inputs like synthetic fertilizers and a cocktail of different pesticides being most likely the reason for the observed loss in heterogeneity and reduction of AMF abundance and diversity.

Our NGS approach revealed that *Piriformospora indica* was only detected in roots of trap cultures supplemented with permaculture soil. A review by Varma et al. (2012) highlighted the multifunctional role of *P. indica* as plant growth promoting fungus. Besides its abilities to promote plant growth (Achatz et al., 2010; Fakhro et al., 2010) and nutrient provisioning (Yadav et al., 2010), *P. indica* was also shown to confer tolerance against biotic and abiotic stresses (Fakhro et al., 2010; Sun et al., 2010; Kumar et al., 2012). Improved P acquisition by naranjilla in the growth response experiment might also have resulted from the interaction of this fungus with naranjilla and/or native AMF strains of the Perm soil.

Further, we identified abundant sequences that map taxonomically to *Malassezia* spp., i.e., *M. globosa* and *M. restricta* mainly inhabiting naranjilla roots supplemented with the Conv soil. A review by Amend (2014) described the two *Malassezia* spp. which were originally known as mammalian skin pathogens as cosmopolitan organisms present in different marine habitats, on the exoskeleton of nematodes and in Antarctic soils. Only one study performed by Roy et al. (2009) reported the occurrence of *Malassezia* spp. colonizing the roots of orchids sampled in the rainforest of Thailand. However, the relevance of this association with plant roots and potential impacts on plant growth performance is not known and would require further research including isolation and characterization of strains of this fungal group.

The high homogeneity in soil fungal communities and the loss in AMF abundance in the conventional system compared to the organic one once more demonstrates how strong and rapid intensive management practices can affect soil biota, considering that only one and 1.5 year passed after converting the natural forest into the conventional and organic plantations, respectively. In this way, our findings highlight again the importance of highly diverse belowground systems including AMF for the stability and productivity of agroecosystems and ecosystems' multifunctionality (Van der Heijden et al., 2008; Wagg et al., 2014). Indeed, belowground systems characterized by a low biodiversity are more susceptible to the spread of diseases (Brussaard et al., 2007) and nutrient leaching (Wagg et al., 2014) and harbor a lower potential for nutrient cycling (De Vries et al., 2013; Wagg et al., 2014). Hence, less intensive cultivation systems like permacultures and agroforestry as well as organic farming should be favored to promote divers below-ground soil-microbial communities.

CONCLUSION

Our study is the first of its kind comparing the effectiveness of exotic AMF strains versus local soil inoculation for naranjilla cultivation with a combined assessment of soil fungal communities in differentially managed naranjilla plantations. This study shows the potential of combining both the use of local soils as inoculants at the nursery stage and the application of less intensive practices as a promising approach to improve and maintain sustainable and resilient naranjilla production systems.

AUTHOR CONTRIBUTIONS

SS was the leading author and contributed to all study related tasks mentioned below. CT contributed to research and supervised MG. The M.Sc. student MG performed all practical work in the frame of her M.Sc. thesis. AK, KS, and MV contributed in research and editing of the manuscript. PM and TB are senior author who guided the research and contributed by editing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01263/full#supplementary-material

DATA S1 | Bioinformatic analysis files.

DATA S2 | Statistical data analysis files for fungal community profiling in R.

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Inoculant of Arbuscular Mycorrhizal Fungi (*Rhizophagus clarus*) Increase Yield of Soybean and Cotton under Field Conditions

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Nutrient availability is an important factor in crop production, and regular addition of chemical fertilizers is the most common practice to improve yield in agrosystems for intensive crop production. The use of some groups of microorganisms that have specific activity providing nutrients to plants is a good alternative, and arbuscular mycorrhizal fungi (AMF) enhance plant nutrition by providing especially phosphorus, improving plant growth and increasing crop production. Unfortunately, the use of AMF as an inoculant on a large scale is not yet widely used, because of several limitations in obtaining a large amount of inoculum due to several factors, such as low growth, the few species of AMF domesticated under *in vitro* conditions, and high competition with native AMF. The objective of this work was to test the infectivity of a *Rhizophagus clarus* inoculum and its effectiveness as an alternative for nutrient supply in soybean (*Glycine max* L.) and cotton (*Gossypium hirsutum* L.) when compared with conventional chemical fertilization under field conditions. The experiments were carried out in a completely randomized block design with five treatments: Fertilizer, AMF, AMF with Fertilizer, AMF with 1/2 Fertilizer, and the Control with non-inoculated and non-fertilized plants. The parameters evaluated were AMF root colonization and effect of inoculation on plant growth, nutrient absorption and yield. The results showed that AMF inoculation increased around 20 % of root colonization in both soybean and cotton; nutrients analyses in vegetal tissues showed increase of P and nitrogen content in inoculated plants, these results reflect in a higher yield. Our results showed that, AMF inoculation increase the effectiveness of fertilizer application in soybean and reduce the fertilizer dosage in cotton.

Keywords: nutrient uptake, seed inoculation, rhizosphere, *Glycine max* L., *Gossypium hirsutum* L.

INTRODUCTION

Nutrient availability is crucial to plant growth and crop production. This is influenced by several factors such as the chemical and physical properties of soil, climate and crop type. Crop production in tropical soils requires large amounts of chemical fertilizers, which enhance nutrient release and availability for plant nutrition (Miransari, 2011). Soybean (*Glycine max* L.) is a legume plant, of Fabaceae family. It is cultivated on large scale because has good adaptability to different soil and climatic conditions. Brazil is the second largest world producer of soybeans after the United States, and the total planted area reached 30,105 thousand hectares. Cotton (*Gossypium hirsutum* L.) is a dicotyledonous plant of Malvaceae family, also cultivated in large scale, its cultivation is an economically important activity for the country and reached around of 1,102.8 thousand hectares of planted area (IBGE, 2014). Therefore, cotton and soybean are two important crops in Brazil and, represent around 58% of the total cultivated area (CONAB, 2014) requiring a large amounts of chemicals fertilizers.

The large use of chemical fertilizers has a serious impact on the environment (Tilman et al., 2002) and the agricultural practices influence soil microorganisms greatly, decreasing soil fertility and organic matter turnover (Altieri, 1999). However, the more crucial issue for modern agriculture is that the natural reservoir of some nutrients as phosphorus (P) is decreasing in the world, leading to increase in fertilizer prices in the last decade (Cordell et al., 2009). The challenge for crop production is change to sustainable practices, by finding alternatives for increasing nutrient availability for plant nutrition as organic fertilization. Some these alternatives for organic fertilization include the use de soil microorganisms (Barrios, 2007; Miransari, 2011). Soil microorganisms play an important role by contributing significantly to nutrient availability through biochemical transformations. Some of these microorganisms act directly on plant nutrition by establishing symbiotic associations with plant roots (Bardgett, 2005). The symbiosis between arbuscular mycorrhizal fungi (AMF) and plant roots is one of the most known beneficial interactions occurring in soil (Smith and Smith, 2011), playing an important role in crop production and nutrient turnover (Andrade, 2004).

Arbuscular mycorrhizal fungi increase the uptake of soil inorganic nutrients, mainly P (Neumann and George, 2010). In addition, other benefits related to AMF are the stabilization of soil aggregates (Rillig, 2004), increased resistance to water stress (Garg and Chandel, 2010) and protection against pathogens (Jung et al., 2012). The use of biofertilizer is considered a good alternative to replace or reduce chemical fertilizer use. In example, other symbiotic microorganisms have been successfully used in soybean, and currently, *Bradyrhizobium* and other genera of symbiotic N-fixing bacteria are extensively used as biofertilizer in intensive soybean culture (Deaker et al., 2004) but not for AMF inocula.

In recent years, interest in AMF has focused on finding a viable method to optimize the production of AMF inoculum to use as inoculant in crop systems (Gianinazzi and Vosátka, 2004; Ijdo et al., 2011). The AMF inoculation in field conditions was been evaluated by some authors as Romero and Bago (2010), Pellegrino et al. (2011, 2012), and Ortas (2012) showing a high potential to increase crops yields. However, the success of AMF inoculation in agricultural soils can be determined by many factors such as species compatibility, habitat niche availability for AMF and competition with native fungi (Verbruggen et al., 2013), these aspects need to be evaluated under local conditions for a more appropriate assessment of the viability of AMF use as biofertilizer in crops.

The potential of colonization in soil of *in vitro Rhizophagus clarus* inoculum was first assessment in cotton and soybean in greenhouse conditions. No differences were found between *R. clarus in vitro* and pot culture inoculums for root colonization, plant biomass and P uptake. These results showed the successful of this AMF isolate in pure culture and the potential of this species for large-scale inoculum production (Cely, 2014).

The objective of this work was to determine the effectiveness of AMF (*R. clarus*) inoculation in two crops soybean (*Glycine max* L.) and cotton (*Gossypium hirsutum* L.), assessing its effect on plant growth, nutrient uptake and yield when compared with conventional chemical fertilization under field conditions. Our hypothesis is that AMF inoculation can be an alternative for total nutrient supply or more effective nutrient absorption, when combined with chemical fertilization.

MATERIALS AND METHODS

Experimental Area

The experiments were carried out in Londrina city – PR, Brazil (23°55′46″ S and 51°19′11″ W) during summer (November to June). The climate is humid subtropical, with rainfall during all seasons, relative humidity around 69% and about 2,000 mm of annual precipitation, and the average summer temperature is around 29.5°C.

Two experimental areas were used (A1 and A2) with a Rhodic Ferralsol soil type according FAO (1994). Soil chemical composition and the number of indigenous AMF were determined before sowing by wet sieving and decanting (Gerdemann and Nicolson, 1963) (**Table 1**).

AMF Inoculum Production and Seeds Inoculation

The *R. clarus* inoculum was produced *in vitro* conditions. The monoxenic culture was obtained using carrot (*Daucus carota* L.) Ri T-DNA transformed roots as host organs (Supplementary Figure S1). The *R. clarus* cultures were maintained by continuous subculture of young colonized root fragments (every 4–5 weeks at 25°C, in the dark) in modified Strullu–Romand medium (MRS; Declerck et al., 1998). Petri dishes with massive growth (mycelia and spores) of *R. clarus* and colonized roots were used as crude inoculum. The inoculation methods consist in the seeds palletization with different propagules (colonized roots, hyphae fragments, and spores) from *in vitro* pure cultures of *R. clarus* helped by an organic matrix and turf. The procedure to obtain

TABLE 1 | Soil properties of the experimental areas.

Area	P (mg dm^3)	C (g dm^3)	pH	cmol$_c$ dm^{-3}							%		MP (Spores/g)
				Al	H$^+$ Al	Ca	Mg	K	S	CEC	V	SA	
A1	12.2	18.42	4.8	0.17	6.20	5.02	1.76	0.84	7.62	13.8	55.13	2.18	3
A2	17.3	17.45	5.0	0.00	5.76	4.15	1.72	0.56	6.43	12.2	52.74	0.00	4
P–K: Mehlich I				Ca–Mg–Al: KCl M							pH: $CaCl_2$ 0.01 M		

S, bases sum; CEC, cation-exchange capacity; V, saturation for bases; SA, Saturation for Al; MP, mycorrhizal propagules.

massive inoculum and seeds inoculation is described in the patent INPI BR 10 2014 017389 7 – July 15, 2014 (Andrade et al., 2014).

Experimental Design

Soybean Experiments

Two experiments were carried out with soybean, first in the harvest 2012/13 (E1), using a conventional soybean var. BRS 133 and the second in the harvest 2013/14 (E2) with a transgenic soybean var. BRS 359 RR. Both experiments were composed by the following treatments: Control (non-AMF inoculation and non-fertilizer application); Fertilizer (200 kg ha^{-1} NPK 0:20:20); AMF (*R. clarus* inoculation plus 65 kg ha^{-1} KCl); AMF + Fertilizer (*R. clarus* inoculation plus 200 kg ha^{-1} NPK 0:20:20); and AMF + 1/2 Fertilizer (*R. clarus* inoculation plus 100 kg ha^{-1} NPK 0:20:20). The fertilizer dosage (200 kg ha^{-1} NPK 0:20:20) was according with agronomic recommendations and chemicals analyses of soil in experimental areas (**Table 1**). The nitrogen (N) supply in all treatments was a commercial inoculant (Rizo Plus® Rhizobacter) that contain two lines of *Bradyrhizobium japonicum* (SEMIA 5079 and SEMIA 5080) and its inoculation was according the manufacturer's recommendation.

The treatments were arranged in a completely randomized block design with five replicates (Supplementary Figure S2). Each replicate consist in plots of 5 × 8 m (40 m^2) with 10 rows with spacing 0.45 m and, a density of ten plants per linear meter (approx. 200,000 plants ha^{-1}). The plots were separated by two lateral lines as edge.

Cotton Experiment

Cotton experiment was carried out in the harvest 2013/14 (December–June) with the following treatments: Control (Non-AMF inoculation and non-fertilizer application); Fertilizer (200 kg ha^{-1} PK 20:20 + 200 kg ha^{-1} urea); AMF (*R. clarus* inoculation plus 65 kg ha^{-1} KCl + 200 kg ha^{-1} urea); AMF + Fertilizer (*R. clarus* inoculation plus 200 kg ha^{-1} PK 20:20 + 200 kg ha^{-1} urea); and AMF + 1/2 Fertilizer (*R. clarus* inoculation plus 100 kg ha^{-1} PK 20:20 + 200 kg ha^{-1} urea). The cotton variety used was Bayer® FM 975WS and the fertilizer dosage (200 kg ha^{-1} PK 20:20 + 200 kg ha^{-1} urea) was according agronomic recommendation for experimental area based in chemical analyses of soil (**Table 1**). The treatments were arranged in a completely randomized block design with five replicates as described above for soybean experiments.

Evaluations and Harvest

The effect of *R. clarus* inoculation in soybean and cotton experiments was assessed by the quantification of effective mycorrhizal colonization of roots and their effect in nutrient uptake (N and phosphorus), biomass production (shoot dry weight), and yield grain (soybean) and lint (cotton).

In soybean experiments, roots of 10 plants per plot were sampled randomly at 30 and 80 days after emergence (DAE) to evaluate the mycorrhizal colonization. In sampled plants at 80 DAE was made the evaluations of biomass and quantification of N and P in plant tissues for variety BRS 133. The percentage of mycorrhizal colonization was estimated by the grid-line method (Giovanetti and Mosse, 1980) after fresh roots were stained (Phillips and Hayman, 1970). N and P in shoot tissues were quantified according to Murphy and Riley (1962) and Sarruge and Haag (1974), respectively. For biomass quantification, plants were cut at the ground level; the total fresh shoot height was measured and shoot dry weight was determined after drying at 50°C for 72 h. For cotton, plants and roots were sampled at 120 DAE. Five plants per treatment of each plot were randomly collected, and evaluated for AMF colonization, fresh and dry shoot height, and N and P quantification.

Relative mycorrhizal dependency (RMD) was determined by the given below (Plenchette et al., 1983).

$$\text{RMD} = \frac{\begin{array}{c}(\text{Dry weight } \textit{R. clarus} \text{ inoculated plants}) - \\ (\text{Dry weight native mycorrhizal plants})\end{array}}{(\text{Dry weight } \textit{R. clarus} \text{ inoculated plants})} \times 100$$

Soybean grains were harvested at 120 DAE. For yield estimation were sampled four linear meters (2 m^2) in central area of each plot; after sampling the grains were cleaned, dried, and weighted. Cotton yield was estimated at 190 DAE by counting and collecting opened bolls in 20 plants in the central rows of each plot.

Statistical Analysis

The statistical analyses of AMF root colonization were performed using the Friedman test at a significance level of $p \leq 0.05$. Plant growth parameters, nutrient uptake and field were analyzed by analysis of variance (ANOVA) and the Tukey test (HSD) at a significance level of $p \leq 0.05$. The analysis was carried with BioEstat 5.0 and STATISTICA 7.0 software.

RESULTS

Soybean Experiments

The first evaluation (30 DAE) of AMF colonization for two soybean varieties (BRS 133 and BRS 359 RR) showed that *R. clarus* inoculation increased root colonization about 20% more than non-inoculated plants; although not statistically significant, this difference indicates that inoculation have a positive effect (**Figures 1A** and **2A**). At 80 DAE the roots colonization showed higher values, around 70%, in inoculated plants with the addition of half dose of fertilizers (AMF + 1/2 Fertilizer), in this time these differences were statistically significant by Friedman test ($p < 0,05$) when compared with non-inoculated plants for two soybean varieties (**Figures 1B** and **2B**). When analyze the AMF root colonization of two soybean varieties, is possible observing that the transgenic variety BRS 359 RR had a highest early colonization (around 50% at 30 DAE) that the conventional variety BRS 133 (around 30% at 30 DAE).

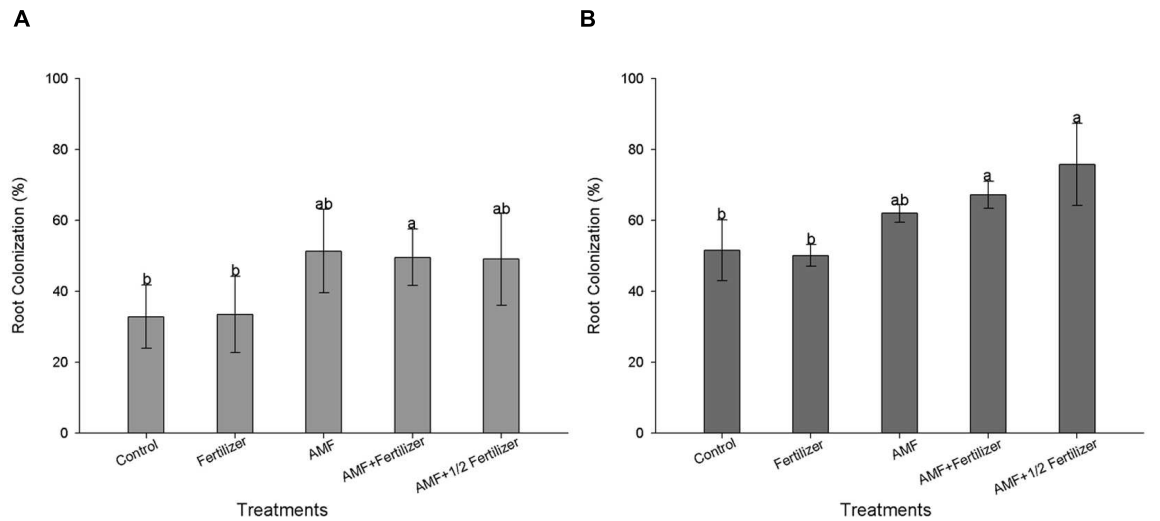

FIGURE 1 | Root colonization of soybean variety BRS 133 at 30 days (A) and 80 days (B). Control (Non-arbuscular mycorrhizal fungi (AMF) inoculation and non-fertilizer application); Fertilizer (200 kg ha^{-1} NPK 0:20:20); AMF (*Rhizophagus clarus* inoculation plus 65 kg ha^{-1} KCl); AMF + Fertilizer (*R. clarus* inoculation plus 200 kg ha^{-1} NPK 0:20:20); and AMF + 1/2 Fertilizer (*R. clarus* inoculation plus 100 kg ha^{-1} NPK 0:20:20). Columns followed by the same letter are not significantly different among treatments by Friedman test ($n = 5$) at $p < 0.05$. Bars represent standard error of means.

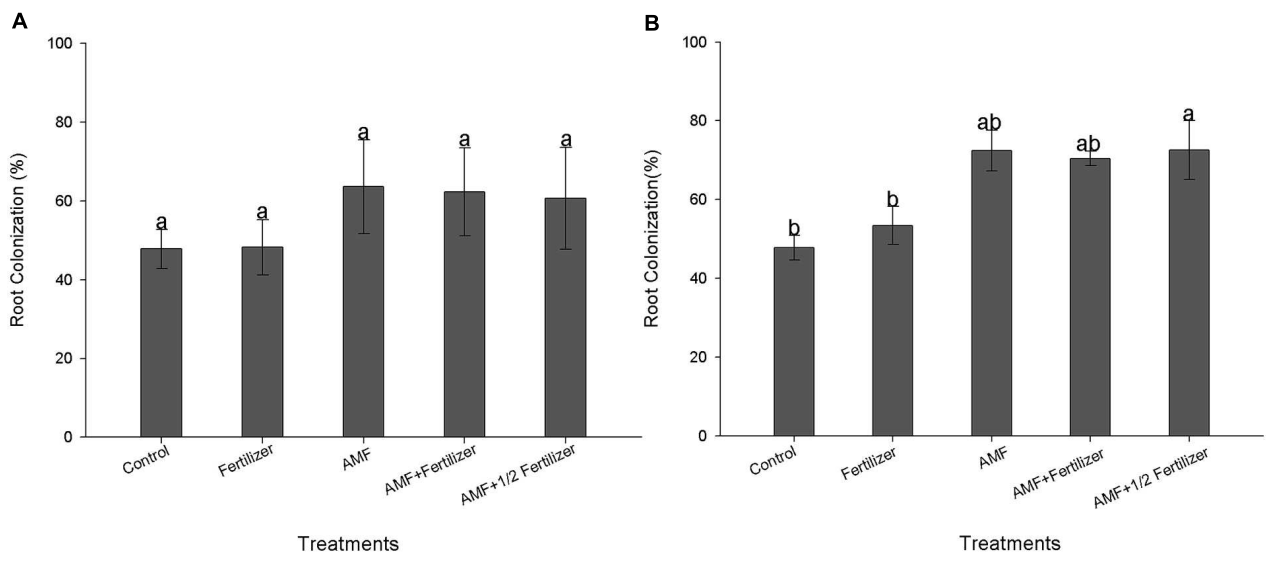

FIGURE 2 | Root colonization of soybean variety BRS 359 RR at 30 days (A) and 80 days (B). Control (Non-AMF inoculation and non-fertilizer application); Fertilizer (200 kg ha^{-1} NPK 0:20:20); AMF (*R. clarus* inoculation plus 65 kg ha^{-1} KCl); AMF + Fertilizer (*R. clarus* inoculation plus 200 kg ha^{-1} NPK 0:20:20); and AMF + 1/2 Fertilizer (*R. clarus* inoculation plus 100 kg ha^{-1} NPK 0:20:20). Columns followed by the same letter are not significantly different among treatments by Friedman test ($n = 5$) at $p < 0.05$. Bars represent standard error of means.

The response of soybean at *R. clarus* inoculation was assessment in variety BRS 133 at 80 DAE and are show in **Table 2**. No differences were observed in plant height between the control and fertilizer or inoculated treatments. Plant biomass and nutrients (N and P) uptake showed that *R. clarus* inoculation (AMF) had the same effect that the conventional fertilization (Fertilizer) and *R. clarus* inoculation with half dose of fertilizer (AMF + 1/2 Fertilizer). These treatments presented statistic differences regarding control (Control) according Tukey test ($p < 0.05$) but not among them. In the other hand, the highest values for these variables were observed in the treatment with *R. clarus* inoculation in combination with conventional fertilization (AMF + Fertilizer). This combination increased highly N and P uptake, around 24%, when compared with soybean only fertilized.

Reflecting the increase in nutrients uptake, *R. clarus* inoculum increased grain yield in cultivar BRS 133, the higher yield was observed in AMF+ Fertilizer treatment, the statistical analysis showed the yield could be equivalent between conventional fertilization, AMF and AMF + Fertilizer treatments (**Figure 3A**). Soybean BRS 359 showed the best grain yield in AMF + Fertilizer and AMF + 1/2Fertilizer treatments (**Figure 3B**). The effect of *R. clarus* inoculation showed high correlation between yield of soybean BRS 133 and P ($r = 0.98$; $p = 0.01$) and N ($r = 0.96$; $p = 0.03$) tissue contents (**Figure 4**).

Cotton Experiment

The *R. clarus* inoculation increased root colonization (80%) when compared with plants without inoculation (50%) at 120 DAE (**Figure 5A**) and, this difference in the colonization was statistically significant according Friedman test ($p < 0.05$), showing that just as soybean, the cotton inoculation with a *R. clarus* had a positive response.

The AMF inoculation does not show a significant effect in plant height. Others parameters as plant biomass and nutrients uptake showed differences in control plants when compared with fertilizer and AMF + Fertilizer combinations. Statistical analysis of these parameters suggest that conventional fertilization in cotton have the same effect that only AMF inoculation, in other hand the fertilization in combination with AMF inoculation (AMF + Fertilizer and AMF + 1/2 /Fertilizer) not differ among them (**Table 3**). Lint cotton yield was significantly higher in plans with AMF inoculation without fertilization and in treatment with AMF inoculation with half dose of

TABLE 2 | Effect of AM inoculation on height, biomass and nutrients uptake in soybean plants at 80 DAE.

Treatments	Height (cm)	Biomass (g plant^{-1})	P (mg plant $^{-1}$)	N (mg plant $^{-1}$ (×10))	RMD (%)
Control	64 ± 5^{a}	21.4 ± 11^{c}	52 ± 29^{c}	190 ± 90^{c}	–
Fertilizer	64 ± 8^{a}	32.4 ± 10^{ab}	82 ± 30^{ab}	300 ± 80^{ab}	–
AMF	62 ± 7^{a}	28.9 ± 7^{ab}	73 ± 13^{ab}	280 ± 50^{ab}	26
AMF+ Fertilizer	57 ± 6^{a}	40.0 ± 8^{a}	102 ± 25^{a}	380 ± 90^{a}	47
AMF+ ½Fertilizer	65 ± 11^{a}	28.6 ± 9^{ab}	75 ± 24^{ab}	300 ± 90^{ab}	26

Control (Non-AMF inoculation and non-fertilizer application); Fertilizer (200 kg ha^{-1} NPK 0:20:20); AMF (Rhizophagus clarus inoculation plus 65 kg ha^{-1} KCl); AMF + Fertilizer (R. clarus inoculation plus 200 kg ha^{-1} NPK 0:20:20); and AMF + ½ Fertilizer (R. clarus inoculation plus 100 kg ha^{-1} NPK 0:20:20). (RMD) relative mycorrhizal dependency. Means followed by the same letter are not significantly different ($p < 0.05$) as determined by Tukey test.

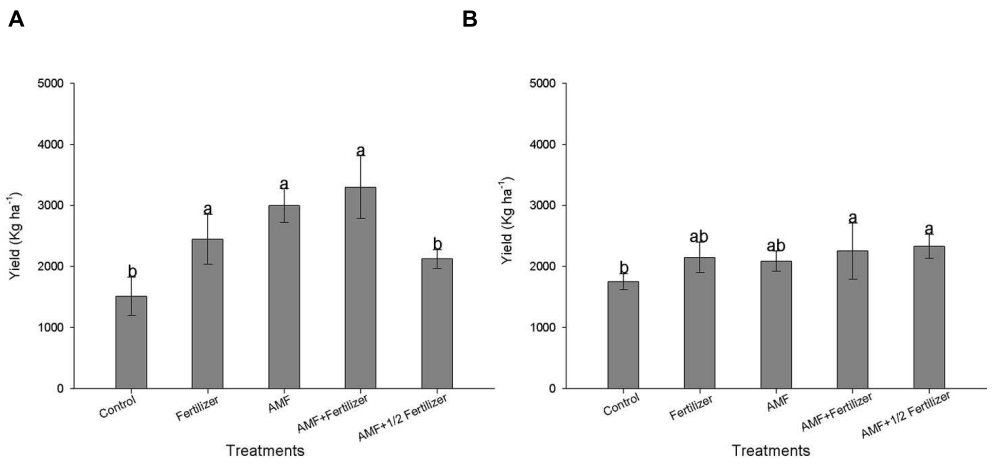

FIGURE 3 | Effect of AMF inoculation on grain yield of soybean BRS 133 (A) and BRS 359 RR (B). Control (Non-AMF inoculation and non-fertilizer application); Fertilizer (200 kg ha^{-1} NPK 0:20:20); AMF (*R. clarus* inoculation plus 65 kg ha^{-1} KCl); AMF + Fertilizer (*R. clarus* inoculation plus 200 kg ha^{-1} NPK 0:20:20); and AMF + 1/2 Fertilizer (*R. clarus* inoculation plus 100 kg ha^{-1} NPK 0:20:20). Columns followed by the same letter are not significantly different between treatments ($p < 0.05$) was determined by Tukey test. Bars represent standard error of means.

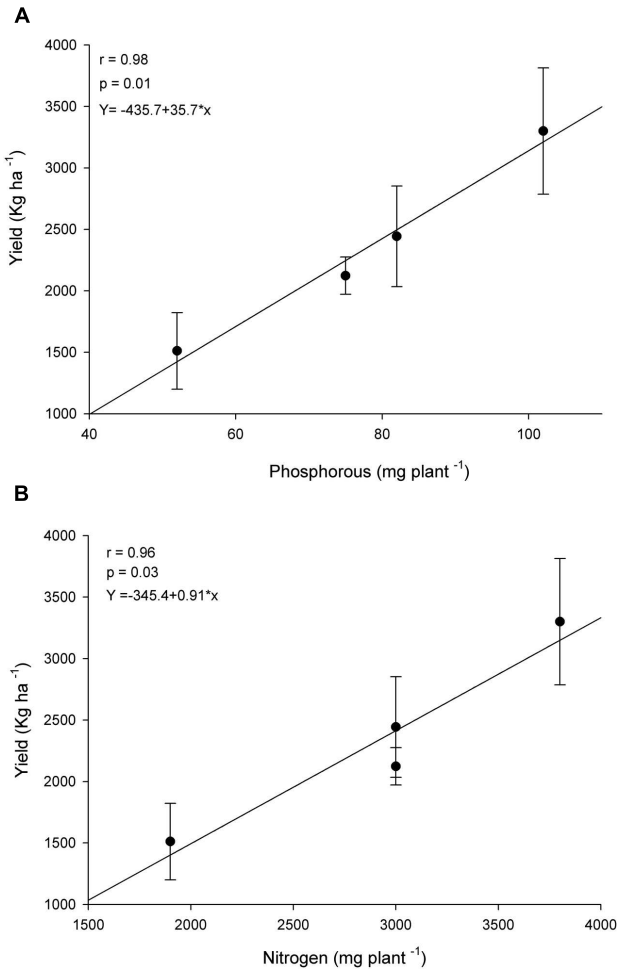

FIGURE 4 | Correlation between shoot nutrients uptake and grain yield of soybean var. BRS133. (A) Phosphorous (P) uptake and grain yield. **(B)** Nitrogen (N) uptake and grain yield.

fertilizer (**Figure 5B**). Nutrients uptake showing significantly high correlation with lint cotton yield, therefore for P uptake the correlation coefficient was $r = 0.90$ (**Figure 5C**), and for N uptake $r = 0.96$ (**Figure 5D**), both statistically significant ($p < 0.05$).

DISCUSSION

The inoculation of *R. clarus* increased plant growth and yield of two varieties of soybean and cotton. Apparently, the inoculum produced *in vitro* was more competitive against native AMF, since inoculated plants showed increased AMF colonization and shoot uptake of P and N. Soybean and cotton showed different responses for *R. clarus* inoculum. First, in soybean, there was a triple interaction (*Bradyrhizobium* – *R. clarus* – plant root), and the inoculum tested was infective and effective, since symbiotic bacteria were already present.

The success of AMF inoculation in agricultural soils can be determined by many factors such as species compatibility, habitat niche availability for AMF and competition with native fungi (Verbruggen et al., 2013). Compatibility is an important point for AMF inoculation, where some isolates could be host "specialists," while others "generalists" (Öpik and Moora, 2012). The inoculum of *R. clarus* tested showed a generalist nature, since it enhanced both plant growth and yield. Accordingly, AMF that are considered plant host generalists have a high establishment rate in several crops (Öpik and Moora, 2012); the results showed that soybean and cotton were effectively colonized, indicating a low specificity by the host plants for *R. clarus*.

In the experiments, mycorrhizal colonization in control plant was around 50% indicating that the agricultural soils support an active indigenous AMF community. The adaptation of *R. clarus* and its competition capacity against indigenous AMF were high. The problem in obtaining an effective AMF inoculum to use on large scale concerns these factors exactly; the inoculum showed good infectivity and high competition capacity under field conditions.

As well known, soil P availability is one the most important factors of AMF regulation, and this characteristic is directly related to the role of P uptake in the AMF symbiosis (Smith et al., 2003; Breuillin et al., 2010; Gutjahr and Parniske, 2013). Our results showed that in soybean and cotton, the moderate soil P availability in the experimental areas (12 and 17 mg dm^{-3}) did not inhibit root colonization of the native AMF population and inoculum of *R. clarus*. The effectiveness of AMF inoculation in greenhouse experiments with phosphate fertilization showed that moderate phosphate availability can allow mycorrhizal colonization, promoting plant growth (Schroeder and Janos, 2005; Taffouo et al., 2013; Xie et al., 2014), and the same responses were found in a field conditions in soybean (Maddox and Soileau, 1991; Karaca et al., 2013).

On other hand, soil P availability can be determined by soil chemical characteristics that influence phosphate solubility. In acid soils, P is less available because of immobilization, even with fertilizer applications, making it unavailable to plants (Busman et al., 2002). Rhodic Ferralsol soils in the experimental area showed low pH, where they can adsorb phosphate, and AMF has an important role in enhancing P uptake and availability, including P from chemical fertilization.

Plants with high P requirements show a high RMD index (Plenchette et al., 1983). Cotton showed a higher RMD (45%) than did soybean (26%) when inoculated with *R. clarus* in the presence or absence of fertilizer. In contrast, when P was added at the recommended dose in combination with AMF inoculation, this index decreased to 41% in cotton and increased to 47% in soybean, suggesting that the gain in biomass was related to the availability of P from the fertilizer, which *R. clarus* provided for the plant roots. Thompson et al. (2012) obtained the same results.

The finding that P and N uptake increased in both crops may be related to *R. clarus* association as observed by other authors (Allen et al., 2003; Barea et al., 2005). AMF improved

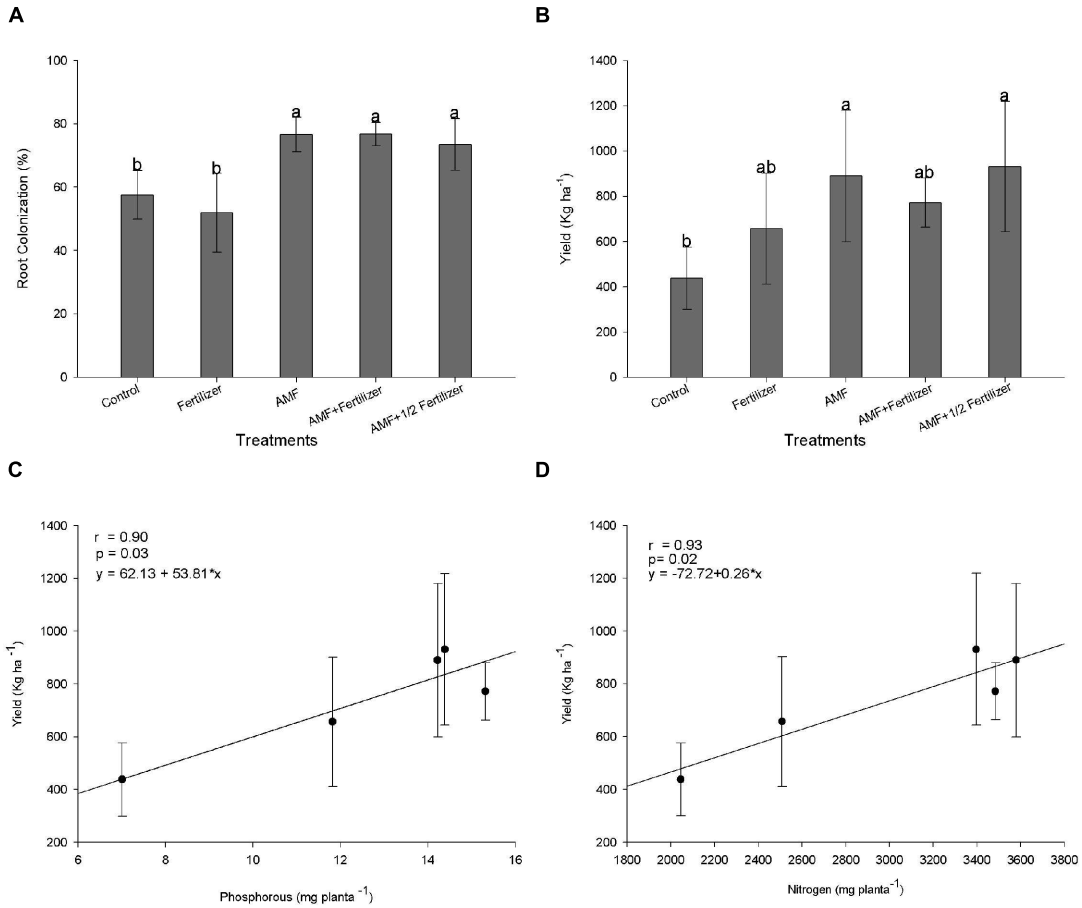

FIGURE 5 | Effect of AMF inoculation in cotton. (A) Root colonization at 120 days after emergence (DAE); **(B)** lint cotton yield; **(C)** correlation between shoot P uptake and lint cotton yield; **(D)** correlation between shoot N uptake and lint cotton yield. Control (Non-AMF inoculation and non-fertilizer application); Fertilizer (200 kg ha^{-1} PK 20:20 + 200 kg ha^{-1} urea); AMF (*R. clarus* inoculation plus 65 kg ha^{-1} KCl + 200 kg ha^{-1} urea); AMF + Fertilizer (*R. clarus* inoculation plus 200 kg ha^{-1} PK 20:20 + 200 kg ha^{-1} urea); and AMF + 1/2 Fertilizer (*R. clarus* inoculation plus 100 kg ha^{-1} PK 20:20 + 200 kg ha^{-1} urea). Columns followed by the same letter are not significantly different ($p < 0.05$) between treatments by Friendman test (for root colonization) and Tukey test (for lint yield). Bars represent standard error of means for each treatment.

TABLE 3 | Effect of *R. clarus* inoculation on total shoot height, biomass, P and N shoot uptake of cotton plants at 120 DAE.

Treatments	Height (cm)	Biomass (g plant^{-1})	P (mg plant $^{-1}$)	N (mg plant $^{-1}$ (×10))	RMD (%)
Control	139.7 ± 15^{a}	48 ± 12^{b}	7 ± 2^{b}	204.6 ± 50^{b}	—
Fertilizer	137.7 ± 11^{a}	59 ± 15^{ab}	12 ± 4^{ab}	250.8 ± 60^{ab}	—
AMF	129.0 ± 14^{a}	87 ± 15^{a}	14 ± 2^{a}	357.8 ± 50^{a}	45
AMF + Fertilizer	143.0 ± 5^{a}	81 ± 18^{a}	15 ± 4^{a}	348.4 ± 90^{a}	41
AMF + 1/2 Fertilizer	136.3 ± 21^{a}	82 ± 24^{a}	14 ± 3^{a}	339.7 ± 90^{ab}	41

Control (non-AM and non-fertilizer); Fertilizer (200 kg ha^{-1} PK 20:20 + 200 kg ha^{-1} urea); AMF (R. clarus plus 65 kg ha^{-1} PK 0:20 + 200 kg ha^{-1} urea); AMF + Fertilizer (R. clarus plus 200kg ha^{-1} PK 20:20 + 200 kg ha^{-1} urea); AMF + 1/2 Fertilizer (R. clarus plus 100 kg ha^{-1} PK 20:20 + 200 kg ha^{-1} urea); RMD, relative mycorrhizal dependency. Means followed by the same letter are not significantly different ($p < 0.05$) as determined by Tukey test.

plant nutrition, leading to an increase in grain yield in soybean and cotton lint production, showing a positive correlation between plant nutrition and yield. Mahanta et al. (2014) also observed a positive linear relationship between P and yield in soybean when inoculated with AMF. The effect of *R. clarus* on cotton growth and yield found here agrees with Thompson et al. (2012) who found an increase in seed cotton yield with *Glomus mosseae* inoculation. The effect of AMF inoculation. However, this is the first time that *R. clarus* inoculum obtained under axenic conditions was tested under field conditions. Ceballos et al. (2013) showed that inoculation of *Rhizophagus irregularis* increased the cassava yield in field and suggest this practice as alternative for improve this crop in several countries.

CONCLUSION

The inoculum of *R. clarus* evaluated was very competitive against endogenous AMF and also increased plant growth and yield. *R. clarus* obtained *in vitro* and tested in the field was efficient in starting early AMF infection in seedlings, improving AM colonization in soybean and cotton. The inoculum of *R. clarus* helped plants to take up P from fertilizer and showed high potential for use in combination with conventional fertilization, for intensive agriculture system in large areas in tropical soils, increasing P absorption and more efficient fertilization use, this is fundamental for the actual challenge of crops production.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00720

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frontiers in Plant Science

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Inoculation of *Schizolobium parahyba* with Mycorrhizal Fungi and Plant Growth-Promoting Rhizobacteria Increases Wood Yield under Field Conditions

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Schizolobium parahyba var. *amazonicum* (Huber ex Ducke) occurs naturally in the Brazilian Amazon. Currently, it is being planted extensively because of its fast growth and excellent use in forestry. Consequently, there is great interest in new strategies to increase wood production. The interaction between soil microorganisms and plants, specifically in the roots, provides essential nutrients for plant growth. These interactions can have growth-promoting effects. In this way, this study assessed the effect of the inoculation with arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) on growth of *S. parahyba* var. *amazonicum* under field conditions. We used two native species of arbuscular mycorrhizal fungi, *Claroideoglomus etunicatum* (Ce), and *Acaulospora* sp. (Ac); two native strains of *Rhizobium* sp. (Rh1 and Rh2); and a non-native strain of *Burkholderia* sp. Different combinations of microorganisms were supplemented with chemical fertilizers (doses D1 and D2) in two planting methods, seed sowing and seedling planting. In seed sowing, the results showed that treatments with Ce/Rh1/Fertilizer D2 and Ac/No PGPR/Fertilizer D2 increased wood yield. In seedling planting, two combinations (Ac/Rh2/Fertilizer D1 and Ac/Rh1/Fertilizer D1) were more effective in increasing seedling growth. In these experiments, inoculation with AMF and PGPR increased wood yield by about 20% compared to the application of fertilizer alone.

Keywords: microorganism interaction, reforestation, Amazon forest, *Schizolobium parahyba*, mycorrhizal inoculant

INTRODUCTION

The negative impacts of agro-industrial development and wood exploitation in native forest areas have encouraged the development of projects focused on reforestation with homogeneous stands or intercropped species of rapid growth and high commercial value. These strategies are directed at degraded areas with the objective of forest restoration or wood production. The family Leguminosae is one of the most representative in terms of number and frequency of plant species in the Amazon region of Brazil (Silva et al., 1988). Some tree species (nodulating and non-nodulating)

of this family are used or have high potential uses for timber production (Sprent and Parsons, 2000) and land restoration (Faria et al., 2010).

Schizolobium parahyba var. *amazonicum* (Huber ex Ducke), belonging to the family Leguminosae and subfamily Caesalpinioideae, is a non-nodulating species native to the Amazon. It is considered an ecologically and economically important species due to its significant wood potential; its commercial potential has been exploited since the 1970s. Today, it is the native species most planted in the Brazilian states of Amazonia, Pará, Maranhão, and Rondonia, covering 87,901 ha (ABRAF, 2012). Due to its fast growth, *S. parahyba* var. *amazonicum* can reach an annual wood yield of 30 m^3 ha^{-1} $year^{-1}$ with 6 years of age (Carvalho, 2007). Moreover, it is considered an important species for carbon sequestration because it produces high levels of biomass in a short period of time (Siviero et al., 2008). The quality of its wood is suitable for furniture and plywood production.

The choice of plant species that are used for restoration and wood production in degraded lands represents a great challenge, because these species need to be able to survive under conditions of low soil fertility. These restrictive factors for plant growth can be attenuated by the action of efficient soil microorganisms such as plant growth-promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF; Chaer et al., 2011). The microbial community in the soil plays an important role in the sustainability of plant communities (Andrade, 2004). The interaction between microorganisms and plants, specifically in the roots, provides for important nutritional requirements of plants and also the microorganisms associated with them. Thus, as the roots directly affect the surrounding microbial populations, the microorganisms present in the rhizosphere can also influence plant growth (Giri et al., 2005).

PGPR are microorganisms that colonize the rhizosphere and promote plant growth. Among them, the N-fixing bacteria (NFB) such as *Rhizobium* species can establish symbiosis with leguminous plant species, resulting in a beneficial interaction for plant growth. Some diazotrophic bacteria can help plant nutrition through biological fixation of N_2 or production of phytohormones (Vessey, 2003). AMF, associated with plant roots, increase the uptake of soil inorganic nutrients, mainly P (Neumann and George, 2010). In addition, other benefits related to AMF are the stabilization of soil aggregates (Rillig, 2004), increasing resistance to water stress (Garg and Chandel, 2010) and protection against pathogens (Jung et al., 2012). In the mycorrhizosphere, the soil surrounding the roots and fungal hyphae (Artursson et al., 2006), AMF can interact with PGPR bacterial species, as well as with endophytic bacteria. Some belong to the genus *Burkholderia* (Bianciotto and Bonfante, 2002). These interactions can provide potential benefits for plant development. The inoculation of compatible combinations of PGPR and AMF in forest and agricultural systems may result in a significant increase in plant growth (Biró et al., 2000; Nadeem et al., 2014; Hashem et al., 2016). Many studies (Marques et al., 2001; Valdenegro et al., 2001; Patreze and Cordeiro, 2004) have demonstrated the synergistic effect of the inoculation of *Rhizobium* and AMF in promoting nodulated legume tree species. However, little research has been carried out on this subject with legume trees of the subfamily Caesalpinoideae (Bryan et al., 1996) with the formation of nodules observed in a few cases (Sprent, 1983).

S. parahyba var. *amazonicum* is a non-nodulating legume, and *Rhizobium* bacteria may promote plant growth in this species in two ways. Some authors suggest that non-nodulating species of the family Leguminoseae can profit from N fixed by root-associated bacteria (rhizosphere bacteria or endophytes) like nodulating species (Bryan et al., 1996; Van Sambeek et al., 2007). On the other hand, it can be assumed that the *Rhizobium* act as plant growth-promoting bacteria in the rhizosphere and release phytohormones (Mehboob et al., 2012).

The use of growth-promoting microorganisms in *S. parahyb*a var. *amazonicum* was assessed by Siviero et al. (2008), who showed that this species displays a positive response to inoculation with AMF in combination with N-fixing bacteria isolated from another plant species (exogenous, i.e., non-native bacteria). The authors observed differences between planting methods (seeds or seedlings) in inoculated plants. In the planting method with seeds, only AMF (*Glomus intrarradices*) inoculation increased biomass and wood production. In the planting method with seedlings, the dual inoculation of AMF (*Glomus clarum*) and PGPR (LEM6 or Rhi1 *Rhizobium* strains) was more effective. In this work, the authors suggested that the selection of native microorganisms is very important to obtain the best results in the field.

Our hypothesis was that the inoculation with indigenous microorganisms is more effective in promoting plant growth of *S. parahyba* var. *amazonicum*, and that the presence of inoculum would help plant roots to be more effective in using the chemical fertilizer applied. Therefore, this study assessed the effect on wood production, comparing inoculation with two indigenous AMF (*Claroideoglomus etunicatum* and *Acaulospora* sp.) isolated from *S. parahyba* var. *amazonicum* roots in interaction with three bacterial strains (two indigenous *Rhizobium* spp. and one exogenous *Burkholderia* sp.). The inoculation with different combinations of microorganisms and the addition of chemical fertilizer was investigated using a completely randomized block experiment. The effect of these factors on *S. parahyba* var. *amazonicum* growth was determined *in situ* over 2 years.

MATERIALS AND METHODS

Experimental Field

The experiments were conducted in the municipality of Dom Eliseu – Pará State (Brazil) [4°17′36″ S and 47°33′15″ W]. Its climate is classified as humid mesothermic, with an average annual temperature of 25°C and annual rainfall of 2500 mm. The region in the wet season shows extensive rain from January to June, and a relative humidity of around 85%. The vegetation is a *terra firme* type with dense forest (da Silva et al., 2011). However, continuous deforestation had destroyed the original vegetation, leading to the emergence of large areas of savannas and secondary forest (SEICOM, 2012).

The soil in the experimental area was a Xanthic Ferralsol according to the FAO classification (FAO, 1994). Prior to

experimentation, the soil was chemically analyzed using a composite sample collected from a depth of 0–20 cm and the physical–chemical analysis showed the following results: pH ($CaCl_2$) 4.8, H^+Al 2.9 $cmol_c\ dm^{-3}$, Al^{+3} 0.2 $cmol_c\ dm^{-3}$; Ca^{+2} 3.3 $cmol_c\ dm^{-3}$, Mg^{+2} 1.0 $cmol_c\ dm^{-3}$, K^+ 0.24 $cmol_c\ dm^{-3}$; P (Mehlich I) 10.0 $cmol_c\ dm^{-3}$, C 19.0 g dm^{-3}; S-SO_4^{-2} 4.2 $cmol_c\ dm^{-3}$, Na^+ 4.0 $cmol_c\ dm^{-3}$, B 0.3 $cmol_c\ dm^{-3}$, Fe^{2+} 99.0 $cmol_c\ dm^{-3}$, Mn 7.3 $cmol_c\ dm^{-3}$, Cu 0.2 $cmol_c\ dm^{-3}$, and Zn 3.0 $cmol_c\ dm^{-3}$. Samples of soil collected in the experimental area showed a low number of AMF spores (3 spores/g of soil) when compared with other soils.

Experimental Design

Two experiments were conducted, each using different planting methods, seeds, and seedlings. The inoculation of each planting method occurred by using different combinations of two species of AMF (*C. etunicatum* and *Acaulospora* sp.), and three PGPR strains (*Rhizobium* sp1, *Rhizobium* sp2, and *Burkholderia* sp.). Additionally, two doses of chemical fertilizer (NPK formulation 10:20:20-N: Urea; P: P_2O_5; K: K_2O) were applied. Dose 1 (D1) was 75 g of fertilizer per plant and dose 2 (D2) was 150 g of fertilizer per plant. The resulting 36 treatments of combinations of these three factors (AMF, PGPR, and Fertilizer) are described in **Table 1**. The treatments were arranged as a completely randomized block design with three repetitions. In the block, each treatment was represented by a row with 10 plants. The spacing was 3 × 2 m between plants and 6 m between blocks. The buffer area in the experiment was composed of three rows with non-inoculated and non-fertilized plants.

Plant Inoculation

The seeds of *S.parahyba* var. *amazonicum* were collected from native forest in Pará state, where the tree occurs naturally. Before sowing, the seeds were scarified mechanically at one end. In the seed system, two seeds were sown in each pit and in the seedling system, one 30 day-old seedling (cultivated in a nursery in plastic bags of 1000 mL with non-sterile soil) was planted before being taken out of the plastic bag.

Microorganism Strains and Growth Conditions

Spores of AMF (*C. etunicatum* and *Acaulospora* sp.) were isolated from the rhizosphere of *S. parahyba* var. *amazonicum* in the Amazon Forest in Dom Eliseu, Pará, and propagated in pots with *Urochloa decumbens* as plant host. Ten grams of inoculum extracted from pots containing 50 spores/g of soil, colonized roots, and mycelia were added before seed sowing or seedling planting in the field.

The bacterial strains used as inoculum were two native ones [*Rhizobium* sp1 (Rh1) and *Rhizobium* sp2 (Rh2)] isolated from roots of *S. parahyba* var. *amazonicum* in the Amazon Forest in Dom Eliseu, Pará. In addition, an exogenous strain of *Burkholderia* sp. was used (Raimam et al., 2007). The *Rhizobium* strains were grown in Petri dishes with TY medium (Beringer, 1974) and the *Burkholderia* sp. strain in Nfb medium (Döbereiner and Day, 1976). For inoculation in the field, the bacteria were re-suspended in sterile saline (0.85% NaCl) plus carboxymethyl cellulose (0.1%) and adjusted by visual comparison with a McFarland standard scale to obtain a final cell concentration of $\sim 10^9$ cells mL^{-1}. Before sowing, the seeds were inoculated by immersion in a bacterial suspension. Seedlings were inoculated with 10 mL of bacterial suspension around the plant.

TABLE 1 | Description of treatments.

Treatment	Description
T1	No AMF/No PGPR/No fertilizer
T2	No AMF/No PGPR/Fertilizer D2
T3	No AMF/No PGPR/Fertilizer D1
T4	No AMF/Burk/No fertilizer
T5	No AMF/Burk/Fertilizer D2
T6	No AMF/Burk/Fertilizer D1
T7	No AMF/Rh1/No fertilizer
T8	No AMF/Rh1/Fertilizer D2
T9	No AMF/Rh1/Fertilizer D1
T10	No AMF/Rh2/No fertilizer
T11	No AMF/Rh2/Fertilizer D2
T12	No AMF/Rh2/Fertilizer D1
T13	Ac/No PGPR/No fertilizer
T14	Ac/No PGPR/Fertilizer D2
T15	Ac/No PGPR/Fertilizer D1
T16	Ac/Burk/No fertilizer
T17	Ac/Burk/Fertilizer D2
T18	Ac/Burk/Fertilizer D1
T19	Ac/Rh1/No fertilizer
T20	Ac/Rh1/Fertilizer D2
T21	Ac/Rh1/Fertilizer D1
T22	Ac/Rh2/No fertilizer
T23	Ac/Rh2/Fertilizer D2
T24	Ac/Rh2/Fertilizer D1
T25	Ce/No PGPR/No fertilizer
T26	Ce/No PGPR/Fertilizer D2
T27	Ce/No PGPR/Fertilizer D1
T28	Ce/Burk/No fertilizer
T29	Ce/Burk/Fertilizer D2
T30	Ce/Burk/Fertilizer D1
T31	Ce/Rh1/No fertilizer
T32	Ce/Rh1/Fertilizer D2
T33	Ce/Rh1/Fertilizer D1
T34	Ce/Rh2/No fertilizer
T35	Ce/Rh2/Fertilizer D2
T36	Ce/Rh2/Fertilizer D1

AM fungi: Ac, Acalospora sp.; Ce, Claroideoglomus etunicatum. PGPR: Burk, Burkholderia sp.; Rh1, Rhizobium sp1; Rh2, Rhizobium sp2.

Data Collection, Biomass, and Wood Yield Determination

Plant growth was determined by shoot diameter (at soil surface), shoot total height (TH), and biomass. Data were collected at 180, 280, 480, and 720 days after planting. At 720 days, we evaluated the diameter at breast height (DBH), TH, and height up to the first leaf (HFL). Biomass (BIO) was determined as described below and was determined for each plant based on the volume

of the stem (Brow, 1997) and multiplied by the correction factor for *S. parahyba* var. *amazonicum* as suggested by Colpini et al. (2009).

$$BIO = [\pi(DBH/2)HFL) \times (0.7)]$$

Wood yield was determined by BIO-value multiplied by the wood specific density of *S. parahyba* var. *amazonicum* and the number of plants per hectare.

$$\text{Wood yield } (m^3\ ha^{-1}) = BIO\ (0.39)\ \times\ d;\ \text{where d is plants ha}^{-1}$$

TABLE 2 | Analysis of variance of plant growth of *S. parahyba* var. *amazoicum* at 180, 280, and 480 days after sowing seeds.

FACTOR	(*p*-values)								
	Shoot Diameter			Total Height of Plant			Biomass		
	180 days	280 days	480 days	180 days	280 days	480 days	180 days	280 days	480 days
AMF	0.167	0.016	0.046	0.072	0.030*	0.514	0.718	0.118	0.030*
PGPR	0.167	0.240	0.000*	0.371	0.873	0.001*	0.734	0.434	0.000*
Fertilizer	0.000*	0.000*	0.000*	0.000*	0.001*	0.843	0.000*	0.000*	0.000*
AMF*PGPR	0.211	0.405	0.396	0.641	0.897	0.628	0.469	0.315	0.474
AMF*Fertilizer	0.167	0.493	0.745	0.211	0.324	0.993	0.069	0.326	1.000
PGPR*Fertilizer	0.032*	0.019*	0.015*	0.303	0.192	0.513	0.057	0.021*	0.092
AMF*PGPR*Fertilizer.	0.569	0.919	0.360	0.914	0.921	0.861	0.430	0.637	0.400

AMF, arbuscular mycorrhizal fungi; PGPR, plant growth-promoting rhizobacteria; Fertilizer.
**Significant difference according to ANOVA ($p < 0.05$).*

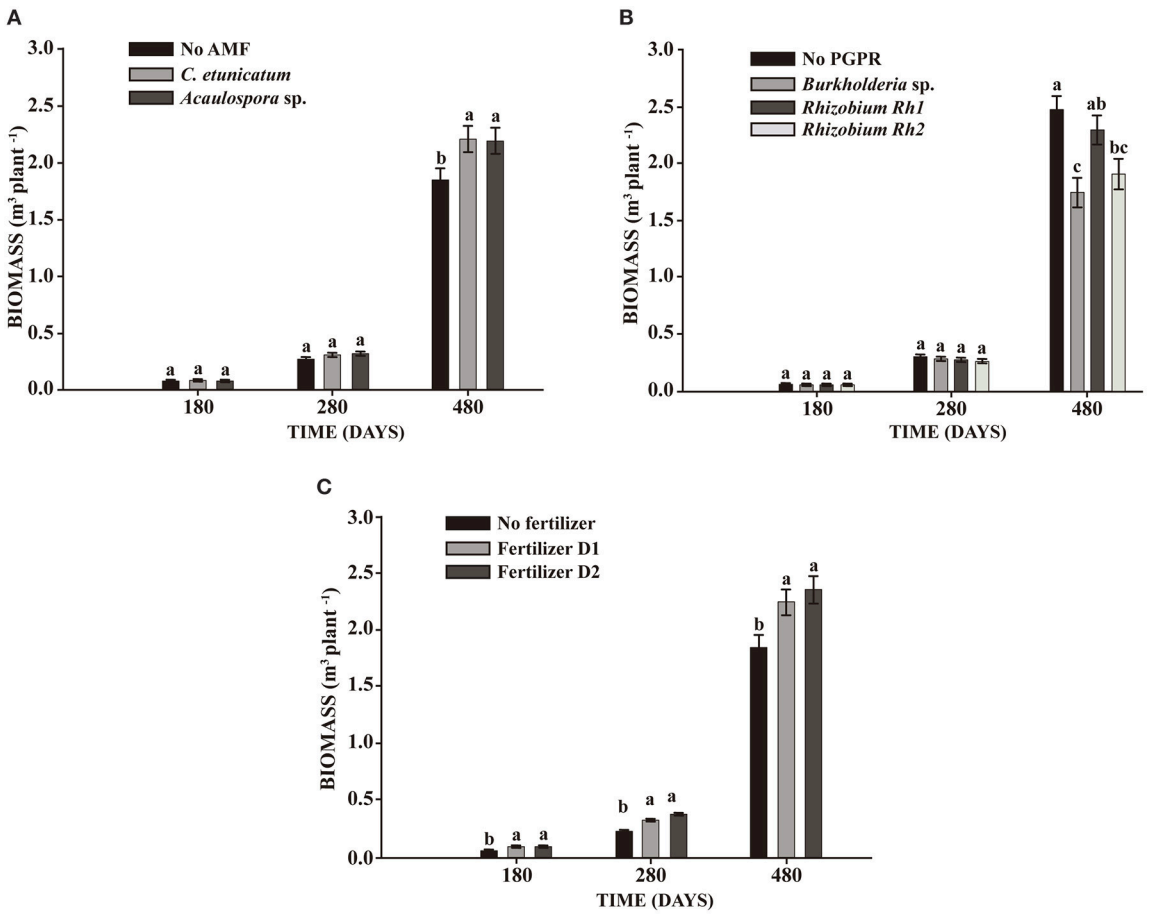

FIGURE 1 | Effect on biomass production of *S. parahyba* var. *amazonicum* after 180, 280, and 480 days of sowing seeds. (A) Arbuscular mycorrhiza fungi (AMF) *C. etunicatum* and *Acaulospora* sp.; **(B)** PGPR strains *Burkholderia* sp., *Rhizobium* Rh1, and *Rhizobium* Rh2; **(C)** two doses of fertilizer (D1: 75 g plant^{-1} and D2: 150 g plant^{-1}). Bars sharing the same letter are not statistically significantly different according to Tukey test ($p < 0.05$).

Statistical Analysis

Statistical analysis was performed using Statistica 7.0 (Statsoft Inc Statistica, 2004). Data were tested for normality using the Shapiro-Wilk test. ANOVA on the data sets (DBH, TH, and biomass) was carried out to determine the interactions of the factors AMF, PGPR, and Fertilizer. Differences between treatments were determined by Tukey's means test (HSD) at $p \leq 0.05$ significance level. A principal component analyses (PCA) was carried out with all data. Time was considered a cofactor, and the treatments were grouped according to AMF inoculation to facilitate the interpretation.

RESULTS

Seed Experiment

In the seeds planting experiment, AMF showed significant effects on DBH and TH at 280 days and BIO at 480 days. PGPR increased DBH and TH at 480 days. Fertilizer addition showed a significant effect on plant growth at all sampling times. The interaction between PGPR and fertilizer effect resulted in increased DBH (**Table 2**). BIO was significantly enhanced by both AMF *C. etunicatum* and *Acaulospora* sp. at 480 days (**Figure 1A**). *Rhizobium* strain Rh1 increased BIO by around 30% when compared with *Burkholderia* (**Figure 1B**). Both doses of fertilizer increased plant growth during the whole experiment (**Figure 1C**).

After 720 days, there was a significant effect on DBH and BIO in AMF plants from sown seeds and in fertilized plants. Bacterial inoculation, especially with *Rhizobium* Rh1, increased DBH, TH, and BIO. The interaction between AMF and bacteria also increased DBH, TH, and BIO. The interaction between *Acaulospora* sp. and *Rhizobium* Rh1 resulted in greater diameter and height of *S. parahyba* in fertilized and non-fertilized plants. On the other hand, the same treatment increased BIO but

TABLE 3 | Effect of arbuscular mycorrhizal fungi (AMF) *Acaulospora* sp. (Ac), *Claroideoglomus etunicatum* (Ce), and PGPR strains *Burkholderia* sp. (Burk), *Rhizobium* Rh1 and Rh2 on diameter at breast height (DBH), total height (TH), and biomass (BIO) after 2 years of sowing seeds.

ANOVA							
FACTOR	**df**	**DBH**		**TH**		**BIO**	
		F	***P-value***	***F***	***P-value***	***F***	***P-value***
AMF	2	7.39	0.0007*	2.50	0.0830	5.69	0.0036*
PGPR	3	9.33	0.0000*	4.75	0.0028*	9.26	0.0000*
Fertilizer	2	3.20	0.0416*	2.00	0.1361	3.66	0.0265*
AMF* PGPR	6	5.18	0.0000*	3.80	0.0010*	4.26	0.0003*
AMF*Fertilizer	4	2.23	0.0646	3.35	0.0101*	1.76	0.1350
PGPR*Fertilizer	6	1.60	0.1430	0.84	0.5336	1.57	0.1515
AMF*PGPR* Fertilizer	12	0.59	0.8454	1.22	0.2651	0.59	0.8451

MEAN TEST									
NFB	**Chemical Fertilizer/AM**								
	No chemical fertilizer			**Chemical fertilizer D1**			**Chemical fertilizer D2**		
	No AM	**Ac**	**Ce**	**No AM**	**Ac**	**Ce**	**No AM**	**Ac**	**Ce**
DBH (cm)									
No NFB	9.79 A,a	9.98 A,a,b	11.23 A,a	11.06 A,a	11.87 A,a	11.64 A,a	10.77 A, a	12.33 A,a	11.72 A,a
Burk	9.48 A,a	8.58 A,b	10.06 A,a	9.60 A,a	9.32 A,b	10.44 A,a,b	8.72 A,b	9.97 A,a	9.67 A,a
Rh1	8.78 B,a	10.83 A, a	10.99 A,a	8.26 B, a	11.79 A,a	10.80 A,b,a	6.51 B,b	11.24 A,a	12.6 A,a
Rh2	9.55 A,a	8.59 A, b	9.32 A,a	9.84 A,a	9.96 A,a,b	8.94 A,b	9.56 A,a	10.86 A,a	9.82 A,a
TH (m)									
No NFB	8.35 A,a	7.92 A,a,b	8.61 A, a	9.00 A,a	9.49 A,a	8.00 A,a	8.71 A,a	9.35 A,a	9.16 A,A,b
Burk	8.10 A,a	7.12 A,b	8.26 A,a	8.00 A,a,b	7.72 A,b	8.42 A,a	6.78 A,b	8.06 A,a	7.70 A,b
Rh1	7.54 B,a	9.02 A,a	8.40 B,a	6.44 B,b	9.48 A,a	8.28 A,b,a	6.13 B,b	8.66 A,a	10.16 A,a
Rh2	8.32 A,a	7.19 A,a,b	7.50 A,a	8.28 A,a,b	8.32 A,a,b	7.88 A,a	7.12 A,A,b	8.71 A,a	8.10A, A,b
BIO dm$^{(3)}$									
No NFB	57.94 A,a	61.36 A,a,b	76.72 A,a	74.04 A,a	91.20 A,a	82.76 A,a	65.75 A,a	102.09 A,a	83.69 A,a
Burk	58.39 A,a	40.63 A,b	64.91 A,a	49.79 A,a	51.84 A,b	70.36 A,a	47.24 A,a	57.71 A,b	53.01 A, a
Rh1	40.92*B, a*	75.33 A,a	76.28 A,a	57.42 A,a	91.49 A,a	70.37 A,a	42.94 B,a	80.68 A,b,b	103.5 A,a
Rh2	62.88 A,a	41.37 A,b	48.28 A,a	54.19 A,a	65.28 A,a,b	48.56 A,a	54.16 A,a	74.10 A,a,b	54.67 A, a

**Significantly different according to ANOVA ($p < 0.05$). Means sharing the same letter are not significantly different according to Tukey HSD test ($P < 0.05$). Capital letters refer to comparisons of AM fungi at each dose of fertilizer (rows), and the small letters refer to comparisons between bacterial treatments (columns).*

only in non-fertilized plants (**Table 3**). The interaction between *Rhizobium* Rh1 and *C. etunicatum* also increased DBH and BIO in non-fertilized and D2 plants, and TH was increased only in D2 plants in the interaction of *C. etunicatum* and *Rhizobium* Rh1 (**Table 3**).

PCA of No AMF plants (**Figure 2A**) revealed that principal component 1 (PC1) and principal component 2 (PC2) accounted for 60.2 and 21.3% of the data variation, respectively. PC1 comprised treatments with *Rhizobium* Rh1 and fertilizer (D1 and D2), and it showed a strong relation with TH, when compared with plants that were only fertilized. In PC2, D2 showed more influence on HFL and BIO. For plants inoculated with *C. etunicatum* (**Figure 2B**), PC1 accounted for 65.3% of data variation and PC2 for 13%. PC1 allowed comparison of two treatments: *Rhizobium* Rh1/No Fertilizer and No PGPR/Fertilizer D2. Both treatments showed more influence on HFL and BIO. PC2 showed that the combination of *Rhizobium* Rh1 and fertilizer D2 had a significant influence on BIO. With regard to inoculation with *Acaulospora* sp., PC1 accounted for 63.8% of data variation and PC2 for 20.4 % (**Figure 2C**). The treatments that showed the highest impact on TH and DBH were fertilizer D1 plus *Rhizobium* Rh1, and the application of D1 or D2 as well. This analysis showed that the inoculation with microorganisms was compatible with the application of fertilizer and that it had a significant effect on plant growth. Therefore, the more effective treatments were: No AMF/*Rhizobium* Rh1/Fertilizer D2; *C. etunicatum*/*Rhizobium* Rh1/No fertilizer; *C. etunicatum*/No PGPR/Fertilizer D2;

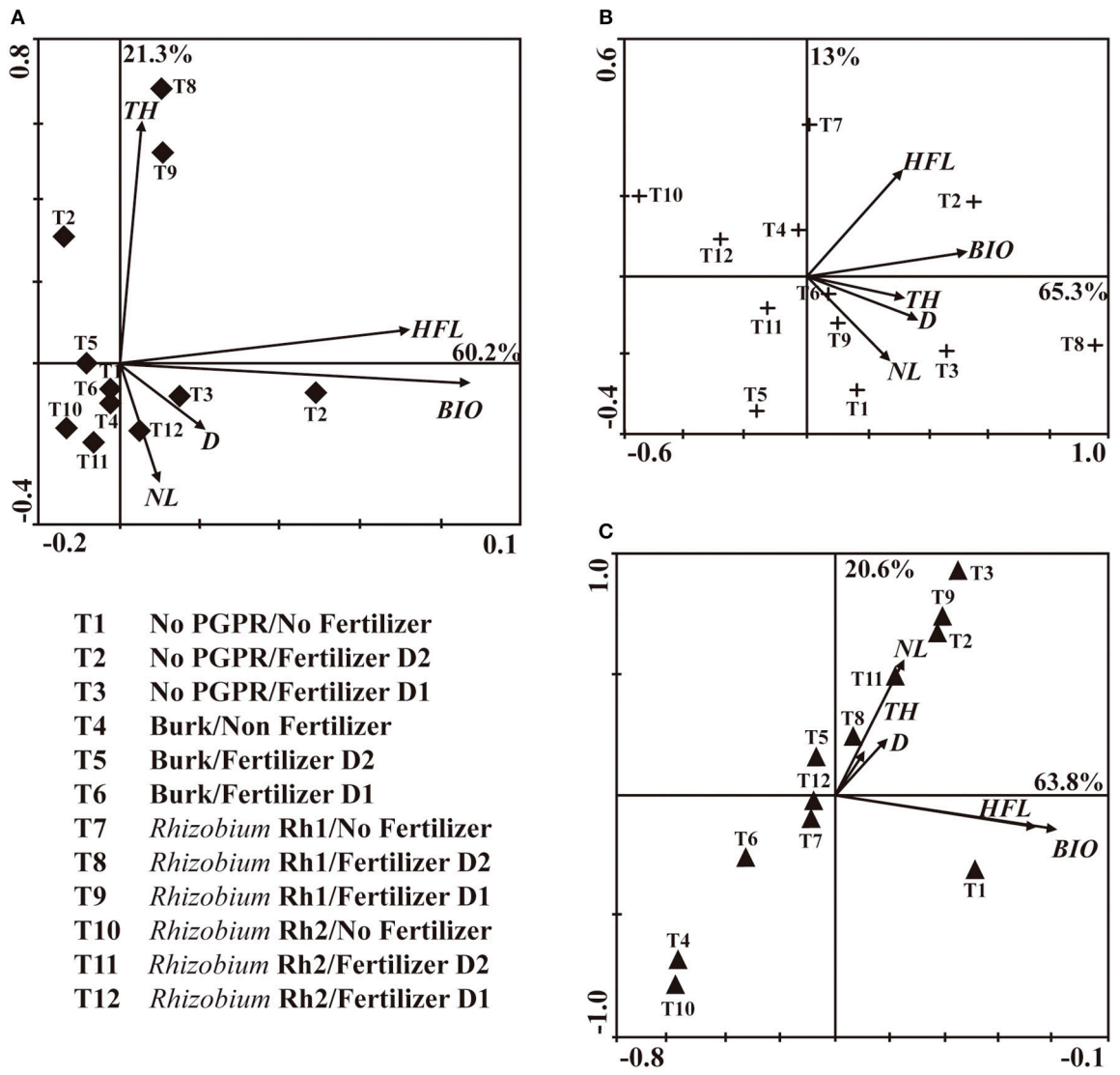

FIGURE 2 | Principal component analysis (PCA) among AM fungi and PGPR bacteria (*Burkholderia* sp, Rhizobium (Rh1 and Rh2) on the shoot diameter (D), total height (TH), height at the first leave (HFL), number of leaves (NL) and biomass (BIO) two years after seeds sowing. (A) No AMF; **(B)** *Claroideoglomus etunicatum*; **(C)** *Acaulospora* sp.

Acaulospora sp./No PGPR/Fertilizer D1 and *Acaulospora* sp./*Rhizobium* Rh1/Fertilizer D2 compared to control plants.

In terms of wood yield in plants from sown seeds, only Ac/No PGPR/No fertilizer was more effective (up to 20% increase in wood production) compared control plants (No AMF, No PGPR, No Fertilizer). In addition, four treatments increased wood yield by 30% (No AM/Rh2/No fertilizer; Ce/*Burkholderia* (Burk)/No fertilizer; Ac/Rh2/Fertilizer D1; and No AMF/No PGPR/Fertilizer D2), and in seven treatments, there was a more than 40% increase (Ce/Burk/Fertilizer D1; Ce/Rh1/Fertilizer D1; Ce/Rh1/No fertilizer; No AM/No PGPR/Fertilizer D1; Ac/Rh2/Fertilizer D2; Ac/Rh1/No fertilizer; and Ce/No PGPR/No fertilizer). Four treatments increased wood yield by more than 50% (No AM/Rh1/Fertilizer D2; Ac/Rh1/Fertilizer D2; Ce/mon-PGPR/Fertilizer D1; Ce/No PGPR/Fertilizer D2), and three treatments by more than 60% (Ac/No PGPR/Fertilizer D1); Ac/Rh1/Fertilizer D1; and No AMF/Rh1/Fertilizer D1). Two treatments increased wood yield by 100% (Ac/No PGPR/Fertilizer D2 and Ce/Rh1/Fertilizer D2) (**Figure 3**), when compared to the control.

The presence of AM fungi was more effective by up to 40% with *C. etunicatum* (Ce/non PGPR/No Fertilizer). On the other hand, when PGPR alone were used for inoculation, wood yield increased only by 30% in the presence of *Rhizobium* Rh2, and with other PGPR no effect was observed. When yield assessed, with increasing more than 50%, the addition of fertilizer (D1 or D2) was needed as well as AMF (*Acaulospora* or *C. etunicatum*).

Seedling Experiment

In the experiment with seedlings, mycorrhizal inoculation showed a significant effect on TH after 280 days. The inoculation of PGPR showed significant differences in all parameters assessed, increasing plant growth at 180, 280, and 480 days. The interaction between AMF and fertilizer showed significant differences in DBH during the whole experiment and in TH and BIO at 180 and 280 days (**Table 4**). No difference in BIO was observed between AMF and control plants (**Figure 4A**). On the other hand, inoculation of *Rhizobium* Rh1 increased BIO (**Figure 4B**). The addition of fertilizer showed a positive effect on BIO at all times evaluated (**Figure 4C**).

Two years (720 days) after planting and inoculated with AMF and PGPR and/or addition of fertilizer, trees in this experiment showed elevated DBH, TH, and BIO values. Plant growth increased in non-fertilized plant inoculated with *Rhizobium* Rh1 and *C. etunicatum* when compared with No AMF plants. D1-fertilized plants plus *Acaulospora* sp. and *Burkholderia* sp. or *Rhizobium* Rh1 increased TH. BIO was increased in non-fertilized plants when inoculated with *C. etunicatum* and

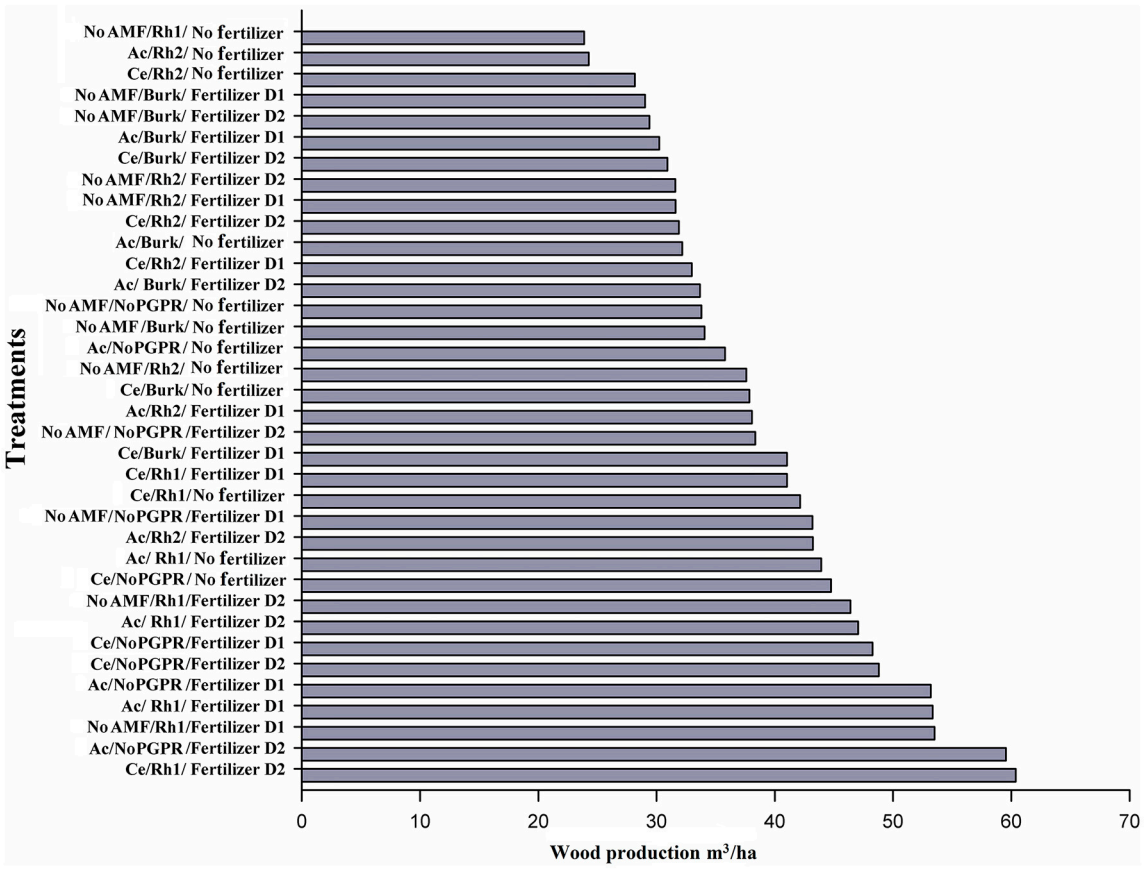

FIGURE 3 | Wood production by different combinations of AMF [*Acaulospora* sp. (Ac) and *Claroideoglomus etunicatum* (Ce)], PGPR [*Burkholderia* sp. (Burk), *Rhizobium* Rh1, and *Rhizobium* Rh2], and chemical fertilizer [D1: 75 g plant^{-1}, D2: 150 g plant^{-1}] 2 years after sowing seeds.

TABLE 4 | Analysis of variance of plant growth of *S. parahyba* var. *amazoicum* at 180, 280, and 480 days after seedling planting.

FACTORS	(*p*-values)								
	Shoot diameter			Total height of plant			Biomass		
	180 days	280 days	480 days	180 days	280 days	480 days	180 days	280 days	480 days
AMF	0.856	0.890	0.424	0.384	0.027*	0.225	0.246	0.799	0.368
PGPR	0.000*	0.000*	0.000*	0.000*	0.000*	0.035*	0.000*	0.000*	0.000*
Fertilizer	0.000*	0.000*	0.009*	0.000*	0.000*	0.001*	0.000*	0.001*	0.037*
AMF*PGPR	0.030*	0.456	0.066	0.222	0.383	0.114	0.101	0.641	0.055
AMF*Fertilizer	0.128	0.153	0.044*	0.112	0.139	0.012*	0.542	0.151	0.015*
PGPR*Fertilizer	0.000*	0.001*	0.029*	0.020*	0.000*	0.684	0.031*	0.004*	0.061
AMF*PGPR*Fertilizer	0.606	0.489	0.095	0.975	0.580	0.066	0.881	0.694	0.259

AMF, arbuscular mycorrhizal fungi; PGPR, plant growth-promoting rhizobacteria; Fertilizer.
**Significant difference according to ANOVA ($p < 0.05$).*

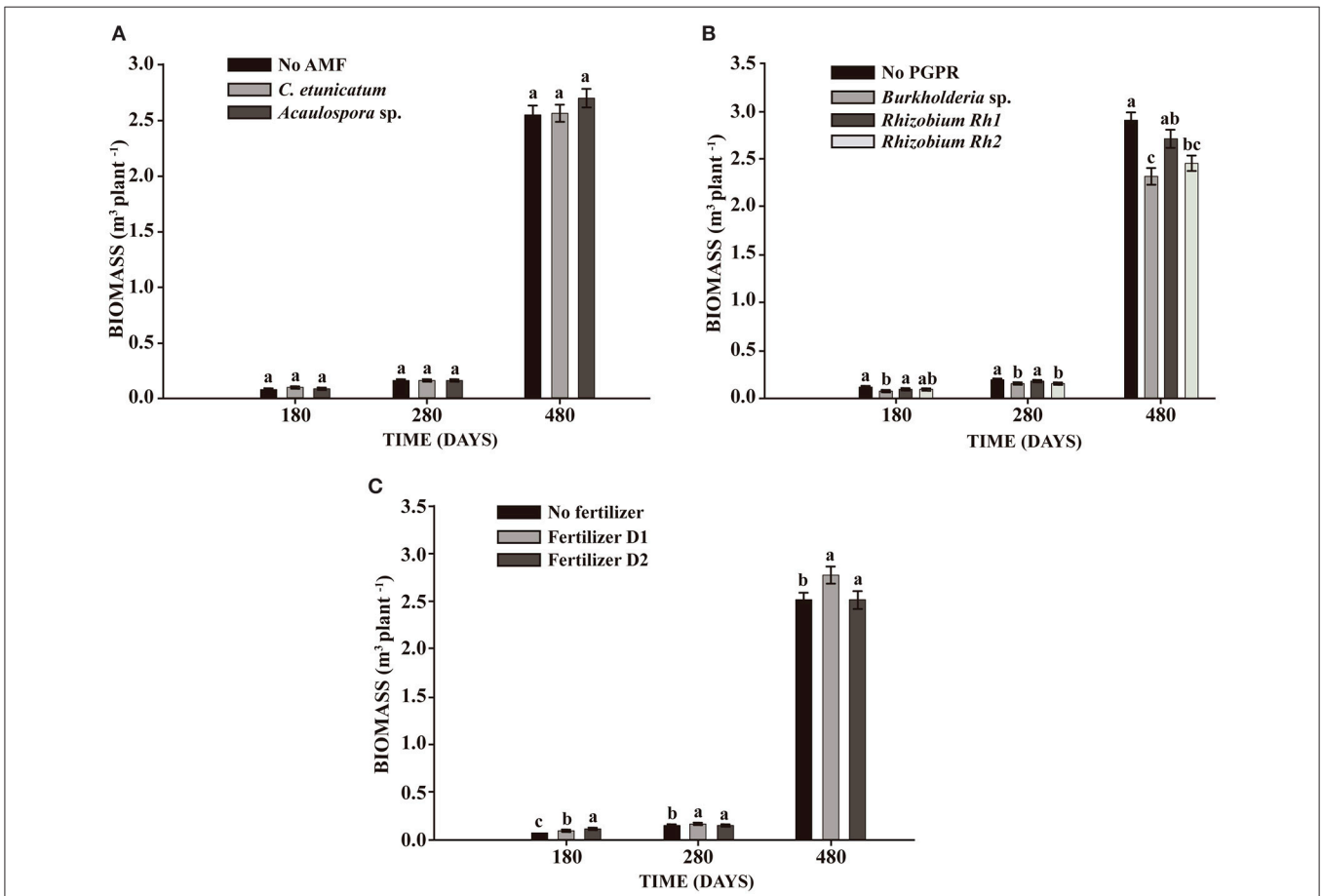

FIGURE 4 | Effect on biomass production of *S. parahyba* var. *amazonicum* 180, 280, and 480 days after seedling planting. (A) Arbuscular mycorrhiza fungi (AMF) *C. etunicatum* and *Acaulospora* sp.; **(B)** PGPR strains *Burkholderia* sp., *Rhizobium* Rh1, and *Rhizobium* Rh2; **(C)** two doses of fertilizer (D1: 75 g plant^{-1} and D2: 150 g plant^{-1}). Bars sharing the same letter are not statistically significantly different according to Tukey test ($p < 0.05$).

Rhizobium Rh1, and in plants fertilized with D1 plus inoculated with *Acaulospora* sp. and *Rhizobium* Rh1 (**Table 5**).

PCA of No AMF treatments allowed us to determine the first principal component (PC1) as accounting for 65.8% of data variation and the second principal component (PC2) for 17.2% of data variation. With PC1 comprising BIO, the treatments did not show significant influence on other variables. On the other hand, in the PC2, comprising No PGPR and Fertilizer D2 (T2), showed more relation with plant height. Inoculation with *Rhizobium* Rh1 without fertilizer (T7) also showed relation with TH (**Figure 5A**).

TABLE 5 | Effect of AMF *Acaulospora* sp. (Ac), *Claroideoglomus etunicatum* (Ce), and PGPR strains *Burkholderia* sp. (Burk), *Rhizobium Rh1* and *Rh2* on diameter at breast height (DBH), total height (TH), and biomass (BIO) 2 years after seedling planting.

ANOVA							
FACTOR	**df**	**DBH**		**TH**		**BIO**	
		F	***P-value***	***F***	***P-value***	***F***	***P-value***
AMF	2	0.08	0.9156	0.85	0.7036	0.13	0.8749
PGPR	3	7.10	0.0001*	10.72	0.0000*	7.37	0.0001
Fertilizer	2	7.36	0.0007*	7.71	0.0005*	6.65	0.0014
AMF* PGPR	6	0.58	0.7397	0.20	0.9768	0.38	0.8892
AMF*Fertilizer	4	1.89	0.1102	3.76	0.0049*	4.03	0.0031
PGPR* Fertilizer.	6	1.62	0.1385	3.35	0.0029*	2.10	0.0511
AMF*PGPR*Fertilizer	12	1.52	0.1098	2.25	0.0086*	1.71	0.0606

MEAN TEST

NFB	**Fertilizer dose/AM1f**								
	No Fertilizer			**Fertilizer D1**			**Fertilizer D2**		
	No AMF	**Ac**	**Ce**	**No AMF**	**Ac**	**Ce**	**No AMF**	**Ac**	**Ce**
DBH (cm)									
No PGPR	11.042 A,b	11.12 A,a	10.67 A,A,b	11.13 A,a	11.28 A,a	11.84 A,a	11.22 A,a	10.80 A,a	11.01 A,a
Burk	9.36 A,b	10.17 A,a	10.53 A,A,b	10.86 A,a	11.17 A,a	10.02 A,b	9.70 A,a	9.60 A,a	10.77 A,a
Rh1	11.92 A,a	10.39 B,a	11.60 A,a	11.32 A,a	11.98 A,a	10.87 A,A,b	10.83 A,a	10.60 A,a	10.80 A,a
Rh2	10.30 A,A,b	10.01 A,a	9.34 A,b	10.76 A,a	12.02 A,a	11.41 A,A,b	10.42 A,a	10.49 A,a	11.15 A,a
TH (m)									
No PGPR	8.71 A,A,b	9.41 A,a	8.38 A,A,b	9.03 A,a	9.16 A,a	9.64 A,a	10.17 A,a	9.28 A,a	9.50 A,a
Burk	7.64 A,b	8.12 A,a	8.05 A,b	9.26 A,b,a	9.58 A,a	8.28*B*, *a*	7.75 A,c	7.80 A,b	8.83 A, a
Rh1	9.72 A,a	8.61 A,a	9.65 A,a	8.67 B, a	10.0 A,a	8.57 B,a	9.37 A,A,b	9.05 A,A,b	9.58 A,a
Rh2	8.42 A,A,b	8.23 A,a	7.70 A,b	9.11 A,a	9.71 A,a	8.78 A,a	8.76 A,b,c	8.61 A,b	9.38 A,a
BIO (dm^3)									
No PGPR	79.56 A,A,b	85.27 A,a	64.31 A,A,b	83.29 A,a	90.90 A,a	93.11 A,a	93.74 A,a	75.19 A,a	93.82 A, a
Burk	49.67 A,b	55.23 A,a	68.73 A,A,b	82.13 A,a	85.49 A,a	66.38 A,a	52.73 A,b	60.61 A,a	72.93 A,a
Rh1	98.73 A,a	65.25 B,a	93.90 A,a	77.07 B,a	101.2 A,a	67.00*B*, *a*	76.39 A,A,b	75.17 A,a	79.92 A, a
Rh2	63.39 A,b	59.04 A,a	55.24 A,b	74.66 A,a	103.6 A,a	79.27 A,a	70.86 A,A,b	66.60 A,a	85.72 A,a

**Significantly different according to ANOVA ($p < 0.05$). Means sharing the same letter are not significantly different according to Tukey HSD test ($P < 0.05$). Capital letters refer to comparisons of AM fungi at each dose of fertilizer (rows), and the small letters refer to comparisons between bacterial treatments (columns).*

For inoculation with *C. etunicatum*, PC1 explained 66.2 % of data variation and PC2 19.3 %. PC1 did not show significant relation with other variables. For PC2, the combination of *Rhizobium* Rh1 and No fertilizer (T7) showed more influence on D, TH, and BIO than did other treatments (**Figure 5B**). When *Acaulospora* sp. was used as inoculum, PC1 grouped 58.9% of data variation and PC2 19.2 %. The treatment with highest impact on plant growth was the combination between *Rhizobium* Rh1 and fertilizer D1 (T9). As for PC2, *Rhizobium* Rh2 plus fertilizer D1 (T12) showed a significant influence on BIO production (**Figure 5C**).

Wood yield (720 days after seedling planting) increased with *Acaulospora* sp. in combination with *Rhizobium* Rh1 or *Rhizobium* Rh2 strains plus D1. Wood production was around 60 m^3 ha^{-1} (**Figure 6**). Single inoculation with *Rhizobium* Rh1 and No AMF/No Fertilizer produced 57 m^3 ha^{-1} (**Figure 6**). In this experiment, only two treatments (No AMF/Rh1/No Fertilizer and Ac/Rh1/Fertilizer D1) increased wood production by more than 20% (**Figure 6**). The Ac/Rh2/Fertilizer D1 treatment resulted in a 30% increase in wood production (**Figure 6**) when compared to controls. The results showed that wood production varied with inoculation with growth-promoting microorganisms. No PGPR/No Fertilizer produced 46.3 m^3 ha^{-1}, and values for only fertilization with D1 or D2 were 48.5 and 54 m^3 ha^{-1}, respectively (**Figure 6**).

DISCUSSION

Several biotic and abiotic factors influence the structural and functional diversity of bacterial communities (Berg and Smalla, 2009). In the relationship between plant and microbial rhizosphere communities, root exudates play an important

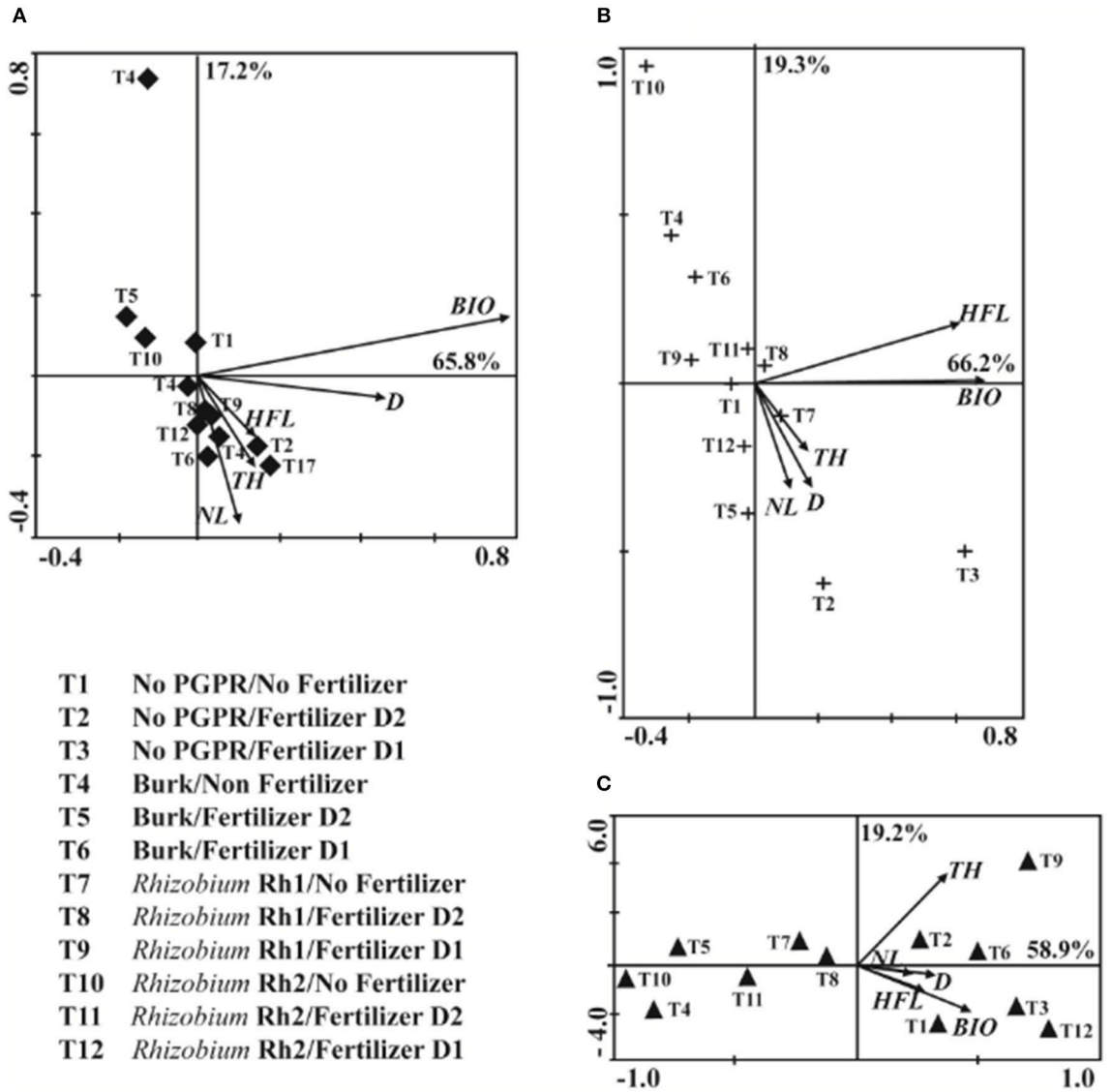

FIGURE 5 | Principal component analysis (PCA) among AM fungi and PGPR bacteria (*Burkholderia* sp, Rhizobium Rh1 and Rh2) on the shoot diameter (D), total height (TH), height at the first leave (HFL), number of leaves (NL) and biomass (BIO) two years after seedling planting. (A) No AMF; **(B)** *Claroideoglomus etunicatum*; **(C)** *Acaulospora* sp.

role in selecting specific microbial populations (Bais et al., 2006). Hence, different plant species are associated with microorganisms that exhibit different responses in terms of survival and activity. In this way, it is necessary to evaluate and select microorganisms from site-specific plant associations, to optimize the inoculant for applications in plant production. The physiological characteristics of the inoculant organism determine to a great extent its fate and activity in soil (Van Veen et al., 1997). In the present study, *Rhizobium* strains promoted the growth of *S. parahyba* var. *amazonicum* when used alone or in combination with *C. etunicatum* or *Acaulospora* sp. in two planting methods. The indigenous isolates of *Rhizobium* were more effective than the exogenous strain of *Burkholderia* sp.

The interaction between AMF and *Rhizobium* improved the development of *S. parahyba* var. *amazonicum* trees from seeds and seedlings. It is believed that the mycorrhiza increase the effectiveness of *Rhizobium* as a result of the general increase in nutritional supply of the host plant (Barea et al., 2002; Bhowmik and Singh, 2004). The ability of *Rhizobium* bacteria to act as endophytes (Spencer et al., 1994; Lupwayi et al., 2004) and PGPR in non-nodulated plants has been confirmed by several studies in other plant species (Yanni et al., 2001; Hossain and Martensson, 2008). Acting as PGPR, rhizobia can support plant growth by solubilizing organic and inorganic phosphates and releasing phytohormones, enzymes, siderophores, exopolysaccharides, and riboflavin (Deshwal,

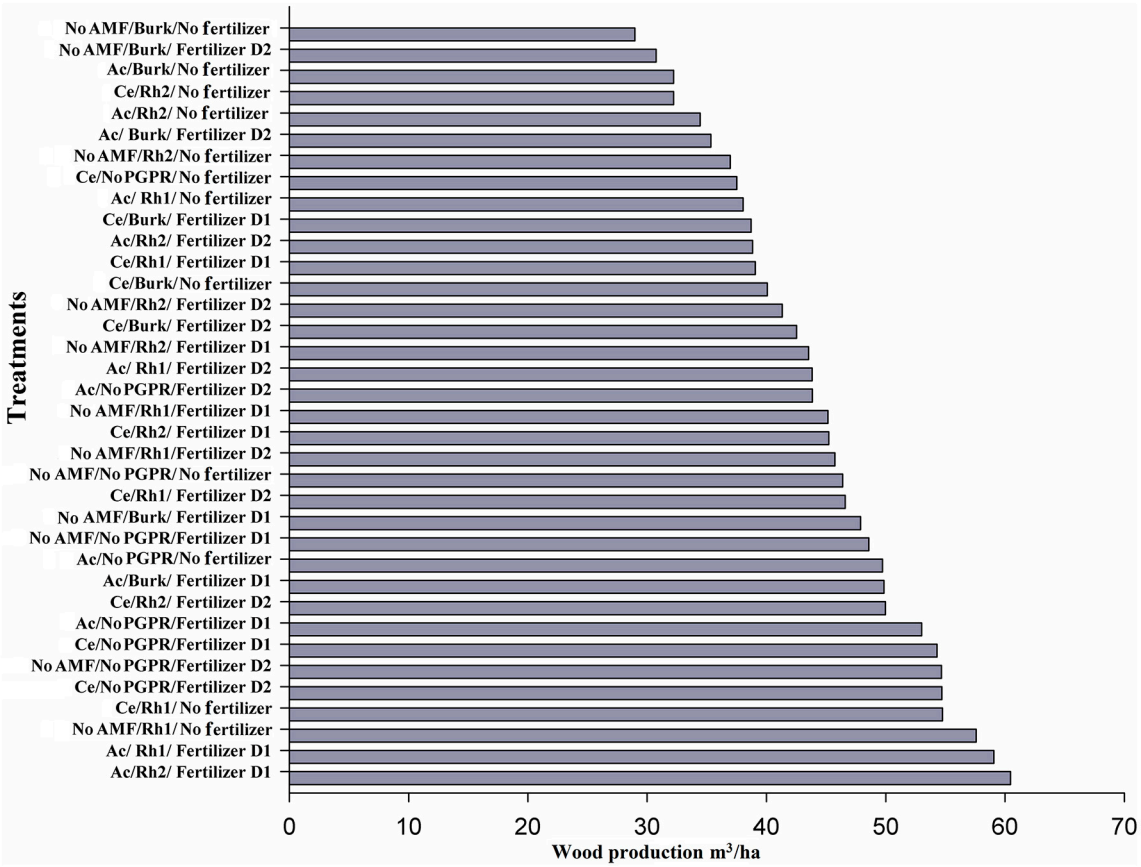

FIGURE 6 | Wood production by different combinations of AMF [*Acaulospora* sp. (Ac) and *Claroideoglomus etunicatum* (Ce)], PGPR [*Burkholderia* sp. (Burk), *Rhizobium* Rh1, and Rh2], and chemical fertilizer [D1: 75 g plant^{-1}, D2: 150 g plant^{-1}] 2 years after seedling planting.

2013). They can also promote growth by inhibiting the growth of pathogens by the release of antibiotic compounds and/or iron immobilization by siderophore production (Mehboob et al., 2012). The double inoculation of *Rhizobium* and AM fungi has been shown to improve plant growth by increasing the nitrogen and phosphorus contents in plant biomass, resulting in improved soil nutrient availability (Matias et al., 2009).

Mycorrhizal inoculation can be integrated into nursery propagation of forestry species, thereby improving planting performance (Herrera et al., 1993). A more appropriate management of mycorrhizal symbiosis in poor soils would allow substantial reduction in the amount of minerals resulting in minimizing losses in productivity, while at the same time permitting a more sustainable production management (Soka and Ritchie, 2014). Due to the low fertility of the soil in the experimental area (**Table 2**), application of chemical fertilizers significantly promotes tree growth. This is a common practice in forestry systems in the area, even though this increases the cost of wood production. The main objective of this work was to reduce or improve efficiency of chemical fertilizer application by *in situ* microorganism inoculation of tropical legume trees.

This was demonstrated by the positive effect on plant growth and wood production with the application of combinations of AMF and *Rhizobium*, which were complemented with the addition of low doses of chemical fertilizer, especially in the seed system. The doses applied in the experiment were lower than those reported by Viégas et al. (2007) who used 255–272 g/plant for *S. parahyba* var. *amazonicum* cultivation in the Amazon area. Diameter, height and biomass of *S. parahyba* var. *amazonicum* after 480 days were equal to or greater than values obtained in plants fertilized with the recommended amount of fertilizer, suggesting a favorable and synergistic action between low fertilization and inoculation with *Rhizobium* and/or AMF.

S. parahyba var. *amazonicum* shows fast growth, reaching a volumetric production of up to 30 m^3 ha^{-1} year^{-1} after 6 years of growth (Carvalho, 2007). In this study, the estimation of wood yield with inoculation of microorganisms reached more than 60 m^3 ha^{-1} in 2 years with the best treatments (Ce/Rh1/Fertilizer D2 in seed sowing and Ac/Rh2/Fertilizer D1 in seedling planting) reaching the maximum yield in 2 years. The promotion of growth of *S. parahyba* var. *amazonicum* by microorganism inoculation has a secondary benefit: carbon sequestration. *S. parahyba* var. *amazonicum* has a low to moderate wood specific density (0.40 g cm^{-3}), with carbon representing around 50% of dry matter. The amount of fixed carbon increases when wood production increases.

CONCLUSION

The use of microorganisms combined or not with fertilizer was more effective in plant growth and wood production in the seeds experiment as compared to the seedling experiment. Wood yield was almost the same in the two systems. However, when using seeds, many treatments increased plant growth and wood yield, and in the seedling system, only three treatments were more effective compared to control plants.

The use of native microorganisms as an inoculant for S. *parahyba* var. *amazonicum* was very effective, especially when combined with low doses of fertilizer, resulting in increased plant growth and wood yield under field conditions. In addition, the inoculation of *Acaulospora* sp. and bacteria improved the absorption of chemical fertilizer, enhancing wood yield. When compared with non-fertilized trees, the best treatments increased wood production by more than 50%. The inoculation of *Acaulospora* sp. and *Rhizobium* Rh1 with D1 fertilizer was the most effective treatment in both systems.

AUTHOR CONTRIBUTIONS

MC: Experimental design and collect of data in the field. MS: Plant culture in the field. JE, FS, VF, AB, EG, GL: Field team control pest, average of plants in the field. IS, AO: Statistical analysis. GA: General coordinator, Head leader team.

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Selecting Native Arbuscular Mycorrhizal Fungi to Promote Cassava Growth and Increase Yield under Field Conditions

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The use of arbuscular mycorrhizal fungal (AMF) inoculation in sustainable agriculture is now widespread worldwide. Although the use of inoculants consisting of native AMF is highly recommended as an alternative to commercial ones, there is no strategy to allow the selection of efficient fungal species from natural communities. The objective of this study was (i) to select efficient native AMF species (ii) evaluate their impact on nematode and water stresses, and (iii) evaluate their impact on cassava yield, an important food security crop in tropical and subtropical regions. Firstly, native AMF communities associated with cassava rhizospheres in fields were collected from different areas and 7 AMF species were selected, based upon their ubiquity and abundance. Using these criteria, two morphotypes (LBVM01 and LBVM02) out of the seven AMF species selected were persistently dominant when cassava was used as a trap plant. LBVM01 and LBVM02 were identified as *Acaulospora colombiana* (most abundant) and *Ambispora appendicula,* respectively, after phylogenetic analyses of LSU-ITS-SSU PCR amplified products. Secondly, the potential of these two native AMF species to promote growth and enhance tolerance to root-knot nematode and water stresses of cassava (Yavo variety) was evaluated using single and dual inoculation in greenhouse conditions. Of the two AMF species, it was shown that *A. colombiana* significantly improved the growth of the cassava and enhanced tolerance to water stress. However, both *A. colombiana* and *A. appendicula* conferred bioprotective effects to cassava plants against the nematode *Meloidogyne* spp., ranging from resistance (suppression or reduction of the nematode reproduction) or tolerance (low or no suppression in cassava growth). Thirdly, the potential of these selected native AMF to improve cassava growth and yield was evaluated under field conditions, compared to a commercial inoculant. In these conditions, the *A. colombiana* single inoculation and the dual inoculation significantly improved cassava yield compared to the commercial inoculant. This is the first report on native AMF species exhibiting multiple benefits for cassava crop productivity, namely improved plant growth and yield, water stress tolerance and nematode resistance.

Keywords: AMF, ecological engineering, cassava yield, tolerance, *Meloidogyne*, drought

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INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a Central and South American native plant with tuberous roots rich in starch. It is a staple food for over 800 million people living in developing countries (Burns et al., 2010). In Côte d'Ivoire, it is the second most important food crop with an estimated annual production in 2013 of 2.5 million tons (FAO, 2014). This crop plays an important role in food security and income generation for many smallholder families. Despite its importance, cassava productivity is low in Côte d'Ivoire where yields are around 6 to 8t/ha compared to a global average level of 13t/ha (FAO, 2014). This low yield appears to be due to several factors. Firstly cassava cropping systems in Côte d'Ivoire are intensive and result in a rapid loss of soil fertility usually requiring long fallow periods (up to 7 years) to restore phosphorus and nitrogen levels. Secondly, cassava productivity is affected by pests, with root-knot nematodes being of major importance (Caveness, 1982; McSorley et al., 1983; Jatala and Bridge, 1990). Cassava yield losses due to nematode damage can be up to 87% (Caveness, 1982; IITA, 1990). Root-knot nematode damage can now be considered as a threat to the production of this major crop plant in Côte d'Ivoire where producers are mainly small farmers who cannot afford to buy nematicides. A third problem is the effect of climate change on crop productivity; notably the impact of drought, as unreliability of rainfall is a factor limiting cassava cultivation in tropical and subtropical areas (N'Guettia and Bernard, 1986). Consequently, although known for its ability to withstand drought, the net biomass production of cassava is reduced in times of water stress, irrespective of variety (Connor et al., 1981). Therefore, in order to sustain cassava productivity for farmers in tropical regions, it is important to develop a technology that can confer simultaneously on this plant (i) better growth and yield, (ii) a better tolerance to nematodes and (iii) a better tolerance to water deficit. Compounding the effects of disease and water stress is the increased vulnerability of rural families and smallholder cassava producers who often do not have access to appropriate technologies, services and markets. In developed countries, farmers rely extensively on industrial fertilizers to maximize crop productivity. Unfortunately, because of their financial and environmental costs, industrial fertilizers are not a solution for underdeveloped countries (Sanchez, 2002).

Cassava farmers could benefit from the multiple services offered by soil microorganisms such as arbuscular mycorrhizal fungi (AMF). Indeed, AMF belonging to the phylum Glomeromycota (Schüßler et al., 2001), constitute a multifunctional partner in the mutualistic interaction they develop with most land plants. The major function of AMF is to provide the mycorrhizal plant with water and essential nutrients such as phosphorus and nitrogen (He et al., 2003; Smith and Read, 2008). In addition to this nutritional function they provide, AMF can enhance plant tolerance to both biotic and abiotic stresses (Augé, 2001, 2004; Ortas et al., 2001; Plenchette et al., 2005; Al-karaki, 2006; Pozo and Azcón-Aguilar, 2007; Porcel et al., 2011; Augé et al., 2015). This multifunctional ability of the partner fungi has led to the development of mycorrhizal inoculants as biofertilizers in agriculture. Mycorrhizal inoculation has been applied for decades to promote better plant growth for various crop plants (Osonubi et al., 1995; Carretero et al., 2009). Cassava is highly mycorrhizal (Sieverding, 1989; Oyetunji and Osonubi, 2007) and there is evidence that AMF play an important role in increasing the productivity of cassava (Sieverding, 1989; Cardoso and Kuyper, 2006; Ceballos et al., 2013). Despite this positive impact of AMF inoculation on cassava productivity, and the known positive impact of mycorrhizal inoculation on root-knot nematode infection in crops such as yam and grapevine (St-Arnaud and Vujanovic, 2007; Tchabi, 2008; Hao et al., 2012; Veresoglou and Rillig, 2012), studies of AMF effects on root-knot nematode and water deficit in cassava remain scarce. In other studies, the impact of AMF on water stress has been documented for several crop plants, including cassava (Augé, 2001; Oyetunji et al., 2007). Although these studies point out the importance of AMF, there is no report of an AMF species that can (i) promote cassava growth and yield (ii) alleviate root-knot nematode damage and (iii) alleviate water stress. Therefore, the identification of AMF exhibiting these three traits could be a step forward to sustain cassava productivity in tropical regions.

The objective of this work was to recover native AMF species from smallholder farms and evaluate their potential to promote cassava growth and enhance resistance to root-knot nematode and water stress. Criteria such as ubiquity and relative abundance in field soils and baited soils were used to select native AMF species that were subsequently further evaluated for plant growth promotion in greenhouse and field conditions.

MATERIALS AND METHODS

Plant Material

The improved cassava variety TME 7 "Yavo" provided by the National Agency for Rural Development Support (ANADER) in Yamoussoukro was used for the experiment. This variety has an 8-month cycle and is known to be resistant to the African cassava mosaic virus. In general, cassava leaves and roots are well developed after 4 months. At this stage, biotic and abiotic stresses can affect cassava growth parameters (Connor et al., 1981). Therefore all the experiments in greenhouse were run on 4-month-old cassava plants.

Methods

Selecting Potential Arbuscular Mycorrhizal Fungi for Inoculum Development

Recovery of AMF species from field soils

Soil samples were collected from cassava fields during the dry period in December 2012 in three agro-ecological zones (Azaguié, Yamoussoukro and Abengourou), which are considered important cassava production areas in Côte d'Ivoire (Chaleard, 1988; Kouadio et al., 2010). Twelve soil samples (1 kg each) were collected at a depth of 0–20 cm from cassava plant rhizospheres, using the sampling method of Huang and Cares (2004), from four fields in each agro-ecological zone (**Table 1**). AMF were extracted from 50 g of field soils by wet sieving (Gerdemann and Nicolson, 1963) using 4 sieves (45, 90, 125, and 500 μm). AMF species were identified as described

TABLE 1 | Geographic coordinates of fields.

Zone	Field	Area (ha)	Point	Geographic coordinates		
				North	West	Altitude (m)
ABENGOUROU	Aniansué 1 (AB1)	1–2	Ab 1/1	06°40′20.10″	003°38′57.72″	166
			Ab 1/2	06°40′20.64″	003°38′56.34″	164
			Ab 1/3	06°40′20.28″	003°38′58.56″	164
	Aniansué 2 (AB2)	2–3	Ab 2/1	06°39′51.96″	003°41′07.80″	170
			Ab 2/2	06°39′53.82″	003°41′06.66″	167
			Ab 2/3	06°39′50.76″	003°41′06.06″	164
	Dramanekro 1 (AB3)	1–2	Ab 3/1	06°42′38.40″	003°37′03.36″	176
			Ab 3/2	06°42′37.44″	003°37′04.80″	176
			Ab 3/3	06°42′37.32″	003°37′05.34″	177
	Dramanekro 2 (AB4)	1–2	Ab 4/1	06°41′48.96″	003°38′19.08″	151
			Ab 4/2	06°41′50.82″	003°38′17.94″	154
			Ab 4/3	06°41′51.60″	003°38′16.50″	152
AZAGUIE	Ahoua 1 (AZ1)	1–2	Az 1/1	05°40′21.06″	004°02′33.42″	51
			Az 1/2	05°40′22.38″	004°02′32.64″	50
			Az 1/3	05°40′22.86″	004°02′31.38″	50
	Ahoua 2 (AZ2)	1–2	Az 2/1	05°38′36.30″	004°03′24.54″	48
			Az 2/2	05°38′34.32″	004°03′18.36″	47
			Az 2/3	05°38′35.88″	004°03′21.36″	46
	M'Bromé 1 (AZ3)	2–3	Az 3/1	05°39′38.28″	004°09′00.00″	53
			Az 3/2	05°39′37.14″	004°08′57.60″	49
			Az 3/3	05°39′35.94″	004°08′57.54″	47
	M'Bromé 2 (AZ4)	2–3	Az 4/1	05°40′04.98″	004°08′43.44″	46
			Az 4/2	05°40′01.56″	004°08′43.32″	47
			Az 4/3	05°39′58.74″	004°08′43.32″	52
YAMOUSSOUKRO	Logbakro 1 (YA1)	1–2	Ya 1/1	06°44′13.50″	005°12′24.60′	223
			Ya 1/2	06°44′14.28″	005°12′24.60″	225
			Ya 1/3	06°44′15.42″	005°12′23.10″	225
	Logbakro 2 (YA2)	1–2	Ya 2/1	06°44′01.68″	005°11′44.22″	207
			Ya 2/2	06°44′02.64″	005°11′45.60″	210
			Ya 2/3	06°44′02.34″	005°11′46.86″	210
	Céman (YA3)	1–2	Ya 3/1	06°53′14.46″	005°17′54.96″	237
			Ya 3/2	06°53′15.06″	005°17′54.90″	233
			Ya 3/3	06°53′15.42″	005°17′54.00″	235
	Zambakro (YA4)	2–3	Ya 4/1	06°43′30.12″	005°24′15.48″	162
			Ya 4/2	06°43′30.36″	005°24′14.52″	159
			Ya 4/3	06°43′28.68″	005°24′14.16″	159

below, and selected according to abundance, occurrence and ubiquity. Species occurrence was determined as the number of fields in which a particular species was found divided by the total number of fields. Each morphotype was maintained in monoculture using variety "Yavo" as a host, in 2-L pots containing soil + sand (3:1 v/v) sterilized by autoclaving.

Isolation of abundant AMF species by trapping

Field soils were used to trap AMF species using the cassava variety "Yavo." The collected soils were mixed with a substrate composed of a mixture of soil and sand (3:1,v/v) sterilized at 120°C and 2 bars for 1 h on two successive days (Bâ et al., 2000) in a 1:1 ratio (v/v). Soils were placed in 10-L pots. The pots were watered every other day with 400 ml of water without fertilizer. After 4 months, cassava plants showed good physiological development. Soils were carefully recovered using a spatula after 4 months of cultivation. 50 g of soils were used to identify abundant and ubiquitous AMF morphotypes.

Morphological Identification of Selected AMF Spores

Spores were extracted by wet sieving and mounted between slide and coverslip in polyvinyl-lacto-glycerol and Melzer's solution

(Morton et al., 1993). They were observed under a microscope and morphologically identified based on their color, shape, and composition of their walls (Schenck and Perez, 1990; see http://invam.wvu.edu/, http://www.zor.zut.edu.pl/ collection websites). Spores were photographed using a Motic BA310 Trinocular compound microscope.

Molecular Identification of Selected AMF Morphotypes

In order to confirm the morphological identification of the selected AMF species, PCR amplification was performed using primers LR1-LSUmAr/LR1- LSUmBr. For each selected species, 10 spores were collected in a 1.5 ml microfuge tube for DNA extraction using the DNeasy Plant Mini Kit (Qiagen). A first PCR amplification using primers LR1 and LSUmAr (van Tuinen et al., 1998; Stockinger et al., 2009) and a nested PCR using LR1 and LSUmBr primers (Krüger et al., 2009; Stockinger et al., 2010) were performed in 30 cycles (95°C 5 min; 94°C 1 min; 58°C 30 s; 72°C 45 s; 72°C 5 min; 25°C 1 s). The size of PCR products were checked on 1% agarose gels. For sequencing, the amplified PCR products were purified using a commercial kit (Nucleospin Extract II) and cloned using the TOPO TA Cloning® Kit (Invitrogen) according to the manufacturer's instructions. Three positive clones were selected for sequencing by GATC Biotech (Konstanz, Germany) using the directional Sanger method. Sequence analyses were done by Blast with NCBI and MAARJAM databases and phylogenetic analyses were performed using the software MEGA 6.06 and the neighbor-joining method (Saitou and Nei, 1987).

Mycorrhizal Inoculum Production

To produce inoculum, the selected strains were grown individually in the greenhouse in a sterile substrate containing cassava plants. For single inoculation, the inoculum (S1: *A. colombiana* or S2: *A. appendicula*) was in the form of 50 g of sterile substrate (soil + sand; 3:1, v/v) containing pieces of mycorrhizal roots, hyphae and about 350 AMF spores. The soil characteristics were (pH = 7.1; organic matter = 2.81%; total nitrogen = 0.15%; available phosphorus = 55 mg/kg) and for the sand (pH = 6.7; organic matter = 0.17%; total nitrogen = 0.01%; available phosphorus = 2 mg/kg). For dual inoculation, the two inocula (25 g each) were mixed to make 50 g.

Evaluation of the Impact of Selected AMF on Cassava Growth and Phosphorus Status in Greenhouse

Experimental design and culture condition

The greenhouse experiment was conducted comparing three AMF combinations (S1, S2, and S1S2) plus the control S0, and 6 replicates (completely randomized blocks) over 4 months. Pots were filled with 8 kg of substrate (soil + sand; 3:1, v/v). Each pot contained one cassava plant that was watered every other day with 400 ml of water without fertilizer.

Assessment of mycorrhizal development

For assessment of root colonization by AMF, fine cassava roots were sampled 4 months after planting, with three replicates per treatment. Each treatment contained three plants. Roots were rinsed and cut into 1–2 cm fragments. These roots fragments were cleared by boiling in 10% (w/v) KOH and stained with 0.05% (v/v) trypan blue in lactoglycerol according to the method of Phillips and Haymann (1970). Ten pieces of roots per plant were placed in glycerol (50%) between slide and coverslip (Kormanik and McGraw, 1982) and observed under an optical microscope. The colonized roots were observed and evaluated according to Trouvelot et al. (1986).

Assessment of the Mycorrhizal Inoculation on Cassava Growth and Phosphorus Levels in Greenhouse

Plant growth was assessed by measuring plant height and foliar surface area using Connor's et al. methods (Connor et al., 1981), and total fresh and dry matter. Plant total fresh matter was determined using an OHAUS balance and the dry matter after oven drying at 80°C for 48 h. Eight young cassava leaves were analyzed for P content after 4 months by the mineralization and calcination method using a Tecator model 40 instrument (Sidney, 1984). All measurements were done in triplicate.

Evaluation of the Impact of Selected AMF Species on Cassava Tolerance and Resistance to Root-Knot Nematode *Meloidogyne* spp. in Greenhouse

Preparation of nematode inoculum

The nematode inoculum was made using a population of *Meloidogyne* spp., isolated from tomato galls grown in a greenhouse. The inoculum was prepared by finely cutting infected tomato roots that were soaked in a jar containing NaClO (0.25%) and shaken for 2 min (Hussey and Barker, 1973). Nematode eggs and juveniles were collected on a 25 μm sieve, rinsed in sterile water and counted under a 40x binocular magnifier. A suspension of 1000 nematodes (juveniles + eggs) was added to each cassava plant (each pot).

Experimental design and culture condition

A 4 × 2 factorial experiment with three replicates and completely randomized design was carried out in the greenhouse over a 4-month period. One factor was the AMF treatment: each selected AMF strain was used either in single inoculation (S1, S2) or dual inoculation (S1S2) and a non-inoculated control (S0). The other factor was inoculation with root knot nematodes, either at the same time as the AMF inoculation (I2), or 1 month after AMF inoculation (I4). Inoculation with nematodes was achieved by loading aliquots of 1000 freshly hatched juveniles and eggs suspended in distilled water into 5-cm-deep holes equidistant around each plant. Pots were filled with 8 kg of substrate (soil + sand; 3:1, v/v). Each pot contained one cassava plant that was watered every other day with 400 ml of water without fertilizer.

Assessment of the mycorrhizal inoculation impact on nematode population

At the end of the experiment, nematodes and eggs were counted according to Daykin and Hussey (1985). The total content of phenols, which are an indicator of plant defense compounds against nematode attack, in roots was estimated using a colorimetric method (Singleton et al., 1999). Total

phenol content was measured after 2 h at room temperature incubation by absorbance at 765 nm, measured in a Jenway 7315 Spectrophotometer. The quantification was done using a gallic acid calibration curve. Biomass (total fresh matter, total dry matter) and phosphorus in cassava leaves were also determined as described above.

Impact of the Selected AMF Inoculation on Cassava Resistance to Water Stress in Greenhouse

Experimental design and culture condition

Before the main experiment, a pot containing 8 kg of soil was filled with water until saturation. Excess water was then allowed to drain over 2 days and field capacity (FC) was measured according to Colombani et al. (1973). A 4 × 2 factorial experiment with three replicates and a completely randomized design was carried out in a greenhouse over a 4-month period. One factor was the AMF treatment: each selected AMF strain applied either in single inoculation (S1, S2) or dual inoculation (S1S2), and an non-inoculated control (S0). The other factor was water regime. All plants were watered to 100% of FC for 2 months after planting. They were then divided in two groups for the remaining 2 months. One group was regularly watered to 100% FC while the other was watered to 10% of FC. That watering regime corresponded to 400 mm of water/year, which can be considered a severe water stress to cassava (FAO, 2013). The total number of plants for the experiment was 48. Mycorrhizal abundance was estimated on roots harvested monthly using the Trouvelot et al. (1986) method. Foliar surface areas were measured on the 3rd and 4th month, and biomass was determined after 4 months, as described previously. The chlorophyll a content of young plant leaves was determined using the method of Arnon (1949). Soluble sugar content (TS) of young leaves, a measure of osmoprotection during water stress, was determined according to Dubois et al. (1956) using the Jenway 7315 spectrophotometer.

Cassava Plant Inoculation under Field Conditions

Study area

An experimental area of about 2500 m^2 was set up in Duokro, 15 km from Yamoussoukro in Côte d'Ivoire, to test the effect of local and commercial strains of mycorrhizae on colonization, and cassava yield during the 2015–2016 season. The average temperature in this region over the season was $32 \pm 2°C$, average total annual rainfall is 1495 mm and average annual humidity is $79 \pm 12\%$.

Experimental design

The field experiment was established using a randomized complete block design with five inoculation treatments: *Ambispora appendicula* (T1), *Acaulospora colombiana* (T2), the dual inoculant *A. colombiana–A. appendicula* (T3), a commercial inoculum Mykepro P501 produced by PremierTech biotechnologies (T4), and a non-inoculated control (T5). The commercial inoculant is composed of a single species *Rhizophagus intraradices*. Mineral fertilizer (30 kg N/ha, 20 kg P/ha and 50 kg K/ha) was applied to the non-inoculated control plots. For each treatment, there were three replicates, resulting in a total of 18 plots. Cassava was planted in ridges 80 cm wide and 20 cm high, separated by 20-cm wide furrows, following the contour. The blocks were arranged perpendicular to the slope. Each plot contained 40 plants, including 16 inoculated plants and 2 lines of 24 plants curbs to limit edge effects. The planting density was 10,000 plants/ha. 25-cm long cassava cuttings, 1.5–2.5 cm thick, with 5–6 nodes were planted in 20-cm deep holes. Cuttings were inserted diagonally in order to promote sprouting. No irrigation water or pesticides were applied. Cassava plant inoculation was done on farm. For the native inoculum, each plant was inoculated with 100 g of inoculums containing 1000 spores + mycorrhizal roots. For the commercial inoculum 6 g containing 3000 spores + mycorrhizal roots was added to each plant, corresponding to triple the dose applied in temperate zones.

Assessment of cassava tuber yield

Tubers were harvested on March 10, 2016. Fresh tubers were weighed and the yield converted to t/ha. When a significant difference was observed in yield compared to plots without AMF, the gain (G) in yield was calculated according to the formula:

$$G(\%) = 100 * \left(\frac{\text{Yield with AMF} - \text{Yield without AMF (control)}}{\text{Yield without AMF (control)}} \right)$$

Statistical Analyses

All experimental data in greenhouse were subjected to statistical analyses by performing either one or two-way analysis of variance (ANOVA) using Statistica 7.1. The significance of the treatment effects was determined using LSD Fisher test with $P = 0.05$.

All field experiment data were analyzed by ANOVA. Fisher's LSD test was also used to determine whether or not treatments were different from each other at $P < 0.05$.

RESULTS

Selection and Identification of Potential Useful AMF Species for Cassava Crop Inoculation

Using spore characteristics, several AMF species were identified (**Table 2**) in the three agricultural zones. The species *Acaulospora scrobiculata*, *A. colombiana*, *A. appendicula, Claroideoglomus etunicatum, Glomus glomerulatum* and an unidentified species *Glomus Sp*2 were abundant at various levels in all three zones. However, when the cassava cultivar Yavo was inoculated with field soils in greenhouse, only *A. colombiana*, *A. appendicula* were confirmed in all soils, with *A. appendicula* having low abundance (**Table 2**). These two morphotypes (LBVM01 and LBVM02), which were present in all soils and also abundant in trapped communities, were considered as good candidates for cassava inoculation. They were initially identified based on morphological criteria using PVLG and Melzer's reagent as *Acaulospora* sp. and *Ambispora* sp. (**Figure 1**). An expected 700 bp fragment was amplified from each morphotype. Both BLAST and phylogenetic analyses allowed the identification of the morphotype LBVM01 as *A. colombiana* (Genbank accession number KX168435) and the other LBVM02 as *A. appendicula* (Genbank accession number KX168436) (**Table 3**; **Figure 2**).

TABLE 2 | Abundance of efficient arbuscular mycorrhizal fungi (AMF) species.

AMF species	AB1	AB2	AB3	AB4	AZ1	AZ2	AZ3	AZ4	YA1	YA2	YA3	YA4	Occurrence (%)
NATIVE AMF SPECIES RECOVERED FROM CASSAVA FIELD SOILS													
Acaulospora excavata	+	+	+	+	–	–	–	–	++	+	+	+	67
Acaulospora scrobiculata	++	+++	+++	++	++	+	++	+	++	++	++	++	100
Acaulospora columbiana	**+**	**+**	**++**	**++**	**+++**	**++**	**+**	**++**	**++**	**+**	**++**	**++**	**100**
Ambispora apendicula	**+++**	**++**	**++**	**+++**	**+++**	**+++**	**+++**	**+++**	**+**	**++**	**+**	**++**	**100**
Claroideoglomus etunicatum	++	++	++	++	+	+	+	+	++	++	++	++	100
Glomus aureum	–	+	+	+	+	+	+	+	–	–	–	–	58
Glomus glomerulatum	++	+	++	++	++	++	++	++	+++	+++	+++	++	100
Glomus clavisporum	–	+	+	–	–	++	++	–	–	–	–	++	42
Glomus sp.1	++	+	++	+	–	–	–	–	+	++	++	++	67
Glomus sp.2	++	+++	++	++	+++	++	+++	+++	+++	++	++	++	100
Funneliformis mossae	–	–	–	–	+	–	–	–	–	–	+	–	17
Rhizophagus intraradices	++	++	++	+++	–	–	–	–	+	+	+	+	67
Rhizophagus manhiotis	–	–	–	–	+	+	–	–	–	–	–	–	17
Sclerocystis sinuosum	+	–	–	++	+	+	++	–	+	–	+	+	67
Septoglomus constrictum	–	–	–	–	+	++	+	–	–	–	+	–	33
*Gigaspora*sp.1	–	–	–	–	–	+	–	–	–	–	–	–	8
Racocetra africana	–	–	–	–	+	+	+	+	+	+	+	+	67
Scutelospora sp.	+	–	+	+	+	+	+	+	–	+	+	+	83
AMF SPECIES TRAPPED AFTER 4 MONTHS FROM FIELD SOILS USING THE CASSAVA CULTIVAR YAVO													
Acaulospora scrobiculata	++	+	+++	–	–	–	+	+++	+	–	+++	–	58
Acaulospora columbiana	**+++**	**+++**	**+++**	**+++**	**+++**	**+++**	**+++**	**+++**	**++**	**+++**	**+++**	**++**	**100**
*Acaulospora*sp.1	+	+	–	–	+	+	–	–	–	–	–	–	33
Ambispora appendicula	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**100**
Glomus clavisporum	++	++	–	–	–	+++	++	++	+	–	–	+	58
Rhizophagus intraradices	++	++	++	+++	–	–	–	–	+	+	+	+	67
Gigaspora sp.1	++	++	+	+	++	+	–	+	–	–	–	–	58

AB, Abengourou; AZ, Azaguié; YA, Yamoussoukro; Field number, 1–2–3–4. –, absent (0 spore/g); +, present (1–2 spores/g); ++, abundant (3–5 spores/g); + + +, highly abundant (6–8 spores/g). bold indicates abundant and ubiquitous AMF species in both field soils and trapped culture.

Effect of *A. colombiana* and *A. appendicula* Single and Dual Inoculation on Cassava Growth and P Uptake

After 4 months of culture in greenhouse conditions (**Table 4**), all cassava plants inoculated (singly or dually) with *A. colombiana* and *A. appendicula* were mycorrhizal. Frequencies and intensities of mycorrhization did not differ significantly between single and dual inoculation (frequency of 26.7 and 48.3%, and intensity of 14.5 and 38.7%, respectively). No mycorrhizal structures were observed in cassava control plants. The foliar P content of cassava plants inoculated with *A. colombiana* was significantly ($p = 0.002$) improved (1.3-fold) compared to the non-inoculated control, whereas the *A. appendicula* single inoculation had no impact. The foliar P content of dual inoculated plants was significantly improved (1.5-fold) compared to the control. The growth parameters (plant height, foliar surface area, total dry and total fresh matter) of *A. colombiana* singly inoculated cassava plants were significantly improved ($p = 0.040$; $p = 0.008$; $p = 0.000$; $p = 0.001$, respectively) after 4 months in the greenhouse. The *A. appendicula* single inoculation had significant impact only on total fresh matter. However, dual inoculation significantly improved all parameters.

Susceptibility of *A. colombiana* and *A. appendicula* Inoculated Cassava Plants to Root-Knot Nematode *Meloidogyne* Spp

Four months after single or dual inoculation with *A. colombiana* or *A. appendicula*, in the presence of the nematode *Meloidogyne* spp., all treated cassava plants were mycorrhizal (**Table 5**). However, the frequencies and intensities of mycorrhization were significantly lower ($p = 0.002$ for both) in the roots of cassava plants that were co-inoculated with the nematode *Meloidogyne* spp. In this condition, the presence of *A. colombiana* and *A. appendicula* as single or dual inoculant significantly reduced nematode egg and population densities. In this experiment, none of the three mycorrhizal inoculation methods significantly affected foliar P contents. However, the single inoculation using *A. appendicula* and the dual inoculation significantly increased cassava total fresh biomass. When the nematodes were added 1 month after AMF inoculation, mycorrhizal root colonization levels were still high after 4 months (frequency 20 and 36.7%,

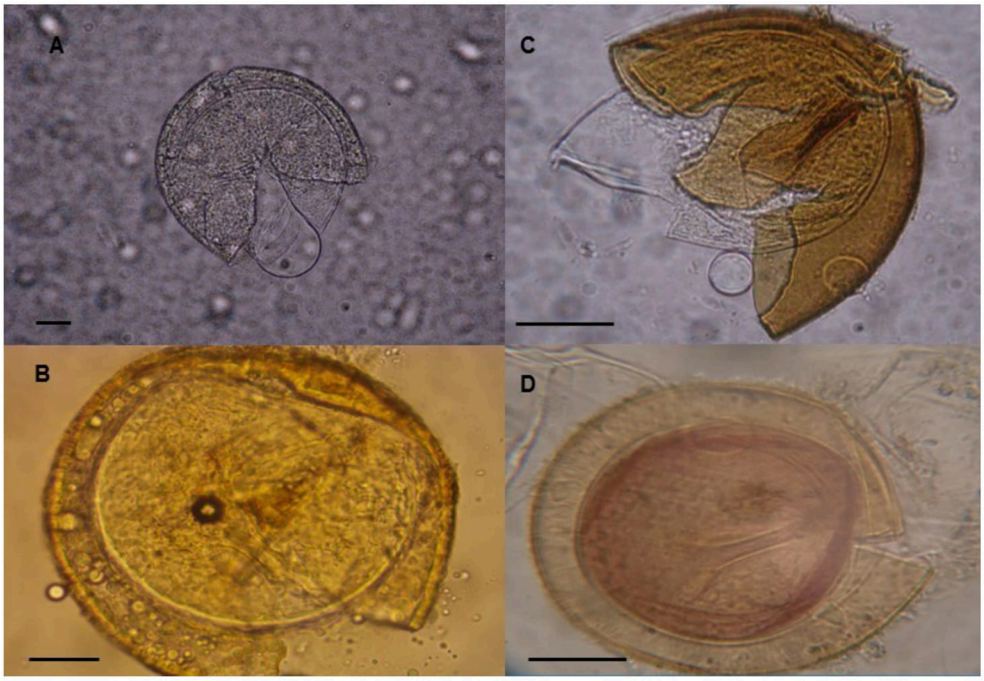

FIGURE 1 | Spores of arbuscular mycorrhizal fungi used in this study. (A) *Acaulospora colombiana* and **(C)** *Ambispora appendicula* stained with lactoglycerol polyvinyl. **(B)** *Acaulospora colombiana* and **(D)** *Ambispora appendicula* stained with Melzer's reagent. Scale bar = 1/25 μm.

TABLE 3 | Consensus identification of the two native species of arbuscular mycorrhizal fungi.

Morpho-species	Morphological identification	Molecular identification	Consensus species	Species accession number in databases
S1	*Acaulospora* sp.	*A. colombiana*	*A. colombiana*	KX168435
S2	*Ambispora* sp.	*A. appendicula*	*A. appendicula*	KX168436

and intensity 15 and 21.5% for single and dual inoculation, respectively). In this case, only the single inoculation with *A. colombiana* significantly ($p = 0.006$) reduced the number of nematode eggs in the cassava roots. Also, only *A. colombiana* as a sole inoculants significantly increased the foliar P content. However, only the dual inoculation increased cassava plant fresh and dry biomass. Phenol contents of AMF pre-inoculated cassava roots were significantly higher than the controls (not inoculated with AMF).

Development of Single and Dual *A. colombiana* and *A. appendicula* Inoculated Plants during Water Stress

When cassava plants were well watered (100% FC), mycorrhizal colonization significantly increased during the first 2 months when inoculated with *A. colombiana* (from 23 to 46.7%) and with the dual inoculation (10 to 23.7%), but not with *A. appendicula* (constant at 10%) (**Table 6**). One month after water stress was initiated (10% FC), mycorrhizal colonization declined significantly in the cassava roots for all mycorrhizal treatments (**Table 6**). After 2 months of water stress the same trend was observed for all mycorrhizal treatments. However, *A. colombiana* colonization of cassava roots remained stable at 26.7% in the fourth month and this was significantly higher ($p = 0.049$) than *A. appendicula*. Under water stress, the presence of *A. colombiana* had a significant positive impact on the growth parameters (total dry matter, fresh matter and foliar surface area) (**Table 7**). In comparison, neither the single inoculation with *A. appendicula* nor the dual inoculation positively impacted these growth parameters. Moreover, under this severe water stress, the cassava plants inoculated with *A. colombiana* significantly improved all functional traits measured, including chlorophyll a (0.209 mg/g FM) and total sugar content (496 μmol/mg FM), compared to non-inoculated plants (**Table 7**).

Impact of Mycorrhizal Inoculation on Cassava Yield under Field Conditions

The impact on cassava yields of the native AMF in single and dual inoculation was assessed in comparison to the commercial inoculant MykePro and the standard chemical fertilizer application (**Figure 3**). The results showed that the chemical fertilizer NPK significantly improved cassava yield (11.38 t/ha) compared to non-inoculated control (8.21 t/ha). This represents a yield gain of 38.5%. Of the AMF treatments, only *A. colombiana* single inoculation and the dual inoculation significantly ($p = 0.003$) improved cassava yield (9.58 and 9.81 t/ha, respectively) compared to non-inoculated control

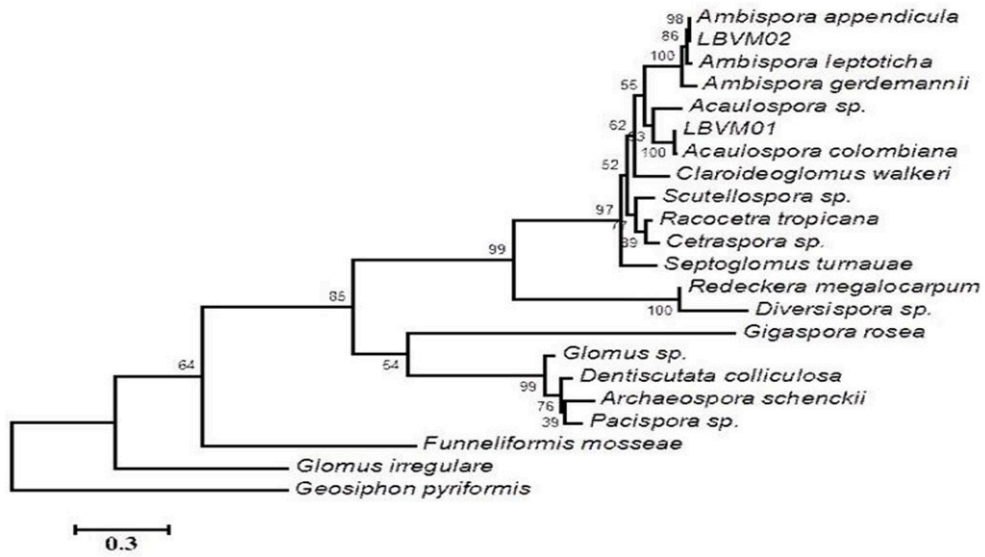

FIGURE 2 | Phylogenetic identification of LBVOl and LBVM02 isolated from field soils. SSU-ITS-LSU gene sequences from AMFs species *Ambispora appendicula* (FN547527), *Ambispora leptoticha* (KC166277), *Ambispora gerdemanii* (KC166282), *Acaulospora* sp. (HF56794), *Acaulospora colombiana* (FR750063), *Scutellospora* sp. (AF396818), *Racocetra tropicana* (GU385898), *Cetraspora* sp. (HM565946), *Septoglomus tumauae* (KF060327), *Redeckera megalocarpum* (NR121478), *Diversispora* sp. (KJ850185), *Gigaspora rosea* (U60451), Glomus sp. (AB326023), *Dentiscutata colliculosa* (GQ376067), *Archaeospora schenckii* (KP144303), *Claroideoglomus walker* (KP191492); *Pacispora* sp. (JQ182768), *Funneliformnis mossae* (KM360085), *Glomus irregulare* (GU585513) and *Geosiphon pyriformis* (JX535577) were used for comparison. The tree was constructed by the neighbor-joining method using Mega version 6.

TABLE 4 | Impact of *A. colombiana* and *A. appendicula* single and dual inoculation on phosphorus nutrition and cassava growth after 4 months.

Treatment	Frequency of mycorrhization (%)	Intensity of mycorrhization (%)	Plant height (cm)	Foliar surface area (cm^2)	Total fresh matter (g)	Total dry matter (g)	*P* (%dm)
Control (S0)	0 ± 0	0 ± 0	34.1^{c} ± 0.9	898.3^{c} ± 38.96	47.4^{c} ± 2.5	15.9^{c} ± 0.2	0.17^{c} ± 0.008
A. colombiana (S1)	48.3^{a} ± 11.8	25.4^{a} ± 7.5	41.5^{ab} ± 0.7	1375.6^{ab} ± 162.3	56.9^{ab} ± 3.2	18.3^{b} ± 0.7	0.22^{b} ± 0.0
A. appendicula (S2)	26.7^{a} ± 4.5	14.5^{a} ± 3	36.7^{bc} ± 2.1	1219.1^{bc} ± 80.36	55^{b} ± 1	17.4^{bc} ± 0.6	0.19^{bc} ± 0.0
A. colombiana-A. appendicula (S1S2)	46.7^{a} ± 15.6	38.7^{a} ± 12.8	44.33^{a} ± 2.6	1711.6^{a} ± 54.01	60.7^{a} ± 2.9	20.3^{a} ± 2.2	0.26^{a} ± 0.016
***P* and *F*-VALUE**							
	$p = 0.018$	$p = 0.024$	$p = 0.040$	$p = 0.008$	$p = 0.000$	$p = 0.001$	$p = 0.002$
	$F = 4.19$	$F = 3.91$	$F = 4.454$	$F = 8.1851$	$F = 14.58$	$F = 7.93$	$F = 12.267$

dm, dry matter. All the values are means of the three replications (n = 3). Means with different letters were significantly different at 5% level.

(8.21 t/ha). This represents a yield gain of 19.4% for the dual inoculation and 16.6% for *A. colombiana*. *A. appendicula* and the commercial inoculant had no significant impact on cassava yield compared to the non-inoculated control.

DISCUSSION

This work aimed to select an abundant native AMF capable of improving cassava crop productivity via several mechanisms, namely improved plant growth, water stress tolerance and nematode resistance. This is an improvement on previous studies, which tended to focus on one aspect affecting cassava yield, without studying the possible interactions with nematodes and drought.

During this study, there was a difference in the way the two native AMF species impacted cassava plant growth in greenhouse conditions. It was shown that only *A. colombiana* significantly increased the plant growth parameters, such as foliar surface area, plant height and biomass (**Table 3**). It has been reported that several factors, such as environmental conditions and functional diversity, can affect nutrient exchange between the fungi and plant partners (Walder and van der Heijden, 2015). The experimental conditions used in this work might have been favorable to *A. colombiana*, which significantly improved P uptake compared to *A. appendicula*. Variable effects among endogenous single species due to the use of different culture media were also observed in other studies (Williams et al., 2012; Ortas and Ustuner, 2014). Also, the two native AMF species may differ in terms of regulation of genes involved

TABLE 5 | Impact of *A. colombiana* and *A. appendicula* single and dual inoculation on nematode communities and cassava plant growth.

Treatment	Frequency of mycorrhization (%)	Intensity of mycorrhization (%)	Egg density in the roots/g	Density of nematodes in the roots/g	Foliar P content	Biomass (g)		Phenols
					P (%dm.)	Total fresh matter (g)	Total dry matter (g)	Phenol concentration (mg EGA/l)
CO-INOCULATION WITH AMF AND NEMATODES (I2)								
Control (S0)	0 ± 0	0 ± 0	$3.7^{c} \pm 0.47$	$7^{b} \pm 4.24$	$0.21^{ab} \pm 0.03$	$46^{bc} \pm 1.5$	$14.1^{ab} \pm 0.6$	$95^{de} \pm 4.7$
Acaulospora colombiana (S1)	$6.7^{b} \pm 4.7$	$0.07^{b} \pm 0.04$	$2^{ab} \pm 0.0$	$2^{a} \pm 0.8$	$0.21^{ab} \pm 0.01$	$53.7^{abc} \pm 2.4$	$15.3^{ab} \pm 0.2$	$116.7^{cd} \pm 12$
Ambispora appendicula (S2)	$13.3^{b} \pm 9.4$	$5.5^{b} \pm 3.67$	$2^{ab} \pm 0.81$	$2.33^{a} \pm 1.24$	$0.22^{abc} \pm 0.00$	$51.5^{a} \pm 5.9$	$17.3^{a} \pm 4.4$	$98.3^{de} \pm 3.6$
A. colombiana—A. appendicula (S1S2)	$3.3^{b} \pm 4.7$	$0.03^{b} \pm 0.04$	$1.7^{a} \pm 0.94$	$2^{a} \pm 0.82$	$0.237^{ab} \pm 0.0$	$56.3^{a} \pm 7.2$	$15.6^{a} \pm 2.26$	$115.7^{cde} \pm 4.5$
INOCULATION WITH NEMATODES 1 MONTH AFTER AMF (I4)								
Control (S0)	0 ± 0	0 ± 0	$3.3^{bc} \pm 1.24$	$5.17^{ab} \pm 1.65$	$0.203^{b} \pm 0.01$	$45.1^{c} \pm 3.25$	$11.7^{b} \pm 0.22$	$88.5^{e} \pm 4$
Acaulospora colombiana (S1)	$30^{a} \pm 8.16$	$15.07^{a} \pm 3.2$	$1.7^{a} \pm 0.47$	$2.17^{a} \pm 0.13$	$0.255^{a} \pm 0.00$	$53.5^{abc} \pm 1.55$	$15.36^{ab} \pm 0.05$	$169.5^{a} \pm 4$
Ambispora appendicula (S2)	$20^{a} \pm 8.16$	$15.07^{a} \pm 6.3$	$2^{ab} \pm 0$	$2.67^{a} \pm 0.47$	$0.213^{ab} \pm 0.00$	$54.34^{ab} \pm 4.94$	$14.85^{ab} \pm 1$	$126^{bc} \pm 13.8$
A. colombiana—A. appendicula (S1S2)	$36.7^{a} \pm 9.4$	$21.4^{a} \pm 6.08$	$2^{ab} \pm 0$	$2.17^{a} \pm 0.13$	$0.227^{ab} \pm 0.01$	$55.5^{a} \pm 3.75$	$17.9^{a} \pm 1.13$	$150.17^{ab} \pm 4.24$
***P* and *F*-VALUE**								
AMF	$p = 0.002$	$p = 0.002$	$p = 0.006$	$p = 0.012$	$p = 0.47$	$p = 0.02$	$p = 0.045$	$p = 0.000$
	$F = 7.729$	$F = 7.8$	$F = 5.89$	$F = 5.044$	$F = 0.887$	$F = 4.35$	$F = 3.364$	$F = 12.52$
Nematodes	$p = 0.000$	$p = 0.000$	$p = 0.806$	$p = 0.741$	$p = 0.97$	$p = 0.91$	$p = 0.5$	$p = 0.000$
	$F = 22.5625$	$F = 41.77$	$F = 0.0625$	$F = 0.113$	$F = 0.001$	$F = 0.013$	$F = 0.47$	$F = 17.72$
AMF × Nematodes	$p = 0.010$	$p = 0.004$	$p = 0.874$	$p = 0.785$	$p = 0.234$	$p = 0.92$	$p = 0.24$	$p = 0.033$
	$F = 5.23$	$F = 6.483$	$F = 0.223$	$F = 0.357$	$F = 1.573$	$F = 0.17$	$F = 1.53$	$F = 3.7$

dm, dry matter, EGA, Equivalent Gallic Acid. All the values are means of the three replications (n = 3). Means with different letters were significantly different at 5% level.

TABLE 6 | Mycorrhizal frequency evolution from well-watered regime (100% of field capacity) at 1 to 2 months, to drought (10% of field capacity) at 2–4 months.

AMF frequency	1 month	2 months	3 months	4 months
Acaulospora colombiana	$23.3^{a} \pm 7.2$	$46.7^{a} \pm 11.86$	$23.3^{a} \pm 4.714$	$26.7^{a} \pm 4.7$
Ambispora appendicula	$10^{a} \pm 0$	$10^{b} \pm 0$	$20^{a} \pm 2.72$	$10^{b} \pm 4.7$
A. colombiana-A. appendicula	$10^{a} \pm 0$	$23.3^{ab} \pm 5.44$	$23.3^{a} \pm 7.2$	$16.7^{b} \pm 1.3$
Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0
***P*- and *F*-VALUE**				
	$p = 0.035$	$p = 0.016$	$p = 0.048$	$p = 0.006$
	$F = 4.714$	$F = 6.377$	$F = 4.121$	$F = 9.067$

Means with different letters were significantly different at $p < 0.05$.

in *P* uptake. Such observations were made when maize plants were individually inoculated with different AMF species (Tian et al., 2013). In our study, single inoculation with *A. appendicula* had no effect. Meanwhile dual inoculation with both species positively improved P uptake and cassava plant growth. Similar observations were made when citrus was treated with different AMF species using a dual inoculation approach (Ortas and Ustuner, 2014). It could mean that when used together as dual inoculants, the two native AMF species induce phosphate transporters in cassava plants, as reported for different AMF species used to inoculate maize plants (Tian et al., 2013).

The study on the interaction between the two native AMF and *Meloidogyene* spp. in greenhouse revealed that negative effects of the AMF against the nematode (reduction of egg and nematode densities) were clearly observed, whether or not AMF and nematodes were co- or post-inoculated (1 month later). Interestingly, the presence of the nematode exerted a negative effect on the AMF, by reducing mycorrhizal intensities and frequencies in the case of simultaneous inoculation. Both types of interactions between nematode and AMF have already been reported. These mutual negative effects occur when fungi and nematodes are competing for space and nutrients (Schouteden et al., 2015). For example, the fungus *Scutellospora heterogama* exerted a biocontrol effect on the sedentary endoparasitic nematode *Meloidogyne incognita* (reproduction was reduced) only when it was pre-inoculated whereas co-inoculation had no effect (Dos Anjos et al., 2010). Such observations have also been made for migratory endoparasitic nematodes. For example, it was shown that *Radopholus similis* and *Pratylencus coffeae* affected the frequency of *Funneliformis mosseae* colonization in banana, but not the intensity (Elsen et al., 2003a,b). In contrast, root colonization by *R. irregularis in vitro* banana plantlets was not affected either by *R. similis* (Koffi et al.,

TABLE 7 | Impact of *A. colombiana* and *A. appendicula* single and dual inoculation on cassava plant growth and physiological traits 2 months after initiation of water stress.

AMF	Foliar surface area (cm^2)		Total fresh matter		Total dry matter		Chlorophyll a (mg/g FM)		Total sugar content (µmol/mg FM)	
	Well-watered	Drought	Well-watered	Drought	Well-watered	Drought	Well-watered	Drought	Well-watered	Drought
Control (S0)	$4362.67^{b} \pm 496$	$39.4^{c} \pm 50.9$	$218.9^{b} \pm 4.2$	$139^{d} \pm 4.6$	$106.74^{c} \pm 6.7$	$88.7^{d} \pm 3.6$	$0.178^{bc} \pm 0.0$	$0.123^{cd} \pm 0.0$	$518^{a} \pm 43.7$	$362.5^{b} \pm 7$
A.colombiana (S1)	$6179.9^{a} \pm 608.3$	$145.5^{c} \pm 23.2$	$243.53^{a} \pm 14.5$	$180^{c} \pm 8.9$	$149.3^{a} \pm 8.32$	$109.07^{c} \pm 9.97$	$0.164^{abc} \pm 0.0$	$0.209^{a} \pm 0.0$	$523.4^{a} \pm 23.5$	$496^{a} \pm 32.4$
A.appendicula (S2)	$4872.3^{b} \pm 589.3$	$13.3^{c} \pm 17.17$	$231.35^{ab} \pm 2.56$	$156^{d} \pm 6.1$	$130.97^{b} \pm 4.96$	$78.37^{d} \pm 4.6$	$0.17^{bc} \pm 0.01$	$0.078^{d} \pm 0.0$	$480.4^{ac} \pm 10.7$	$435.6^{abc} \pm 26$
A.colombiana—A.appendicula (S1S2)	$4617.3^{b} \pm 419.4$	$52.3^{c} \pm 49.5$	$217.03^{b} \pm 12.4$	$153^{d} \pm 11.8$	$109.7^{c} \pm 3.3$	$87.87^{d} \pm 8.7$	$0.18^{bc} \pm 0.01$	$0.15^{bc} \pm 0.01$	$446.5^{abc} \pm 29.8$	$351.5^{bc} \pm 25$
P and F-VALUE										
AMF	*p = 0.002*		*p = 0.000*		*p = 0.000*		*p=0.017*		*p=0.032*	
	F = 5.7652		*F = 9.970*		*F = 19.240*		*F = 4.7855*		F=3.769	
Water regime	*p = 0.000*		*p = 0.000*		*p = 0.000*		*P = 0.010*		*p=0.009*	
	F = 763.3963		*F = 238.107*		*F = 97.697*		*F = 8.7654*		F=8.748	
Strain × Water regime	*p = 0.008*		*p = 0.492*		*P = 0.007*		*P = 0.009*		P = 0.449	
	F = 4.4874		*F = 0.838*		*F = 5.815*		*F = 5.6544*		F=0.928	

FM, Fresh matter. All the values are means of the three replications (n = 3). Means with different letters were significantly different at 5% level.

2013) or by *P. coffeae* in transformed carrot roots (Elsen et al., 2003c).

Overall, in the presence of these native AMF, cassava plants continued to grow even though nematodes were present. It appears that the mycorrhizal cassava plants were either resistant (e.g., suppression or reduction of the nematode reproduction) or tolerant (low or no suppression in cassava plant growth) to nematodes, as reported in other studies (Hussey and Roncadori, 1982; Affokpon et al., 2011). However, the mechanism of the bioprotection conferred to cassava plants by the native AMF against the root-knot nematode *Meloidogyne* spp. is not yet understood. It may be due to the production of phytochemical inhibitors of nematodes, as was observed elsewhere. Indeed, in this work it was observed that phenolic compounds were significantly increased in cassava plant roots when nematodes were post-inoculated. Previous work has shown production of phenolic compounds to be a plant defense mechanism against nematode attacks (Zhu and Yao, 2004; Xu et al., 2008). Elsewhere, accumulation of phenolic compounds has been observed in mycorrhizal *Impatiens balsamina,* an ornamental plant, in presence of *M. incognita* (Banuelos et al., 2014). Singh et al. (1990) concluded that the pre-inoculation of plants, coupled with biochemical changes are responsible for resistance to nematodes. In contrast, when the cassava plants were co-inoculated with the AMF and the nematodes, there was no significant increase in phenolic compounds. Obviously there may be another mechanism involved in the inhibition of *Meloidogyne* spp. activity. For example, there was an up regulation of mycorrhiza-induced plant defense genes against the ectoparasitic nematode *Xiphinema index* in grapevine plants pre-inoculated with *R. intraradices* (Hao et al., 2012).

Besides its capacity to promote cassava growth and enhance resistance and tolerance to the root-knot nematode, *A. colombiana* also conferred water stress tolerance to cassava plants under severe drought condition. This AMF species significantly improved cassava plant growth under water stress. It was observed that mycorrhizal frequencies decreased gradually during the period of drought for all treatments, compared to the 100% FC water regime. However, despite severe water stress, the mycorrhizal colonization frequencies of *A. colombiana* remained higher than the control and stable over time. This water stress tolerance could be the result of *A. colombiana* promoting specific plant stress resistance response during the drought period, as suggested by others (Augé, 2001). For example the presence of this AMF may enhance photosynthetic activity due to the high levels of chlorophyll a and total sugars in *A. colombiana* colonized cassava plants compared to non-mycorrhizal plants (Mathur and Vyas, 1995).

Overall, this study clearly showed the multiple functions of the native AMF species *A. colombiana*. Importantly, *A. colombiana* was dominant in all three study areas and was persistently found and easily produced in trap culture. Abundance and persistence of AMF species are very important for efficient AMF species selection to ensure potential inocula are not lost during trap culture propagation (Trejo-Aguilar et al., 2013). This is essential, as the most widespread method for inoculum propagation is the use of trap plants (Berruti et al., 2016).

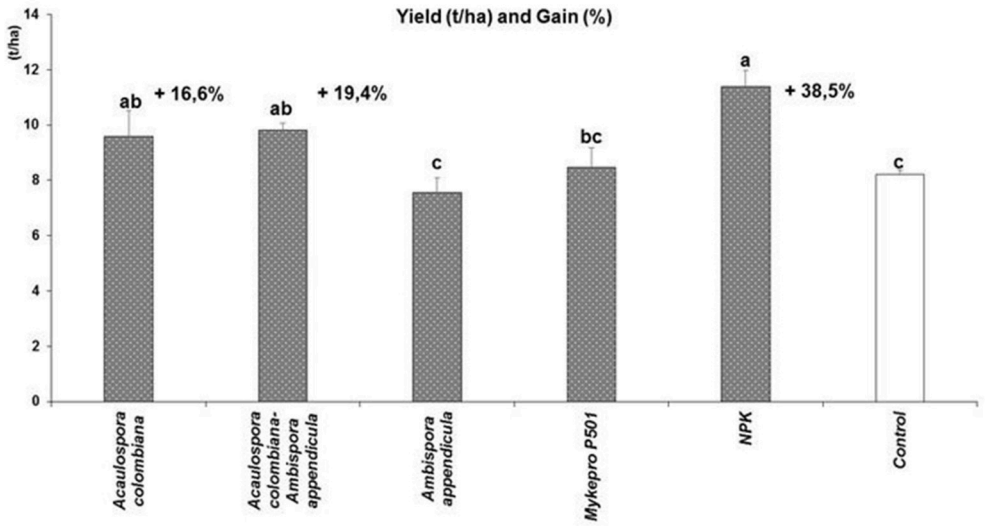

FIGURE 3 | Yield and yield gain of fresh cassava tubers as affected by inoculation with arbuscular mycorrhizal fungi or application of chemical fertilizer, under field conditions. Columns with the same letter are not significantly different at $P = 0.05$.

Under field conditions, *A. colombiana* showed a good potential for improving cassava productivity. The dual inoculation using the two native AMF species also increased cassava yield under field conditions. This opens up the possibility of using single and dual inoculation of these two native AMF species to improve cassava productivity in the field. During this study the native inoculants performed better than the commercial inoculant. Indeed, the origin and the composition of AMF are very important factors to take into account for inoculum development (Berruti et al., 2016). It has been shown that native AMF have higher efficiency in terms of plant protection against nematode (Affokpon et al., 2011) and stress tolerance (Ruiz-Lozano and Azcón, 2000) than commercial inoculants generally used in the field. Commercial inoculants are generally comprised of AMF species that can be considered as exotic species in tropical and subtropical regions (Oliveira et al., 2005; Schreiner, 2007). One main drawback in the use of commercial inoculants is the fact that the species used might not survive the competition with local AMF communities. Rodriguez and Sanders (2015), who discussed this issue, recommended research to understand local communities through metagenomics and genetic studies. The use of native inoculants comprised of native AMF like *A. colombiana* is highly recommended as an alternative to exotic species (Oliveira et al., 2005). As a persistent and abundant generalist, *A. colombiana* may have been a good competitor under field conditions, as in the greenhouse. Moreover, since commercial inoculants can be either ineffective (Faye et al., 2013) or badly formulated (Corkidi et al., 2004), the use of *A. colombiana* is more likely to be affordable and effective for cassava farmers in tropical and subtropical regions.

In conclusion, this study clearly points out the potential of *A. colombiana* as a native AM fungus suitable for inoculating cassava. The process developed in this study to select the multipurpose (plant growth improvement, water stress tolerance and nematode resistance) AMF species *A. colombiana* for cassava could be applied to efficiently select effective AMF inocula for other crops.

AUTHOR CONTRIBUTIONS

This work is done is the scope of a project in the Laboratoire de Biotechnologie Végétale et Microbienne under the supervision of ZA. SJ designed and run all the experiments as a Ph.D. student. KC helped in designing the PCR amplification protocols and sequence analyses. VR was a cosupervisor of this work as a collaborator on this project. ZA is the coordinator of the project and SJ supervisor.

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