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Indigenous arbuscular mycorrhizal fungi associated with tree species of the agroforestry systems of Rwanda and their potential to colonize maize roots

Jean de Dieu Habiyaremye[1,2*], Catherine Muthuri[3], Viviene Matiru[4], John Nyaga[3], Athanase Mukuralinda[5], Vicky Ruganzu[6], Jean Damascene Ngabonziza[6] and Fergus Sinclair[3]

[1]Pan African University Institute for Basic Sciences, Technology and Innovation, Nairobi, Kenya.
[2]Department of Mathematics, Science and Physical Education, School of Education, University of Rwanda, Kigali, Rwanda.
[3]World Agroforestry Centre, Nairobi, Kenya.
[4]Botany Department, School of Biological Sciences, Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.
[5]World Agroforestry Centre, Kigali, Rwanda.
[6]Rwanda Agriculture Board, Kigali, Rwanda.

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Arbuscular Mycorrhizal Fungi (AMF) form symbiotic associations with plant roots and can help mobilize nutrients from soil to the plant. The current study hypothesized that agroforestry systems of Rwanda harbor AMF with the potential to colonize roots of crops and hence enhance productivity. AMF spores were extracted from soil samples collected around most dominant tree species in Bugesera and Rubavu districts, respectively, representing semi-arid and sub-humid agro-ecological zones of Rwanda. The spores were morphologically identified and trap cultures for the most three predominant AMF spore morphotypes were made. This was followed by in-situ inoculation of maize with the fresh inocula from the trap cultures. Four different AMF genera were detected; *Glomus*, *Gigaspora*, *Scutellospora* and *Acaulospora.* All genera were found in both agroecological zones and in soil samples from all the host tree species with *Glomus* being the predominant group. All the maize inoculated with AMF had their roots colonized and *Gigaspora* performed best. The mean percentage root colonization varied between 40 and 70%. The study showed that soils under agroforestry systems of Rwanda harbor AMF with capability to colonize maize roots. These findings could be exploited in a view of selecting and developing well performing and adapted inocula to be used as bio-fertilizer.

Key words: Arbuscular Mycorrhizal Fungi, agroforestry system, root colonization, maize.

INTRODUCTION

Crop productivity is decreasing in Rwanda mainly due to the decline in soil fertility associated with many other constraints such as, the overexploitation of lands caused by high population density, land degradation and

*Corresponding author. E-mail: jdhabiyaremye@gmail.com.

fragmentation, deforestation, and water scarcity (Habiyaremye et al., 2015). Maize, as one of the most important crops in Rwanda, was identified among the priority crops by the Government of Rwanda within the context of the National Crop Intensification Programme. The plant plays an important role in food security and income generation for the majority of Rwandese and the whole sub-Saharan Africa (Nyaga et al., 2017). Maize is planted in most parts of Rwanda but requires substantial inputs of nutrients to produce high yield. While most options to improve crop productivity involve the use of expensive inputs that inherently increase environmental risks that farmers are often unable or unwilling to bear, it is necessary to investigate alternative eco-efficient options that farmers can afford in order to raise their production systems. In this perspective, much focus should be given to better understanding of the impact of trees on soil microorganisms with specific emphasis on Arbuscular Mycorrhizal Fungi (AMF).

AMF, plant root-inhabiting soil fungi, form obligate symbiotic associations with over 80% of terrestrial plant families (Smith and Read, 2008; Harley and Smith, 1983). AMF are ubiquitous in almost all plant communities in both natural and managed ecosystems, even though their number has decreased due to tillage, removal of topsoil, erosion, fumigation and over-fertilization (Rajah and Tang, 2005). They are widespread in tropical soils and are associated with a wide variety of plant species, including both crops and trees (Sieverding, 1991; Atayese et al., 1993; Adjoud-Sadadou and Halli-Hargas, 2000). AMF form an interface between soil and plant roots (Power and Mills, 1995; Ingleby, 2007) and increase the absorptive surfaces of the roots (Manjunath and Habte, 1988). This is due to the extra-radical hyphae of the AMF extending beyond plant roots and acting as their extensions in acquiring nutrients from the soil (Rhodes and Gerdemann, 1975). AMF therefore absorb mineral nutrients from soil through their extended hyphal network and deliver them to their host plants in exchange for carbohydrates (Oehl et al., 2003). AMF provide other benefits to the host plants like enhancing their tolerance against abiotic stresses such as drought and metal toxicity (Meharg and Cairney, 2000).

As AMF are not host-specific (Ingleby, 2007), the same fungi associated with trees can colonize crop species and therefore enhance both tree and crop growth in agroforestry systems. In this regards, the tree species can act as a ‘reservoir’ of AMF, from which roots of growing crop seedlings can quickly form mycorrhizal associations. All the soils harbor AMF spores despite the different structural and chemical differences of the cropping fields (Don-Rodrgue et al., 2013).

Plant root colonization by AMF is an important key and a strong basis for all the benefits the plant can expect to get from the fungi. This has been studied and shown to improve productivity of several field crops, including maize (Chen et al., 2004). Plant root colonization by AMF depends on plant species (Panja et al., 2014). This was observed among AMF isolates belonging to different species, as well as among isolates of the same species (van der Heijden et al., 1998; Klironomos, 2003). On the other side, for the same plant species, the effects and contribution of AM fungi vary according to the fungal isolates, reflecting the differences in the symbiotic efficiency of the fungus (de Novais et al., 2014). Plant responses to AMF depend also on environmental conditions such as pH, soil nutrient availability, water, light intensity and temperature (Porras-Soriano et al., 2009; Smith and Smith, 1996). Colonization is restricted to root cortex and does not enter the vascular cylinder. The nature and abundance of propagules of these fungi determine their resistance during periods of inactivity, response to disturbance, and resistance to predation by other soil organisms (Brundrett and Abbott, 1994).

AMF are proven essential to increase the sustainability of agricultural systems (Cardoso and Kuyper, 2006). Even though numerous studies have reported the positive effect of AMF inoculation on crop production (Nyaga et al., 2014), a majority of past field AMF inoculation attempts have focused on the use of exotic strains, disregarding the potential of the indigenous strains (Njeru et al., 2014), yet native species have been regarded as more adapted to the soil environment than introduced strains (Klironomos, 2003). This may be cited among the possible reasons behind failure in the field inoculation attempts. In this regards, use of native AMF species can constitute an environmentally friendly method of soil fertility amendment over time (Nyaga et al., 2015). The current study aimed therefore to identify indigenous AMF species of agroforestry systems of Rwanda and investigate their potential to colonize maize crop.

MATERIALS AND METHODS

Description of the study sites

Bugesera is a district located in Eastern province of Rwanda. The district altitude varies between 1300 and 1667 m with soft slopes and its relief is mainly constituted of a succession of low plateau, valleys and swamps. It has an annual precipitation ranging from 700 to 900 mm and the mean atmospheric temperature is between 21 and 29°C. Soils in the region are sandy-loam of moderate fertility (Habiyaremye et al., 2015; JICA, 2006; MINITERE, 2003). Dominant crops of the region are banana, maize, beans and cassava; and trees are *Acacia* species, *Senna spectabilis*, *Grevillea robusta*, and *Eucalyptus* species (Kiptot et al., 2013; CRA, 2005).

Rubavu, one of the Western province districts of Rwanda, is characterized by an altitude ranging between 2000 and 3000 m with higher slopes (the mean slope is 35%). The atmospheric temperatures are generally cool with an average of 10°C. The region annual mean rainfall is 1800 mm. Dominant crops in the region include maize, Irish potatoes, climbing beans, wheat and vegetables such as carrots and cabbages along with tea plantations on valley bottoms. The major trees are *Alnus acuminata* along the contours, *Markhamia lutea* on farm*, Eucalyptus* spp. woodlots, *G. robusta*, bamboo, avocado and some indigenous trees such as ficus (Kiptot et al., 2013).

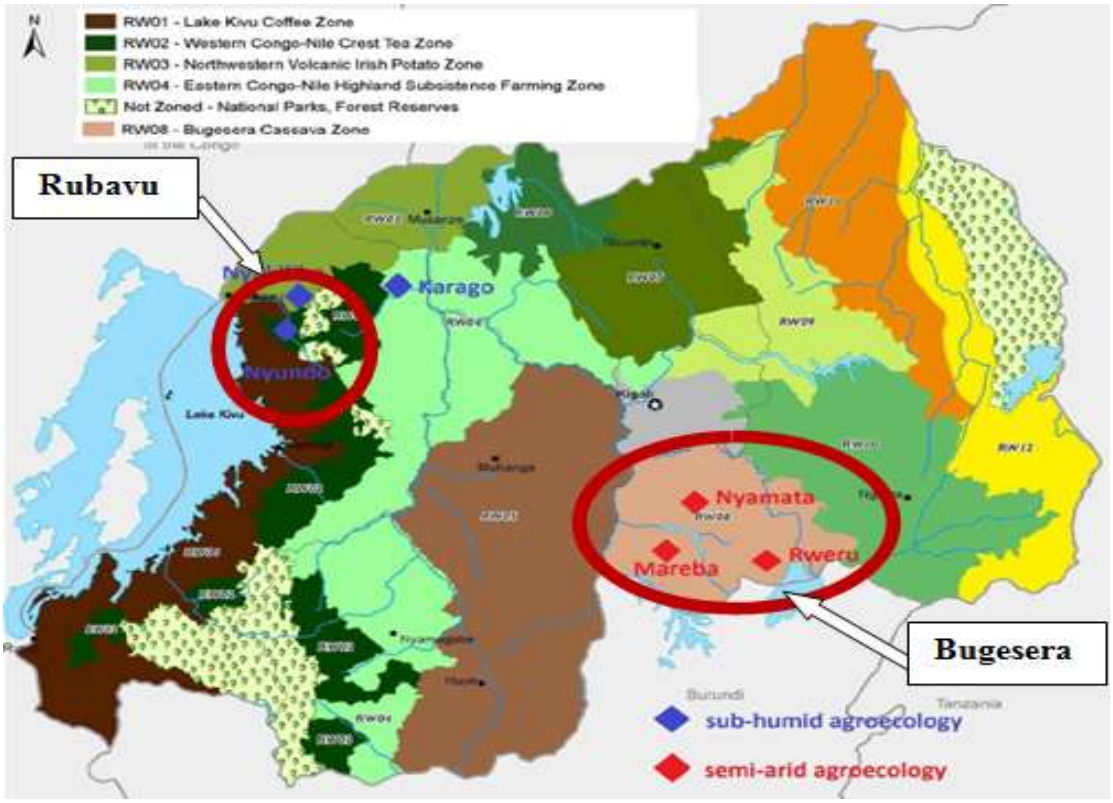

Figure 1. Agro-ecological map of Rwanda - Selected sites (sectors).

Sampling

Soil was sampled around most dominant tree species selected in Nyundo and Rweru sites of Rubavu and Bugesera districts, respectively, representing semi-arid and sub-humid agro-ecological zones of Rwanda (Figure 1). The soil pH and phosphorus which are important in AMF presence and colonization ranged, respectively of 5.0 to 6.5 and 25.0 to 58.4 mg/kg at Bugesera and of 4.9 to 5.8 and 10.1 to 19.7 mg/kg at Rubavu. The two sites were chosen because they represent areas with ongoing complimentary project activities on tree-crop interactions with a wide range of participatory trials by the farmers.

Soil samples were collected using a soil auger at 0 to 10 cm depth, around individual trees of the four most common tree species found in the study area. Sampled AMF host trees species were *M. lutea, A. acuminata*, *G. robusta* and *Eucalyptus* spp. for Rubavu and *S. spectabilis, Acacia polyacantha, G. robusta* and *Eucalyptus* spp. for Bugesera.

Three tree replicates were sampled and soil samples were collected at three distances from the tree trunk: 0.5 m from the tree trunk, the edge of the tree canopy and 3 m from the edge of the tree canopy. At every distance, the soil was sampled in the east and west directions of the tree and the two samples were pooled into a composite sample, so to have a total of 72 soil samples. The collected soil samples were stored and transported in plastic bags to the laboratory and kept at 4°C until processing.

Extraction of AMF spores

The extraction of AMF spores was done using the method adapted from Gerdemann and Nicolson (1963), Habte and Osorio (2001) and Ingleby (2007). This consists of mixing 50 g soil with water to obtain a 1 L suspension, which was then strongly agitated to disperse the soil aggregates and release AMF spores. The liquid was then poured onto a nest of sieves (200 µm pore size on top to allow flow of spores by retaining large soil and organic matter particles, and 45 µm on the bottom to retain AMF spores yet allow passage of the finest soil particles). The collected residue in the smallest sieve was washed and transferred into 50 ml centrifuge tubes and centrifuged with water for 5 min at 1,800 rpm. The supernatant was then discarded and the pellet re-suspended in 48% (w/v) sucrose and centrifuged again for 1 min at 1,800 rpm. The supernatant (with spores) was poured onto 45 µm sieve and rinsed with water to remove the sucrose. The remaining residues on the sieve were transferred to a Petri dish for initial observation and collection of AMF spores under dissecting microscope with 40× magnification.

Morphological identification of AMF spores

Thirty AMF spores were randomly picked from a pool of spores extracted around individual host tree species of each agro-ecological zone. This resulted in a total of 240 spores prepared for identification. The morphological identification of AMF spores was done using the method adapted from INVAM (2004) and Ingleby (2007). Spores were grouped into different morphotypes according to their morphological characteristics. After the uniformity of the morphological groups was confirmed under a dissecting microscope, microscopy slides were prepared for each different spore morphotype with polyvinyl-alcohol and polyvinyl-alcohol plus Melzer's solution with 1:1 ratio. The different morphotypes were examined under a stereomicroscope at 400× and tentatively

identified to the genus level. This morphological spore identification was mainly based on spore size, shape, color, wall structure, hyphal attachment, ornamentation and Melzer's solution reaction.

AMF spores multiplication and maize inoculation

AMF spores propagation through trap cultures development

The aim of the cultures was to maintain a living collection of the organisms under study and obtain fresh spores for further inoculation. The trap cultures were set in pots and soil sampled from Bugesera agroforestry system was randomly chosen to be used. Three pots were filled with a mixture of sterilized soil and sand in a ratio 1:1 at ¾. The three most predominant isolated morphotypes of AMF spores were sown in the three pots, respectively. Seeds of sorghum were sown in each pot as symbiotic partner plant to AMF. As one of the most effective symbiotic partners of AMF, sorghum had been previously used for multiplication of AMF spores. Fast germination and growth as well as large root density of this plant favor rapid formation of numerous infection points which result in contact with greater number of spores (Carrenho et al., 2002). The trap cultures were maintained in green house for 8 weeks with regular watering.

Inoculation of maize with AMF

The experiment was conducted in 5 L pots in a greenhouse with natural lighting and temperature. Treatments were factorial combinations of two factors; including AMF inoculation (AMF inoculum vs. non-mycorrhizal control) and P addition (0, 0.9, 1.9 and 2.9 g P per pot). The experiment was arranged in a design with three replicates for each treatment. A total of 96 pots including 84 pots of maize plants treated with AMF inoculum and 12 controls were organized into 32 treatments and tested for AMF colonization. Fertilizers N and K were added as 1.9 g of N per pot in the form of urea and 1.9 g per pot in the form of KCl. All the amounts of added N, P and K per pot were determined based on a fertilizer's NPK ratio % of 17-17-17 at 300 kg NPK fertilizer per hectare as being applied in maize farming in Rwanda. The maize variety used in the experiment was ZM607 because of its high productivity, rapid growth and resistance against various diseases.

Mycorrhizal inoculum consisted of soil, spores, mycelium and infected root fragments picked from the trap cultures. Each pot was inoculated with 100 g inoculum for the AMF treatment. Each pot was filled with 5 kg of autoclaved soil. The inoculum was placed 20 mm below the seeds prior to sowing. Maize seeds were surface sterilized in a 70% alcohol solution for 5 min then washed several times with distillated water. Five seeds of maize were sown in each pot and thinned to three after seedling emergence. Watering was done daily and plants were harvested 60 days after germination.

Evaluation of maize roots colonization

To be able to observe the infection of roots by AMF, washed root samples were to be cleared in potassium hydroxide, bleached in alkaline hydrogen peroxide, acidified in hydrochloric acid and stained with trypan blue. The roots were then de-stained, mounted on a glass microscope for microscopic observation, and the frequency of AMF colonization recorded and expressed in percentage (Ingleby, 2007; Habte and Osorio, 2001).

Collection of maize roots samples

Entire roots of maize were picked from the soil, washed free of soil and tertiary roots were collected to obtain a representative sample. Roots samples were stored in plastic vials within 70% ethanol before staining for AMF assessment.

Roots clearing, staining and de-staining

Maize roots previously stored in 70% ethanol were stained for AMF assessment. Ethanol was poured and 2.5% KOH was added for clearing root samples. The roots were heated in an oven at 70°C for 1 h; KOH was poured and roots were rinsed with tap water. Alkaline hydrogen peroxide (comprised of 60 ml of 20 to 30% NH_4OH and 90 ml of 30% H_2O_2 and 840 ml distilled water) was added to remove the phenolic substances. The roots were placed in the oven at 70°C for 20 min. The roots were then rinsed with tap water, 1% HCl was added and the root samples were left for 24 h. HCl was poured and without rinsing the roots, 0.05% trypan blue (500 ml glycerol, 450 ml water, 50 ml of 1% HCl and 0.5 g trypan blue) staining reagent was added and placed in the oven for 1 h at 70°C. The stain was then poured and de-staining solution, acidic glycerol (500 ml glycerol, 450 ml water, 50 ml of 1% HCl) was added.

Slide preparation and roots analysis for AMF colonization

Roots were removed from the de-staining solution and placed in a Petri dish. A small amount of water was added into the Petri dish, and with forceps and a surgical blade on a holder, roots were cut into approximately 1 cm pieces. Pieces of roots (10) for each specimen were mounted on a glass microscope slide and a drop of lactic acid added as a mounting reagent. The cover slip was gently lowered from the edge and roots gently squashed. Slides were examined under the compound microscope at 100× magnification and the frequency of AMF colonization (arbuscules, vesicles, internal and external hyphae) was recorded for each sample.

Statistical analyses

To evaluate the data on maize roots colonization by AMF, the analysis of variance (ANOVA) was used. Comparison among different morphotypes' performance in colonizing maize roots was carried out at $p = 0.05$ significant level.

RESULTS

Morphological characterization of AMF Spores

Based on major differences in spore morphological appearance, four different types (genera) of spores were detected from the agroforestry systems in the semi-arid and sub-humid agroecologies of Rwanda. The characteristics shown by the different types of spores are indicated in Table 1. These morphotypes AMF1, AMF2, AMF3 and AMF4 were identified into four genera, that is, *Glomus*, *Gigaspora*, *Scutellospora* and *Acaulospora,* respectively (Figure 2) based on their morphological features.

All spore types were found in soil samples from all the host tree species. In general, of the assessed AMF spore from Bugesera agro-ecological zone, 45.83% were *Glomus*, 25.00% *Gigaspora*, 15.83% *Scutellospora* and 13.33% *Acaulospora*. From Rubavu agro-ecological

Table 1. AMF spores morphological characteristics.

Rapid diagnosis (under dissecting microscope)			Deep diagnosis (Under compound microscope)				Genus
Morphotype	**Size**	**Color**	**Reaction in Melze's reagent**	**Hyphae**	**Wall layers**	**Ornamentation**	
AMF1	Small	Light yellow to brown	Yes	+	1 or 2 laminated	No	*Glomus*
AMF2	Big	White to gray	No	+	3 laminated	No	*Gigaspora*
AMF3	Small	Brown	Yes	+	2 Non-laminated	Yes	*Scutellospora*
AMF4	Big	Brown to black	No	-	2 or 3 non-laminated	Yes	*Acaulospora*

+Presence of subtending hyphae; - absence of subtending hyphae (sessile).

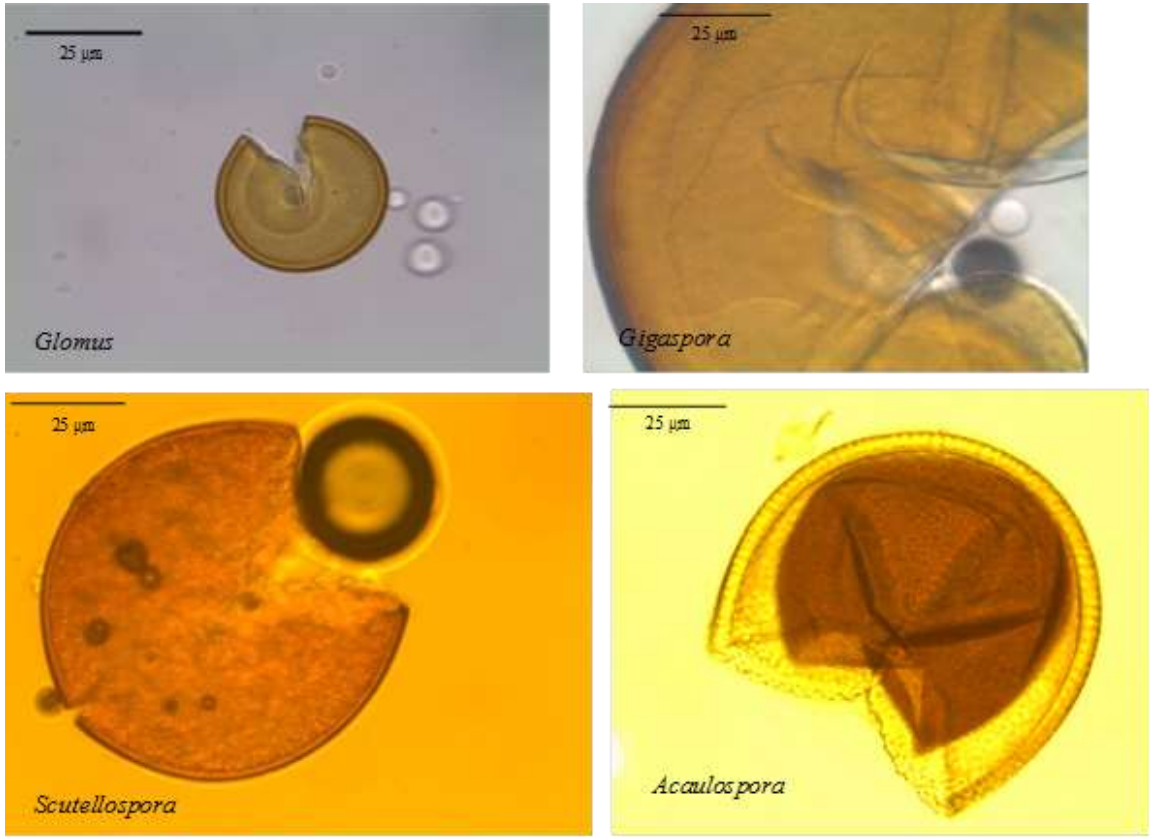

Figure 2. Photomicrographs of AMF spores (400× magnification).

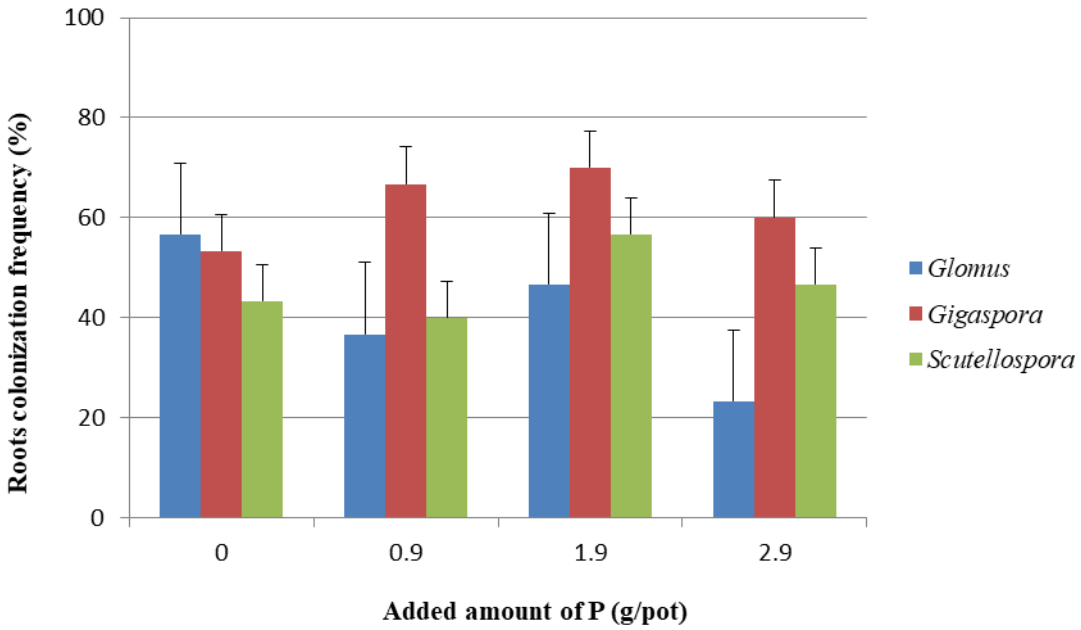

Figure 3. Mycorrhizal root colonization frequency of tested AMF genera in response to P fertilizer addition.

zone, 46.66% were *Glomus*, 31.66% *Gigaspora*, 14.16% *Scutellospora* and 7.50% *Acaulospora*. Combining data from both agro-ecological zones, 46.25% were *Glomus*, 28.33% *Gigaspora*, 15.00% *Scutellospora* and 10.4 % *Acaulospora*.

In all, *Glomus* was the predominant genus; the second dominant AMF genus encountered was *Gigaspora*. The third was genus *Scutellospora* and the last was the genus *Acaulospora.*

Maize roots colonization by AMF

Potential of the tested AMF genera to colonize maize was evaluated in terms of root colonization frequency. All the plant roots in the treatments with AMF were colonized by abuscules and/or vesicles of the AMF. The percentage of root colonization detected ranged from 10 to 100% with a mean colonization of 40% for the least performing AMF treatment, and 70% for the most performing. Root colonization frequency in all treatments without mycorrhizal inoculation was always zero. Mycorrhizal root colonization frequency versus phosphorus fertilization application for *Glomus*, *Gigaspora* and *Scutellospora* is as shown in Figure 3. In this study, the tested levels of P fertilization did not show significant effect on AMF root colonization and the noticed fluctuations of the fungi performance were not consistent (Figures 3, 4, 5, and 6).

The analysis of variance (ANOVA) showed that *Gigaspora* performed better than *Glomus* (p=0.004) and *Scutellospora* (p=0.022), respectively. When combined to *Glomus* and *Scutellospora* (Figure 4), the root colonization frequency of *Gigaspora* statistically decreased with p = 0.03 and 0.008, respectively. When all the genera *Glomus*, *Gigaspora* and *Scutellospora* were united to test their combined potential, the root colonization frequency of *Gigaspora* was not significantly affected. When similar combinations were applied to *Glomus* and *Scutellospora,* their individual performance was not significantly affected. Figures 5 and 6 show different combinations involving *Glomus* and *Scutellospora* as well as the fluctuations observed on their performance, but no significant difference was tested.

DISCUSSION

Characterization of AMF spores

Taxonomy of AMF can be done based upon the morphology of large asexual spores the fungi produce in the soil (Mohammadi et al., 2011). In this research, four AMF genera were morphologically recovered from soil samples and *Glomus* was the predominant taxonomic group. The predominance of *Glomus* was also reported in dry afromontane forests of Ethiopia (Tesfaye et al., 2003b), in tropical rain forest of Xishuangbanna, China (Zhao et al., 2001), in tropical rain forest in Mexico (Guadarrama and Alvarez-Sanchez, 1999), and in arid and semi-arid lands of North Jordan (Mohammad et al., 2003). *Glomus* spp. were also the most frequently encountered AMF in the fecal samples collected from terrestrial and arboreal small mammals in a Panamian

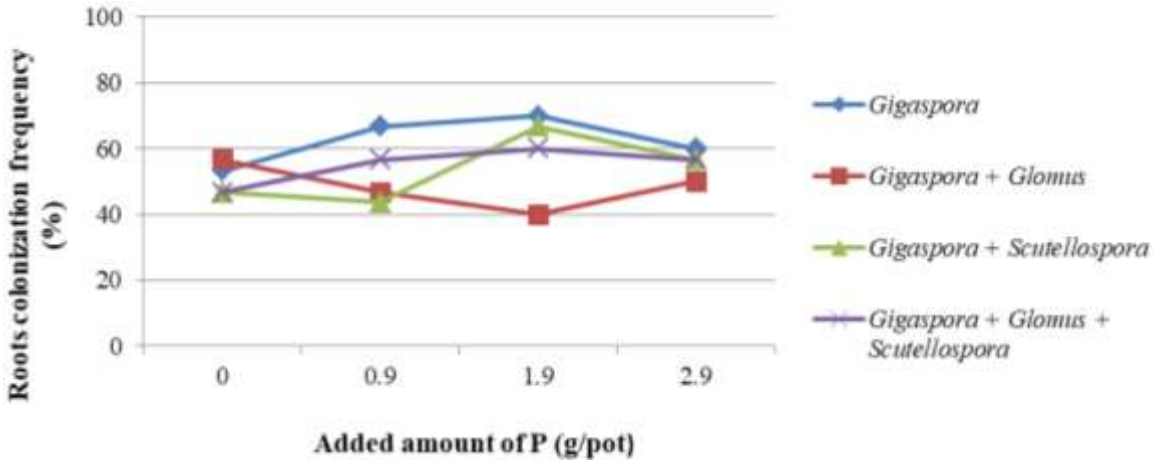

Figure 4. *Gigaspora* root colonization frequency in various combinations with other genera.

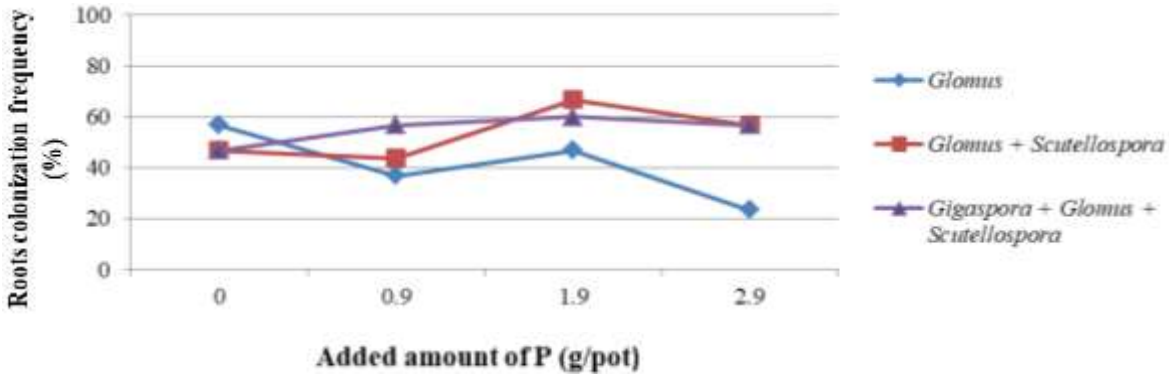

Figure 5. *Glomus* root colonization frequency in various combinations with other genera.

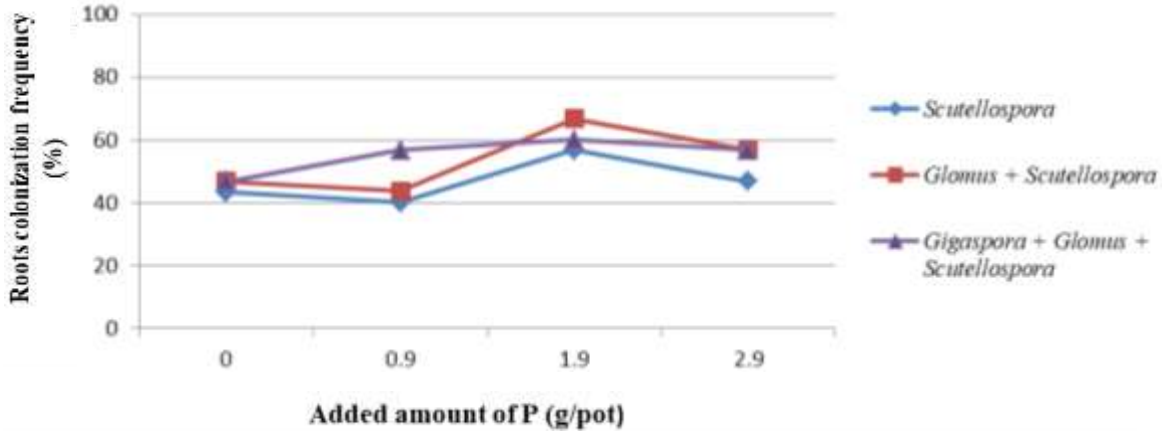

Figure 6. *Scutellospora* root colonization frequency in various combinations with other genera.

cloud forest with 87% frequency of occurrence in the samples (Mangan and Alder, 2000). Million (2002) also reported that more than 80% spore extracted beneath *Acacia tortilis* was *Glomus*. Similarly Munro et al. (1998)

found out that *Glomus* spores were the dominant beneath the host tree *A. tortilis*. According to Frioni et al. (1999), acidic soil favors *Glomus* abundance than other genera. The sampled soils of a pH ranging between 5 and 6.5 in Bugesera and between 4.9 and 5.8 in Rubavu may explain why *Glomus* dominated other genera in the present findings. *Gigaspora*, the second dominant AMF genus in the sampled agroforestry systems, was also reported to be abundant at lower pH (Frioni et al., 1999). The least dominant spore type was the genus *Acaulospora*. Less occurrence of Acaulospora was also noticed by Sewnet and Tuju (2013) in their research on AM fungi associated with shade trees and *Coffea arabica* L. in a coffee-based agroforestry system in Bonga, Southwestern Ethiopia.

Even though the fungi encounter different soil environment and different host plant species, and despite large geographic separation and agro-ecological differences between Rubavu and Bugesera, diversity of AMF spores was similar in all the sampled soils and in both agro-ecological zones. Bugesera and Rubavu contained similar communities of AMF spores; all genera *Glomus*, *Gigaspora*, *Scutellospora* and *Acaulospora* were found in both habitats. The present results concur with previous findings that the AMF community can adapt to different environmental conditions and host plants (*Yang et al., 2009*). In addition, the two agroforestry systems have fairly similar pH. Furthermore, AMF are ubiquitous and non-host specific (Don-Rodrgue et al., 2013; Ingleby, 2007; Rajah and Tang, 2005).

Colonization of maize roots by AMF

Indigenous AMF associated with tree species of the agroforestry systems in the semi-arid and humid agroecologies of Rwanda demonstrated ability of infecting maize roots. One of the reasons to explain this observation may be the noticed ability of AMF to form symbiotic associations with plant roots which is generally non-specific (Carrenho et al., 2002); the fungi can consequently form associations with a wide range of plant species. In this regards, the same fungi associated with a tree can infect roots of crop species (Ingleby, 2007). The study also confirmed results from many previous researches that maize can easily and fast form mycorrhizal associations (Mohammadi et al., 2011), and permits wide proliferation of AMF in its roots. This may be partly due to the larger root density of the plant, extension and branching (Robertson et al., 1980), allowing therefore contact with a greater number of AMF propagules. Compatibility between AMF and maize could also be explained by anatomic characteristics of the plant roots which favor the early stages of the plant-fungus interaction (Brundrett and Kendrick, 1990). For instance, maize possesses a root surface covered by two kinds of mucilage: a gelatinous material produced by the root cap and another firmer and uniformly thickened, attached to the epidermal cells. When the roots elongate in maize, the mucilaginous mantle is detached only with epidermal and hypodermic cells contrary to some other plants in which this mucilaginous mantle is detached with the cortical cells. These anatomical root characteristics may influence AMF development and be responsible for the high maize roots infection with AMF since the roots keeps the sites where symbiosis is established (cortex) (Mc Cully, 1987).

Contrarily to negative effects of P on AMF reported in many previous researches, in this study P fertilization did not show significant effects on AMF root colonization. Similar results of no significant P fertilizer effect were reported by Wang et al. (2018) in their investigation on the effect of N-P fertilization on AMF root colonization. Results of the current study are also in agreement with Grant et al. (2005) who reported that P fertilization does not always reduce mycorrhizal association. This lack of consistency among various research findings on impact of fertilization on AMF plant root colonization pushed Wang et al. (2018) to suggest that the effects of fertilization on AMF may be context-dependent and many other factors may be involved. However, much experimental evidence shows that a high level of plant P status rather than that of the soil regulates mycorrhizal colonization (Lu et al., 1994; Koide and Li, 1990).

Root colonization frequency by *Gigaspora* was significantly higher than that of the other tested genera. This may be an indication that the phenomenon is related to inoculum infectivity. However, the current study was not able to show the reason behind the noticed higher performance of *Gigaspora*. Although AMF colonization was significantly lower for *Glomus* and *Scutellospora*, it was never zero. Thus, all the tested AMF may still be able to colonize roots of crops and contribute to crop nutrition regardless of the genus.

Therefore, the ability of AMF native of Rwanda to colonize maize roots as an important key and a strong basis for all the benefits the plant can expect to get from the fungi were noticed. This is also a proof that, once well studied, the indigenous AMF of Rwanda can be exploited as a bio-fertilizer and extensively used by farmers.

Conclusion

The current study demonstrated that agroforestry systems of Rwanda harbor AMF with the potential to colonize roots of crops and hence enhance productivity. AMF native to Rwanda could be considered to be a future tool in agriculture especially as a bio-fertilizer. Therefore, there is a need for an inventory of AMF in all agroforestry systems of Rwanda with a deep study on their ecology and host range before application. Their relation with nutrient dynamics and other soil characteristics of Rwanda territory should also be evaluated.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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