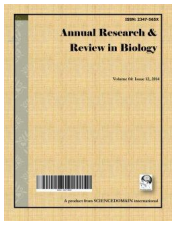

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Effect of Quercetin and Pectinase on Spore Germination and Hyphal Growth of Arbuscular Mycorrhizal Fungi and Biomass Production of *Nicotiana tabacum* L. and *Calopogonium mucunoides* desv

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Authors' contributions

This work was carried out in collaboration between all authors. Authors ELMN and DN designed the study. Authors ELMN and OB reviewed the experimental design, enabled the practical realization of this work. Author ELMN wrote the protocol and the first draft of the manuscript. Authors RT, AFM and TANM managed the analyses of the study. Authors ELMN, LM and AFM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The aim of this study was to evaluate the ability of mycorrhizal fungi to rapidly colonize roots of host plants and to predict their competitive advantage over native strains. Different concentrations of quercetin and pectinase were used to stimulate hyphal growth and to determine the germination rate of arbuscular mycorrhizal fungi (AMF) in a Petri dish. The best strains were used to inoculate two host plants under green house conditions in the presence of high concentrated polyphenol and

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enzyme. The essays were carried out in the laboratory of biotechnology center; University of Yaounde I. Five strains of AMF from Cameroonian's soils were identified and compared with exotic strain. Water agar 0.7% was mixed with three different concentrations of quercetin (0, 2.5 and 5 µM) and pectinase (0, 1.6 and 3.2 units/ml). *Nicotiana tabacum* and *Calopogonium mucunoides* were used to determine the root colonisation percent of AMF and shoot biomass after inoculation. Results showed that for most species of AMF, 5% quercetin and 3.2 units/ml of pectinase significantly stimulate *in vitro* spore germination and hyphal growth. Treatment combining quercetin and pectinase could lead to a synergetic effect on mycorrhizal symbiosis and plant performance. These compounds which can modify the symbiosis physiology may be of great importance in the regulation of plant and soil microbe interaction. They could also contribute in stimulating the growth of these strict biotrophic fungi under *in vitro* culture, and to enhance an increase in plant biomass.

Keywords: Mycorrhizal fungi; root colonization; competition; spore germination; plant Inoculation.

1. INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are symbiotic and obligate biotrophic micro-organisms which are associated with about 90% - 95% plant species [1,2], they cannot grow in an axenic media. Many trials did not succeed in growing them without a living plant, only on root organ culture transformed using *Agrobacterium rhizogenes.* Fungi responsible for this type of symbiosis are Glomeromycota [3]. They had been revealed to play an important role in mineral nutrition [4], drought tolerance [5,6] and resistance to pathogens in host plant diseases [7]. These endophytes differ widely according to their potential to improve plant growth [8]. Some genus such as *Gigaspora, Glomus, Scutellospora and Acaulospora* were found in Cameroon soils [9,10,11].

A program on beneficial micro-organisms for sustainable agricultural production initiated at the Biotechnology Centre, University of Yaoundé I in Cameroon, required the isolation of AMF from different ecosystems, their characterization and the analysis of their germination ability and their symbiosis activity in order to select some species for bio fertilizer production [12,13]. Field trials for instance using different species of *Glomus* showed that, an inoculated AMF strain can be excluded completely from plant root system by other indigenous species if it is less competitive. In contrast, if some highly competitive strains, it will be sufficient to robe seeds coat with these strains inoculums in other to have good root colonization after spores' germination [14,15] Studied the level of competition between fungi species and suggested that, species which first colonize plant roots system have a competitive advantage over other species. Whereas, [16], suggests that, competitive interactions should focus on intraradial colonization dynamics as well as the mechanism of fungal mediation to establish the interaction. However, root colonization strictly depends on the viability of spores in the soil. But the spores need a long time to germinate. Studies on the germination of asexual spore of AMF had shown variable results [17]. Several factors had been reported to affect spore germination [18] such as; the pH of the medium, dormancy rate, type of soil, root exudates, the presence of micro-organisms in the soil. In that respect, first studies suggested that; after a period of storage, spores became senescent showing the influence of dormancy [19]. In an attempt to determine specific compounds capable to induce spore germination, [20] showed that some flavonoids such as apigenin, hesperitin and naringenin could stimulate an early *in vitro* spore germination of *Gigaspora margarita* Becker and Hall. The author thinks that; modifying chemical composition of the environment while monitoring the pH, could induce fungi growth within growth media. Similarly, [21,22] showed that flavonoids are considered as hyphal growth stimulators and influence pre-infective phase of AMF root colonization. These flavonoids increase roots colonization by inducing rapid spores germination, favor hyphal extension, during the colonization process. The germination rate, the type of germination and the quantity of mycelium produced are criteria which are considered to promote a rapid colonization of species introduced in a soil containing native AMF. This study was carried out to evaluate the effect of a flavonoid compound and a hydrolytic enzyme on six species of arbuscular mycorrhizal fungi for spores' germination potential, hyphal growth ability as well as roots colonization percent and plant biomass production in two host plants: *Nicotiana tabacum* (Solanaceae) and *Calopogonium* sp under greenhouse condition.

2. EXPERIMENTAL DETAILS

2.1 Origin of Native AMF Spores and Strains Identification

Arbuscular mycorrhizal fungi spores originated from four agro ecological zones of Cameroon and one was obtained from the University of Hawaii, USA (Table 1). Spores were extracted from the soil after trapping with pearl millet (*Pennisetum americanum* L.) and the cowpea (*Vigna unguiculata* L.) by wet sieving and decantation reframe method [23]. After isolation and purification, spores were surface-sterilised in a 2% (w/v) chloramine solution mixed with 0.02% (w/v) streptomycin sulfate according to the modified two-step procedure described by [24]. Immediately after sterilisation the propagules were transferred in Petri dishes containing 0.7% water agar and stored in darkness at 30°C for germination. All the Petri dishes were sealed with paraffin. Then germinated spores were grown on *Brachiaria decumbens* plantlet in pots containing sterilized mixture of soil-sand (2:2). After root colonisation, spores from Bafia (GACB1), Ngaoundéré (GMVN1 and SGMN1), Ndupe (GGRN1), Ekona (GMDE1) and Hawaii (GCXH) were preserved in a culture substrate at 4°C in the resource microbial bank in the Soil Microbiology laboratory of the University of Yaoundé I.

2.2 Assays

2.2.1 Effect of quercetin and pectinase on spore germination and hyphal growth

Water agar 0.7% was mixed with three different concentrations of quercetin: 0, 2.5 and 5 µM [25] previously sterilised by filtration through a Millipore membrane of 0.22 µm and on the other hand, different concentrations of pectinase: 0, 1.6 and 3.2 units/ml were used. Each Petri dish was filled with 50 ml of the solution and six AMF species were tested. Ten sterilised spores were used per Petri dish with three replicates. The Petri dishes were incubated at room temperature (20-30°C) in full darkness and humidity varying between 70-80%. The dishes were sealed with paraffin. The parameters measured were: the germination percentage, the length of the hyphae as well as the anatomical description on the different phases of spore germination. The spore germination was observed using a stereomicroscope (x 40) and a compound microscope. The hyphal length was measured by the grid line and intersects method [26]. A spore was considered to have begun germination when different hyphae protrude other than the residual hyphae and an attachment emerges through the spore wall.

2.2.2 Effect of quercetin and pectinase on root colonization and on production of *Nicotiana tabacum* and *Calopogonium mucunoides* under green house conditions

Three spore strains namely, GACB1, GMVN1 and GCXH were inoculated into *Nicotiana tabacum* and *Calopogonium mucunoïdes* under green house conditions in the presence of 5 µM of quercetin and 3.2 units/ml of pectinase. The seeds of *Nicotiana and Calopogonium* were first disinfected in 10% calcium hypochlorite for 10 minutes, washed on sterilised water and allowed to germinate in basins containing sterilised sand. The basins were placed in the culture room and all plants received the nutritive solution of Rorison twice a week. After a month,

Table 1. Origin of native arbuscular mycorrhizal fungi and soil characterization

Codes[1]	Mycorrhizal strains	Host plant and origin[2]	Soil pH	Available phosphorus (ppm)[3]	Root colonization (%)[4]
GMVN1	*Gigaspora margarita*	*Vigna* (Ngaoundéré)	5.0	2.5	75
SGMN1	*Scutellospora gregaria*	*Manihot* (Ngaoundéré)	5.0	3.4	62
GGRN1	*Glomus geosporum*	*Rinorea* (Ndupe)	4.2	9.0	20
GMDE1	*Gigaspora margarita*	*Dioscorea* (Ekona)	5.8	18.0	63
GCXH	*Glomus clarum*	unknown (Hawaii)	-	-	60
GACB1	*Glomus albidum*	Crotalaria (Bafia)	6.3	2.5	55

[1]*Species internal code in the laboratory of microbiology (UMAB)*
[2]*Host plant in brackets and geographical origin*
[3]*According to Olsen (1954)*
[4]*Stained with acid fuchsin (Komanick and McGraw, 1982) and grown on millet*

the plants (seedlings) were transferred under green house conditions in black polyethylene sachets containing 2.20 lbs of a mixture of sand and soil (2:2). The soil was sterilised twice in an autoclave at 120°C for 40 minutes with an interval of 48 hours. Each plastic bag received 100 spores/plant and 1 ml of quercetin or pectinase. Each treatment was performed on 2 plants with 3 replicates. The plants were also given 100 ml of nutritive solution of Rorison twice a week. At the end of two-month period, all plants were harvested, the roots were stained using acid fuchsin 0.05% for root colonisation assessment [27]. The shoot of the host plants was dried in an oven at 80°C during 48 hours and weighed.

2.2.3 Data analyses

Data were subjected to statistical analysis using analysis of variance (ANOVA) and means were separated using least significant difference (L.S.D.) at 5% probability level.

3. RESULTS AND DISCUSSION

3.1 Effect of Pectinase and Quercetin on Spore Germination and Growth of AMF

The germination percentage in GMVN1, SGMN1 GMDE1 and GCXH strains increased as the concentration of pectinase increased (Table 2). The maximum germination was observed with 3.2 unit/ml of pectinase treatment. GMVN1 and SGMN1 strains showed the highest percentage (96.7%) followed by GCXH (86.7%). Spore germination rate of GACB1 and GGRN1 strains were not influenced by any concentration of pectinase at all. The concentration of 3.2 units/ml of pectinase influenced the germination of strain GMDE1 (66.7% vs. 46.7% in control without pectinase).

As far as hyphae growth is concerned, except in GGRN strain, the results in Table 3 indicates that, the length of the hyphae increases with increase in the concentration of pectinase. Maximum growth is observed in strains GMVN1 (2000 µm) and in SGMN1 (3000 µm) receiving both 3.2 units/ml of pectinase. Similar work was done by [28] in assessing *in vitro* hyphal growth of *Glomus mosseae* to chitinase and β-1,3-glucanase enzymes and observed that, the nature of AMF cell wall, the isoform state some enzymes, enzymes interaction, the localization of hydrolytic enzymes on the mycelium (apical or sub apical regions) influence growth of AMF hyphae of the genus *Glomus*. In addition to these factor highlighted by this author, other factors seem to be critical as reveal by this study, that is; the AMF genotype as well as the environmental factors. In this respect, the genus *Gigaspora* (GMVN1, GMDE1) appear to response positively as far as the growth of the hyphae is concerned. The most plausible hypothesis could be as a result of the modification of the molecular composition of the cell wall. In this line, strains GMVN1 and GMDE1 from Ngaoundéré and Ekona respectively could have similar abiotic factors that brought about similar changes with time. This hypothesis is supported by the fact that, according to [29] who think that there is a high sensibility of the genus *Gigaspora*. Similarly, [30,31] identified the presence of β-1,3- glucan in the cell wall in the order of Glomales. Also, [28] suggest that the presence of chitin in the cell wall of *Glomus mosseae* and thus explains the growth inhibition observed when chitinase alone or when combined with β-1,3- glucanase were added. However, Pectinase enzymes have a good reaction with Gigasporineae and Glomineae as well. This enzyme could play an important role in mycelium production.

Table 2. Effect of different pectinase concentrations on spore germination (%) of six arbuscular mycorrhizal fungi strains after 6 days growth at pH 4.5

Codes	Spore germination (%)		
	Pectinase concentrations (unit/ml)		
	0	1.6	3.2
GACB1	30.0[a]	30.0[a]	33.0[a]
GMVN1	73.0[a]	73.0[a]	96.7[b]
SGMN1	50.0[a]	56.7[a]	96.7[b]
GGRN1	16.7[a]	13.3[a]	13.3[a]
GMDE1	46.7[a]	66.7[b]	66.7[b]
GCXH	63.0[a]	73.3[b]	86.7[c]
Mean	46.6	52.2	65.5

[a, b, c]: Within each line means followed by the same letter are not Significantly different at the 5% level of confidence

In contrast, [17] using 3.2 units/ml of cellulase showed a spore germination enhancement in the order of 80% at day 9 with *Gigaspora rosea*. Some hydrolytic enzymes such as cellulase, pectinase and xyloglucanase seem to be involved in the penetration and development of AM fungi in plant roots. Their activities have been reported in colonized roots and in the external mycelium of AM fungi [32]. A correlation between

endo-xyloglucanase activity of the external mycelia and root colonization and plant growth was noted by the same authors. This result indicates that the activity of this enzyme is an important factor influencing fungal colonization and plant growth [33].

Table 3. Effect of different pectinase concentrations on the hyphal length (µm) of six arbuscular mycorhizal fungi strains after 6 days growth at pH 4.5

Codes	Hyphal length (µm)		
	Pectinase concentrations (unit/ml)		
	0	1.6	3.2
GACB1	140[a]	140[a]	170[b]
GMVN1	800[a]	850[b]	2,000[c]
SGMN1	1,000[a]	1,600[b]	3,000[c]
GGRN1	130[a]	117[b]	100[a]
GMDE1	100[a]	123[b]	125[b]
GCXH	400[a]	403[a]	475[b]
Mean	428	539	978

[a, b, c]: Within each line means followed by the same letter are not significantly different at the 5% level of confidence

The effects of quercetin on spore germination depend on the species of AMF and the concentrations used as well. The results are shown on Table 4. The percentage of spore germination was low in the absence of quercetin and increased at 2.5 µM concentration and reached the maximum at 5 µM in all AMF species except in GGRN1 in which there was no effect. Previous study on the diversity of AMF in Cameroon' soil [11] revealed that strain GGRN1 isolated from Ndupe (Edea) show a low colonization percentage (25%) with Millet and Niebe host plants as compare to strains GMVN1 and SGMN1 from Ngaoundéré (98%). This result partially explains that the number of spore and hyphal growth of strain GGRN1 is conditioned by the host plant used, in the sense that, according to [29] who observed that, the concentration of flavonoids in leguminous roots varies with root colonization with specific AMF strains while [34] explained that the stages of interaction between the plant and the symbiont as well as the Nitrogen and/or Phosphorus deficiency respectively are significantly impaired with flavonoid concentration. In the same line, [35] think that the metabolism of flavonoids is strongly affected with the AMF colonization.

Quercetin concentration of 5 µM stimulates spore germination in all AMF species but not in GGRN and as well as in mycelia growth of all species but not in GGRN and GMDE1. Flavonoid produced by plant roots such as quercetin have a stimulating effect on the development and production of hyphae from spore germination tubes in *Gigaspora margarita* and in some species of *Glomus* [36,37]. [38] Showed that the addition of quercetin led to an increase of the root colonization by the fungi but their effect on the production of abundant mycelium was not observed. [37] Obtained a mycelium development of more than 500 mm/spore from *Gigaspora margarita* which was at least ten times higher as compared to the control with a concentration of 10 µM of quercetin combined with 2% of CO_2. The maximum concentration of 5 µM used in this study gave a mycelium production of 3.5 times higher as compared to the control in GMVN1 and 4.8 times higher in SGMN1 but with a constant CO_2 concentration. This result suggests that the mixture of 2% of CO_2 with quercetin has an obvious beneficial effect on mycelium development of fungi [37,25].

Table 4. Effect of different quercetin concentrations on spore germination (%) of six arbuscular mycorhizal fungi strains after 6 days growth at pH 4.5

Codes	Spore germination (%)		
	Quercetin concentrations (µM)		
	0	2.5	5
GACB1	30[a]	36.7[a]	70[b]
GMVN1	73[a]	73[a]	90[b]
SGMN1	50[a]	53[a]	97[b]
GGRN1	16.7[a]	20[a]	20[a]
GMDE1	46.7[a]	56.7[a]	70[b]
GCXH	63[a]	63[a]	76.7[b]
Mean	46.7	50.4	70.6

[a, b]:Within each line means followed by the same letter are not significantly different at the 5% level of confidence

The effect on hyphal growth increased in all AMF species as the concentration of quercetin increased except in species GGRN1. The maximum growth was observed at 5 µM in SGMN1 (4800 µm) followed by strain GMVN1 (2800 µm) (Table 5). Fig. 1A shows that quercetin-treated mycorrhizal fungi (GACB1) spores germinated and produced more abundant hyphae as compared to the non-treated roots in Fig. 1B. This result concurs with that of [39] who identified a concentration of 2.5 µM favorable to increased hyphal elongation in *Glomus etunicatum* after 21 days with hyphal length of 7 mm. The very low hyphal growth recorded may

be partially genotype related. However, Hyphal branching in the presence of 2.5 µM quercetin was much greater than in the untreated control after 21 days because according to the author, quercetin may prevent new spore germination within 7 days following inoculation.

Table 5. Effect of different quercetin concentrations on the hyphal length (µm) of six arbuscular mycorrhizal fungi strains after 6 days growth at pH 4.5

Codes	Hypha length (µm)		
	Quercetin concentrations (µM)		
	0	2.5	5
GACB1	140[a]	148[a]	296[b]
GMVN1	800[a]	955[b]	2,800[c]
SGMN1	1,000[a]	1,500[a]	4,800[b]
GGRN1	130[a]	135[a]	135[a]
GMDE1	100[a]	123[b]	129[b]
GCXH	400[a]	401[a]	700[b]
Mean	428.3	543.7	1,476.7

[a, b, c]:Within each line means followed by the same letter are not significantly different at the 5% level of confidence

3.2 Effect of Quercetin and Pectinase on Root Colonisation and Shoot Biomass of Nicotiana and Calopogonium

The assay was performed with three AMF species, *Glomus albidum* (GACB1), *Gigaspora margarita* (GMVN1) and *Glomus clarum* (GCXH) on *Nicotiana* and *Calopogonium*. The results shown on Tables 6 and 7 clearly indicate that quercetin and pectinase significantly stimulates root colonisation and production of plant biomass in the two host plant. That stimulation varied with the AMF species used.

As far as the host plant *Nicotiana* is concerned, on receiving quercetin, the percentage increased in root colonisation was observed with GACB and GMVN1 strains. No effect was observed with GCXH strain. When GMVN1-inoculatum with 5 µM of quercetin in *Nicotiana* an important number of auxiliary cells (echinulate type) were produced in the soil associated with abundant arbuscules in the roots (Fig. 1C). Quercetin also increased root colonisation in *Calopogonium* with all AMF strains. Pectinase greatly increased root colonisation of *Nicotiana* with GMVN1 strain (47%); this increase was significant with strain GCXH but not for GACB1.

The increase in root colonization is followed by the production of plant shoot biomass, consequently quercetin led to a significant increase shoot biomass in *Nicotiana* by 92% with GACB1, by 44% with GMVN1 and by 40% with GCXH strains. There was equally an increase of 27% due to quercetin in non-inoculated *Nicotiana* but it was not significant. However, the biomass increase over the non-mycorrhizal control was of the order of 445%, 530% and 296% in inoculated *Nicotiana* with GACB1, GMVN1 and GCXH strains respectively. Increase of 22% and 61% was observed

Table 6. Effect of quercetin on *Nicotiana tabacum* and *Calopogonium* root colonized by three arbuscular mycorrhizal fungi strains after 2 months growth

Strains	Root colonization (%)			
	Nicotiana		*Calopogonium*	
	Quercetin -	Quercetin+	Quercetin-	Quercetin+
GACB1	30[a]	68[b]	42[a]	54[b]
GMVN1	68[a]	95[b]	81[a]	100[b]
GCXH	51[a]	55[a]	63[a]	74[b]

[a, b, c]:Within each line means followed by the same letter are not significantly different at the 5% level of confidence

Table 7. Effect of pectinase on *Nicotiana tabacum* and *Calopogonium* root colonized by three arbuscular mycorrhizal fungi after 2 months growth

Strains	Root colonization (%)			
	Nicotiana		*Calopogonium*	
	Pectinase-	Pectinase+	Pectinase-	Pectinase+
GACB1	30[a]	38[b]	42[a]	44[a]
GMVN1	68[a]	100[b]	81[a]	93[b]
GCXH	51[a]	65[b]	63[a]	70[a]

[a, b]:Within each line means followed by the same letter are not significantly different at the 5% level of confidence

with GMVN1 and GCXH strains respectively in *Calopogonium*. The biomass increase was significant only inoculated plants. With GACB1 strains the biomass increase was not significantly different from the non-mycorrhizal control. The increase was of the order of 108%, 195% and 102% with GACB1, GMVN1 and GCXH strains respectively when compared to non-mycorrhizal control (Fig. 2). In inoculated *Calopogonium* with 5 µM quercetin, the production of intra-root spores and vesicles was abundant (Fig. 1D).

As far as the effect of pectinase is concerned; the biomass production was not significantly different in *Nicotiana* inoculated with all species of AMF. In *Calopogonium*, pectinase effect was significantly different with GMVN1 and GCXH (Fig. 3).

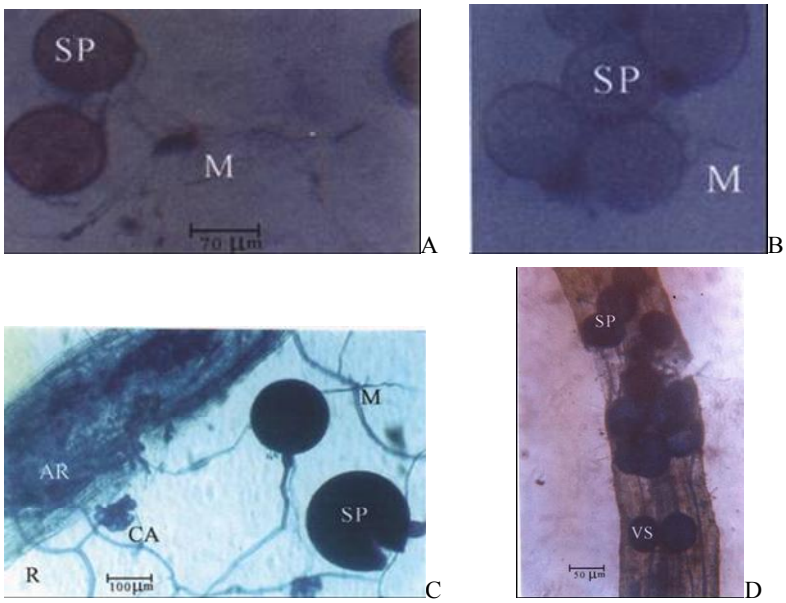

Fig. 1. Effect of quercetin treatment on the germination of spores and root infection. A: *In vitro* germination of spores from *Glomus albidum* (GACBI) with quercetin

Note the abondance of hyphae; B: In vitro germination of spores from Glomus albidum (GACBI) without quercetin: Hyphae is less profuse; C: Tobacco roots infected by Gigaspora margarita (GMVI) with quercetin (5µM); D: Calopogonium sp. root infected by Glomus clarum (GCXH) with quercetin: (AR: arbuscules CA: auxiliary cells, SP: spores, VS: vesicles, M: hyphae)

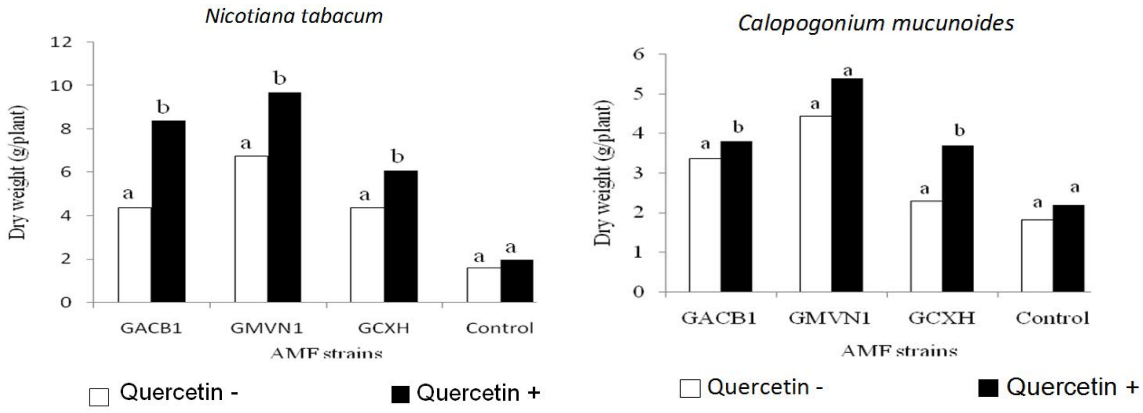

Fig. 2. Response of AMF inoculation on *Nicotiana tabacum* and *Calopogonium mucunoides* shoot dry weight in presence or absence of Quercetin after two-month growth under greenhouse conditions. The same letters are not significantly different at 5% probability level

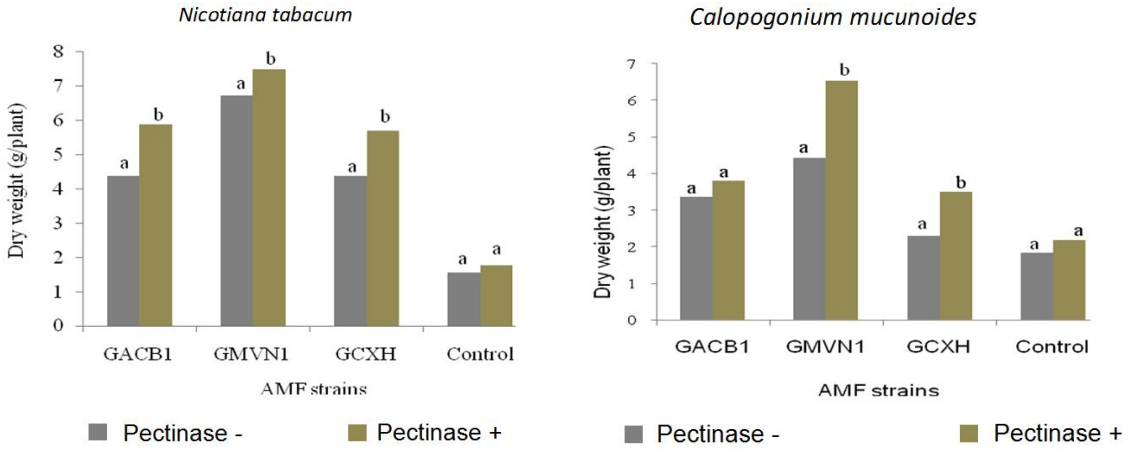

Fig. 3. Response of AMF inoculation on *Nicotiana tabacum* and *Calopogonium mucunoides* shoot dry weight in presence or absence of pectinase enzymes after two-month growth under greenhouse conditions. The same letters are not significantly different at 5% probability level

Selected strains with the aim of choosing the most acid tolerant AMF strains which should easily germinate and adapt to an acidic environment [14]. The strains GACB1, GMVN1 and GCZH showed different results on both *Nicotiana* and *Calopogonium* plants under greenhouse conditions depending on the presence or the absence of quercetin or pectinase. Either with the enzyme or the polyphenol the stimulation of root colonization and biomass increase was observed in both plants. The results of other studies [38,40] had also shown that fungi penetrate the roots by an enzymatic mechanism producing hydrolases as pectinases. [41] Found higher concentrations of polyphenols in mycorrhizal roots compared to non-mycorrhizal ones. Some authors demonstrated that higher concentrations of polyphenols mainly protect the roots against pathogenic micro-organisms and pests [42]. Other authors showed using apigenin that a concentration of 2-8 uM was able to double the hyphal growth of *Gigaspora margarita*, *Glomus mossae* and *G. intraradices*; while the glycosydated form resulted in loss of activity of theses AM fungi [43].

4. CONCLUSION

The efficiency of AMF inoculation in plants depends on the aptitude of mycorrhizal fungus to develop rapidly in soil with the presence of native strains. The survival of all efficient AMF species in the soil depends on the ability of the mycelium to rapidly colonize host plant root and mainly to have a competitive advantage on the native strains. The results of this study have permitted the selection of *Gigaspora margarita* (GMVN1), *Scutellospora gregaria* (SGMN1) and *Glomus clarum* (GCXH) which had shown important spore germination ability, an efficient mycelium growthlength and good root colonization. Pectinase which hydrolyses plant cell walls and quercetin an isoflavonoid, which both are found in soil micro-organisms and in legumes respectively could alter spore germination, hypha growth, root colonization and also plant biomass production. Treatment combining quercetin and pectinase could lead to a synergetic effect on mycorrhizal symbiosis and plant performance. These compounds which had modified the symbiosis physiology may have a great importance in the regulation of plant soil microbe interaction. They can equally contribute to stimulate the growth of these strict biotrophic fungi under culture or *in vitro* and thus favoring plant biomass production. Their potential in a sustainable agriculture system could be of particular interest for agriculture in the tropics.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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