



## Potentialisation of the biocontrol efficacy of arbuscular mycorrhizas fungi against cocoa black pod rot causing *Phytophthora megakarya* with natural flavonoid

Louise Wakam Nana<sup>1,3</sup>, Virginie Thérèse Ekounda<sup>1,3</sup>, Pierre Mkounga<sup>2</sup>, Pierre Eke<sup>1,3</sup>, Augustin Ephraïm Nkengfack<sup>2</sup>, Dieudonné Nwaga<sup>\*1</sup>

<sup>1</sup>Laboratory of Soil Microbiology, University of Yaoundé I, Cameroon

<sup>2</sup>Department of Organic Chemistry, University of Yaoundé I, Cameroon

<sup>3</sup>Department of Biochemistry, University of Yaoundé I, Cameroon

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### Abstract

Cocoa (*Theobroma cacao* L.) black pod rot, caused by oomycetes Chromista *Phytophthora megakarya* is the major constraint to cocoa production in Cameroon, causing substantial yield losses (up to 100%). As mean to alter the yield shortage, priority is given to chemical fungicides, though Arbuscular Mycorrhizals Fungi (AMF) have been pointed out to offer a friendly alternative. Moreover, exudation of flavonoids in the mycorrhizosphere could modulate the symbiotic efficiency of these symbionts. Thus, the single and associative effects of two AMF strains (*Gigaspora margarita* and *Glomus intraradices*) and a natural flavonoid (3,5,7,3',4',5'-hexahydroxy flavanone) were evaluated for their ability to induce tolerance in two cocoa (*T. cacao*) clones (SNK 10 and ICS 84) against *P. megakarya* under greenhouse conditions. Also, as biochemical resistance markers, qualitative (TLC) and quantitative changes in total phenol and flavonoid were assessed Twenty Weeks After Sowing (WAS). The results indicated that, by adding the flavonoid, the AMF significantly improved the growth, total phenol and flavonoid contents as well as the susceptibility of both clones towards *P. megakarya*. The TLC revealed an enhanced biosynthesis of flavones and anthocyanidins in fresh leaves from the ICS 84 clone which was found to be the least sensitive to *P. megakarya*. Our results reveal that the dual application of AMF and flavonoid significantly suppresses the black pod disease on cocoa (*T. cacao*) seedlings, thereby supporting their used to improve the tolerance of cocoa plant against *P. megakarya*.

\*Corresponding Author: NWAGA Dieudonné ✉ [dnwaga@Yahoo.fr](mailto:dnwaga@Yahoo.fr)

## Introduction

Cocoa (*Theobroma cacao* L.) is a perennial crop of high economic importance in several cocoa-producing countries (Efombagn *et al.*, 2013; Nyadamu *et al.*, 2013). However, black pod disease caused by *Phytophthora* induces substantial yield losses worldwide, particularly in Africa. In Cameroon, pods loss due to the disease ranges from 50 to 80 % (Ndoumbe-Nkeng *et al.*, 2004). Despite much effort on breeding for resistance to *Phytophthora* in Cameroon, cocoa cultivars completely resistant have not been found to date (Omokolo *et al.*, 2002; Efombagn *et al.*, 2013).

So far, the chemical control using metalaxyl and copper-based contact fungicides is the most widely used control method (Opoku *et al.*, 2007; Deberdt *et al.*, 2008). But, the practice remains too expensive and constraining for most smallholders (Ndoumbe-Nkeng *et al.*, 2004). Furthermore, in the long run, chemical pesticides have adverse environmental impact and the exacerbating risk of resistance development (Ndoumbe-Nkeng and Sache, 2003; Deberdt *et al.*, 2008; Nyadamu *et al.*, 2013).

As alternative, the biological control have gained wide acceptance during the last decades as they have shown a relatively wide spectrum of activity against a large numbers of plant pathogens under greenhouse and field conditions (Wehner *et al.*, 2009). In this connection, Arbuscular Mycorrhizal Fungi (AMF) known as major components of the rhizosphere of most plants, play an important role in decreasing plant disease incidence (Akthar and Siddiqui, 2008). Several AMF species have been found to control plant pathogens such as species of *Aphanomyces*, *Cylindrocladium*, *Fusarium*, *Macrophomina* and *Phytophthora*, (Harrier and Watson, 2004; Askar and Rashad, 2010; Küçükyumuk *et al.*, 2014). In this regards, Tchameni *et al.* (2012) showed the capacity of the AMF *Gigaspora margarita* and *Glomus mossae* to stimulate the growth of cocoa (*T. cacao*) plants and reduce their susceptibility towards *P. megakarya*. The induction of host plant defense system through the enhancement of the biosynthesis of defense-related enzymes and molecules are among

the mechanism thought to be involved in the reduction of plant disease incidence and/or virulence by AMF (Nana *et al.*, 2002; Fokom *et al.*, 2010). Currently, most studies focused on the interactions between a single pair of species (i.e. one beneficial and one pathogen) and do not take into consideration the vast microbial diversity within the functional groups coexisting on and near plant roots. Therefore, we hypothesized that assemblages of AMF derived from multiple species may exhibit greater potential to protect host plants against pathogens than single AMF species (Maherali and Klironomos, 2007; Wehner, 2009; Singh, 2014).

In addition, the colonization of plant roots by AMF involves a molecular dialogue between plant and AMF (Harrison, 1998; Bais *et al.*, 2006; Cesco *et al.*, 2012; Lu *et al.*, 2015). This dialogue starts during the presymbiotic stage and might be regulated by secondary metabolites. In fact, plant-derived flavonoids might act as regulators in plant-fungus interactions during the precolonization and the cell-to-cell stage of the development of the symbiosis (Vierheilig and Piche, 2002; Scervino *et al.*, 2009; Hassan and Mathesius, 2012). Scervino *et al.* (2009), revealed an increased hyphal length, number of hyphal branches, number of clusters of auxiliary cells, number of entry points and the percentage of tomato (*Lycopersicon esculentum* L.) root colonized by *Gigaspora margarita* when applied in combination with 3-methoxy-5,6,7,8-hydroxy-4-hydroxy flavone isolated from shoots of non arbuscular mycorrhizal inoculated clover (*Trifolium repens*). A closed relationship between such events and the percentage of root colonization by *Gigaspora* or *Glomus* symbionts in presence of the flavonoids tested was found by Scervino *et al.* (2005a). Besides, the influence of flavonoids on the AMF seems to be different not only at genera but also at the species level (Vierheilig *et al.*, 1998).

In our previous work Tchameni *et al.* (2012), we observed that the AMF, *Gigaspora margarita* and *Glomus intraradices* used alone could significantly reduce the cocoa (*T. cacao*) black pod disease through leaf disc assay under greenhouse conditions.

In order to address these limitations, the present study was undertaken to assess the combined effect of those AMF alone or in dual application with the 3,5,7,3',4',5'-hexahydroxy flavanone on the induction of growth and tolerance in two cocoa (*T. cacao*) clones against *P. megakarya*.

## Materials and methods

### *Microbial strains and inoculum production*

The AMF strains (*Gigaspora margarita* and *Glomus intraradices*) used was obtained from the Laboratory of Soil Microbiology, Biotechnology Centre, University of Yaoundé I, Cameroon. They were isolated from maize (*Zea mays*) root and grown in sterile coarse sand (Nwaga *et al.*, 2010). These AMF strains were selected according to their capacity to enhanced nutrient uptake by their host plants to a larger extent (*Gigaspora margarita*) and to increase tolerance to pathogenic infections (*Glomus intraradices*) (Ngonkeu, 2009; Tchameni *et al.*, 2012). The inoculum consisted of a mixture of spores, mycelium, root fragments and coarse sand. The prepared inoculum was stored under laboratory conditions at 15 to 20°C after drying.

The *P. megakarya* isolate, obtained from the Regional Laboratory of Biological Control and Applied Microbiology of the Institute of Research Agricultural for Development (IRAD) Yaoundé, Cameroon, was isolated on cocoa (*T. cacao*) pods exhibiting black pod symptoms and stored in sterile distilled water on small pieces of V8 agar at 4°C. Before the leaf disc assay, the pathogen was grown on fresh V8 agar medium. Zoospores were obtained from sporangia of artificially infected cocoa (*T. cacao*) pods maintained in humid plastic boxes at room temperature (28°C) for 5 days. Sporangia were scraped from their surfaces and placed in sterile distilled water. The suspension was then placed in a refrigerator at 4°C for 5 min and transferred to room temperature for 15 min. The concentration of released zoospores was adjusted to 10<sup>6</sup> zoospores/ml using a hemacytometer (Nwaga, 1984; Nyassé *et al.*, 1995).

### *Substrate preparation and seedling production*

The soil used for the pot experiments was collected from the A horizon of cocoa (*T. cacao*) farms around Yaoundé. This soil was air-dried, passed through a 5 mm sieve to remove stones and conspicuous plant debris before mixing at the ratio of 2:1 (v/v soil: sand) proportion with coarse sand. Nutrients analysis of a soil subsample yielded the following results: pH (water) 6.38; Ca (3.97 cmol/kg); Mg (0.92 cmol/kg); K (0.28 cmol/kg); Na (0.023 cmol/kg); P (1.54 µg/g); organic carbon (0.75 %); N total (0.070 %); C/N (11.17). The soil-sand mixture was then autoclaved three times for 1hour at 121°C.

Six-month-old cocoa pods (cultivar SNK 10 and ICS 84) were collected from trees at the cocoa (*T. cacao*) research station in Nkoemvone (N 2°90', E 11°20'), South-Cameroon and transported to the Laboratory of Soil Microbiology, Biotechnology Centre (University of Yaoundé I, Cameroon). The SNK 10 and ICS 84 trees located in this experimental field were separated by a 3.5 m grass trace. Therefore the ICS 84 flowers could have been pollinated with pollen from ICS 84, same to SNK 10. These clones represent deferent levels of susceptibility to black pod disease according to Blaha and Lotode (1976). SNK10 is highly sensitive to *P. megakarya* with 99% of successful infection, while ICS 84 is less sensitive with a successful infection rate of 29% (Nana, 1991). For pre-germination, 25 cocoa pods in each clone cocoa seeds were extracted from the pods and washed with distilled water. The seeds were then transferred to a plastic tray (30 x 30 x 15 cm) half-filled with sterile soil and incubated at room temperature for ten days.

### *Flavonoid extraction and test solution preparation*

The flavonoid (3,5,7,3',4',5'-hexahydroxy flavanone) used in this study was extracted at the Laboratory of Phytochemistry and analytic Chemistry of the Department of Organic Chemistry from the bark of *Pycnanthus angolensis*, a forest tree. Barks were crushed after drying at 70°C for 72 hours and the flavonoid was extracted by dissolving 10 g of bark powder in 3 liter of concentrated methanol.

After concentrated with vacuum evaporator, the crude extract was separated and purified by column chromatography and identified based on their UV and RMN spectra (Satnami and Yadava, 2011). The flavonoid crystals were dissolved in absolute ethanol and added to sterile distilled water to obtain 5  $\mu$ M solution and stored at 4°C in darkness until used (Scervino *et al.*, 2005a).

#### *AMF inoculation and cocoa seedlings sowing*

The sterile soil-sand mixture was transferred in clean plastic pots (3 l). For inoculation select AMF strains, 10 g of mixture (containing 100 spores of *Gigaspora margarita* and *Glomus intraradices*) were placed at 5 cm below the surface of the growth medium. Thereafter, a single pre-germinated cocoa (*T. cacao*) seed of each cultivar was sowed into each pot. For the controls, cocoa (*T. cacao*) seed were planted into non-inoculated soil.

#### *Flavonoid application and growth condition*

Two weeks after sowing, flavonoid solution was applied 1 cm around the plant collar region at the rate of 20 ml/plant. The experiment design was a 3 x 3 factorial combination with four treatments in a randomized complete block design with three replicates and each treatment consisting of six replicates was repeated twice.

For each cocoa (*T. cacao*) clone, treatments were:

My-Fl- = treatment without AMF nor flavonoid:

My+Fl- = treatment with AMF without flavonoid:

My-Fl+ = treatment with flavonoid without AMF:

My+Fl+ = treatment with AMF and flavonoid.

Plants were watered after two days with distilled water and grown for 20 weeks. Twenty weeks after sowing (WAS) five plants of each treatment were carefully harvested, the roots were washed with tap water to remove soil particles and evaluated for the following growth parameters: number of leaves, shoot height (cm) and shoot and root fresh weight (g).

#### *Assessment of AMF root colonization frequency*

The root subsamples (1g) were cleared using 10% potassium hydroxide (KOH) and stained with acid fuchsin (Kormanick and McGraw, 1982)

They were then mounted on slides and observed using a light microscope. The percentage of root length colonized by AMF was calculated by the gridline intersect method (Trouvelot *et al.*, 1986).

#### *Extraction and determination of total phenol and flavonoid content in cocoa plant leaves*

Total phenol was extracted as described by Bastide *et al.* (1988), modified by Nana *et al.* (1995). A mixture of 0.5 g grounded leaves samples was dissolved in 25 ml of 70% methanol at room temperature for 45 min. The mixture was filtered and evaporated at reduced pressure. The aqueous phase obtained was adjusted to 25 ml with sterile distilled water, and depigmented by adding 12.5 ml of 40% ammonium sulphate [(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>], 0.35 ml of 80% ortho-phosphoric acid and 25 ml of petroleum. The ether phases were discarded and the aqueous phases were extracted four times with 25 ml ethyl acetate. The aqueous phases were discarded and the organic phases of the same sample were combined, dried by addition of 5 g of magnesium sulphate (MgSO<sub>4</sub>) and filtered after 5 min with Whatman n°1 paper. The salt residue was discarded and the clear organic phase was dried at 40°C under vacuum using a rotary evaporator. This extract was submitted to total phenolic and flavonoids quantification.

Folin-Ciocalteu reagent (diluted 10-fold) was used to determine the total phenolic

contents of the leaf samples as described by Jaafar *et al.* (2010). For that, 0.5 ml of the sample extract was mixed with 2 ml of NaCO<sub>3</sub> (20%), before adding Follin-Ciocalteu reagent (2.5 ml). After two hours at 22°C, absorbance was measured at 725 nm. Total phenol content was expressed in mg of chlorogenic acid per gram of leaves of a group of plants.

For total flavonoids compounds, 1 ml of sample was mixed with 0.3 ml NaNO<sub>3</sub>, the mixture was placed in a test tube covered with aluminum foil. After 5 min, 0.3 ml of AlCl<sub>3</sub> (10%) was added followed by 2 ml of 1 M NaOH. The absorbance was measured at 510 nm using rutin as a standard. The results were expressed in term of mg rutin/g dry sample (Jaafar *et al.*, 2010).

*Thin layer chromatography (TLC) of flavonoids extract*

The extracts of flavonoids were subjected to thin layer chromatographic analysis on silica gel as solid phase (Harborne, 1998), using butyl acetate/acetic acid/water in the proportions (4/1/5) (v/v/v) as mobile phase (Macheix, 1974). After the development of the chromatogram, the plates were dried at room temperature and detection was carried out using UV trans illuminator at 254 nm. The colors of spots and retention factor (Rf) value were recorded, corresponding to those produced by the reference: flavonol, Rf: (0.5-0.75) yellow; anthocyanidin, Rf: (0.54-0.90) orange-red; flavone, Rf: (0.00-0.50) purple (Markham, 1982; Bandyukova and Shinkarenko, 1973).

*Leaf disc inoculation assay*

The degree of resistance of the *T. cacao* clones was assessed by the leaf disc assay (Nwaga, 1984; Nyassé *et al.*, 1995; Efombagn *et al.*, 2013). In fact, preliminary test using inoculations on alternative organs such as leaves showed significant correlations with resistance levels of the pods in the field (Nyassé, 1997; Tahi *et al.*, 2000), suggesting that selection can be readily done by using leaf inoculations. Young cocoa (*T. cacao*) leaves (20 weeks-old) were collected from the seedlings and disc were made using cork borer. Leaf discs were randomly selected and placed in humidified petri dishes. For each treatment, five petri dishes were used. A total of 10 discs were inoculated per petri dish. Inoculation was carried out in the laboratory by depositing a 10 µl drop of suspension of a 10<sup>6</sup> zoospores/ml suspension of *P. megakarya* (Nyassé *et al.*, 1995). In each treatment, one control made up of 10 discs inoculated with 10 µl of sterile distilled water was done.

The inoculated discs were then incubated in the dark at 25°C. Disease expression was rated six days after inoculation using the rating scale developed by Nyassé *et al.* (1995) where 0: no symptom development; 1: penetration points observed at the inoculation site; 2: connected points; 3: reticulate necrotic aspect; 4: marbled necrosis; and 5 : true necrosis. This experiment was repeated twice and the infection intensity index was determined for each treatment by calculating the ratio of the sum of individual score over the total number of leaves used. The infection intensity index was used to express the tolerance level as follows: highly resistant (HR) (0 < index < 1), resistant (R) (1< index<2), moderately resistant (MR) (2<index<2.5), susceptible (S) (2.5<index< 3.5), highly susceptible (HS) (index>3.5) (Paulin *et al.*, 2008).

*Statistical analyses*

Statistical analyses of the data were performed with SPSS software (version 17.0). All the tested parameters were analyzed by ANOVA followed by Least Significance Difference (LSD) test to differentiate treatments, at 5% probability.

**Results**

*Effect of AMF and flavonoid application on T. cacao roots colonization*

Plants grown in substrates inoculated with AMF were successfully colonized compared to non-inoculated plants 20 WAS (Table 1). As shown in this table, the root colonization varied significantly depending on the treatments tested and *T. cacao* clone involves. Plants treated with dual application flavonoid-AMF, significantly increased AMF root colonization of SNK 10 clone (76.67%) when compared to mycorrhizae alone (70.00%) and the ICS 84 clone.

**Table 1.** Effect of AMF and flavonoid (3,5,7,3',4',5'-hexahydroxy flavanone) application on cocoa (*T. cacao*) seedling root colonization (SNK10 & ICS 84) at 20 WAS.

Treatments	Cocoa clones	
	SNK 10	ICS 84
My-Fl-	0.00 <sup>c</sup>	0.00 <sup>c</sup>
My-Fl+	0.00 <sup>c</sup>	0.00 <sup>c</sup>
My+Fl-	70.00 <sup>b</sup>	66.67 <sup>a</sup>
My+Fl+	76.67 <sup>a</sup>	63.34 <sup>b</sup>

Means values with the same letter within a column are not significantly different at P < 0.05.

*Effect of AMF and flavonoid application on growth parameters*

Globally, and no matter the clone enrolled, the growth of *T. cacao* seedlings was not affected by 3,5,7,3',4',5'-hexahydroxy flavanone application alone except a reduction for the number of leavers and shoot of fresh weight in SNK 10 clone (P<0.05; Table 2).

The AMF inoculation increased the shoot fresh weight and the root fresh weight for the clone SNK 10 and the shoot fresh weight for ICS 84 clone. AMF effect is greater on SNK 10 clone (62-79%) biomass than ICS 84 (34-35%). Dual application of AMF and flavonoid showed a mark significance increase on cocoa biomass (shoot and root) from 61.61% to 83.29% on SNK 10 clone and 51.17% to 93.04% on ICS 84 clone compared to control.

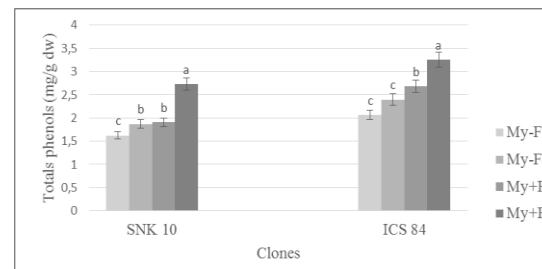
**Table 2.** Effect of AMF and 3,5,7,3',4',5'-hexahydroxyflavanone application on growth parameters of *T. cacao* seedlings (SNK10 & ICS 84) 20 WAS.

Treatments	Number of leaves		Shoot height (cm)		Shoot fresh weight (g)		Root fresh weight (g)	
	SNK 10	ICS 84	SNK 10	ICS 84	SNK 10	ICS 84	SNK10	ICS 84
My-Fl-	12.00 <sup>b</sup>	11.00 <sup>ab</sup>	23.90 <sup>b</sup>	24.83 <sup>b</sup>	12.66 <sup>b</sup>	11.52 <sup>c</sup>	8.62 <sup>b</sup>	6.61 <sup>b</sup>
My-Fl+	7.00 <sup>c</sup>	10.00 <sup>b</sup>	23.90 <sup>b</sup>	24.66 <sup>b</sup>	5.96 <sup>c</sup>	14.61 <sup>b</sup>	12.14 <sup>ab</sup>	8.15 <sup>b</sup>
My+Fl-	15.00 <sup>ab</sup>	11.00 <sup>ab</sup>	27.93 <sup>a</sup>	26.40 <sup>a</sup>	20.46 <sup>a</sup>	15.65 <sup>a</sup>	15.46 <sup>a</sup>	8.94 <sup>b</sup>
My+Fl+	16.00 <sup>a</sup>	13.00 <sup>a</sup>	27.60 <sup>a</sup>	27.40 <sup>a</sup>	20.46 <sup>a</sup>	17.50 <sup>a</sup>	15.80 <sup>a</sup>	12.76 <sup>a</sup>

Means values with the same letter within a column are not significantly different at P < 0.05.

*Effect of AMF and flavonoid application on total phenol content*

The effect of AMF inoculation and flavonoid application on the total phenol contents of cocoa (*T. cacao*) seedlings (ICS 84 and SNK 10) 20 WAS is shown in Fig. 1. We found a significant variation of phenolic contents depending on the treatments.



Means values with the same letter within a column are not significantly different at P < 0.05.

**Fig. 1:** Effect of AMF and 3,5,7,3',4',5'-hexahydroxy flavanone application on *T. cacao* seedlings total phenol content 20 WAS.

Compared to control conditions, phenol concentrations were not affected by flavonoid applications alone at ICS 84 whereas increased at SNK 10 clone. As compare with control treatment, total phenol contents increased with AMF inoculation alone and in combination with flavonoid for both clones.

*Effect of AMF and flavonoid application on T. cacao seedlings flavonoids contents*

AMF inoculation induced increasing of total phenol contents by 17.42% on SNK 10 clone and 29.86% on ICS 84. Dual application of AMF and flavonoid increases total phenol by 68.16% on SNK 10 clone and 57.86% on ICS 84 clone compared to control. Globally, ICS 84 cocoa (*T. cacao*) plants treated with the AMF alone or and dual flavonoid application showed increase records by 6.28% and 6.37% respectively than plant of SNK 10 clone.

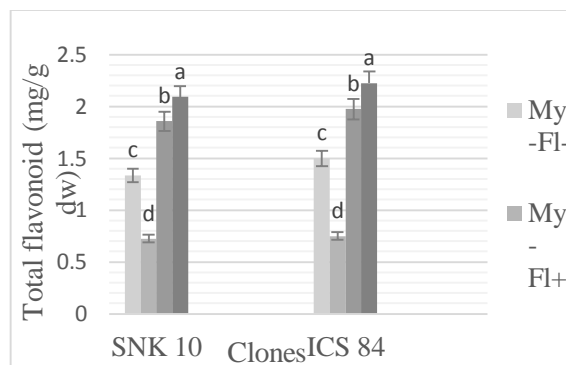
The contents of fiavanoids in the leaves of cocoa (*T. cacao*) seedlings treated with AMF and flavonoid alone and in combination 20 WAS are shown in Fig. 2. Compared to non-inoculated seedlings, flavonoids concentrations were significantly increased after the treatments with AMF either single or in association with flavonoid (P<0.05). When 3,5,7,3',4',5'-hexahydroxy flavanone applied alone,

the flavonoids content in young *T. cacao* seedlings leaves significantly decreased in both clones compared to control. AMF inoculation alone increased flavonoid contents by 39.36% on SNK 10 clone and by 31.67% on ICS 84. The combined action of AMF and flavonoid showed the highest records of flavonoids contents than in AMF inoculated plants by 56.88% on SNK 10 clone and 48.33% on ICS 84 clone compared to control. Between the clones, ICS 84 plants treated with AMF and flavonoid alone and in combination had the highest amount of flavonoids either 40.56% and 19.30% respectively compared to SNK 10 clone.

*Effect of AMF and flavonoid application on T. cacao seedlings of flavonoids analysis by Thin Layer Chromatography (TLC)*

The qualitative analysis of flavonoid content was made by thin layer chromatography. For SNK 10 clone, results shows 6 spots (Rf 0.21; 0.29; 0.54; 0.64; 0.75 and 0.83) were observed in the control treatment (Table 3). After single and dual application of AMF and flavonoid, 5 and 6 spots was observed respectively.

However, 2 news spots of Rf 0.71 and 0.78 after AMF treatment and also the spots of Rf 0.14 and 0.43 after coupling AMF and flavonoid. A single specific spot yellow colour was observed with all the treatments of the SNK 10 clone; all identified as flavonols.



Means values with the same letter within a column are not significantly different at P < 0.05.

**Fig. 2:** Effect of AMF and flavonoid application on cocoa (*T. cacao*) seedling (SNK10 and ICS 84) total flavonoid content 20 WAS.

**Table 3.** Thin layer chromatographic analysis of flavonoid extract from SNK 10 (*T. cacao*) plants.

TRT No	My-Fl-			My-Fl+			My+Fl-			My+Fl+			Identified Flavonoids
	Colour	Int	Rf	Colour	Int	Rf	Colour	Int	Rf	Colour	Int	Rf	
1										Yellow	++	0.14	Flavonols
2	Yellow	++++	0.21							Yellow	++	0.21	Flavonols
3	Yellow	+++	0.29	Yellow	+++	0.29	Yellow	++	0.29				Flavonols
5				Yellow	++	0.36							Flavones
6										Yellow	+++	0.43	Flavonols
7							Yellow	++	0.50				Flavonols
8	Yellow	++++	0.54	Yellow	+	0.54							Flavonols
9	Yellow	++++	0.64	Yellow	++	0.64							Flavonols
10							Yellow	++++	0.71	Yellow	++++	0.71	Flavonols
11	Yellow	++++	0.75	Yellow	++	0.75							Flavonols
12							Yellow	+++	0.78	Yellow	++	0.78	Flavonols
13	Yellow	+++++	0.83	Yellow	+++	0.83	Yellow	++++	0.83	Yellow	++++	0.83	Flavonols

My-Fl-: control; My-Fl+: flavonoid application; My+Fl-: inoculation with AMF; My+Fl+: dual inoculation AMF and flavonoid application; TRT: treatments; Int: intensity; Rf: retention factor.

For ICS 84 clone, results shows 3 spots of Rf 0.43; 0.64 and 0.86 with yellow colour observed in the control treatment 20 WAS and identified as flavonols (Table 4). AMF inoculation as well as combination shows 5 and 9 spots respectively.

Compared to the control, 2 news spots with Rf 0.21 and 0.36 were observed simultaneously in two treatments with yellow colour and identified as

flavonols. However, 2 news spots of Rf 0.53 and 0.57 steady at one spot of Rf 0.53 only in mycorrhiza treatment and identified also as flavonols.

Alone after dual application AMF and flavonoid, 4 spots of Rfs 0.28 (yellow); 0.50 (purple); 0.57 (red) and 0.78 (yellow) were observed and identified as flavonols, flavones, anthocyanidins and flavonols respectively.

**Table 4.** Thin layer chromatographic analysis of flavonoid extract from ICS 84 (*T. cacao*) plants.

Nº	My-Fl-			My-Fl+			My+Fl-			My+Fl+			Identified Flavonoid
	Colour	Int	Rf	Color	Int	Rf	Colour	Int	Rf	Colour	Int	Rf	
1							Yellow	++	0.21	Yellow	+++	0.21	Flavonols
2										Yellow	+++	0.28	Flavonols
3							Yellow	++	0.36	Yellow	+++	0.36	Flavonols
4	Yellow	+	0.43	Yellow	+++	0.43							Flavonols
5										Purple	+++++	0.50	Flavones
6							Yellow	+++	0.53				Flavonols
7										Red	++++	0.57	Anthocyanidin
8	Yellow	+	0.64	Yellow	+++	0.64				Yellow	+++++	0.64	Flavonols
9				Yellow	+++	0.71	Yellow	+++	0.71	Yellow	++++	0.71	Flavonols
10										Yellow	+++	0.78	Flavonols
11	Yellow	++++	0,86							Yellow	++++	0,86	Flavonols

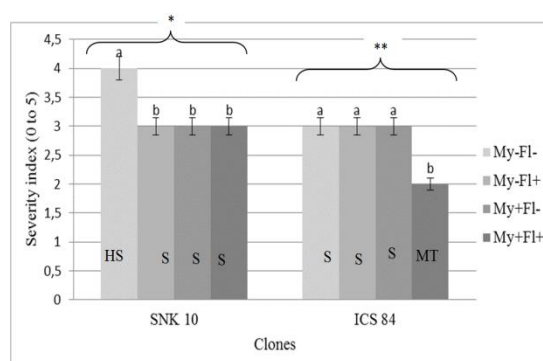
*Effect of AMF and flavonoid application on black pod disease severity*

Six days after leaf inoculation, necrotic lesions were observed on *T. cacao* leaf discs inoculated with zoospores, while no symptom was seen on foliar disc inoculated with sterile distilled water (Fig. 3).

The analysis of variance revealed that disease expression was significantly different among clones and treatments ( $P < 0.05$ ). The highest level of infection intensity index (lowest level of resistance) was observed with treatments of SNK 10 clone without.

AMF and flavonoid (3,5,7,3',4',5'- hexahydroxy flavanone); these plants were therefore classified as highly susceptible (HS) (index>3.5). But all treatment in this clone induced increase level of tolerance, they are susceptible (S) ( $2.5 < \text{index} < 3.5$ ). Plants of ICS 84 clone inoculated with both AMF and flavonoid presented lowest disease index (highest level of resistance) were classified as moderately tolerant (MT) ( $2 < \text{index} < 2.5$ ) (Fig. 3). After dual application.

AMF and flavonoid, the level of tolerance on leaves at ICS 84 clone increases 2 times in comparison to SNK 10 clone. In this experiment, mycorrhizal colonization was not correlated with disease severity and in same treatment, the levels of tolerance vary on clone.



Means values with the same letter within a column are not significantly different at  $P < 0.05$ .

**Fig. 3.** Effect of AMF and flavonoid application on *P. megakarya* cocoa black pod rot severity six days after inoculation. MR: Moderately Tolerant; S: Susceptible; HS: Highly Susceptible

**Discussion**

The use of beneficial microorganisms such as *Arbuscular mycorrhizal* fungi (AMF) for biopesticides to reduce diseases on various important crops is considered as one of the most promising methods in agricultural management practices (Toua *et al.*, 2013). AMF have been reported to be effective in controlling plant diseases (Agrios, 1997; Askar and Rashad, 2010; Tchameni *et al.*, 2011; Küçüküyük *et al.*, 2014). In addition, the assemblages of AMF derived from multiple species may exhibit greater potential to protect host plants against pathogens than a single AMF species (Maherali and Klironomos, 2007; Wehner *et al.*, 2009).



Besides, abundant data have shown the effect of flavonoids on different pre-symbiotic stages of AMF. Nevertheless, the effectiveness has been dependant of the type and the concentrations of flavonoids involved as well as the AMF species (Cesco *et al.*, 2010; Hassan and Ulrike, 2012).

#### *Effect of AMF and flavonoid application on T. cacao roots colonization*

The results recorded from this study indicated and increase in AMF root colonization (from 70 to 76%) with the clone SNK 10 upon application of 3,5,7,3',4',5'-hexahydroxy flavanone. A reverse trend was recorded with the clone ICS 84 (from 66.67 to 63.34%). Similarly, Tchameni *et al.* (2008), showed that the kaempferol and 3-O-D glucopyranosyl kaempferol could significantly increase the French bean root colonization by *Glomus clarum* and *Gigaspora margarita*. These results are in line with the findings of Scervino *et al.* (2006) who reported that the *Gigaspora margarita* and *Glomus intraradices* hyphal branching entry points and colonization percentage of tomato (*Lycopersicon esculentum* L.) roots nearly doubled upon the application of 0.5 µM apigenin.

An earlier result by the same author (Scervino *et al.*, 2005a) revealed the inhibitory effects of the acacetin and rhamnetin on the number of entry points and root colonization of the same plant by *Gigaspora rosea* during and *in-vitro* experiment. These findings further highlight the fact that several factors including the host-AMF genera correspondence have to be taken into consideration while selecting a biocontrol agent (Vierheilig *et al.*, 1998; Scervino *et al.*, 2005b; AL-Ghamdi and Jais, 2013).

Once symbiosis is established, the plant has to regulate the level of fungal proliferation within the roots to prevent excessive colonization and carbon drainage, thus maintaining the interaction at mutualistic levels (Jung *et al.*, 2012). For example, under conditions of high exogenous phosphate supply, the plant actively inhibits proliferation of the fungus within the roots (Breuillin *et al.*, 2010).

#### *Effect of AMF and flavonoid application on growth parameters*

As consequence of a successful colonization of cocoa root by AMF, exacerbated by the application of the flavonoid, and increased root, shoot and root fresh weight of ICS 84 and SNK 10 seedlings was recorded respectively. These results may be explained by the branching of the extra-mycelia of AMF under the influence of the 3,5,7,3',4',5'-hexahydroxy flavanone that have subsequently explored in a larger extent the rhizospheric soil, inducing an improvement in the absorption of more nutrients (Harley, 1989; Smith and Read, 2008). It is thus evident that as compare to treatments with AMF alone, mycorrhized plants treated with 3,5,7,3',4',5'-hexahydroxy flavanone take up a larger amounts of nutrients; increasing therefore the biomass production and photosynthetic rates that have been estimated up to 20 % by mycorrhized tomato (*L. esculentum*) seedlings Bago *et al.* (2000). These results are in agreement with a previous study which showed that by combining the spores of *Glomus clarum* and *Gigaspora margarita* with kaempferol, the French bean biomass could increase up to 84% as compare to mycorrhized plant alone 45 days after sowing (Tchameni *et al.*, 2008).

#### *Effect of AMF and flavonoid application on total phenol content*

In both clones, treatments with AMF (My+Fl- and My+Fl+) showed and increased amount of phenolic compounds in young *T. cacao* leaves as compared to non treated control (My-Fl-). The highest content of phenolics was registered with the dual application of AMF and 3,5,7,3',4',5'- hexahydroxy flavanone (My+Fl+). These results further strengthen the fact that the biosynthesis of phenolic compounds in cocoa (*T. cacao*) plants can be stimulated by several factors, especially the AMF as previously shown (Tchameni *et al.*, 2011). In this connection, and considering the treatment without flavonoid (My+Fl-), Nana *et al.* (2002); Al-Askar and Rashad (2010) and Lu *et al.* (2015) reported that the inoculation with AMF promoted phenol biosynthesis in cowpea (*Vigna unguiculata*), common bean (*Phaseolus vulgaris* L.) and yam (*Dioscorea* spp.) respectively.

In fact, there is accumulating literature showing the involvement of mycorrhizal fungi in the enhancement of host plants defense responses against pathogen attacks (Volpin *et al.*, 1995; Al-Askar and Rashad, 2010; Tchameni *et al.*, 2011). They often lead to a significant increase in the phenolic content and the activities of defense related enzymes such as phenylalanine ammonia-lyase (PAL) and polyphenol oxidase (PPO) implicated in disease resistance induction (Volpin *et al.*, 1995; Al-Askar and Rashad, 2010). An enhanced biosynthesis of phenolic compounds was also recorded by Fokom *et al.* (2010). This author discovered that mycorrhized cowpea (*Vigna unguiculata*) accumulated lower content of phenolic compounds. When compared with the application of a mixture of AMF (*Gigaspora margarita*, *Glomus intraradices*, *Glomus clarum*, *Scutellospora gregaria*) and 3-O-glucoside kaempferol (13%). Literally, and once more, after the application of flavonoid, there is an increased probability of successful infections by acting as chemoattractants and growth enhancers for microsymbiont. This interaction induced the formation of the number of clusters of auxiliary cells and number of entry point structure for AMF hyphae branches into the root (Scervino *et al.*, 2006; 2009). Consequently, these events would have apparently increased the *T. cacao* seedlings defense response mediated by the AMF.

#### *Effect of AMF and flavonoid application on T. cacao seedlings flavonoids contents*

Compared to the negative control, AMF alone (39.36% and 31.67% respectively for SNK 10 and ICS 84) and in combination with 3,5,7,3',4',5'-hexahydroxy flavanone (56.88% and 48.33% respectively for SNK 10 and ICS 84) increased the flavonoids content. The dual application of AMF and flavonoid showed greater concentration in both clones (13.51% and 14.87% respectively for SNK 10 and ICS 84) when compared with mycorrhized plants alone. Various studies have reported that the amount of synthesized flavonoids increases before AMF colonize the plant roots (Larose *et al.*, 2002; Lu *et al.*, 2015). For example, Volpin *et al.* (1995) showed that the concentration of formononetin and formononetin-7-O-glycoside of alfalfa (*M. sativa* L.)

roots was increased when *Glomus intraradix* was present in the rhizosphere, and the increase was greater when the AMF was helped by the flavonoid presence. Inversely, cocoa (*T. cacao*) seedlings treated with 3,5,7,3',4',5'-hexahydroxy flavanone alone significantly decreased the content in flavonoid independently on the clones as compared to control. Such decrease could be attributed to the fact that, flavonoids can indirectly affect nutrient cycles in the soil, interfering with the plant nutrition and metabolism. Indeed, once in the rhizosphere, the fate of flavonoids differs depending to the surrounding microorganism (Hassan and Ulrike, 2012). Since the present experiment was conducted in sterile soil, the 3,5,7,3',4',5'-hexahydroxy flavanone rather than undergoing the interaction with the soil mycoflora or its transformation into monocyclic acid such as protocatechuic acid and incorporated into organic matter during the humification process (Barz and Höesel, 1975; Bollag *et al.*, 1997; Dec *et al.*, 2001), would have acted as metal ion chelators altering the soil nutrient cycle. In addition, Cesco *et al.* (2012) showed that, chelation and reduction of metals can alter nutrient concentration in the soil and this might have importance especially for the availability of phosphorus and iron and thus the plant metabolism.

#### *Effect of AMF and flavonoid application on T. cacao seedlings of flavonoids analysis by Thin Layer Chromatography (TLC)*

The thin layer chromatography of the flavonoid groups synthesized upon single and dual application of AMF and flavonoid reveal tree main types in *T. cacao* leaves: flavonols, flavones and anthocyanidins. The flavonols being the most widely spread, were detected in all treatment for both clones. But, anthocyanidins and flavones groups were identified only in ICS 84 clone after dual application of AMF and flavonoid indication the additive effect of the flavonoid application. The qualitative analysis realized by Djocgoue *et al.* (2007) in cocoa (*T. cacao*) leaves showed a higher accumulation of flavone (luteolin derivatives) and flavanone (apigenin derivatives),

similar results were observed by Nana *et al.* (2011) who identified by HPLC the anthocyanidins and flavanes (epicatechin) in cocoa (*T. cacao*) pods husk of SNK 413 and related them to its resistance against *P. megakarya* attack.

*Effect of AMF and flavonoid application on black pod disease severity*

*Phytophthora* species attacks all parts of the cocoa (*T. cacao*) tree, however, the major economic loss is from infection of the pod (Evans and Prior, 1987). Since *T. cacao* is a perennial tree, of fruits production usable for resistance evaluation needs a long time period. However, the *in vitro* early screening tests using inoculations on alternative organs such as leaves had already shown similar ranking between leaf disc and attached pod test results for parental clones (SNK 10, SNK 413, ICS 84, ICS 95, IMC 67 and UPA 134), suggesting that selection can be readily done by using leaf inoculations (Nwaga, 1984; Nyassé *et al.*, 1995; Nyassé, 1997). Other earlier studies including some SNK accessions have shown good correlation between leaf discs tests and attached pod resistance tests and among between leaf disc tests, detached pod tests and field observations of *Phytophthora* pod rot incidence (Efombagn *et al.*, 2011). In this study, the dual application AMF and flavonoid increase level of tolerance in both clones: SNK 10 plants moved from a highly susceptible to a susceptible clone, while ICS 84 moved from a susceptible clone to a moderately tolerant. Globally, the tolerance level of ICS 84 leaves was increased 2 times in comparison to SNK 10 clone after dual application of AMF and flavonoid.

Plant defense responses are coordinated by small molecules that act as signal transducers and tailor the coordinated expression of genes that encode for defense-related proteins and others compounds (Jones and Dangl, 2006). Among these molecules, the phenol compounds such as flavonoids play key roles including the regulation of defense reactions inside the root, and it has been hypothesized that mycorrhizal invasion triggers a temporary defense response in the root that involves induction of flavonoid phytoalexins (Harrison and Dixon, 1993; Hassan and Ulrike, 2012).

This finding could explain the decrease in the index severity of the two clones (SNK 10 and ICS 84). The lowest disease index (highest level of tolerance) observed at ICS 84 clone after treated with both AMF and flavonoid was in agreement with high diversity of flavonoid groups identified in these plants. This result indicates that the inhibitory effect of flavonoids on *P. megakarya* development depends to the level and the type of these compounds in the plant tissues. Accordingly, the addition of flavonoid in the medium may have changed the qualitative content of flavonoid in both clones leading to an increased resistance to *P. megakarya*. Previous studies have shown tolerance of ICS 84 clone to the black pod disease compared to SNK 10 clone both in field and laboratory conditions (Blaha and Lotohe, 1976; Nwaga, 1984; Nana *et al.*, 1995 and Tchameni *et al.*, 2011).

The contrasting response in each clone in their natural condition may be due to various flavonoids content identified by TLC, which might have induced tolerance in selective clones and involve the stimulation of the resistance against *P. megakarya*. This is in agreement with the result of Nana *et al.* (2011); Nyadamu *et al.* (2013) who also reported that the resistance of *T. cacao* against *P. megakarya* is directly linked to the amount and diversity of bioactive compounds such as flavonoids. Moreover, Tchameni *et al.* (2011); (2012) showed that the leaves susceptibility of the young seedlings of *T. cacao* 18 weeks old decreased with the inoculation using the AMF *Gigaspora margarita* and *Glomus intraradices*. These results showed the implication of flavonoids compounds in cocoa (*T. cacao*) defense system against *P. megakarya*. The protection of the host plant from pathogens by mycorrhizal fungi has also been partially attributed to the enhanced synthesis of flavonoid (phytoalexins) in response to the mycorrhizal symbiont (Morandi, 1996; Hassan and Ulrike, 2012). The presence of AMF associated to flavonoid can lead to *de novo* synthesis of flavonoid phytoalexins that exhibit antifungal activity (Larose *et al.*, 2002; Mierziak *et al.*, 2014).

## Conclusion

The present study clearly demonstrated that: the treatment of *T. cacao* plants roots by dual application of AMF and flavonoid induced and increase concentration of defense related metabolites, such as phenolic and flavonoid compounds in both tested clones. Moreover, this association induced disease tolerance in cocoa (*T. cacao*) seedlings against the infection with *P. megakarya*. This phenomenon was confirmed in this study by the enhancement of the growth and the highest production of phenolic and flavonoid compounds. Thus, the treatment of AMF with the 3,5,7,3',4',5'-hexahydroxy flavanone could be an environmentally safe approach in controlling *P. megakarya* causing black pod of *T. cacao*. But, before a proper field application it is necessary to verify the action spectrum of our flavonoid with different AMF species and to seek for an inexhaustible source. Since the AMF used in this study has been formulated and is being used by farmers as biofertilisers for several economically important crop in the country. Therefore, a screening of the tested flavonoid with other AMF species and other pathosystem remain a research field to explore before trials.

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