

Full Length Research Paper

Precocious screening tests of the resistance of two varieties of cocoa seedlings (*Theobroma cacao* L.) from combinations of fertilizers against *Phytophthora megakarya* (Brasier and Griffin) in nursery

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Experiments were conducted to appreciate the resistance level of cocoa seedlings aged three months from combinations of fertilizers against *Phytophthora megakarya* based on artificial leaf disk inoculation test. The two cocoa varieties involved were: Tafo 79/501 (V_1) and SNK 13 (V_2). For each variety, 11 treatments with three replicates were used on 33 randomly-selected plants. These include Arbuscular Mycorrhizal Fungi (M), *Trichoderma asperellum* strain PR11 (T), Organic Fertilizer Gro-wild (O), Chemical fertilizers (NPK), the following combinations (TO), (MT), (MO), (MOT), (MOTNPK) as well as a negative control made by original substrate only (T-) and sterile control with sterilized substrate (TS). Results show that the resistance level of cocoa seedlings in nursery to *P. megakarya* varied with the cocoa variety and for a given variety with the fertilizer. Fertilizers were classified into four categories according to the variation of the infection severity index from 0 (V_1 MOT) to 4.72 (V_1 TS) and from 1.27 (V_2 MOT) to 4.86 (V_2 T-). The most efficient treatment for both V_1 and V_2 was MOT that allowed a dualistic action between Arbuscular Mycorrhizal Fungi and *T. asperellum* in the presence of organic fertilizer Gro-Wild.

Key words: Cocoa, *Phytophthora megakarya*, leaf disk, infection severity index, resistance.

INTRODUCTION

Theobroma cacao L. is one of the most important cash crops in Cameroon, and other countries in Central and West Africa (Assoumou, 1997). Africa is the main producer of this product worldwide (Lass, 2004). Indeed,

about 73% of world production comes from the Ivory Coast, Ghana, Nigeria and Cameroon (ICCO, 2011).

Since 1990, Cameroon has suffered a drastic drop in its cocoa production (Mossu, 1990) whose main causes,

among others, are the rural exodus, the stopping of state subsidies to cocoa farmers, the falling price of cocoa kilogram, the aging of cocoa plantations, pests and various diseases (Tchameni et al., 2012).

Fungal diseases are one of the most important limiting factor of cocoa production (Kouakou et al., 2011). *Phytophthora megakarya* is one of the Oomycete pathogens reported on *Theobroma cacao* (Tchameni et al., 2012). It is the most virulent of *Phytophthora* species that was first reported as the causal agent of black pod disease in 1979 (Akrofi, 2015).

P. megakarya is only endemic to West and Central Africa (Tondje et al., 2007). Nyassé et al. (1999) used isozyme and random amplified polymorphic DNA (RAPD) markers to estimate the genetic diversity and structure among *Phytophthora* isolates from Ghana, Togo, Nigeria, Cameroon, Gabon and Sao Tomé. This pathogen has become the main yield-limiting factor in cocoa production in the sub region (Akrofi, 2015) with yield losses of 50 to 80% in Cameroon (Ndoumbé-Nkeng et al., 2004).

Thus, the menace of *P. megakarya* on cocoa is of great concern to cocoa farmers and scientists. *Phytophthora* spp. control is a major challenge for world cocoa cultivation, and selection of resistant material is a priority research theme for many producing countries (Nyassé et al., 1995). Indeed, yield losses of this crop and cost of controlling black pod disease affecting cocoa constitute a significant financial burden on agricultural enterprises and has serious socio-economic and environmental consequences wherever these pathogens are found (Akrofi, 2015).

Besides, many genetic and environmental factors on growth and quality of seedling were reported in different plant species (Bilir et al., 2004; Yazici, 2010; Dilaver et al., 2015; Tebes et al., 2015; Cercioğlu and Bilir, 2016; Yilmazer and Bilir, 2016; Cetinkaya and Cercioğlu, 2017).

The challenge for the next 50 years is to double agricultural production by respecting the rules of sustainable agriculture with low inputs and without risk to human health (Tilman et al., 2002). Thus, there is increased need for fundamental knowledge in order to develop effective and sustainable methods for the control of crop diseases. One possible strategy is to develop agricultural practices based on ecological processes such as the relationship between flowers and pollinators (Klein et al., 2007; Azo'o et al., 2011, 2012, 2017), the use of natural soil resources, plant nutrient recycling, plant material selection, and management of organic and inorganic inputs (Duponnois et al., 2012). Overall, the general policy for sustainable agricultural and rural development consists in diversifying production systems to make the best possible use of available local

resources (De Silguy, 1997).

The main objective of this study is to find the protection methods of cocoa seedlings in the nursery by biological fertilization process. More specifically, the study leads us to:

1. Evaluate *in vitro*, the resistance level of cocoa seedlings against *P. megakarya* by testing leaf disks of plants from various combinations of fertilizers and
2. Compare the level of susceptibility between two studied cocoa varieties from these combinations.

MATERIALS AND METHODS

Study site

The study was conducted in a nursery of the experimental station of the Institute of Agricultural Research for Development (IRAD) of Nkolbisson (11°36' East and 3°44' North) during the small rainy season. Nkolbisson is a suburb which belongs to the Yaoundé 7th Sub-Division, the Mfoundi Division and the Centre Region of Cameroon.

The Centre Region of Cameroon has Yaoundé as its chief town; this city is also the political capital of Cameroon and the capital of the Mfoundi Division. The Centre Region extends between latitudes 3°47' to 3°36' north and between longitudes 11°10' to 11°45' east. The average altitude is 760 m (Létouzey, 1968).

The Centre Region belongs to the forest zone of Cameroon. The climate here is Guinean type including four seasons with two differently dry and rainy seasons: the brief rainy season (March to June) is followed by the short dry season (July to August) and the longer rainy season (September to November) is followed by the more extended dry season (November to March). The mean annual rainfall is about 2000 mm and the mean annual temperature 26.6°C. These climatic conditions are favorable for cocoa cultivation in the region.

Plant material

Two varieties of cocoa namely Tafo 79/501 and SNK 13 collected from the Centre SODECAO (Cocoa Development Company) of Mengang (Centre Region, Cameroon) were used. The first variety is resulting from the hybridization between Nanay 32 and Parinai 7 varieties, and the second one from Trinitario and Forastero (Blahe and Lotodé, 1976). Both cocoa varieties are among the most disseminated by the Centre SODECAO of Mengang.

Biological, organic and chemical materials

Biological, organic and chemical materials used are reported in Table 1.

Combinations of different treatments

The different combinations of treatments applied per pot for each

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Table 1. List of biological, organic and chemical materials.

Biological material		
Designation	Function	Origin
<i>Gigaspora margarita</i>	Biofertilizer	
<i>Acaulospora tuberculata</i>	Biofertilizer	Laboratory of Biological Control and Applied Microbiology
<i>Trichoderma asperellum</i>	Biofertilizer	IRAD, Nkolbisson
<i>Phytophthora megakarya</i>	Pathogen	
Organic and chemical materials		
Gro-wild	Organic fertilizer	
Ridomil	Fungicide	Shop of phytopharmaceutical products, Yaoundé
NPK	Chemical fertilizer	

variety were as follows:

1. Arbuscular Mycorrhizal Fungi or AMF (M) of two strains (*Gigaspora margarita* and *Acaulospora tuberculata*)
2. *Trichoderma asperellum* strain PR11 (T)
3. Organic Fertilizer made from fish namely Gro-wild (O)
4. Chemical fertilizers or positive control (NPK or T₊)
5. *T. asperellum* strain PR11 + Organic fertilizer (TO)
6. AMF + PR11 (MT)
7. AMF + Organic fertilizer (MO)
8. AMF + Organic fertilizer + PR11 (MOT)
9. AMF + PR11 + Organic fertilizer + Chemical fertilizer (MOTNPK))
10. Negative control: original substrate without any treatment (T₋)
11. Sterile control: sterilized substrate without any treatment (TS)

Preparation of pots

The soil used for experimentation was brought from the forest undergrowth of Messassi, a neighborhood of Yaoundé. This substrate is the same as that supplied to Nkolbisson nurserymen. The pots were made from polythene bags 20 x 17 x 65 mm. Three randomized blocks of 11 treatments with three replicates each were used per variety.

A total of 66 pots were constituted, including 33 per variety. The seeds of V₁ and V₂ were pre-germinated for 18 days before being transplanted one per pot. 10 g of an inoculum containing AMF spores (*Gigaspora margarita* and *Acaulospora tuberculata*) were brought into 30 pots when transplanting of the pre-germinated plants.

Conidia of *T. asperellum* (PR11) harvested from the seven-day old cultures were introduced into beakers containing 200 ml of distilled water. After mechanical stirring for 1 minute, 5 ml of conidia solution of 5.10⁻⁷ mol/ml concentration were scraped into 11 Petri dishes and added into 30 pots provided for this treatment using a micropipette (Tchameni et al., 2011).

A mixture of the organic fertilizer (Gro-wild) in 15 L of water was carried out; 80 ml of this solution was added in each of the 30 pots provided for this purpose. The chemical fertilizer NPK (20-10-10), regarded as a positive control (T₊), has been spread into 12 pots at a dose of 10 g/pot, with 2 g per pot weekly. For the sterile substrate (TS), 6 pots were concerned; in these pots, the soil was sterilized by autoclaving at 121°C for 1 h with 2 replications in an interval of 24 hours. Finally, 6 pots were set up as the negative control (T₋); here, the pots received no treatment.

The nursery was housed inside a greenhouse of the Regional Biocontrol and Apply Microbiology laboratory of IRAD-Nkolbisson (Yaoundé, Cameroon). Watering was done twice a week, with one-

half litre of water measured per pot at the dawn and at the twilight.

In vitro evaluation of leaf disks test

The leaf test is an artificial inoculation method to assess the resistance of genotypes against infections (Nyassé et al., 1995). It was here to search for the incidence and severity of the disease on the leaves of cocoa plants in petri dishes following the protocol of Nyassé et al. (1995).

Three months old leaves were collected early in the morning before sunrise for both varieties. Leaf disks of 15 mm in diameter were laid in petri dishes. It was subsequently inoculated by depositing one drop of 10 µl of the zoospore suspension in the middle of each one. The device consisted of 11 treatments for varieties Tafo 79/501 and SNK 13 respectively, with 3 replicates per treatment.

Overall, 33 petri dishes each containing 12 leaf disks were constituted per variety as function of the treatment received by the seedling. Only, leaf disks issued from the positive controls (T₊) received each an additional drop of 10 µl of Ridomil (R). To monitor and describe disease expression, observations were made 6 days after incubation at ambient temperature according to the recommendations of Tchameni et al. (2011). The rating scale from 0 to 5 developed by Nyassé et al. (1995) was used for evaluation:

1. No symptom development was rated 0
2. Penetration point observed at the inoculated site was rated 1
3. Network of points was rated 2
4. Weblike patch was rate 3
5. Mottle patch was rated 4
6. True patch (necrosis) was rated 5.

The disease severity was determined for each treatment by calculating the ratio of the sum of individual scores over the total number of leave disks used (Tchameni et al., 2012). The infection severity index was used to express the resistance level as follows: 1) highly resistant: 0 < index ≤ 1; 2) resistant: 1 < index ≤ 2; 3) moderately resistant: 2 < index ≤ 2.5; 4) susceptible: 2.5 < index ≤ 3.5; 5) highly susceptible: 3.5 < index ≤ 5 (Paulin et al., 2008).

Statistical analysis

Data collected was keyed into an Excel sheet and analysed using Statistica 6.0 software. The analysis of variance (ANOVA) was used for multiple comparisons of means; when the overall difference between the means was found significant, the analysis was

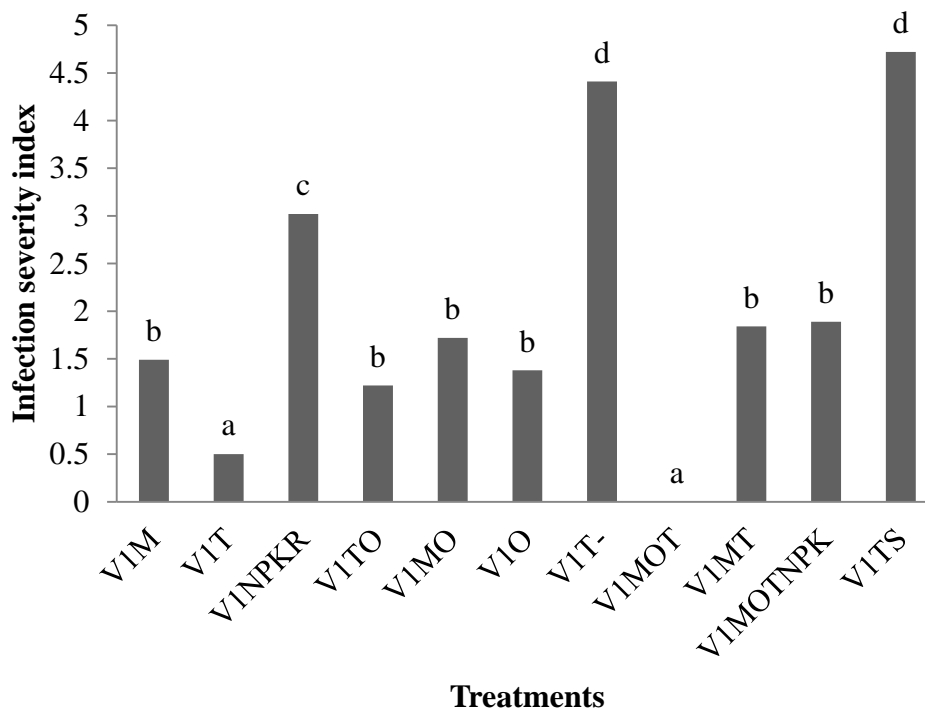


Figure 1. Infection severity index as a function of the treatments in Tafo 79/501 (Values followed with the same letter are not significantly different at $P > 0.05$, by HSD test).

Table 2. Categorization of treatments depending on the degree of susceptibility of V_1 to *P. megakarya*.

Treatments	Infection severity index	Degree of susceptibility
V_1 MOT	0	Highly resistant
V_1 T	0.5	
V_1 TO	1.22	Resistant
V_1 O	1.38	
V_1 M	1.49	
V_1 MO	1.72	
V_1 MT	1.84	
V_1 MOTNPK	1.89	
V_1 NPKR	3.02	
V_1 T-	4.41	Highly susceptible
V_1 TS	4.72	

continued by comparing the average pairwise using the HSD test. Means are reported as significantly different if P was less than or equal to 0.05.

RESULTS

From this study results, susceptibility of seedlings of cocoa of three months ageing in nursery varied with the different treatments applied. Figure 1 shows the variation

of the infection severity index due to *P. megakarya* *in vitro*, depending on treatments in Tafo 79/501 (V_1) variety.

Analysis of variance shows that in Tafo 79/501, the manifestation of the disease is significantly different between treatments ($F = 7.66$, $P < 0.05$). Treatments are thus classified into four categories depending on the variation of the infection severity index of *P. megakarya* which determined the degree of susceptibility of different plants to the black pod disease. Smaller the index, largest

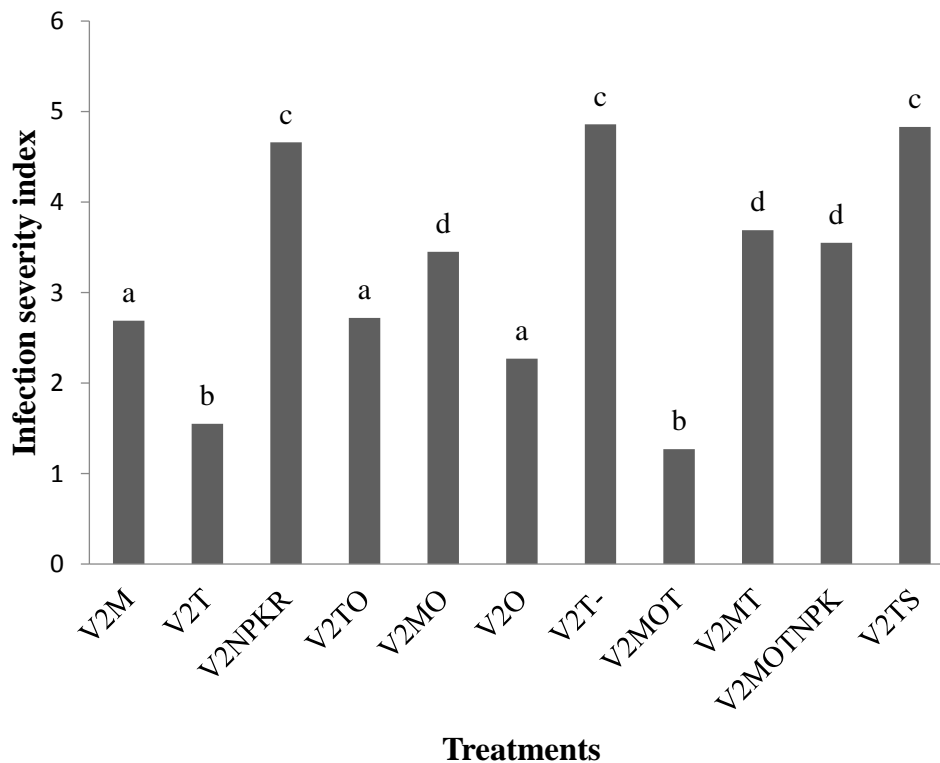


Figure 2. Infection severity index as a function of the treatments in SNK 13 variety (values followed with the same letter are not significantly different at $P > 0.05$, by HSD test).

the faculty of resistance acquired by the corresponding seedling plants; conversely, high index values determine the high susceptibility of plants to black pod disease (Table 2).

The treatment with *T. asperellum* (V₁T) and the mixture *T. asperellum*-Mycorrhizae-Organic fertilizer (Gro-wild) (V₁MOT) are those offering cocoa seedlings in the nursery high resistance to leaf development of *P. megakarya*. As it concerns treatment V₁MOT, no symptoms (0) were observed on correspondent leaf disks six days after inoculation of zoospores; about V₁T, beyond healthy leaf disks, some showed penetration points (1) of the germ *P. megakarya*.

Other treatments with Mycorrhizae and/or PR11 strain of *T. asperellum* following V₁M, V₁MO, V₁MOT, V₁MT, V₁MTONPK and V₁TO as well as those based on organic fertilizer only (V₁O) make seedlings corresponding resistant to the development of *P. megakarya*. For those treatments, the leaf disks showed symptoms ranging from penetration points (1) to network of points (2).

The contribution of a drop of Ridomil on leaf disks of seedlings of cocoa which received chemical fertilizer NPK (V₁NPKR) gives unsatisfactory results. Here we observed the disks with network points (2), weblike patch (3) and mottle patch (4).

Finally, the substrate receiving no treatment (V₁T-) and the sterilized one (V₁TS) offer no protection to young

corresponding plants and thus predispose them to a greater susceptibility to *P. megakarya*. This is why the bulk of the corresponding leaf disks showed true patch or necrosis (5) overall. Figure 2 shows the variation of the infection severity index due to *P. megakarya* *in vitro*, depending on treatments in SNK 13 (V₂) variety.

As regards of this variety, the differences were also significant between treatments ($F = 6.43$; $P < 0.05$). The results reported in Table 3 show the categories of each treatment. The treatment V₂T and V₂MOT offer cocoa seedlings resistance to the development of the germ of black pod disease, despite the presence of penetration points (1) and connected points (2) observed on leaf disks.

The treatment based on Organic fertilizer Gro-wild (V₂O) has conferred to cocoa seedlings a moderate resistance against *P. megakarya*; here weblike patches (3) were prominent on leaf disks compared with penetration points (1) and connected points (2).

The Mycorrhizae-based treatment (V₂M) and organic fertilizer combinations with Mycorrhizae (V₂MO) or *T. asperellum* strain PR11 (V₂TO) are less efficient in the variety SNK 13 and cause the susceptibility of young plants of this variety to *P. megakarya*. The infection severity index here is up to 2.5 and then conferred to leaf disks the presence of symptoms from scales 2, 3 and 4. Finally, the presence of treatment V₂MOTNPK, V₂MT,

Table 3. Categorization of treatments depending on the degree of susceptibility of *V₂* to *P. megakarya*.

Treatments	Infection Severity Index	Degree of susceptibility
V ₂ MOT	1.27	Resistant
V ₂ T	1.55	
V ₂ O	2.27	Moderately resistant
V ₂ M	2.69	Susceptible
V ₂ TO	2.72	
V ₂ MO	3.45	
V ₂ MOTNPK	3.55	
V ₂ MT	3.69	Highly susceptible
V ₂ NPKR	4.66	
V ₂ TS	4.83	
V ₂ T-	4.86	

V₂NPKR, V₂T- and V₂TS do not prevent the seedlings of cocoa correspondent that are highly susceptible to infection. Leaf disks from these treatments were affected generally by mottle patch (4) and true necrosis (5) and then increased the infection index.

DISCUSSION

The results confirm the good response of leaf disks of seedlings of the variety Tafo 79/501 issued from treatments V₁MOT, V₁T, V₁TO, V₁O, V₁MO, V₁M, V₁MT and V₁MOTNPK compared with the variety SNK 13 where only V₂MOT, V₂T and V₂O showed resistance against *P. megakarya*. Our results corroborated those from Blaha and Lotodé (1976) which classified the variety Tafo 79/501 ahead of SNK 13 in terms of their ability to tolerate the pathogen of cocoa black pod disease, *P. megakarya*.

The results also show the good behavior of the leaf disks derived from plants inoculated by the two biofertilizers namely PR11 and AMF, including very good results in the case of isolated inoculations as dualistic. Our results are in agreement with those of several authors who have conducted research works on the effect of both biofertilizers on resistance *in vitro* of seedlings of cocoa against *P. megakarya* using the techniques of leaf disks (Nyassé et al. 1995; Tahi et al., 2006; Tondje et al., 2007; Tchameni et al., 2011).

Furthermore, research carried out by Paulitz and Belanger (2001), Harrier and Watson (2004), Jemo et al. (2007), Tondje et al. (2007), Nwaga et al. (2007, 2010) and Tchameni et al. (2011) showed that AMF and members of the genus *Trichoderma* have emerged as promising groups of microbial inoculants that can induce plant growth and resistance to diseases. Indeed, the roots of more than 80% of plant species are generally

colonized by the AMF which are beneficial in combination with their host (Harley and Smith, 1983).

According to Sikes et al. (2009), AMFs have a protective effect against some plant pathogens. The results of Harrier and Watson (2004) found that colonization of roots of cocoa by the AMF reduces the susceptibility of the crop to 50-70% of diseases; in the same order, Nwaga et al. (2007, 2010) have shown that the action of the fungi on the host plant reduces root and foliar diseases caused by pathogens.

As well, the fungi of the genus *Trichoderma* are commonly used in the biocontrol of diseases that affect plant species (Jemo et al., 2007; Vinale et al., 2008). In addition, *Trichoderma* spp. in their interactions with plant roots provide nutrients to their hosts, promote the growth of these, increase their productivity and improve their disease resistance capacity (Martinez et al., 2001; Harman, 2008; Tchameni et al., 2011).

The combination of the two strains of Mycorrhizae namely *Gigaspora margarita* and *Acaulospora tuberculata* with the strain PR 11 of *Trichoderma asperellum* (V₁MT and V₂MT) give unsatisfactory results than when each biofertilizer is inoculated individually. The study results agree with those on cocoa seedlings in the nursery by Tondje et al. (2007).

According to these authors, the dualism between mycorrhizae and *Trichoderma* fungi causes an antagonistic effect between the two types of biofertilizers. Active agents of mycoparasitism, it is possible in the association that, the PR 11 strain of *T. asperellum* develops a negative interference effect with mycorrhizae strains used, as suggested by Rousseau et al. (1996). Similar results within the antagonism between the two biofertilizers were also found on bean (Martinez et al., 2001).

Indeed, the ability of dual inoculation with AMF and *T. asperellum* to enhance growth and induce systemic

resistance of seedlings was less functional, showing their possible incompatibility to occupy the same rhizosphere (Tchameni et al., 2011).

However, organic fertilizer (Gro-wild) gave interesting results in terms of protection of cocoa seedlings against *P. megakarya*. Indeed, according to Cantin (2001), organic fertilizers stimulate soil biological activity. Because of their ability to inoculate thousands of microorganisms in the soil and increase their biodiversity, organic fertilizers contribute to phenomena such as plant protection against certain fungal infections and bacterial in nature (Cantin, 2001).

Furthermore, it is known that organic fertilizers are sources of nutrients for microorganisms in the soil (Cantin, 2001). It is therefore possible to think that the presence of Gro-wild in the dualism between Mycorrhizae and *Trichoderma asperellum* strains PR11 (V₁MOT and V₂MOT) would be the cause of a synergistic action of the two microorganisms; that would explain why these combinations provide treatment more efficiency to both cocoa varieties studied.

Conclusion

The main objective was to evaluate *in vitro* resistance of cocoa seedlings of both varieties Tafo 79/501 and SNK 13 to the agent of black pod disease *P. megakarya*. Inoculation of biofertilizers in experimental pots gave good protection of cocoa seedlings against the development of *P. megakarya*. The latter confirmed their status as crop protection microorganisms. However, their effectiveness was increased in their association with organic fertilizer Gro-wild. Cocoa farmers can benefit from the use of biofertilizers like the AMF and PR11 strains of *T. asperellum* instead of the usual pesticides; indeed, the foliar resistance of cocoa to *Phytophthora* spp. is an indicator of the resistance of the pods in the field.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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