International Journal of Microbiology and Mycology | IJMM |
pISSN: 2309-4796
http://www.innspub.net
Vol. 14, No. 3, p. 1-10, 2022

Open Access RESEARCH PAPER

Biocontrol of black pod disease in Côte d'Ivoire through the selection of cocoa tree (*Theobroma cacao* L.) endophytic bacteria antagonist of *Phytophthora* spp.

Ouattara Adama[1,2], Coulibaly Klotioloma[*2], Konate Ibrahim[1], Gogbe Françoise[2], N'guessan Walet Pierre[2], Acka Kotaix[2], Kouame Norbert[2], Tahi Mathias[2], Guiraud Brigitte[2], Assi Maryse[2], Kone Daouda[3], N'guessan François[2], Tidou Abiba Sanogo[1], Abdelkarim Filali-Maltouf[4]

[1]Laboratory of Agrovalorization, Department of Biochemistry and Microbiology, Faculty of Agroforestry, Jean Lorougnon Guédé University, Daloa, Côte d'Ivoire [2]National Center of Agronomic Research, Divo, Côte d'Ivoire

[3]Laboratory of Plant Physiology, Faculty of Biosciences, Félix Houphouët-Boigny University, Abidjan, Côte d'Ivoire

[4]Faculty of Sciences, Laboratory of Microbiology and Molecular Biology, University Mohammed V-Agdal, Rabat, Morocco.

Keywords: *Theobroma cacao* L., Black pod disease, *Phytophthora*, Biocontrol, Endophytic bacteria

Publication date: March 09, 2022

Abstract

This study aimed at selecting cocoa tree endophytic bacteria antagonistic to *Phytophthora* spp. in view to produce a new biofungicide capable of controlling black pod disease. Endophytic bacteria were isolated from healthy organs (roots, leaves and stems) of young nurseries of two clones NA32 and P7. These isolates were confronted *in vitro* with two *Phytophthora* species (*Phytophthora palmivora* and *Phytophthora megakarya*). Leaf and detached pod tests were carried out in a four-factor split-plot randomized experimental design. At total, 116 endophytic bacteria were isolated. These bacteria inhibited the radial growth of *Phytophthora* by 25.3±1.5 to 70.54±2.14%. Four isolates 48P, 60P, 23P and 18N were more effective in *in vitro* tests. The susceptibility index of the clone NA32 was reduced from 3.0 to 0.97 on leaf discs and from 7.57 to 1.27 on detached pods. These endophytic bacteria induced resistance to clone NA32 and increased the intrinsic resistance of clones PA150 and SCA6. Endophytic bacteria can be used for biocontrol of black pod disease. However, field trials are needed to confirm the stability of these laboratory results .

* **Corresponding Author:** Coulibaly Klotioloma ✉ coolklotiolo@yahoo.fr

Introduction

Black pod disease is a major constraint for cocoa production, in West Africa and particularly in Côte d'Ivoire (Ploetz, 2016; Coulibaly *et al.*, 2018). Control of this disease is therefore priority (Mpika *et al.*, 2009). Several integrated control approaches have been suggested to eradicate this disease. Systemic contact and metalaxyl-based copper fungicides have been commonly used (Pohe *et al.*, 2013). Various agronomic control methods such as sanitary harvesting and use of resistant or tolerant varieties have been applied (Tahi *et al.*, 2006; Albert *et al.*, 2017). But, so far none of them have shown conclusive results. Biological control agents have been considered as an alternative approach to control various plant diseases (Tjamos *et al.*, 2010). The exploitation of endophytes as biological control agents for plant diseases has attracted much interest in scientific research (Tondje *et al.*, 2006). Indeed, their ability to colonize host plant tissues has made them valuable and effective for sustainable agriculture (Nur *et al.*, 2016; Ouattara *et al.*, 2019). They are considered as a tool to improve crop yield compared to other biological agents (Soylu *et al.*, 2005; Nur *et al.*, 2016). As internal colonizers of the root system, endophytes can compete within the vascular system, inhibiting pathogens to obtain both nutrients and space for their proliferation. Various endophytic bacteria belonging to the genera *Bacillus* and *Pseudomonas* have strong antifungal activity to control *Phytophthora* diseases (Rika *et al.*, 2014). This study aimed at selecting cocoa tree endophytic bacteria antagonistic to *Phytophthora* spp., in view to produce a new biofungicide capable of controlling black pod disease. In this study, the fungicidal effect of the endophytic bacteria was assessed on detached cocoa leaves and pods in the laboratory.

Materials and methods

Fungal strains

Two strains of *Phytophthora* were used, *Phytophthora palmivora* BL7.11.2 and *Phytophthora megakarya* 13P30.1 isolated in 2000 and 2013, respectively, from pods naturally affected by black pod disease. They were purified and then stored in a fungus bank in test tubes on agar pea medium. These strains were reactivated on fresh agar pea medium and their aggressiveness was reactivated on detached pods.

Bacterial isolates

Four bacteria antagonistic to strains *Phytophthora palmivora* BL7.11.2 and *Phytophthora megakarya* 13P30.1 previously selected in *in vitro* confrontation tests were used for the leaf and pod tests. These isolates were coded according to their origin from cocoa clone NA32 for the first one and from clone P7 for the last three.

Plant material

Symptoms less leaves and pods of three cocoa clones (NA32, PA150 and SCA6) of varying susceptibility to *Phytophthora* spp. were used for these tests. The clones NA32, PA150, SCA6 are known susceptible, moderately resistant and resistant to cocoa black pod disease. They were all collected in the experimental plots at the research station of Divo (National Center of Agronomic Research).

Isolation of endophytic bacteria from cocoa

Isolation of endophytic bacteria from cocoa (*Theobroma cacao* L.) was carried out from symptoms less of two cocoa clones young plants (NA32, susceptible and P7, resistant to black pod disease) This isolation was carried out according to the method of Konaté *et al.*, (2015). The organs (roots, stems and leaves) were surface sterilized, then triturated in distillated water and finally the grinds were spread on a yeast extract mannitol agar (YEM) medium.

In vitro antagonism test

All bacterial isolates were tested *in vitro* on solid agar pea medium for their antagonistic activity against both strains *P. palmivora* and *P. megakarya*. Bacteria were inoculated as

rectilinear streaks dividing Petri dishes into two equal parts (Melnick *et al.*, 2011). Two discs of mycelium calibrated to 5mm in diameter from a pure culture of each *Phytophthora* strain were removed with a Bunsen burner sterilized cookie cutter and placed on each side of the bacterial streak, 1cm from the edge of the Petri dish. Controls were not inoculated with the bacteria. The cultures were then incubated in the dark at 26°C. After 24 hours, the first observations were done. The incubation period was extended to 30 days to check the stability of the results obtained in these tests. All tests were carried out in three replicates. Radial growth measurements were taken daily and stopped when one of the mycelial colonies covered the total Petri dishes surface. Estimation of *Phytophthora* growth, consisted of daily (every 24 hours) measurements of the diameter of mycelial colonies using a ruler. Evaluation of the inhibition rate (I.R.) exerted by endophytic bacteria on the radial growth of *Phytophthora* strains was estimated using the following formula (Melnick *et al.*, 2011; Ouattara *et al*., 2020):

Dn: Average diameter of *Phytophthora* mycelial growth in the presence of bacteria;

Dc: Average diameter of *Phytophthora* mycelial growth in the absence of bacteria (Control)

Preparation of bacterial inoculum

Pre-culture of each bacterial isolate was carried out on liquid pea culture medium. After 48 hours of incubation, the suspensions (10^9 CFU/mL) were submitted to a serial of dilution (1:10) to obtain a final concentration of 10^6 or 10^3 CFU/mL (Konaté *et al.*, 2015).

Preparation of Phytophthora zoospores inoculum

Suspensions of zoospores of both *Phytophthora* strains were obtained from ten-day-old cultures in Roux flasks. After 3 days of incubation in the dark, the cultures were exposed to a 12-hour photoperiod and then placed at a temperature of 4°C for 15 minutes and exposed to incandescent lamp light for 45 minutes. Finally, the zoospores suspensions were counted using a Malassez cell and adjusted to a concentration of 3×10^5 zoospores/mL for inoculation onto leaf discs (Nyassé *et al.*, 1995).

In vivo antagonism tests on leaf discs and detached pods

Experimental design and treatments

These tests were conducted for each *Phytophthora* strain in a 4-factor split-plot randomized experimental design. The first factor assessed the effect of three cocoa clones (NA32, PA150 and SCA6) which susceptibilities to *Phytophthora* are known (susceptible, moderately resistant and resistant). The second factor assessed the effect of four bacterial isolates (60P, 48P, 18N and 23P) selected for their antagonism *in vitro* tests. The third factor tested the effect of 3 concentrations ($C_1=10^3$, $C_2=10^6$, $C_3=10^9$ CFU/mL) of each bacterial inoculum. Finally, the fourth factor assessed the effect of the bacterial inoculum application periods. Three varying periods were tested (the first period named (AV) consisted of applying the bacterial inoculum one hour (01h) before the inoculum of *Phytophthora* spp. The second period (P) consisted to inoculate simultaneously the bacterial inoculum and that of *Phytophthora* spp. The third period (AP) consisted to inoculate the bacterial inoculum one hour (01h) after the inoculum of *Phytophthora* spp. A total of thirty six (36) treatments with nine (09) treatments per bacterial isolate were applied to leaf discs and pods of the three cocoa clones.

Leaf discs tests

For these tests, cocoa leaf discs were arranged in trays on foam (1cm thick) soaked with distilled water. The leaf discs were inoculated with a suspension of each bacterial pre-culture at different concentrations 10^3, 10^6 and 10^9 CFU/mL. Each leaf disc received 10 µL of a suspension of *Phytophthora* zoospores, calibrated at 3×10^5 zoospores/mL. Controls were inoculated only with zoospores suspensions of the

Phytophthora strains. After inoculation of leaf discs, each tray was covered with a black tarpaulin and incubated at 26 ±2°C for 7 days. Symptom reading was conducted according to Blaha scale (Nyassé *et al.*, 1995). In this scale cocoa pods susceptibility index to black pod disease ranged from 0 to 5 (0: no symptom development; 1: penetration points; 2: connected points; 3: reticulate necrotic spots; 4: marbled necrotic spots; 5: true necrotic spots).

Detached pod tests

For the detached pod test, cocoa pods were inoculated by spraying with 1mL of each bacterial pre-culture suspension at different concentrations 10^3, 10^6 and 10^9 CFU/mL. Symptom reading was conducted according to Iwaro *et al.* (2005) [16]. The absolute controls were treated with a suspension of *Phytophthora* zoospores calibrated at the concentration of 5×10^5 zoospores/mL. The reference controls were treated with a chemical fungicide (FanticPlus 50g/L active ingredient composition). Table 1 summarizes the different criteria of Iwaro scale (Iwaro *et al*., 2005).

Table 1. Scale of cocoa clones susceptibility indices to black pod disease (Iwaro *et al.*, 2005).

[a]Susceptibility indices	Level of infection	[b]Susceptibility indices	Level of infection
1	No symptoms	5	1 to 5 true lesion spots
2	1 to 5 points of attack	6	6 to 15 true lesion spots
3	6 to 15 points of attack	7	more than 15 true lesion spots
4	more than 15 points of attack	8	true spot of expanding lesion

[a]Based on the absence of visible lesions (rating 1) and the number of non-expanding (localised). lesions (rating 2–4).

[b]Based on the number of expanding, countable lesions (rating 5–7) and expanding coalesced lesions (rating 8)

Statistical analysis

All data were entered in Excel and analyzed in SAS® version 9.4 software (SAS Institute Inc. 1995). Data underwent an arcsinus√ transformation to fit the normal distribution (Gomez & Gomez, 1984) and then subjected to analysis of variance (ANOVA) using the PROC GLM (Process of General Linear Model) procedure. Multiple comparisons were performed using the Student-Newmanes-Keuls method at probability $p \leq 0.05$.

Results and discussion

In vitro selection of endophytic bacteria antagonistic to Phytophthora

In Dual culture tests, 116 cocoa tree endophytic bacteria inhibited the mycelial growth of both *Phytophthora* strains. Inhibition rates (I.R.) of mycelium growth recorded after 30 days, varied from 25.3 to 70.54%. A total of 4 bacterial isolates (18N, 42P, 47P and 60P) showed high antagonistic activity towards both *Phytophthora* strains with an inhibition rate above 60% (Table 2). The bacteria inhibited *in vitro* mycelial growth of both *Phytophthora* strains.

A zone of inhibition was observed on both sides of the bacterial streaks. This provides evidence that the bacterial isolates that inhibited *Phytophthora* strains produced inhibitory substances (Yuan *et al*., 2012; Acebo-Guerrero *et al*., 2015; Iqbal *et al*., 2019). *Phytophthora* antagonistic microorganisms can be isolated from the cocoa ecosystem (Kébé *et al*., 2009). Epiphytic bacteria isolated from the pods of three cocoa clones SCA6, T85/799 and IFC5 (all resistant to black pod disease) were able to highly inhibit the growth of *P. palmivora* in *in vitro* tests, with inhibition rates of 69.7 to 65.8% (Akrofi *et al*. , 2017). According to Mejia *et al*. (2008), the results of *in vitro* antagonism tests do not necessarily reflect those obtained in *in vivo* or *in planta* tests. Some microorganisms showed to be antagonistic *in vitro* may be ineffective in *in vivo* tests or under field conditions. Because in real field conditions, many ecological parameters such as temperature, wind, sun, rain, relative air humidity can influence the development of the antagonistic microorganism tested (Deberdt *et al.*, 2008).

Table 2. *In vitro* inhibitory effect of some endophytic bacteria on mycelial growth of *Phytophthora* at 30 days of incubation.

Bacterial isolates	*Phytophthora palmivora*		*Phytophthora megakarya*	
	Mean diameter ±Sd (mm)	Inhibition rate ±Sd (%)	Mean diameter ±Sd (mm)	Inhibition rate ±Sd (%)
48P	24,75±3,59	70,54±2,14	34,5±2,13	58,94±2,63
18N	28,25±1,25	66,37±1,49	33±2,94	60,72±1,75
42P	30,25±1,70	63,99±2,03	30±1,80	64,29±1,68
60P	30,5±0,57	63,69±0,68	32,75±2,98	61,02±2,44
47P	31,25±0,95	62,8±1,13	31,25±0,95	62,8±1,40
23P	31,75±1,25	62,2±1,49	35,75±2,94	57,45±1,75
68P	53,75±3.30	36,01±3.93	62,75±3.20	25,3±3.81

Sd: Standard deviation.

Effect of bacterial treatments on cocoa clones leaf discs and detached pod susceptibility

Bacterial isolates 48P, 18P and 23P applied to leaf discs reduced black pod disease necrosis symptoms induced by *P. palmivora* and *P. megakarya* strains. Leaf discs of all three cocoa clones (NA32, PA150 and SCA6) treated with these bacterial suspensions showed no symptoms (susceptibility index value: 0) or penetration points (susceptibility index value: 1) in contrast to the untreated control NA32.

The control NA32 showed an index value of 3.0 (reticulate necrosis symptoms) for both *Phytophthora* strains (Fig. 1). Thus, isolates 48P and 18N showed higher inhibitory effects on both *Phytophthora* strains. The bacterial isolate 23P showed a medium inhibitory effect on both *Phytophthora* strains.

In contrast to the three isolates listed above, isolate 60P showed no inhibitory effect against both *Phytophthora* strains.

The inoculum concentration of 10^9 CFU/mL of isolates 48P, 18P, and 23P was most effective. These isolates effectively reduced black pods disease necrosis symptoms on the leaf discs of all three cocoa clones particularly the susceptible clone NA32.

This clone showed susceptibility indices of 0.97 to 1.00 (no symptom or penetration points) when treated with the bacterial suspensions (Fig. 1).

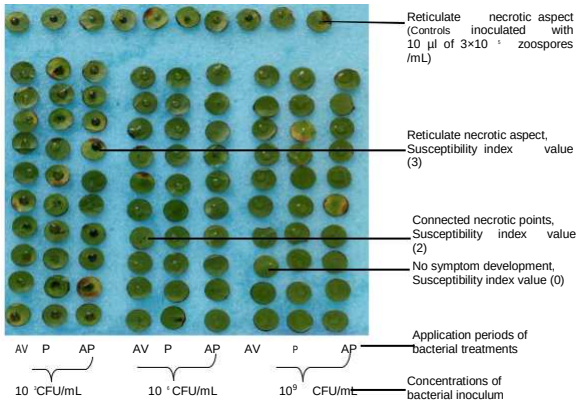

Fig. 1. Fungicidal effect of endophytic bacteria (18N and 48P) against *Phytophthora* on leaf discs of the clone NA32.

AV: treatments where bacterial inoculum was applied one hour before *Phytophthora palmivora* or *Phytophthora megakarya* zoospores; P: treatments where bacterial inoculum and *Phytophthora* zoospores were applied simultaneously; AP: treatments where *Phytophthora* zoospores were applied one hour before bringing in bacterial inoculum; CFU/mL: Colony-forming unit per milliliter.

The results of confrontation tests on detached pods of cocoa endophytic bacteria (48P, 18N, 60P and 23P) and both *Phytophthora* strains (*P. palmivora* and *P. megakarya*) were summarized in

Tables 3, 4 and 5. A significant difference between the susceptibility indices induced by bacterial treatments applied on the three cocoa clones (NA32, PA150 and SCA) and controls was observed. Indeed, treatments with the four bacteria (isolates: 48P, 18N, 60P and 23P) suspensions induced a reduction of the three cocoa clones' susceptibility to *P. palmivora.* With This *Phytophthora* strain, cocoa clones showed mean susceptibility indices ranging from 4.14 (more than 15 points of attack) to 5.94 (6 to 15 true lesion spots) against a mean index value of 7.57 (more than 15 true lesion spots or true spot of expanding lesion) for the absolute control NA32. Similarly, a reduction of the three cocoa clones' pods susceptibility to *P. megakarya* strain was observed. With this *Phytophthora* strain, the susceptibility mean indices were ranged from 1.22 (No symptom) to 4.46 (more than 15 points of attack) compared to the mean index value of 5.51(6 to 15 true lesion spots) recorded for the untreated control NA32. In these biotests, isolates 48P and 18P effectively reduced black pod disease necrosis symptoms on leaves and pods of all three cocoa clones, particularly for the susceptible clone NA32. This showed that these bacterial isolates induced resistance to clone NA32 on the one hand, and on the other hand enhanced the intrinsic resistance of clones PA150 and SCA6 (Bowers & Tondje, 2006). These results corroborate those of Kébé *et al* (2009) who showed the existence of rhizobacteria indigenous to cocoa farm soils capable of reducing the foliar susceptibility of two cocoa clones P7 and SCA6 to *P. palmivora* attack. The effectiveness of the bacterial treatments applied one hour before the fungal inoculum, proves that the bacterial endophytes once installed create a barrier against the penetration of the pathogens on the stomata. This could reflect the induction of resistance to the susceptible clone NA32 (Nana *et al*., 2016).

Furthermore, bacterial isolates certainly produce antifungal substances that neutralize *Phytophthora* zoospores development on the leaf discs and pods even when *Phytophthora* zoospores inoculum is applied one hour of time before to apply the bacterial suspensions (Tondje *et al*., 2006; Gadji *et al*., 2018).

Table 3. Fungicidal effect of cocoa tree endophytic bacteria against *Phytophthora* on the clone NA32.

Clone		NA32							
Phytophthora spp		*Phytophthora palmivora*				*Phytophthora megakarya*			
Bacterial isolates / Concentrations	Periods	48P	18N	60P	23P	48P	18N	60P	23P
$C_1 = 10^3$ CFU/mL	AV	$6,35^{d}$	$7,45^{d}$	$6,40^{d}$	$6,86^{d}$	$5,52^{e}$	$6,37^{e}$	$3,64^{d}$	$4,56^{e}$
	P	$5,25^{c}$	$4,69^{c}$	$7,32^{d}$	$6,17^{d}$	$5,73^{e}$	$6,80^{f}$	$3,64^{d}$	$4,56^{e}$
	AP	$5,80^{d}$	$5,25^{c}$	$7,32^{d}$	$6,40^{d}$	$6,16^{e}$	$2,97^{c}$	$3,34^{c}$	$3,34^{c}$
$C_2 = 10^6$ CFU/mL	AV	$5,25^{c}$	$6,63^{d}$	$6,63^{d}$	$7,32^{d}$	$6,80^{f}$	$6,58^{f}$	$2,43^{bc}$	$3,34^{c}$
	P	$5,25^{c}$	$7,18^{d}$	$5,26^{c}$	$6,63^{d}$	$6,37^{e}$	$6,80^{f}$	$2,43^{bc}$	$2,43^{bc}$
	AP	$6,63^{d}$	$4,97^{c}$	$6,40^{d}$	$6,63^{d}$	$6,16^{e}$	$1,27^{a}$	$2,12^{b}$	$3,64^{d}$
$C_3 = 10^9$ CFU/mL	AV	$7,46^{d}$	$4,14^{b}$	$6,17^{d}$	$6,40^{d}$	$6,80^{f}$	$6,80^{f}$	$3,04^{c}$	$1,22^{a}$
	P	$6,90^{d}$	$5,52^{c}$	$5,49^{c}$	$5,94^{d}$	$4,46^{d}$	$4,88^{e}$	$2,12^{b}$	$1,82^{ab}$
	AP	$4,97^{c}$	$4,97^{c}$	$7,09^{d}$	$6,17^{d}$	$4,88^{e}$	$3,40^{c}$	$2,43^{bc}$	$2,73^{c}$
Absolute control		$7,57^{e}$	$7,57^{e}$	$7,57^{e}$	$7,57^{e}$	$5,51^{e}$	$5,51^{e}$	$5,51^{e}$	$5,51^{e}$
Reference control		$1,49^{a}$	$1,49^{a}$	$1,49^{a}$	$1,49^{a}$	$1,81^{D}$	$1,81^{D}$	$1,81^{D}$	$1,81^{D}$

Means followed by the same letters are not significantly different at 5% threshold according to the Student-Newman-Keuls test.

AV: treatments where bacterial inoculum was applied one hour before *Phytophthora palmivora* or *Phytophthora megakarya* zoospores; P: treatments where bacterial inoculum and *Phytophthora* zoospores were applied simultaneously; AP: treatments where *Phytophthora* zoospores were applied one hour before bringing in bacterial inoculum ; CFU/mL: Colony-forming unit per milliliter.

Table 4. Fungicidal effect of cocoa tree endophytic bacteria against *Phytophthora* on the clone PA150. Eans followed by the same letters are not significantly different at 5% threshold according to the Student-Newman-Keuls test.

Clone		PA150							
Phytophthora spp		*Phytophthora palmivora*				*Phytophthora megakarya*			
Bacterial isolates / Concentrations	Periods	48P	18N	60P	23P	48P	18N	60P	23P
$C_1 = 10^5$ CFU/mL	AV	1,66^{ab}	2,76^{ab}	2,74^{ab}	1,83^{ab}	2,55^{ab}	1,27^{a}	1,52^{a}	1,52^{a}
	P	2,76^{ab}	4,14^{b}	1,83^{ab}	1,37^{a}	3,18^{b}	1,27^{a}	1,52^{a}	1,52^{a}
	AP	1,66^{ab}	3,04^{b}	1,83^{ab}	1,60^{ab}	0,85^{a}	0,85^{a}	1,22^{a}	1,22^{a}
$C_2 = 10^6$ CFU/mL	AV	1,38^{a}	2,21^{ab}	2,06^{ab}	1,83^{ab}	1,27^{a}	1,70^{a}	1,52^{a}	1,22^{a}
	P	2,21^{ab}	2,21^{ab}	1,14^{a}	1,37^{a}	1,27^{a}	2,12^{a}	1,22^{a}	1,22^{a}
	AP	1,66^{ab}	4,14^{b}	1,37^{a}	1,83^{ab}	1,48^{a}	0,85^{a}	1,52^{a}	1,22^{a}
$C_3 = 10^9$ CFU/mL	AV	1,11^{a}	2,21^{ab}	1,60^{ab}	2,29^{b}	1,06^{a}	0,85^{a}	1,22^{a}	1,52^{a}
	P	1,11^{a}	2,48^{ab}	1,14^{a}	1,60^{ab}	1,06^{a}	0,85^{a}	1,22^{a}	1,22^{a}
	AP	1,11^{a}	2,48^{ab}	1,83^{ab}	2,29^{b}	1,70^{a}	0,85^{a}	1,22^{a}	1,22^{a}
Absolute control		3,99^{b}	3,99^{b}	3,99^{b}	3,99^{b}	1,40^{a}	1,40^{a}	1,40^{a}	1,40^{a}
Reference control		1,15^{a}	1,15^{a}	1,15^{a}	1,15^{a}	1,04^{a}	1,04^{a}	1,04^{a}	1,04^{a}

AV: treatments where bacterial inoculum was applied one hour before *Phytophthora palmivora* or *Phytophthora megakarya* zoospores; P: treatments where bacterial inoculum and *Phytophthora* zoospores were applied simultaneously; AP: treatments where *Phytophthora* zoospores were applied one hour before bringing in bacterial inoculum; CFU/mL: Colony-forming unit per milliliter.

Table 5. Fungicidal effect of cocoa tree endophytic bacteria against *Phytophthora* on the clone SCA6

Clone		SCA6							
Phytophthora spp		*Phytophthora palmivora*				*Phytophthora megakarya*			
Bacterial isolates / Concentrations	Periods	48P	18N	60P	23P	48P	18N	60P	23P
$C_1 = 10^5$ CFU/mL	AV	1,66^{b}	2,21^{b}	2,06^{b}	2,06^{b}	4,88^{c}	5,73^{c}	1,82^{ab}	1,52^{a}
	P	1,66^{b}	2,21^{b}	1,37^{a}	1,60^{b}	5,52^{c}	5,1^{c}	1,22^{a}	2,43^{b}
	AP	1,66^{b}	2,48^{b}	1,83^{b}	1,14^{a}	3,18^{c}	2,76^{ab}	1,82^{ab}	1,52^{a}
$C_2 = 10^6$ CFU/mL	AV	1,66^{b}	2,21^{b}	1,60^{b}	1,37^{a}	5,73^{c}	5,95^{c}	1,22^{a}	1,22^{a}
	P	1,93^{b}	1,93^{b}	1,37^{a}	1,37^{a}	5,52^{c}	5,52^{c}	1,82^{ab}	1,22^{a}
	AP	1,66^{b}	1,66^{b}	1,14^{a}	1,14^{b}	3,18^{c}	0,85^{a}	1,22^{a}	1,22^{a}
$C_3 = 10^9$ CFU/mL	AV	1,38^{a}	1,38^{a}	1,60^{b}	0,92^{a}	4,25^{c}	5,1^{c}	1,22^{a}	1,22^{a}
	P	1,11^{a}	1,38^{a}	1,14^{a}	0,92^{a}	3,4^{c}	4,89^{c}	1,22^{a}	1,22^{a}
	AP	1,38^{a}	1,38^{a}	1,14^{a}	1,14^{a}	4,04^{c}	0,85^{a}	1,22^{a}	1,22^{a}
Absolute control		1,70^{b}	1,70^{b}	1,70^{b}	1,70^{b}	1,56^{a}	1,56^{a}	1,56^{a}	1,56^{a}
Reference control		1,01^{a}	1,01^{a}	1,01^{a}	1,01^{a}	1,04^{a}	1,04^{a}	1,04^{a}	1,04^{a}

Means followed by the same letters are not significantly different at 5% threshold according to the Student-Newman-Keuls test.

AV: treatments where bacterial inoculum was applied one hour before *Phytophthora palmivora* or *Phytophthora megakarya* zoospores; P: treatments where bacterial inoculum and *Phytophthora* zoospores were applied simultaneously; AP: treatments where *Phytophthora* zoospores were applied one hour before bringing in bacterial inoculum ; CFU/mL: Colony-forming unit per milliliter.

Conclusion

This study showed that cocoa trees harbor endophytic bacteria antagonistic to both *Phytophthora* species (*P. palmivora* and *P. megakarya*). These bacteria significantly inhibited *in vitro* the mycelial growth of both *Phytophthora* strains. In addition, they reduced the susceptibility of clones NA32, PA150 and SCA6 to *Phytophthora* attacks on leaves and detached pods. This induction of resistance was more remarkable for the clone NA32 which is naturally susceptible to black pod disease.

This study therefore proved the efficacy of cocoa endophytic bacteria and their possible use as new biofungicide of black pod disease.

However, these endophytic bacteria need to be tested in the field conditions in order to confirm the stability of their fungicidal effect against *Phytophthora* spp.

Acknowledgements

We sincerely thank Misters Coulibaly Bakary, Research technician, and Touré Laurent, Research assistant at the Laboratory of Plant pathology, National Center of Agronomic Research (CNRA), Research Station of Divo (Côte d'Ivoire), for the appreciable help provided during leaf and pod inoculation tests.

References

Acebo-Guerrero Y, Hernandez-Rodrıguez A., Vandeputte O, Miguelez-Sierra Y, Heydrich-Perez M, Ye L., Cornelis P., Bertin P and El Jaziri M. 2015**.** Characterization of *Pseudomonas chlororaphis* from *Theobroma cacao* L. rhizosphere with antagonistic activity against *Phytophthora palmivora* (Butler). Journal of Applied Microbiology **119,** 1112-1126.

Akrofi AY, Terlabie JL, Amoako-Attah I, Asare EK. 2017. Isolation and characterization of bacteria from different cacao progenies and their antagonistic activity against the black pod disease pathogen, Phytophthora palmivora. Journal of Plant Diseases and Protection **124,**143-152.

Albert SLC, Mohd JAK, KHIM PC, CHONG M. H. 2017. Assessing the cocoa genotypes for resistance to black pod using the area under the disease-progress curve (AUDPC). Bulgarian Journal of Agricultural Science **23,** 972-979.

Bowers JH, Tondje PR. 2006. Screening biocontrol candidates for *Phytophthora megakarya* using the leaf disk test. *In:* Tondje P. R., Hebbar P. K., Samuels G. J., Bowers J. H., Evans H. C., Holmes A K., Onguene N. A. & Foko J. (Editors). Microbial biocontrol methods for *Phytophthora megakarya* cacao black pod disease in Africa 183p.

Coulibaly K, Aka RA, Camara B, Kassin E, Kouakou K, Kébé BI, Koffi NK, Tahimg, Walet NP, Guiraud SB, Assi ME, Kone B, N'Guessan KF, Koné D. 2018. Molecular Identification of *Phytophthora palmivora* in the Cocoa Tree Orchard of Côte d'Ivoire and Assessment of the Quantitative Component of Pathogenicity. Internationale journale of science **7,** 7-15.

Deberdt P, Mfegue CV, Tondje PR, Bonmc, Ducamp M, Hurard C, Begoude BAD, Ndoumbe-Nkeng M, Hebbar PK, Cilas C. 2008. Impact of environmental factors, chemical fungicide and biological control on cacao pod production dynamics and black pod disease (*Phytophthora megakarya*) in Cameroon. Biological Control **4,** 149-159.

Gadji AAG, Kouamékg, Coulibaly K, Yapo OB, Aka AR, Brar KS, Tyagi R, Abo K. (2018). Effect of *Bacillus thuringiensis var. kurstaki* HD-1-based biopesticide on the pathogenicity of Phytophthora palmivora. Journal of Biodiversity and Environmental Sciences **12,** 456-464.

Gomez KA, Gomez AA. 1984. Statistical Procedures for Agricultural Research. 2nd ed. Chapter 2. New York: John Wiley & Sons 7-83.

Iqbal Z, Ghazanfar MU, Raza W, Ahmad S, Anjum MZ. 2019. Efficacy of bio control agents for management of *Phytophthora megasperma* causes of collar rot of peas. International Journal of Biosciences **14**, 281-285.

Iwaro AD, Thévenin J.-M, Butler DR, Eskes AB. 2005. Usefulness of the detached pod test for assessment of cacao resistance to *Phytophthora* pod rot. European Journal of Plant Pathology **113**, 173-182.

Kébé IB, Mpika J, N'Guessan KF, Hebbar PK, Samuels GS, Ake S. 2009. Isolement et identification de microorganismes indigènes de cacaoyères en Côte d'Ivoire et mise en évidence de leurs effets antagonistes vis-à-vis de *Phytophthora palmivora*, agent de la pourriture brune des cabosses. Sciences & Nature **6,** 71-82.

Konate I, Ouattara A, Coulibaly B, Guei NKR, Amani K, Kouakou IK, Filali-Maltouf A, Koffi M. 2015. Phenotypic Diversity of Associative Bacteria Isolated from Roots and Stems of Cacao (*Theorem cacao*) Tree in Daloa, Côte d'Ivoire. International Journal of Current Microbiology and Applied Sciences **4,** 560-570.

Mejia LC, Rojas EI, Maynard Z, Bael SV, Arnold AE, Hebbar P, Samuels GJ, Robbins N, Herre EA. 2008. Endophytic fungi as biocontrol agents of Theobroma cacao pathogens. Biological Control **46,** 4-14.

Melnick RL, Suárez C, Bailey BA, Backman PA. 2011. Isolation of endophytic endospore forming bacteria from *Theobroma cacao* as potential biological control agents of cacao diseases. Biological Control **57,** 236-245.

Mpika J, Kebe IB, Druzhinina IS, Komon-Zélazowska M, Kubicek CP, Aké S. 2009. Inhibition de *Phytophthora palmivora*, agent de pourriture brune des cabosses de cacaoyer en Côte d'Ivoire, par *Trichoderma* sp. Sciences & Nature **6,** 49-62.

Nana LW, Ekounda VT, Mkounga P, Eke P, Nkengfack AE, Nwaga D. 2016. Potentialisation of the biocontrol efficacy of arbuscular mycorrhizas fungi against cocoa black pod rot causing *Phytophthora megakarya* with natural flavonoid. International Journal of Agronomy and Agricultural Research **9,** 165-181.

Nur RR, Maizatul SM, Idris AS, Madihah AZ, Nasyaruddin M. 2016. The Potential of Endophytic Bacteria as a Biological Control Agent for *Ganoderma* Disease in Oil Palm. Sains Malaysiana **45,** 401-409.

Nyassé S, Cilas C, Herail C, Blaha G. 1995. Leaf inoculation as an early screening test for cocoa (*Theobroma cacao* L.) resistance to Phytophthora black pod disease. Crop Protection **14,** 657-663.

Ouattara A, Coulibaly K, Konate I, Kebe BI, Beugre GAM, Tidou AS, Abdelkarim F.-M. 2020. Screening and Selection *in vitro* and *in vivo* of Cocoa Tree (*Theobroma Cacao* Linn) Endophytic Bacteria Having Antagonistic Effects against *Phytophthora* Spp. Fungal Agents Responsible of Black Pod Disease in Côte d'Ivoire. Journal of Applied & Environmental Microbiology **8,** 25-31.

Ouattara A, Coulibaly K, Konate I, Kebe BI, Tidou AS, Filali-Maltouf A. 2019. Selection of Cocoa Tree (*Theobroma cacao* Linn) Endophytic Bacteria Solubilizing Tri-Calcium Phosphate, Isolated from Seedlings Grown on Soils of Six Producing Regions of Côte d'Ivoire. Advances in Microbiology **9,** 842-852.

Ploetz R. 2016. The Impact of Diseases on Cacao Production: A Global Overview in: B.A. Bailey, L.W. Meinhardt (eds.), Cacao Diseases. *Springer International Publishing Switzerland,* Beltsville, USA 33-59.

Pohe J, Pohe SSW, Okou SFF. 2013. Association oxyde de cuivre et metalaxyl dans la lutte contre la pourriture brune des cabosses de cacaoyer en Côte d'Ivoire. Journal of Animal & Plant Sciences **16,** 2362-2368.

Rika FNB, Aris Tri W, Nurita TM. 2014. Control activity of potential antifungal-producing *Burkholderia* sp. in suppressing *Ganoderma boninense* growth in oil palm. Asian Journal of Agricultural Research **8,** 259-268.

SAS. 1995. Guide to the use of PC-SAS Version 9.4 for DOS for Statistical Analysis. SAS Institute, Cary, North Carolina.

Soylu S, Soylu EM, Kurt S, Ekici OK. 2005. Antagonistic potentials of rhizosphere-associated bacterial isolates against soil-borne diseases of tomato and pepper caused by *Sclerotinia sclerotiorum* and Rhizoctonia solani. Pakistan Journal of Biological Sciences **8,** 43-48.

Tahi GM, Kébé BI, N'Goran AKN, Sangare A, Mondeil F, Cilas C, Eskes AB. 2006. Expected selection efficiency for resistance to cacao pod rot (*Phytophthora palmivora*) comparing lead disc inoculations with field observations. Euphytica **149,** 35-44.

Tjamos EC, Tjamos SE, Antoniou PP. 2010. Biological management of plant diseases: Highlights on research and application. Journal of Plant Pathology **92,** 17-21.

Tondje PR, Hebbar PK, Samuels GJ, Bowers JH, Evans HC, Holmes KA, Onguene NA, Foko J. 2006. Microbial biocontrol methods for *Phytophthra megakarya* cacao black pod disease in Africa. Institute of Agricultural Research for Development 183p.

Yuan J, Li B, Zhang N, Waseem R, Shen Q, Huang Q. 2012. Production of Bacillomycinand macrolactin-type antibiotics by *Bacillus amyloliquefaciens* NJN-6 for suppressing soilborne plant pathogens. Journal of Agricultural and Food Chemistry **60,** 2976-2981.