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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

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Abstract We are investigating in this study the screening and selection of cocoa tree (*Theobroma cacao* L.) endophytic bacteria having antagonistic effects against the fungi *Phytophthora* spp. and to assess their inhibitory potential as biological control agents against black pod disease in Côte d'Ivoire. A total of One hundred and sixteen bacteria were isolated from symptomless root, leaf and stem tissues of two cocoa clones NA32 (susceptible) and P7 (resistant) young seedlings. These isolates restricted the radial growth of the mycelium of *P. palmivora* (strain BL7.11.2) and *P. megakarya* (strain 13P30.1) with percentages inhibition of 28.58 to 70.54% and 25.3 to 64.29% respectively. Four isolates, 48P, 60P, 23P and 18N were more effective in *in vitro* test and selected for *in vivo* confrontation test on leaf discs. Treatments based on two isolates S1 (48P) and S2 (18N) applied at the concentration 10^9 CFU/mL significantly ($p < 0.05$) reduced the black pod necrosis induced by the two *Phytophthora* strains on the leaf discs of three cocoa clones (NA32, PA150 and SCA6). In addition, simultaneous (C3PS1 and C3PS2) or separate (C3AVS1 and C3AVS2) inoculations of these two bacteria and *Phytophthora*, induced resistance to the clone NA32 and increased the intrinsic resistance of the clones PA150 and SCA6. This study showed that endophytic bacteria antagonistic to *Phytophthora* spp. can be an alternative solution in the biocontrol of black pod disease. *In situ* pod tests should be performed to confirm these preliminary results and propose effective bacterial inoculum.

Keywords: *black pod, phytophthora spp, endophytic bacteria, cocoa tree, biocontrol*

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1. Introduction

The high incidence of black pod is a major constraint to cocoa production in West Africa, particularly in Côte d'Ivoire [1,2]. The control of this disease is therefore both a necessity and a priority [3,4]. Several integrated control approaches have been suggested to eradicate this disease. Copper and metalaxyl-based fungicides have been widely used [5]. Other agronomic methods such as harvesting diseased pods and using partially resistant and/or tolerant varieties have been also applied [6,7]. However, none of them have given conclusive results. Biological control agents have been considered as an alternative approach to control various plant diseases [8]. The use of endophytic bacteria as biological control agents against plant diseases has attracted considerable interest in scientific research [9]. Their ability to colonize the host plant tissues has made them valuable and effective for sustainable agriculture and has provided a tool to improve crop yield compared to other biological agents [10,11]. 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. Many bacteria belonging to the genera *Bacillus* sp, *Pseudomonas* sp, *Burkholderia* sp and fungi such as *Trichoderma* spp and *Aspergillus* spp have effective antifungal activity to control *Phytophthora* diseases [12,13,14]. The aim of this study was to screen and to select cocoa tree (*Theobroma cacao* L.) endophytic bacteria with antagonistic effects against *Phytophthora* spp. and to assess their potential as biological control agents against black pod disease in Côte d'Ivoire.

2. Material and Methods

2.1. Material

2.1.1. Fungal Strains

Two strains BL7.11.2 and 13P30.1 of *Phytophthora palmivora* and *Phytophthora megakarya* respectively isolated in the year 2000 and 2013 from naturally infected cocoa pods were used. These strains were stored in a mycobank at National Agronomic Research Center (CNRA)'s Phytopathology Laboratory in Bingerville (Côte d'Ivoire) and were periodically isolated once more from infected cocoa pods to maintain their virulence.

2.1.2. Bacterial Isolates

One hundred and sixteen (116) endophytic bacteria were isolated from young seedlings organs (roots, stems and leaves) of two clones (NA32 and P7 respectively susceptible and resistant to black pod) as described by Konaté et *al*. [15] and Rayda et *al*. [16].

2.1.3. Plant Material

Young symptomless leaves of three cocoa clones NA32, PA150 and SCA6 respectively susceptible, moderately resistant and resistant to black pod, were collected. The sampling was done from non-lignified twigs of ten trees per clone at the Divo research station (CNRA). A total of seven hundred and twenty (720) leaves were taken, at the rate of two hundred and forty (240) leaves per clone and twenty four (24) leaves per tree. The collected leaves were stored in moistened plastic bags and transported in a cooler.

2.2. Methods

2.2.1. *In vitro* Antagonism Assay

In vitro pairing assays were conducted on carrot extract agar against pathogens *Phytophthora palmivora* and *P. megakarya*. Bacteria were streaked in line dividing the agar plates into two equal parts [17]. Two discs of mycelium calibrated to 5 mm in diameter from a five-day-old culture of each strain of *Phytophthora* grown on pea extract agar were placed apart on each side of the bacterial streaks (01 cm from the edge of the Petri dish). Control plates consisted of the mycelial discs without bacterial streaks. Plates were then incubated in darkness at 26 ± 2°C and were observed after twenty four hours. Three replicate plates were prepared for all bacteria and for each pathogen strain.

2.2.2. Calculation of Percentage Inhibition (P.I.)

Growth of *Phytophthora* strains was recorded by measuring the diameter of the colonies each day using a ruler, as shown in Figure 1 and ended when one of the mycelium colonies covered the entire plate. The percentages inhibition (P.I.) exerted by endophytic bacteria on the radial growth of *Phytophthora* mycelium were estimated according to the following formula [18].

$$\text{Percentage inhibition}\left(\text{P.I}\right)=\left(1-\frac{\text{Dn}}{\text{Dt}}\right)*100.$$

Where P.I. (%) is the average percentage inhibition; Dn is the average diameter of mycelial colonies in the presence of bacteria and Dt is the average diameter of control plates mycelial colonies.

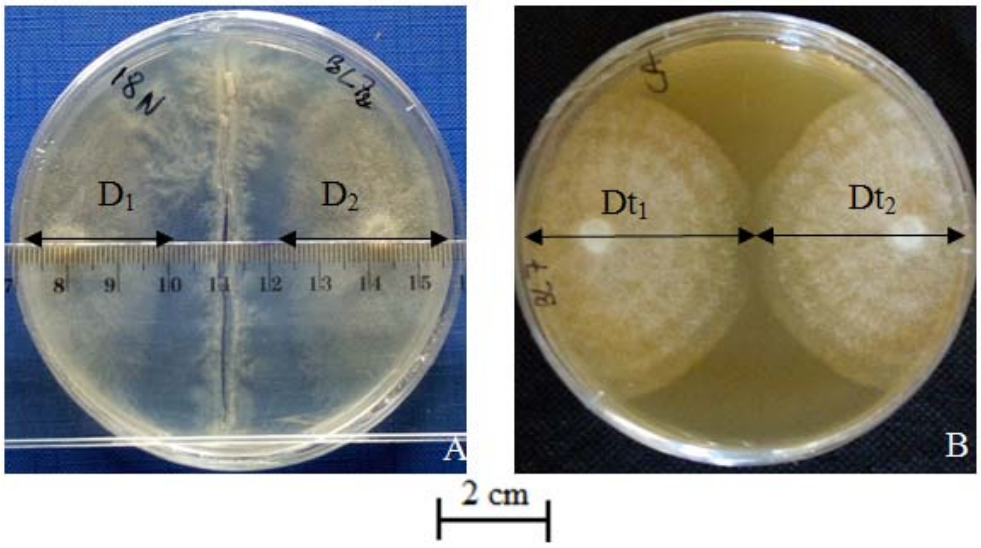

Figure 1. Measurement of *Phytophthora* spp. mycelium radial growth (A) in the presence of endophytic bacteria; (B) in the absence of endophytic bacteria. Where: **Dn** = $(D_1+D_2)/2$; **Co=** $(Dt_1+Dt_2)/2$; **Dn** = average diameter of mycelium in the presence of bacteria; **Dt=** average diameter of control mycelial colonies

2.2.3. Survival Test of *Phytophthora* spp. on Wounded Pods

After thirty days of *in vitro* confrontation, seven bacteria (11P; 18N; 23P; 42P; 47P; 48P; 48P and 60P) having a percentage inhibition greater than or equal to 59 % with the two strains of *Phytophthora* were tested on wounded pods to see the survival and aggressiveness of the pathogen. Mycelium explants (05 mm in diameter) of each *Phytophthora* strain were collected along a line on the contact fronts of mycelium and bacterial colonies. The explants were inoculated in wounded pods at a rate of four pods per bacterial isolate. The pods were then placed in bins on foams soaked in distilled water and closed with black tarpaulins and were incubated at 26 ± 2°C for five days.

2.2.4. Bacteria Inoculum Preparation

Fresh cultures of each bacterial isolate were performed in a pea extract broth. After forty eight (48) hours of incubation, the suspensions (10^9 CFU/mL) were diluted to a final concentration of 10^6 and 10^3 CFU/mL.

2.2.5. *Phytophthora* Inoculum Preparation

A suspension of zoospores of each strain was obtained from a sixteen-day-old culture on pea extract agar in Roux's vials. After six days of incubation in darkness, the cultures were exposed to a twelve-hour photoperiod (an alternation of 12 h/12 h darkness and fluorescent light) for ten days. In order to obtain zoospores release, cultures of *P. palmivora* and *P.megakarya* were flooded with sterile distilled water at 4°C and exposed to incandescent light for forty five minutes. The zoospores suspension was then adjusted with a Malassez hemacytometer (SOVIREL, France) to 3 x 10^5 zoospores/mL [19].

2.2.6. *In vivo* Antagonistic Effect of Endophytic Bacteria against *Phytophthora palmivora* and *P. megakarya*

The *in vivo* antagonist effect of endophytic bacteria against *Phytophthora palmiv*ora and *P. megakarya* was evaluated on leaf discs (15 mm in diameter). This test was conducted for each *Phytophthora* strain by a randomized four-factor split-plot experimental design. The first factor was the cocoa clone and three clones (NA32, PA150 and SCA6) which susceptibility to *Phytophthora* is known (susceptible, moderately resistant and resistant) were used. The second factor was the four bacterial isolates (60P; 48P; 18N and 23P) selected for good percentage inhibition. The third factor was the test of three concentrations (C_1=10^3, C_2=10^6, C_3=10^9 UFC/mL) of each bacterial inoculum. Finally, the fourth factor was the variation of three application periods of the bacterial inoculum. First period named (AV) consisted in applying the bacterial inoculum one hour before the inoculum of *Phytophthora* spp. The second period (P) consisted in simultaneously applying the bacterial inoculum and that of *Phytophthora* sp. Finally, the third period (AP) consisted in applying the bacterial inoculum one hour after the *Phytophthora* spp. The leaf discs placed in bins on foam of (01cm) thickness soaked in distilled water were inoculated with aliquots of 10 µL of suspension of each bacterial fresh culture at different concentrations 10^3, 10^6 and 10^9 CFU/mL. Each leaf disc then received, according to the type of treatment, 10 µL of a suspension of a strain of *Phytophthora* calibrated at 3×10^5 zoospores/mL. Controls were inoculated only with zoospore suspensions of the *Phytophthora* strains used. A total of thirty six treatments at the rate of nine treatments per bacterial isolates were applied to the leaf discs of the three cocoa clones used. Ninety leaf discs per clone arranged in three lines of ten discs per type of treatment were inoculated with each bacterial suspension and distributed in four bins each constituting a repeat. After inoculation of the leaf discs, each bin was covered with a black tarpaulin and incubated at 26 ± 2°C for seven days. The results were read according to the Blaha scale [19,20], whose black pod symptom score ranged from 0 to 5 (0: no symptoms; 1: point of penetration; 2: network points; 3: cross-linked task; 4: marbled task; 5: real task).

2.2.7. Statistical Analyses

All data were analyzed using SAS® software 9.4 [21]. The P.I. were transformed at arcsinus $\sqrt{}$ to adjust them to the normal distribution [22] and then submitted with the data obtained in leaf discs tests to ANOVA according the PROC GLM program (Process of General Linear Model) followed by Student-Newman-Keuls test at the threshold $\alpha = 5$ %.

3. Results and Discussion

3.1. Results

3.1.1. *In vitro* Antagonism Assay

Bacterial isolates of cocoa tree significantly inhibited mycelial growth of both *Phytophthora* strains in confrontation tests, as shown in Figure 2. The value of the P.I. of mycelium radial growth recorded after thirty days, varied significantly ($p < 0.05$) from 25.3 to 70.54 % depending on the bacterial isolates and the two fungal strains tested, as shown in Table 1. In fact, the isolates 48P; 18N; 42P; 60P; 47P; 47P and 23P were highly effective against *P. palmivora* with P.I. of 70.54 to 62.20% or diameters between 24.75 and 31.75 mm of mycelial growth. The isolates 42P; 47P; 60P; 18N; 22N; 66P and 63P were highly effective against *P. megakarya* with P.I. varying from 64.29 to 60.13% or diameters between 30 to 33.5 mm of mycelial growth. A total of four bacterial isolates 18N; 42P; 47P and 60P showed effective antagonistic activity against these two *Phytophthora* strains with P.I. above 60%.

3.1.2. Inhibitory Effect of Endophytic Bacteria on the Survival of *Phytophthora* spp

The results of survival and virulence tests of *Phytophthora* after thirty days of *in vitro* confrontation test with the seven effective bacterial isolates (11P; 23P; 42P; 47P; 48P; 60P and 18N) were variously conclusive. Indeed, mycelial plugs collected from the plates containing isolates 18N; 23P; 48P and 60P did not induce any symptoms of black pod disease in the wounded pods during five days of incubation. However, the other three isolates (11P; 42P and 47P) did not reduce this aggressiveness.

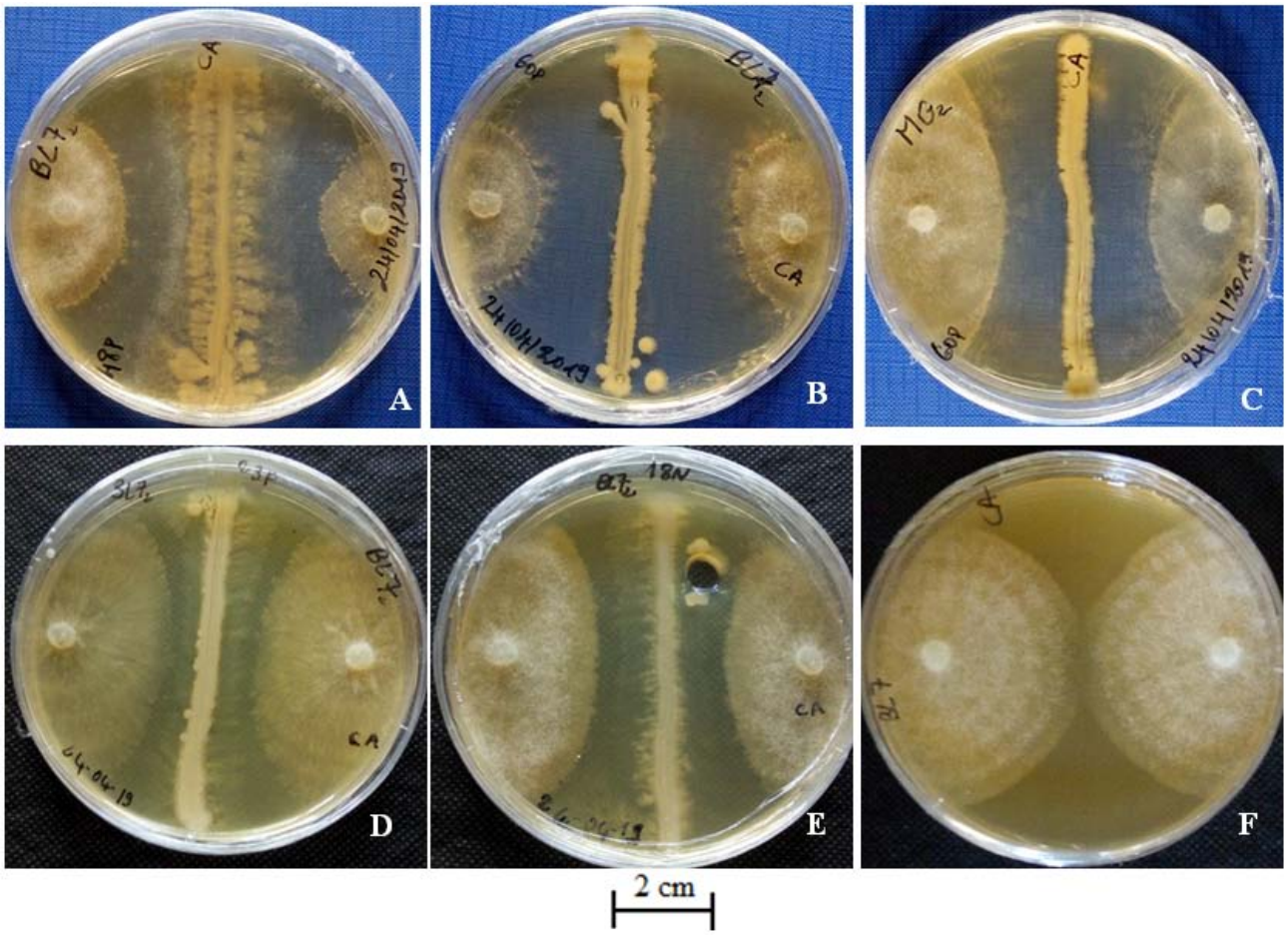

Figure 2. *In vitro* confrontation test of some cocoa endophytic bacteria against *Phytophthora* spp. (A) Isolate 48P; (B) Isolate 60P; (D) Isolate 23P; (E): Isolate 18N against *P. palmivora*; (C): isolat 60P against *P. megakarya*; (F): Control *P. palmivora BL7.11.2*

Table 1. *In vitro* inhibitory effects of some endophytic bacteria on mycelial growth of *Phytophthora* spp at thirty days of incubation

N°	Isolates	*P. palmivora*			Isolates	*P. megakarya*		
		M.D±sd	P.I (%)±sd (%)±sdd(%)±sd	P.I trsf		M.D±sd	P.I (%)±sd	P.I trsf
1	48P	24.75±3.59	70.54±4.28	0.787^{a}	42P	30.00±1.41	64.29±1.68	0.703^{a}
2	18N	28.25±1.25	66.37±1.49	0.727^{b}	47P	31.25±0.95	62.80±1.13	0.683^{ab}
3	42P	30.25±1.70	63.99±2.03	0.697^{bc}	60P	32.75±4.11	61.02±4.89	0.660^{abc}
4	60P	30.50±0.57	63.69±0.68	0.695^{bcd}	18N	33.00±2.94	60.72±3.50	0.658^{abcd}
5	47P	31.25±0.95	62.80±1.14	0.682^{cde}	22N	33.00±3.36	60.72±4.00	0.658^{abcd}
6	23P	31.75±1.25	62.20±1.49	0.675^{cdef}	66P	33.50±2.38	60.13±2.83	0.650^{bcde}
7	11P	34.00±2.00	59.53±2.38	0.645^{defg}	63P	33.50±2.38	60.13±2.83	0.650^{bcde}
8	40P	34.00±2.44	59.53±2.91	0.642^{efgh}	40P	33.75±2.06	59.83±2.45	0.645^{cdef}
9	14P	34.00±2.94	59.53±3.50	0.642^{efgh}	52N	34.00±3.65	59.53±4.34	0.643^{defg}
10	20N	34.50±4.50	58.93±5.35	0.635^{efghi}	65P	34.00±2.44	59.53±2.91	0.640^{defg}
11	50N	35.00±3.36	58.34±3.36	0.627^{fghi}	48P	34.50±4.43	58.94±5.27	0.633^{efg}
12	17N	36.75±1.82	56.25±2.63	0.600^{ghi}	23P	35.75±1.50	57.45±1.78	0.618^{fgh}
	Control	84.00	0.00	0.00	Control	84.00	0.00	0.00
	ANOVA	F=47.12	p<0.05	CV=6.92	ANOVA	F=25.19	p<0.05	CV=5.67

M.D (mm) = mycelium mean diameter; **P.I** (%) = percentage inhibition of mycelial growth; **P.I trsf** = percentage inhibition in transformed values (y = arcsin√x); **sd=** standard deviation**; CV** (%) =coefficient of variation; the means with the same letter are not very different according to the Student Newman-Keuls test at the threshold α = 0.05.

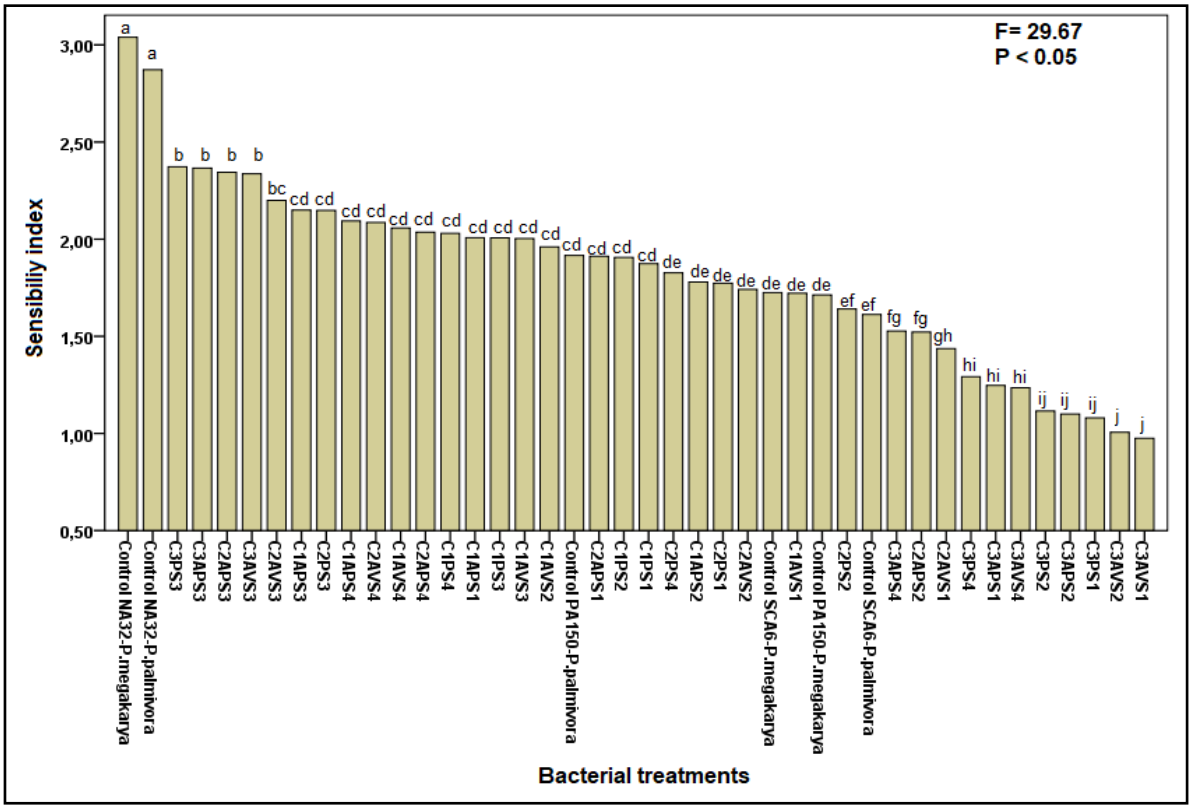

Figure 3. Effect of bacterial treatments on the susceptibility of three cocoa clones (NA32, PA150 and SCA6) to *Phytophthora* spp. C_1= Concentration 10^3 CFU/ml; C_2= Concentration 10^6 CFU/ml; C_3= Concentration 10^9 CFU/ml; S1= Isolate 48P; S2= Isolate 18N; S3= Isolate 60P; S4= Isolate 23P; Averages with the same letter are not very different according to the Student Newman-Keuls test at threshold 5 %

3.1.3. *In vivo* Antagonist Effect of Endophytic Bacteria against *Phytophthora palmivora* and *P. megakarya*

The application of bacterial treatments significantly reduced ($p < 0.05$) the susceptibility to black pod disease of the three clones. Significant effects ($p < 0.05$) of factors clone and timing of bacterial inoculum application were noted. Similarly a significant interaction effect ($p < 0.05$) of clone and bacterial factors on the black pod disease symptoms was observed. Treatments based on bacteria S1 (48P), S2 (18P) and S4 (23P) applied to foliar discs significantly reduced black pod disease necrosis induced by both *Phytophthora* strains to the three clones (NA32; PA150 and SCA6). Leaf discs treated with these bacteria showed mostly no symptoms or simple penetration point (indices values 0 or 1), unlike the clone NA32 controls where necrosis network (indices values 2.8 and 3.0) were observed for *Phytophthora palmivora* and *P. megakarya*, as shown in Figure 3. Bacterial isolates S1 (48P) and S2 (18N) highly inhibited both *Phytophthora* strains. Furthermore, bacterial isolate S4 (23P) moderately inhibited both *Phytophthora* strains. Unlike the three bacterial isolates cited above, the isolate S3 (60P) showed no inhibitory effect towards the two *Phytophthora* strains. Because, treatments based on this bacteria were statistically close to the two controls of the clone NA32. Treatments at the concentration 10^9 CFU/ml of the two bacterial isolates S1 (48P) and S2 (18N) were highly effective. These bacterial isolates effectively reduced black pod necrosis on the foliar discs of the three clones, particularly the susceptible clone NA32, as shown in Figure 3.

3.2. Discussion

Endophytic bacteria isolated from the vegetative organs of cocoa tree exerted *in vitro* a high inhibitory activity on the mycelial growth of the both strains of *Phytophthora*. After thirty days of confrontation *in vitro*, an effective reduction of the mycelium diameter and an inhibition zone on both sides of bacterial streaks were observed. That demonstrate isolates which hindered the growth of *Phytophthora* produced inhibitory substances [23]. Survival tests conducted on wounded pod confirmed the antagonistic effect of isolates 18N; 23P; 60P and 48P. However, isolates 42P; 47P and 11P were found to be ineffective. These isolates simply inhibited the growth of *Phytophthora* by competing for nutrients in the culture medium. Their antagonistic effects were simple fungistatic effects [3]. In contrast, isolates 18N; 23P; 60P and 48P could have fungicide effects against *Phytophthora palmivora* and *P. megakarya*. Those effects would be more or less effective depending on the bacterial isolate and the strain of *Phytophthora*. Studies have shown the existence of *Phytophthora* antagonistic microorganisms in the cacao tree ecosystem [20,23].

These isolates inhibited the radial growth of *P. palmivora* (BL7.11.2) and *P. megakarya* (13P30.1) mycelium on carrot extract agar plates with P.I. of 28.58 % to 70.54 % and 25.3 to 64.29 % respectively. Similar results were obtained by Akrofi et *al*. [18] who showed that epiphytic bacteria isolated from pods surface of three resistant cocoa clones (SCA6, T85/799 and IFC5), highly inhibited the growth of *Phytophthora palmivora* with P.I. of 69.7 and 65.8 %. Isolates S1 (48P) and S2 (18N) effectively reduced black pod symptomatic necrosis onto all three

clones, particularly onto the susceptible clone NA32. This showed that these isolates induced resistance to the clone NA32 on the one hand, and increased the intrinsic resistance of the two clones (PA150 and SCA6) on the other hand. Kébé et *al*. [20] also isolated indigenous rhizobacteria from cocoa tree soils. These bacteria inhibited *Phytophthora palmivora* on leaf discs of two resistant cocoa clones (P7 and SCA6). According to Mejia et *al*. [24] the results of *in vitro* tests do not necessarily reflect those obtained *in vivo* or in field tests. Some microorganisms found to be highly antagonistic *in vitro* may be ineffective in *in vivo* or in field tests due to many ecological parameters.

The effectiveness of bacterial treatments applied for one hour on leaf discs before the introduction of the fungal inoculum proves that these endophytic bacteria, once installed, create a barrier against the penetration of pathogens in the stomata. This could explain the induction of resistance to the susceptible clone NA32. In addition, these isolates certainly produce antifungal substances that neutralize the development of zoospores on the foliar discs even when the inoculum of *Phytophthora* spp was applied one hour before the inoculum of the bacterial isolates was applied.

4. Conclusion

The cocoa tree naturally hosts endophytic bacteria that may have antagonistic effects against certain fungal pathogens such as *Phytophthora palmivora* and *Phytophthora megakarya*. In fact, these bacterial isolates significantly inhibited, *in vitro*, the mycelial growth of these two strains of *Phytophthora* and induced resistance against *Phytophthora* spp. to different cocoa clones. This resistance induction was more remarkable to the clone NA32, susceptible to black pod disease. This study therefore proves the antagonistic potential of cocoa endophytic bacteria and their possible use in biological management of black pod disease. These bacterial isolates may be tested in pod tests and field trials to confirm the stability of their antagonistic effects against *Phytophthora* spp.

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