IN VITRO ASSESSMENT OF BIOPESTICIDE *BACILLUS THURINGIENSIS* VAR. *KURSTAKI* HD-1 EFFECTIVENESS ON *PHYTOPHTHORA PALMIVORA*, AGENT OF COCOA BLACK POD ROT IN CÔTE D'IVOIRE

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Abstract

Phytophthora Palmivora is responsible for the cocoa black pod rot in Côte d'Ivoire, with production losses varying from 20 to 40% depending on the regions. Effectiveness of the environmentally friendly biopesticide *Bacillus thuringiensis* var. *kurstaki* HD-1 was evaluated *in vitro* on the pathogen. The medium pea supercooled has been incorporated with the bioproduct at different concentrations: C1 = 100%, C2 = 50%, C3 =25%, C4 = 12.5% and C5 = 6.25%. This medium into Petri dishes was inoculated either by spreading, with 0.1 mL of *P. palmivora* inoculum at10^3 zoospores /mL, or with a mycelial disc of *P. palmivora* calibrated at 6 mm diameter in the center of the box. The results showed that the germination of zoospores was inhibited at the rate of 100% in the presence of the concentrations C1 and C2. This rate was 74.82 ± 21.53%, 73.17 ± 20.88% and 59.12 ± 39.05%,

respectively, for C3, C4 and C5. The efficacy of the bioproduct is revealed by a significant reduction of mycelia growth ranging between 57.77± 2.4 to 100% in the presence of the concentrations C5 to C1. The IC_{50} of germination was 3.36% of concentration and the IC_{90} was 43.22%; while those which inhibit 50% and 90% of mycelial growth were 5.67% and 77.94% respectively. The biopesticide Btk HD-1 has a proven fungistatic effect against *P. palmivora*. Its effectiveness increases with its concentration. So it can be a valuable component of an integrated cocoa black pod management.

Keywords: Biopesticide; *Bacillus thuringiensis* var *kurstaki* HD-1; *Phytophthora palmivora*; Black pod disease; Cocoa tree; Starch industry wastewater; Biocontrol

Introduction

Cocoa beans' export represents, for many developing countries, one of the main sources of income (Oxfam International, 2009). Among theses countries, Côte d'Ivoire remains the greatest provider worldwide for more than three decades (Tano, 2012). Unfortunately, Ivorian cocoa production is facing many constraints among which cocoa black pod rot caused by *Phytophthora* spp is prominent (Mpika *et al*., 2009).The National Agricultural Research Center in Côte d'Ivoire, estimated that the production losses due to *Phytophthora* spp range between 20% and 40% (CNRA, 2013). According to the International Cocoa Organization (ICCO, 2009), these damage vary from 30% to 40% around the word.

To control this disease, chemical fungicides, usually based on copper, have long been used (Pohé, 2012). But their massive use in agriculture is a real global threat today. Indeed, they are currently classified as major environmental pollutants causing toxic residues in air, soil and water (Jawich, 2006). Additionally, there is the appearance of the pathogen's resistant strains as persistent organic pollutants (Pohé and Agnéroh, 2013).

Because of the disadvantages of chemical control on the one hand, the environment protection, the health quality of agricultural products (no pesticide residues) and the populations health requirements on the other hand, research for biological control are increasingly recommended and encouraged (AGCan and CCNUPA, 2001; NBPBCO 2001). In this context, biological control methods using biopesticides based especially on *Bacillus thuringiensis* (Bt), have been developed; *Bacillus thuringiensis* (Bt) have been developed; and their use in agriculture appears particularly promising (Joung and Côté, 2000). These biopesticides are suspensions formulated from mixtures of bacterial spores and protein crystals obtained after

culturing, growth and sporulation of the strain in a bioreactor (Adjallé, 2009).

Bt has advantages related to its specificity, the biodegradability of its protein crystals, making them harmless for non-target organisms and for human being. Moreover, they are non-toxic for the environment (Joung and Côté, 2000). Yet, there are limited data on the use of Bt against phytopathogenic microorganisms. However, it is recognized that certain synthetic Bt products such zwittermicin A, a fungistatic antibiotic, is highly active against Oomycetes (Zhou *et al*., 2008) and chinases have fungicidal activity (Jaoua *et al*, 2007). In this study, the effectiveness of var. *kurstaki* HD-1, biopesticide made of Bt against *P. palmivora*, pathogen agent of cocoa black pod rot in Côte d'Ivoire, was evaluated *in vitro*.

Material and methods

Fungus materials and culture medium of *Phytophthora palmivora*

A strain of *Phytophthora palmivora*, whose aggressiveness is proven on the plant material, was used to conduct *in vitro* tests. This strain, called BL7 / 11.2, was isolated from a pod naturally infected by brown rot. This pod was harvested on the cacao tree instead of cocoa tree 2, line 11 of the experimental plot BL7, planted in 1986 at the National Agricultural Research Center (CNRA), Station of Bingerville, in southern Côte d'Ivoire. The isolation was performed by taking a piece of cocoa pod infected by black rot and filing it on a water agar medium at1.5%. After formation of the thallus, a mycelia fragment was taken from the front of the crop growth and transferred to petri dishes on agar medium containing pea (Coulibaly *et al*., 2013).The incubation was performed at 26 °C for 4 to 5 days. To avoid the obvious heterogeneity of zoospores, cloning of single zoospore isolation strain was performed according to the technique described by Babacauh (1980).

The inoculum was composed of *P. palmivora* zoospores. The zoospore suspension was obtained from a pure culture aged of 5 to 6 days in the middle pea Roux flasks. The Incubation was made in a photoperiod of 12 hours for three days. The culture was then inoculated with 40 mL of distilled water and placed for 15 min at a temperature of 4 °C and then exposed to light of an incandescent lamp of 60 W for 45 minutes. The zoospore suspension obtained in the roux flask was counted with a Malassez cell and adjusted to a concentration of 10^3 zoospores /ml.

Biological control material

The biological control material used was a biopepticide made of *Bacillus thuringiensis* variety *kurstaki* HD-1 (Btk HD-1). It is made in the Bioconversion of wastewater and wastewater sludge into value added

products laboratory of Water-Earth-Environment, National Scientific Research Institute of Quebec University, Canada. This bioproduct was synthesized in a bioreactor using the starch industry wastewater (SIW) as raw material. It is in liquid form and contains spores ($2.46.10^9$ CFU / mL) and cells ($3.13.10^9$ CFU / mL) (Vu, 2009).

The biopesticide based on Btk HD-1 was mixed up in sterile distilled water in order to generate a range of concentrations of 100%, 50%, 25%, 12.5% and 6.25 % (v/v), respectively designated C1, C2, C3, C4 and C5. These concentrations were chosen after a preliminary laboratory tests.

In vitro Bacillus thuringiensis* strain (Btk HD-1) effect on the germination of zoospores of *P. palmivora

Five Erlenmeyer bottles containing each, 54 ml of agar medium made of supercooled pea and completed to 60 mL with a concentration of the tested biopesticide made of Btk HD-1 (100%, 50%, 25%, 12.5% and 6.25%). After homogenization of the medium on a magnetic stirrer for 2 min, the content of each vial was distributed equally into 4 Petri dishes of 90 mm diameter; each box constituting a replication. With a rake Pasteur pipette, an amount of 100 µL of inoculum of *P. palmivora* with a zoospore concentration of 10^3/mL was plated after the solidification of the medium. The tested biopesticide was not added to the control culture medium (To). The inoculated plates were placed in a crystallizer before being incubated at 26 ± 1 °C during 24 hours.

The estimation was made 24 hours after the spreading of zoospores on the culture medium. The germinated zoospores were counted per Petri dish depending on the concentrations and in comparison to the control medium culture. The germination rate was calculated according to the formula modified by Shuman (2001):

$$\mathbf{G}\ (\%) = \left(\frac{\mathbf{Ng}}{\mathbf{Nt}}\right) \times \mathbf{100}$$

G (%) is the germination rate, **Ng** is the number of germinated zoospores in the presence or absence of a concentration (C) of the Btk HD-1 biopesticide and **Nt** is the total number of zoospores spread or grown on medium control and embedded culture.

The germination inhibition rate was determined according to the formula of Bouigoumane *et al.* (2008):

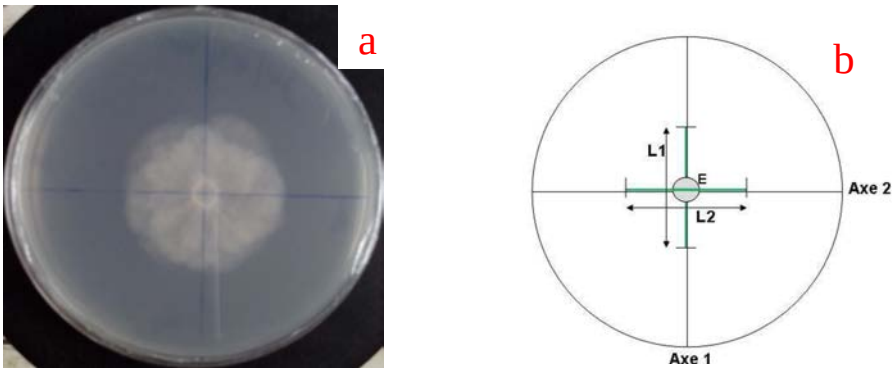
$$\mathbf{Ig}\ (\%) = \left(\frac{\mathbf{No} - \mathbf{Nc}}{\mathbf{No}}\right) \mathbf{x\ 100}$$

Ig (%) is the germination inhibition rate, **No** is the number of germinated zoospores on control medium culture and **Nc** denotes the number of germinated zoospores in the presence of a concentration (C) of the Btk HD-1biopesticide.

In vitro* biopesticide (Btk HD-1) effect on the mycelial growth of *P. palmivora

The mycelial discs of *P. Palmivora* (6 mm in diameter) were obtained from actively growing colonies aged of 4 days on pea extract agar. The procedure described previously was used. A mycelial disc was transferred in the center of the pea extract agar plates. The control was performed under the same conditions but without addition of biopesticide. The incubation was carried out like previously.

The mycelial growth of *P. palmivora* explant was measured from Petri dishes according to culture medium treated with each test concentration (C) and the untreated culture medium (control). The measurements were made with a graduated ruler every 24 hours considering two perpendicular axes drawn at the base of each Petri dish and which intersect in the middle of the explant (Figure 1).



(Camara, 2011)

Figure 1: Diagram illustrating the method of measuring the growth of fungal colonies in a Petri dish

E: explant of known mean diameter (X) at time t_0

L1: diameter of the explant E on the y-axis (axis 1) at time t_1

L2: diameter of the explant E on the y-axis (axis 2) at time t_1

The mean diameter (Dm) was calculated according to the formula below:

$$\mathbf{Dm}\ (\text{mm}) = \frac{\mathbf{L1 + L2}}{\mathbf{2}}$$

Dm (mm) indicates the mean diameter of the mycelia

The inhibition rate of mycelial growth (Ic) versus control was calculated using the following formula (Kwazou *et al*., 2009):

$$\mathbf{Ic}\ (\%) = \left(\frac{\mathbf{Do - Dc}}{\mathbf{Do}}\right)\mathbf{x\ 100}$$

Do indicates the mean mycelial growth of *P. palmivora* (mm) on the control culture medium and **Dc** is the mean mycelial growth of *P. palmivora* (mm) on the culture medium added with a concentration (C) of Btk HD-1 based biopesticide.

Determination IC_{50} and IC_{90} of Biopesticide

The IC_{50} and IC_{90} are concentrations that induce 50% and 90% reduction of the mycelia growth and the germination of parasite, respectively. They were calculated after 5 days of culture for the mycelia growth and 2 days for the germination of zoospores. The inhibition rate of mycelia growth and germination of zoospores were transformed into probit values. The regression lines were established as follows: **y = alog (x) + b**, where **a** is the regression coefficient, **b** a constant, **x** the concentration of biopesticide, **y** the probit and log the decimal logarithm.

From linear regression lines, IC_{50} and IC_{90} values were determined by simple projection (Serghat *et al*., 2004).

***In vitro* fungicidal or fungistatic activity of Btk HD-1**

At the end of each test, the explants, in the culture medium incorporated with the biopesticide, which growth value was zero, were transferred to new dishes containing agar medium made of pea without the biopesticide. This helped to determine the nature of the inhibitory effect of each concentration (C) of the biopesticide. The explants were observed every day over 3 to 5 days and those having resumed growth were noted. Thus, the recovery rate was evaluated. When the explants transferred to new culture media grow, the concentration is termed fungistatic, as it prevented the growth of the fungus without killing him. Otherwise, it is said fungicide (Kouamé, 2014).

Statistical analysis

The data collected for each test were submitted to analysis of variance (ANOVA) using Statistica 7.1 software. In case of significant differences, the averages were compared according to the Newman-Keuls test at the 5% threshold.

Results

Effect of Btk HD-1 on the germination of zoospores

The incubation of zoospore *P. palmivora* on untreated culture medium (control) and on the culture medium treated with the biopesticide was done for 2 days. The germination rate (G) of zoospores belonging to the control culture medium (To) was 2 times higher than those treated with culture media concentrations (C1 = 100%, C2= 50%, C3 = 25% and C4 =

12.5%) of Btk HD-1 the first and second day. This rate is much higher than that of the culture medium treated with the concentration C5 of Btk HD-1 during the 2 days (Table I).

Table I: Germination average rate (% G) per day depending on the concentrations of the biopesticide Btk HD-1

	Day 1	Day 2
Control (To)	52,75 ± 10,04 d	56 ± 10,39 d
Btk HD-1		
C1	0,00 ± 0,00 a	0,00 ± 0,00 a
C2	0,00 ± 0,00 a	0,00 ± 0,00 a
C3	22,5 ± 2,51 b	26 ± 3,16 b
C4	23,5 ± 4,5 b	27,75 ± 5,12 b
C5	37 ± 4,76 c	41,5 ± 3,69 c

The averages of each column followed by the same letter are not significantly different at a level of 5% (Newman-Keuls test).

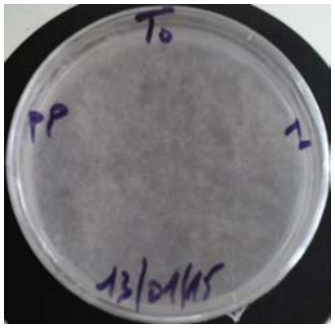
The applied treatment was effective during the 2 days of incubation at rates ranging from 54.70 ± 8.43 to 100% the first day for the concentrations C4 to C1 and 91.63 ± 6.12 to 100% the second day for all of the concentrations tested (Table II). The concentration that inhibited 50% (IC_{50}) and 90% (IC_{90}) the germination was 13.39% and 56.38% at 1 day. In the second day of incubation, the IC_{50} and the IC_{90} were respectively the concentrations $5.67.10^{-5}$ % and 5.22% of Btk HD-1.

Table II: Inhibition rate of germination (% Ig) of zoospores versus concentrations of Btk HD-1

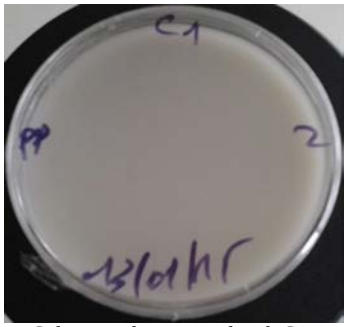
Btk HD-1	Day 1	Day 2
C1	100 ± 0,00 b	100 ± 0,00 b
C2	100 ± 0,00 b	100 ± 0,00 b
C3	55,92 ± 10,77 a	93,72 ± 3,72 a
C4	54,70 ± 8,43 a	91,63 ± 6,12 a
C5	26,19 ± 25,81 c	92,05 ± 1,11 a

The averages of each column followed by the same letter are not significantly different at a level of 5% (Newman-Keuls test).

The biopesticide Btk HD-1 showed a strong fungitoxic action on the germination of zoospores (Figure 2).

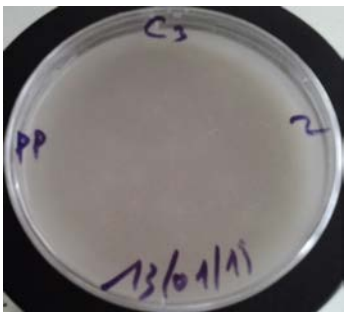


Untreated culture medium (To)
(Germination of zoospores)



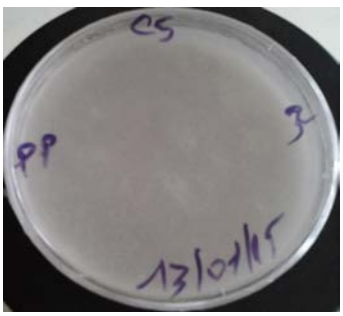
Culture medium treated with C1
(No germination)

Culture medium treated with C2
(No germination)



Culture medium treated with C3
(Inhibition at over 74%)

Culture medium treated with C4
(Inhibition at 73%)



Culture medium treated with C5
(Inhibition at 59%)

Figure 2: Inhibition of zoospores germination on culture medium treated with biopesticide Btk HD-1 at various concentrations after 4 days

All tested concentrations induced more than 50% reduction of the zoospores' germination of zoospores. The concentrations C1 and C2 reduced to 100% the germination of zoospores. More specifically, C1 and C2 provoked 100% reduction while C5, C4 and C3 reduced the germination of zoospores from 59.12 ± 39.05 to 74.82 ± 21.53 (Table III). After 2 days of incubation the IC_{50} was 3.36% and the IC_{90}, 43.22% of Btk HD-1 concentration.

Table III: Germination rate (G) and inhibition (Ig) after 2 days incubation under the five concentrations of Btk HD-1

	G (%)	Ig (%)
Témoin (To)	54,37 ± 9,62 d	-
Btk HD-1		
C1	0,00 ± 0,00 a	100 ± 0,00 a
C2	0,00 ± 0,00 a	100 ± 0,00 a
C3	24,25 ± 3,24 b	74,82 ± 21,53 b
C4	25,62 ± 5,01 b	73 ± 20,88 b
C5	39,25 ± 4,62 c	59,12 ± 39,05 b

The averages of each column followed by the same letter are not significantly different at a level of 5% (Newman-Keuls test).

Effect of Btk HD-1 on mycelial growth of *P. palmivora*

The mean diameter of *P. palmivora* varied depending on the concentrations of biopesticide used. Thus, at the first day the of incubation, the growth of mycelium of the control culture medium (To) which was 24.37 ± 0.47 mm reached up to 90 mm diameter on the 5th day.

The mycelial growth was reduced up to 100 % with C1 concentration during the 5 days of incubation, thus leading to the absence of the parasite growth on the culture medium. However, with the concentration C2 the mycelial growth was respectively of 9 ± 1.54 mm on the second day on the culture medium treated and 16.5 ± 2.64 mm on the 5th day.

Concerning the C3, C4 and C5 concentrations, the mycelium has progressed every day to a kinetic from 11.87 ± 0.47 to 18.62 ± 1.79 mm for the day one of incubation and 32.87 ± 0.85 to 38 ± 2.16 mm on the 5th day of incubation (Figure 3).

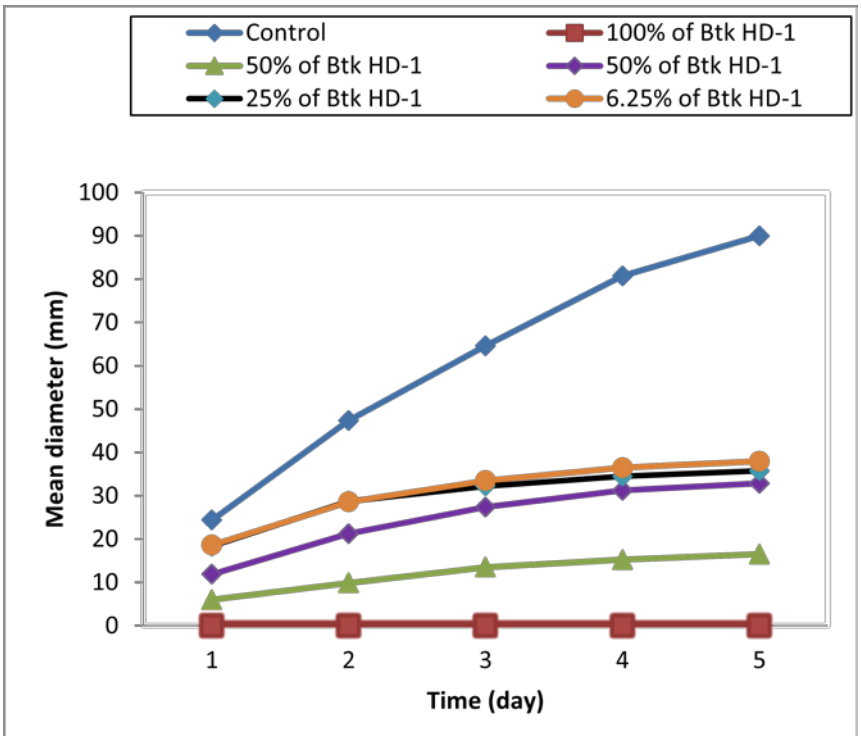
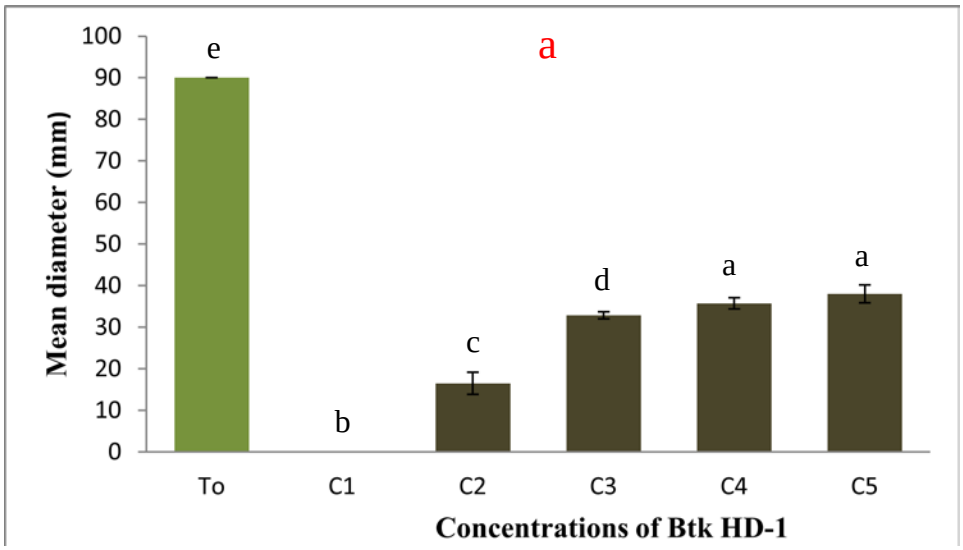


Figure 3: Effect of biopesticide Btk HD-1 on *P. palmivora* mycelial growth

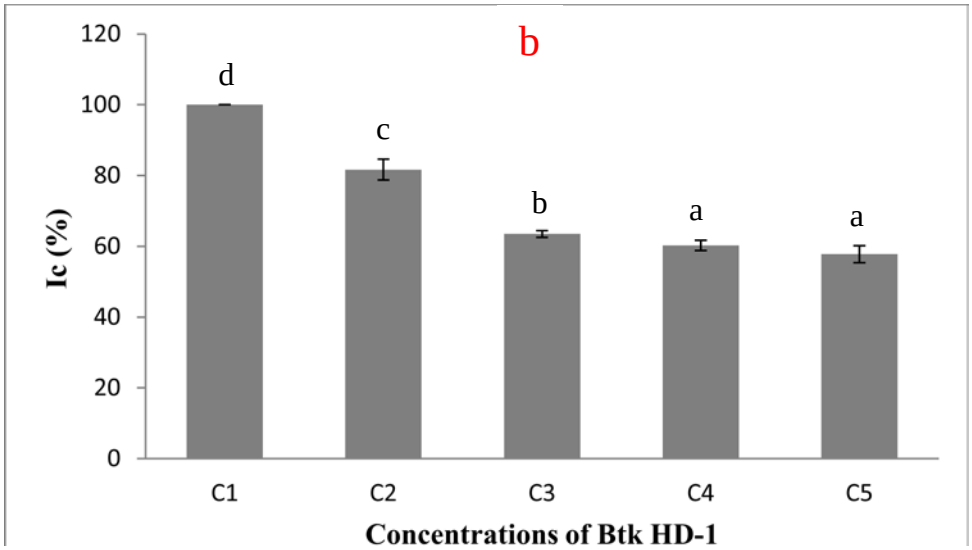
Mean diameter (Dm) and inhibition rate of mycelial growth (Ig)

The mean diameter (Dm) and the inhibition rate of mycelial growth (Ic) of the pathogen evolved in parallel (Figure 4a and b).

After five days of incubation, the inhibition rate of mycelial growth in the presence of the concentration C1 was 100%.The medium treated with the concentration C2 was 81.66 ± 2.93%. Concerning the media treated respectively with the concentrations C5, C4 and C3, the inhibition rate were between 57.77 and 63.47 ± 2.4% ± 0.94% of C5 to C3 (Figure 4b). The concentration which inhibits 50% (IC_{50}) of mycelial growth after 5 days was 5.67% and the IC_{90} was 77.94% of Btk HD-1 concentrations.



The bars surmounted by the same letter are not significantly different at the level of 5% (Newman-Keuls test).



The bars surmounted by the same letter are not significantly different at the level of 5% (Newman-Keuls test).

To: control; C1: 100% concentration of Btk HD-1; C2: 50% of Btk HD-1
C3: 25% of Btk HD-1; C4: 12.5% of Btk HD-1; C5: 6.25% of Btk HD-1

Figure 4: Mean diameter (Dm) and inhibition rate of mycelial growth (Ic) *P. palmivora* versus concentrations of Btk HD-1 biopesticide

Phytopathogenicity ability of *P. palmivora* after its *in vitro* confrontation with biopesticide (Btk HD-1)

The explants (mycelial discs) taken from the culture medium treated with 100% of the concentration of Btk HD-1 and transferred into untreated culture medium resumed growth at 25%. In the other hand, those taken on the same box at 5 mm on either side of the mycelial disk did not grow.

On the culture medium supplemented with 50% of the concentration (C2) of Btk HD-1, the explants taken at 10 mm on either side of the inoculum (mycelial disks), and transferred into untreated culture medium, did not grow. On the other hand, those taken between 5 and 9 mm grew with a rate of 50%.

On the explants taken between 10 and 16 mm on either side of the inoculum on the medium treated with 25% concentration (C3) of Btk HD-1, there was regrowth with a rate greater than 50%. Beyond 17 mm, there was no growth.

The explants, taken at between 20 and 30 mm on either side of the inoculum on the medium incorporated with 12.5% and 6.25% concentrations (C4 and C5) of Btk HD-1, did not grow on the untreated medium culture. Below this radius (between 15 and 20 mm), the explants grew with a rate of 75%.

So, the tested concentrations of Btk HD-1 based biopesticide did not have a fungicidal effect on the growth of *P. palmivora* instead, they had an established fungistatic effect.

Discussion

This study showed a significant inhibitory effect of *Bacillus thuringiensis* var. *kurstaki* HD-1 (Btk HD-1) on the germination of zoospores and mycelial growth of *Phytophthora palmivora* on agar medium containing pea. On germination of *P. palmivora*, zoospores, concentrations at 100% (C1) and at 50% (C2) of biopesticide Btk HD-1 induced the same inhibition rate (100%) of zoospores germination. The same result was observed in the case of inhibiting the mycelial growth with the concentration C1. These results confirm the action, already mentioned by many other researchers, of *Bacillus thuringiensis* (Bt) against certain phytopathogenic microorganisms. Zhou *et al.* (2008) reported that Bt has a very high activity against Oomycetes and their relatives. Silo-Suh *et al.* (1994) reported that Bt is effective against the melting of alfalfa seedlings caused by *Phytophthora medicaginis*. Shang *et al.* (1999) showed that Bt exerts greater inhibition of cysts germination and germ tubes *Phytophthora torulosum* elongation. This Bt inhibitory action on the germination of zoospores and mycelial growth of *P. palmivora* is certainly due to Bt active components synthesized by bio-fermentation during its growth. This view is shared by Zhou *et al.* (2008),

who claim that Bt produces other active components with good application prospects for the control of plant diseases, apart from the crystal protein substances and insecticides. The same authors mention that zwittermicin A, a linear aminopolyol antibiotic produced by Bt, is very active against some plant pathogens. Indeed, to study the mechanism of the activity of zwittermicin A against weaker eukaryotes, such as Oomycetes including *Phytophthora* spp which is part, and a variety of bacteria, Milner *et al*. (1996) have built a *Bacillus cereus* UW85 genomic library. The results revealed the presence of a 1.2 kb fragment of DNA from *B. cereus* UW85 called Zmar. This gene confers resistance to zwittermicin A and encodes for a protein Zmar 43.5 kDa. This protein attaches own significant resistance to *Bacillus* group that produces zwittermicin A. However, the fungicidal activity of this linear aminopolyol antibiotic remains an enigma (Stabb and Handelsman, 1998).

Apart from zwittermicin A, chitinases are active elements which maximize the action against some Bt plant pests. This enzyme has been mentioned by some authors as possessing fungicidal activity (Jaoua *et al.*, 2007). On the other hand, others consider it as a proven fungistatic activity enzyme against several soil fungi (Arora *et al.*, 2013). According to these authors, this enzyme interacts with the protein Cry1Ac to maximize the insecticidal activity and to facilitate propagation of Btk HD-1 in an environment by inhibiting the growth of fungi. This statement of the authors Arora *et al*. (2013), is confirmed by our results. Indeed, the mycelial growth of the pathogen, on culture medium supplemented with different concentrations of Btk HD-1 suspension, was reduced significantly at rates ranging from 57.77 to 100% from the lowest to the highest concentration of Btk HD-1. If chitinases contribute to the reduction of mycelial growth, their mode of action is not yet well defined. This is what led Schnellman *et al.* (1994) to argue that the biological function and the utility of chitin binding protein are not well clarified, but they are suspected to facilitate the attachment of microbial chitin for it subsequent degradation.

In addition to its insecticidal activity on a wide range of insect pests, the fungistatic and bactericidal activity of Bt has been proven. The Authors Dong *et al.* (2002 and 2004) and Zhou *et al.* (2006) had already revealed that the acyl homoserine lactone (AHL), lactonase produced by this bacteria blocks bacterial pathogenicity, including that of *Erwinia carotovora* responsible for soft rot of Chinese cabbage. Other authors go further to say that Bt has bactericidal activity (Jaoua *et al*., 2007). This is certainly what allows Zhou *et al.* (2008) to conclude that Bt has an enormous biocontrol potential against plant diseases.

The Bt biopesticides, able to control both pests and reduce the pressure of plant pathogenic microorganisms, open integrated control

channel. They could be a promising alternative to chemical control because, unlike synthetic pesticides, their use would present a safety for the environment, and would have no effect on beneficial fauna and Are non-toxic for humans.

Conclusion

This study highlighted the effectiveness, in laboratory, of Btk HD-1 biopesticide on *P. palmivora*, pathogen of cocoa black pod rot. The results showed that Btk HD-1 is a fungistatic agent effective against *P. palmivora*, able to inhibit the germination of zoospores with a rate of 100% and to reduce significantly its growth with a rate beyond 80% for a Btk HD-1concentration of 50%.

This *in vitro* assay gives good prospects for the management of cocoa black pod rot. This alternative to chemical control is possible and further studies, including *in vivo* tests and field experiments, should strengthen its implementation.

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