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Inhibition of *Phytophthora palmivora*, causal agent of cocoa black pod disease, by using three (3) species of *Trichoderma* in three (3) cocoa-producing regions in Côte d'Ivoire

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Abstract

Cocoa black pod disease, caused by *Phytophthora* sp. leads to yield losses. In order to remedy such losses, the use of an antagonistic fungus such as *Trichoderma* is recommended. This study aims at contributing to cocoa yield improvement in Côte d'Ivoire by controlling *Phytophthora* sp. To this end, healthy cocoa pods, affected by black pod disease and soil from cocoa trees' rhizosphere were collected. *Trichoderma* sp. and *Phytophthora* sp. were respectively isolated from the soil and from the pods affected by black pod disease. PCR and amplicon sequencing analyses were used to characterize *Phytophthora* and *Trichoderma* species. *In vitro* and *in vivo* comparisons were carried out between *Trichoderma* sp. and *Phytophthora* sp. Three species of *Trichoderma* (*T. harzianum*, *T. asperellum* and *T. virens*) and three morphotypes of *Phytophthora palmivora* found in the cocoa orchards were characterized. *Trichoderma* species showed antagonistic activity towards *Phytophthora palmivora* with inhibition rates ranging from 65 to 87.5% and from 75 to 88.7% on detached pods and on culture medium, respectively. These *in vitro* and *in vivo* tests showed that these *Trichoderma* strains have a strong inhibitory action in the presence of *Phytophthora palmivora* strains, in particular *Trichoderma virens* (with 87.5% inhibition rate).

Keywords: Cocoa, Black pod disease, *Phytophthora* sp., *Trichoderma* sp.

Introduction

In Côte d'Ivoire, cocoa cultivation plays an important role in the social and economic life of populations. It generates approximately 46% of export revenues. Moreover, it constitutes a substantial source of income for thousands of cocoa farmers (Dembélé *et al.* 2009). It alone accounts for 15% of Ivorian GDP (Dufumier, 2016). As the world's leading producer, with more than 43% of global yield (ICCO, 2003), the Ivorian orchard is threatened by several parasitic attacks. Such infections cause significant damage to plantations and economic losses for the producer. Thus, among these infections are classified cryptogamic diseases which cause damage that can reach 20 to 60% of yield losses (Coulibaly *et al.* 2017; Koua *et al.* 2018), including cocoa black pod disease. This alteration of the cocoa pod (*Theobroma cacao*), is caused by two (2) species of *Phytophthora* in Côte d'Ivoire. The most widespread species is *Phytophthora palmivora*, which has caused 30% of losses (Mpika *et al*. 2009). As for *Phytophthora megakarya*, it was discovered in the eastern part of the country, inducing losses of up to 60%. In order to combat this disease, chemical control was considered following the convincing results of the research. Chemical solutions, in spite of their effectiveness, are relatively expensive, pollute the environment and waterways, and harm human health. The search for biological solutions appears to be essential to curb this plague. The use of fungal antagonists, especially species of the *Trichoderma* genus. This approach requires the isolation and establishment of a collection of *Trichoderma* from the cocoa farm and the assessment of its antagonistic potential (Bowers *et al.* 2001). The use of *Trichoderma* sp. for the control of *Phytophthora* sp. makes it possible to reduce black pod disease incidence in Latin America (Krauss and Soberanis, 2002; Krauss *et al*. 2003). However, the selectivity of the antagonistic power in *Trichoderma*, and the specificity of the target and its environment would require the choice of *Trichoderma* adapted to the agroclimatic conditions of the country. In this study, *Trichoderma* sp. and *Phytophthora* sp. species found in Ivorian orchards were isolated and characterized. The antagonistic power of *Trichoderma* sp. species on *Phytophthora* sp. species was assessed both *in vitro* and *in vivo*, to allow the selection of *Trichoderma* sp. for *P. Palmivora* control trials in plantations.

Materials and Methods

Material

The microorganisms used in the study were derived from the cocoa farming ecosystem, including pods affected by black pod disease, and the cocoa rhizosphere soil. Apparently healthy pods for *in vivo*test and the PDA medium ‘Potato Dextrose Agar’, suitable for a broader spectrum of fungi, were used for *in vitro* antagonist test between such microorganisms.

Methods

Determination of the molecular characteristics of fungal strains

The different fungal strains stemming from the primary cultures were successively subcultured separately on new PDA media. After 3 to 4 successive monospore or monocolony subcultures on the culture media, the DNA of the pure and individualized strains (Davet and Rouxel, 1997) was extracted and amplified by PCR. After amplification, PCR products were migrated by electrophoresis on a 2% agarose gel. For that purpose, 0.6 g of agarose (Bioshop, Canada) was dissolved in 30 mL of 1X TAE (Tris, Acetate EDTA) buffer for 2 minutes in the microwave (iwave). Then, 1.5 µL of ethidium bromide (0.5 µg/mL) was added to the supercooled agarose gel before being poured into the mold. Once solidified, the gel was immersed in 0.5X TAE buffer in a chamber (Wide Mini-Sub Cell GT). Then, 5 µL of each PCR product was dropped into the agarose gel wells. A 2-µL volume of the size marker (BenchTop 100 bp DNA ladder, Promega, USA) was deposited in a well so as to determine the size of the different amplified DNA fragments. The migration was done at 70 V for 60 min. Finally, the gel was visualized under UV

light using a gel reader (E-BOX VX5, France). PCR products were sequenced at Macrogen (Netherlands).

Determination of the *in vitro* antagonistic activity of *Trichoderma* against *Phytophthora palmivora*

The series of direct confrontation experiments, on culture medium, were carried out between *Phytophthora palmivora* and *Trichoderma* species. Three (3) species of *Trichoderma* were used to assess their capacity to inhibit *Phytophthora palmivora's* mycelial growth.

This confrontation by direct contact on culture medium consisted in placing in the same Petri dish containing a PDA medium of mycelial explants, one carrying the *Trichoderma* species and the other, *Phytophthora palmivora*.

The 2 mycelial explants were placed on either side so as to be diametrically opposite at equidistance from the center of the Petri dish (Benhamou and Chet, 1996). Notes on the inhibition of diametrical growth of *Phytophthora* colonies and their invasion by *Trichoderma* mycelium were made daily. In addition, microscopic observations of the direct effect of the antagonist on the condition of the *Phytophthora* mycelium were made.

Measurement of the mycelial growth rate of fungal strains

The data collected included the dates of mycelium appearance from day one today. This was used to determine the average diameter. The growth rate was then calculated. The diameter was measured with a graduated ruler along two perpendicular axes while assuming that the mycelia grow in a circular way according to the following formula (Shuman, 2001) :

$$Dm = \frac{(L+l)}{2}$$

Dm: Average diameter
L: Mycelium size along the x-axis
l: Mycelium size along the y-axis

The growth rate (GR) was assessed every 24 hours while following the same diameter measurement procedure. The growth rate of each mycelium per Petri dish was determined by the formula below (Shuman, 2001):

$$VC = \frac{\sum(DLn - DLn - 1)}{jn}$$

VC: Growth rate of each mycelium (cm/day)
Dn: Average diameter measured on day n
DLn-1: Average diameter measured before day n
Jn: Number of days of measurement of average diameters from day 1 to day n

Assessment of the inhibition rate of mycelial growth of *Phytophthora palmivora* by *Trichoderma*

Scoring of the mean diameter of treated colonies was performed every day for 7 days. The assessment of inhibition was estimated by calculating the percentage of inhibition of mycelial growth according to the formula described by Whipps (1997):

$$I\,(\%) = \left(1 - \frac{Dn}{Do}\right) x100$$

I (%): Average inhibition of mycelial growth
Dn: Mean diameter of *Phytophthora palmivora* in the presence of *Trichoderma* sp.
Do: Average diameter of *Phytophthora palmivora* control

Determination of the *in vivo* antagonistic activity of *Trichoderma* against *Phytophthora palmivora*

The method for assessing the effect of *Trichoderma* sp. on *Phytophthora palmivora* strains was performed on cocoa pods. This test was carried out by spraying 30 ml of spore suspension of the antagonist agent into superficial wounds made on cocoa pods. Two (2) hours after this treatment, a 6-mm diameter mycelial explant of the PDA medium on which *Phytophthora palmivora* mycelium was found was taken and

placed inside each wound. Control pods were inoculated in the same way with a fragment of the pathogen culture in the wounds made on the pods without *Trichoderma* species. The pods thus treated were placed in boxes for five (5) days at a temperature of 25°C and in the dark. After these five (5) days, the diameter of the lesions caused by *Phytophthora palmivora* on cocoa pods was measured (Ben-Daniel *et al.* 2009).

Statistical analysis

The data obtained in this study were analyzed using R studio software version 4.3.0. The one-way ANOVA test was performed to compare the means of inhibition rates of each *Phytophthora palmivora* morphotype in the presence of *Trichoderma* species. In case of significant difference, Fischer's LSD test was used for the distinction of homogeneous groups.

Results and Discussion

Results

Characterized fungal strains

Thirty-eight (38) *Phytophthora* isolates from pods showing black pod disease symptoms and 47 *Trichoderma* isolates from the rhizosphere of cocoa trees were purified. Fungi from which DNA was extracted and amplified by PCR produced amplicons of an estimated size of 500 bp (**Fig.1**). Sequencing results revealed a specific diversity of fungal strains. Sequence similarity searches with the NCBI (National Center for Biotechnology Information) database allowed the identification of its fungal species (**Table 1**). These isolates belonging to the genus *Phytophthora* were identified as *Phytophthora palmivora* and classified into three (3) morphotypes. They included *T.harzianum* (14 isolates), *T. virens* (18 isolates), *T.asperellum* (15 isolates).

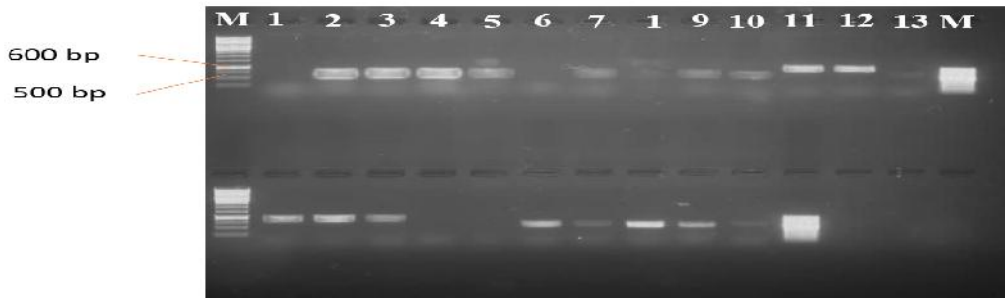

Fig.1 : Electrophoretic profile for DNA PCR products of fungal strains with the primer pair ITS1 and ITS4
M: molecular weight marker
1 to 13: sample number

Table: 1 Sequence characteristics of fungal strains

Code	Name of similar sequences	Homology rate	Accession number
CK14	*Phytophthora palmivora*	100%	JX155790
CK13	*Phytophthora palmivora*	100%	KY447326
CK11	*Phytophthora palmivora*	100%	MH401199
CK7	*Trichoderma virens*	100%	MN102106
CK2	*Trichoderma asperellum*	100%	MT529846
C5	*Trichoderma harzianum*	99,45%	OL604510

In vitro antagonistic activity of *Trichoderma* against *Phytophthora palmivora*

The mean colony diameter of *Phytophthora palmivora* was reduced in the presence of the 3 *Trichoderma* species after 4 days of incubation compared to the control. Indeed, there was a significant difference between the 3 *Trichoderma* species on *Phytophthora palmivora* mycelial growth with ($P = 0.0098 < 0.05$) (**Fig.2**). Each *Phytophthora palmivora* strain mycelial growth was inhibited by *Trichoderma harzianum*, T. *asperellum* and T.*virens* strains. The inhibition rates of *Trichoderma* species ranged from 75 to 88.7% with a high antifungal activity for *Trichoderma virens* strain (88.7%). However, among the 3 *Phytophthora palmivora* morphotypes, strain *Phytophthora palmivora* 1 was more inhibited in the presence of *Trichoderma* species (**Fig.3 and 4**).

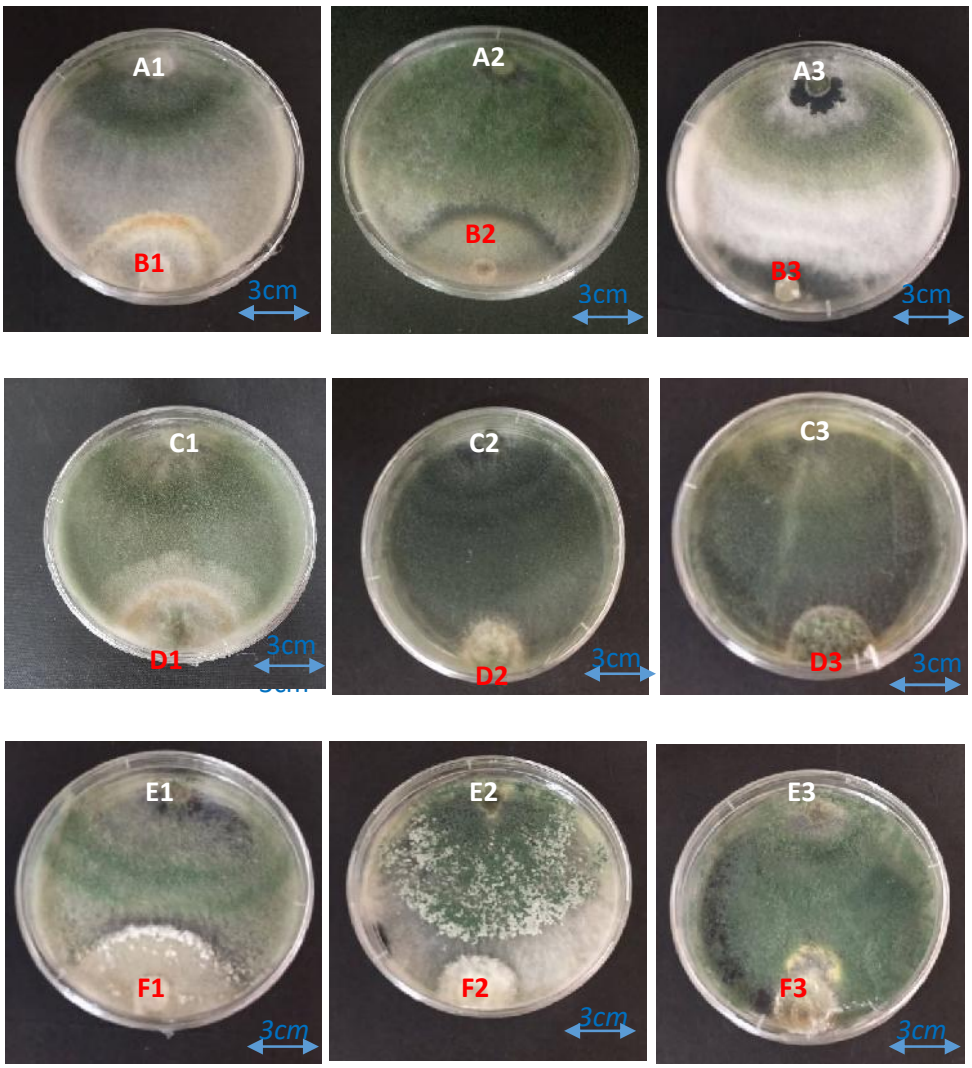

Fig. 2 Confrontations between *Trichoderma* and *Phytophthora* strains in *in vitro* culture

A1: Explant of*Trichoderma asperellum* ;
A2: Explant of*Trichodermaasperellum* ;
A3: Explant of*Trichodermaasperellum* ;
C1: Explant of *Trichoderma virens* ;
C2: Explant of *Trichoderma virens* ;
C3: Explant of *Trichoderma virens* ;
E1: Explant of *Trichoderma harzianum* ;
E2: Explant of *Trichoderma harzianum* ;
E3: Explant of*Trichoderma harzianum* ;

B1: Explant of *Phytophthora Palmivora* 1 ;
B2: Explant of *Phytophthora Palmivora* 2 ;
B3: Explant of *Phytophthora Palmivora* 3 ;
D1: Explant of *Phytophthora Palmivora* 1 ;
D2: Explant of *Phytophthora Palmivora* 2 ;
D3: Explant of *Phytophthora Palmivora* 3 ;
F1: Explant of *Phytophthora Palmivora* 1 ;
F2: Explant of *Phytophthora Palmivora* 2 ;
F3: Explant of *Phytophthora Palmivora* 3 ;

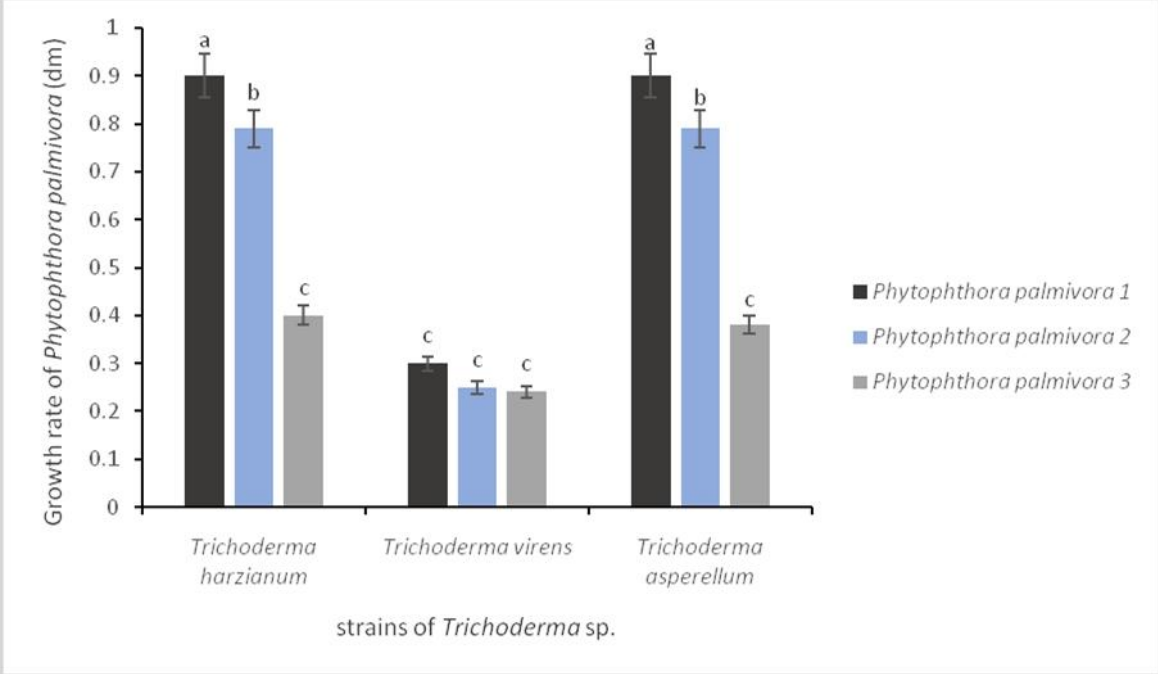

Fig. 3: *Phytophthora palmivora* mycelial growth rate in relation to *Trichoderma* strains

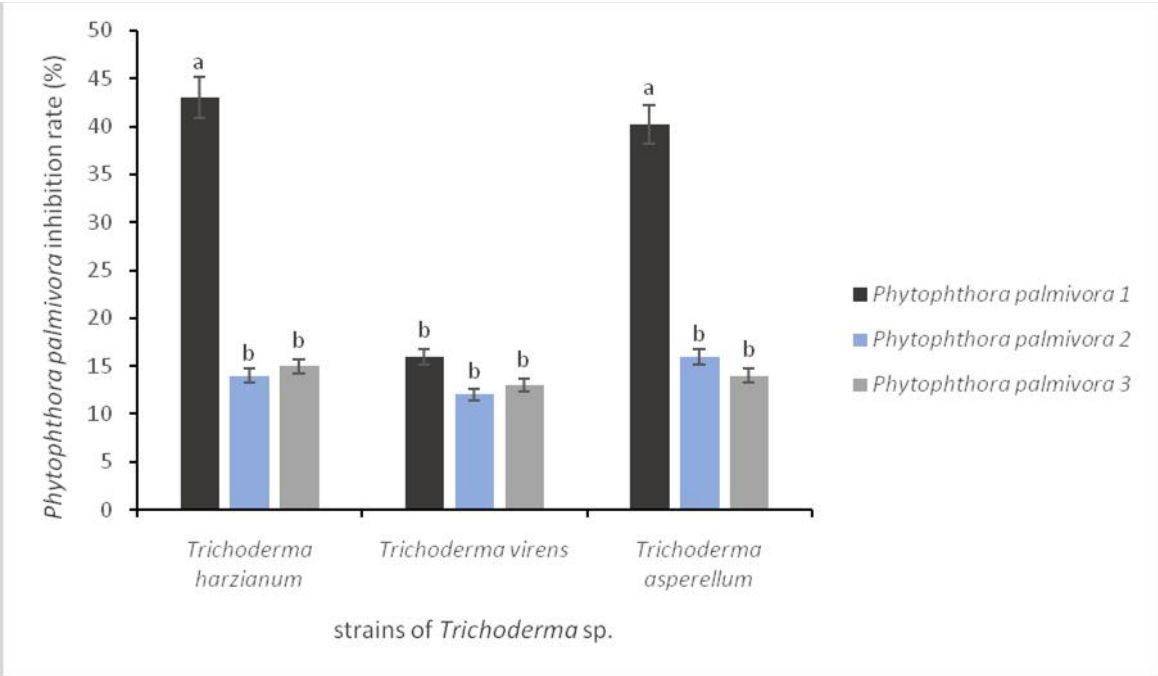

Fig. 4: *In vitro* inhibition rate of *Phytophthora Palmivora* mycelial growth by *Trichoderma*

In vivo antagonistic activity of *Trichoderma* species on *Phytophthora palmivora* strains

The pods inoculated with *Phytophthora Palmivora* mycelial fragments developed symptoms after five (5) days of inoculation. Those inoculated with *Trichoderma* species showed no symptoms after five (5) days of inoculation. *Trichoderma* species showed an average inhibition rate of 65 to 87.5% on *Phytophthora palmivora* strains. *Trichoderma harzianum* and *Trichoderma asperellum* species showed inhibition rates in the range of 69.7% and 65% respectively while *Trichoderma virens* showed an inhibition rate in the range of 87.5%.

In the presence of *Phytophthora palmivora* strain, statistical analysis showed that there was a significant difference between the 3 *Trichoderma* species (P = 0.0395099 < 0.05) (**Fig. 5 and 6**).

Fig. 5: Cocoa pods, five (5) days after inoculation with fungal strains
A: with *Phytophthora Palmivora* strains only
B: with *Phytophthora Palmivora* strains + *Trichoderma*

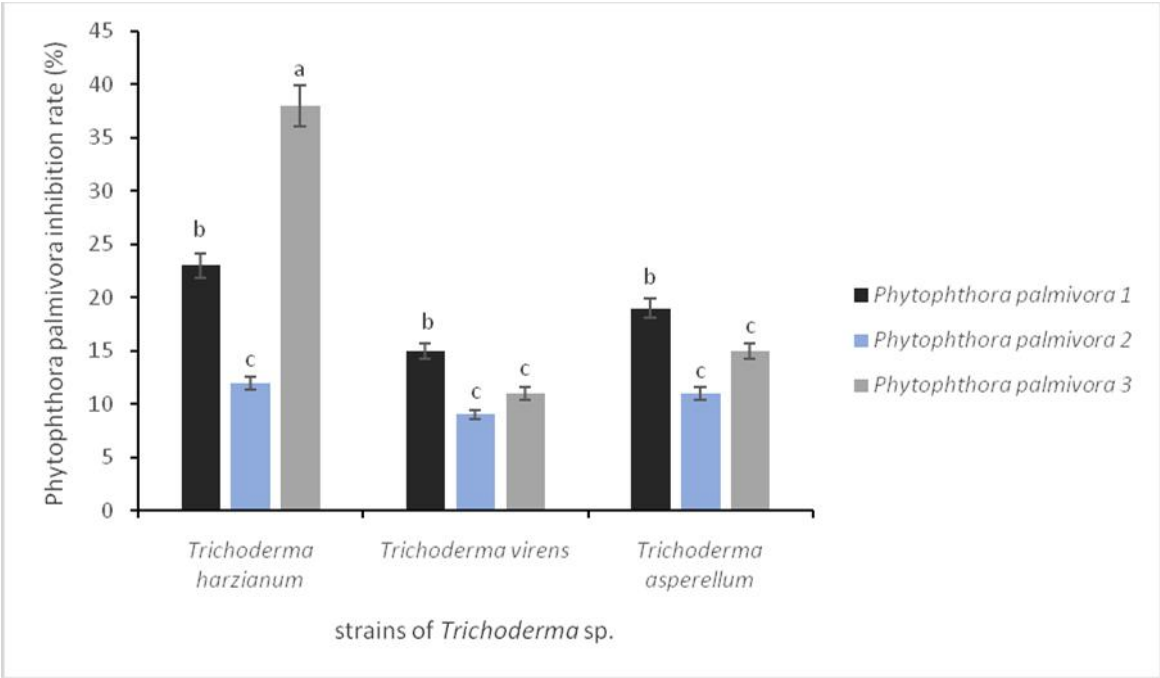

Fig. 6: Inhibition rate of *Trichoderma* species on *Phytophthora palmivora* isolates

Discussion

A diversity of *Phytophthora* sp. and *Trichoderma* sp. strains was isolated from cocoa pods affected by cocoa black pod disease and from the rhizosphere soils of cocoa trees in the two cocoa production zones of Côte d'Ivoire. This diversity of *Phytophthora* sp. strains could be explained by the climatic and agronomic conditions favorable to the development of the fungus which varies from one locality to another. In fact, the development of the fungi is favored by the shading, heat and high inhibition rate prevailing in cocoa farms (Pohé *et al.* 2013). These

conditions may favor the presence and/or absence of certain *Phytophthora* sp. strains. These results are similar to those of Mfegue in 2012. Indeed, these two authors showed the existence of two distinct morphological types of *Phytophthora palmivora* on cocoa. The presence of *Trichoderma* sp. strains isolated from the soil from the cocoa rhizosphere could be explained by the abundance of this fungus in the soil and on decaying plants. These results are similar to those of (Singh *et al.* 2007) who showed that fungi of the genus *Trichoderma* sp. are present in all agricultural soils and on other environments.

The antifungal activity of *Trichoderma* sp. against *Phytophthora palmivora* showed a reduction in the diameter of *Phytophthora* sp. colonies in the presence of *Trichoderma* sp. strains and an inhibition rate from 26.29% to 28.02%.

This result could be explained by the fact that fungi of the genus *Trichoderma* are very competitive for food and space, as these fungi can grow rapidly and utilize food sources efficiently. The pathogenic fungi are thus deprived of the space and growth factors they need for their development (Daami-Remadi *et al.* 2001). This antagonistic power was also observed by Benhamou and Chet (1997) by performing a direct confrontation on culture medium between *Trichoderma harzianum* and *Pythium ultimum* after 4 to 5 days of inoculation. These results revealed that when testing the antagonistic activity of *Trichoderma harzianum* towards two Pythium species, the Petri dish is totally invaded by Pythium sp. after the first three days. *Trichoderma harzianum* does not begin to exert its antagonistic activity until the fourth day of incubation.

The inhibitory activity of *Trichoderma* sp. species observed on pods after inoculation attested that these fungal species showed a high inhibition rate of 55% of *Phytophthora palmivora* strains. This action of *Trichoderma* sp. would be due to the penetration in the sporocysts of *Phytophthora*, to the rolling on the hypha of this fungus leading to its destruction.

This antagonistic activity of *Trichoderma* strains is in line with those obtained by (Tondje *et al.* 2007) who reported a strong inhibitory action on *Phytophthora megakarya*, the more aggressive agent of black pod disease. Daami-Remadi *et al*. (2001) also showed the antagonistic effect of *Trichoderma harzianum* isolate towards *Fusarium* sp. causal agent of dry rot of potato tubers. This inhibition increased to 93% when the antagonist was added as a spore suspension to the culture medium.

Pods pre-inoculated by spraying with a suspension of *Trichoderma* sp. were inoculated with a suspension of *Phytophthora* sp. zoospores. Results revealed heterogeneity in the inhibitory action of *Trichoderma* sp. population. This heterogeneity reported by Howell *et al*. (2000) is believed to be due to the different ability of *Trichoderma* sp. strains to secrete antifungal substances and to be mycoparasitic. Strain *Trichoderma* sp. 1 showed a strong ability on all *Phytophthora* sp. strains. Similar results were obtained by Tondje *et al*. (2005) who revealed that after pre-inoculation of *Trichoderma asperellum* on pod fragments in Petri dishes, necrotic lesions caused by *Phytophthora megakarya* were reduced.

Conclusion

This study aimed at controlling the causal agent of cocoa black pod disease. To this end, an inventory by molecular characterization of *Trichoderma* and *Phytophthora* species was carried out. The inhibitory power of *Trichoderma* species was tested. At the end of our study, three (3) strains of *Phytophthora palmivora* and three (3) species of *Trichoderma* (*T. harzianum*, *T. asperellum* and *T. virens*) were isolated respectively from cocoa pods with black pod disease and from the rhizosphere of cocoa orchards and characterized. The *in vitro* and *in vivo* confrontation tests carried out between *Trichoderma* species and *Phytophthora palmivora* strains revealed a strong antifungal activity on *Phytophthora* sp. strains. The application of *Trichoderma* sp. on cocoa pods allowed the reduction or even the absence of black pod

disease on cocoa pods. *T. virens* isolates with strong antagonistic power towards *Phytophthora palmivora* were selected from the collection for field trials. The use of *Trichoderma* sp. opens a promising door to biological control of *Phytophthora* sp., the causal agent of cocoa black pod disease in Côte d'Ivoire.

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