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Impact of environmental factors, chemical fungicide and biological control on cacao pod production dynamics and black pod disease (*Phytophthora megakarya*) in Cameroon

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Abstract

The impact of environmental factors and microbial and chemical control methods on cacao pod production dynamics and spread of black pod disease caused by *Phytophthora megakarya* was assessed over three consecutive years in a smallholder's plantation. Significant positive correlations were found between rainfall records and pod rot incidence when assessed after a 1-week lag. Disease distribution across various pod developmental stages showed that immature pods were the most susceptible to *P. megakarya* attack. Weekly observations of the pod distribution and disease progression at various developmental stages on cacao trees sprayed with fungicide Ridomil, *Trichoderma asperellum* biocontrol agent (strain PR11), or untreated control trees indicated that the total pod production and the incidence of black pod rot was significantly different between the treatments. The disease rates were 1.73%, 47.1% and 71.23% in the plots treated with fungicide, PR11 and untreated, respectively, in 2004, and 0.67%, 11.35% and 34.04% in the same plots in 2005. A significant carry over effect of the biological agent was noticed up to 1 year after the sprays with strain PR11 were halted. The disease incidence averaged 16.67% in the plots previously sprayed with PR11 and 35.88% in the control plot, although both the plots were sprayed with the same contact fungicide, Kocide. Monitoring through enumeration and molecular typing revealed that *Trichoderma* propagules remained consistently more abundant in flower cushions of cacao trees than in soil or bark. This study showed that microbial control of black pod disease with PR11 was promising but not as effective as the chemical methods under the current high disease pressure, and therefore needs to be integrated with other control methods to establish a sustainable management system for black pod disease in Cameroon.

Keywords: Black pod disease; Cocoa; *Theobroma cacao*; Epidemiology; Fungicide; *Phytophthora megakarya*; Microbial control; *Trichoderma asperellum*; Monitoring

1. Introduction

Black pod disease is a major constraint to the cocoa production in West Africa (Flood et al., 2004). In Cameroon, various species of *Phytophthora* exist but the most prevalent and aggressive one is *Phytophthora megakarya*

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(Nyassé, 1997). Losses can reach up to 100% of the cocoa production in smallholders' plantations when no control measures are taken (Despréaux et al., 1988; Berry and Cilas, 1994). In Cameroon, management of black pod disease using currently-available genetic resistance, chemical control, and cultural practices has been unsatisfactory. Despite much effort on breeding for resistance to *Phytophthora* in Cameroon, cacao clones completely resistant have not been found to date. Although chemical control using systemic and copper-based contact fungicides is the most widely-used control method, it remains too expensive for the majority of smallholders. In the long run, chemical spraying also will have adverse environmental impact (Ndoumbè-Nkeng and Sache, 2003). Cultural methods such as the phytosanitary pod removal, which consists of eliminating the diseased pods, have proven relatively efficient in reducing the secondary inoculum (Ndoumbè-Nkeng et al., 2004). Biological control using the biocontrol fungus *Trichoderma* naturally antagonistic to *Phytophthora* spp. pathogens represents a new alternative for the control of the black pod disease. Microorganisms belonging to the genus *Trichoderma* have proven to be effective in the control of some fungal plant pathogens (Papavizas, 1985; Samuels, 1996; Elad and Freeman, 2004; Holmes et al., 2004; De Souza et al., 2006). Previous studies undertaken in Cameroon have led to the isolation of a few fungal strains of *Trichoderma asperellum* from the agricultural soils under cacao trees or organs of other crops with potential for controlling *P. megakarya* (Tondje et al., 2005).

However, the above methods, when used alone, do not provide a satisfactory level of disease control on smallholder farms. Evidence is now accumulating that microbial control of cacao pathogens could be more effective if they are combined with others methods, such as phytosanitation, chemical sprays, and in combination with resistant or tolerant germplasm (Krauss and Soberanis, 2001). Developing an integrated approach also means addressing issues such as understanding the environmental parameters, epidemiology of the disease and the fate of the biological control agent, as well as the targeted pathogen. In particular, factors such as genetic background of the pathogen and ability of the biocontrol agent to be established and to spread could have an impact on the efficiency of this integrated approach.

The aim of the current study was to assess the impact of environmental factors, and treatments including a potential biocontrol fungus, *T. asperellum*, strain PR11 and Ridomil fungicide on cacao pod production dynamics and progression of black pod incidence (*P. megakarya*). This study was conducted in Cameroon over three consecutive years in one smallholder's plantation. In the third year, no biocontrol agent was applied to the plots previously sprayed with the biocontrol agent in order to assess potential post-treatment effect. In addition, the main climatologic parameters, which are known to contribute to disease development, were determined, and the existence of a susceptible pod developmental stage was also studied. Moreover, fate of the released microbial *Trichoderma* agent was also monitored using enumeration and molecular typing methods.

2. Materials and methods

2.1. Meteorological measurements

Total rainfalls (mm), as well as minimum and maximum temperatures (°C) were recorded daily at 6.00 am from April 2004 until November 2006. Both rain gauge and thermometer were installed about 200 m away from the cacao plantation. The rain gauge and the thermometer were placed at 1.5 m above ground in open area and under shade on a trunk, respectively.

Three production cycles were represented by three seasons in our study. Except for the third season, each season began as soon as the first pods appeared (April) until to the end of production cycle including the intercropping season (December–March). The third season started in April (2006) until to the end of the production cycle (November 2006).

For both seasons 2004 (April 2004–March 2005) and 2005 (April 2005–March 2006), the mean minimum and maximum temperatures were estimated as the means of the daily minimum and maximum temperatures, respectively, recorded during a week. Total rainfall of both first and second seasons was expressed as the weekly sum of daily precipitations (Fig. 2).

2.2. Experimental design

The study was conducted in a smallholder's plantation located near Bokito in the Northwest of the Central Province in Cameroon over three consecutive years. This area is located in a forest-savanna transitional zone receiving an annual rainfall of 1300–1500 mm with a mean annual temperature of 24 °C (maximum 28.7 °C, minimum 20.5 °C). The experimental trial was at least 2 ha in size with cacao germplasm consisting of a mixture of hybrids and Amelonados (''German Cocoa'') (Sounigo, personal communication). The spacing between cacao trees within a plot varied from 2.5×2.5 m to 3×3 m, giving densities of 1111–1600 trees/ha. As dead trees were not systematically replaced, there were numerous gaps in the different plots, thereby reducing the density. Trees were on an average 50 years old and intercropped with other wild and domesticated fruit trees. The tree height is about 4–6 m and shading was generally light.

Prior to the setting up of the trial, weeds were cleared manually, trees were lightly pruned and diseased pods were removed. The phytosanitary pod removal was done 4–6 m above the ground in the canopy that is generally dense if unmanaged. Rotten pods were harvested using special pruning shears. No fertilizer was applied. The experimental trial was divided in three experimental plots of 30 useful cacao trees. In each experimental plot, 30 replicate trees were randomly chosen. Experimental plots are separated

from each other by a "sanitary cordon" of 10 m width, sprayed with fungicide Ridomil Plus Gold 66 WP (metalaxyl + copper oxide, 3.33 g l^{-1}) every 4 weeks. In the chemical control plot (MET), pods on trees were treated with Ridomil Plus Gold 66 WP (6% metalaxyl + 60% copper oxide) using a knap-sack sprayer at the rate of 200 ml per tree every 2 weeks from April 2004 to March 2005 (season 2004) and then every 4 weeks from April 2005 to November 2006 (seasons 2005 and 2006). In the second plot (PR11), trees were sprayed using a knap-sack sprayer at the rate of 250 ml per tree of a *Trichoderma* spore suspension every 2 weeks starting from April 2004 when the first cherelles appeared till March 2006 and then the spraying stopped during the third season from April to October 2006. The third plot (CONT) remained untreated during both 2004 and 2005 seasons. During the 2006 season, except the chemical control plot (MET), the others plots (PR11 and CONT) were treated every 2 weeks with Kocide 2000, a contact and polyvalent fungicide formulated in wettable granules (53.8% copper hydroxide). This fungicide, previously tested *in vitro* is compatible with the bioagent *T. asperellum* PR11 (Tondje, personal communication). Cultural control methods, which consisted of weekly removal of diseased pods and regular harvesting and pruning, were practiced in all experimental plots, as recommended by Ndoumbè-Nkeng et al. (2004).

Previous results from assays of the genotypic and phenotypic variability of the population of the pathogen *P. megakarya* from these three plots revealed the presence of two distinct genotypes that were characterized by a moderate aggressiveness level (Ducamp, personal communication).

2.3. Microbial treatment

The mycoparasite of *P. megakarya*, *T. asperellum* strain PR11, was used in this study. It was isolated from *Xanthosoma sagittifolium* at Etoug Ebe, Cameroon, described by Tondje et al. (2005) and was identified microscopically as *T. asperellum* by the USDA-ARS Systematic Botany and Mycology Laboratory (SBML) in Beltsville, Maryland, USA. Microscopic identification was confirmed through sequences of translation-elongation factor 1-alpha (*tef1*) and sequences were deposited in GenBank under the No. EF186002 (Begoude et al., 2007), and cultures have been deposited in conserved core collections (IRAD, Yaoundé, Cameroun, USDA-SBML, Beltsville, USA and USDA-EBCL, Monferrier-le-Lez, France). Working cultures were routinely cultured on half strength potato dextrose agar (PDA) (Difco, MD). *T. asperellum* strain PR11 was mass-produced by solid-state fermentation according to Hebbar and Lumsden (1999) with rice as a solid matrix. Incubation was at 26 °C in constant light (ambient light during the day, fluorescent light at night). Fungal spores were extracted from the solid matrix using a 100 μm of diameter sieve and mixed with sterile cassava flour to obtain 2×10^9 Colony Forming Unit (CFU) per gram before being sprayed on to the trees. Each tree was sprayed with 250 ml of a conidial suspension (2×10^{11} CFU l^{-1}).

2.4. Monitoring of PR11 through enumeration and molecular typing

At the start of the trial and at its completion, the population of the PR11 propagules and confirmation of their identity in soil, bark and flower cushions of cacao trees in the field was verified through enumeration using serial dilution method and molecular typing, respectively. Soil samples were collected from the upper 8 cm of organic layer around five trees selected at random. Bark and flower cushion samples were collected on each tree within the plot. One to five-gram samples from a blended mix for flower, bark and soil, respectively, were air-dried at room temperature and blended in 20–100 ml of water. A 10-fold dilution series in sterile water was then plated on a semi-selective medium for *Trichoderma* following Hebbar and Lumsden (1999) for CFU-counting. Individual colonies were purified on PDA plates and 25 mg of mycelium were scraped from these plates and ground with 25 mg of glass sand, 500 μl of preheated cetyltrimethyl ammonium bromide (CTAB) 2X lysis buffer, 57 μl of CTAB 10% in 0.7 M NaCl, and 70 μl of NaCl 5 M. Tubes were incubated for 1 h at 65 °C with occasional vortexing. Ten microliter of Proteinase K (20 mg/ml) and 10 μl of RNase A (10 mg/ml, Macherey Nagel) were then added. Tubes were incubated for 1 h at 60 °C while being mixed occasionally by inverting the tube and let to be decanted. DNA in the supernatant was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), followed by an equal volume of chloroform-isoamyl alcohol (24:1).DNA was precipitated with 1/10 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of ice absolute ethanol and washed with 70% ethanol. The pellet was dried using Speed Vac and suspended in 50–100 μl of 5 mM Tris, pH 8.

SCAR markers used in the present study were primarily developed for assignment of strain identity. When applied to a large-scale sampling, they had only value in partitioning the intra-species diversity of *T. asperellum* in different genetic groups, including the PR11 SCAR group. They did not enable us to discriminate PR11 strain from the other isolates belonging to the same genetic SCAR group. For that reason, we referred only to the PR11 SCAR group as PR11 throughout the paper. A multiplex PCR approach was developed for typing PR11 using 96-well PCR microplate and Perkin-Elmer 9700 thermocycler. The sequences of all primers of SCAR markers used in this study can be obtained upon request to the USDA-ARS-EBCL laboratory in Montpellier, France. The PCR reaction of a 25 μl final volume consisted of 5–200 ng genomic DNA, 0.15 μM of each of the primer set (4-3-111F2 and 4-3-111 R) and 0.2 μM of the primer set (GACA101R and GACAcomF), 1X Qiagen Taq Buffer, 0.2 mM each dNTP and 1 U of Taq polymerase. The amplification con-ditions consisted of an initial denaturation step of 3 min at 94 °C, followed by 5 cycles of 30 s at

94 °C, 30 s at 65 °C, 50 s at 72 °C, 35 cycles of 30 s at 94 °C, 30 s at 63 °C, 50 s at 72 °C and a final extension step of 7 min at 72 °C. 6 μl of the PCR products generated were analyzed on 2% agarose gel.

2.5. Epidemiological survey and observations

The observations were recorded from the first week of treatments (April 2004). Healthy fruits at various developmental stages including cherelles (i.e. less than 10 weeks old), immature pods (IP) (10–20 weeks old), and ripe pods (RP) were counted on all 30 replicate trees each week. The ripe (RP) pods were counted and harvested each week, and the pods for which the fruit developmental stage that could not be categorized was labeled as indeterminate stage (IS). The number of cherelles or wilted pods (W), rotten pods (R), pods damaged by rodents or other cacao disease pathogens (OD) also were recorded during each week before being discarded.

2.6. Data analysis

Disease rate for black pod rot (RR) was calculated for the three seasons (2004, 2005 and 2006) according to:

$$RR = (R/N1) * 100 \quad (1)$$

where R is the number of rotten pods and $N1$ is the total number of pods recorded at the completion of each season excluding wilted cherelles. $N1$ was calculated according to:

$$N1 = R + OD + \text{last week IP} + RP$$

For 2004 and 2005 seasons, the potential pod production (N) was calculated each week (i), according to:

$$Ni = Ri + ODi + IPi + RPi \quad (2)$$

The black pod disease dynamic was also studied by calculating the cumulative weekly pod rot rates and the disease temporal progression.

The pod rot incidence at different developmental stages was determined in order to analyze the effect of the pod age on disease incidence and to identify a particularly susceptible stage. The number of IS pods was included in the total number of rotten pods recorded.

Analyses were carried out using the generalized linear model, applying the binomial distribution. The comparison of adjusted means was completed according to the Wald Chi-Square test (McCullagh and Nelder, 1989; Agresti, 1990) in order to assess the effect of treatment on the incidence of the rotten pods rate. At the same time, the interactions between pod age × treatment were also equally studied. A cross correlation analysis between the various meteorological factors and the pod rot rate was done in order to highlight the factors involved in the onset of the black pod disease epidemics. The factors tested were rainfall, rain frequency and temperature. Data were analyzed using SAS, version 8.2 (SAS Institute Inc, 2001) software package.

3. Results

3.1. Dynamics of the pod production

A kinetic scheme of the weekly potential production per tree was estimated over the two consecutive years corresponding to 2004 and 2005 seasons (Fig. 1). In 2004 (Fig. 1A), the duration of the pod production period was from week 16 (July 30) to week 36 (December 17) for all treatments. During the 2004 season, the production was higher in the fungicide treated (MET) plot, with a production peak reaching 28 pods per tree on week 26 (October 08), compared to those obtained in CONT and PR11 plots which were 13 pods per tree on week 24 and 17 pods per tree on week 26, respectively. It was observed that the production peak was 2 weeks later in the MET and PR11 plots (the most productive plots) compared with the CONT plot. In 2005 (Fig. 1B), the production period started earlier on week 9 (June 6) and extended until week 37 (December 19) for all treatments and with a production peak on week 19 (August 15). However, the potential production in this second season was lower compared to the first season. Once again, the production was higher in the fungicide treated (MET) plot, with a production peak reaching 14 pods per tree on week 19, compared with the production peaks obtained in CONT and PR11 plots which were 9 and 8 pods per tree, respectively.

3.2. Dynamics of the pod rot progression

The kinetics of weekly rainfall and pod rot disease progression was estimated over the two seasons in 2004 and 2005 (Fig. 2). The disease progression curves were highly variable with respect to treatment and the cropping season. The incidence of black pod was higher in the control plots during both years with disease incidence being higher in 2004 (Fig. 2A) season than in 2005 (Fig. 2B) season. Although, the disease onset was earlier in 2005, the first symptoms appeared in the CONT plots during both years. In the MET plot, we observed a very low disease incidence during the two seasons.

During the first (2004) season, the PR11 plot disease progression followed a double-S curve (Fig. 2A). In this experimental plot, after a relative latent phase, the epidemic outbreak started on week 16 before reaching a first plateau at week 23–27, during a low rainfall period. Then, this was followed by a second exponential phase that reached a second plateau on week 35. On the contrary in the CONT plot, the disease progression was linear during this low rainfall period and reached a plateau on week 35. On week 35 the cumulative rot rates reached 71.23%, 47.10% and 1.73% in the CONT, PR11 and MET plots, respectively, and remained unchanged until the completion of the observations (week 52; Fig. 2A). During the second season, the PR11 plot disease progression followed an almost flat curve (Fig. 2B). In this experimental plot, the epidemic outbreak started on week 11 followed by a disease

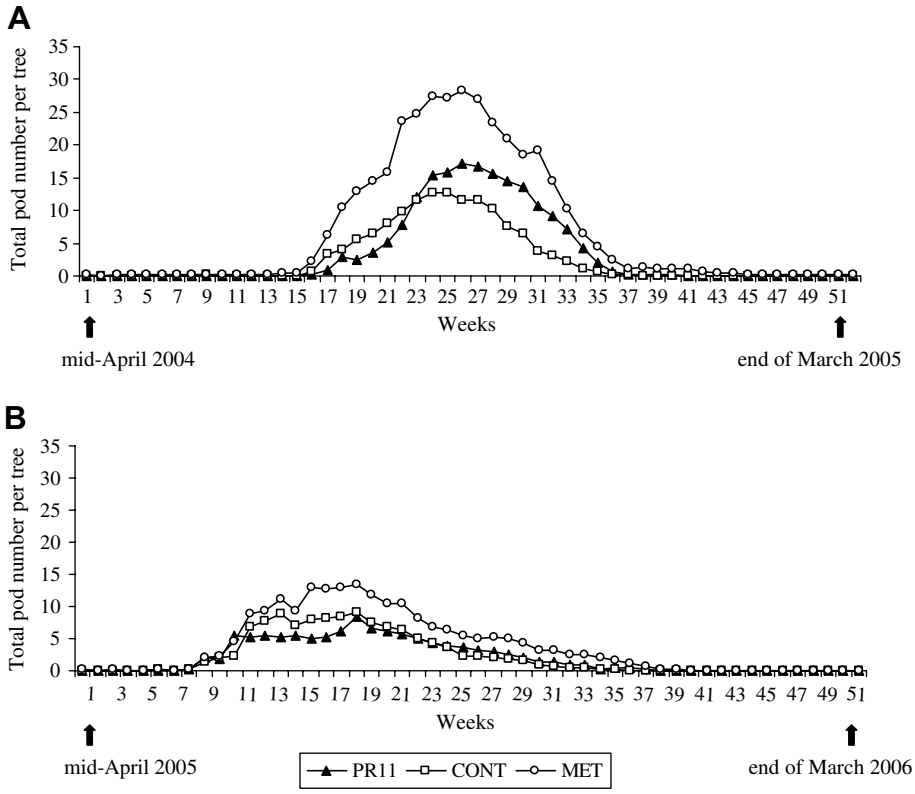

Fig. 1. Average pod production per tree in plots treated with *Trichoderma asperellum* strain PR11 (PR11), the fungicide metalaxyl (MET), and untreated control plots (CONT), in years (A) 2004 and (B) 2005.

incidence which remained low even during the high rainfall season before reaching a plateau on week 32. On the contrary in the CONT plot, the disease progression was constant during the high rainfall season and reached a plateau on week 34. On week 34 the cumulative rot rates reached 34.04%, 11.35% and 0.67% in the CONT, PR11 and MET plots, respectively, and remained unchanged until the completion of the observations (week 52; Fig. 2B).

3.3. Study of the putative factors triggering the development of the black pod disease

The cross correlation analysis between the black pod disease rates (%) and meteorological parameters recorded weekly in CONT and PR11 plots is presented in Fig. 3. The results of the MET plot were not taken into account in this study because of the extremely low rot rate recorded in this experimental plot in both seasons.

In 2004 (Fig. 3A), the highest significant correlation between pod rot rate and rainfall was obtained after 1-week lag, ($r = 0.53$; $P = 0.0002$ and $r = 0.56$; $P = 0.0001$ for CONT and PR11, respectively. In 2005 (Fig. 3B), the highest significant correlation between pod rot rate and rainfall was obtained at 1-week lag within the PR11 treatment ($r = 0.42$; $P = 0.0029$) and no significant correlation was obtained within the CONT treatment ($r = 0.26$; $P = 0.0697$). In 2004, the coefficient of correlation obtained between the number of recorded rains per week and the incidence of pod rot rate, at 1-week lag were $r = 0.30$ ($P = 0.0511$) for CONT plot and $r = 0.38$ ($P = 0.0108$) for PR11 plot. Also, a significant correlation was obtained for these parameters at 2-weeks lag ($r = 0.33$; $P = 0.0295$ and $r = 0.33$; $P = 0.0283$ for CONT and PR11, respectively). No correlation between the number of recorded rains per week and the incidence of pod rot rate was observed in 2005 season. Moreover, for both seasons, no significant correlation was observed between the temperature (T° minimal, T° maximal or T° average) and pod rot rate.

3.4. Effect of the developmental stage on the black pod disease incidence

The interaction of treatment × developmental stage was highly significant ($P < 0.0001$ and $P = 0.0276$ for 2004 and 2005 season, respectively), suggesting that the disease incidence for each developmental stage is likely to vary from one treatment to another. For each season 2004 and 2005, the statistical analysis was thus performed treatment by treatment.

The results revealed a significant effect of development stage of pods on the disease incidence (Table 1). In the CONT experimental plot, the disease incidence is significantly higher in the ''immature pod'' stage compared to the ''cherelle'' and to the ''Ripe pod'' stages ($P < 0.0001$)

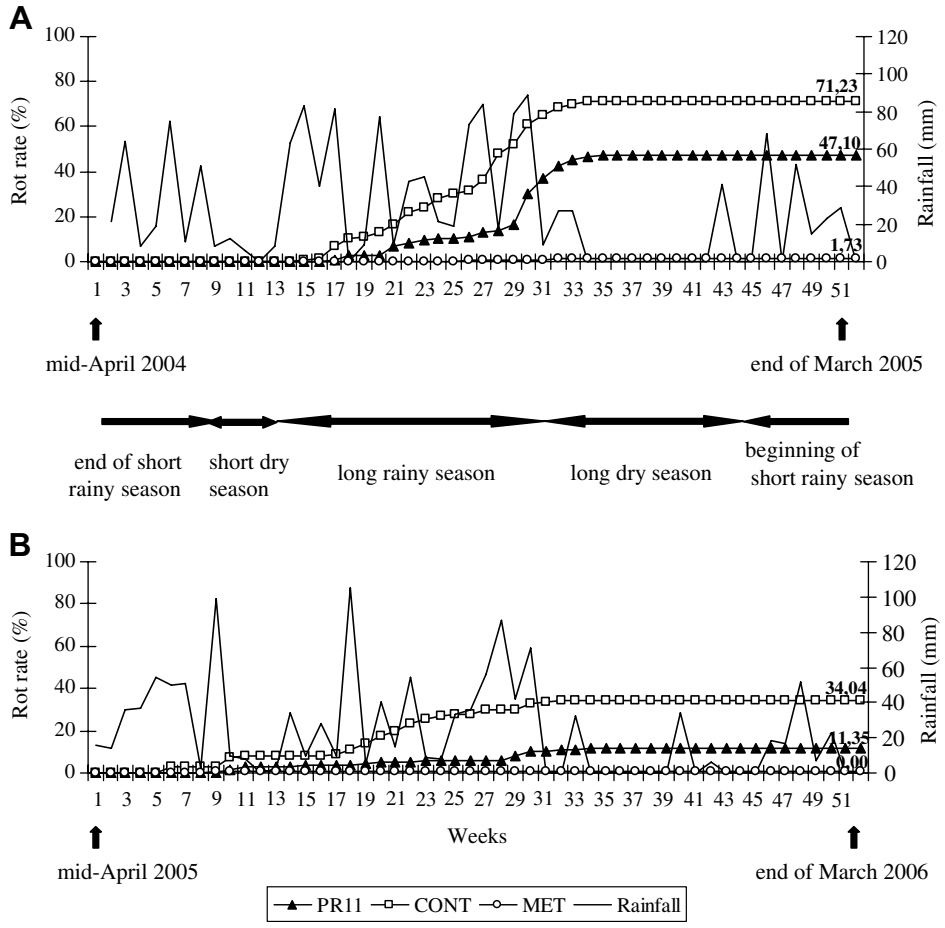

Fig. 2. Weekly rainfall (mm) and pod rot values (%) for trees treated with *Trichoderma asperellum* strain PR11 (PR11), the fungicide metalaxyl (MET), and untreated control plots (CONT), in years (A) 2004 and (B) 2005.

for both 2004 and 2005 seasons. In the PR11 experimental plots too, the disease incidence was significantly higher in the "immature pod" stage compared to the "cherelle" stage ($P < 0.0001$) and to the "Ripe pod" stage in both 2004 ($P = 0.0415$) and 2005 season ($P < 0.0001$). However, no clear differences were found in the disease incidence among the developmental stages of pods within the MET experimental plot because of the extremely low rot rate recorded in this experimental plot in both seasons. The black pod disease incidence on the various developmental stages indicated that losses due to the disease were higher at the "immature pod" stage in CONT and PR11 plots.

3.5. Effect of the three treatments on the disease incidence

The three treatments CONT, PR11 and MET were ranked according to decreasing disease incidence observed during the three seasons 2004, 2005 and 2006 (Table 2). For both seasons 2004 and 2005, the results showed a highly significant effect of the treatments on the disease incidence ($P < 0.0001$). The results highlighted the potential of the PR11 treatment to reduce the disease incidence in the field in comparison to the CONT treatment. In the PR11 plot, the disease rate was reduced by 24% and 23% for the 2004 and 2005 season, respectively. MET treatment was the best treatment among the three applied as regards disease incidence with pod rot rate of 1.73% and 0.67% in 2004 and 2005, respectively.

Analysis of the data obtained in the third (2006) season, where the microbial applications were stopped and replaced by application of a less expensive and *T. asperellum*-compatible contact fungicide Kocide in PR11 and CONT plots, to evaluate the post-treatment effects of biological agent, also revealed a significant carry-over effect of the biological treatment. The effect of Kocide treatment associated with the post-treatment effects of PR11 on the disease incidence was significant ($P < 0.0001$) with pod rot rates averaging 35.88% and 16.67% in the CONT and PR11 plots, respectively (Table 2), whereas the pod rot rate in the MET plot (spraying with fungicide Ridomil Plus Gold 66 WP only) was 1.45%. During this season, the CONT and the PR11 plots, which were treated only with the contact fungicide Kocide, could be compared; the disease rate in the PR11 plot was reduced by 19% compared to the CONT plot, which remained untreated in 2004 and 2005 seasons.

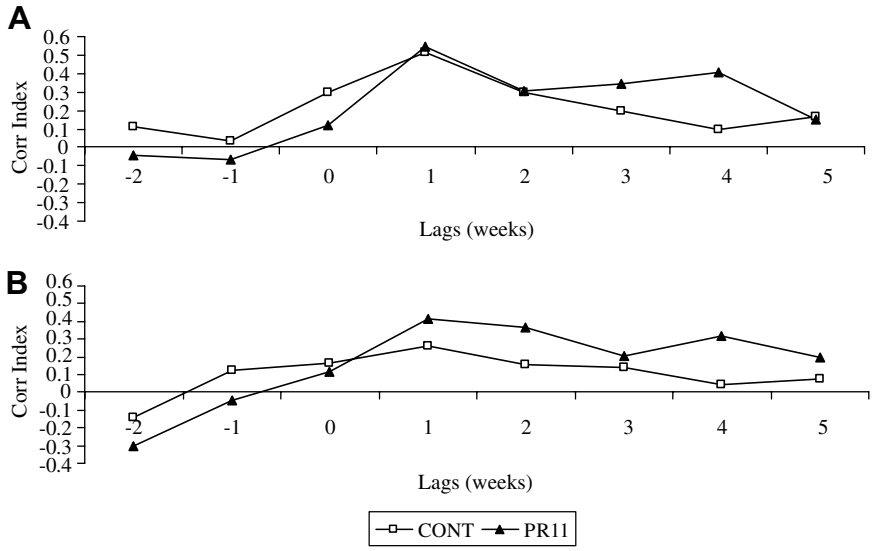

Fig. 3. Correlation index values between pod rot rate and rainfall in plots treated with *Trichoderma asperellum* strain PR11 (PR11) and untreated control plots (CONT), in years (A) 2004 and (B) 2005.

Table 1
Incidence of the developmental stage on the black pod disease in plots treated with *Trichoderma asperellum* strain PR11, the fungicide metalaxyl MET and untreated control plots CONT, in years 2004 and 2005

Developmental stages	Rate of R pods (%)*					
	CONT[a]	PR11[b] 2004	MET[c]	CONT[a]	PR11[b] 2005	MET[c]
Cherelle	11.37b	4.15c	0.09b	6.67b	1.42b	0.44a
Immature pod	43.52a	21.23a	0.55ab	24.91a	7.09a	0.00a
Ripe pod	8.53b	16.08b	0.82a	2.11c	2.48b	0.22a

R pods, rotten pods.
* Numbers followed by the same letter are not significantly different at $P < 0.05$. The data were analyzed according to the general linear model using binomial law. Mean separation involved adjusted means according to the Wald Chi-Square test.
[a] Untreated control plots.
[b] Plots treated with *Trichoderma asperellum.*
[c] Plots treated with the fungicide metalaxyl.

Table 2
Total yield and incidence of black pod disease in plots treated with *Trichoderma asperellum* strain PR11, the fungicide metalaxyl MET and untreated control plots CONT, in years 2004, 2005 and 2006

Treatment	Number of recorded pods			Rate of R pods (RR %)*		
	2004	2005	2006	2004	2005	2006
CONT[a]	563	285	800	71.23a	34.04a	35.88a
PR11[b]	603	282	366	47.1b	11.35b	16.67b
MET[c]	1101	451	622	1.73c	0.67c	1.45c

R pods, rotten pods.
* Numbers followed by the same letter are not significantly different at $P < 0.05$. The data were analyzed according to the general linear model using binomial law. Mean separation involved adjusted means according to the Wald Chi-Square test.
[a] Untreated control plots.
[b] Plots treated with *Trichoderma asperellum.*
[c] Plots treated with the fungicide metalaxyl.

3.6. Incidence of cherelle wilt

The percentages of wilted pods ranged from 49.87% to 55.98% in 2004 and from 45.53% to 58.99% in 2005 with no clear treatment effects. This seems to be more a physiological effect occurring when cherelles are especially numerous on the trees.

Table 3
Mean number of *Trichoderma asperellum* propagules distributed in the upper organic soil layers around five trees selected at random and in flower cushions of 30 cacao trees in plots treated with *T. asperellum* strain PR11, the fungicide metalaxyl MET and untreated control plots CONT, in years 2005 and 2006

Treatment	$\log_{10}$CFU g^{-1} of dry weight of soil sample[a]		$\log_{10}$CFU g^{-1} of dry weight of flower cushion sample[a]	
	2005	2006	2005	2006
CONT[b]	1.66 ± 1.02	0	0	0
PRII[c]	4.03 ± 1.01	3.46 ± 0.09	6.54 ± 0.15	4.26 ± 0.38
MET[d]	0.6 ± 0.6	0	0	0

[a] Mean number ± standard error.
[b] Untreated control plots.
[c] Plots treated with *Trichoderma asperellum.*
[d] Plots treated with the fungicide metalaxyl.

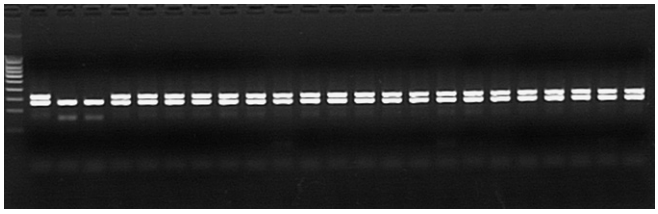

Fig. 4. Molecular typing of *Trichoderma asperellum* isolates sampled from soil and flower cushions in the *T. asperellum* strain PR11-treated plot by multiplex PCR assay using SCAR primer set. Lane 1, 1.000 bp Molecular Weight Marker SF (Eurogentec); lane 2, PR11 type strain; lanes 3 and 4, an other type of isolate; lanes 5–24, PR11 type isolates.

3.7. Field monitoring of the PR11 agent through enumeration and molecular typing

The molecular typing approach used in our study was primarily used for quality control of the PR11 microbial formulation produced at IRAD prior to spraying. Prior to field spraying, random sampling of soil was performed to assess the natural *Trichoderma* species richness. Results showed that it was possible to find propagules of *T. asperellum* in these plots representing 11% (15/136) of all *Trichoderma* species inventoried. Most of them (85%) belong to a different SCAR group than PR11. After spraying, random sampling of soil, bark and flower cushion was performed for both enumeration and molecular typing of the *Trichoderma* propagules isolated. Bark sampling in all plots including PR11-treated samples resulted in a residual number of *Trichoderma* progagules and was thus not taken into account. Average number of *Trichoderma* propagules in the PR11-treated plot for 2 years ranged from 4.03 to 6.54 $\log_{10}$CFU/g (dry weight of the sample) in the soil and flower cushion samples, respectively (Table 3). In the third year even in absence of spraying, these values are still high in both soil and flower cushions samples although they declined by 15% and 35% in soil and flower cushions, respectively, compared to year 2005. The multiplexed SCAR primers generated a typical pattern for PR11 with two diagnostic amplicons of 321 and 276 bp (Fig. 4). All the isolates sampled in soil and flower cushions were typed using the multiplexed SCAR markers and were identified as *T. asperellum* PR11 SCAR group. No *Trichoderma* propagules were found in flower cushions in CONT and MET plots. However, based on SCAR marker technique, except one isolate from MET plot, all the *Trichoderma* isolates recovered from the CONT and MET soils, did not have the characteristic banding pattern of *T. asperellum* PR11 SCAR group.

4. Discussion

This current study provides a better perspective of cacao production dynamics and black pod disease dynamics as affected by various environmental conditions such as rainfall and temperature, as well as the impact of two major control methods, fungicide or microbial treatment, over two consecutive years. A third year was performed to assess the microbial post-treatment impact on the disease incidence associated with a compatible fungicide.

Our study confirms the beneficial effect of Ridomil Plus Gold 66 WP on the pod yield potential of a commercial cocoa farm, with the 2004 season more productive than the following 2005 season. A beneficial effect of *T. asperellum* PR11 treatment on both the flower and total pod productions was also observed during the first season. Previous studies have also reported biological control sprays resulting in improved pod production (Krauss and Soberanis, 2001).

The black pod incidence of the disease in the field is often influenced by environmental conditions. Numerous studies were carried out previously to study the correlation between climatic parameters and the incidence of the black pod disease caused by *Phytophthora* spp. (Thorold, 1967; Wood, 1974). Rainfall, high moisture, and low temperature are known to create favorable conditions for the development of black pod disease, especially in high rainfall areas in Cameroon (Ndoumbè-Nkeng, 2002). The analysis of crossed correlations between rainfall (mm) and the weekly rates of pod rot carried out in this study demonstrated that highest significant correlations were obtained in untreated control CONT and *Trichoderma* PR11-treated plots with rainfall readings recorded 1 week before the disease ratings. In addition, the number of cumulative rains per week was also a determining factor of the expression of the black pod disease. This result shows clearly that, in our study, rainfall

is an important determining factor for black pod disease outbreak, with disease symptoms expressed approximately 1 week later. However, temperature was not a major factor in triggering black pod disease under the conditions prevailing in this study.

The pod rot rate progression curve showed the important role of primary and second inocula in the disease dynamics. The initial flat phase of the disease progression curve in the *Trichoderma* PR11-treated plots, over the first two seasons, did not show an exponential increase as observed in the control plots. This can be explained by the possible reduction in the development of primary *Phytophthora* inoculum source by *T. asperellum* strain PR11. The progression of the disease dynamics observed in the PR11 plot, although lower, was comparable to the one observed in the control plots. Interestingly, a second exponential growth of pathogens was observed in both plots at the onset of rainfall, especially in 2004 season which resulted in higher disease incidence. Such resumption of disease progress curve confirms the important role of climatic conditions in both the interaction between the cacao-host and the pathogen and between the biocontrol agent and the pathogen. Moreover, besides the pods, there are other sources of pathogen inoculum in the field such as soil, soil–root interface and alternate host-trees such as *Cola nitida* (Nyassé, 1997). Therefore, the differences observed with the disease incidence after the different treatments can be associated with those observed with the primary and secondary inoculum dynamics. Research on black pod disease epidemiology has shown that the disease progresses due to the presence of several primary inoculum sources. These are pathogen spores mainly present in the soil, on bark, on flower cushions, on the sporulating mummified or ripe pods present on the trees, and also on the shade trees that are secondary hosts of the black pod pathogen (Muller, 1974a,b; Medeiros, 1976; Babacauh, 1980; Maddison and Griffin, 1981; Opoku, 1994; Opoku et al., 2002). When the environmental conditions are favorable, the dissemination of the infectious propagules can occur from the soil, rain water splash, wind, insects (Babacauh, 1980), and other living organisms such as rodents (Muller, 1974a,b) and ants. The disease progression on the same tree remains primarily from pod to pod (Maddison and Idowu, 1981).

This current study has allowed us to better describe the various phases of disease progression in the experimental plots. In the CONT and PR11 plots where disease incidence was high, we have shown that the "immature pod" stage was the most susceptible stage to *P. megakarya* attack. Preliminary studies on tissue sensitivity of cacao tree towards *Phytophthora* demonstrated the influence of the pod physiological stage on the success of the infection (Blaha and Lotodé, 1976). These results were confirmed by Efombagn et al. (2004) who found the 2–3 month old developmental stage of the pod to be the most susceptible to black pod disease. The current study confirms the results of Ndoumbè-Nkeng et al. (2004) and Tondje et al (2005) on the potential suppressive effect of *T. asperellum* against *P. megakarya* in combination with cultural practices such as regular harvesting and pruning.

The association of cultural practices with biological control was previously compared with cultural practices alone by Krauss and Soberanis (2001). This study performed on managing the frosty pod rot disease caused by *Moniliophthora roreri* in Peru reported better control of the disease if biological control was applied in conjunction with cultural control than either method by itself.

Similar to the previous studies, the present study has also shown the positive impact of the fungicide treatment on black pod disease (Ndoumbè-Nkeng and Sache, 2003). When compared to the control plots, disease impact was significantly reduced (by 23–24% in 2004 and 2005 seasons, respectively) in the biocontrol treated PR11 plots, representing a net benefit of ~5 pods per tree. Assuming that 1200 trees can be planted per ha in this zone, this represents a substantial increase of approximately 6000 pods ha^{-1} $year^{-1}$ for the local farmer, and about 200 kg/ha of commercial cocoa beans.

Results obtained during the third season in the PR11 plot clearly showed a positive effect of post-microbial control treatment or in other words the carry-over effect. The disease impact was significantly reduced (by 19%) in the PR11 plot in comparison to the control plot. The suppressive effect of *T. asperellum* was maintained for almost a year after spraying.

Ridomil Plus Gold 66 WP is known to have a direct toxic action on *P. megakarya*, mainly through persistence of the metalaxyl, which, after spraying, penetrates inside the pods and so prevents the infection. Despite its efficiency whatever the application frequency (every 2 or 4 weeks), its market price is considered to be too expensive for smallholders in Africa and its toxicity towards human and environment remains an issue.

The biocontrol strain PR11 used in this study belongs to the genus *Trichoderma* species which are well-documented as effective biocontrol agents of soil-borne plant diseases. It has been suggested that *Trichoderma* species could act against target organisms in several ways. They can produce extra cellular enzymes and antifungal antibiotics, compete with other fungal pathogens, promote plant growth, induce plants resistance or also be an opportunistic, avirulent plant symbionts living within superficial cells of the roots (Harman et al., 2004).

Monitoring methods used in this study have enabled us to directly verify the presence of the biocontrol agent and its distribution in the plot up to 2 years after the treatments. For the first time, our results demonstrated that *Trichoderma* propagules remained consistently more abundant in flower cushions of cacao trees than in soil or even the bark. Interestingly, flower cushions are supposedly a source of primary inoculum of *P. megakarya*. The soil-borne phase of the *P. megakarya* life cycle is in close association with the roots and soil surface water. From here, zoospores spread stepwise up to the canopy and the path-

ogen is able to remain viable in bark and flower cushions for several months (Flood et al., 2004). Water splash resulting from rainfall or dew deposition on the flower cushions along with optimum temperatures provide the ideal conditions for disease development. Although common environmental factors such as wind, rain, and temperature, favor disease propagation, water is the major factor that drives disease epidemics caused by *P. megakarya*. There is indirect evidence on the importance of the flower cushion in disease spread. Appiah et al. (2004) confirmed that cushion-borne cankers result in surprisingly high number of stem cankers, which is known to play an important role in primary infection of cacao pods. This is the first report of colonization of the cacao flower cushion by *Trichoderma* species, where it is likely that mycoparasitism of the mycelium of *P. megakarya* present in the cushions is occurring, and thus suppressing pathogen growth and subsequent pathogen load. In the laboratory, plates precolonized with *Phytophthora* isolates were shown to be mycoparasitized by *T. asperellum* strains (Tondje et al., 2005).

The use of antagonistic microorganisms is frequently considered as one of the safest and most affordable control strategies. Research on the biological control against *P. megakarya* is promising but still requires further confirmation. It is necessary to set up multiple trials under different agro-ecological conditions, in order to develop an effective and durable method. In parallel, an economic survey needs to be carried out in order to show the benefit generated by the use of such microbial agents compared to the current chemical control. It will be interesting to evaluate the performance of *Trichoderma* strain PR11 with improved formulations in order to reduce the wash-off of the biocontrol agent during heavy rains.

Although meaningful results were obtained in this study as regards microbial control of black pod disease, we are far from reaching a complete control of this disease in Cameroon. The combination of biological and chemical methods, genetic control, and adequate cultural methods in an integrated program appears the best alternative for a sustainable management of cacao black pod disease in Cameroon.

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References

Agresti, A., 1990. Categorical Data Analysis. John Wiley & Sons, Inc. Press, New York.

Appiah, A.A., Opoku, I.Y., Akrofi, A.Y., 2004. Natural occurrence and distribution of stem cankers caused by *Phytophthora megakarya* and *Phytophthora palmivora* on cocoa. European Journal of Plant Pathology 110, 983–990.

Babacauh, K.D., 1980. Structure et dynamique des populations de *Phytophthora* sp. parasite du cacaoyer (*Theobroma cacao* L.). Paris, France: Université de Paris-Sud, Centre d'Orsay, Ph.D. Thesis.

Begoude, B.A.D., Lahlali, R., Friel, D., Tondje, P.R., Jijakli, M.H., 2007. Response surface methodology study of the combined effects of temperature, pH, and aw on the growth rate of *Trichoderma asperellum*. Journal of Applied Microbiology 103 (4), 845–854.

Berry, D., Cilas, C., 1994. Etude génétique de la réaction à la pourriture brune des cabosses chez des cacaoyers (*Theobroma cacao* L.) issus d'un plan de croisement diallèle. Agronomie 14, 599–609.

Blaha, G., Lotodé, R., 1976. Un caractère primordial de sélection du cacaoyer au Cameroun: la résistance à la pourriture brune des cabosses. Café Cacao Thé 20, 97–116.

De Souza, J.T., Pomella, A.W.V., Bowers, J.H., Pirovani, C.P., Loguercio, L.L., Hebbar, K.P., 2006. Genetic and biological diversity of *Trichoderma stromaticum*, a mycoparasite of the cacao witches'-broom pathogen. Phytopathology 96, 61–67.

Despréaux, D., Cambony, D., Clément, D., Nyassé, S., Partiot, M., 1988. Etude de la pourriture brune des cabosses du cacaoyer au Cameroun : définition de nouvelles méthodes de lutte. In: Cocoa Producers' Alliance (Eds.), Proceedings of the 10th International Cocoa Research Conference, 1987. Santo Domingo, Dominican Republic: ICRC, pp. 407–412.

Efombagn, M.I.B., Marelli, J.P., Ducamp, M., Cilas, C., Nyassé, S., Véfonge, D., 2004. Effect of fruiting traits on the field resistance of cocoa (*Theobroma cacao* L.) clones to *Phytophthora megakarya*. Journal of Phytopathology 152, 557–562.

Elad, Y., Freeman, S., 2004. Biological control of fungal plant pathogens. In: Kempken, F. (Ed.), . The Mycota, A Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research. Agricultural Applications, XI. Springer, Berlin-Heidelberg, Germany, pp. 93–109.

Flood, J., Guest, D., Holmes, K.A., Keane, P., Padi, B., Sulistyowati, E., 2004. Cocoa under attack. In: Flood, J., Murphy, R. (Eds.), Cocoa Futures: A Source Book of Some Important Issues Facing the Cocoa Industry. Chinchina, Colombia: CABI-FEDERACAFE, USDA, pp. 33–53.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M., 2004. Trichoderma species—opportunistic, avirulent plant symbionts. Nature Reviews Microbiology 2, 43–56.

Hebbar, K.P., Lumsden, R.D., 1999. Formulation and fermentation of biocontrol agents of cocoa fungal pathogens: example of *Trichoderma* species. In: Krauss, U., Hebbar, K.P. (Eds.), Research Methodology in Biocontrol of Plant Diseases with Special Reference to Fungal Disease of Cocoa. CATIE, Costa Rica, pp. 63–68.

Holmes, K.A., Schroers, H.J., Thomas, S.E., Evans, H.C., Samuels, G.J., 2004. Taxonomy and biocontrol potential of a new species of *Trichoderma* from the Amazon basin of South America. Mycological Progress 3 (3), 199–210.

Krauss, U., Soberanis, W., 2001. Biocontrol of cocoa pod diseases with mycoparasite mixtures. Biological Control 22, 149–158.

McCullagh, P., Nelder, J.A., 1989. Generalized Linear Models, second ed. Chapman and Hall, London.

Maddison, A.C., Griffin, M.J., 1981. Detection and movement of inoculum. In: Gregory, P.H., Maddison, A.C. (Eds.), Epidemiology of *Phytophthora* on Cocoa in Nigeria. Commonwealth Mycological Institute, Kew, England, pp. 31–49.

Maddison, A.C., Idowu, O.L., 1981. The epidemic on sprayed cocoa at Owena. In: Gregory, P.H., Maddison, A.C. (Eds.), Epidemiology of

Phytophthora on Cocoa in Nigeria. Commonwealth Mycological Institute, Kew, England, pp. 163–172.
Medeiros, A.G., 1976. Sporulation of Phytophthora palmivora (Butl.) in relation to epidemiology and chemical control of cocoa Black Pod disease. University of California, Riverside, USA, Ph.D. Thesis.
Muller, R.A., 1974a. Effects on prophylactic measures on dissemination of *Phytophthora palmivora*. In: Gregory, P.H. (Ed.), *Phytophthtora* Disease of Cocoa. Longman, London, UK, pp. 169–178.
Muller, R.A., 1974b. Integrated control methods. In: Gregory, P.H. (Ed.), *Phytophthora* disease of cocoa. Longman, London, UK, pp. 259–268.
Ndoumbè-Nkeng, M., 2002. Incidence des facteurs agro-écologiques sur l'épidémiologie de la pourriture brune des fruits du cacaoyer au Cameroun: contribution à la mise en place d'un modèle d'avertissements agricoles. INA-PG, Paris, France, Ph.D. Thesis.
Ndoumbè-Nkeng, M., Sache, I., 2003. Lutte contre la pourriture brune des cabosses du cacaoyer au Cameroun. Phytoma. La Défense des Végétaux 562, 10–12.
Ndoumbè-Nkeng, M., Cilas, C., Nyemb, E., Nyasse, S., Bieysse, D., Flori, A., Sache, I., 2004. Impact of removing disease pods on cocoa black pod caused by *Phytophthora megakarya* and on cocoa production in Cameroon. Crop Protection 23 (5), 415–424.
Nyassé, S., 1997. Etude de la diversité de *Phytophthora megakarya* et caractérisation de la résistance du cacaoyer (*Theobroma cacao* L.) à cet agent pathogène. INPT, Toulouse, France, Ph.D. Thesis.
Opoku, I.Y., 1994. Survival of Phytophthora palmivora and *Phytophthora megakarya* in soil and cocoa roots. University of London, London, UK, Ph.D. Thesis.
Opoku, I.Y., Akrofi, A.Y., Appiah, A.A., 2002. Shade trees are the alternative hosts of the cocoa pathogen *Phytophthora megakarya*. Crop Protection 21, 629–634.
Papavizas, G.C., 1985. Trichoderma and Giocladium: biology, ecology, and potential for biocontrol. Annual Review of Phytopathology 23, 23–54.
Samuels, G.J., 1996. Trichoderma: a review of biology and systematics of the genus. Mycological Research 100, 925–935.
SAS Institute Inc., 2001. SAS User's Guide: Statistics Institute Inc., Release 8.2. Cary, NC, USA.
Thorold, C.A., 1967. Black Pod disease of *Theobromae* cacao. Review of Applied Mycology 46, 225–237.
Tondje, P.R., Begoude, D., Samuels, G.J., Hebbar, P.K., Foko, J., 2005. Biological control of the cocoa Black Pod disease causative agent *Phytophthora megakarya* with *Trichoderma asperellum*. In: Cocoa Producers'Alliance (Eds.), Proceedings of the 14th International Cocoa Research Conference, 2003. Accra, Ghana, vol. II, pp. 677–682.
Wood, G.A.R., 1974. Black pod and meteorological factors. In: Gregory, P.H. (Ed.), Phytophthora Disease of Cocoa. Longman, London, UK, pp. 153–159.