Journal of Plant Protection Research eISSN 1899-007X

ORIGINAL ARTICLE

The potential of *Trichoderma asperellum* organic extract and its emulsion to inhibit cocoa (*Theobroma cacao* L.) black pod disease and induce biochemical defenses against *Phytophthora megakarya*

Yves Olivier Youassi Youassi[1], Henri Tibo Ambata Ambata[1], Mylaure Benisa Jiogue[1], Fabiola Jeannette Ndondoni Dikongue[1], Moïse Ntah A Ayong[1], Marie Amperes Boat Bedine[2], Séverin Nguemezi Tchameni[1]*, Modeste Lambert Sameza[1]

[1] Department of Biochemistry, Faculty of Science, University of Douala, Douala, Cameroon

[2] Department of Agriculture, Faculty of Agronomy and Agronomic Sciences, University of Dschang, Dschang, Cameroon

Vol. 64, No. 1: 19–28, 2024

DOI: 10.24425/jppr.2024.149159

Received: September 08, 2023
Accepted: December 04, 2023
Online publication: March 06, 2024

*Corresponding address:
tchameni1@yahoo.fr

Responsible Editor:
Kallol Das

Abstract

This work aimed to evaluate the potential of *Trichoderma asperellum* organic extract and its emulsion to control cocoa black pod disease caused by *Phytophthora megakarya*. Organic extract was obtained after fermentation of *T. asperellum* and its emulsion prepared by emulsification. The *in vitro* antimicrobial assays of organic extract and its emulsion were evaluated and the *in situ* tests were carried out on detached cocoa pods. *T. asperellum* inhibited the mycelia growth of *P. megakarya* at the rates of 52% and 100%, respectively, on dual culture and the cellophane plate. This antagonist produced lytic enzymes such as cellulase, amylase, lipase and protease. The organic extract contained alkaloid, flavonoid and phenol compounds. The emulsion obtained was stable. At 100 $\mu g \cdot ml^{-1}$, the extract and its formulation completely inhibited the mycelial growth of *P. megakarya*. Similarly, when infected detached cocoa pods were sprayed with extract or emulsion, there was a significant reduction of necrosis both for healing and prevention with the latter being the most efficient. For the preventive tests, the total inhibition was recorded at 3000 $\mu g \cdot ml^{-1}$ and 1000 $\mu g \cdot ml^{-1}$, respectively, with crude organic extract and its emulsion· For curative tests, total inhibition was obtained at 4000 $\mu g \cdot ml^{-1}$ and 3000 $\mu g \cdot ml^{-1}$, respectively, for preventive and curative tests. There was a significant and positive correlation between the content of biochemical markers and the reduction of necrosis on cocoa pods after treatment with the extract or its formulation. *T. asperellum* organic extract emulsion could be used as an alternative in the bio- protection of cocoa black pods disease.

Keywords: black pod disease, emulsion, induction of biochemical defense, organic extract, *Trichoderma asperellum*

Introduction

Cocoa (*Theobroma cacao* L.) belongs to the Malvaceous family and is one of the most important cultures in tropical countries. In fact, its beans, the raw material used in the chocolate industry, are rich in unsaturated fatty acids, vitamins, minerals, as well as phenolic and flavonoid compounds. In traditional medicine, cocoa is used to fight against infectious diseases and metabolic disorders (Scudder *et al.* 2022).

Despite its importance, cocoa faces several diseases which can significantly reduce yield, along with its marketable and nutritional value. Black pod disease, caused by the Oomycete *Phytophthora megakarya,* is the most devasting constraint. In the absence of any treatment, yield losses can reach 80–100% (Ndoumbe-Nkeng *et al.* 2004).

In order to control this pathogen, farmers commonly use chemical pesticides like copper hydroxide

and metalaxyl-M + mancozeb. However, the repeated use of these products is toxic to humans and the environment, has carcinogenic effects and possibly induces the pathogen's resistance (Bateman 2004). An alternative is the use of biological control agents which are more eco-friendly. Among these, *Trichoderma* spp. are one of the most studied and have been successfully used as biopesticides and biofertilizers (Woo and Pepe 2018). Their biocontrol properties are due to different mechanisms of action such as competition for space and nutrients (Rabuske *et al.* 2023), the production of cell wall-lytic enzymes, antibiosis, mycoparasitism as well as the induction of systemic resistance to pathogens in plants. The latter is characterized by the synthesis of biochemical substances such as proteins, amino acids, phenolic compounds, sugars and enzymes [phenyl alanine ammonia lyase (PAL)] and polyphenol oxidases (PPO) (Manzar *et al.* 2022). The antagonistic properties of *Trichoderma* spp in terms of antibiotics production have been intensively studied. These compounds, called secondary metabolites, belong to several chemical groups (alkaloids, phenols, terpenes, polyketides, etc.) and may be able to affect the interactions of plants with their pathogens (Woo *et al.* 2023). All these strategies used by *Trichoderma* spp. are often complementary to neutralize the pathogen and reduce the disease incidence.

The use of *Trichoderma* as a biological agent is mainly based on spores or an organic extract (Vinale and Sivasithamparam 2020). Although research has shown the effectiveness of *Trichoderma* organic extracts in the control of plant pathogens, their application is limited due to their instability. However, it may be possible to increase their efficacy as an oil-formulation or emulsion (Fraceto *et al.* 2018). An emulsion is a very small (micro or nano-particle), colloidal particulate system which is thermodynamically and kinetically stable (Chhipa 2017). It consists of two non-miscible liquids such as water and organic extract, stabilized by an interface of surfactant(s) and co-surfactant(s) able to form a small particle size phase. It is used in many domains including pharmaceutical, agrochemical and food industries (Chhipa 2017). In agro-chemistry, oil--formulations or emulsions are used for the production of biopesticides which possess many advantages over the crude extract (Singh *et al.* 2023).

In our previous studies, many species of *Trichoderma* were isolated from the plant rhizosphere. The screening of these isolates for their antagonism against plant pathogens revealed that *T. asperellum* was one of the most effective (Bedine *et al.* 2020). However, there is no previous information concerning the potential use of an organic extract of this antagonist to control black pod disease. Therefore, this study aimed to evaluate the effect of an organic extract of *T. asperellum* and its emulsion on the mycelial growth of *P. megakarya* and its ability to reduce cocoa black pods disease and induce biochemical resistance.

Materials and Methods

Plant pathogen

Phytophthora megakarya was isolated from cocoa pods showing the typical symptoms of black pod disease. Briefly, pod pieces (3–5 mm) were taken from the edge of necrosis and sterilized in 1.0% sodium hypochlorite for 2 minutes. Pod pieces were washed three times with sterile distilled water and inoculated on V_8 agar plates. After 3–5 days of incubation at 28°C in the dark, the emerged mycelia from the pod fragments were transferred to fresh medium and purified by successive transfers. The pathogenicity test was carried out using Koch's postulates. *Phytophthora megakarya* was identified on the basis of physiological and morphological traits as described by Ristaino (2012).

Trichoderma asperellum strain

The strain of *Trichoderma asperellum* (gene bank accession, KF040478) used in this study came from the core culture of the Laboratory of Biochemistry of the University of Douala (Cameroon). It was previously isolated from the common bean rhizosphere (Soa Subdivision, center region of Cameroon) and identified by Bedine *et al.* (2020).

In vitro antagonism test

In vitro antagonism tests were done on paired culture and by the cellophane plate method, both in 9 cm Petri dishes containing 15 ml of Potato Dextrose Agar (PDA) medium.

For dual culture, *T. asperellum* and *P. megakarya* preculture discs were seeded at the edge of a PDA plate, on opposite sides, equidistant from the center. A plate inoculated only with the pathogen served as the control. After 7 days of incubation at 25°C, the inhibition (%I) of the mycelial growth was assessed by comparison to the growth of the control plates. Furthermore, agar discs were randomly picked from the intermingling region, and transferred to a PDA plate supplemented with benomyl in an attempt to recover *P. megakarya*.

The cellophane plate method was carried out by laying 6 cm sterilized membrane on a PDA plate, and inoculated with a *T. asperellum* agar disc. After 2 days of incubation at 25°C, the cellophane membrane with *T. asperellum* was removed and *P. megakarya* was incubated at the center of the medium. The inhibition of the mycelial growth of *P. megakarya* was evaluated by

comparison to the growth of the control plates (plates inoculated with the pathogen). Each treatment consisted of three replicates and the experiments were repeated twice.

Production of lytic enzymes by *Trichoderma asperellum*

For the production of lytic enzymes, *T. asperellum* was incubated on different specific agar media and incubated at 28°C for 3 days. The size of a clear halo around the fungal colony (indicating the enzyme activity) was measured (Bedine *et al.* 2020).

For cellulase activity, the detection medium (pH 7) consisted ($g \cdot l^{-1}$) of NaCl (0.5), H_2PO_4 (1.0), $MgSO_4$, $7H_2O$ (0.5), $MnSO_4$, H_2O (0.01), NH_4NO_3 (0.3), $FeSO_4$,$7H_2O$ (0.01), carboxymethylcellulose (10), and agar (15). After incubation, Congo red solution (0.1%) was poured on the surface of the colony for 10 min, and 1.0M NaCl for 10 min.

For amylase activity, GYP (glucose yeast extract peptone) medium containing 2% of soluble starch was used. A chromogenic medium (pH 7.4) was used for lipase activity. This medium contained 0.01% phenol red, 1% lipid substrate (Tween 80), 10 mM $CaCl_2$, and 2% agar. Finally, protease was evaluated on a medium containing ($g \cdot l^{-1}$) K_2HPO_4 (2), 10 g of glucose, 5 g of peptone, 15 g of gelatin and 15 g of agar. After incubation, plates were flooded with 10% of sodium sulphate solution.

Production of *Trichoderma asperellum* crude organic extract

Four *T. asperellum* explants (5 mm) taken from the margin of 2 days precultures were inoculated in a 1liter Erlenmeyer flask containing 200 ml sterilized Potato Dextrose Broth (PDB). After 30 days of incubation at room temperature under stationary conditions, the culture was filtered and the filtrate extracted three times with ethyl acetate (1l of the filtrate per 350 ml solvent). The organic phase was concentrated by a rotary evaporator ((BUCHI brand) at 60°C and the crude extract dried at 40°C, weighed and stored at 4°C until used.

Chemical screening of *Trichoderma asperellum* crude organic extract

The chemical screening involved highlighting the major chemical constituents (alkaloids, phenols, flavonoid, anthocyanins and quinones) in the organic extract, according to the method described by Bruneton (2016)

For alkaloids, the test was performed by adding 1 ml Dragendoorff's reagent to 2 ml of extract. An orange red precipitate indicated the presence of alkaloids.

Concerning the phenols**,** 2 ml of 5% neutral ferric chloride solution was added to 1 ml of extract dissolved in ethanol. A dark blue color indicated the presence of phenolic compounds and tannins.

For flavonoid, two to three drops of sodium hydroxide (NaOH) were added to 2 ml of extract. A deep yellow color appeared but gradually became colorless by adding a few drops of diluted HCl, indicating that flavonoid was present.

Fifteen ml of HCl 1% was added to 2 ml of extract and the mixture was boiled. An orange blue color indicated the presence of anthocyanins.

Two ml sodium hydroxide (10 M) were added to 2 ml of extract. After vigorous shaking, an orange-red color indicated the presence of quinones.

Preparation of *Trichoderma asperellum* organic extract emulsion and characterization (OEE)

Trichoderma asperellum organic extract emulsion (OEE) was prepared as described by Aloke *et al.* (2020) with some modifications. Two phases were used, namely, the organic phase (10% of the emulsion) consisting of a mixture of the extract and tween 80 (1 : 1 ratio) and the aqueous phase (90% of the emulsion) containing palm oil and polypropylene glycol. Briefly, the extract and tween were first mixed with a magnetic stirrer at 500 rpm for 10 min, then the aqueous phase was added and the agitation continued for 2 hours. Physicochemical characteristics of the obtained emulsion were assessed based on criteria such as the physical appearance, pH, foam persistency, centrifugation and storage stability tests (Nirmala *et al.* 2020).

In vitro antimicrobial activities

The *in vitro* antimicrobial assay of *T. asperellum* crude extract and its emulsion was carried out against *P. megakarya*, using an agar plate incorporation method. The organic extract was first diluted in DMSO (DiMethyl SulfOxide) ($1000\ \mu g \cdot ml^{-1}$) prior to incorporation into PDA medium.

For a volume of 10 ml of culture medium, 250, 500, 1000 and 2000 µl of crude extract ($1000\ \mu g \cdot ml^{-1}$) were incorporated to obtain concentrations of 25, 50, 100 and 200 $\mu g \cdot ml^{-1}$, respectively. Similarly, Organic Extract Emulsion (OEE) was mixed directly into the culture medium to obtain the desired concentration (25 to 200 $\mu g \cdot ml^{-1}$). The control plates were supplemented with both DMSO and the mixture of water-tween-polypropylene glycol, instead of the extract or emulsion. Tests were performed on 90 mm Petri dishes containing 10 ml PDA medium, on which

a 5 mm preculture plug of the pathogen was seeded. After 7 days of incubation at 25°C, the inhibition of the mycelial growth (IMG) was calculated according to the formula:

$$\%\ \text{IMG} = \frac{D_0 - D_x}{D_0} \times 100,$$

where: D_0 – the diameter of the pathogen growth on control plates and D_x – the diameter of the pathogen growth on the treated plates containing extract or emulsion. Each treatment consisted of three replicates and the experiment was repeated twice.

Necrosis inhibition tests on detached cocoa pods

The detached cocoa pod test was done by the method described by Iwaro *et al.* (2000) with some modifications. Mature ICS84 cocoa clone known as moderately sensitive to black pod disease was used (Efombagn *et al*. 2011). The pods were first washed with distilled water and disinfected with alcohol (70%) for 5 min. The upper side of each pod was sprayed with 5 ml of the extract/formulation at various concentrations (1000, 2000, 3000 and 4000 $\mu g \cdot ml^{-1}$) with a hand-held pressurized sprayer. Pods were then wounded with a syringe and infected with 200 µl of *P. megakarya* zoospore (3×10^5 zoospores $\cdot$ ml^{-1}) suspension, 24 hours after extract/formulation application for prevention of the disease and 48 hours before for healing properties. Zoospores were obtained using the method previously described by Sameza *et al.* (2014). For control, pods were treated with DMSO or the mixture of water-tween-polypropylene glycol. Treated pods were incubated in plastic trays with absorbent paper at 25 ± 2°C in the dark for 7 days. Each treatment consisted of four cocoa pods and the experiment was repeated three times. The evaluation of necrosis inhibition (%NI) was carried out using the following formula:

$$\text{NI}\ (\%) = \frac{D_0 - D_t}{D_0} \times 100,$$

where: D_0 – diameter of the control; D_t – average diameter of the treated pods.

Evaluation of the biochemical parameters on cocoa pods

One gram (1 g) of cocoa pod (taken from 2 cm of a necrosis area) was ground at 4°C in a porcelain mortar by adding a pinch of blue fountain sand and 2 ml of sodium phosphate buffer (0.1 M; pH 7). The ground material obtained was centrifuged at 3000 rpm at 4°C for 20 minutes. The supernatant was recovered and stored at 4°C until use for proteins content and enzymatic assays.

Total soluble proteins and polyphenol oxidase (PPO) assay

Soluble proteins content was determined according to the method of Bradford (1976). In each tube, 20 µl of protein extract was added to 2 ml of Bradford's reagent (Coomassie G_{250} + orthophosphoric acid + ethanol 95°). After 10 min of incubation, the optical density was determined at 595 nm by using a spectrophotometer (BKUV-1600PC BIOBASE). The protein content was expressed as $mg \cdot g^{-1}$ of fresh material by comparison with Bovine Albumin Serum (0.01 to 0.1 µg) as standard.

The activity of the PPO was evaluated according to the protocol described by Mayer *et al.* (1966). The reaction mixture consisted of 1.5 ml of sodium phosphate buffer (0.1 M; pH 7.0) and 200 µl of catechol (0.01 M). Briefly, 20 µl of the enzyme extract was added to the mixture and incubated for 2 minutes at room temperature. The reaction was stopped by cooling the tubes in ice for 5 minutes. Optical density (OD) was measured at 420 nm against a blank where the enzymatic extract was substituted by the buffer solution. The specific enzyme activity was expressed as a function of the change in optical density ($OD \cdot min^{-1} \cdot mg^{-1}$) of proteins $\cdot$ g^{-1} of fresh material).

Phenolic and flavonoid compounds assay

One gram (1 g) of cocoa pod was ground with mortar and pestle in 4 ml of methanol (70%) and centrifuged at 4000 rpm for 15 minutes. The supernatant was used to evaluate the total polyphenol and flavonoid contents (Jaradat *et al.* 2020).

Polyphenol content was evaluated using the Folin--Ciocalteu colorimetric method. The reaction mixture contained 100 µl of supernatant (extract), 2000 µl of sodium carbonate (20%), and 250 µl of Folin-Ciocalteu's reagent. After 60 min of incubation at room temperature in the dark, the absorbance was measured at 760 nm against the blank tube where polyphenol was replaced by a methanol (70%). The calibration curve (10–100 $\mu g \cdot ml^{-1}$) was plotted and the total polyphenol content was expressed as µg of catechol equivalent per g of fresh weight of pod

Flavonoid content was determined by using the aluminium chloride colorimetric method. The reaction mixture contained 100 µl of supernatant, 400 µl of distilled water, and 30 µl of sodium nitrite (5%). After 5 minutes of incubation at room temperature, 20 µl of aluminium chloride (10%), 200 µl of Na_2CO_3 (1 M), and 250 µl of distilled were added. The mixture was homogenized with a vortex and the optical density measured at 510 nm. The standard curve was plotted (10–100 $\mu g \cdot ml^{-1}$) and the flavonoid content was expressed as µg of quercetin equivalent per g of cocoa pod.

Statistical analysis

Data were analyzed with SPSS software version 18.0 for Windows (SPSS, Inc., Chicago, IL, USA) version. The results were presented as means ± standard deviation (SD). One way ANOVA (analysis of the variance) was used to study the effect of the organic extract and its emulsion concentration on mycelial growth of *P. megakarya* and biochemical parameters. Duncan's post hoc test was used for multiple comparison. The degree of significance was fixed at 5%, and the Pearson ccorrelation was used to evaluate the link between the parameters

Results

In vitro confrontation of *Trichoderma asperellum* against *Phytophthora megakarya*

In dual culture *T. asperellum* significantly ($F_{3.10} = 66.79$; $p < 0.001$) inhibited (52%) the mycelial growth of *P. megakarya*. At the intermingling region, any attempt to re-isolate *P. megakarya* failed. On the other hand, the growth of this pathogen was also completely inhibited (100%) by non-volatile compounds released by *T. asperellum* using the cellophane plate method.

Production of hydrolytic enzymes

Hydrolytic enzyme activities were evaluated on the basis of halo formation around a *T. asperellum* colony. For cellulases, 4.03 ± 0.15 cm, 7.03 ± 1.75 cm, 8.23 ± 0.25 cm and 7.01 ± 0.13 cm, respectively, amylases, lipases and proteases were obtained.

Chemical content of organic extract of *Trichoderma asperellum*

Qualitative screening of *T. asperellum* extract showed the presence of chemical constituents namely alkaloids, flavonoids and phenolic compounds, while no anthocyanins and quinones were detected.

Characterization of *Trichoderma asperellum* organic extract emulsion

The results of physicochemical characteristics of *T. asperellum* organic extract emulsion are presented in Table 1. The formulation had a clear and bluish reflection appearance, a 7.6 pH with 0.7 ml of foam. Moreover, it did not show any destabilization processes such as blending, sedimentation and flocculation.

Table 1. Physicochemical characteristics of *Trichoderma asperellum* organic extract emulsion

Characteristics and stability tests	Results
Appearance	clear and bluish reflection
pH	7.6
Foam formed after 1 min	0.7 cm
Stability after 1 h at rest and cold	+
Centrifugation at 5000 rpm for 30 min	+
Storage stability tests after 14 days	
At 4°C	+
At 25°C	+
At 55°C	+

(+) – good (no phase separation)

Effect of crude organic extract of *Trichoderma asperellum* and its emulsion on mycelial growth of *Phytophthora megakarya*

The organic crude extract and its emulsion significantly exhibited ($F_{2.76} = 26.61$; $p < 0.001$) concentration dependent antimicrobial activity against *P. megakarya* with total inhibition occurring at 100 $\mu g \cdot ml^{-1}$ (Table 2). Statistical analysis revealed that, there were significant and positive correlations between the inhibition of the mycelial growth of the pathogen and the concentration of organic extract ($p = 0.001$; $r = 0.95$) and emulsion ($p = 0.001$; $r = 0.97$).

Necrosis inhibition tests on cocoa pods

When infected detached cocoa pods were treated with *T. asperellum* organic extract or its emulsion, there was significant reduction of necrosis diameter both for preventive ($F_{2.87} = 14.61$; $p < 0.001$) and healing tests

Table 2. Effect of organic extract of *Trichoderma asperellum* and its emulsion on mycelial growth of *Phytophthora megakarya*

	Organic extract	Emulsion
Concentration [$\mu g \cdot ml^{-1}$]	inhibition [%]	
0.00	0.00 a ± 0.00	0.00 a ± 0.00
25.00	56.00 b ± 0.04	61.46 b ± 1.54
50.00	87.2 c ± 0.02	84.20 c ± 1.05
100.00	100.00 d ± 0.00	100.00 d ± 0.00
200.00	100.00 d ± 0.00	100.00 d ± 0.00

Values are the means of three replicates and according to Duncan test, in the same column, means ± SE with the same letters are not significantly different at $p < 0.05$

($F_{3.98}$ = 14.61; $p < 0.01$) (Fig. 1 and Fig. 2). For the preventive tests, the total inhibition was recorded at 3000 µg · ml^{-1} and 1000 µg · ml^{-1}, respectively, with crude organic extract and its emulsion (Fig. 1). Meanwhile, for curative tests, total inhibition was obtained at 4000 µg · ml^{-1} and 3000 µg · ml^{-1}, respectively (Fig. 2). Statistical analysis revealed that the preventive tests were more efficient than the curative test ($p \leq 0.05$).

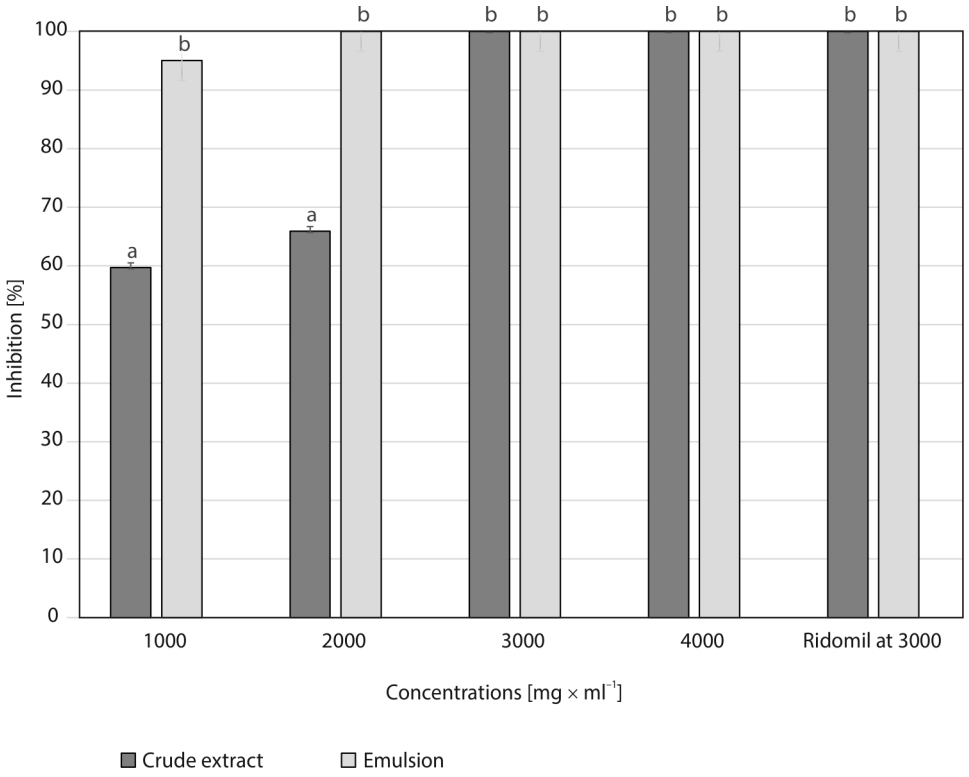

Fig. 1. Effect of crude organic extract of *Trichoderma asperellum* and its emulsion on cocoa pod necrosis: Preventive test

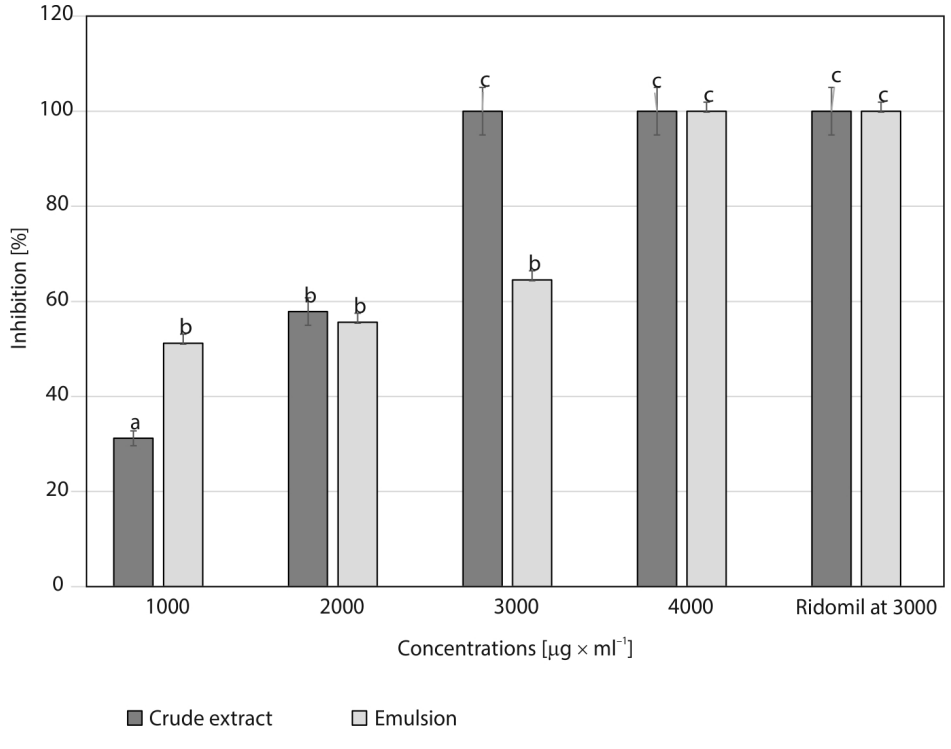

Fig. 2. Effect of crude organic extract of *Trichoderma asperellum* and its emulsion on cocoa pod necrosis: Curative test

Total soluble proteins content

The data presented in Table 3 show that there was no significant difference in total soluble content between uninfected cocoa pods sprayed with pure organic extract of *T. asperellum* (58.6 mg · g^{-1}) and its emulsion (60.20 mg · g^{-1}), compared to the control (62.1 mg · g^{-1}). Total soluble proteins content was significantly ($F_{3.76}$ = 121.69; $p < 0.001$) increased with *P. megakarya* infection. In the control infected cocoa pods, the total soluble protein was 68.33 mg · g^{-1}. For the cocoa pods sprayed with organic extract and infected with *P. megakarya*, the total soluble proteins were 68.13 mg · g^{-1} and 64.68 mg · g^{-1}, respectively, for curative and preventive tests. For the cocoa pods sprayed with emulsion and infected, the concentrations of soluble proteins were 68.0 mg · g^{-1} and 77.07 mg · g^{-1} for both preventive and healing tests.

Table 3. Effect of organic extract of *Trichoderma asperellum* and its emulsion on total soluble protein content of cocoa pods

	Soluble protein content [mg · g^{-1} of fresh material]		
	uninfected pods	infected pods	
		preventive test	curative test
Control	62.10 a ± 0.26	68.33 b ± 0.40	68.33 b ± 0.39
Organic extract	58.60 a ± 0.43	68.13 b ± 0.34	64.68 ab ± 0.97
Emulsion	60.20 a ± 0.13	68.00 b ± 0.43	77.06 c ± 0.76

Values are the means of three replicates and according to Duncan test, in the same column, means ± SE with the same letters are not significantly different at p <0.05

Polyphenol oxidase activity

The data presented in Table 4 revealed that there was no significant difference of polyphenol oxidase activities between the non-infected cocoa pods treated with organic extract of *T. asperellum* (25.88 ± 0.45 mg · g^{-1}) and its emulsion (27.10 ± 0.80 mg · g^{-1}), compared to the control (26.93 ± 0.67 mg · g^{-1}). The infection significantly ($p < 0.05$) increased the polyphenol oxidase activity. However, in the infected cocoa pods, no significant difference was obtained after spraying with organic extract and its emulsion both for preventive and curative tests.

Table 4. Effect of organic extract of *Trichoderma asperellum* and its emulsion on polyphenol activity of cocoa pods

	Polyphenol activity [optical density · $minute^{-1}$]		
	uninfected pods	infected pods	
		preventive test	curative test
Control	26.93 a ± 0.67	35.57 b ± 0.98	35.57 b ± 0.65
Organic extract	25.88 a ± 0.45	34.9 b ± 0.86	35.70 b ± 0.84
Emulsion	27.10 a ± 0.80	31.10 b ± 0.90	38.33 bc ± 0.76

Values are the means of three replicates and according to Duncan test, in the same column, means ± SE with the same letters are not significantly different at p <0.05

Polyphenol content

Results showed significantly decreased ($F_{3.11}$ = 112.31; $p < 0.001$) amounts of polyphenol content between the sprayed healthy cocoa pods with organic extract (68.1 µg · g^{-1}) and its emulsion (60.2 µg · g^{-1}), compared to the non-sprayed (83.87 µg · g^{-1}) (Table 5). Infection significantly increased the polyphenol content in the control pods ($F_{2.17}$ = 13.91; $p < 0.001$) and in the pods treated with emulsion ($F_{3.45}$ = 32.71; $p < 0.001$). However, the content of polyphenol significantly decreased ($F_{2.27}$ = 23.50; $p < 0.001$) in infected pods treated with organic extract during preventive and curative tests. For pods sprayed with emulsion, a significant decrease ($p < 0.001$) was obtained during the preventive test (Table 6). There was a significant and positive correlation between the polyphenol content and the reduction of necrosis. In the infected and treated pods with organic extract, the correlations were $p = 0.001$; $r = 0.82$ and $p = 0.001$; $r = 0.79$, respectively, for preventive and curative tests. For the pods sprayed with emulsion, they were $p = 0.001$; $r = 0.91$ and $p = 0.01$; $r = 0.78$, respectively.

Table 5. Effect of organic extract of *Trichoderma asperellum* and its emulsion on polyphenol content of cocoa pods

	Polyphenol content [µg · g^{-1}]		
	uninfected pods	infected pods	
		preventive test	curative test
Control	83.87 c ± 0.53	134.53 f ± 1.20	134.53 f ± 0.76
Organic extract	68.10 b ± 0.58	79.20 c ± 0.79	69.53 b ± 0.89
Emulsion	60.20 a ± 0.86	95.20 d ± 0.90	113.35 e ± 1.09

Values are the means of three replicates and according to Duncan test, in the same column, means ± SE with the same letters are not significantly different at p <0.05

Flavonoid content

The results (Table 6) showed a significant decrease in the flavonoid content of uninfected sprayed cocoa pods with extract ($F_{2.67}$ = 16.19; $p < 0.001$) and its emulsion ($F_{2.21}$ = 11.13; $p < 0.001$). For the control, this content was 61.69 µg · g^{-1}, while it was 36.78 µg · g^{-1} and 27.1 µg · g^{-1}, respectively, for pods treated with organic extract and its emulsion. A markable increase of this flavonoid content after infection was noted. Also, there was a significant decrease after treatment with the organic

Table 6, Effect of organic extract of *Trichoderma asperellum* and its emulsion on flavonoid content of cocoa pods

	Flavonoid content [μg · g^{-1}]		
	uninfected pods	infected pods	
		preventive test	curative test
Control	61.69 d ± 0.75	113.73 f ± 0.61	113.73 f ± 1.60
Organic extract	36.78 b ± 0.63	44.87 bc ± 0.77	58.43 d ± 0.90
Emulsion	27.10 a ± 0.39	50.85 c ± 0.85	80.46 e ± 0.68

Values are the means of three replicates and according to Duncan test, in the same column, means ± SE with the same letters are not significantly different at $p < 0.05$

extract ($p < 0.001$) or emulsion ($p < 0.001$) for both preventive or curative tests. Flavonoid content was significantly and positively correlated with the necrosis reduction on cocoa pods after infection. Correlation rates obtained were $p = 0.001$; $r = 0.92$ and $p = 0.000$; $r = 0.96$, respectively, for preventive and curative tests for pods treated with extract, while they were $p = 0.000$; $r = 0.94$ and $p = 0.000$; $r = 0.98$ after treatment with emulsion.

Discussion

In this study, *T. asperellum* exhibited significant antagonistic activity against *P. megakarya* and released lytic enzymes able to act on specific substrates such as cellulose, starch, lipids and proteins, some of which are important constituents of oomycetes. The antagonism of *T. asperellum* and other species against plant pathogens including Oomycete is well known (Tchameni *et al.* 2017; Mendez *et al.* 2020). This could be explained by their ability to use several mechanisms such as antibiosis, competition and mycoparasitism as well as the production of lytic enzymes (Tchameni *et al.* 2020; Bedine *et al.* 2020). However, these enzymes and secondary metabolites may have a synergistic effect (Tchameni *et al.* 2020). Organic extract obtained from *T. asperellum* contained flavonoid and alkaloid compounds, family groups known for their biological activity. This extract and its emulsion significantly reduced the mycelial growth of *P. megakarya* and inhibited the necrotic lesions on cocoa pods at various concentrations. Organic products formulated from various extracts are known for their fungicidal, bactericidal and insecticidal actions (Waligóra *et al.* 2023). Limited research on the evaluation of the activity of biopesticides based on organic extract of *Trichoderma* has been carried out. However, under optimal conditions *P. megakarya* is able to move through the cortex and infect the beans which is the major stage of spoilage as the beans are intended for consumption. In response, the formulation could act through an antibiotic effect of its metabolites, able to diffuse through the cell surface and affect the cell activity of the pathogen by preventing the repair of previous damage to the wall or even death (Martinez *et al.* 2023). This death by contact is linked to the necrotrophic action of antibiotic molecules which prevent formation of the cell membrane, formation of vesicles, reduction in the diameter of the hyphae and degradation of the cell wall. However, at certain concentrations, the presence of various necrotic lesions is observed on the surface, at the level of the second internal layer of the pods or even at the level of the beans. After treatment with cocoa at 3000 μg · ml^{-1}, no lesions were observed. This could be explained by the systemic effect of the product which penetrates into the first layers of the cortex, exerting an antimicrobial action and conferring better protection on the pods. The inhibitory effects of the oil-formulation could be related to the better stabilization and conservation of the components and their ability to diffuse into the mesocarp of cocoa pods (Soumanou and Adjou 2016). In addition, the oily nature allows better fixation of the active ingredients on the surface and could optimize the diffusibility of the latter through the cells of the cortex which are lipidic in nature. Thus, the preventive treatment could promote the diffusibility of the product and the stimulation of natural defense molecules. Associated with the active ingredients following a concerted mechanism would lead to systemic resistance before infection by *P. megakarya*. The resistance of the detached pods was expressed visually by the reduction or even the absence of necrosis. There are many mechanisms involved in induced systemic resistance, including hypersensitivity responses, lignification, callose deposits, papillae formations as well as the accumulation of proteins, phenolic compounds, activation of peroxidases and phenylalanine ammonia lyase (Woo *et al.* 2023). Our results showed a significant increase in PPO activity for the preventive treatment compared to curative tests. Along the same line, this enzyme activity was significantly higher in the oil-formulation treatment than in the crude extract.

Following oxidative stress by accumulation of reactive oxygen species (O_2, OH^- and H_2O_2) produced by infected pods, they release PPO which reacts with reactive oxygen species by oxidation to give various molecules such as quinones. These quinones are highly reactive molecules that cause covalent modifications and cross-linking of nucleophilic substituents of amino acids and proteins. These modifications could exert an anti-nutritive defense against pathogens. In addition, quinones produced by PPO may possess direct antibiotic and cytotoxic activities against pathogens. Our study also showed a protein production that increased significantly only during treatment with biopesticides, while no difference was observed with preventive or curative treatments. A significant increase in the production of polyphenols was also observed during the preventive treatment compared to the curative

treatment with the emulsion. However, this production was lower than for uninfected controls. However, the protein levels would reflect their involvement in the defense process of the pods. After infection, the plant synthesizes a whole range of structural and enzymatic proteins which aim to ensure its defense and integrity. This suggests that the accumulation of proteins constitutes the basic substrate for the formation of polyphenols, flavonoids, tannins and defense enzymes such as PPO, PO and PAL (Fadiji and Babalola 2020). Thus, a strong production of phenolic compounds was observed due to a strong stimulation of the elicitors resulting from the action of *P. megakarya.* This could be due to a better diffusibility of the active ingredients of the emulsion than the pure extract, inducing a hypersensitivity reaction and early stimulation of resistance mechanisms involving acquired systemic resistance (Morán-Diez *et al.* 2023). Saxena *et al.* (2016) showed that the total phenol content in the leaves of plants treated with *Trichoderma* spp. which were infected with the pathogen was higher than that of plants treated only with *Trichoderma* spp. isolates. However, as seems to be the case in this work, the resistance is not always correlated to the quantity of phenols, but rather to the nature of the accumulated phenols. In this study, flavonoids were studied. Thus, the antimicrobial effect of phenolic compounds (flavonoids, tannins, etc.) is mainly linked to the content of its main classes and their involvement during plant-microorganism interaction. For its part, the production of flavonoids increases significantly during the preventive treatment with biopesticide compared to the curative treatment. As for the polyphenols, this production was lower than for the uninfected controls.

Conclusions

Trichoderma asperellum organic extract and its emulsion can significantly inhibit cocoa black pod disease. This could be due to various mechanisms such as the presence of bioactive compounds in the extracts and the induction of biochemical defense in treated cocoa pods. Therefore, organic extract from *T. asperellum* and its emulsion could be used as a biopesticide for eco- friendly control of cocoa black pod. However, field trials are needed.

Acknowledgement

The authors are grateful to the Laboratory of Biochemistry, Faculty of Science, University of Douala, for providing research facilities.

References

Aloke P., Rodas E.W., Deewakar B., Dipak K.H., Birendra N.P., Pabitra K.B., Ramen K.K. 2020. Development of nanoemulsion formulation of mustard oil, its chemical characterization and evaluation against post-harvest anthracnose pathogens. Indian Phytopathology 73: 449–460. DOI: 10.1007/s42360-020-00237-8

Bateman R. 2004. The use of narrow-angle cone nozzles to spray cocoa pods and other slender biological targets. Crop Protection 23: 989–999. DOI: 10.1016/j.cropro.2004.02.014

Bedine B.M.A., Sameza M.L., Iacomi B., Tchameni N.S., Fekam B.F. 2020. Screening, identification and evaluation of *Trichoderma* spp. for biocontrol potential of common bean damping-off pathogens. Biocontrol Science and Technology 30 (3): 228–242. DOI: 10.1080/09583157.2019.1700909

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Annal of Biochemistry 72 (1–2): 248–254.

Bruneton J. 2016. Pharmacognosie, Phytochimie, Plantes médicinales. Edition Lavoisier, 51 pp.

Chhipa H. 2017. Nanofertilizers and nanopesticides for agriculture. Environomental Chemistry Letters 15 (1): 15–22. DOI: 10.1007/s10311-016-0600-4

Efombagn M.I.B., Biyesse D., Nyasse S., Eskes A.B. 2011. Selection for resistance to Phytophthora pod rot of cocoa (*Theobroma cocoa* L.) in Cameroon: repeatability and reliability of screening tests and field observations. Crop Protection 30: 105–110. DOI: 10.1016/j.cropro.2004.02.014

Fadiji AE., Babalola, O.O. 2020. Elucidating mechanisms of endophytes used in plant protection and other bioactivities with multifunctional prospects. Frontiers in Bioengineering and Biotechnology 8: 467. DOI: 10.3389/fbioe.2020.00467

Fraceto L.F., Maruyama C.R., Guilger M., Mishra S., Keswani C., Singh H.B., De Lima R. 2018. *Trichoderma harzianum*-based novel formulations: potential applications for management of Next-Gen agricultural challenges. Journal of Chemical Technology and Biotechnology 93: 2056–2063. DOI: 10.1002/jctb.5613

Iwaro A.D., Sreenivasan T.N., Butler D.R., Umaharan P. 2000. Rapid screening for Phytophthora pod rot resistance by means of detached pod inoculation. p. 109–113. In: "Working Procedures for Cocoa Germplasm Evaluation and Selection". IPGRI, Rome.

Jaradat N., Qneibi M., Hawash M., Sawalha A., Qtaishat S., Hussein F., Issa L. 2020. Chemical composition, antioxidant, antiobesity, and antidiabetic effects of *Helichrysum sanguineum* (L.) Kostel. from Palestine. Arabian Journal for Science and Engineering 46 (1): 41–51. DOI: 10.1007/s13369-020-04707-z

Manzar N., Kashyap A.S., Goutam R.S., Rajawat M.V.S., Sharma P.K., Sharma S.K., Singh H.V. 2022. *Trichoderm*a: advent of versatile biocontrol agent, its secrets and insights into mechanism of biocontrol potential. Sustainability 14: 12786. DOI: 10.3390/su141912786

Martinez Y., Ribera Y., Schwarze F.W.M.R., France D. K. 2023. Biotechnological development of *Trichoderma*-based formulations for biological control. Applied Microbiology and Biotechnology 107: 5595–5612. DOI: 10.1007/s00253-023-12687-x

Mayer A.M., Harel E., Ben-Shaul R. 1966. Assay of catechol oxidase: a critical comparison of methods. Phytochemistry 5 (4): 783–789. DOI: 10.1016/S0031-9422(00)83660-2

Mendez R.W., Obregon M., Maran-Diiez E.M., Hermosa R. 2020. *Trichoderma asperellum* biocontrol activity and induction of systemic defenses against Sclerotium cepivorum in onion plants under tropical climate conditions. Biological Control 141: 104145. DOI: 10.1016/j.biocontrol.2019.104145

Morán-Diez M.E., Martínez de Alba Á.E., Rubio M.B., Hermosa R., Monte E. 2021. *Trichoderma* and the plant heritable priming responses. Journal of Fungi 7: 318. DOI: 10.3390/jof7040318

Ndoumbe-Nkeng M., Cilas C., Nyemb E., Nyasse S., Bieysse D., Flori A., Sache I. 2004. Impact of removing diseased pods on cocoa black pod caused by *Phytophthora megakarya* and on cocoa production in Cameroon. Crop Protection 23: 415–424. DOI: 10.1016/j.cropro.2003.09.010

Nirmala M.J., Durai L., Gopakumar V., Nagarajan R. 2020. Preparation of celery essential oil-based nanoemulsion by ultrasonication and evaluation of its potential anticancer and antibacterial activity. International Journal of Nanomedicine 15: 7651–7666. DOI: 10.2147/IJN.S252640

Rabuske E.J., Muniz B.F.M., Brun T., Saldanha A.M., Sarzi S.J., Savian G.L., Walker C., Rolim M.J., Zabot L.G., Mazutti A.M. 2023. *Trichoderma asperellum* in the biocontrol of *Lasiodiplodia theobromae* and *Pseudofusicoccum kimberleyense*. Journal of Plant Protection Research 63 (4): 488–498. DOI: 10.24425/jppr.2023.147832

Ristaino J.B. 2012. Methods of isolation of *Phytophthora* species. A Lucid Key to the common species of *Phytophthora*. Plant Disease 96: 897–903. DOI: 10.1094/PDIS-08-11-0636

Sameza M.L., Bedine Boat M.A., Tchameni N.S., Nguemnang M.C.L., Jazet D.P.M., Fekam, B.F., Menut, C. 2014 Potential use of *Eucalyptus globulus* essential oil against *Phytophthora colocaciae*, the causal agent of taro leaf blight. European Journal of Plant Pathology 140: 243–250. DOI: 10.1007/s10658-014-0457-y

Saxena A., Raghuwanshi R., Singh H.B. 2016. Elevation of defense network in chilli. Scientific Research 6 (5): 4270–4280. DOI: 10.3390/jof7040314

Scudder M., Wampe N., Waviki Z., Applegate G., Herbohn J. 2022. Smallholder cocoa agroforestry systems; is increased yield worth the labour and capital inputs? Agricultural Systems 196: 103350. DOI: 10.1016/j.agsy.2021.103350

Singh S.P., Keswani C., Minkina T., Sensinenea F. 2023. Nanoinputs: a next-generation solution for sustainable crop production. Journal of Plant Growth Regulator 42: 5311–5324. DOI: https://doi.org/10.1007/s00344-023-10943-y

Soumanou M.M., Adjou E.S. 2016. Sweet fennel (*Ocimum gratissimum*) Oils. p. 765–773. In: "Essential Oils in Food Preservation, Flavor and Safety" (Preedy V.R., ed.). Academic Press.

Tchameni N.S., Sameza M.L., O'donovan A., Fokom R., Mangaptche N.E.L., Wakam N.L., Etoa, F.X, Nwaga D. 2017. Antagonism of *Trichoderma asperellum* against *Phytophthora megakarya* and its potential to promote cacao growth and induce biochemical defence. Mycology 8 (2): 84–92. DOI: 10.1080/21501203.2017.1300199

Tchameni S.N., Cotarleī M., Ghinea I.O., Bedine B.M.A., Sameza M.L., Borda D., Bahrim G., Dinica, R.M. 2020. Involvement of lytic enzymes and secondary metabolites produced by *Trichoderma* spp. in the biological control of *Pythium myriotylum*. International Microbiology 23 (2): 179–188. DOI: 10.1007/s10123-019-00089-x

Vinale F., Sivasithamparam K. 2020. Beneficial effects of *Trichoderma* secondary metabolites on crops. Phytotherapy Research 34: 2835–2842. DOI: 10.1002/ptr.6728

Waligóra M., Nowicka S., Idziak R., Ochodzki P., Szulc P., Majchrzak L. 2023. The total phenolic compound and sorgoleone content as possible indirect indicators of the allelopathic potential of sorghum varieties (*Sorghum bicolor* (L.) Moench). Journal of Plant Protection Research 63 (4): 450–458. DOI: 10.24425/jppr.2023.146869

Woo S.L, Pepe O. 2018. Microbial consortia: Promising probiotics as plant biostimulants for sustainable agriculture. Frontier in Plant Science 9: 180. DOI: 10.3389/fpls.2018.01801

Woo S.L., Hermosa R., Lorito M., Monte E. 2023. *Trichoderma*: a multipurpose,plant-beneficial microorganism for ecosustainable agriculture. Nature Review Microbiology 21 (5): 312–326. DOI: https://doi.org/10.1038/s41579-022-00819-5