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Antagonistic Effect and Antifungal Activity of Organic Extracts of *Trichoderma harzianum* and *Trichoderma asperelloides* toward *Fusarium oxysporum elaedis*, the Causal Agent of Fusariose Oil Palm

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Abstract This study aimed to evaluate the antagonistic effet and organic extract of *T. harzianum* and *T. asperolloide*s against *Fusarium oxysporum elaedis*, agent responsible of fusariose of oil palm (*Elaeis guineensis Jacq*). *In vitro*, the antagonistic activities were carried out by direct confrontation and antibiosis. The ability of *Trichoderma* spp to produce hydrolytic enzymes was determined in specific solid media. After fermentation, organic extracts were obtained, phenolic and flavonoid compounds were evaluated and their antifungal activity was done *in vitro* against mycelial growth of the pathogen. The results obtained show that the both antagonists used have significantly reduced the mycelian growth of the pathogen. In direct cofrontation, the inhibition of myelian growth was 64.58% and 70.8% respectively, for *T. harzianum and T.asperelloides*. The non-volatile compounds produced the inhibition of mycelial growth of *Fusarium oxysporum.* The inhibition *was* 75.0% and 71.0%, respectively. The inhibition percentage of volatile compounds was 43.75% and 25.00% respectively. *T. harzianum* and *T. asperelloides* produced respectively, cellulases (62.3mm and 66.3mm), lipases (43mm and 66.7mm), amylases (44.6 mm and 68.3 mm) and proteases (54.6 mm and 52mm). The organic extract of these antagonists' content phenolic and flavonoids compounds. At 400 µg/ml these organic extracts significantly inhibited the mycelial growth of *F. oxysporum*. The inhibition was 69.29% and 40.69% respectively for crude extract of *T. harzianum* and *T. asperelloides*. There were significant and positive correlation between polyphenol and flavonoid content and the inhibition of mycelial growth of the pathogen. These results showed that, *T. harzianum* could be used to develop a bioproduct to protect oil palm.

Keywords: *Antagonistic activity, hydrolytic enzymes, Trichoderma, Fusarium oxysporum elaedis, oil palm*

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1. Introduction

The oil palm (*Elaeis guinnensis* Jacq.) is an important subtropical crop widely cultivated for its fruits which are one of the highest oils yielding in the word. It is also used for diverse purposes, including the production of food, oilseed products, dietary supplements, biofuels as well as pharmaceutical and cosmetic. Despite their importance, oil palm faces to many constrains including diseases such as basal stem rot, bud rot and vascular wilt which reduce the yield of the oil and affect growth [1]. In Cameroon, vascular wilt disease caused by *Fusarium oxysporum* is one of the most devasting diseases [2]. It is characterized by discoloration and blockage of the xylem vessels. This vascular discoloration occurs at the stems and can spread systemically to the petiole. Vascular wilt disease seriously affects small farms and several oil palm and can cause more than 70% mortality [3].

To menage the disease, farmers commonly used many control measures such as resistant plant material selection and chemical pesticides [1]. However, repeat and hazardous used of chemical product is toxic for humans and their environment and above all its ability to cause development of trains of resistant of pathogens [4]. An

alternatives that are overcome the limits show is the use of natural substances. The use of biological control agents such as *Trichoderma* genus fungi would be a promising pathway [5,6,7]. *Trichoderma* genus fungi are filamentous and saprophytic microorganisms that are found naturally in the rhizosphere of plants and subwoods [8]. They are able to fight several plant pathogens by using a diversiy of mechanisms action such as direct confrontation, mycoparasitism, antibiosis and stimulation of the defense and growth mechanisms in the plant [9]. To our knowledge, very few studies aimed at assessing the protective potential of oil palm through the use of antagonistic microorganisms of the *Trichoderma* genus have been conducted. The objective of this work is to study the antagonistic potential of *T.harzianum and T.asperelloides* isolates against *Fusarium oxysporum elaedis,* agent responsible for the fusariose oil palm.

2. Material and Methods

2.1. Microbial Isolates

The microbial isolates used in this study were obtained from the collection of the Laboratory of Biochemistry of University of Douala. The antagonists (*T. harzianum* and *T. asperelloides*) were previously isolated from the rhizosphere of Cameroonian fields and identified by Bedine (2020) [7]. *Fusarium oxysporum elaedis* was isolated from root tissues of infected oil palm showing typical symptoms of fusariose and the pathogenicity test was carried out according to Koch's postulate [11].

2.2. *In vitro* Antagonist Potentiel of *Trichoderma*

The antagonistic activities of the *Trichoderma* spp. were carried out *in vitro*, on the PDA (Potato Dextrose Agar) medium by direct confrontation and by ability to produce bioactive volatile and non-volatile substances.

2.2.1. Direct Confrontation

The antagonistic potential of *Trichoderma* isolates was evaluated against *F. oxysporum* using dual culture. Mycelial discs (5 mm diameter) were taken from 3 days old cultures of the antagonist and the pathogen. The discs were then paired on PDA agar plate in 90 mm Petri dishes. Plates inoculated only with the antagonist or pathogen served as control. All culture plates were incubated at room temperature (25±2°C) after 7 days and the data expressed as the inhibition (%I) of *F. oxysporum*. estimated according to the following formula: %I = 100(Ro – Rt)/Ro, with Ro *:* radial growth of pathogen in the control plates and Rt :radial growth of pathogen in dual culture. Each treatment was done in triplicate and the experiment was repeated three time.

2.2.2. Assessment of the Effect of Volatile and Non-volatile Substances

The effect of non-volatile and volatile substances released by *Trichoderma* on *F. oxysporum* was carried out according to the method used by Bedine et al [7]. In the case of volatile compounds, 5 mm of mycelial disc taken from 2-days of pre-culture of *Trichoderma* was inoculated in the center of a 9 cm PDA plate. The lid of the Petri dish was replaced with a centrally inoculated dish with *F. oxysporum*. The 2 plates were sealed with a paraffin plug and incubated at 28°C for 4-days. In the control plate, the antagonist was replaced by an agar disc. For non-volatile compounds, each antagonist was cultured for 2-days on a sterile cellophane disc placed on PDA in a 9 cm Petri dish. In the control plate, *Trichoderma* has been replaced by an agar disc. The cellophane with the mycelium was then removed and the test pathogen inoculated for a 7-days incubation period at 28°C. For both tests, each treatment consisted of 3 plates and the experiments were repeated 3 times. The radial growth of *F. oxysporum* was measured and the inhibition of mycelial growth was evaluated according to the formula %I = 100(Do – De)/Ro, with Do*:* radial growth of pathogen in the control plates and De: radial growth of pathogen in dual culture.

2.3. Assessment of Extracellular Enzyme Production

2.3.1. Lipases Activity

Tween 80 (polyoxyethylene sorbitan monooleate) agar medium as substrate lipid described by Sierra (1957) and containing 0.01% phenol red was used to detect the production of lipolytic enzymes. Enzymatic activity is characterized by the appearance of an opaque halo around the colonies [11].

2.3.2. Proteases Activity

Presence of proteolytic activity was carried out on milk agar (20% milk agar). The strains after seeding were incubated at ambient laboratory temperatures for 3 days. The hydrolysis of milk casein is characterized by the observation of a clear and transparent zone around the colony [12].

2.3.3. Amylases Activity

Amylase activity was done on PDA medium containing 1% soluble starch [13]. After inoculation, the dishes are incubated at 30±2°C. for 3 days. The revelation was made by flooding the agar medium with a lugol solution for 30 seconds followed by rinsing with distilled water. Starch hydrolysis is indicated by the appearance of a clear zone around the colony [13].

2.3.4. Cellulases Activity

Cellulolytic activity was evaluated on carboxymethylcellulose agar (CMC) medium [14]. After growth, the dishes are stained with a Congo red solution (0.1%). for 30 minutes of reaction at ambient temperature and washed with a solution of NaCl (1M) for one hour. The appearance of clear zones around the colonies allows the demonstration of cellulolytic activity producing cellulase [14].

2.4. Extraction and Evaluation of Antifungal Activity of Organic Extract of *Trichodema sp*.

2.4.1. Extraction of Organic Extract

Each isolate of *Trichoderma* was fermented in solid medium in stationary mode for 30 days. Unhusked rice was used as substrate [15]. The extraction of secondary metabolites was carried out by maceration with ethyl acetate for 7 days at room temperature. The organic extract was obtained by evaporation of solvent under reduced pressure at 40°C. The crude extracts obtained were stored at 4°C until use.

2.4.2. Assays of Total Polyphenol and Flavonoid Content

The assay of total polyphenol was carried out using Folin-Ciocalteu's reagent according to the method described by Wood et al. (2002) [16]. To a volume of 100 µL (1mg/ml) of each extract were added 2.5 mL of Folin-Ciocalteu reagent diluted to 1/10th. The resulting mixture was kept for 2 min in the dark at room temperature and then 2 mL of 2% sodium carbonate solution was added. The resulting solution was then incubated at 50°C for 30 min. The absorbance was read using a UV-visible spectrophotometer at a wavelength of 760 nm against a constituted blank or the extract was replaced by methanol. The quantification of the total polyphenol content expressed in mg of gallic acid equivalent per gram of extract (mg EAG/g of extract) from the calibration curve of type Y = ax+b using gallic acid as standard.

The total flavonoid content was determined according to the method described by Marinova et al [17]. 0.75mL of 5% (m/v) sodium nitrite solution and 0.75mL of 10% (m/v) aluminum chloride solution were added to 2.5mL of extract solution. After 5 min of incubation, the mixture was brought into contact with 5 mL of a 1 M sodium hydroxide solution. The volume obtained was adjusted to 25 mL then stirred vigorously. Absorbance was measured at 510 nm against a blank containing no extract. Quantification of the total flavonoid content expressed in milligrams of quercetin equivalent per gram of extract (mg EQ/g of extract). The flavonoid content of the hydroalcoholic extract and the fractions were determined from the Y= ax+b type calibration curve using quercetin as a standard.

2.4.3. Antifungal Activity of *Trichoderma* Organic Extracts

Inhibition of mycelial growth of *F. oxysporum* was assessed by the food poisoning method. A five mm diameter disc was taken from the 2-day-old pathogen preculture and incubated in the center of a 9 cm diameter PDA medium containing the crude extract (previously dissolved with tween 80 at a 1:9 ratio) of each antagonist at varying concentrations from 50 to 400 µg/ml. The control was made by plate containing PDA with Tween 80. Plates were incubated at 25 ± 2°C and radial growth was evaluated 7 days after incubation. Each treatment consisted of four Petri dishes and the experiment was repeated twice. Inhibition (%I) of the mycelial growth was calculated using the following formula: %I = ((Do – De)/Do) 9100 where Do was the diameter of the mycelial growth in the control and the diameter of the growth of th mycelial in the plates supplemented with crude extract. [10]

3. Results

3.1. Antagonistic Effects of *T.harzianum* and *T. asperelloides* on Mycelial Growth of *F. oxysporum*

In direct confrontation, the mycelial growth of *Fusarium oxysporum* was significantly inhibited by the both antagonists (Table 1 and photography 1). The inhibition percentage was 64.58% and 70.8% respectively, for *T. harzainum* and *T. asperelloides*.

Table 1. Antagonists effects of *Trichoderma* isolates on mycelial growth inhibition of *F. oxysporum*

	Dual culture	Volatile compounds	Non volatile compounds
	Inhibition (%)		
T. harzianum	64.58±3.60[a]	75.0±0.0[b]	43.75±6.25[b]
T. asperelloides	70.83±3.0[b]	71.0±0.0[a]	25.0±0.0[a]

Treatments with the same letter are not significantly different at the 5% level.

The non-volatile compounds produced by the antagonist significant inhibited the mycelial growth of *Fusarium oxysporum*. The inhibition was 75% for *T.harzianum* and 71% and for *T. asperelloides*.

The volatile compounds released by *T.harzianum* and *T.asperelloides* inhibited the growth of the pathogen at 45.75% and 25.00%, respectively.

3.2. Production of Lytic Enzymes

Lytic enzymes activities were characterized by halo zone formation around *Trichoderma* colony. The both *Trichoderma* isolates used produced cell wall degrading enzymes, depending of enzyme and the isolate (Figure 1 and Photography 2). The diameter of the halos of cellulases were 62.33mm and 66.33 mm for *T. harzianum* and *T. asperelloides*, respectively. The zone diameter of lipases was 43.5 mm for *T. harzianum* and 66.6mm for *T. asperelloides*. The halo diameter of amylases was 68.33 mm for *T. harzanum* and 44.67 for *T. asperelloides*. In the case of proteases, the halo diameters were 54.7 mm for *T. harzianum* and 52.0 for *T. asperelloides*. The *T. harzianum* activity approximates that of *T. asperelloides*.

3.3. Polyphenols and Flavonoids Content

The results showed that, the crude extract of *T. harzianum* and *T. asperelloides* are rich in polyphenols and flavonoids (Figure 2). The polyphenols content were 0.13mg/ml for the extract of *T. harzianum* and 0.067 mg/ml for the extract of *T. asperelloides* while, the flavonoids content was 0.18 mg/ml and 0. 14mg/ml, respectively.

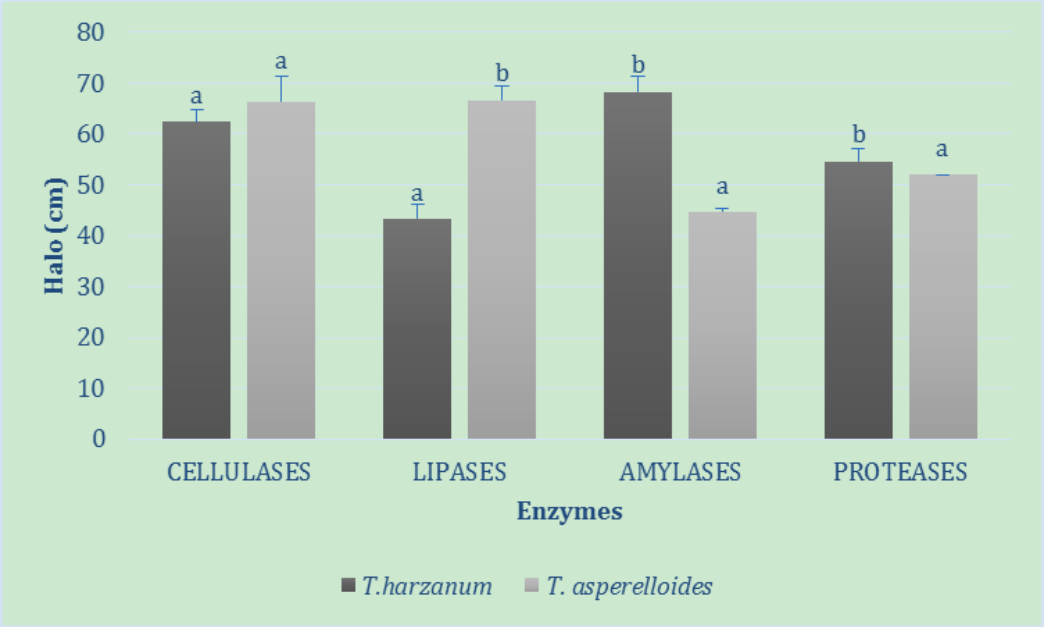
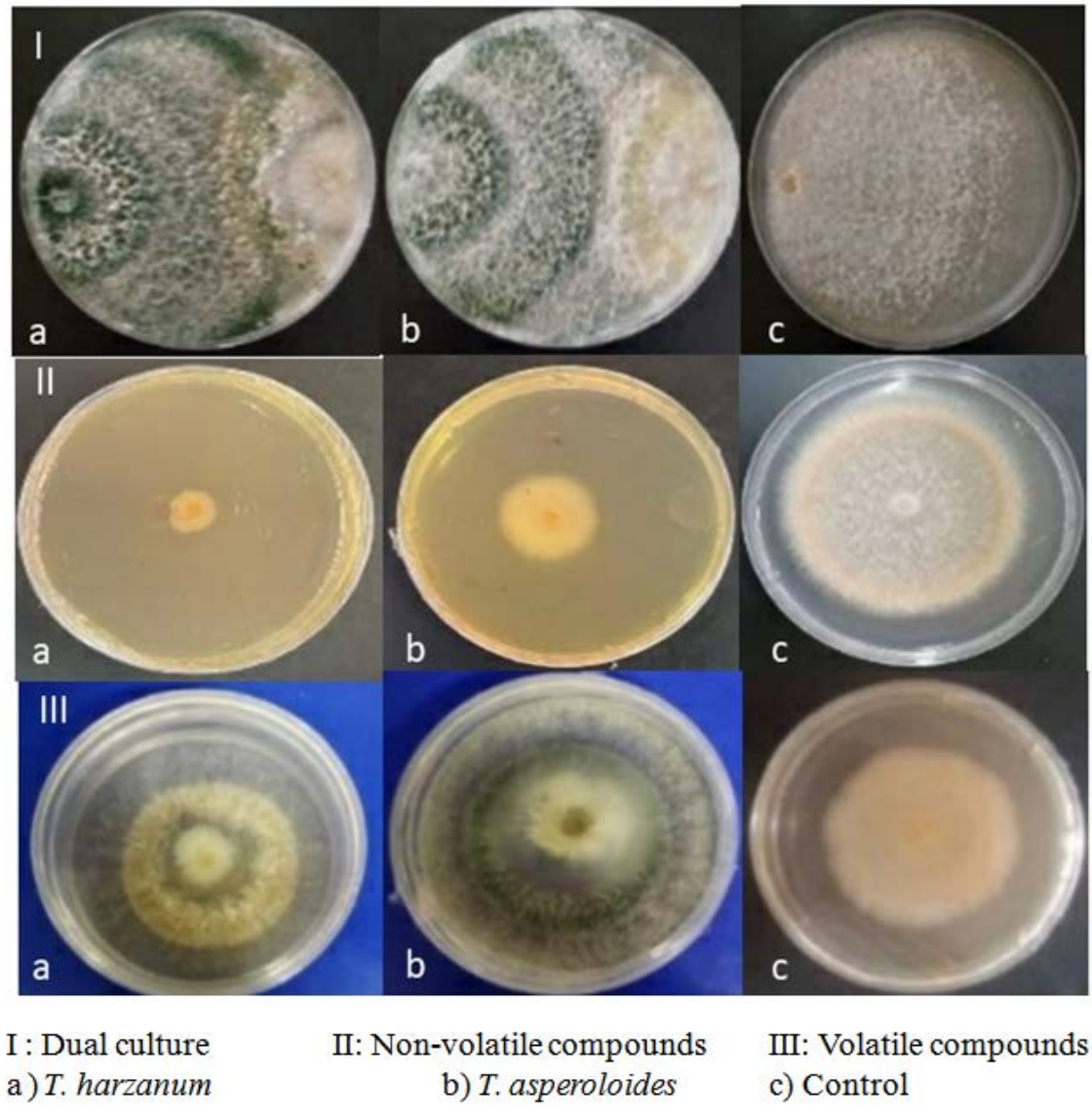


Figure 1. Production of hydrolytic enzymes by *Trichoderma* isolates (In each histogram, treatments with the same letter are not significantly different at $p \leq 0.05$)



Photography 1. Antagonist effects of Trichoderma isolates on mycelial growth of *F. oxysporum*

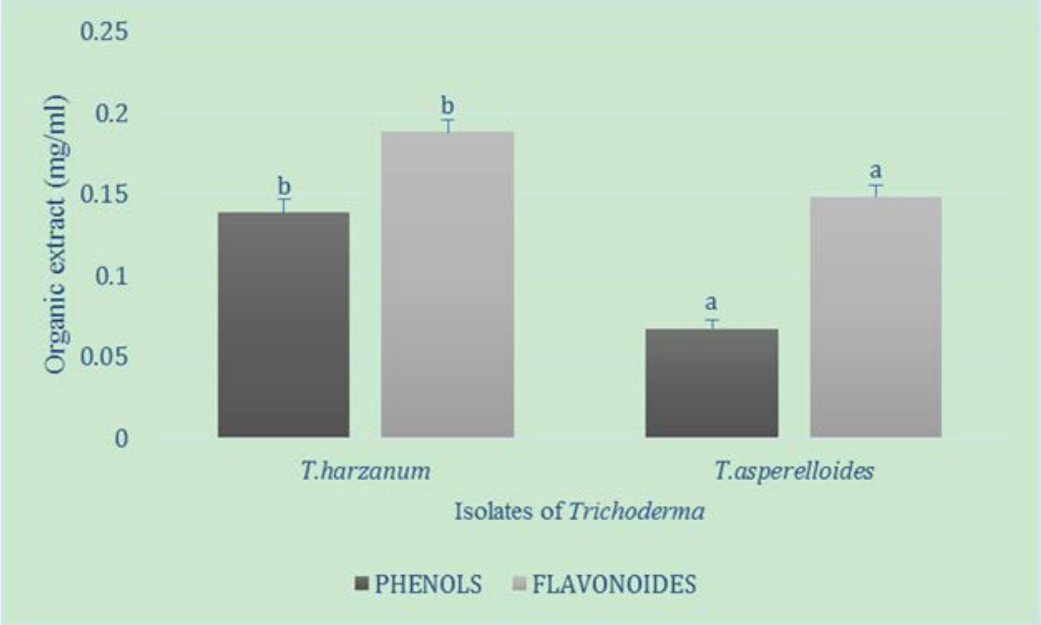
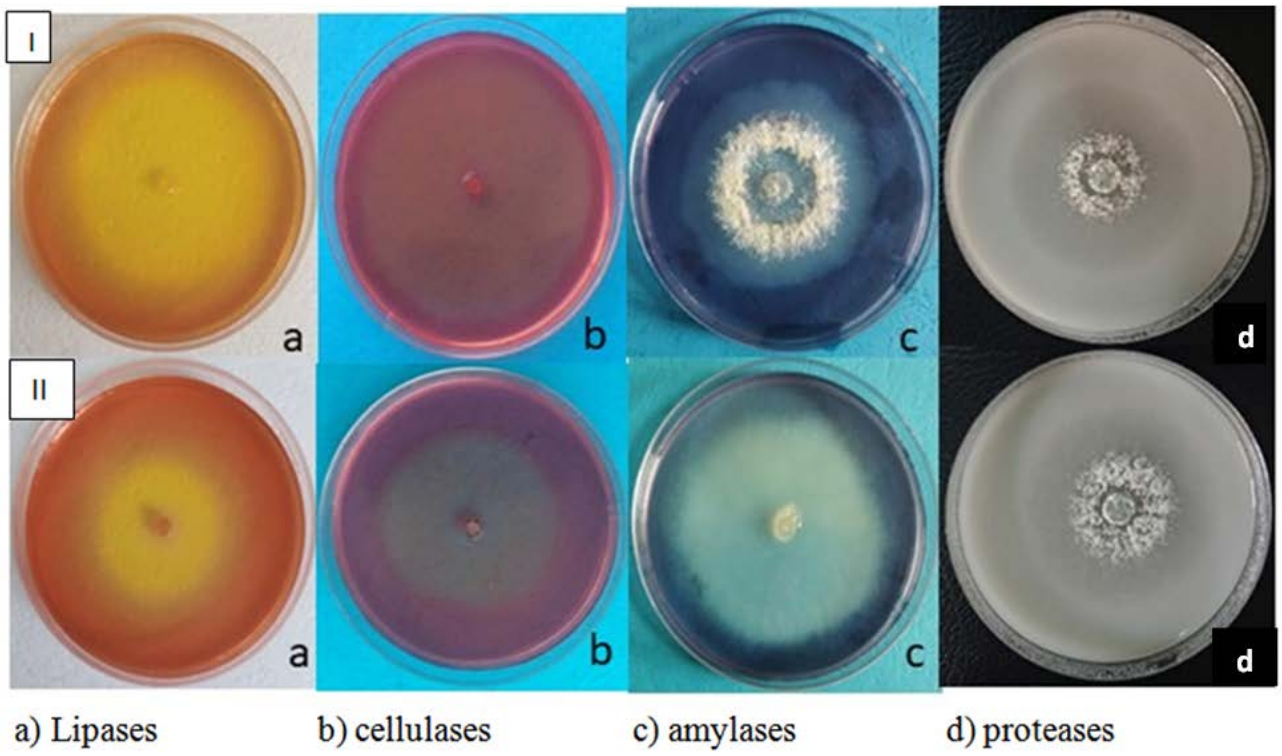


Figure 2. Phenols and flavonoids content of organic extracts of Trichoderma isolates (In each histogram, treatments with the same letter are not significantly different at $p \leq 0.05$)



Photography 2. Release of hydrolytic enzymes by *Trichoderma* isolates (I: *T. harzianum*, II: *T. asperelloides*)

3.4. Antifungal Assay

The crude extract of *T.harzianum* and *T. asperelloides* significantly inhibited the mycelial growth of *F. oxysporum* in a dose dependent manner (Figure 3). At 4000µg/ml, the inhibition was 69.29% and 40.69% respectively for crude extract of *T.harianum* and *T. asperelloides.* From extract of *T.harianum*, there were significative and negative correlation between the inhibition of *F. oxysporum* and the polyphenol content (p=0.00; r = 0.85) and the inhibition of mycelial growth of th pathogen and the flavonoid content (p = 0.01; r = 0.78).

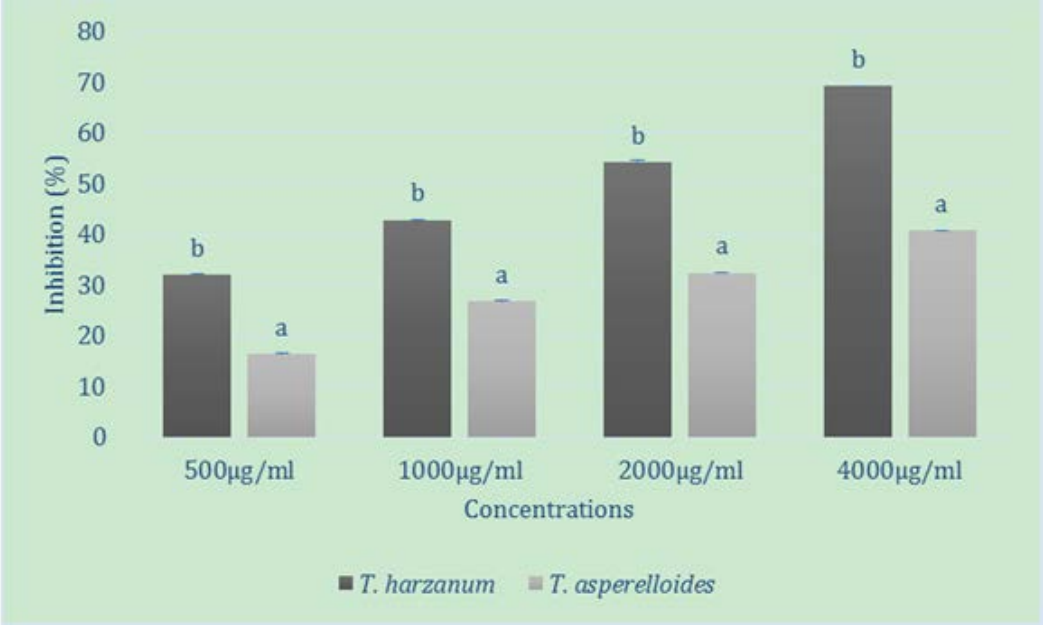


Figure 3. Efficacy of organic extracts of *Trichoderma* on mycelian growth of *F. oxysporum* (In each histogram, treatments with the same letter are not significantly different at $p \leq 0.05$)

4. Discussion

The *in vitro* antagonistic activity showed the potential of the isolates tested to inhibit the mycelian growth of *F. oxysporum.* This could be due to their ability to release volatile and non-volatile secondary metabolites but also to their high enzymatic activity. The production of secondary metabolites plays an important role in the biological control process as they can affect plant interactions with their pathogens [18,19]. Reino *et al.*, 2008 [20], highlighted the antibiotic properties of secondary metabolites of biological control agents allowing them to inhibit other phytopathogenic micro-organisms. Some of these secondary metabolites produced by *Trichoderma* would be able to inhibit the bioactive molecules produced by other fungi [6]. Secondary metabolites are also thought to be involved in inducing plant resistance (Peptaibols, 6-pentyl-2H-pyran-2-one) and improving their growth (Koninginins, harzianopyridone, harzianolide, harzianic acid) [19].

Trichoderma isolates have shown strong lytic enzyme producing potential. De Marco et al [21] revealed the potential of *Trichoderma harzianum* isolate to produce hydrolytic enzymes. Other authors have demonstrated the ability of different isolates of *Trichoderma* to release hydrolytic enzymes such as N-acetyl-β-D-glucosaminidase, chitin 1,4-β-chitobiosidase, glucan 1,3-β-glucosidase, cellulase, xylanase and protease [7,22,23]. These enzymes are thought to be involved in the degradation of the cell walls of phytopathogens and interactions with the plant. The effectiveness of *Trichoderma* fungi is based on their mode of action, which includes competition for nutrients (iron, nitrogen, carbon), mycoparasitism or hyperparasitism, induction of systemic resistance in the plant, the production enzymes that degrade the cell membrane and antibiosis through the production of a variety of compounds with several biological activities [6]. Antagonistic activity are well known to characterize by production of non-volatile and volatile organic metabolites [19]. These compounds play a key role during the pathogen attack. Non-volatile organic metabolites, such as phenolic compounds and flavonoids, can significantly inhibit the mycelial growth of soil borne pathogens [24,25]. The present study showed that the non-volatile organic compounds produced by *T. harzianum* and *T. asperelloides* significantly reduced the mycelial growth of *F. oxysporum eladies*. This suggests that during the antagonism, these compounds may be involved in the suppression of the pathogen. The results of Pakora et al. [25] showed that crude organic extracts from *T. harzanum* and *T. asperelloides* significantly inhibited the mycelial growth the pathogen causing cocoa black pod. Moreover, Leylaie and Zafari [26] obtained a significant correlation between the anthraquinones (polyphenolic compounds) produced by *Trichoderma* and the antimicrobial activities against a panel of fungus pathogens. Our results showed a significant correlation between the antifungal effects and the production of total polyphenols and flavonoids. Polyphenol compounds such as flavonoids could act by attaching to the cell wall of pathogens and disorganizing their membrane structure. They could also be able to inhibit the activity of lytic enzymes produced by the pathogen [19]. The antifungal activities of organic extract could be due to synergistic effects of non-volatile and volatile organic compounds. Many species of *Trichoderma* produce volatile organic compounds that have inhibited soil borne pathogens [19,21]. Volatile secondary metabolite could confer resistance to *Trichoderma* against biotic stress factors and could protect its cell walls against the action of cell wall enzymes released by pathogens. In addition, in paired culture, the production of volatile compounds is better when *Trichoderma* was confronted with pathogens [27].

5. Conclusion

This study showed the antagonistic and antifungal effect of organic extract of *T. harzianum* and *T. asperelloides* against *Fusarium oxysporum*. These could be explained by production of hydrolytic enzymes and secondary metabolites by the both antagonists. The better antagonist was *T.harzianum* and it could be used as alternative to control fusariose oil palm disease.

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