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Appraisement of antimicrobial potential of organic extracts of *Trichoderma virens* and *Trichoderma asperellum* against *Phytophthora colocasiae*, the causal agent of taro leaf blight

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Moïse Ntah A Ayong[1], Henri Tibo Ambata Ambata[1], Marie Ampère Boat Bedine[2], Eveline Siebatcheu[1], Olivier Youassi Youassi[1], Severin Nguemezi Tchameni[1]*, Modeste Lambert Sameza[1] and Jean Duplex Wansi[3]

[1]Laboratory of Biochemistry, Department of Biochemistry, Faculty of Sciences, University of Douala, Cameroon, P. O. Box 24 157 Douala, Cameroon.
[2]Phytopathology and Agricultural Zoology Research Unit, Department of Agriculture, Faculty of Agronomy and Agronomic Sciences, University of Dschang, P. O. Box 96, Dschang, Cameroon.
[3]Laboratory of Chemistry, Department of Chemistry, Faculty of Sciences, University of Douala, Cameroon, P. O. Box 24 157 Douala, Cameroon.

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ABSTRACT

This work aimed to evaluate the bioactive potential of organic (ethyl acetate) crude extracts from two *Trichoderma* strains against *Phytophthora colocasiae*, the causal agent of taro leaf blight. *Trichoderma* sp. were isolated from taro rhizosphere by the soil dilution method and identified by amplifying the sequences of ITS1 and ITS4. Organic extracts were obtained from liquid fermentation, and the secondary metabolites were quantified using a spectrophotometer. Antimicrobial activities of the extracts were evaluated on mycelial growth, zoospores, and sporangia germination, as well as the reduction of leaf necrosis on detached taro leaves. The results obtained show that molecular typing identified antagonist fungal isolates as *Trichoderma virens* (MN833411.1) and *Trichoderma asperellum* (MN452804.1) with 99.2% and 100% similarity, respectively. All the organic extracts contained high amounts of polyphenols, flavonoids, tannins, and significantly inhibited the mycelial growth, zoospores, and sporangia germination of *P. colocasiae*. The total inhibition of mycelial growth was obtained at 100 µg/plug and 200 µg/plug respectively, for organic extracts of *T. virens* and *T. asperellum*. For both organic extracts, at 4 µg/plug, total inhibition of the zoospores' germinations were obtained while at 2 µg/plug, we observed total inhibition of sporangia germination. At 800 µg/mL, these organic extracts also significantly reduced necrosis development on detached taro leaves, both for healing (55.35 and 37.92% respectively, for extracts of *T. virens* and *T. asperellum*) and preventive testing (55.35 and 37.92% respectively, for the extracts of *T. virens* and *T. asperellum*). This findings suggest that organic extracts from *T. virens* and *T. asperellum* could be used in an eco-friendly way to manage taro leaf blight.

INTRODUCTION

Due to its nutritional value and socioeconomic impact, taro (*Colocasia esculenta*) is an important subsistence food in subtropical countries including Cameroon (Rao et al., 2010). However, its cultivation faces several constraints

throughout the world, leading to a yield loss of 50-100%. Among them, taro leaf blight (TLB) caused by an Oomycete namely *Phytophthora colocasiae* is the most devastating pathogen (Tyson, 2012). The pathogen appeared in Cameroon in 2009 and a severe epidemic ensued in 2010, with an alarming loss of crop yield reaching 90% (Mbong et al., 2013). The disease has been persistent and is typically devastating.

The management of the taro leaf blight is based on the repeated use of chemical fungicides, which are potentially harmful to the environment, humans, and animals and could result in the development of resistant strains (Otieno, 2020; Tabi et al., 2021). The excessive use of synthetic chemicals in farming systems led governmental and international institutions to limit their use and encourage interest in sustainable food and agriculture (Kalfas et al., 2022), using alternative healthcare methods. One of them is the use of fungal antagonists, especially *Trichoderma*.

The genus *Trichoderma* includes important biocontrol agents that have been successfully applied as biopesticides worldwide and are well-known for their multi-pronged action against several economically important plant pathogens (Harman et al., 2004). They use several mechanisms of action, such as competition for nutrients and space, mycoparasitism, promotion of plant growth and/or defense responses, and antibiosis (Woo et al., 2014; Tchameni et al., 2020; Bedine et al., 2021). The latter could be defined as the secretion of biological metabolites by certain microorganisms to inhibit the growth or development of other microbes. The efficacy of beneficial *Trichoderma* strains has often been related to the great array of secondary metabolites that they are able to produce, belonging to various classes of chemical compounds and showing different biological activities such as phytopathogen growth inhibition or stimulation of plants' defense mechanisms (Arjona-Girona et al., 2014; Wu et al., 2015; Tchameni et al., 2017). The organic crude extract of *Trichoderma* contains a mixture of secondary compounds. These molecules may show antimicrobial potential and have been used for biological control. Among these compounds, cyclopentenone derivatives (Zhu et al., 2021), octahydronaphthalene derivatives (Sofian et al., 2021), a-pyrone and decalin derivatives (Nuansri et al., 2021) sorbicillinoids (Ma et al., 2021), (Z)-5-amino-5-(1,1,2-trihydroxybuta-1,3-dienyloxy)pentane-6,7,8,9-tetraol (Siebatcheu et al., 2021) and others have been recently described to protect plants from diseases. However, very little work has focused on the ability of *Trichoderma* extracts to control taro leaf blight. This work aimed to evaluate the effect of organic extracts of these two *Trichoderma* strains against *P. colocasiae*, the causative agent of taro leaf blight.

*Corresponding author. E-mail: tchameni1@yahoo.fr

MATERIALS AND METHODS

Plant pathogens

Phytophthora colocasiae isolate used in this study was obtained from the core culture collection of the Laboratory of Biochemistry (University of Douala). This pathogen was previously isolated from taro field crops in Cameroon by Ntah et al. (2018) and was routinely maintained on PDA slants at 4°C and sub-cultured bimonthly until use.

Isolation and molecular identification of *Trichoderma*

Isolation

Trichoderma isolates were purified from soil samples collected from rhizosphere of taro plants taken from Littoral Region of Cameroon. Each collected soil sample was thoroughly mixed, grounded in a mortar and passed through a 0.5 mm diameter soil screen sieve. One gram (1g) of soil sample was added to 9 mL of sterile distilled water. The mixture was homogenized with magnetic stirrer and a serial dilution prepared at 10-2 and 10-3. One milliliter of each dilution was inoculated on Potato Dextrose Agar (PDA) medium supplemented with antibiotic (streptomycin at 250 mg/L) in 9 cm Petri dishes and spread out. Emerging mycelia of fungal colonies were replicated after 48 h of incubation into new PDA medium and purified by successive transfers.

Molecular identification of *Trichoderma* isolates

Extraction of DNA

Mycelial samples (10 mg) of the various isolates were taken from the 5-days preculture on PDA medium by mat scraped from the surface of culture and introduced into eppendorf tubes. These eppendorf tubes received 100 µl of lysis buffer (Tris-HCl; pH 7.2 in 50 mM EDTA, 3% SDS, 1% 2-mercatoethanol). Then the entire mixture was shaken for 1 min and introduced in the microwave for 15, 10 and 5 seconds respectively with 5 to 10 second intervals. The eppendorf tubes thus treated received 300 µL of lysis buffer and were incubated at 80°C. for 10 mins in a sea bath. After incubation, 400 µL of a Phenol-Chloroform-Isoamyl-alcohol mixture (25:24:1) was added to the tubes and the whole was shaken for 15 seconds then centrifuged at 15,000 g for 15 mins. The aqueous phase of each tube was recovered and introduced into a new tube containing 10 µL of potassium acetate (5 M) and 180 µL of isopropanol. The whole was then incubated for 5 mins on ice and centrifuged for 10 mins at 10000 g. The supernatant was removed and the pellet was washed with 250 µL of 80% ethanol, then centrifuged again for 10 mins

at 1000 g. The pellet thus obtained, representing the isolated DNA, was dried for 10 mins using a SpeedVac solvent evaporator. DNA was then suspended in 100 µL of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored at -20°C until use.

Molecular Identification

Identification of *Trichoderma* isolates was performed by amplification and analysis of the Internal Transcribed Spacer (ITS) region of ribosomal DNA (rDNA). The amplification was made using the universal primer pairs ITS-1 (5'-TCCGTTCCTCAACCAGCGG-3' (19bp)) and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3'(20 bp)). The reaction mixture for PCR amplification consisted of 45 µL of a blend (5 µL buffer, 0.4 dNTPS (10 mM), 2.5 µL of ITS1 and ITS4 primer each, 0.25 µL of Taq polymerase (5 U/µL), 34.35 µL of sterile distilled water) and 5 µL of genomic DNA (5 to 40 ng). The amplification reaction was carried out in a Progene Thermocycler from Tech (Cambridge) according to the following program: A denaturation step at 94°C for 4 mins, A pairing step (Hybridization) of the primers with the DNA template at 55°C for 2 mins, one elongation step at 72°C for 1 min (Kumar et al., 2016). The products from the PCR amplification were sequenced using an automatic DNA sequence analysis system (Beckman Coulte Hair Sequencer 2000 XL) for 15 hours and raw data were then analyzed by the CEQ XL software version 4.3. The sequences obtained were subjected to a sequence homology search using the BLAST (Basic Local Alignment Search Tool) program on the NCBI (National Center for Biotechnology Information) site to search for the closest sequences deposited in GenBank (http: //www.ncbi.nlm.gov/BLAST) (Feitosa et al., 2019).

Evolutionary relationships of taxa

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of base differences per site. This analysis involved 18 nucleotide sequences. All positions with less than 100% site coverage were eliminated, i.e., fewer than 0% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There was a total of 470 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

***In vitro* antagonism test**

Dual culture test

Dual culture was carried out by pairing *Trichoderma* and *Phytophthora* on V8 agar medium. Hence, 5 mm mycelial disc of 3-days precultures of *Trichoderma* and *Phytophthora* were inoculated in diametrically opposite positions, equidistant to the center of 9 cm Petri dish containing 10 mL of V8 agar medium. *P. colocasiae* was inoculated two days before the antagonist. Petri dishes inoculated only with pathogen served as control. The dishes thus seeded were incubated at room temperature (25° C) for 7 days and the radial growth of each microorganism measured. Each treatment consisted of three replicates and the experiment repeated twice. The inhibition of *P. colocasiae* mycelial growth by *Trichoderma* was estimated using the following formula:

$$\% I = [(Do - Dx) / Do] \times 100$$

Where % I: inhibition percentage of radial mycelial growth; Dx: radial mycelial growth of *P. colocasiae* in the presence of *Trichoderma*; Do: radial mycelial growth of *P. colocasiae* in the absence of *Trichoderma*.

Antibiosis

Eighty-millimeter diameter sterile cellophane discs was aseptically deposited on the surface of 10 mL of V8 agar medium in 90 mm Petri dishes and 5 mm *Trichoderma* explant inoculated at the center. After 2 days of incubation at room temperature (25°C), the cellophane containing *Trichoderma mycelia* was removed and a *P. colocasiae* disc inoculated. *P. colocasiae* plug was replaced by an agar disc in the control treatment. The Petri dishes were further incubated at room temperature (25°C) for 3 days and the radial growth of *P. colocasiae* measured. The inhibition percentage was estimated by the formula:

$$\% I = [(Do - Dx) / Do] \times 100,$$

Where % I: inhibition percentage of mycelial growth; Dx: mycelial diameter of *P. colocasiae* in the test dish; Do: mycelial diameter of *P. colocasiae* in the control.

The microbicidal or microbiostatic effect of non-volatile compound was estimated by transferring the *P. colocasiae* plug from the Petri dishes presenting 100 % inhibition to fresh media.

Evaluation of the antimicrobial activity of *Trichoderma* organic extract

Preparation of the organic extracts

Five mycelial plugs collected from the margin of 2-days *Trichoderma* precultures were inoculated into 1000 mL conical flasks containing 250 mL of sterile Potato Dextrose Broth (PDB) and the whole incubated for 4 weeks at 28°C under stationary condition. Cultures were then filtered under vacuum through Whatman N° 4 filter paper and the filtrates extracted three times with ethyl acetate (EtOAc) as solvent. The organic fraction was evaporated under reduced pressure at 35°C and the crude extracts obtained were stored at 4°C until use.

Secondary metabolites assay of organic extracts

Polyphenol

Five hundred microliters of the crude extract (1 mg/mL) were added to a mixture of 2.5 mL of Folin-Ciocalteu's reagent (10%) and 2.5 mL of aqueous calcium carbonate (Na_2CO_3) solution 7.5%. After 45 mins of incubation in the dark, the absorbance was measured at 760 nm in a spectrophotometer against a blank in which the extract was replaced by 500 µL of distilled water. The calibration curve was obtained using gallic acid as the standard solution. The amount of pplyhenols was estimated from the regression equation of the standard range established with gallic acid and was expressed as microgram of gallic acid equivalent per gram of extract (Jaradat et al., 2020).

Flavonoids

One milliliter of ethyl acetate extract (1 mg/mL) was mixed with 0.3 mL of $NaNO_2$ (5%), 0.3 mL of aluminum trichloride solution $AlCl_3$ (10%) and 1 mL of NaOH (2 M). After incubation in the dark for 30 mins at room temperature (25°C), the absorbance of the mixture was measured at 415 nm against blank using distilled water instead of the extract. The total flavonoid contents of the extracts were estimated from a linear calibration plot, established from quercetin (reference standard) at concentrations of 5; 10; 15; 20; 25 and 30 mg/L. The flavonoid contents of the extracts were expressed in milligrams of quercetin equivalents per gram of extract (Jaradat et al., 2020).

Tannins

The tannin content was determined according to the method described by Mignanwandé et al., (2020). Four hundred microliters of ethyl acetate extract (1 g/L) were mixed with 3 mL of vanillin (4%) and 1.5 mL of hydrochloric acid (37%) The blank was prepared by replacing the extract with methanol. The tubes thus treated were stirred at 30°C for 20 mins in the dark. The absorbance was read at 500 nm. The calibration curve was obtained using catechin as reference. Tannin concentrations were expressed in milligrams of catechin equivalent per 100 mg of extract (mg EC/100 mg).

Antimicrobial activity of the organic extracts

Mycelial growth Inhibition

Phytophthora colocasiae mycelia growth inhibition was carried out following the method described by Ntah et al., (2018). Briefly, 5 mm diameter mycelial growth plugs taken from the margin of a 3 days pathogen preculture was placed at the center of a 9 cm Petri dish containing V8 Agar medium. The crude extract was firstly diluted in Dimethyl sulfoxide (DMSO) to give various concentration of 39, 29 and 10 µL/µg and 10 µL of each concentration was applied on the plug. The plates inoculated with the solvent served as control. The Petri dishes thus treated were left open in a laminar flow cabinet to allow the solvent to evaporate and then incubated at room temperature (25°C) for 3 days after which the mycelial growth inhibition (%I) was through the formula:

$$\% I = [(Do - Dx) / Do] \times 100$$

Where, Do represents the pathogen's growth diameter on control plate and Dx is the pathogen's growth diameter on treated plate.

Each treatment consisted of three replicates and the experiment was repeated twice.

Inhibition of zoospores and sporangia germination

Sporangia solution was obtained by brushing taro necrotic leaves in distilled water and then filtered to retain leaf debris. The resulting filtrate was calibrated using a Mallassez cell at 10^4 sporangia/mL.

Zoospores liberation was induced by incubating tubes containing sporangia solution at + 4°C for 4 hours followed by incubation at room temperature (25°C) for 30-60 mins. The zoospore suspension was filtered using Whatman N°2 filter paper to remove sporangia debris. The tubes were vortexed to induce encystment and the zoospore concentration adjusted to 10^4 zoospores/mL.

The inhibition of the germination of sporangia was carried out on the basis of the method adapted from Jones et al., (1991) by using microplate.

The effects of extracts on sporangia and zoospores were

assessed by the microdilution in liquid medium in 96-well microplates (NUNC TM). Fifty microliters (50 µL) of sterile distilled water were distributed in microcupules. The same volume (50 µL) of crude extract dissolved in DMSO with concentration of 32 µg/mL was added to the upper wells (first raw). Then, a series of 6 dilutions was made according to a geometric sequence of ratio ½ from the first raw corresponding to the concentration of 16 µg/µL, to the last raw of 0.5 µg/µL. The content of each well (50 µL) was diluted by adding 50 µL of sporangia or zoospore inoculum, while control wells received DMSO. The plates were incubated at 13°C for 16 hours to induce direct germination (sporangium germination) and at 20°C for 24 hours for zoospore germination. The tests were repeated three times for each substance to be assayed. After incubation, the aliquots from wells were examined under a line microscope at 40x magnification.

Direct germination was assessed as the number of sporangia emptied of their zoospores with a dissolved papilla. Germination percentages were based on a count of at least 100 sporangia in each replicate. Counts did not include visually damaged or abnormally shaped sporangia, or the few sporangia which germinated abnormally by indirect germination.

The reduction in the viability of the sporangia in response to treatments was evaluated by counting sporangia with coagulated or disturbed protoplasmic content, unlike healthy sporangia which exhibited normal protoplasmic fluidity.

The percentage of germinated zoospores was evaluated by counting a total of 100 zoospores per well. A zoospore was considered germinated when the length of the germ tube was at least equal to the length of the zoospore cyst. The reduction in viability of zoospores was assessed by counting dead zoospores. Zoospores are considered dead due to degradation of their structure.

Effect of *Trichoderma* organic extracts on taro leaf blight disease

The extract potential to reduce necrosis was evaluated as described by Sameza et al. (2014). Briefly, the second oldest leaves of *Colocasiae esculenta* plants (30-days-old) clear of any visible infection, from an experimental field at the University of Douala, Cameroon were removed. Their surface was sterilized with a 1 % sodium hypochlorite solution for 2 mins and rinsed twice with sterile distilled water. Thereafter the leaves were cut into 90 mm diameter discs. One hundred microliters of various crude extracts concentrations (2000; 4000; 6000 and 8000 µg/mL) obtained by dissolution of extract in 20 % DMSO were sprayed 24 hours before (preventive test) or after (healing test) inoculation of the pathogen. This inoculation was carried out by placing a 5 cm diameters explant of *P. colocasiae* on the upper surface of the leaves discs. Control leaf discs were treated with DMSO (20%). The leaf discs thus treated were placed in Petri dishes containing humidified filter paper and incubated at room temperature (25 ± 2°C) for 4 days. After, the diameters of necrosis as well as the production of sporangia were evaluated.

The inhibition of necrosis (In%) was estimated using the formula:

$$In\% = (1 - Dx/Dm) \times 100.$$

Where Dm: diameter of necrosis on control leaf discs; Dx: diameter of necrosis on tests leaf discs.

In order to assess the production of sporangia, each leaf disc was stirred in 2 mL of distilled water for 10 mins and then centrifuged at 3000 rpm for 5 mins. the recovered supernatant was used for enumeration of sporangia using the Malassez cell under optical microscope. Inhibition of sporangia production (Is%) was obtained according to the formula:

$$Is\% = (1- Sx/So) \times 100$$

Where So: number of sporangia on the control leaf disc; Sx: number of sporangia on the treated leaf disc.

Statistical analysis

Data were analyzed using SPSS software version 16 for Windows (SPSS, Inc., Chicago, IL, USA) and Stat View version 5.0 for Windows (SAS Institute, Inc., IL, USA).

Qualitative and quantitative data are presented as percentages or mean ± standard deviation (SD) respectively. The one factor analysis of the variance (ANOVA) was used to compare the means and Duncan's post hoc test was used to establish the significance of the differences between the values at $p<0.05$.

RESULTS

Molecular identification

According to the comparison of the sequences of both *Trichoderma* isolates (Tri 001 and Tri 003), the NCBI database showed degrees of similarity of 99.20 to 100% (Table 1 and Figure 1). The two species were identified as *Trichoderma virens* and *Trichoderma asperellum* respectively, under accession MN833411.1 and MN452804.1

Antagonism assay

Two strains were tested for their antagonism toward of *P. colocasiae* in dual culture and results showed that, they

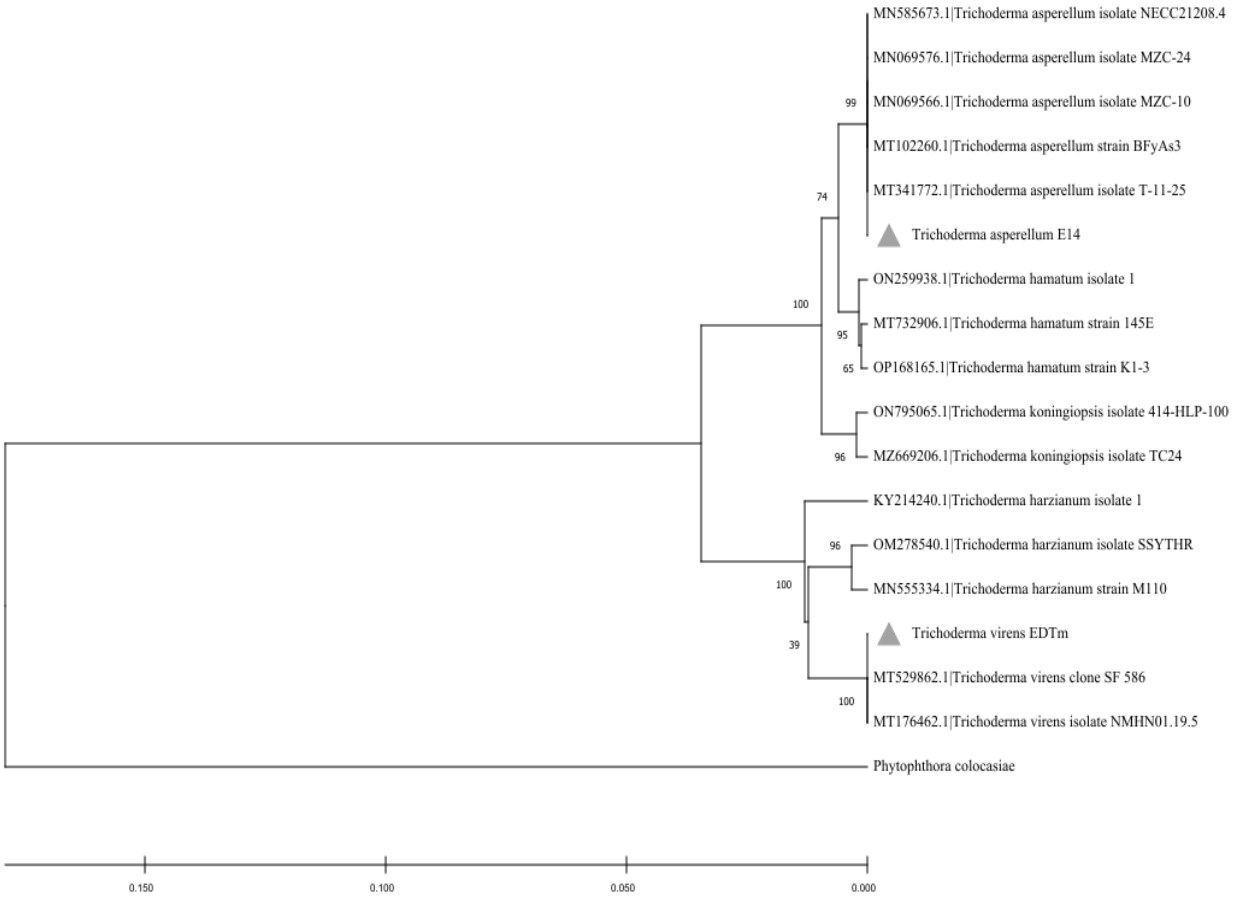

Figure 1. Phylogenetic analyses of *Trichodema* strains.

Table 1. Identification of *Trichoderma* isolates with NCBI gene bank.

Code	Similarity (%)	Organism with the highest sequence identity with GenBank Acc. no.
Tri 001	99.20	*Trichoderma virens* MN833411.1
Tri 003	100	*Trichoderma asperellum* MN452804.1

significantly inhibit the mycelial growth of the pathogen (Table 2). The percentage of inhibition was 43.66 % and 54.57 % respectively, for *T. asperellum* and *T. virens*. During the antagonism, both strains of *Trichoderma* grew on the pathogen and sporulate (Figure 2). They also produced diffusible substance that completely (100 %) inhibited *P. colocasiae* growth.

Secondary metabolites content of organic extracts

The phenolic compounds, flavonoid and tannin content of organic crude extracts of the two *Trichoderma* strains was evaluated. Results revealed that, these extracts contained secondary metabolites (Table 3). The organic extract from *T. virens* contained 197.00 mg/g of polyphenol, 24.05 mg/g of flavonoids and 262.4 mg/g of tannins while, the organic extracts of *T. asperellum* contained 227.00 mg/g, 46.86 mg/g and 699.9 mg/g, respectively.

Inhibition of mycelia growth, zoospore and sporangia germination

All the organic crude extracts from the two *Trichoderma* strains significantly inhibited the mycelial growth of *P. colocasiae* (Figure 2) as well as zoospore and sporangia inhibition (Table 4). Depending on extract concentration,

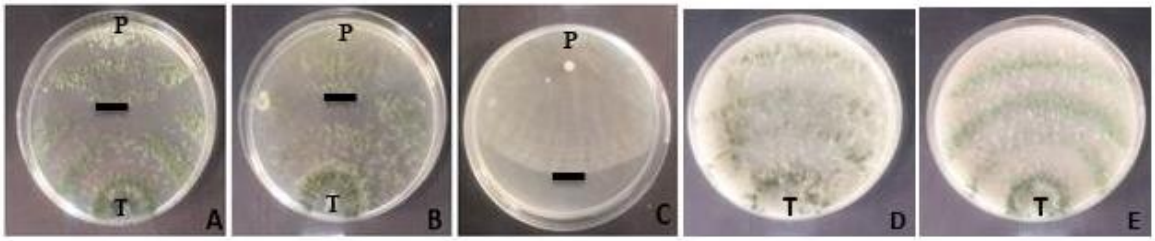

Figure 2. Dual culture of *Trichoderma* strains against *P. colocasiae* after 3 days of incubation. P, deposite point of *P. colocasiae* plug; T, deposite point of *Trichoderma* sp plug ; (▬) growth arrest of pathogène; (A) *T. asperellum*; (B) *T. virens*; (C) Control pathogen; (D) Control *T. virens*; (E) Control *T. asperellum*.

Table 2. Inhibition of mycelial growth of *P. colocasiae* by *Trichoderma* strains in dual culture.

Variable	Control	*T. asperellum*	*T. Virens*
Mycelial growth (mm)	47.33 ± 0.58[a]	26.67 ± 0.58[b]	21.5 ± 0.71[d]
Inhibition (%)	0.00[d]	43.66 ± 1.22[c]	54.57 ± 1.06[a]

In each line, means ± standard deviation followed by the same letter are not significantly different according Duncan's test at 5%.

Table 3. Secondary metabolites content of *Trichoderma* organic extracts.

Microbial species	Phenolic compounds (mg/g)	Flavonoids (mg/g)	Tanins (mg/g)
T. virens	197.00±2.00[b]	24.05±1.76[b]	260.24±1.73[b]
T. asperellum	227.00±0.00[a]	46.86±1.37[a]	690.99±1.66[a]

Means ± SD followed by the same letter in the same column are not significantly different by ANOVA's test (p≤0.05).

Table 4. Inhibition of zoospores and sporangia germination by the crude extracts of *Trichoderma* strains.

Extract (µg/ml)	Zoospores inhibition (%)		Sporangia inhibition (%)	
	T. virens	*T. asperullum*	*T. virens*	*T. asperullum*
0.00	16.00 ± 1.00[h]	16.00 ± 1.00[h]	16.00 ± 1.00[g]	16.00 ± 1.00[g]
0.25	37.67 ± 0.58[g]	44.33 ± 1.00[f]	40.67 ± 0.58[f]	77.67 ± 0.58[d]
0.50	49.67 ± 0.58[e]	50.00 ± 0.58[e]	65.67 ± 0.58[e]	89.67 ± 0.58[c]
1.00	62.67 ± 0.58[d]	75.00 ± 1.00[c]	89.67 ± 0.58[c]	95.67 ± 0.58[b]
2.00	75.00 ± 1.00[c]	88.00 ± 1.00[b]	100.00 ± 0.00[a]	100.00 ± 0.00[a]
4.00	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]	100.00 ± 0.00[a]

Means ± SD followed by the same letter in the same test (zoospores inhibition or sporangia inhibition) are not significantly different by Duncan's test (p ≤ 0.05).

inhibition increased with the extract concentration. The total inhibition of pathogen mycelia growth occurring at 100 and 300 µg/plug respectively for *T. Virens* and *T. asperellum* extract while for sporangia and zoospore the highest inhibition (100 %) occurred at 2 ug/mL and 4 ug/mL respectively. However, death of zoospores and sporangia was observed at concentrations greater than or equal to 2 µg/mL. These concentrations induced the death of all (100

Table 5. Effect of *Trichoderma* crude extracts on leaf disc necrosis inhibition.

	Extracts (µg/ml)	Necrosis inhibition (%)	
		T. virens	***T. asperellum***
Preventive	0.0	0.00 ± 0.00[a]	0.00 ± 0.00[a]
	200.0	0.00 ± 0.00[a]	0.00 ± 0.00[a]
	400.0	18.59 ±1.63[b]	27.05 ± 1.36[c]
	600.0	30.06± 1.00[d]	38.2 ± 1.00[e]
	800.0	70.52± 1.62[f]	75.38 ± 0.82[g]
Healing tests	0.0	0.00 ± 0.00[a]	0.00 ± 0.00[a]
	200.0	0.00 ± 0.00[a]	0.00 ± 0.00[a]
	400.0	11.27±1.4[c]	3.97 ± 1.64[b]
	600.0	26.95± 0.23[e]	16,10 ±0.80[d]
	800.0	55.35±1.41[g]	37.92 ± 0.70[f]

Means ± SD followed by the same letter in the same test (preventive or curative) are not significantly different according Duncan's test (p≤0.05).

Table 6. Effect of *Trichoderma* crude extracts on sporangeogenesis inhibition.

	Organic extract (µg/ml)	sporangeogenesis Inhibition (%)	
		T. asperulum	***T. virens***
Préventive	0.0	0.00[a]	0.00[a]
	200.0	33.21±0.75[c]	25.22± 1.5[b]
	400.0	69.43±1.5[d]	64.44±5.75[d]
	600.0	77.5 ±3.80[f]	74.20±2.50[e]
	800.0	94.10 ± 1.33[h]	91.94±0.00[g]
Healing tests	0.0	0.00[a]	0.00[a]
	200.0	12.05±1.00[b]	17.8±1.01[b]
	400.0	38.23±3.03[c]	49.16±3.96[d]
	600.0	43.29±2.01[e]	53.10±2.01[f]
	800.0	51.49±3.40[g]	61.54±5.48[f]

Means ± SD followed by the same letter in the same test (preventive or curative) are not significantly different according Duncan's test (p≤0.05).
%) sporangia and zoospores.

%) sporangia and zoospores.

Inhibition of taro leaves necrosis by *Trichoderma* extract

The application of *Trichoderma* organic extracts on taro leaves significantly reduced necrosis induced by *P. colocasiae* (Table 5), with the highest inhibition occurring at 800 µg/mL. During preventive test the inhibition percentages were 70.52 % and 75.38 % respectively, with the extract of *T. virens* and *T. asperellum*. Meanwhile for curative test, they were 55.35% and 37.92%. Table 6 showed a significant reduction of sporangiogenesis in all treatment. The preventive test presented a significant inhibition varying from 17.40 to 94.10 compared to the curative treatment which was from to 0.00 to 7.92. *T. asperellum* crude extract showed the greatest necrosis and sporangiogenesis inhibition in preventive test. In healing test, *T. virens* showed an ability to inhibit necrosis and sporangiogenesis.

DISCUSSION

Trichodema spp. are an important source of secondary metabolites, some of which have antibiotic properties and may play a significant role in biological control (Tyskiewicz et al., 2022). This work evaluated the bioactive potential of crude extracts of two *Trichoderma* species isolated in Cameroon against taro leaf blight caused by *P. colocasiae*. Coculture tests showed that *Trichoderma* strains identified by molecular analysis as *T. virens*, and *T. asperellum* grew faster than *Phytophthora colocasiae* on V8-Agar medium. In fact, members of the *Trichoderma* genus have a strong invasiveness, which makes them not only a good competitor for the substrate but also for the nutrients and therefore has antagonistic potential against other microorganisms (Sheridan et al., 2020). Moreover, the antagonistic potential varies from one species to another (Woo et al., 2020). The present study showed an inhibition of pathogen, varying according to the antagonist tested. Similar results were obtained by Nath et al., (2014) who showed that the antagonistic potential of *Trichoderma* against *P. colocasiae* varies according to the species of the antagonist (inhibition varying from 36.66 to 100%) and within the same species of *T. asperellum* (varying inhibition from 64.80 to 100%). Indeed, the antagonistic potential against plant pathogens could differ from the effectiveness of the antagonism, which itself depends on the genetic profile of the isolate. The inhibition observed during the coculture could be explained by the fact that *Trichoderma* can produce enzymes such as glucanases, proteinases, chitinases, and cellulases that can degrade the cell walls of other microorganisms (Tchameni et al., 2020; Muniroh et al., 2019). The cell wall of *P. colocasiae* is composed mainly of beta-(1-3) and beta-(1-6)-glucans as well as cellulose and therefore can be degraded by glucanases and cellulases (Harman, 2006; Gajera et al., 2012; Naglot et al., 2015; Parmar et al., 2015). These enzymes then play an important role in the lysis of the cell wall of the phytopathogen allowing control agents to establish their mycoparasitism mechanism (Gajera et al., 2012). The *Trichoderma* strains tested in this study showed an ability to grow on the pathogen and sporulate. This observation could be a morphological manifestation of mycoparasitic activity (Abo-Elyousr et al., 2014). This activity could be supplemented by the action of antibiotic molecules which could prevent the formation of the cell

membrane. The antibiosis test using the cellophane membrane method showed the ability of both *Trichoderma* strains tested to totally inhibit the mycelial growth of *P. colocasiae*. This activity would also depend on the species of *Trichoderma*. In fact, Nath et al., (2014) were isolated several species of *T. asperellum* which showed inhibition of *P. colocasiae* under antibiosis test ranging from 71.43 to 79.63%. The absence of resumption of mycelial growth after transfer of explants of the pathogen in a fresh medium suggests the death of the pathogen. Therefore, the diffusible metabolites produced by these strains of *Trichoderma* could be microbicidal. These data suggest one of the main mechanisms of action of both species of *Trichoderma* is antibiosis which involves the production of secondary metabolites with antimicrobial properties. Hua et al., (2021) demonstrated that these antibiotics could be involved in the mechanism of *Trichoderma's* antagonistic action.

Crude organic extracts from *T. virens* and *T. asperellum* showed significant and variable inhibition of *P. colocasiae* mycelial growth. Ntah et al., (2018) showed that *T. hazianium*, *T. atroviride* and *T. viride* extracts possess potential to completely inhibit the mycelial growth of *P. colocasiae*. The antibiotic metabolites contained in these extracts could prevent the formation of the cell membrane. This growth inhibition could be mainly due to the disruption of membrane permeability. The extracts presented the same groups of compounds but at different concentrations. The difference in activity observed with the extracts could be explained by the presence of different compounds belonging to the major chemical groups or to identical compounds but of variable concentration (Mironenkaa et al., 2020).

The quantitative study of the organic extracts showed the presence of flavonoids, polyphenols and tannins. These compounds have properties to inhibit mycelial growth of filamentous organisms. These compounds have different mechanisms of action on pathogens. Flavonoids by their composition in phenolic hydroxyls can penetrate the cell membrane, thus the hydroxyls combine, precipitate and denature protoplasmic proteins (Ho and Tan, 2015). Tannins form complexes with enzymes and other proteins causing enzyme inhibition. Indeed, tannins can inhibit the transport of electrons through membranes, and they can alter ions such as iron and copper by inhibiting the activity of certain enzymes essential to the life of microorganisms (Ho and Tan, 2015).

Moreover, these crude extracts significantly reduced the number of germinated propagules (sporangia and zoospores) (0% germination at 4 and 8 µg/mL) as well as the number of viable propagules (0% viable at 4 and 8 µg/mL) in comparison with the control. Ye et al., (2014) working on the ability of crude extracts of *T. harzianum* to reduce the germination of *Ciboria carunculoides* showed that a total inhibition (100%) of spore germination was obtained at 5000 µg/mL compared to the control which showed a germination of 97.6%. In addition, Boregowda et al., (2021) showed that *T. asperellum* and *T. virens* have sporangiosporicidal activity at 83% and 81% respectively, against *Sclerospora graminicola*. Sporangia and zoospores are a primary source of infection and the use of *Trichoderma* extracts is a means of controlling their proliferation and the disease.

Application of ethyl acetate extracts of different *Trichoderma* sp. to leaf discs resulted in variable inhibition of necrosis and sporangiogenesis. The secondary metabolites contained in these extracts can therefore manage taro leaf blight. Boregowda et al., (2021) showed that extracts of *T. asperellum* and *T. viens* present high potential to inhibit sporulation of *Sclerospora graminicola* on leaf disc. Michael et al., (2004) demonstrated that the antibiotic 6-pentyl-a-pyrone produced by *Trichoderma* spp. reduced conidiation of *F. oxysporum* by 44.4%. Moreover, Intana et al., (2011) showed that crude ethyl acetate extracts (2 %) of two *T. virens* isolates could reduce the severity of disease caused by *Alternaria brassicicola* on detached leaves by 22.5 and 32.0%.

Conclusion

This study showed that crude extracts obtained from T. virens and T. asperellum exhibited significant mycelial growth inhibition and propagule germination inhibition in P. colocasiae. In addition, application of these extracts to the leaf discs significantly reduced sporangiogenesis and necrosis. Therefore, crude extracts from Trichoderma virens (MN833411.1) and Trichoderma asperellum (MN452804.1) offer a promising alternative to synthetic pesticides. Future investigations should therefore focus on the formulation of a biopesticide based on extracts and spores of Trichoderma as well as the determination of the chemical composition of all the organic extracts.

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