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Research Article

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Phytochemical characterization of sub-fractions of *Thevetia peruviana*, *Azadirachta indica* and their antifungal efficacy on *Pythium myriotylum*, the causal agent of cocoyam root rot disease (*Xanthosoma sagittifolium* L. Schott)

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ABSTRACT

Pythium myriotylum is a telluric Oomycete, the causal agent of cocoyam root rot disease. In Cameroon, it can cause crop losses of up to 100% without sanitary measures. The aim of this study was to evaluate the antifungal efficacy of bioactive compounds from *Thevetia peruviana* (yellow oleander) and *Azadirachta indica* (neem) sub-fractions obtained by GC/MS against *P. myriotylum*. The antifungal activities of crude extracts (2%; 1%; 0.5% (m/v)) of *T. peruviana* and *A. indica* (2% and 0.5% (m/v)) in water, methanol and acetone were evaluated. The sub-fractions resulting from the roughing of the most active fractions by VLC (Vacum Liquid column) were characterized by GC/MS and their minimum inhibitory concentrations (MIC) were determined, as well as their mode of action on *P. myriotylum*. The results obtained show that GC/MS analysis identified many compounds with antifungal activities such as Oxime-, methoxy-phenyl, Hexadecanoic acid, methyl ester; 9,12-Octadecadienoic acid, methyl ester, (E,E); 7-Octadecenoic acid, methyl ester; 9-Octadecenoic acid, methyl ester, (E); Methyl stearate, Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl. All fractions derived from crude extracts significantly reduced the growth of *P. myriotylum* compared with the negative control, with the highest inhibition rates obtained with the aqueous ethyl acetate phase fractions (87.77% and 100% for *T. peruviana* and *A. indica*, respectively). The most active sub-fractions F1 of VLC2 and F4 of VLC1 inhibited the pathogen's protein synthesis. The lowest MICs were obtained with sub-fractions F1 of *A. indica* and F4 of *T. peruviana* (0.166% and 0.0837%). *T. peruviana* and *A. indica* can be considered as potential substitutes for chemical control.

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INTRODUCTION

Cocoyam (*Xanthosoma sagittifolium* L. Schott), an herbaceous plant of the Araceae family, is the sixth most important root and tuber plant in the world (ReyesCastro *et al.*, 2005). Mainly cultivated for its leaves and roots, it is an important source of carbon due to its richness in starch, protein, amino acids (Pérez *et al.*, 2007), vitamins and antioxidants (Sefa-Dedeh & Agyir-

Sackey, 2004), contributing almost 60% of an adult's protein requirements (Boudjeko *et al.*, 2006). Despite its economic, nutritional and pharmaceutical importance, its production has fallen considerably, from 1.8 million tonnes in 1975 to 0.1 million tonnes in 2019, and remains insufficient to meet the needs of an ever-growing population (FAOSTAT, 2019). Several constraints limit its productivity, such as the poor quality of production equipment, low soil fertility and the preponderance of diseases, of which root rot caused by the Oomycete *Pythium myriotylum* is the most important in Cameroon (Omokolo *et al.*, 2003).

P. myriotylum has been identified as the main cause of Cocayam root rot disease in Cameroon, causing losses of up to 90% despite phytosanitary action (Nzietchueng, 1983; Schafer, 1999; Zhang & Yang, 2000; Boudjeko *et al.*, 2006; Nyochembeng *et al.*, 2007; Gómez-Alpízar *et al.*, 2011). One method of reducing the incidence of Cocoyam root rot disease is chemical control using synthetic pesticides. Although highly effective (Kanda *et al.*, 2013), this method has several drawbacks, including the emergence of resistant strains and adverse effects on the environment and human health (Harir, 2010; Houndété *et al.*, 2010; Agboyi *et al.*, 2016; ten Hoopen & Krauss, 2016; Galani *et al.*, 2020; Manfo *et al.*, 2020). To address these constraints, biological control using microorganisms with antagonistic effects such as fungi (Ambang *et al.*, 2007; Mbarga *et al.*, 2012), and bacteria (Tambong & Höfte, 2001; Kouomou *et al.*, 2019) have proved their worth.

Studies carried out in recent years have demonstrated that many plants have biocidal potential against a wide range of pests and can be used in extract form (Ambang *et al.*, 2010; Mondédji *et al.*, 2015; Anjarwalla *et al.*, 2016; Son *et al.*, 2017; Mboussi *et al.*, 2018; Ndacnou *et al.*, 2020; Essome *et al.*, 2020). Among these plants with biocidal potential, *Thevetia peruviana* from the Apocynaceae family and *Azadirachta indica* from the Meliaceae family has been the subject of several works in which their biocidal activities have been demonstrated. The work of Mboussi *et al.* (2016), Dida *et al.* (2019), Essome *et al.* (2020), Ngatsi *et al.* (2020) and Foka *et al.* (2023) showed that aqueous and organic extracts of *T. peruviana* and A. *indica* seeds significantly reduced the incidence of fungal infections. But to date, very little work has been done on the bio-guided search for their active molecules against phytopathogens such as *P. myriotilum*. The aim of the present work, which falls within the framework of the search for innovative perspectives in biological control, was to characterize the sub-fractions of *T. peruviana* and A. *indica* that are most effective against *P. myriotylum*, the causal agent of cocoyam root rot disease.

MATERIALS AND METHODS

Study Material

The plant material consisted of yellow oleander (*Thevetia peruviana*) leaves harvested in Yaoundé; neem (*Azadirachta indica*) seeds were collected in the Nord-Region of Cameroun. The fungal material consisted of a strain of *Pytium myriotylum* from the phytoprotection and genetic resources development laboratory of the Biotechnology Center in Nkolbisson, at the University of Yaoundé I. The chemical material consisted of the solvents hexane, ethyl acetate, methanol, n-butanol, acetone and Tween80 and a systemic fungicide with metalaxyl and copper oxide as active ingredients.

METHODS

Extraction of Metabolites from *T. peruviana* and *A. indica*

Preparation of T. peruviana crude extracts

Distilled water, acetone and methanol crude extracts of fresh *T. peruviana* leaves were obtained by drying, grinding and maceration in various solvents as described by Ambang *et al.* (2010). The filtrates obtained were concentrated in a rotavapor at 60 °C, and then stored in a refrigerator at 4 °C.

Preparation of crude extracts of A. indica oilcake

Distilled water, acetone and methanol crude extracts of neem seeds were obtained from the oil cake after de-oiling and maceration in various solvents. The aqueous extract was obtained by infusion at 40 °C. The filtrates obtained were concentrated in a rotavapor at 60 °C, and then stored in a refrigerator at 4 °C.

Antifungal activity of T. peruviana and A. indica crude extracts

The inhibitory effect of crude extracts on *P. myriotylum* was determined using the culture medium poisoning method (Flore *et al.*, 2023). PDA culture medium was prepared and, before complete cooling, supplemented with concentrations (2% w/v; 1% w/v; 0.5% w/v; 0.25% w/v) of crude extracts. The positive control was treated with the chemical fungicide at 0.33% as recommended by the manufacturer. Inhibition percentage (I) was calculated in relation to the negative control using the formula.

$$I\% = \frac{Dto - Dxi}{Dto} X100$$

With I(%): Inhibition percentage; Dto is the average diameter of the control batch and Dxi is the average diameter of batches in the presence of the extract.

Fractioning of Crude Extracts of *T. Peruviana, A. Indica* and Antifungal Activities of The Different Fractions

Partitions with etyl acetate and n-butanol

The various extracts (aqueous, methanol and acetone) of *T. peruviana* and A. *indica* were successively partitioned using two organic solvents: ethyl acetate and n-butanol. Extracts were successively dissolved in water (250 mL) and transferred to a separatory funnel. A volume of ethyl acetate (300-400 mL) was made up to recover the ethyl acetate fraction, which was concentrated and stored at 4 °C. From the residual aqueous phase, the n-butanol fraction was obtained as above for ethyl

acetate and stored at 4 °C. The aqueous residue obtained was concentrated and stored at 4 °C.

Antifungal activity of T. peruviana and A. indica fractions

This was determined as previously described with crude extracts of T. peruviana and A. indica, using concentrations of 2% w/v and 0.5% w/v.

Vacuum liquid chromatography (VLC) roughing of fractions

The most active fractions of the *T. peruviana* and A. *indica* extracts were roughing by vacuum liquid chromatography using silica as the stationary phase, and a DCM/methanol/water gradient (90-10-0, 80-20-2, 70-30-5) as the eluent, in order of increasing polarity. Based on the thin-layer chromatographic profiles, the sub-fractions were grouped.

Antifungal activity of T. peruviana and A. indica subfractions

The mycelium of a 14-day-old pure culture of *P. myriotylum* was used to produce the spore suspension by heat shock in 10 mL of sterile ice-cold distilled water before being transferred to a 25°C oven in a dark chamber for one hour. The minimum inhibitory concentrations of the *T. peruviana* and A. *indica* sub-fractions were determined according to the protocol of Cockerill *et al*. (2012). A volume of 100 µL of culture broth (PDB) was added to each microplate well, and wells 1 A to 1 D were supplemented with 68 µL of PDB and 32 µL of the sub-fractions stock solution (25 mg/mL). The microplates were homogenized; a series of dilutions (½ dilutions) was made from well to well. Subsequently, 100 µL of freshly prepared inoculum at 2.5×10³ spores/mL was added to each microplate well, giving a total volume of 200 µL and concentrations of 10000 - 5000 - 2500 - 1250 - 625 - 312.5 - 156.25 - 78.13 - 39.12 - 19.53 - 9.77 - 4.88 µg/mL, varying from the first to the twelfth well. A series of ½ dilutions without inoculum was also carried out with the extract only, and served as a negative control to check its purity. In order to control normal pathogen growth, cultures were grown in wells and served as a negative control. The sterility of the culture medium was checked by seeding 200 µL of culture medium in wells. Finally, the positive control was performed with nystatin (400 µg/mL). Rhezasurin was used as a growth indicator.

Characterization of the Most Active Subfractions of *T. peruviana* and *A. indica*

Mode of Action of the Most Active *T. peruviana and A. indica* Subfractions

Effect on P. myriotylum wall integrity

The lytic action of the most active subfractions on the wall of *P. myriotylum* was carried out according to the protocol established by Limsuwan and Voravuthikunchai (2013). The spore suspension was standardized to 10³ spore/mL in 0.9% NaCl. Thus, a volume of sub-fractions was introduced into different tubes containing 1900 µL of this suspension to have a concentration equivalent to 1 MIC and ½ MIC in the medium. The resulting suspensions were incubated at 37 °C under flurry. At times 0 h, 2 h, 18 h, 24 h and 48 h, absorbance was measured at 620 nm and that at time 0 h was used to evaluate relative absorbance (Ar) at different times. Where $Ar = DOt - DOO$

Effect on protein synthesis by P. myriotylum

The evaluation of the effect of the most active sub-fractions on protein synthesis by *P. myriotylum* was carried out following the protocol described by Upadhyay *et al*. (2008). A volume of 0.5 mL spore suspension (standardized to 10³ spore/mL) was added to tubes each containing 9.25 mL PDB. Then 0.25 mL of sub-fraction solution (10 mg/mL) was added to each previous mixture to obtain 1 MIC and ½ MIC concentrations. The control tube was treated under the same conditions and received 0.25 mL PDB. Tubes were incubated at 27°C with 80 rpm rotation. After 24 hours of incubation, centrifugation at 4000 g for 5 minutes recovered the fungal pellets, which were mixed with 500 µL phosphate buffer (0.1 Mm, pH 6.8) for protein extraction. After 1 hour incubation, centrifugation at 4000 g for 3 minutes at 4 °C recovered the protein-containing supernatant, which was then assayed by the Bradford method (Sigma-Aldrich).

Effect on ATPase-proton pump activity in P. myriotylum

The evaluation of ATPase-H+ pump function in the presence of the most active subfractions was carried out according to the method described by Soukoudjou (2020), by monitoring changes in the pH of the culture medium. Any inhibition of medium acidification in the presence of a sub-fraction was attributed to an inhibitory effect of the latter on ATPase-H+ pump function. A spore suspension was introduced into 20 mL physiological water and standardized to 10³ spore/mL, then 1.5 mL of this inoculum was taken and diluted in 150 mL PDB contained in an Erlenmeyer flask. After 18 hours incubation at 27 °C, with agitation, this suspension was centrifuged at 3,000 rpm for 30 minutes at 4 °C. The pellet was then washed with distilled water, followed by 50 mM KCl and re-suspended in 150 mL 50 mM KCl. The suspension was then kept at 4 °C for 18 hours (for glucose starvation) and the pH was adjusted to 6.5 by adding HCl and/or NaOH. A volume of 9 mL of this solution was added to 0.5 mL of the subfraction solution (5 mg/mL) was added to obtain concentrations equal to ½ MIC and MIC. After 10 minutes of pre-incubation at 27 °C, acidification of the medium was triggered by adding 0.5 mL of a 20% glucose solution, whose rapid catabolism will be accompanied by the release of protons into the medium. The pH of the medium was then measured every 30 minutes for 1 hr 30 min. For the negative control, the sub-fraction was replaced by water. The pH values recorded were used to plot pH versus time curves.

Identification of Metabolites in the Most Active Subfractions of *T. peruviana* and *A. indica*

GC-MS analysis of the extract and the best subfractions was carried out according to the protocol described by Kumar *et al*. (2014). A mass of 10 micrograms (µg) of each sub-fraction

was diluted in methanol, solubilized in an ultrasonic bath and filtered through a 0.45 micron microfilter. The solution obtained was then injected into an Agilent Palo Alto GC-MS chromatograph (GC-MS 5975) with the following parameters: Agilent DB 5 ms column, length 30 m, internal diameter: 0.2 nm, thickness 0.25 mm, temperature 70-300 °C or 10 °C/minute, gas eluent: helium, flow rate: 1.51 mL/minute. The NIST (National Institute of Standards and Technology) database was used for compound identification (Taduri *et al.*, 2022).

Statistical Analysis

The data obtained were subjected to an analysis of variance (ANOVA), followed by a comparison of means using the Tukey's test at the 5% probability threshold with SPSS software (version 25 for Windows). Averages were plotted using Microsoft Excel 2016.

RESULTS

Effect of *T. peruviana* and *A. indica* Crude Extracts

Inhibition percentage of various T. peruviana and A. indica extracts at different concentrations

Percentage inhibition of pathogen mycelial growth revealed that all crude extracts of *T. peruviana* had significant inhibitory activity ($P \leq 0.05$) compared with the negative control. There was no significant difference between these different extracts and the positive control (86.72% inhibition), with the exception of *T. peruviana* extract with 2% methanol (88.38% inhibition), which showed a higher inhibition rate than the latter, and *T. peruviana* with 2% acetone (69.12% inhibition), which showed a lower inhibition rate than both the positive control and the latter (Figure 1).

Inhibition percentage of different A. indica extracts at different concentrations

Aqueous and organic crude extracts of *A. indica* at different concentrations significantly inhibited ($P \leq 0.05$) mycelial growth of *P. myriotylum* after 7 days of incubation (Figure 2). There was also a correlation between inhibition rate and concentration, for both aqueous and methanoic extracts. Indeed, the higher the concentration, the greater is the inhibition rate (80.74% and 93.35% inhibition for aqueous and 2% methanol neem); however, for acetone extracts, the inhibition rate increases with decreasing concentration (54.854% inhibition for 2% concentration). No significant difference from the positive control (86.72% inhibition) was observed compared to the 2% concentration for either aqueous or methanoic extracts (Figure 2).

Inhibition rate of different fractions of T. peruviana aqueous and organic extracts

Inhibition rate of pathogen mycelial growth shows that all *T. peruviana* fractions have a significant effect on the pathogen. The different fractions of the crude extracts with aqueous and organic solvents revealed a clear reduction in mycelial growth of the T aq acetate 2%, T aq aq 2%, T aq acetate 0.5% fractions (87.77%, 77.73% and 87.08% inhibition respectively), as did the positive control (100%, inhibition) compared with the negative control (0.1%, inhibition) (Figure 3). However, there was no significant difference between these fractions and the positive control.

Inhibition rates of different fractions of A. indica aqueous and organic extracts

The inhibition rates of pathogen mycelial growth show that all

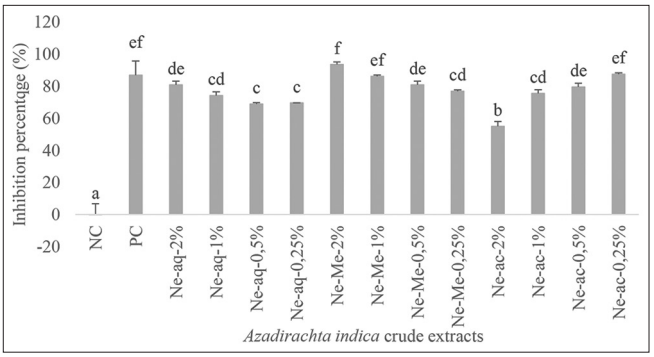

Figure 2: Inhibition percentage of *A. indica* crude extracts on *P. myriotylum* mycelial growth after 7 days. NC: negative control; PC: positive control; Ne: neem; aq: aquous; Me: methanolic; ac: acetone

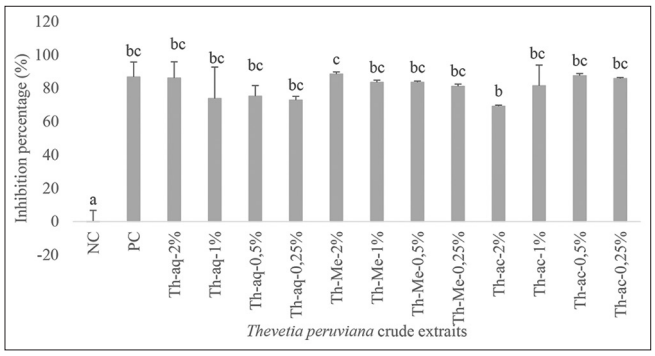

Figure 1: Inhibition percentage of *T. peruviana* crude extracts on *P. myriotylum* mycelial growth after 7 days. NC: negative control; PC: positive control; Th: *T. peruviana*; aq: aquous; Me: methanolic; ac: acetone

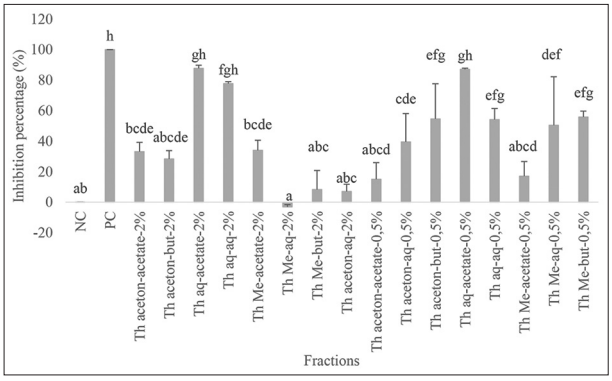

Figure 3: Inhibition percentage of *T. peruviana* crude extract fractions on *P. myriotylum* mycelial growth after 7 days. NC: negative control; PC: positive control; Th: *T. peruviana*; but: n-buthanol; Me: methanolic; aq: aqueous; acetone extract

A. *indica* fractions have a significant effect on the pathogen. The A. *indica* fractions showed no significant difference between the 2% aqueous acetate and butanoic phases and the positive control (100%, 63.32% and 100% inhibition respectively). On the other hand, they significantly inhibited pathogen growth compared with the negative control (0.1% inhibition). In general, the percentage of inhibition depends on the concentration of the different fractions (Figure 4).

Minimum inhibitory concentration of the most active sub-fractions of A. indica and T. peruviana

The minimum inhibitory concentrations of the VLC2 subfraction obtained from the acetate fraction of the aqueous neem extract are statistically very close to the positive control (0.19%), with the F1 fraction showing the lowest value (0.17%) (Figure 5a). However, there was no significant difference between these sub-fractions. On the other hand, the VLC1 sub-fraction obtained from the aqueous fraction of the *T. peruviana* aqueous extract, shows that there is a significant difference between the sub-fractions and the positive control. Indeed, the F3 and F4 subfractions recorded the lowest value (< 0.05%) compared with the other sub-fractions and the positive control, which had 4%, 0.5% and 0.19% respectively (Figure 5b).

Mode of Action of the Most Active Sub-fractions on the Phytopathogen (*P. myriotylum*)

Inhibition of protein synthesis by the pathogen

Analysis of protein production by the pathogen under the effect of the most active sub-fractions F1 of VLC2 (231 µg/g) and F4 of VLC1 (182 µg/g) shows that it inhibits *P. myriotylum* protein synthesis compared with the negative control (430 µg /g) (Figure 6).

Observation of the variation in absorbance of the pathogen spore suspension under the effect of the most active sub-fractions F1 of VLC2 and F4 of VLC1 shows that F1 has a significant effect on wall integrity, unlike F4. In fact, the F4 sub-fraction of VLC1 has no significant effect, while F1 of VLC2 leads to lysis of the pathogen's wall (Figure 7).

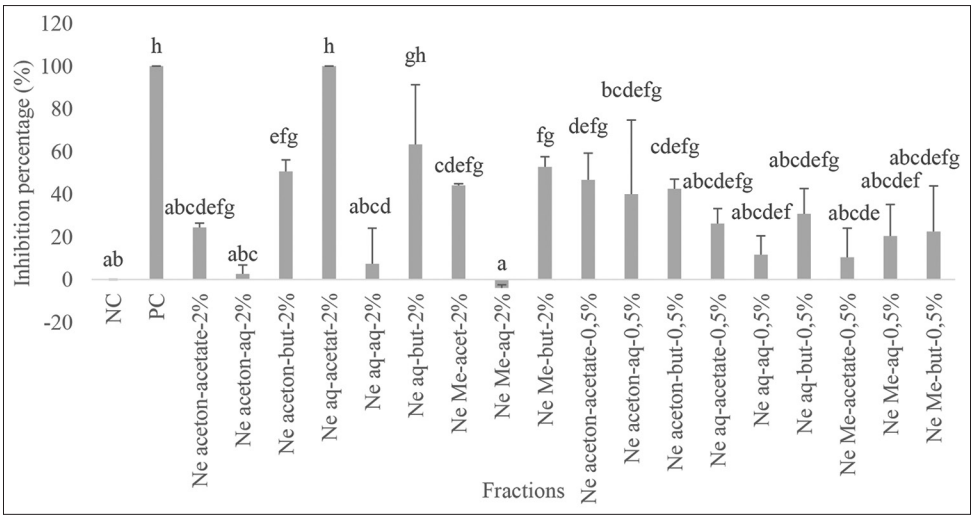

Figure 4: Percentage inhibition of *A indica* crude extract fractions on mycelial growth of *P. myriotylum* after 7 days. NC: negative control; PC: positive control; Ne: neem; but: n-buthanol; Me: methanolic; aq: aqueous; acetone extract

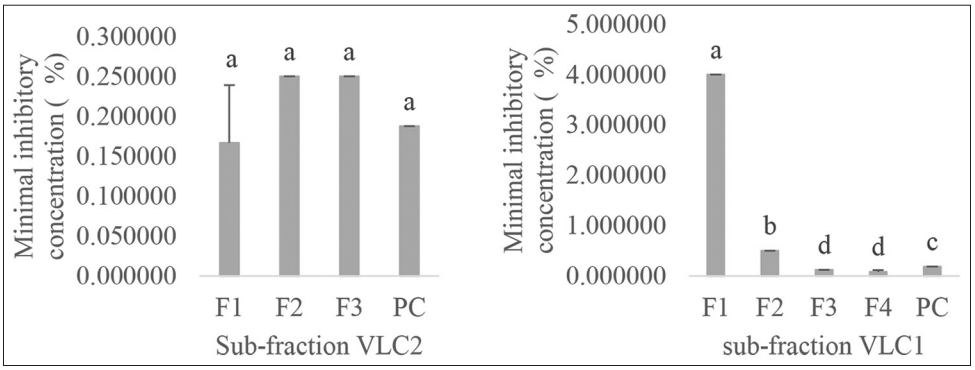

Figure 5: Minimum inhibitory concentration (MIC) of the sub-fractions of the *A indica* and *T. peruviana* fractions most active on *P. myriotylum* spore growth. F: fraction; PC: positive control; VLC2: sub-fractions obtained from the acetate fraction of the *A. indica* aqueous extract; VLC1: sub-fractions obtained from the aqueous fraction of the *T. peruviana* aqueous extract

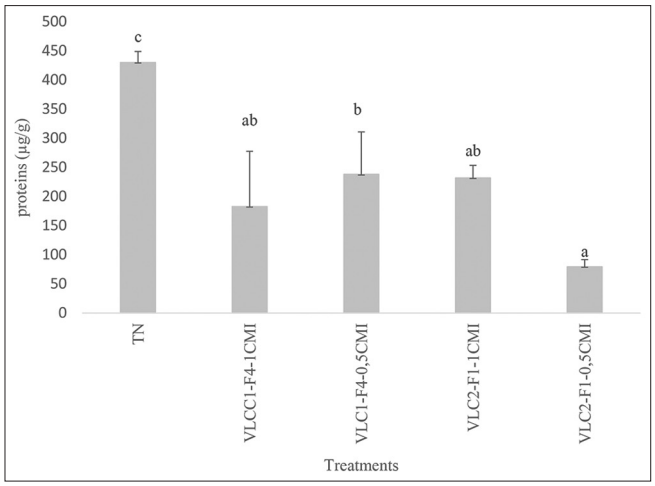

Figure 6: Effect of sub-fractions on protein synthesis by *P. myriotylum*. VLC2: sub-fractions obtained from the acetate fraction of the aqueous neem extract; VLC1: sub-fractions obtained from the aqueous fraction of the aqueous *T. peruviana* extract; F: fraction of the acetate fraction of the aqueous neem extract

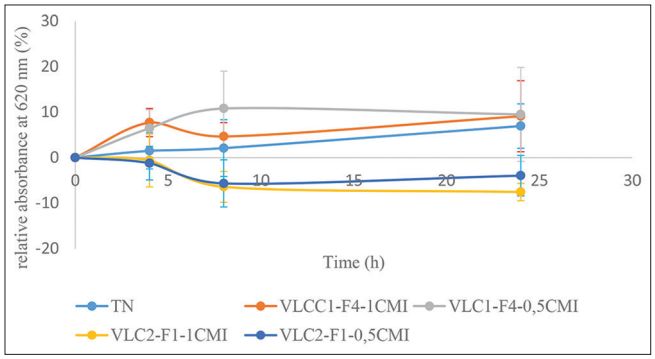

Figure 7: Effect of sub-fractions on wall integrity of *P. myriotylum*. VLC2: sub-fractions obtained from the acetate fraction of the aqueous extract of *A. indica*; VLC1: sub-fractions obtained from the aqueous fraction of the aqueous extract of *T. peruviana*; F: fraction of the acetate fraction of the *A. indica* extract

A study of the variation on pH of the microbial medium, under the effect of sub-fractions F1 VLC2 and F4 VLC1, shows a difference compared to the negative control. Indeed, an inhibition of acidification of the medium by the F1 sub-fraction over time is noted, compared with the control and the F4 sub-fraction. There was no significant difference between the latter and the control. Thus, the F1 sub-fraction inhibits the activity of the pathogen's proton ATPase pumps (Figure 8).

GC/MS Analysis

GC/MC analysis of the F1 sub-fraction revealed 60 compounds, of which the 9 most abundant (peak area>1600000) are listed in the table below, with retention times ranging from 1.607 to 12.691 and from 2.300 to 10.070 respectively (Figures 9, 10 & Table 1).

GC/MC analysis of the F4 sub-fraction revealed 45 compounds, of which the 12 most abundant (peak area>150000) are listed in the table below, with retention times ranging from 1.585 to

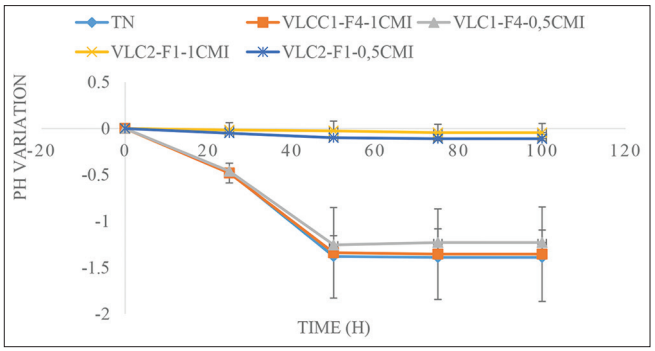

Figure 8: Effect of sub-fractions on ATPase-H+ pumps of *P. myriotylum*. VLC2: sub-fractions obtained from the acetate fraction of the neem aqueous extract; VLC1: sub-fractions obtained from the aqueous fraction of the *T. peruviana* aqueous extract; F: fraction

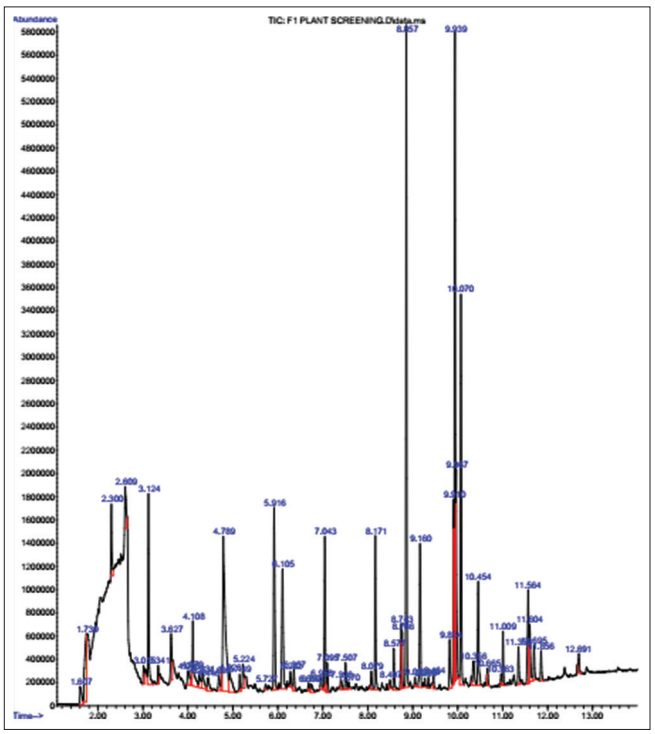

Figure 9: VLC2 F1 sub-fraction chromatograph

12.022 and from 1.602 to 12.022 respectively (Figures 11, 12 & Table 2).

DISCUSSION

The aim of the present work, which is part of the search for innovative perspectives in biological control, was to characterize the sub-fractions of *T. peruviana* and *A. indica* most effective against *P. myriotylum*, the causal agent of cocoyam root rot (*X. sagittifolium*).

Results showed significant inhibition of *P. myriotylum* growth and development by the aqueous and organic crude extracts of *A. indica* and *T. peruviana*, with the highest inhibition rates obtained with 2% methanol (93.35% and 88.37% respectively, versus 86.71% for the positive control). This inhibition would

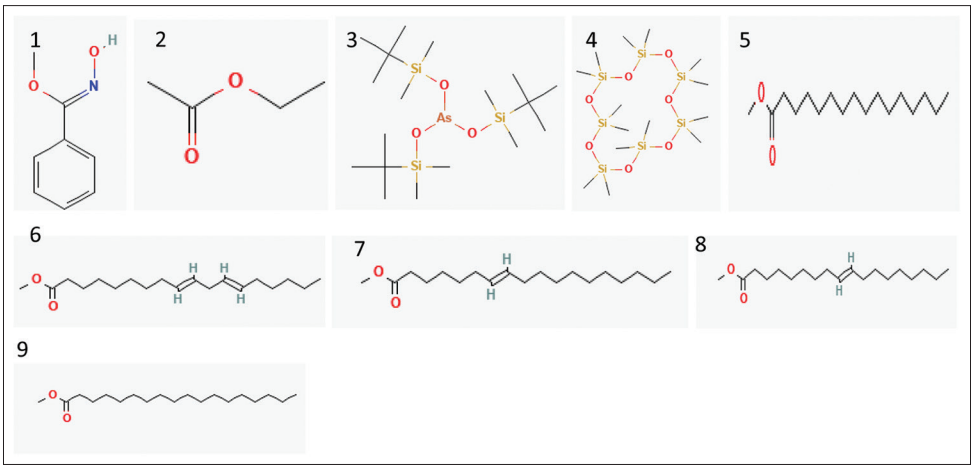

Figure 10: Nine most abundant compounds in the F1 sub-fractionv of VLC2. Oxime-, methoxy-phenyl; Ethyl Acetate; Tris(tert-butyldimethylsilyloxy) arsane; Cycloheptasiloxane, tetradecamethyl; Hexadecanoic acid, methyl ester; 9,12-Octadecadienoic acid, methyl ester, (E,E)-; 7-Octadecenoic acid, methyl ester; 9-Octadecenoic acid, methyl ester, (E)-; Methyl stearate

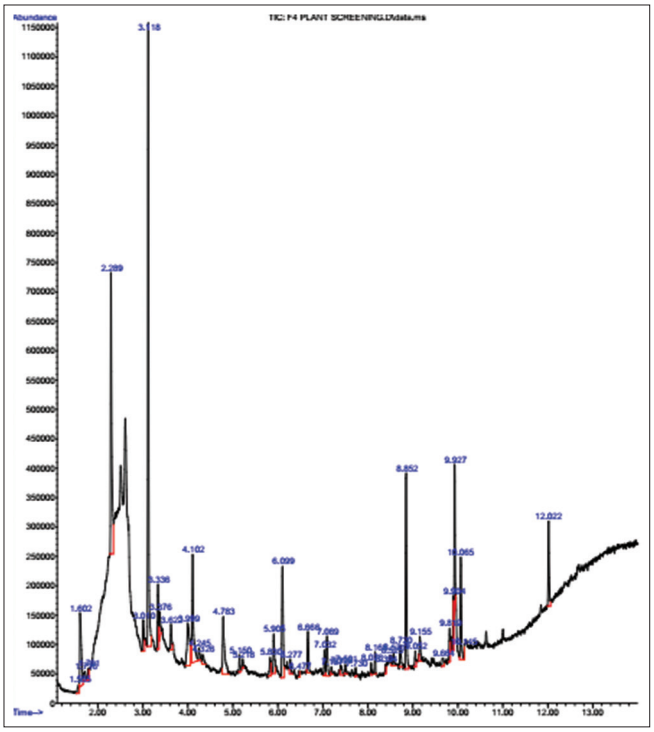

Figure 11: VLC1 F4 sub-fraction chromatograph

be due to the presence of bioactive compounds present in these two plants, as demonstrated by Ambang *et al.* (2010, 2011), Dida *et al.* (2020) and Foka *et al.* (2023). Furthermore, the strong inhibition of methanoic extracts would be due to the ability of methanol to extract more bioactive compounds (Hlokwe *et al.*, 2018). These results are similar to those of Toka *et al.* (2023) working on the antifungal activity of A. *indica* and *Balanites aegyptiaca* extracts against *Fusarium oxysporium* isolated from tomato showed that the methanol extract of these two plants inhibited the mycelial growth of the pathogen. The increase in inhibition rate with increasing concentration would be due to the higher proportions of these bioactive compounds in methanol and aqueous extracts. These results are similar to those of Essome *et al.* (2020), who demonstrated that inhibition rates dependent on dose- (the higher the concentration, the greater the inhibition). Contrary results were obtained for acetone extracts (the higher is the concentration, the lower is the inhibition). This could be explained by the presence of certain compounds acting with the polarity of acetone, which at high doses would inhibit the efficacy of the supposedly bioactive compounds present in these two plants.

Analysis of the inhibition rates of the various fractions enabled us to identify the most active fractions. This revealed that the aqueous acetate phase fractions of *T. peruviana* and A. *indica* at 2% were the most active, with inhibition rates of 87.77% and 100% respectively; followed by Th aqueous acetate phase at 0.5% (87.03%), Th aqueous phase at 2% (77.72) for the

T. peruviana fractions and Ne aqueous butanoic phase at 2% (63.31) for A. *indica*. There was no difference between these fractions and the positive controls. The fractions of aqueous extracts proved more active than those in acetone and methanol, indicating the majority presence of antifungal elements in the latter. Indeed, the binding of plant compounds by a solvent is a function of its polarity and/or its content of polar or apolar compounds, depending on the plant (Ngo *et al.*, 2017). Also, acetate phases present higher inhibition rates than other phases. This would be due to the order of passage of the different solvents in the roughing columns according to a polarity gradient from the least apolar to the most apolar solvent.

The minimum inhibitory concentrations of the VLC1 and VLC2 sub-fractions from the 2% *T. peruviana* aqueous phase extract and the 2% A. *indica* aqueous phase extract were used to identify the most active sub-fractions. Sub-fractions F1 of VLC2 and F4 of VLC1 achieved the lowest minimum inhibitory concentrations (0.166% and 0.0837% respectively). Identification of the secondary metabolites contained in these two sub-fractions of interest by GC/MS revealed the presence of 60 compounds, 9 of which are in the majority (peak area>1600000) for F1 and 45 compounds, 12 of which are in

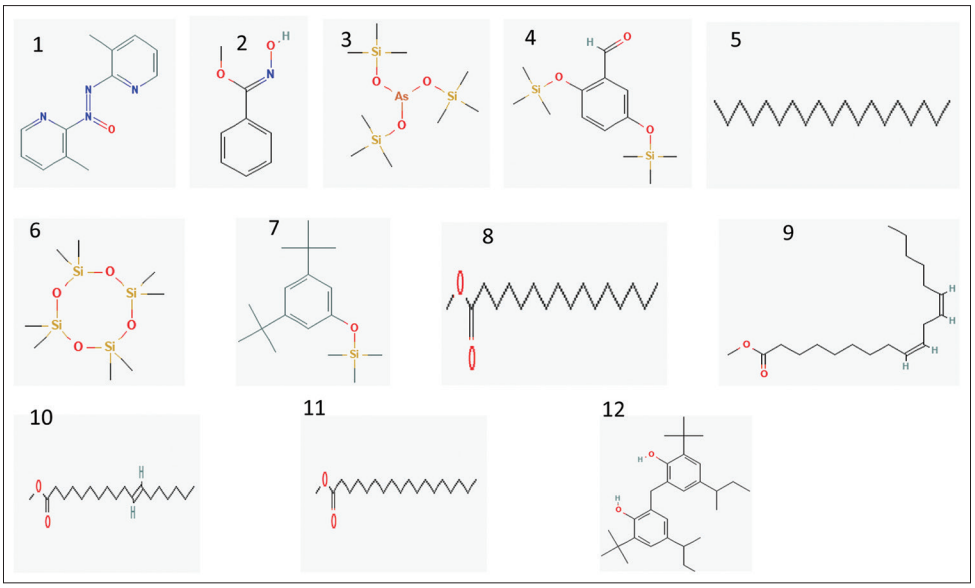

Figure 12: Structure of the nine most abundant compounds in the F4 sub-fraction of VLC1. 2,2'-Azoxybis[3-methylpyridine]; Oxime-, methoxy-phenyl-; Arsenous acid, tris(trimethylsilyl) ester; enzaldehyde, 2,5-bis[(trimethylsilyl)oxy]-; Nonadecane; Cyclotetrasiloxane, octamethyl; Phenol, 2,4-bis(1,1-dimethylethyl)-; Hexadecanoic acid, methyl ester; 9,12-Octadecadienoic acid, methyl ester; 11-Octadecenoic acid, methyl ester; Methyl stearate; Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl.

Table 1: Majority compounds in the F1 subfraction of VLC2

S. No.	RT	Area %	The best hit from library.	Qual	MM g/mol	MF
1	2.300	1.21	Oxime-, methoxy-phenyl-	25	151 0.16	$C_8H_9NO_2$
2	2.609	1.17	Ethyl Acetate	64	88.11	$C_4H_8O_2$
3	3.124	3.16	Tris (tert-butyldimethylsilyloxy) arsane	78	468.7	$C_{18}H_{45}AsO_3Si_3$
4	5.916	4.43	Cycloheptasiloxane, tetradecamethyl	93	519.07	$C_{14}H_{42}O_7Si_7$
5	8.857	10.86	Hexadecanoic acid, methyl ester	99	270.5	$C_{17}H_{34}O_2$
6	9.910	2.88	9,12-Octadecadienoic acid, methyl ester, (E, E)-	99	294.5	$C_{19}H_{34}O_2$
7	9.939	12.32	7-Octadecenoic acid, methyl ester	99	296.5	$C_{19}H_{36}O_2$
8	9.967	3.82	9-Octadecenoic acid, methyl ester, (E)-	99	296.5	$C_{19}H_{36}O_2$
9	10.070	7.04	Methyl stearate	99	298.5	$C_{19}H_{38}O_2$

Table 2: Majority compounds in the F4 sub-fraction of VLC1

S. No.	RT	Area %	The best hit from library	Qual	MM g/mol	MF
1	1.602	4.17	2,2'-Azoxybis[3-methylpyridine]	43	-	-
2	2.289	11.78	Oxime-, methoxy-phenyl-	87	151 0.16	$C_8H_9NO_2$
3	3.118	18.39	Arsenous acid, tris (trimethylsilyl) ester	64	342.49	$C_9H_{27}AsO_3Si_3$
4	3.336	2.43	Benzaldehyde, 2,5-bis[(trimethylsilyl) oxy]-	50	282.48	$C_{13}H_{22}O_3Si_2$
5	3.376	1.12	Nonadecane	35	268.5	$C_{19}H_{40}$
6	4.102	5.33	Cyclotetrasiloxane, octamethyl	83	296.61	$C_8H_{24}O_4Si_4$
7	6.099	5.72	Phenol, 2,4-bis (1,1-dimethylethyl)-	96	278.5	$C_{17}H_{30}OSi$
8	8.852	7.01	Hexadecanoic acid, methyl ester	99	270.5	$C_{17}H_{34}O_2$
9	9.904	1.65	9,12-Octadecadienoic acid, methyl ester	97	294.5	$C_{19}H_{34}O_2$
10	9.927	4.56	11-Octadecenoic acid, methyl ester	99	296.5	$C_{19}H_{36}O_2$
11	10.065	3.88	Methyl stearate	99	298.5	$C_{19}H_{38}O_2$
12	12.022	3.10	Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl	95	340.5	$C_{23}H_{32}O_2$

the majority for F4 (peak area>150000). Among the majority compounds whose probable structures have been proposed, those derived from silica would come from neosynthesis during the production of sub-fractions by VLC. The bioactive compounds contained in the F1 and F4 sub-fractions would be responsible for the antifungal activity observed on the inhibition of the growth and development of *P. myriotylum* by extracts and fractions of A. *indica* and T. *peruviana* (Siddharth & Vittal, 2018). Indeed, Oxime methyl phenyl belonging to the imine family (present in F1 and F4) is generally used in fungicide formulation due to their effective properties against harmful fungi (Kozłowska *et al.*, 2017; Shahbaz, 2017; Dhuguru *et al.*, 2022; Zhmurenko *et al.*, 2020). They are also highly effective against gram- and gram+ bacteria, a powerful anti-carcinogen and antioxidant, as demonstrated by the work of Kozlowska *et al.* (2017), Shahbaz (2017), Zhmurenko *et al.* (2020) and Dhuguru

et al. (2022). In addition, fatty acid esters such as Hexadecanoic acid, methyl ester; 9,12-Octadecadienoic acid, methyl ester; 11-Octadecenoic acid, methyl ester are recognized for their antimicrobial activities and are also thought to be responsible for the inhibition of *P. myriotylum* (Ojinnaka *et al*., 2015; Dida *et al*., 2024). Indeed, Dooh *et al*. (2021), Meena *et al*. (2021), Toka *et al*. (2023) and Dida *et al*. (2024) found similar chemical compounds in organic extracts of *T. peruviana* seeds and leaves that were shown to possess antifungal activity. Pyridines and their derivatives (2,2'-Azoxybis[3-methylpyridine]) are also recognized for their antifungal, antibacterial and even antidiabetic properties (Mishra *et al*., 2021; Mohammad Abu-Taweel *et al*., 2022). Plants are an important source of nutrients for many organisms, including microbes and pathogenic fungi. To defend themselves against pathogen attacks, they develop innate or acquired mechanisms and produce compounds such as fatty acid esters and phenolic compounds, recognized for their antioxidant, anti-carcinogenic and antimicrobial activities (Castillo *et al*., 2012; El-Naggar & El-Ewasy, 2017; Tleubayeva *et al*., 2021) as present in these different fractions.

The F4 sub-fraction had a relatively low MIC compared with F1. This considerable difference could be the result of their mode of action on *P. myriotylum*. Indeed, the F4 sub-fraction acts on *P. myriotylum* by inhibiting protein synthesis, proton ATPase pump activity and by acting on the integrity of the *P. myriotylum* wall, unlike the F1 sub-fraction which merely inhibits protein production. The compounds present in these two fractions act differently on the pathogen. The inhibition of protein synthesis by the two sub-fractions could be explained by the presence of oxime-, methoxy-phenyl, a powerful anti-microbial as demonstrated by Al-Mussawii *et al.* (2022). Moreover, the modes of action of sub-fraction F4 are similar to those obtained by Flore *et al.* (2023) on the effect of plant extract-based formulations for the protection of cocoyam against *P. myriotylum*.

CONCLUSION

The aim of this study was to characterize the bioactive fractions of *A. indica* and *T. peruviana* on the development of *P. myriotylum*. Aqueous and organic crude extracts of *T. peruviana* and *A. indica* as well as their fractions significantly reduced mycelial growth of *P. myriotylum*; GC/MS of the two sub-fractions of interest F1 from *A. indica* and F4 from *T. peruiana* revealed the presence of bioactive compounds with antimicrobial activities, including oxime-, methoxy-phenyl, Phenol, 2,4-bis(1,1-dimethylethyl); Hexadecanoic acid, methyl ester; 9,12-Octadecadienoic acid, methyl ester; 11-Octadecenoic acid, methyl ester; Methyl stearate; Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl and many others; Analysis of the modes of action of the sub-fractions revealed inhibition of pathogen protein synthesis by these two sub-fractions. F4 also acts on wall integrity and proton ATPase pump activity. From all this, these two plants could be used as potential substitutes for synthetic pesticides.

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