Plant Omics Journal POJ

POJ 3(3):70-76 (2010) ISSN:1836-3644

Effect of *Thevetia peruviana* seeds extract on *in vitro* growth of four strains of *Phytophthora megakarya*

Z. Ambang*[1], J. P. Ngoh Dooh[1], G. Essono[1], N. Bekolo[1], G. Chewachong[1], C.C. Asseng[2]

[1] Laboratory of Phytopathology and Microbiology, Department of Plant Biology, Faculty of Science, University of Yaoundé I, Box. 812, Yaoundé-Cameroon
[2]Department of Plant Biology, Faculty of Science, University of Douala, Box 24155, Douala-Cameroon

*** Corresponding author: zachambang@yahoo.fr**

Abstract

Recently, a number of researchers have shown interest in finding out the potential of natural substances of plant origin in crop protection. A study was carried out in Yaounde (Cameroon) to test the antifungal activity of extracts of *Thevetia peruviana* seeds. Crude extracts were obtained after successive maceration with hexane, ethyl acetate and methanol. Seed extracts were tested for inhibition of *P. megakarya*, a causal agent of black pod disease of *Theobroma cacao*. Four strains of *P. megakarya* (BOK 11; BOYO; TA121 and TA123) were used. Antifungal tests were performed by using three concentrations (12.5, 25 and 50 µl ml^{-1}) of crude extracts. Seven days after incubation on pea agar medium, the results showed that the use of 50, 25 and 12.5 µl.ml^{-1} of crude extracts with ethyl acetate resulted in 100, 70 and 50% inhibition rates respectively on *P. megakarya*. Methanol extract on the BOYO, TA121 and TA123 strains led to total inhibition, while 37.5, 43.2 and 63.9 % inhibition rates were recorded for BOK11 strain at 12.5, 25 and 50 µl.ml^{-1} concentrations of the extract respectively. These results suggest that crude extracts from *T. peruviana* seeds are efficient biocide substances with antifungal activity.

Keywords*:* Antifungal activity; plant extracts; *Thevetia peruviana; Phytophthora megakarya*

Abbreviations*:* BOYO_*P. megakarya* strain isolated in a locality of Mbalmayo, Centre Region of Cameroon; BOK 11_*P. megakarya* strain isolated in a locality of BOKITO, Centre Region of Cameroon; CEH_crude extract with hexane; CEEA_crude extract with ethyl acetate; CEM_crude extract with methanol; MIC_minimal inhibitory concentration; TA121 and TA123_*P. megakarya* strains isolated in locality of TALBA, Centre Region of Cameroon

Introduction

In Cameroon, black pod disease, caused by *Phytophthora megakarya*, is the most important field disease in cocoa beans production and causes up to 80% loss when no protection measure is taken (Berry and Cilas, 1994). In cocoa protection, several methods are recommended to fight against *P. megakarya.* Chemical protection which is mostly used is not only inefficient at times, but is also expensive and causes environmental pollution. The pesticides generally used are not always accessible to farmers. The biological method involves the use of plant extracts and antagonistic micro organisms. The use of *Trichoderma* species (Holmes et al., 2004; Tondjè et al., 2003) provided interesting results. However, a number of limitations with respect to the action spectrum of these organisms and their high cost accrue. Recent research reports have shown the importance of natural plant products, not only in human health (Omolara et al., 2007) but also in agriculture, as a rich source of pesticide substances that could be used in crop protection (Mollah and Islam, 2007; Ambang et al., 2007; Wink, 1993).

Yellow oleander (*Thevetia peruviana*) is a small tree commonly used as an ornamental plant in many tropical countries and belongs to the family *Apocynaceae* (Tewtrakul et al., 2002). Seeds, leaves, fruits and roots of *T. peruviana* are considered as potential sources of biologically active compounds (Oderinde and Oladimeji, 1990), such as insecticides (Ambang et al., 2005; Reed et al., 1982), rodenticides (Oji et al., 1993; Oji et al., 1994; Oji and Okafor, 2000), fungicides (Kurucheve et al., 1997; Gata-Gonçalves et al., 2003) and bactericides (Saxena and Jain, 1990; Obasi and Igboechi, 1991). This plant may also produce cardiac glycolsides, such as peruvoside and nerifolin, which have a relatively high therapeutic index compared with that of digoxin (Manthu and Sharma, 1980; Gata-Gonçalves et al, 2003; Omolara et al., 2007). There is paucity of information in many African countries on the antifungal properties of yellow oleander in crop protection. The relationship between crude extract concentrations and growth of *P. megakarya* strains can determine the fungicidal and fungistatic activity of these plant extracts. The main objective of this study was to examine the potential of crude extracts from *T. peruviana* seeds after maceration in different organic solvents in inhibiting the development of *P. megakarya*.

Materials and methods

Plant and fungal materials

Plant material included fruits from yellow oleander (*T. peruviana*). These fruits were harvested in Yaounde (Cameroon). Fungal material included pure strains of *P. megakarya* obtained from isolates collected from diseased cocoa trees that showed a high intensity of black rot in old

Table 1. Relative composition of the constituents found in crude extract *of T. peruviana* seeds (Gata Gonçalves et al., 2003)

Compound	%
3-Methylcyclohexanone	5.0
1-Nonene	0.2
Isopulegol	0.3
2-(2-Butoxyethoxy) ethanol	1.4
Benzoic acid	0.8
Capylic acid	0.9
Pulegone	14.3
Tetrahydrogeraniol	0.3
4-Isopropyl-1,3-cyclohexanedione	2.8
3-butyl-hexa-3-ene-2-one	1.8
Carvacrol	0.7
2-Butoxyethyl acetate	4.7
2-Nonenal	1.0
2-Propyl-1-heptanol	0.9
8-Methyl-1-undecene	3.0
Citonellol	1.0
*C*is-Pulegone oxide	2.7
Spathulenol	1.6
Nerolidol	0.4
Methyl isosterate	2.0
Palmitic acid	6.2
Methyl palmitate	0.2
Methyl elaidate	3.9
Linoleic acid	8.2
Oleic acid	1.0
9-Octadecenal	0.6
9-Octadecenol	0.2
9-Octadecenamide	2.5
B-Ergosterol	1.9
Terpenes	25.2
Fatty acid and derivatives	24.8
Others	20.5
Identified compounds	70.5

farms which had never been subjected to phytosanitary treatment. The strains which were taken from different cocoa producing villages in the Centre Region of Cameroon. include BOYO, TA 121, TA 123 and BOK 11. These strains of different sensitivities were selected following their degree of aggressiveness.

Obtention of crude extracts

Fruits of *T. peruviana* were peeled in order to obtain the kernels. They were next cracked and the grains obtained

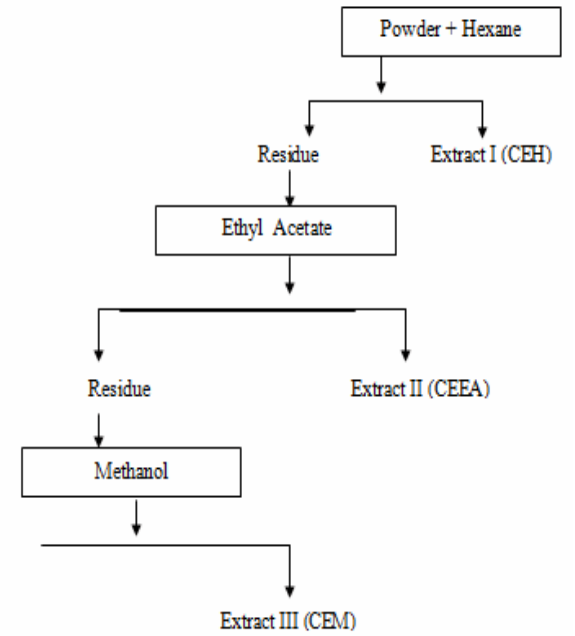

Fig 1. Scheme of plant extract production using different organic solvents

dried at room temperature (24±2 °C) in the laboratory for four weeks. The dried grains were crushed and the powder obtained weighed and macerated at the rate of 285 g L^{-1} of solvent in a 20 L bucket. Hexane, ethyl acetate and methanol were successively used as organic solvents. Maceration in each solvent lasted 48 h. At the end of each process, the residue was reused with another solvent (Fig. 1). After maceration, the solvent, saturated with extracted compounds was filtered and the filtrate dried with a Bûchi rotative evaporator. Extracts obtained were ready for further use.

Preparation of culture medium

To prepare the culture medium, 70 g of pea grains were completely ground in a manual water-mill. The solution obtained was filtered in a 100 ml beaker using a double folded muslin cloth. Next, the solution was adjusted to a volume of 100 ml with distilled water then 15 g of agar were added to the boiling solution and homogenised in a plate with a magnetic stirrer. The pH was adjusted to 6.5. The solution obtained was sterilised in an autoclave at 121 °C for 30 min.

Obtention of different extract concentrations

A mother solution of 500 µl ml^{-1} was prepared by mixing 1 ml of the extract to 0.25 ml of the solvent and 0.75 ml of distilled water (i.e. an initial volume of 2 ml). From this solution, 12.5, 25 and 50 $\mu l.ml^{-1}$ concentrations were obtained by taking from the mother solution, 0.25, 0.5 and 1 ml respectively. These were then added to 9.75, 9.5 and 9 ml of the culture medium to obtain a final volume of 10 ml. The solvents used for dilution were hexane, ethyl acetate and methanol.

Antifungal activity of crude extracts

To determine the inoculum concentration of every strain of *P. megakarya*, Petri dishes were prepared with 15 ml of pea agar (PA) and a 6 mm diameter of mother isolate of *P. megakarya*.

Table 2. Effect of solvents on growth of *P. megakarya* strains (mean diameter in mm ± SE)

Solvents	Doses (µl ml^{-1})	BOK11	TA121	BOYO	TA123
Methanol	0	44.75 ± 3.92 a	56.87 ± 4.33 a	55.86 ± 5.11 a	46.55 ± 4.22 a
	50.0	44.34 ± 3.41 a	57.29 ± 4.47 a	54.86 ± 5.38 a	49.78 ± 4.14 a
Ethyl acetate	0	58.78 ± 3.14 a	51.20 ± 4.20 a	54.69 ± 4.64 a	50.27 ± 4.35 a
	50.0	57.55 ± 3.22 a	50.18 ± 4.70 a	52.50 ± 4.61a	49.68 ± 3.64 a

Means followed by the same letter in a column are not significantly different according to Duncan test (P= 0.05)

After seven days of incubation at 24 °C, fungus suspensions were prepared by disintegrating the mycelium in 200 ml of a liquid medium adequate for *P. megakarya* (Allen and Kuc, 1989). Each fungus suspension was filtered under vacuum through nylon Magna on a Buckner, adding liquid media until a spore count of 3 x 10^6 to 5 x 10^6 per ml was achieved using Malassez Cell. Antifungal tests were performed in Petri dishes on pea agar medium containing 12.5, 25 and 50 µl of crude extracts of *T. peruviana* seeds. Fungi were inoculated by depositing a 6 mm diameter of mother isolate of *P. megakarya* in the middle of the Petri dishes containing the culture medium with crude extracts. Incubation was performed at 24 ±2 °C for seven days in continuous dark (12 h) and under exposure to light (12 h), in laminar box "Captair CRUMA" made in Spain, with a set of two lamps as a light source. The distance between the lamps and the surface of Petri dishes was 60 cm. At the end of each trial, explants of mycelium from the dishes where growth was totally inhibited were taken and deposited aseptically on the culture medium containing no plant extract. After incubating for 7 days and depending on whether growth resumed or not, the initial extract was identified as being fungistatic or fungicidal (Kishore et *al*., 1993; Pandey et *al*., 1982). Microscopic observations were done at the end of each trial in order to visualize any modification in the mycelium or any effect of plant extracts on fungal sporulation. Spores were liberated by thermal shock after passage in cold air for 30 min, and then left in the dark for one hour.

Measurement of fungal radial growth

Radial growth of *P. megakarya* was evaluated by daily measurement (start after two days of incubation) of perpendicular diameters of mycelia growth each day, according to the Singh et al. (1993) formula: $D = \frac{d_1 + d_2}{2} - d_0$

Where: d_0 = diameter of explant; d_1 and d_2 = diameters of culture measured in both perpendicular directions.

Determination of inhibitory percentage

Inhibitory percentage (I %) of different extract concentrations was determined by comparing with the control sample after 14 days of growth according to the following formula (Singh et al., 1993):

$$I(\%) = \frac{D_t - D_x}{D_t} \times 100$$

Where: I (%) = inhibitory percentage; D_t = average diameter of the culture with neither fungicide nor extract; D_x = average diameter of the culture with extract or synthesised fungicide.

Composition of Thevetia peruviana extract

Gata-Gonçalves et al. (2003) in a similar study reported that crude extract of *T. peruviana* seeds after analysis by Capillary gas chromatography contain several classes of components. In the composition of the extracts of *T. peruviana* seeds, they are many terpene compounds and some fatty acids (Table 1).

Table 3. Influence of concentration of CEM on the number of *P. megakarya* spores (BOK11 strain) after seven days of incubation

Doses of the extract (CEM) in µl ml^{-1}	Number of spores ml^{-1}
0 (Control)	20
12,5	1. 10^5
25	3. 10^6
50	5. 10^6

Statistical analysis

The SPSS computer software was used. Extract activity modalities were compared on the basis of growth diameter of different strains with one dimensional analysis of variance test (ANOVA). Duncan's multiple range test permitted the constitution of homogeneous sub-units at a threshold of 5%.

Results

Effect of solvents on fungal growth

The incorporation of methanol and ethyl acetate in the culture medium at high concentrations (50.0 µl) enabled us to prove that dilution agents (organic solvents) have no effect on the growth of *P. megakarya* for all the strains used (Table 2). No significant differences in fungal growth where observed between test and control samples.

Effect of crude extracts with Ethyl Acetate (CEEA)

CEEA generated different degrees of inhibition on the strains tested. The growth of BOYO, TA121 and TA123 strains were highly inhibited. All the treatments proved to be efficient against these strains with inhibitory percentages ranging from 1.82 ± 0.57 for the minimal dose (12.5 µl ml-1) to 97.41 ± 0.23 % for the highest dose (50 µl ml-1). TA121 strain was more sensitive to the 50 µl.ml-1 dose of CEEA. On the other hand, BOK11 strain was resistant to CEEA (Fig. 2). Comparing growth diameters between CEEA (50 µl ml-1) and control samples revealed a significant difference ($P<0.05$). However, the percentage of inhibition reduced with time ranging from 100 % on the third day to 9.44 ± 1.05 % on the 10th day signifying some tolerance of this strain to CEEA (Fig. 2). Total inhibition of TA123 strain was recorded with the 50 µl.ml-1 dose of CEEA until the 7th day after incubation (Fig. 3).

Effect of crude extract with methanol (CEM)

A total repressive effect of the CEM was observed on the TA121, TA123 and BOYO strains of *P. megakarya* for all the concentrations, since there was no fungal growth after the

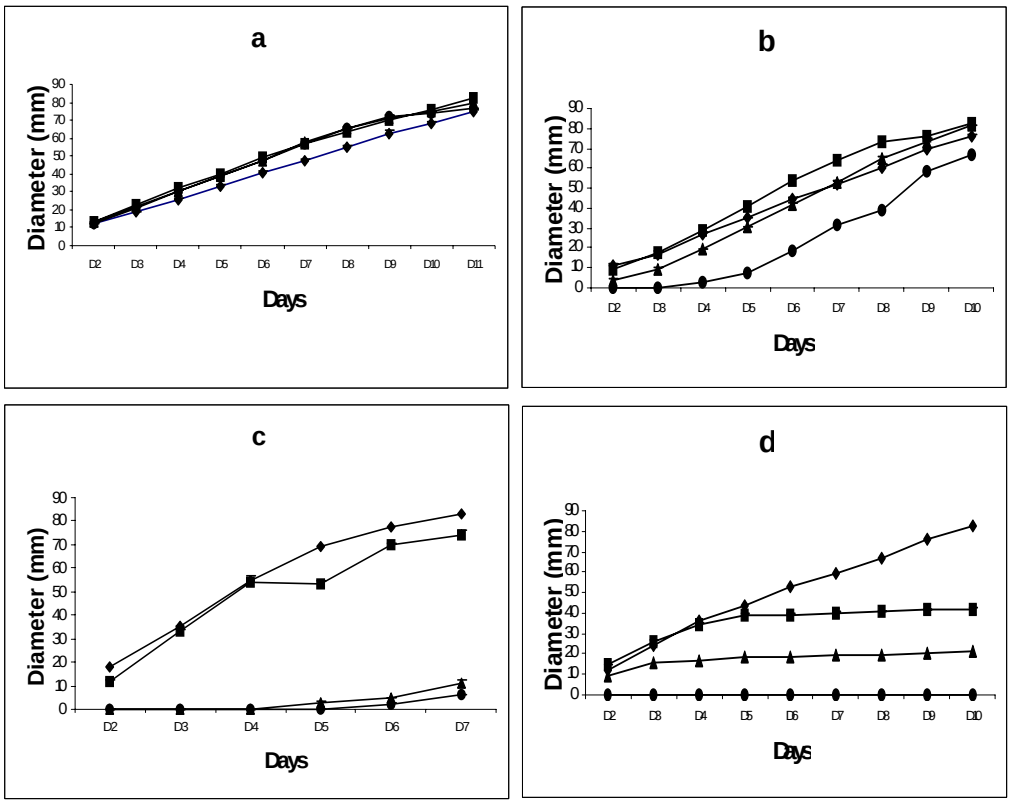

Fig 2. Effect of CEEA on radial growth of different strains of *P. megakarya* on pea agar medium (a) BOK11; (b) TA121; (c) BOYO; (d) TA123; CEEA: crude extract with ethyl acetate.

use of this extract (Fig. 4). The growth diameter obtained with the BOK11 strain also revealed a highly significant effect of CEM ($P < 0.01$) on fungal development when compared with the control sample (Fig. 4). More than 50 % inhibition was observed for the three doses used on BOK11 strain.

Effect of plant extracts on the hyphae morphology and sporulation of P. megakarya

The effect of *T. peruviana* seed extracts on the morphology of *P. megakarya* varied depending of the type and the concentration of extract. Microscopic observations showed that in control dishes, mycelia of *P. megakarya* were characterised by large, free and distinctive hyphae placed in a disordered manner (Fig. 5 A). Contrarily, in dishes with high concentration of extract, the hyphae were few and very small with a large number of sporocysts (Fig. 5 B, C and D). In samples where growth was not totally inhibited, the observation of spores at the end of the growth period did not reveal any inhibitory effect of the extracts on sporulation. The number of spores was higher in the medium with plant extracts than in the control after seven days of incubation. Increase in the number of spores was proportional to the concentration of the extract (Table 3).

Fungicidal activity of crude extracts

All the extracts showed fungicidal effects even at low doses on TA121, TA123 and BOYO strains. However, the activity of CEM at 25 and 50 µl ml^{-1} was respectively fungistatic and fungicidal on BOK 11 strain (Table 4).

Table 4. Fungicidal activity of *T. peruviana* extracts

P. megakarya strains	Plant extracts	MIC (µl)	Effect
TA121	CEM	12.5	fungicidal
BOYO	CEM	12.5	fungicidal
TA123	CEM	12.5	fungicidal
	CEEA	50.0	fungicidal
BOK 11	CEM	12.5	fungistatic
	CEM	50.0	fungicidal

MIC- Minimal inhibitory concentration

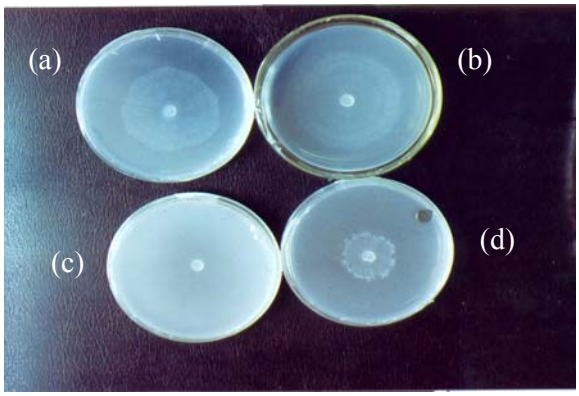

Fig 3. Inhibitory effect of CEEA concentrations on TA123 strain of *P. megakarya* 7 days after incubation on PA medium a- 0 µl ml^{-1} (fungal growth in control); b- 12.5 µl ml^{-1} (low inhibition); c- 25.0 µl ml^{-1} (high inhibition); d- 50.0 µl ml^{-1} (total inhibition).

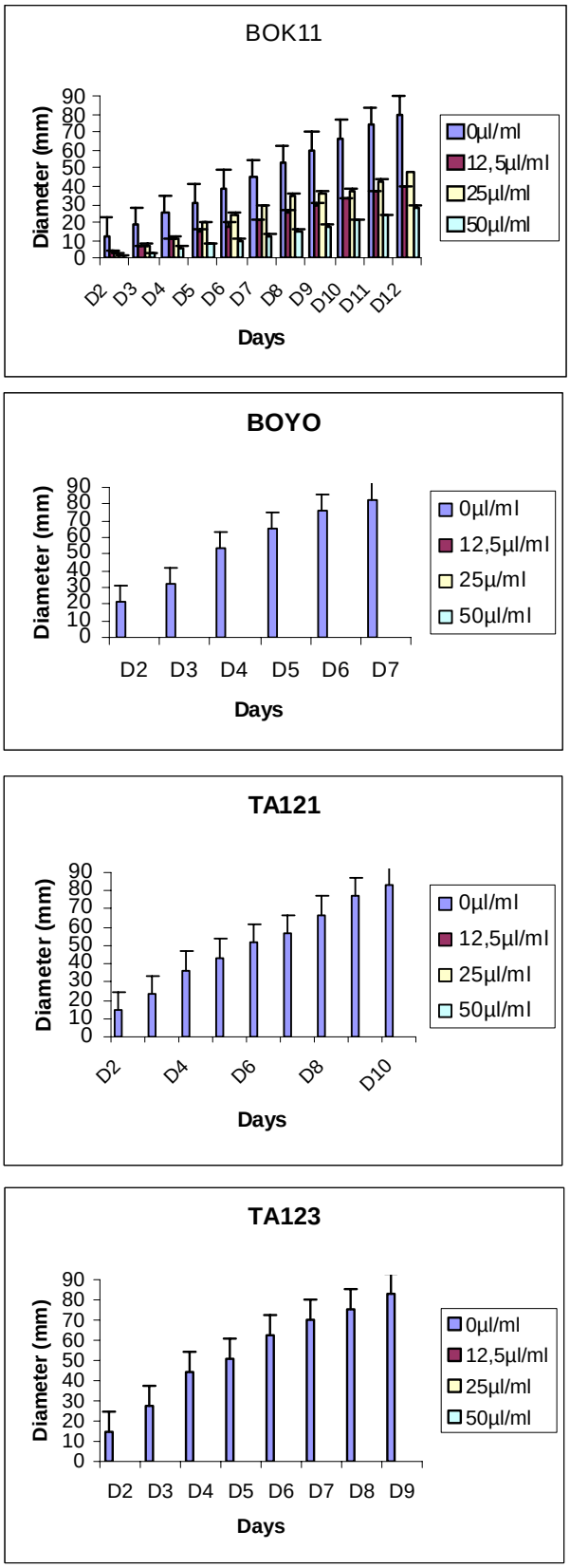

Fig 4. Influence of CEM on the growth of different strains of *P. megakarya* on PA medium

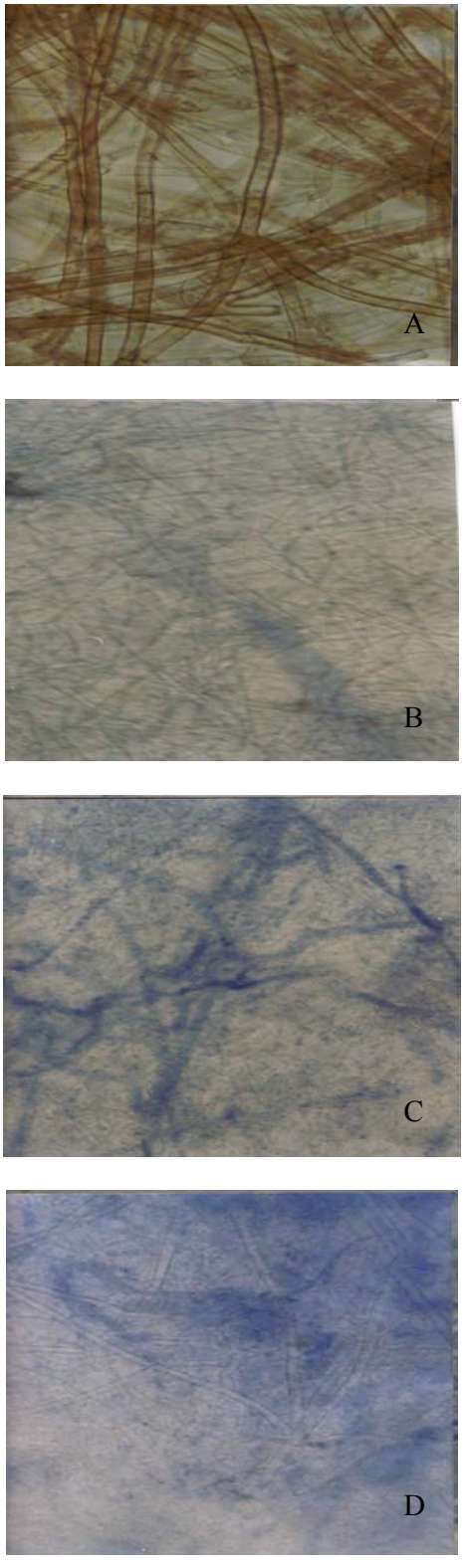

Fig 5. Influence of *T. peruviana* extract on hyphae structure and sporulation of *P. megakarya* A: Control; B: Medium with 12.5 µl of CEM, C: Medium with 25 µl of CEM; D: Medium with 50 µl of CEM

Discussion

The use of natural substances of plant origin in crop protection is an important means of promoting biopesticides in crop production. This study was aimed at testing the antifungal properties of extracts of *T. peruviana* seeds against some strains of *P. megakarya*, causal agent of black pod, a very dangerous disease of *Theobroma cacao* in many African countries. The study includes all biotechnology aspects. The stimulating effect on the growth of fungi observed at the least concentration of plant extracts (12.5 µl ml^{-1}) is close to Roger's (1951) hypothesis that nearly all anti-cryptogamics act as excitants at low doses. CEEA showed a high inhibitory effect against TA121, TA123 and BOYO strains but had a very low influence on the growth of BOK 11 strain which seems to be resistant. Methanol extract showed very high inhibitory percentage and was efficient against BOK 11 strain which was resistant to CEEA. The antifungal activity results of CEM from *T. peruviana* seeds in this study could be explained by the presence of compounds of very high molecular weight (Ibiyemi et al, 2002) such as terpenes (pulegone, cis-pulegone, spathulenol, citronellol, carvacrol, nerolidol, isolpulegol) and fatty acids (palmitic, linoleic acid, benzoic, caprylic and oleic) which were identified using capillary gas chromatography by Gata-Gonçalves et al. (2003) in similar research. Results obtained show that BOK 11 strain of *P. megakarya* was less aggressive when compared with the growth of other strains in control samples. Similar results were obtained by Ondo (2005) after testing the BOK 11 strain on cocoa pods and leaves. However, this strain was noticed to be resistant to the plant extracts used. This might be due to the non specificity that it presents at the level of the fungal membrane. Generally, antifungal substances could be contact or systemic. Depending on the situation, intracellular specific receptors could be indispensable for the expression of the biological activity of antifungal compounds (Juakli, 2003). Some chemical constituents have the capacity to recognise pathogens active sites while others do not. They could be acting through a concentration effect and once fixed on their receptors, may elicit responses such as alteration of the fungal plasmic membrane and inhibition of the general metabolism (fungistatic effect) or an inhibition of respiration (fungicide effect), similar results were obtained by Mutok (2008) The results of this study suggest that strain sensitivity to tested plant extracts is not linked to the production of zoospores. However, TA121 strain was noticed to be most resistant to the tested extracts when compared to TA123 and BOYO strains. This result could be linked to the high production of zoospores by the TA121 strain. The same results were obtained for aggressiveness of the strains tested. Extracts had no effect on sporulation. Crude extracts of *T. peruviana* seeds with methanol (CEM) and ethyl acetate (CEEA) showed a strong fungicidal activity against *P. megakarya* and may constitute a less expensive and efficient biocide that can be used in plant protection, particularly in *Theobroma cacao* protection against black pod disease.

Acknowledgements

Authors are thankful to Prof. Ndongo E. of the Department of Organic Chemistry, University of Yaounde I, for help to obtain the plant extracts used in this work.

References

Allen EH, Kuc J (1989) α-Solanine and α-Chaconine as fungitoxic compounds in extracts of Irish potato tubers. Phytopathol 58: 776-781

Ambang Z, Ndongo B**,** Petga E, Ngoh Dooh JP, Asanga A (2007) Effect of crude extracts of *Thevetia peruviana* seeds on development of leaf spot disease of groundnut (*Arachis hypogaea* L.) caused by *Cercospora* sp. *African Crop Science*. In : Kasem ZA, Mahmoud MA, Shalabi SI (eds) African Crop Science Conference Proceedings, ACSS, October, 27-31 2007. vol 8. El-Minia, Egypt, p 797-800

Ambang Z, Ngoh DJP, Ndongo B, Djilé B (2005) Effet des extraits bruts des graines du laurier jaune (*Thevetia peruviana* Pers) sur les charançons (*Sitophilus zeamais* Motsch), ravageurs des stocks. In: Omokolo ND (ed). Proceeding of XIIth Annual Conference of the Cameroon Bioscience Society, Yaounde, Cameroon, 2005

Berry D, Cilas C (1994) Etude du comportement d'une parcelle diallèle 6x6 vis-à-vis de la pourriture brune des cabosses de cacaoyers due à *Phythophthora* spp. au Cameroun. In: Proceedings of 11th International cocoa Research Conference, Yamoussoukro, Ivory Coast, 1994

Gata-Gonçalves L., Nogueira JMF, Matos O, Bruno De Sousa R (2003) Photoactive extract from *Thevetia peruviana* with antifungal properties against *Cladosporium cucumerinum*. J.Photochem. Photobiol. 70: 51-54

Holmes KA, Schroers HJ, Thomas SE, Evans HC, Samuels GJ (2004) Taxonomy and biocontrol potential of a new species of *Trichoderma* from the Amazon basin of South America. Mycological Progress 3: 199-210

Ibiyemi SA, Fadipe VO, Akinremi O O, Bako SS (2002) Variation in oil composition of *Thevetia peruviana* Juss "Yellow oleander" fruit seed. Journal of applied sciences and environment management 6: 61-66

Juakli MH (2003) La lutte biologique en phytopathologie. In: Lepoivre P (ed) Phytopathologie, 1st edn. De boeck, Bruxelles

Kishore N, Mishra AK, Cham SJPN (1993) Fungitoxicity of essential oils against dermatophytes. Mycoses 36: 211-215

Kurucheve V, Echilan JG, Jayaraj J (1997) Screening of higher plants for fungi toxicity against *R. solani* in vitro. Indian Phytopathol 50: 235-241

Mantu DE, Sharma AK (1980) Cardenolide contents in different genotypes of Thevetia nerifolia and Nerum odorum. Nucleus 23: 218-225

Mollah JU, Islam W (2007) Toxicity of *Thevetia peruviana* (Pers) Schum extract to adults of *Callosobruchus maculates* F. (Coleoptrera: Bruchidae). J Agric Rural Dev 5: 105-109

Mutok BF (2008) Effet de l'extrait brut au methanol des graines de *Thevetia peruviana* sur *Phytophthora megakarya* testé in vivo sur feuilles et cabosses de *Theobroma cacao* L. Mémoire de DEA, Université de Yaoundé I, p 54

Obasi NB, Igboechi AC (1991) Seed-oil distillates of *Thevetia peruviana* (SYN.*T.neriifolia*): analysis and antibacterial activity. Fitoterapia 62: 159-162

Oderinde RA, Oladimeji GR (1990) Oil characteristics of *Thevetia peruviana* (Yellow Oleander) and Pulmeria alba (White frangipani), Riv. Ital. Sostanza Grasse 67: 635-637

Oji O, Okafor QE (2000) Toxicological studies on steam bark, leaf and seed kernel of Yellow Oleander (*Thevetia peruviana*). Phytother. Res 14: 133-135

Oji O, Madubuike F.N, Ojimelukwe PC, Ibeh C.M. (1994) Rodenticide Potential of *Thevetia peruviana*. J. Herbs spices medicinal plants 2: 3-10

Oji O, Madubuike FN, Nwalozie MC (1993) Mortality in rats following Dietary inclusion of *Thevetia neriifolia* seeds. Fitoterapia 64: 137-139

Omolara OO, Ibiyemi AS, Lamidi AU (2007) Effect of detoxification on nutrient content of *Thevetia peruviana* seed cake. Research Journal of Applied Sciences 2 : 188-191

Ondo AS (2005) Caractérisation de quelques isolats de *P. megakarya* agent causal de la pourriture brune des cabosses de cacaoyer (*Theobroma cacao* L). Mémoire de DEA, Université de Yaoundé I, p 58

Pandey DK, Chandra H, Tripathi NN (1982) Volatile fungitoxicity activity in higher plants: special reference to that of *Callistemun lanceolatus* D.C. Phytopathol, 105: 175-182

Reed DK, Freedman B, Ladd TL (1982) Insecticidal and antifeedant activity of neriifolin against Codling moth, Striped cucumber beetle and Japanese beetle. J. Econ. Entomol 75: 1093-1097

Roger L (1951) Phytopathologie des pays chauds. Tome 1. Paul chevalier. Paris VI. France

Saxena VK, Jain SK (1990) *Thevetia peruviana* kernel oil: a potential Bactericidal agent. Fitoterapia 61: 348-349

Singh G, Padhvay RK, Narayaman GS, Padmkumari KP, Rao GP (1993) Chemical and fungitoxic investigation on the essential oil of Citrus. Pers. Z. Deutsche Zeits half fur Pflangenfrankenenen und flanzenschutz 100: 69-74

Tewtrakul S, Nakamura N, Hattori M, Fugiwara T, Suparita T (2002) Flavonone and flavonol glycosides from leaves of *Thevetia peruviana* And their HIV-1 reverse transcriptase and HIV-1 integrase Inhibitory activities. Chem. Pharm. Bull. 50: 630-635

Tondjè PR, Begoudé AD, Samuels GJ, Hebbar PK, Foko J (2003) Biological control the cocoa black pod disease caucausative agent (Phytophthora megakarya) with Trichoderma asperellum. In: Proceedings of 13th International cocoa Research Conference, Accra, Ghana, 2003

Wink M (1993) Production and application of phytochemicals from an agricultural perspective. In: van Beek TA, Breteler H (eds) Phytochemistry and Agriculture, Clarendon Press, England, p 171-212