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Full Length Research Paper

Screening and the effect of extracts of *Thevetia peruviana* on the development of *Colletotrichum gloeosporioides,* causal agent of cassava anthracnose disease

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The objective of this work was to carry out the screening of extracts from seeds of *Thevetia peruviana* (Pers.) K. Schum. and assess the effect of the extracts on the *in vitro* and *in vivo* development of *Colletotrichum gloeosporioides* that causes anthracnose of cassava. Five extracts obtained by soxhlet apparatus (extracts of hexane, ethyl acetate, acetone, methanol and the aqueous extract) were used. One fungicide (mancoxyl) served as control reference. Five strains (DB 1.1, 5a local, ES 4.1, Lit po and Bam 1.1) from five regions of Cameroon were tested. Three extracts concentrations (6.25, 12.5, and 25 µl/ml) and one concentration of fungicide (62 µg/ml) were used in PDA medium. The screening performed revealed the presence of several chemical families such as, coumarins, phenols, alkaloids, sugars, terpenes, sterols and flavonoids. The acetone, methanol and aqueous extracts inhibited the growth of strains with inhibition percentage ranging from 11.90 %-93 %. Only acetone extract proved active in the inhibition of spore germination of *C. gloeosporioides* with a percentage of inhibition of nearly 100 % at all concentrations. The MIC_{50} and MIC_{90} (minimum inhibitory concentrations) were determined. The *in vivo* test carried out on detached leaves has confirmed the effectiveness of these extracts in reducing the severity of the disease. These extracts could be used in the integrated control of the plant pathogens tested.

Key words: *Colletotrichum gloeosporioides*; *Thevetia peruviana*; screening; extracts; inhibition.

Abbreviations: HE_ hexane extract; EAE_ ethyl acetate extract; AE_ acetone extract ; ME_ methanol extract; AqE_ aqueous extract

INTRODUCTION

The protection of plants against crop diseases and pests is a key factor in the increase in crop production (Nguyemban, 1996). Tropical crops are the subject of special attention, due to plant pathogens that attack them and cause huge economic losses to farmers, and also because of their economic importance for these countries.

Cassava (*Manihot esculenta* Crantz) is one of the most important crops utilised as source of energy in the diet for the people in many regions of the world (Ceballos et al., 2004). Moreover, cassava is also used as a raw material in many industrial applications. Cassava anthracnose

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disease caused by the fungus *Colletotrichum gloeosporioides* f. sp. manihotis (Fokunang et al. 2002;IITA, 1990) has been reported to be one of the most important diseases of cassava in many countries especially in Africa (Owolade et al., 2005). This infection is characterised by cankers on stems, branches and fruits, leaf spots and tip die-back (IITA, 1990).The use of chemical fungicides is the most common choice for management of anthracnose disease,but this also causes the development of fungal resistance (Brent and Hollomon, 1998). In addition, continuous and inappropriate use of chemical fungicides to manage anthracnose disease is not considered to be the long-term solution because this can increase the investment expenses, the risk of having high levels of toxic residues, and also the concerns in human health and environmental settings (Latha et al., 2009).

Due to these reasons, there are several attempts to search for alternative measures to control the anthracnose disease effectively. Recent efforts have focused on the development of environmentally safe, long-lasting and effective biocontrol methods for management of anthracnose diseases. The utilization of natural products, especially the plant extracts, has been shown to be effective against many plant pathogens and consider to be safe for consumers and environments. A number of plant species have been reported to possess natural substances that are toxic to a variety of plant pathogenic fungi (Bautista-Banos et al., 2000; Imtiaj et al., 2005).

Seeds, leaves, fruits and roots of *Thevetia peruviana* . are considered as potential sources of biologically active compounds, such as insecticides (Reed et al. 1982; Ambang et al. 2005), rodenticides (Oji and Okafor, 2000) fungicides (Gata-Gonçalves et al., 2003; Ambang et al., 2010), virucides (Tewtrakul et al., 2002) and bactericides (Saxena and Jain 1990) *Thevetia peruviana* has already shown its effectiveness in reducing the inoculum pressure as well as the incidence of brown rot (Ambang et al., 2010). The objective of this work was to evaluate, *in vitro* and *in vivo* antifungal potential of extracts of seeds of yellow oleander (*Thevetia peruviana* (Pers.) K. Schum) on the development of *Colletotrichum gloeosporioides* a causative agent of anthracnose disease of cassava.

MATERIALS AND METHODS

Plant material

The plant material used consisted of *Thevetia peruviana* seeds collected in Yaoundé (Cameroon), and cassava leaves of the sensitive variety LMR from six months old plants obtained at IITA (International Institute of Tropical Agriculture) Nkolbissong-Yaounde.

Obtention of plant extracts

The mature fruits were collected from different locations of the city of Yaounde. The seeds obtained from fruits were dried in the laboratory at room temperature and then crushed using a hand mill (brand: "Victoria"). The resulting powder was loaded into cartridges and placed in a soxhlet apparatus. Extraction solvents with high polarity, hexane, ethyl acetate, acetone and methanol were successively used in the process. Each solvent was circulated through cartridges during 48 to 72 hours. The product obtained was then concentrated with Rota vapor and then kept at 4°C (refrigerator) throughout the experiment (Gata-Gonçalves et al., 2003). The aqueous extract was obtained by maceration of the seed powder in sterile distilled water for at least 12 hours (Stoll, 1994).

Isolation, purification of pathogen strains and obtaining of spores

Isolation was performed from infected cassava stems showing symptoms of anthracnose, collected in the field, in central, southern, littoral, south-west and east regions, and placed in a cooler and transported to the laboratory. The fragments of about one cm, presenting symptoms were cut with a scalpel and soaked in alcohol 70 °C for 2-3 minutes, then rinsed 2-3 times with sterile distilled water. These fragments were transferred to Petri dishes containing water-agar culture medium and incubated at 25°C in the dark. Four to five days after, the mycelium obtained was transplanted in boxes containing PDA medium. This last step was repeated several times in order to obtain pure strains of the fungus.Spores were obtained by scraping the mycelium of a pure culture from 6 to 7 days and homogenizing in a few ml of sterile distilled water.

Evaluation of the effect of the extracts on spore germination and growth of strains

To evaluate the effect of extracts on spore germination and growth, five strains of *C. gloeosporioides* were used (Bam 1.1, Lit po, ES 4.1, DB 1.1 and local).Different concentrations, C_1 (6.25 µl/ml), C_2 (12.5 µl/ml) C_3 (25 µl/ml) were prepared from a stock solution of 500 µl/ml. (Gata-Gonçalves, 2001). The dose equivalent to the highest concentration of the extract was prepared with mancoxyl. For the aqueous extract, the concentrations were 6.25, 12.5 and 25mg/ml obtained in the same manner.

Evaluation of the effect of the extracts on growth of strains

Mycelia disks obtained using a cookie cutter 7 mm in diameter and taken through a loop in cultures from 6 to 7 days of *C.gloeosporioides* were deposited in the center of each Petri dish containing PDA medium enriched with different extracts or fungicide (mancoxyl). A negative control non -supplemented in extract and a solvent control dilution were prepared. Each treatment was repeated 3 times in each of two tests. The plates were incubated at 25 °C and measurements were taken every day from the second day. However, the experiment was stopped when the Petri dishes were completely covered by the fungus. The radial growth of the strains tested was evaluated by measuring daily (48 h after inoculation) and at the same time, the two perpendicular diameters of the tracks on the back of the Petri dish. The average of two perpendicular measurements of the diameter minus diameter of explants represents the measurement of the radial growth of the fungus, according to the formula.

$$D = \frac{d1 + d2}{2} - d_0$$ (Singh et al., 1993)

where do = diameter of the explant, d_1 and d_2 = Culture diameters measured in two perpendicular directions.

Evaluation of the effect of the extracts on spore germination

The different media supplemented with extracts, fungicide and solvent dilution (Tween) were cast on the viewing strip, and after solidification, 20 µl /ml of a spore suspension calibrated at 3-4 10^5 spores/ml using a Mallasez's cell (hematimeter) was spread by means of a micropipette on each preparation. The whole was incubated in the dark for at least 12 hours. Each dose was repeated three times.

The experiment was repeated once. Counting of spores germinated or not was made on a total of 100 spores on three different areas of each blade. 300 spores per repetition and 900 spores for each treatment were counted under an optical microscope (magnification × 20). A total of 1,800 spores per treatment were counted (Wildmer and Laurent, 2006) for both tests.

Determination of minimum inhibitory concentrations

The values obtained after counting the spores and growth were used to determine the percentages of inhibition using the following formula:

IP = [(A-B)/A] x 100 (Leroux et al., 1978).

Where IP = inhibition percentage; A = number of spores or average culture diameter found in control medium; B = number of spores or average culture diameter with plant extract or fungicide.

From the linear regression equation between the natural logarithms of concentrations along the abscissa and the percentage inhibition of germination in ordinate, reducing the concentrations of 50% (MIC50) and 90% (MIC90) was determined to growth and germination spores (Dohou et al., 2004). The minimum concentrations which inhibit totally growth and germination were obtained for extracts that showed complete inhibition (100%).

Phytochemical Screening of extracts

The chemical tests were performed on the extracts of *T. peruviana* to determine their composition using the standard formula described by Sofowara (1993), Edeoga et al. (2005) and Sahuvinod et al. (2010). (Table 1).

Detached leaf bioassay

The ability of the extracts to reduce the frequency and size of lesions caused by *C. gloeosporioides* on leaves was evaluated according to a modification of the method of assessing resistance to *C. gloeosporioides* by leaves parts in the laboratory developed by Suparat et al., (2010). The leaves were surface-sterilised by dipping in 70% ethanol for 1 mn and rinsing two times in sterile distilled water. These changes involve two processing steps: first after spending 24 hours on a distilled water-soaked foam to make them most receptive leaflets (LMR) were sprayed on their underface with 80µl/ml extract solution at the concentration of 6,25µl/ml. Then in the second stage, a second application was made, 12h at least after, by spraying with a conidial suspension of *C. gloeosporioides* calibrated at 3-4 10^5 spores/ ml. For control tests, the leaflets were sprayed or not with a solution of water + tween (1%). An average of 20µl/ml inoculum was deposited per leaflet.

Thus inoculated pods were placed in bins on the foam soaked with distilled water. Incubation was carried out on leaflets inoculated at room temperature (28-30 °C); humidity was 70-75%. Leaflets were arranged in randomized complete block design in the tray. Each leaflet was infected at four different locations. The severity of the infection was assessed by measuring the area of necrosis according to observations of symptoms in the 4th and 6th day following an established scale of 1 to 5 (black dot = 1; 0.1-0.5 cm necrosis = 2, 0.5-1cm necrosis = 3, 1 -1.5 cm necrosis = 4; necrosis> 2cm = 5).

Statistical analyses

Data was analyzed by the Microsoft Excel software that performs the analysis of variance (ANOVA) and with the

Table 1: Chemical screening protocol of extracts

Natural product groups	Mode of operation	Characteristics
Essential oils	Evaporate 2 ml of extract in water	Scent a parfum
Saponifiable oils	Put 2 drops of extract on a filter paper	A transparent spot is formed
Coumarins	3 ml of extract + hot water + 10 % NH_4OH	Fluorescence
Alkaloids	4 ml of extract + 2 ml2% Hcl+ heat drops of Meyer reagent	A yellowish white precipitate is formed
Sterols	2 ml of extract + 4 ml acetic anhydride + 1 ml $CHCl_3$ + few drops of concentrated H_2SO_4	A greening blue color is formed
Triterpenoids	2 ml of extract + 4 ml acetic anhydride + 1 ml $CHCl_3$ + few drops of concentrated H_2SO_4	A purplish red color is formed
Flavonoids	2 ml of extract + 3ml concentrated HCl +some magnesium turnings	Effervescences + reddish brown color formed
Anthraquinones	2 ml of extract + 1 ml of $CHCl_3$ + 1 ml NH_4OH or NaOH	Purplish red coloration is observed
Gallic tannins	1 ml of extract + 1 ml of water + 3 drops of 5 % $FeCL_3$	Blackish- blue coloration
Catechic tannins	1 ml of extract + 1 ml of water + 3 drops of 5 % $FeCL_3$	Greenish-brown coloration
Saponins	1 ml of extracts + 1 ml of water + vigorous shaking	Appearance of foams
Anthocyanins	Extract + NaOH + HCl + pH paper	Colors, red in acid medium, violet in neutral medium and green in basic medium
Sugars	2 ml of extract + 2ml of naphtols in ethanol (molish reagent)+ concentrated H_2SO_4 poured gently and 2 liquid phases are distinct	A violet coloration is formed at the interface of two liquids
Glycosides	3 ml of extract + 3ml of glacial acetic acid + concentrated H2SO4 poured gently	Greenish–blue color for steroid glycosides. Purplish-red color for triterpenoids glycosides
phenol	2ml of extract+ethanol+lead acetate solution	White precipitate appeared

SPSS16.0 software using generalized linear model. Duncan at 5% was used to compare the averages.

RESULTS

Phytochemical screening

The phytochemical screening of extracts revealed the presence of several chemical compounds belonging to different families such as oils, coumarins, saponins, anthraquinones, sterols, alkaloids, tannins and phenol. The hexane extract is the most deficient in chemical compounds, only oils were found in it. Acetone, aqueous and methanol extracts were richer in compounds. Sugars and sterols were very abundant in the aqueous extract. No same chemical compound was found in the five extracts. Sugars, phenols and glycosides were found in traces (table 2).

Effect of extracts on the growth of *C. gloeosporioides* strains

Effect of hexane extract (HE) on the growth of *Colletotrichum strains*

The HE had no effect on the growth of the strains tested. A growth stimulating effect was revealed with some studied strains (ES 4.1, Lit po and Bam1.1) at all concentrations. Mancoxyl inhibited all strains with percentage range from 34.5% - 84.2%, a significant difference was observed between mancoxyl and all others treatment (table 3).

Effect of ethyl acetate extract (EAE) on the growth of *Colletotrichum strains*

The radial development of 5a local, Bam 1.1, Lit po and ES 4.1 strains was stimulated by EAE extract in all concentrations. The percentage inhibition obtained with 5a local was -54.3%, -62.9% and -100% respectively at the C_1, C_2 and C_3 concentrations. Only ES 4.1 was inhibited at the C_2 and C_3 concentrations (table 4).

Effect of methanol extract (ME) on the growth of *Colletotrichum strains*

The ME has been very effective on the growth of strains of *C.gloeosporioides* at all concentrations. The percentages of inhibition ranged from 11.90% to 62.32%. EM presented an inhibition greater than or equal to the

Table 2: Occurrence of natural products of each respective extract.

Solvent Products	Hexane	Ethyl acetate	Acetone	Methanol	Aqueous
Essential oils	T	_	+	+	+
Saponifiable oils	+	+	+	_	+
Coumarines	_	+	+	+	+
Alkaloids	_	_	_	+	+
Sterols	_	+	+	+	+++
Terpenoids	_	_	_	T	-
Flavonoids	_	_	_	_	_
Anthraquinones	_	_	_	_	+
Catechic tannins	_	_	_	+	_
Gallic tannins	_	_	_	_	_
Saponins	_	_	+	+	+
Anthocyanes	_	+	_	+	_
Steroic glycosides	_	T	+	+	+
Triterpenoid glycosides	_	_	_	T	_
Free sugars	_	_	T	T	+++
Phenols	_	_	_	T	_

- Absence of the products, + presence, +++ abundant presence, T presence in traces.

Table 3: Inhibition percentage of growth of *C.gloeosporioides* strains in PDA medium amended with different concentrations of HE and fungicide

isolate	concentrations[a]				
	T-	C_1	C_2	C_3	T+
ES 4.1[b]	0 c	-28.1 a	-5.3 b	-35.1 a	34.5 d
DB 1.1	0 a	2.7 b	2.7 b	4.0 b	54.4 c
Lit po	0 a	-37.0 b	-42.6 b	-42.6 b	44.1 c
Bam 1.1	0 c	-11.6 a	-11.6 a	-5.8 b	84.2 d
5a local	0 a	6.5 b	-1.6 a	-1.6 a	67.4 c

[a]T- control; C_1-6,25µl/ml ; C_2- 12,5µl/ml ; C_3- 25µl/ml ; T+ -mancoxyl
[b]Means within the same strain followed by the same letter are not significantly different (P=<0.05) according to least significant differences

Table 4: Inhibition percentage of growth of *C.gloeosporioides* strains in PDA medium amended with different concentrations of EAE and fungicide

	concentrations[a]				
isolate	**T-**	**C_1**	**C_2**	**C_3**	**T+**
ES 4.1[b]	0 b	-2.9 a	31.4 c	28.6 b	34.5 c
DB1.1	0 b	-10.2 a	-10.1 a	-11.6 a	54.4 c
Lit po	0 c	-8.6 a	-4.3 b	-10.0 a	45.9 d
Bam 1.1	0 b	-25.0 a	-26.7 a	-28.3 a	81.8 c
5a local	0 d	-54.3 c	-62.9 b	-100.0 a	74.4 e

[a]T- control; C_1-6,25µl/ml ; C_2- 12,5µl/ml ; C_3- 25µl/ml ; T+ -mancoxyl
[b]Means within the same strain followed by the same letter are not significantly different (P<0.05) according to least significant differences

fungicide at all doses with isolates ES 4.1 and Lit po, a significant difference was revealed between the different doses and the fungicide (P<0.05). Thus, the following three percentages were obtained 50.65%, 48.1 and 50.65% against 34.55% for the fungicide with the isolate ES 4.1. The local isolate 5a was the most resistant to the extract with percentages of around 11.90% to 28.57% (Table 5).

Table 5: Inhibition percentage of growth of *C.gloeosporioides* strains in PDA medium amended with different concentrations of ME and fungicide

	concentrations[a]				
isolate	**T-**	**C1**	**C2**	**C3**	**T+**
ES 4.1[b]	0a	50.7 c	48.1 c	50.7 c	34.5 b
DB1.1	0 a	12.5 b	31.9 c	50.0 d	54.4 d
Lit po	0 a	42.0 b	50.7 c	62.3 d	45.9 b
Bam 1.1	0 a	12.8 b	40.0 c	45.7 c	84.2 d
5a local	0 a	11.9 b	11.90 b	28.6 c	19.1 c

[a]T- control; C_1-6,25µl/ml ; C_2- 12,5µl/ml ; C_3- 25µl/ml ; T+ -mancoxyl
[b]Means within the same strain followed by the same letter are not significantly different (P<0.05) according to least significant differences

Table 6: Inhibition percentage of growth of *C.gloeosporioides* strains in PDA medium amended with different concentrations of AE and fungicide

	concentrations[a]				
isolates	**T-**	**C_1**	**C_2**	**C_3**	**T+**
ES 4.1[b]	0 a	24.7 c	16.4 b	41.1 e	33.5 d
DB 1.1	0 a	15.8 b	12.3 b	28.1 c	54.4 d
Lit po	0 a	1.3 a	4.1 a	2.6 a	45.9 b
Bam 1.1	0 a	11.7 b	15.6 c	20.8 d	84.1 e
5a local	0 a	20.3 b	31.9 b	46.4 c	67.4 d

[a]T- control; C_1-6,25µl/ml ; C_2- 12,5µl/ml ; C_3- 25µl/ml ; T+ -mancoxyl
[b]Means within the same strain followed by the same letter are not significantly different (P<0.05) according to least significant differences

Table 7: Inhibition percentage of growth of *C.gloeosporioides* strains in PDA medium amended with different concentrations of AqE and fungicide

	Concentrations[a]				
isolates	**T-**	**C_1**	**C_2**	**C_3**	**T+**
ES 4.1[b]	0 a	4,0 b	6,8 b	93,2 d	33,4 c
DB 1.1	0 a	28,9 b	85,5 d	84,1 d	68,3 c
Lit po	0 a	27,3 b	24,7 b	33,8 c	43,2 d
Bam 1.1	0 a	44,0 b	52,0 c	46,7 b	84,1 d
5a local	0 a	59,7 b	92,2 b	93,5 b	67,4 b

[a]T- control; C1-6,25µl/ml ; C2- 12,5µl/ml ; C3- 25µl/ml ; T+ -ridomil
[b]Means within the same strain followed by the same letter are not significantly different (P=<0.05) according to least significant differences

Effect of acetone extract (AE) on the growth of *Colletotrichum strains*

AE showed significant inhibition on the growth. The highest percentage of inhibition was obtained with 5a local and ES 4.1 strains at concentration of 25µl/ml respectively 46.38% and 41.1%. Lit po was least sensitive to extract at any dose tested with the inhibition percentages of 1.3% and 2.6% at doses of C_1 and C_3. The mancoxyl proved more effective than the extract with four strains (P<0.05). AE showed superior efficacy to synthetic fungicide with a percentage inhibition of 41.10 for C_3 dose, against 33.51% for mancoxyl with ES 4.1 strain (Table 6).

Effect of aqueous extract (AqE) on the growth of *Colletotrichum strains*

AqE inhibited all strains used at all doses. For the highest concentration, the inhibition percent age of growth ranged from 13.3 to 93.51%. Isolates ES4.1 and lit po proved less sensitive to the extract to C_3 dose, with 13.33% and 33.77% respectively. The extract showed superior efficacy than mancoxyl with two strains ES4.1 and 5a local with 93.51% against 67.33% for the mancoxyl and 93.24 against 33.43% for the mancoxyl. No strain has shown superior efficacy to fungicide with all doses tested (Table 7).

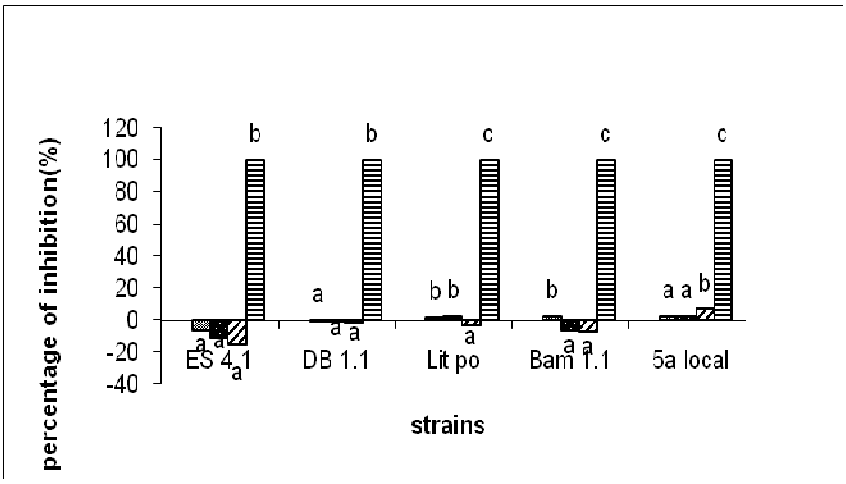

Figure 1: Effect of HE on germination of *C.gloeosporioides* strains For each concentration the values assigned by the same letter are not significantly different according to the Duncan test (P<5%) .

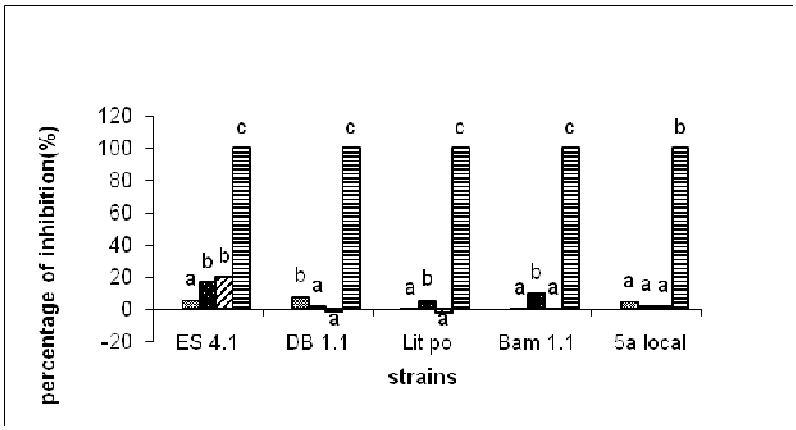

Figure 2: Effect of EAE on germination of *C.gloeosporioides* strains For each concentration the values assigned by the same letter are not significantly different according to the Duncan test at (P<5%).

Effect of extracts on germination of *C. gloeosporioides* spores

Effect of hexane extract (HE) on spores germination

The HE had no effect on the germination of spores of different strains tested. No significant difference was revealed at 5%. Slight stimulation of germination was observed with this extract with some strains (ES 4.1 and Bam 1.1) at some concentrations (negative percentages) (Figure. 1).

Effect of ethyl acetate extract (EAE) on spores germination

EAE did not have a significant inhibitory effect on the germination of spores at the different doses used. ES 4.1 was inhibited with inhibition percentage of 20% at C_3 concentration. But no significance difference was observed between mancoxyl and any concentration of extract (P<5%). Mancoxyl showed an efficiency of 100% with all strains tested (figure 2).

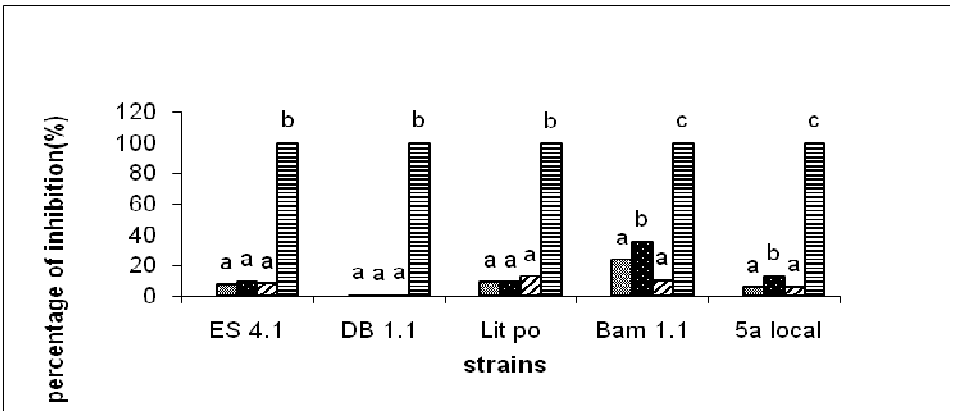

Figure 3: Effect of ME on germination of *C.gloeosporioides* strains
For each concentration the values assigned by the same letter are not significantly different according to the Duncan test (P<5%).

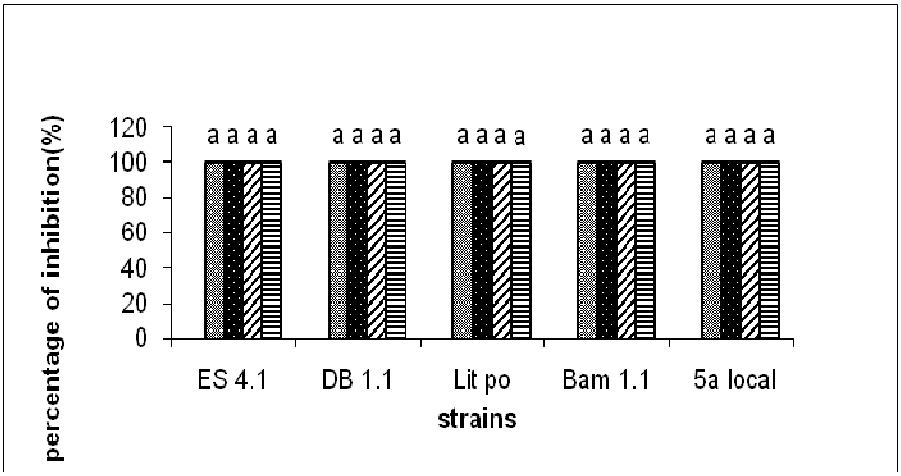

Figure 4: Effect of AE on germination of *C.gloeosporioides* strains
For each concentration the values assigned by the same letter are not significantly different according to the Duncan test (P<5%).

Effect of methanol extract (ME) on spores germination

ME slightly inhibited spore germination. The inhibition percentage ranged from 0.66% to 35, 53%. Bam 1.1 strain was the most sensitive to the extract (figure 3).

Effect of acetone extract (AE) on spore germination

Acetone extract proved to be very effective in inhibiting the germination of spores of the fungus. At all concentrations tested and with all the strains, the percentage of inhibition was 100%. No significant difference was obtained between this extract and the synthetic fungicide used, the mancoxyl (figure 4).

Effect of aqueous extract (AqE) on spore germination

Aqueous extract showed no significant inhibitory effect on spore germination compared to the mancoxyl positive control (P>5%). The highest percentage of inhibition was 3%, and was obtained with Lit po (figure 5).

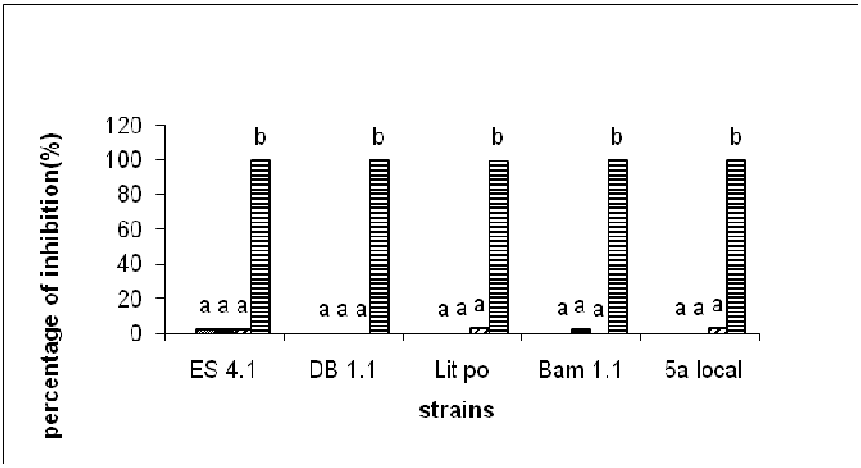

Figure 5: Effect of AqE on germination of *C.gloeosporioides* strains
For each concentration the values assigned by the same letter are not significantly different according to the Duncan test (P<5%).

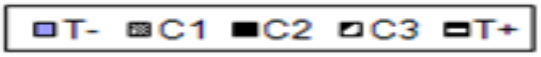

Table 8: MIC_{90} and MIC_{50} (in µl/ml) of the mycelia growth and germination of *C.gloeosporioides* in the presence of *T. peruviana* extract.

Isolate	HE		EAE		AE		ME		AqE	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Minimum inhibitory concentrations (MICs) of the extracts on the germination of spores										
ES4.1	$5.7\ 10^{5}$	$4.5\ 10^{7}$	**		6.25*		1.610^{20}	$8.1\ 10^{36}$	**	
DB1.1	**		**		6.25		$7.9\ 10^{7}$	$5.9\ 10^{14}$	**	
Bam1.1	**		$3.2\ 10^{10}$	1.310^{10}	6.25		**		**	
Lit po	**		**		6.25		$7.9\ 10^{7}$	5.910^{14}	$7.7\ 10^{12}$	3.410^{22}
5a local	$2.2\ 10^{8}$	$6.4\ 10^{13}$	**		6.25		$2.1\ 10^{8}$	2.210^{14}	$1.7\ 10^{7}$	6.310^{29}
Minimum inhibitory concentrations (MICs) of the extracts on the growth of strains										
ES4.1	**		49.27	298.1	84.2	2566	2.6	39.4	15.9	30.6
DB1.1	1.810^{23}	4.010^{41}	**		438.2	42572	1.7	7.8	3.9	27.5
Bam1.1	2.310^{7}	$3.8\ 10^{11}$	**		2376	$117\ 10^{4}$	2.9	14.7	45.4	90.6
Lit po	**		**		$1.6\ 10^{7}$	410^{11}	10.8	175.3	1259.6	7198.3
5a local	**		**		30.90	268.5	191	5602.1	3.2	17.2

*Minimum concentration which inhibited completely germination of spores
** Represents values that are undefined for being at zero statistically

MIC_{50} and MIC_{90} determination

MIC calculated from equations derived from regression was higher for germination compared to growth. This shows the sensitivity of these samples at the stage of growth. The HE, EAE, ME and AqE showed the highest MIC for germination. The minimum concentration which completely inhibited germination of spores of all tested strains (6.25µl/ml) was obtained with the AE. The ME and AqE gave the lowest MIC of growth. The equations with negative slope and non-significant with correlation coefficients less than 0.7 did not allow calculating the corresponding MIC of some isolates (table 8).

The *in vivo* test (detached leaf bioassay)

The test performed with acetone extract (AE) on leaflets showed a significant difference amongst control and dose tested at P<0.05. After 4 day, necrosis appeared on the inoculated leaflets treated with water + tween. Almost no symptoms were observed on the leaflets that were treated with extract. On day 6, a significant difference was obtained with only four of the five strains, Bam1.1 has been assigned (table 9). A highly significant correlation was obtained between the results of 4th and 6th day (matrix of Pearson 0,35**) .

Table 9: effect of the AE on the severity of the disease (anthracnose).

	4th Day		6th Day	
isolate	**Control[a]**	**treated**	**Control**	**treated**
Lit po	3.2±2.0 a	0.2±1.1 b	4.8±0.4 a	2.0±1.0 b
DB 1.1	3.2±0.6 a	0.2±0.4 b	3.2±0.6 a	1.1±0.8 b
ES 4.1	3.4±1.7 a	0.5±1.1 b	4.1±0.7 a	3.4±1.4 b
5a local	2.0±1.0 a	0.9±1.1 b	4.6±0.5 a	2.2±1.5 b
Bam 1.1	1.5±2.0 a	0.8±0.2 b	3.8±0.6 a	3.5±1.5a

[a]The value in the same row and same day affected with the same letter are no significantly different at P<0.05 according to Duncan's test

DISCUSSION

The antifungal properties of five extracts of *T.peruviana* was tested against five strains of *C.gloeosporioides* at different concentrations (6.25µl/ml, 12.5µl/ml and 25µl/ml) in cassava.

The results of the screening performed showed the presence of several families of compounds such as essential oils, sterols coumarins saponins, sugars, terpenes, tannins. Several of these compounds were also obtained by Gata-Gonçalves et al. (2003) and Oderinde et al. (1990) with the same plant.

The HE stimulated the growth of all strains compared to the control. A similar phenomenon was observed by Gata-Gonçalves (2001) with the aqueous extract of the leaves of *Thevetia peruviana* obtained by infusion (80 °C) on the development of *Fusarium culmorum*. The growth stimulating effect of the fungus observed with the HE approximates the assumption of Roger (1951) which states that almost all fungicides act as stimulants in low doses. Thus, Southan and Ehrlich cited by Roger (1951) gave the name of hormesis stimulus caused by harmful substance acting at very low doses.

AqE, AE and ME were effective against the growth of strains with a strong inhibition for methanol and AqE extracts. The antifungal activity of these extracts in this study could corroborate the work of Gata-Gonçalves et al. (2003) who explained it by the presence of the high molecular weight compounds (lactones and sterols). Other compounds in these extracts are known for their antifungal properties, triterpenes, phenols, sugars (Johann et al. 2007). These results are in agreement with those of Ogbebor et al. (2007) who had an inhibition of growth of mycelium of *C.gloeosporioides* which causes anthracnose of Hevea nearly 100% using leaf extracts of *O.bacillicum* Furthermore, these studies confirm those of Ambang et al. (2010) who obtained complete inhibition of the growth of the *P. megakarya* strains with methanol extract of seeds of *T. peruviana.*

The results indicate that the extract with acetone was found to be very active in the inhibition of spore germination for all strains with 100% inhibition of in all concentrations tested. This could be due to possible concentration of compounds responsible in this extracts. These results are in agreement with those obtained by Prapassom et al. (2012) who achieved complete inhibition of spore germination of *C. gloeosporioides* with the methanol extracts of leaves of *Piper sarmentosum* and with chloroform of *Mentha cordifolia* 1.25%. Furthermore the acetone extract of *Piper betle* leaves to 10µg/ml concentration inhibits the germination of spores of *C. gloeosporioides* of 72.91% (Johnny et al., 2011).

Plant extracts have shown their effectiveness against the germination of fungal spores. This is the case with results of Achraf et al. (2012) with the aqueous extract of *Asphodelus tenuifolius* and *Zygophyllum album* that proved effective in inhibiting spore germination of *Penicillium expansum* with a percentage inhibition of 95.48% and 93.82% respectively.

T. peruviana includes bioactive substances such as saponins, tannins, sterols, terpenes, flavonoids in significant amounts which possess antifungal and antimicrobial activity. Boulenouar et al., (2009) demonstrated the antifungal effect of flavonoids and the work of Gata-Gonçalves et al. (2003) showed the effect of extracts of *T. peruviana* against *Cladosporium cucumerinum.*

The low MIC value obtained with AE and ME highlights the effectiveness of these extracts on the germination of fungal spores. These results are in agreement with those of Serghat et al. (2004) who argue that low MIC values inhibit conidial germination of *Pyricularya grisea* causal agent of rice blast.

The leaves detached bioassay has demonstrated the effectiveness of extracts on plant tissues when they are treated by conidia of *C.gloeosporioides*.

The effectiveness of AE on germination could be explained by the presence of a compound that should act on the breath.

The heterogeneity observed in the percentages of inhibition and minimum inhibitory concentrations could be explained by the fact that fungi do not react in the same way with biopesticides.

This study is the first using *T. peruviana* as biopesticide against *C. gloeosporioides* pathogen in cassava.

CONCLUSION

Seed extracts of *T. peruviana* showed significantly effective inhibition rate against strains of *Colletotrichum gloeosporioides* in cassava. Acetone extract, Methanol extract and Aqueous extract are shown as antifungal against growth of *C. gloeosporioides*. Acetone extract has been proven his effective against germination of spore. Minimum inhibitory concentrations which completely inhibited germination of spores were observed with Acetone extract.

This study served as a basis for determining appropriate and effective concentration for the use of the plant for biological control against *Colletotrichum gloeosporioides*. Acetone extract showed its inhibitory potency in reducing severity of anthracnose on the leaf.

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