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Mycoflora associated with cocoa (*Theobroma cacao*) pods in Cameroon and antifungal effect of plant extracts

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Abstract— *Mycoflora associated with the pod rot disease of cocoa (Theobroma cacao) and evaluation of the in vitro efficacy of aqueous and ethanolic extracts of A. conyzoïdes and Chromolaena odorata against the pathogenic fungi, C. gloeosporioides and B. theobromae, isolated from cocoa pods were investigated. After isolation, the fungal species were exposed to various concentrations (5 ; 10 ; 15 ; 20 mg/ml) of aqueous, and ethanolic (1.25 ; 2.5 ; 5 ; 10 mg/ml) extracts. Results obtained showed some variations in isolation frequency of fungi from cocoa pods of each locality. Aspergillus, Colletotrichum, Botryodiplodia, Trichoderma and Verticillium were the most common genera that colonized the cocoa pods from Akonolinga and Tonga with different incidences. Colletotrichum gloeosporioides was present (48.84%) in pods collected in Tonga and in those from Akonolinga (41.46%), followed by Botryodiplodia theobromae which was present on 20.93% and 29.27% respectively. All the used concentrations of extracts of both plants significantly reduced the growth of the fungal pathogens. For ethanolic extracts, Ageratum conyzoïdes completely (100%) inhibited the growth of both fungi at 10 mg/ml and for Chromolaena odorata, total (100%) inhibition was observed on B. theobromae at 5 mg/ml while C. gloeosporioides was completely inhibited at 10 mg/ml. In the case of aqueous extracts, Chromolaena odorata, completely (100%) inhibited the growth of B. theobromae and C. gloeosporioides at 20 mg/ml. Similarly, Ageratum conyzoïdes completely suppressed the growth of B. theobromae at 20 mg/ml, however, this dose was obtained as an inhibition of 78% of C. gloeosporioides. Further investigation of the isolation of active antifungal compound should be done.*

Keywords— A. conyzoides, C. odorata, antifungal effect, Cocoa pods, mycoflora, plant extracts.

I. INTRODUCTION

Theobroma cacao (Cacao tree and cocoa tree), is a small (4 to 8 m) tall evergreen tree in the family Malvaceae (Juan *et al*., 2008) native to the deep tropical regions of Central and South America. Its seeds, cocoa beans, are used to make cocoa mass, cocoa powder and chocolate (Copetti *et al*., 2010). The fruit or cocoa pod is ovoid shape, 15 to 30 cm long and 8 to 10 cm wide, ripening yellow to orange. Cacao is grown both by large agro industrial plantations and small producers, the bulk of production coming from millions of farmers who have a few trees each (Henderson, 2007). In cocoa orchards in Cameroon, cacao pods are threatened by the surge in fungal diseases such as the brown rot caused by species of *Phytophthora* genus (Assoumou, 1997). This disease can cause yield losses between 60 to 100% in field (Luter and Akrofi, 1993, Berry and Cilas, 1994, Opoku *et al*., 2000), when conditions favor the development of disease. Other diseases such as black rot, and Witch's broom respectively caused by *Botryodiplodia theobroma, Moniliophtora roreri* and *Roniophtora perniciosa* take more and more scale (Koné, 1999 ; Koumé, 2006). Over the years there have been reports of fungal attack on cocoa pods rendering the seeds (beans) unfit for human consumption. Fungi such as *Phytophthora palmivora*, *P. capsici, P. kevea* causative agents of (black pod rot), *Lasiodiplodia* spp (*Lasiodiplodia* pod rot) *Macrophoma* spp *(Macrophoma* pod rot), *Phytophthora citropthora* and *P. megakarya* (*Phytophthora* pod rot) have been reported to cause depletion of pods/seeds value in the field (APS, 2011).

Although chemical control was developed by the research scientists, the dissemination of this method to the farmers was little successful. The requirements of the international market in terms of bean quality, environmental constrains, health issues for the consumers (Anonyme, 2006), are numbers of constraints that do not facilitate the development of the chemical control method. Face with this distrust increased with respect to these chemicals, there is a renewed interest in methods such as varietal resistance, use of biofongicide. On one hand, this study aims to analyze the fungi associated with decay of cocoa pods of 3 varieties collected from 2 localities in Cameroon: one located in the central region, belonging to the agroecological area with bimodal rainfall and the other

located in the Western region of country, in the upland area with single rainfall mode. On the other hand, this study also aims the evaluation of antifungal activity of some local plant species in order to offer an alternative of biocontrol.

II. MATERIALS AND METHODS

Sample collection and pathogen identification

One hundred matured infected cocoa pods were obtained from the field at different locations in Akonolinga (Central Region) and Tonga (Western Region) of Cameroon and transported to the Phytopathology Laboratory of the University of Dschang for analyses. The two Local Areas are the major producers of cocoa of the country. Cocoa beans (about 5mm in diameter) from the symptomatic and asymptomatic cocoa pods were removed following surface sterilisation with 70% ethanol for 10secs, blotted dry with sterile paper towel, and plated onto chloramphenicol-amended Potato Dextrose Agar (PDA). The V6 and V8 culture media were also used to promote the *Phythophtora* highlighting. After 3-5 days of incubation at 28˚C microbial growth was assessed microscopically. Cultures of the isolates were transferred to a new culture medium plated on Petri dishes, from where axenic cultures were obtained (Gevens *et al*., 2008). Identification of the isolates was based on morphological characteristics, described in the 1998 illustrated genera of fungi by Barnett and Hunter (1998) and with literature on the identification of pathogenic fungi by Dugan (2006).

Plant extracts

Aerial parts (leaves and stem) of *Chromolaena odorata* and *Ageratum conyzoïdes* L were collected in June 2016 from the locality of Akonolinga, Centre region of Cameroon**.** Their identification were confirmed through consultation in the Herbarium of the Department of Plant Biology, University of Dschang. Plant parts collected were washed three times with running tap water and rinsed with distilled sterile water. They were separately air-dried at room temperature and ground in a mortar. One hundred grams of the resulted dried powder were macerated in 500 ml of distilled water or ethanol and mixed thoroughly. For aqueous extract the mixture was allowed to rest for 48 hours and the supernatant passed through whatman's N°. 1 filter paper to obtain the extract. With regards to ethanolic extract, after maceration for 4 hours in a warring blender (Warring International, New Hartford, CT, USA), the macerate was passed through Whatman's N°. 1 filter paper and evaporated using a Rota vapour at 40°C water bath temperature (Heidolph) (Keuete *et al*., 2015). Extracts were preserved aseptically in a brown bottle at 4°C until further use (Souza *et al*., 1995).

In vitro antifungal activity of plant extracts

The antifungal effect of plant extracts were evaluated on *C. gloeosporioides* et *B. theobromae*, isolated from cocoa pods. The *in vitro* antifungal activity was assessed according to the agar dilution method (Sharma and Trivedi, 2002) on PDA (Difco). Plant extracts were dissolved in dimethylsulphoxide (DMSO) and diluted to give serial dilutions that were incorporated into growth medium. Concentrations of 1.25 ; 2.5 ; 5 and 10 mg/ml for ethanol extracts and 5, 10, 15, 20 mg/ml for aqueous extracts were used. PDA medium supplemented with different concentrations of the extracts were inoculated with 6-mm diameter (plugs) of the test pathogen cut from the margin of 7-day-old cultures. The plates were incubated in duplicates over a period of 10 days for *C. gloeosporioides* and *B. theobromae* at 20 ± 2°C. The radial mycelia growth was measured daily and the fungi toxicity was expressed as percentage inhibition of radial mycelia growth. In order to distinguish between fungicidal and fungi-static activity of the selected plant extract against the test pathogen, the mycelia plugs that did not show any growth were transferred to a freshly poured PDA plate and incubated for 7 days at 20 ± 2°C to observe the recovery of growth. The fungicidal effect was classified as an absence of growth whereas any observed growth was classified as fungi-static.

Statistical analysis

Data collected on percentage inhibition and lesion area were subjected to analysis of variance (ANOVA) using SPSS software version 17. The mean values were separated using Duncan Multiple Range Test (DMRT) at $P \leq 0.05$.

III. RESULTS AND DISCUSSION

Mycoflora associated with cocoa pods

The fungal species listed in Figure1 could be regarded as common post-harvest decay agents of various studied fruits. Through this investigation at 20 ± 2ºC 6 fungal species attributed to six genera were isolated. *Aspergillus, Colletotrichum, Botryodiplodia, Trichoderma* and *Verticillium* were the most common genera that colonized the cocoa pods from Akonolinga and Tonga with different incidences. The most frequent fungi were *Colletotrichum gloeosporioides* 48.84% of pods collected in Tonga and 41.46% in those from Akonolinga, followed by *Botryodiplodia theobromae* which was present in 29.27% of cocoa pods from Akonolinga and 20.93% in those of Tonga. Figures 2 and 3 shows the macro and microscopic characters of *Botryodiplodia theobromae* and *Colletotrichum gloeosporioides*.

In these two production areas, fungal biodiversity affecting the cocoa pods vary qualitatively and quantitatively. The

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isolations made on the different pods collected showed a predominance of three fungal species including *Colletotrichum gloeosporioide, Botryodiplodia theobromae* and *Trichoderma sp*. Other species such as *Fusarium oxysporum, Aspergillus niger* and *Verticillium sp*. appear at low frequency. Similar results have been reported by Evans *et al*. (2003) and Rubini *et al*. (2005) showing that soils under cocoa tree and pods are sites of preliferation of indigenous microorganisms potentially antagonistic of *Phytophtora* such as *Trichoderma sp., Colletotrichum gloeosporioide, Fusarium oxysporum* and *Botryodiplodia theobromae* occupying the same ecological niche. The high proliferation of *Colletotrichum gloeosporioide*, *Botryodiplodia theobromae* and *Trichoderma sp* in these two cocoa ecosystems could justify the scarcity of *Phytophthora megakarya*, causal agent of brown rot. Similar results have been achieved using isolates of *Trichoderma sp.* and *Stromaticum sp*. to fight against the brown rot of cacao tree (Krauss and Soberanis, 2002).

Antifungal effect of plant extracts

Effect of ethanol extracts

Antifungal effects of ethanol extracts of *Chromolaena odorata* and *Ageratum conyzoïdes* L. on fungal growth are presented on Table 1. There were significant differences in the mycelia growth inhibition of plant extract-supplemented samples compared with the negative control (ANOVA and Duncan Multiple Range Test, $P < 0.05$). The effect of extracts with increasing concentrations showed a gradual inhibition of the growth of *C. gloeosporioides* and *B. theobromae*. It was noted that ethanolic extracts of *Ageratum conyzoïdes* completely (100%) inhibited the growth of both fungi at 10 mg/ml. With the ethanolic extracts of *Chromolaena odorata*, 100% inhibition was observed for *B. theobromae* at the dose of 5 mg/ml while *C. gloeosporioides* was completely inhibited at 10 mg/ml.

Effect of aqueous extracts

Antifungal effects of aqueous extracts of *Chromolaena odorata* and *Ageratum conyzoïdes* L. on fungal growth are presented on Table 2. Generaly there are significant differences in the mycelia growth inhibition of plant extract-supplemented samples compared with the negative control (ANOVA and Duncan Multiple Range Test, $P < 0.05$). Aqueous extracts of *Chromolaena odorata*, completely (100%) inhibited the growth of *B. theobromae* and *C. gloeosporioides* at the dose of 20 mg/ml. Similarly aqueous extracts of *Ageratum conyzoïdes* completely inhibited the growth of *B. theobromae* at 20 mg/ml, however this dose was obtained as an inhibition of 78% of *C. gloeosporioides*.

Aqueous and ethanolic extracts of *C. odorata* and *A. conyzoides* showed fungicidal effect at concentrations 20 mg/ml and 10 mg/ml respectively.

The growth inhibition percentages of different fungi by plant extracts proved to be dependent on the concentration, the type of extract and the plant tested. Results obtained from *Ageratum conyzoïdes* extracts are in agreement with previous studies that showed the antifungal activities of this plant against devastating pathogen on variety of economic plants (Mughal *et al*. 1996; Bajwa *et al*., 2001; Sidra and Uzma, 2012). Similarly, the results achieved with leaves extract of *Ageratum conyzoïdes* are similar to those obtained by Tsapi (2000) and Megatche (2011) which showed that these extracts inhibit the development of *Phytophtora megakarya* (responsible for the brown rot of cocoa) and *P. colocasiae* (causative agent of late blight of taro). A wide range of allelochemicals including alkaloids, flavonoids, chromenes, benzofurans and terpe-noids have been isolated from *A. conyzoides* (Okunade, 2002). According to Tran *et al*. (2004), three phenolic compounds were identified in the leaf, stem and root of *A. conyzoides* including gallic acid, coumallic acid and protocatechuic acid and catechin were found only in the stem. Three additional allelochemicals were also found in the leaf consisting of p-coumaric acid, sinapic acid and benzoic acid. The greater number of allelochemicals found might result in the stronger inhibitory activity.

Also, results obtained with *C. odorata* extract are similar to those reported by (Ngono *et al*., 2006) which showed that this extract inhibit the development of yeast, filamentous fungi and that of several multicellular dermatophyte fungi. Kra *et al*. (2009) showed the effect of the leaf extract of *C. odorata* in vitro on two isolates of *F. oxysporum*, causing symptoms of *Fusarium* wilt. A qualitative chemical analysis of the extract and fractions showed the presence of biologically active constituents such as some coumarins, flavonoids, phenols, tannins and sterols, this could justify the antifungal activity.

IV. CONCLUSION

For the two areas investigated, the fungal biodiversity appeared to be highly variable both qualitatively and quantitatively. *Aspergillus, Colletotrichum, Botryodiplodia, Trichoderma* and *Verticillium* were the most common genera that colonized the cocoa pods from Akonolinga and Tonga with different incidences. This study suggests that *A. conyzoides* and *C. odorata* have fungitoxic chemicals against *B. theobromae* and *C. gloeosporioides*, cocoa rot causing pathogen. Ethanolic and aqueous extracts of *A. conyzoides* and *C. odorata* greatly reduced the fungal growth, which can be used for the disease management. Further investigation on the isolation of active antifungal compounds should be done

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and the isolated antifungal compounds should be checked against other pathogenic fungi to control the different diseases.

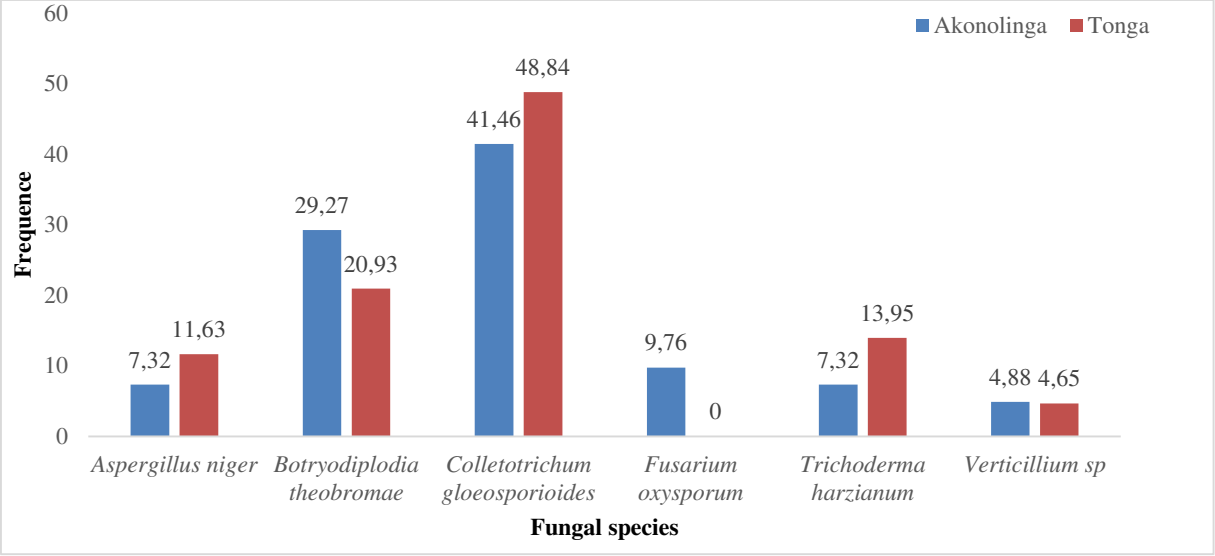

Fig.1: Frequencies of the different fungal species identified with respect to the locality

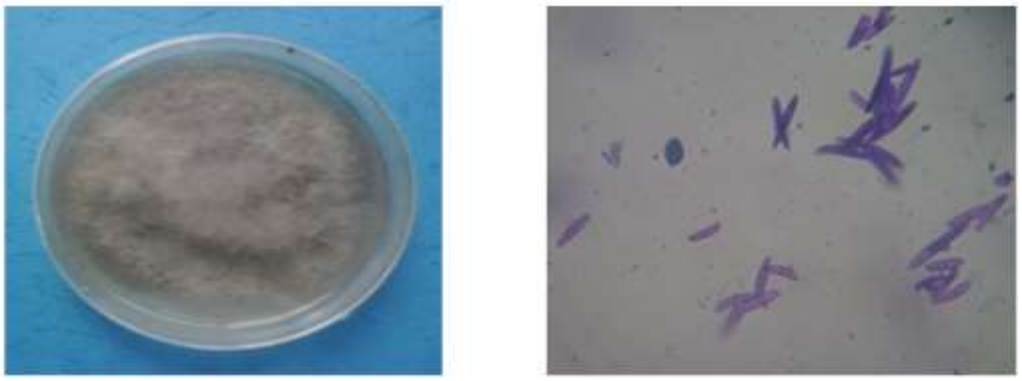

Fig.2: Botryodiplodia theobromae, axenic culture and conidia

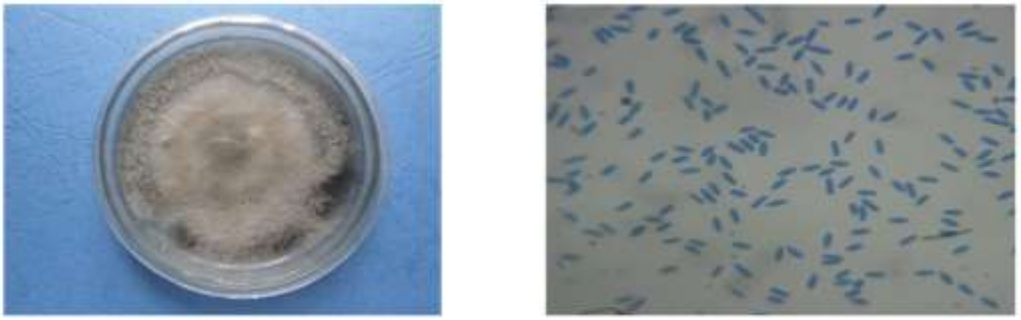

Fig.3: Collectotrichum gloeosporioides, axenic culture and conidia

Table.1: Inhibition Percentage (%) of radial growth of fungal pathogens by ethanol plant extracts

Ethanolic extracts	Concentration	*C. gloeosporioides*	*B. theobromae*
A. conyzoides	T-	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
	1.25 mg/ml	$39.01 \pm 7,40^{b}$	53.72 ± 5.65^{b}
	2.5 mg/ml	$39.41 \pm 11,34^{b}$	40.98 ± 4.75^{b}
	5 mg/ml	$49.02 \pm 18,68^{b}$	48.24 ± 14.01^{b}

	10 mg/ml	100.0 ± 0,00[a]	100.00 ± 0.00[a]
	T+	100.0 ± 0,00[a]	98.60 ± 0.03[a]
C. odorata	T-	0.00 ± 0.00[c]	0.00 ± 0.00[e]
	1.25 mg/ml	62.94 ± 11,93[b]	58.63 ± 5.65[d]
	2.5 mg/ml	69.61 ± 14,04[b]	76.67 ± 4.33[c]
	5 mg/ml	91.17 ± 8,54[a]	100.00 ± 0.00[a]
	10 mg/ml	100.0 ± 0,00[a]	100.00 ± 0.00a
	T+	100.0 ± 0,00[a]	83.33 ± 1.22[b]

Values in the same row followed by different letters are significantly different (P ≤ 0.05).
T- = Negative control (Distilled water) ; T+ = Positive control (Mancozeb).

Table. 2: Inhibition Percentage (%) of radial growth of fungal pathogens by aqueous plant extracts

Aqueous extract	**Concentration**	***C. gloeosporioides***	***B. theobromae***
A. conyzoides	T-	0.00 ± 0.00[e]*	0.00 ± 000[c]
	5 mg/ml	35.29 ± 16.38[d]	26.47 ± 14.70[b]
	10 mg/ml	45.69 ± 8.93[cd]	32.94 ± 12.0 [b]
	15 mg/ml	59.61 ± 5.30[c]	84.90 ± 16.64[a]
	20 mg/ml	78.63 ± 3.40[b]	100.00 ± 0.00[a]
	T+	100.00 ± 0.00[a]	100.00 ± 0.00[a]
C. odorata	T-	0.00 ± 0.00[e]	0.00 ± 0.00 [e]
	5 mg/ml	32.16 ± 4,34 [d]	19.01 ± 5.89 [d]
	10 mg/ml	52.94 ± 4.70 [c]	54.31 ± 8.67 [c]
	15 mg/ml	72.15 ± 8.34 [b]	67.45 ± 1.22 [b]
	20 mg/ml	100.00 ± 0.00[a]	100.00 ± 0.00[a]
	T+	91.76 ± 7.34 [a]	100.00 ± 0.00[a]

Values in the same row followed by different letters are significantly different (P ≤ 0.05).
T- = negative control (Distilled water); T+ = positive control (Mancozeb).

REFERENCES

[1] Anonyme, 2006. New EU regulation on maximum residue levels of pesticides in food : minimising the impact on the cocoa sector. *In*: proceedings of the 15th International Cocoa Research Conference. 9-10 October 2006. San Jose (Costa Rica). pp. 1565-1571.

[2] APS, 2011. Diseases of *Theobroma cacao* in Florida. American Phytopathogical Society, Minnesota, USA. p. 415

[3] Assoumou, J., 1977. Agriculture d'exportation et bataille du développement en Afrique tropicale: Economie *du cacao*. Ed. Jean Pierre De large. Paris. p 350.

[4] Bajwa, R., Akhtar, N. and Javaid, A., 2001. Antifungal activity of allelopathic plant extracts. Effect of aqueous extracts of three allelopathic *Asteraceous* species on growth of aspergilla. Pak. J. Biol. Sci. 4: 503-507.

[5] Barnett, H.L., Hunter, B.B., 1998. Illustrated genera of imperfect fungi 4th edition, APS press, St. Paul Minnesota. p. 32

[6] Berry, D., Cilas, C., 1994. Etude génétique de la réaction à la pourriture brune des cabosses chez des cacaoyers (*Theobroma cacao* L.). Thèse de Doctorat d'Etat. Université de Paris-Sud, centre d'Orsay, 153p.

[7] Copetti, M., Lamanka, B., Frisvad, J., Pereira, J., Taniwaki, M., 2011. Mycobiota of cocoa. from farm and chocolate. Food Microbiol. 28:1499-1504.

[8] Dugan, F.M., 2006. The identification of fungi. APS press, St Paul Minnesota. p. 50.

[9] Evans, H.C., Holmes, K.A. and Thomas, S.E., 2003. Endophytes and mycoparasites associated with an indigenous forest tree, *Theobroma gileri*, in Ecuador and preliminary assessment of their potential as biocontrol agents of cocoa diseases. *Mycological Progress* 2: 149-160.

[10] Gevens, A.J., Donahoo, K.H.L., Hausbeck, M.K., 2008. Characterization of *Phytophthora capsici* causing foliar and pod blight of snap bean in Michigan. Plant Dis. 92(2):198-320.

[11] Henderson, J.S., 2007. Chemical and archaeological evidence for the earliest *Cacao* beverage. Proceed. Nat. Acad. Sci. 104:18937-18940.

[12] Juan, C., Motamayor, P.L., Jay, W.I., Da Silva, E.M., Reg, L., David, N.K., Steven, B.J., Raymond, J.S., 2008. Geographic and Genetic population differentiation of the Amazonian chocolate tree (*Theobroma cacao)* PLos ONE 3(10):1371-1376.

[13] Keuete Kamdoum, E., Tsopmbeng Noumbo, G., Yaouba, A., Djeugap F.J., And Serferbe Signaboubo, 2015. Antifungal potential of some plant extracts against three post-harvest fungal pathogens of avocado (*Persea americana* Mill.) fruits. Int. J. of Multi. Res. and Dev. Vol : 2, Issue:4, 148-152 e-ISSN: 2349-4182

[14] Koné, Y.R., 1999. Etude de la structure actuelle des populations de *Phytophthora* spp., agents de la pourriture brune des cabosses du cacaoyer (*Theobroma cacao* L.) en Côte d'Ivoire. Mémoire de Diplôme d'Agronomie Approfondie, Option Défense des cultures, Ecole Supérieure d4agronomie, Yamoussoukro.111p

[15] Kouamé, K.D., 2006. Structure et dynamique des populations de *Phytophthora* spp., agents de la pourriture brune des cabosses du cacaoyer (*Theobroma cacao* L.) en Côte d'Ivoire. Mémoire de DEA. UFR biosciences. Université de Cocody, Abidjan, Cote d'Ivoire. 74p

[16] Krauss, U., Soberanis, W., 2002. Effect of fertilization and biocontrol application frequency on cocoa pod diseases. Biological Control 24: 82-89.

[17] Luter bacher, M.L., Akrofi, A.Y., 1993. The current status and distribution of *Phytophthora megakarya* in Ghana. *In: Proceedings of the 11th International Cocoa Reseach Conference*, Yamoussoukrou, Côte d'Ivoire, pp.57-58.

[18] Megatche, 2011. Efficacité *in vitro* des extraits de plantes sur la croissance et le développement de *Phytophthora colocasiae*, agent causal de la flétrissure des feuilles de taro (*Colocasia esculenta*). Mémoire de Master II en phytopathologie. Université de Dschang, 164 p.

[19] Mughal, M.A., Khan, T.Z. and Nasir, M.A., 1996. Antifungal activity of some plant extracts. Pak. J. Phytopathol. 8: 46-48.

[20] Ngono Ngane, A., Ebelle Etame, R., Ndifor, F., Biyiti, L., Amvam Zollo, P.H., Bouchet, P., 2006. Antifungal Activity of *C. odorata* (L.) King & Robinson (Asteraceae) of Cameroon. *Chemotherapy*. 2006; 52(2):103-6.

[21] Okunade, A.L., 2002. *Ageratum conyzoides* L. (*Asteraceae)* Fitoterapia. 73: 1-16.

[22] Opoku, I.Y., Appiah A.A., Akrofi A.Y., Owusu G.K., 2000. *Phytophthora megakarya*: a potential threat to the cocao industry in ghana. J. Agri. Sc. 33: 237-248.

[23] Rubini, M.R., Silva-Ribeiro, R.T., Pomella, A.W.V., Maki, C.S., Araujo, W.L., Dos Santos, D.R., and Avevedo, J.L., 2005. Diversity of en dophytic fungal community of cocoa (*Theobroma coca* L.) and biological control of *Crinipellis perniciosa*, causal agent of Withches'Broom Disease. *Int. J Biol Sci*. 1:24-33.

[24] Sharma, N. and Trivedi, P.C., 2002. Screening of leaf extracts of some plants for their nematicidal and fungicidal properties against *Meloidogyne incognita* and *Fusarium oxysporum*. Asian J. Exp. Sci. 16: 21-28.

[25] Sidra Javed, and Uzma Bashir, 2012. Antifungal activity of different extracts of *Ageratum conyzoïdes* for the management of *Fusarium solani*. African Journal of Biotechnology Vol. 11(49), pp. 11022-11029.

[26] Souza, C., Koumaglo, K., Gbeassor, M., 1995. Evaluation des propriétés antimicrobiennes des extraits aqueux totaux de quelques plantes médicinales. Pharma. Med. Trad. Afr. 103-112.

[27] Tran, D.X., Shinkichi, T., Hong, N.H., Khanh, T.D., Min, C.I., 2004. Assessment of phytotoxic action of *Ageratum conyzoides* L. (billy goat weed) on weeds. Crop Prot. 23: 915-922.

[28] Tsapi, 2000. Etude de la composition chimique et évaluation *in-vitro* de l'activité antifongiques des huiles essentielles de *Cupressus lusitanica* Mill. Sur quelques isolats de *Phytophthora megakarya, agent causal* de la pourriture brune des cabosses du cacaoyer (*Theobroma cacao* L.*).* Mémoire de maîtrise de biochimie. Université de Dschang, 1-59 p.