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ORIGINAL ARTICLES

Flavonoïd Compounds Synthesis by Cocoa Fruits (*Theobroma cacao* L.) in Response to *Phytophthora megakarya* Infection

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ABSTRACT

Cocoa black pod is a disease caused by *Phytophthora*, a genus in the oomyceta responsible of the most serious and economically important plant diseases. In Cameroon *Phytophthora megakarya* species is the most virulent pathogen of *Theobroma cacao* L. with about 80 % yield loss. The goal of the present work is to study biological and biochemical molecules used by T*heobroma cocoa* L. to fight agains**t** *Phytophthora megakarya.* Our work focus on immature fruits of two clones of cocoa (SNK 10, very sensible to *P. megakarya* and SNK 413 less sensible). Two groups (control and test) of 10 fruits of each clone were used for the experiment.. Cocoa fruits are infected artificially by the pathogen and the necroses area evaluated after 2, 4, and 6 days. The experiment was repeated twice a year within 3 consecutives years Samples used for analysis were cut 2 cm away from the necrotic area. Phenolic compounds were extracted from these samples with ethyl acetate as solvent and titrated spectrophometrically after dyeing with folin ciocalteu reagent. The so extracted compounds were scan with the spectrophotometer UV, analysed with the high pressure liquid chromatograph and finally used to test the in vitro sensibility to *Phytophthora megakarya*. Results showed that, progression of fruits tissues colonisation by the fungi is directly linked to clone sensibility. SNK 413 clone (less sensible) has the less necrotic area and the high phenolic compounds amount compare to the SNK 10 clone highly sensitive to *Phytophthora megakarya.* The infection of the cocoa fruits with the pathogens did not bring out the synthesis of new phenolic compounds but the accumulation of flavonoid derived compounds. These phenolic compounds significantly inhibit *P. megakarya* growth *in vitro*. Spectrophotometric scaning shows a link between cocoa black pod resistance and its flavanols contents. Analysis by HPLC identified these compounds as epicathechin, procianidin B_2 and procianidin C_1 derivative. These compounds can be used as biochemical resistance marquers of *Theobroma cacao* L against *Phytophthora megakarya*.

Key words: *Theobroma cacao* L, *Phytophthora megakarya*, black pod disease, phenolic compounds, flavanols.

Introduction

About 6 000 000 tonnes of chocolates are produced every year in the world **(**Lass, 2004a). Cocoa seed, the main ingredient used in the chocolate production has been sale at around 3900 billions US dollars in the year 2003/2004. This devise can highly contribute to redress developing country economy. In the same years, the cocoa production was estimated at 3.4 million of US dollars (FAO 2005) and 69% of this production belongs to the sub Saharan countries like Ivory Coast, Ghana and Cameroon (Lass 2004b). In those countries, around 80% of the production come from the small scales production and constitute the main income for small producer (Laird *et al*. 2007). Cocoa is the second important crop that highly contributes to redress developing countries economy after banana plantain. Ivory Coast who is the first producer across the world got 35% of these devices from cocoa. Cameroon is the 6th produced arrowed the word with around 200 000 tones which can generate about 356 billions US dollars (FAO 2009).

However cocoa production has many problems that considerably drop down it yield. Losses due to parasitical attacks are the main causes of yield lost. Black pod disease causes by *Phytophthora sp*, frosty pod causes by *Crinipellis rorei and* witches broom cause by *cripellis perniciosa* are examples of pathogens that decrease cocoa productivity (Wood and Lass 1985). Cocoa black pod, caused by oomyceta of the genus *Phytophthora*, causes substantial yield losses worldwide, particularly in Africa with the species *Phytophthora megakarya*, which is the most damaging species for the cocoa industry. Yield lost per year can reach 40% of the world production and vary according to the geographic area as well as the *Phytophthora* species (Flood *et al*. 2004). In optimum conditions, Losses can reach 80 % (Bowers *et al*., 2001; Nyassé *et al*. 2007).

Fungicides are used to control the disease and mummified pods are removed at the beginning of the season, followed by weekly phytosanitary removal. Chemical control is expensive, commercially non-viable and

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environmentally harmful. For example, *Phytophthora sp* can develop a resistance against metalasyl which is the principal fungicide used for the control of cocoa black pod disease (Bateman 2004). Others methods available for controlling cocoa black pod are the use of resistant cultivars and appropriate cultural practices such is phytosanitary pod removal which is a potentially efficient control method (Ndoumbe-Nkeng *et al*. 2004).

Another way of fight against this pathogen is to find resistant strains of cocoa as well as to increase the resistance of the cultivated varieties. Many studies shown that there is a correlation between the resistance of *Theobroma cacao* L against *Phytophthora sp* and the necrotic area on the leaf and shoot infected artificially with *P. megakarya*. (Alemano *et al*. 2003, Djocgoué *et al*. 2006; Paulin *et al*.2008). In general, resistance can be attributed to the accumulation of fungitoxic compounds in the infected point. Those compounds for the majority of plant are present before the infection and remain in the cell in the conjugated form. Secondary metabolic compounds are the main group implicated in the defence process (Brunneton 1999). Phenolics present in healthy, uninfected plant tissues, as preformed antimicrobial compounds, that inhibit the growth of fungi, may include simple phenols, phenolic acids, flavonols, some isoflavones… Those that are induced in response to fungal infection include phenolic phytoalexins, isoflavonoids, pterocarpans, furocoumarins, flavans, stilbenes, phenanthrenes… (Lattanzio *et al* 2001).

The role of phenolic compounds in plant defence is well documented (Tan *et al*. 2004; Cherif *et al*. 2007). Generally, phenolics accumulate at different levels in infected tissues in response to pathogen invasion. The resistance of apple (*Malus domestica*) to *Ventura inaequalis* is related to the higher content of catechin and proanthocyanidins in leaves (Treuter and Feucht 1990). Daayf *et al*. (1997) reported the accumulation of a methylester, *p*-coumaric acid, in leaves of cucumber (*Cucumis sativus*) infected by *Sphaerotheca fuliginea*. In the date palm–*Fusarium oxysporum* f. sp. *albedinis* pathosystem, there is a higher accumulation of non-constitutive hydroxycinnamic acid derivatives in resistant cultivars (El Hadrami *et al*. 1997). Some plants can inducible form a large variety of phenolic phytoalexins. Kodama *et al*. (1992) listed some 16 phytoalexins produced by rice (*Oryza sativa*) in response to pathogen attack.

The goal of this work is to draw a relationship between the amount and the nature of soluble phenolic compounds of fruits and the level of sensibility of cocoa against *Phytophthora megakarya* causal agent of the black pod disease. We have evaluated the necrotic area of fruits following artificial infection by *Phytophthora megakarya,* the amount of phenolic compounds, and their differentiation using spectrophotometer, analysis using high pressure liquid chromatograph and the fungi toxicity. This will enable us to understand the role of phenolic compounds and precisely flavanols in defence process and know whether they can be used as biochemical marquers of resistance.

1. Material :

a. Plant material:

It is fruits of 2 clones of cocoa belonging to the sensibility scale establish by Blaha et Lotode (1976). Those clones are:

- SNK 10, highly sensitive to *Phytophthora megakarya* with 99% of successful infection
- SNK 413 less sensitive to *Phytophthora megakarya* with 37% of successful infection

Analysis is done on the cortex part of immature fruits pod, which have adult size. Those clones come from the national institute of agricultural research for development (IARD) at Nkolbisson-Cameroon.

b. Fungi material:

It is made with L_2C_2 strain of *Phytophthora megakarya*. We got it from the laboratory of phytopathology of the IRAD at Nkolbisson (Cameroon). His mean diameters of growth within a day vary from 2 to 10 mm (Despreaux 1988; Nyassé 1997). This strain was identifying to have an appreciable infective rate on cocoa clones in Cameroon (Nyassé 1992, Nyassé *et al*. 1997).

2. Methods:

a. Fruits infections with P. Megakarya:

Two groups (control and test) of 10 fruits of each clone were used for the experiment. The experiment was repeated twice a year within 3 consecutives years

Fruits were infected manually in the laboratory conditions. 100µl of zoospores suspension concentrated at 10^{+5} zoospores per ml was deposited in small wells previously dig on the cocoa pod area. Incubation was done at 25 °C in dark conditions. We follow the infection process for 6 more days and the necrotic area evaluated using the formula

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S= D x d x ∏ (Blaha et Lotode, 1976) S: necrotic area (mm^2)
D: necrotic diameter (mm) d: small diameter (mm)

b. Extraction and titration of soluble phenolic compounds:

We sample on the cortex part of cocoa fruit for healthy fruit and at 2 cm from the necrotic area for infected fruits. The collected sample was freeze at -20°C, lyophilized with the ALPHA CHRIST 1-5 apparatus, crushed and store in desiccators. Extraction of soluble phenolic compounds was done following the method described by Nana (1991): 1 g of powder was extracted with 70 % methanol, depigmented with petroleum ether in acid (orthophosphoric acid 85%) and saline (ammonium sulphate concentrated at 40%) medium. The extraction of soluble phenolic compound was done with pure ethyl acetate.

The titration of soluble phenolic compound was evaluated by the method describe by Marigo (1973) using the folin ciocalteu reagent. To 1ml of pure phenolic extract, add 1ml of folin ciocalteu reagent and 2 ml of sodium carbonate at 20 % and read the optical density of the mixture at 725nm with the spectrophotometer (BECKMAN DU 68). Results are expressed in equivalent gramme of chlorogenic acid, a pure phenolic used as standard.

3. Spectrophotometric UV scaning and high pressure liquid chrommatography (HPLC) analyses of phenolic compounds:

Pure soluble phenolic compounds were scaned with the BECKMAN DU 68 spectrophotometer between 200 and 400nm wave length. The interested wave length is 280 nm for flavanols and 320 nm for hydrocynamic derivative compounds.

HPLC was performed on INESTIL RP 18 colon and the chromatogram collected with DIODE ARRAY WATERS 994, at the two wave lengths.

d. Fongitoxicity test of soluble phenolic compouds:

We used 1.37 mg of soluble phenolic compounds and 2.5 ml of *Phytophthora megakarya* zoospores suspensions for 100ml of agar culture media made with pigeon pea. A round of 7 mm wathman paper containing soluble phenolic compound diluted at 1% was put in the Petri dishes with zoospores of *Phytophthora megakarya*. After 48 h of incubation at 25°C in dark conditions, the inhibition of *Phytophthora megakarya* was appreciated by the clear circular area with opacity fund. The estimation of necrotic area is done using the formula

$S = \prod \times R^2$. S: necrotic area (mm^2) R: half diameter of the necrotic area (mm^2)

e. Statistical analysis.

Statistical analysis was done using the SPSS program. Comparison of mean was done by student Fischer test.

Results:

1- Evolution of the necrotic area on the cocoa pod (Theobroma cacao L) infected by Phytophthora megakarya:

We have evaluated the necrotic area during 2, 4 and 6 days after inoculation with the zoospores on fruits. Results presented in figure 1 show that infection begins 2 days after inoculation for SNK10 and SNK 413 clones. Necrotic development showed no difference for the two clones 2 days after infection. However the difference appears after 4 and 6 days. The necrosis appear rapidly on SNK10 compare to SNK413 clones.

2- Evolution of the soluble phenolic compounds of cocoa pod of Theobroma cacao L infected or not by Phytophthora megakarya:

Impact of the infection on the amount of phenolic compound has been tested on the mesocarp of fruits of SNK10 and SNK413 after 2, 4 and 6 days of inoculation. Results obtain show that amount of soluble phenolic compound is high in SNK413 compare to SNK10 infected or not (Table 1). However this amount is constant for SNK413 and SNK10 infected or not. Statistic analysis shown that interaction between clones is highly significantly ($p<0,0001$) for healthy clones($F = 922,9$), after 2 days ($F = 308,2$) after 4 days ($F = 1218,0$) after 6 days ($F = 790,5$) after infection.

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3- Differentiation of total phenolic compounds by UV spectrophotometer of Theobroma cacao L pod infected or not by Phytophthora:

Spectrophotometric analysis point out two groups of phenolic compounds. The first group absorb at 280 nm, which the wave length of absorption of flavanols and its derivatives. The second group absorb at 320 nm which is the wave length of absorption of hydroxycinnamic derivatives. The pic observed at 220 nm is not

Table 1: Amount of soluble phenolic compounds (mg/g of fresh matter) in mesocarp of fruits healthy or infected of cocoa pod of *Theobroma cacao* L.

Days after infection	0	2	4	6
SNK10	34,5b	36,3c	31,5a	36,3b
SNK413	59,3c	50,6a	59,9c	59,2c
Clone effect	922,9***	308,2***	1218***	790,5***

SNK10: high sensitive clone with 99 % of successful infection
SNK413: less sensitive clone with 34 % of successful infection
Letters with the same sign are not significantly different at the level of P<0.005
*** Significant at p<0,001 ** significant at p<0, 01 * significant at p<0, 1 ns not significant

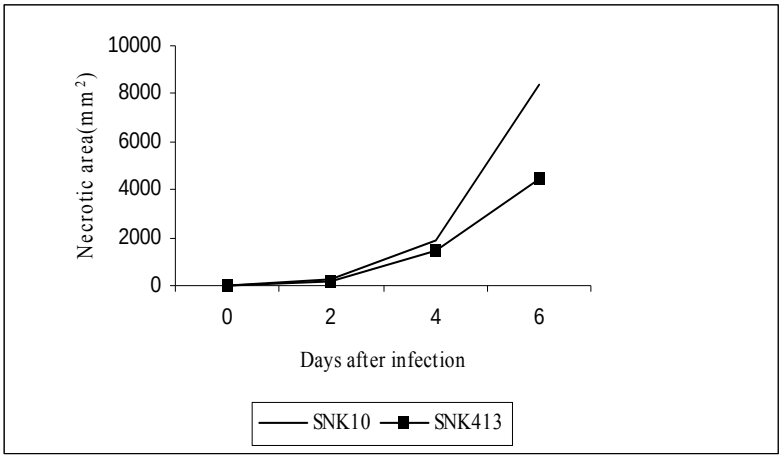

Fig. 1: Evolution of necrotic area from mesocarp of fruits cocoa pod after infection with *P. Megakarya.*

characteristic of phenolic compounds and has not been consider in this study. Fruits mesocarp of SNK413 clone has more flavanols compare to the SNK10 clone. For SNK413, there is accumulation of flavanols and it derivatives following infection by *Phytophthora megakarya*.

4- Identification of phenolic compounds using high pressure liquid chromatography (HPLC):

HPLC chromatogram of the phenolic compound of the two clones of cocoa are presented in figure 3. The wave lengths of appreciation are 280 nm and 320 nm.

On these chromatograms, we observe major compounds represented 75 to 80% of the total phenolic compounds present with the characteristic of caffeic derivative. There are also 20 other compounds grouped as follow:

One represented essentially flavonols and its derivatives (280nm)

Another group represented by the hydroxycinamic derivatives (320nm)

Identification done by comparison of the phenolic compounds of cocoa seed (Villeneuse *et al*., 1988), show that the major flavanols have the spectral properties of :

- procyanidin B_2
- procyanidin C_1 derivative
- epicatechin.

Analysis of the flavanic compouds compare to the total phenolic compounds is shown in table 2. The cocoa clone SNK 413 has a relative high amount of flavanols compare to the clones SNK10. There is accumulation of flavanol in the cocoa fruit of SNK 413 following infection by ***Phytophthora megakarya*** and not for the SNK10 fruits. 4 and 6 days after infections, SNK413 clone accumulates 3 times more flavonols compare to the healthy fruits.

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5- In vitro fongitoxicity biological tests of soluble phenolic compounds:

Phenolic extract, of fruits pod of SNK413 and SNK10 clones (healthy or infected) has been tested for their fungitoxicity versus *Phytophthora megakarya*. Results are presented in table 3.

Phytophthora megakarya growth is inhibited by phenolic extract of SNK413 and SNK10 clones. The rate of inhibition of *Phytophthora megakarya* growth is higher with SNK413 extract compare to the SNK10 extract. Interaction between clones is highly significantly for healthy clones (p<0,00, F = 16844), 2 days after infection (p<0,01, F = 459,9), 4 days after infections (p<001, F = 74,2) and 6 days after infections (p<0,001, F = 194067).

Table 2: Relative percentage of flavanols (%) from fruits mesocarp healthy or infected of cocoa pod of *Theobroma cacao* L.

Days after infection	0	2	4	6
SNK10	3,1b	1,7a	1,9a	2,9b
SNK413	6,8a	9,5a	23,1b	20,1b
Clone effect	ns	92,9**	701,6***	451,1***

SNK10: high sensitive clone with 99 % of successful infection
SNK413: less sensitive clone with 34 % of successful infection
Letters with the same sign are not significantly different at the level of P<0.005
*** Significant at p<0,001 ** significant at p<0, 01 * significant at p<0, 1 ns not significant

Table 3: Biological test of fongitoxicity of soluble phenolic extrait (mm^2) from fruits mesocarp healthy or infected of cocoa pod of *Theobroma cacao* L.

Days after infection	0	2	4	6
SNK10	336,7a	336,7a	346,3a	411,0a
SNK413	664,0b	496,7a	555,7a	770,0c
Clone effect	16844***	459,9**	74,18*	194067***

SNK10: high sensitive clone with 99 % of successful infection
SNK413: less sensitive clone with 34 % of successful infection
Letters with the same sign are not significantly different at the level of P<0.005
*** Significant at p<0,001 ** significant at p<0, 01 * significant at p<0, 1 ns not significant

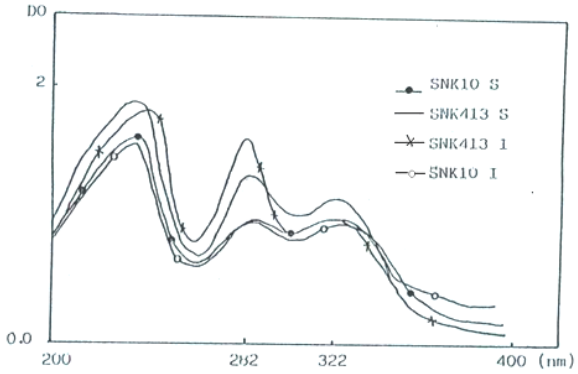

Fig. 2: Ultraviolet spectre of phenolic compounds in *Theobroma cacao* L. fruits of SNK10 and SNK413 which were healthy (S) and infected (I) with *P. Megakarya.*

Discussion:

We try to establish a relationship between soluble phenolic compounds of cocoa fruits, their composition as well as their antifungal capacity through the diameter of the necrotic area.

Infection begins 2 days after inoculation of fruits mesocarp of SNK413 and SNK10 clones. This period is the time used by the pathogen to gain host tissues. Difference between necrotic area is appreciable 4 days after inoculation and considerable 6 days after. Necrosis appear rapidly on SNK10 fruits pod compare to SNK413. we hypothesise that SNK10 clone is more sensible to *Phytophthora megakarya* attacks than SNK413 clones. Similar observation was made by Nana *et al*. (1995); Nyassé *et al*. (1995); Paulin *et al*. (2005) who shown SNK10 and SNK413 react differently to *Phytophthora megakarya* attacks in field and laboratory conditions respectively. The amount of total phenolic compounds is high for SNK413 clone compare to SNK10 clone when they are infected or not. Those results showed the implication of phenolic compounds in cocoa defence system versus *Phytophthora megakarya*. Those observations are similar to those of Nana *et al*. (1995) and Boudjeko *et al*. (2006), who showed the importance of phenolic compounds in the defence process of *Theobroma cacao* against *P. megakarya.* In general, elicitation of plant brings out the synthesis and the accumulation of phenolic compounds. Nana *et al*. (2003) showed the accumulation of phenolic compounds in cowpea grain following attack by insect. Conceicao *et al*. (2006) also showed the induction of phenolic compounds in cell of *Hyerium*

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perforatum L.following elicitation by *Colletotrichum gloeosporioides*. Thukkaram *et al*. (2009) showed a relative high amount of phenolic compound in hybrids and parents plants of Musa resistant to *Fusarium oxysporum* f. sp. *cubense* race 1 compare to the sensible plants. Different studies showed that there are often large increases in phenolic synthesis in plants after attack by plant pathogens (Matern *et al*. 1995; De Ascensao 2003). Phenolics that occur constitutively and function as preformed inhibitors are generally referred to as phytoanticipins, and those that are produced in response to infection by the pathogen are called phytoalexins and

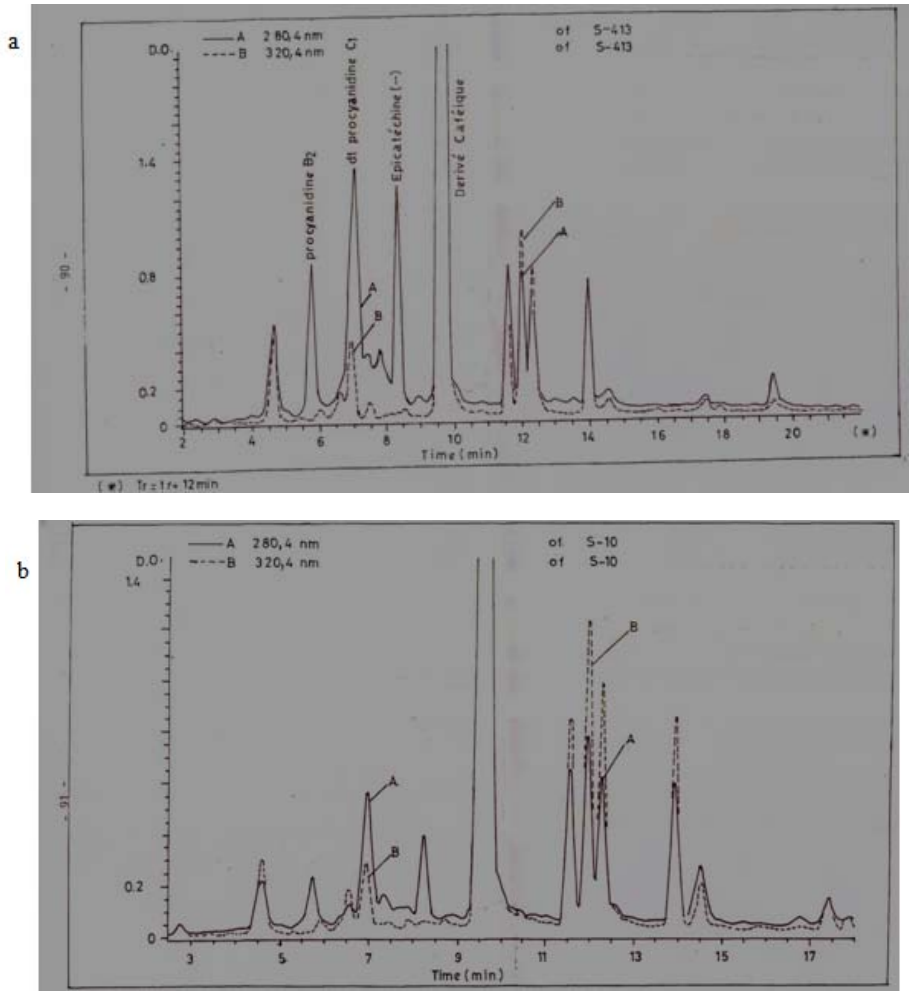

Fig. 3: HPLC chromatogram of phenolic compounds in cocoa healthy fruits of *Theobroma cacao* L : a) SNK413 genotype b) SNK10 genotype.

constitute an active defense response. In resistant plants, phenolic based defense responses are characterized by the early and rapid accumulation of phenolics at the infection site resulting in the effective isolation of the pathogen (Fernandez *et al*. 1998; Cherif *et al*. 2009).

UV spectrophotometer showed 2 groups of compounds: flavanols with its derivatives and hydroxynamic derivatives in the two clones of cocoa healthy or not. There was no synthesis of new group of compounds following infections (figure 1). Those results had shown the implication of phenolic compounds and precisely flavonol and its derivatives in the resistance of cocoa versus *Phytophthora megakarya*. Similar results were obtained Treuter and Feucht (1990) who showed the implication of flavonols in the resistance of pineapple versus invaders. The antimicrobial property of phenolic compounds and especially those of the flavonol group is well documented (Smith and Banks 1986; Hahlbrock and Scheel 1989). Isoflavanoids with antimicrobial activity have been characterized in *Medicago* species (Latunde-Dada *et al*. 1987); the pterocarpan medicarpin has been implicated in the resistance of Alfalfa to *Colletotricum trifolii* and several leaf spot diseases (Kessmann *et al*. 1990; Ebel and Cosio 1994). This antimicrobial role of phenolics is based on indirect evidence,

such as correlations between the timing of phytoalexin accumulation in resistant and susceptible interactions and the potency of phenolics as antimicrobial agents *in vitro*.

Identification by comparison of phenolic compounds of cocoa seed following Villeneuse *et al*. (1989), showed that the principle flavanol has the spectral characteristic of procyanidin B_2, a derivative of procyanidin C_1, and epicatéchin. Those observations prove that flavonols in generals and precisely procyanidine and epicathechine are implicated directly in the resistance of cocoa versus *Phytophthora megakarya*. This is in accordance to the results of Rizwana Banu *et al*. (2007) showing the accumulations of benzoic acid, paranitrophénol and orcinol in resistant cultivars of *Cajanus cajan* à *Helicoverpa armigera*. Fongitoxicity results confirm the above results and shown that flavanols and precisely procyanidins and epicathechin can be used as biochemical marquers of cocoa resistance versus *Phytophthora megakarya*.

Conclusion:

SNK413 the less sensible clone has the less necrotic area and the highest amount of phenolic compounds compare to SNK10 clone. Infection did not bring up synthesis of new phenolic compounds, but the accumulation of compounds that absorb at 280nm. There is a direct correlation between the resistance of *Theobroma cacao* L. and its amount of phenolic compounds. Flavanols and its derivative can be used as biochemical marquers of resistance of *Theobroma cacao* L against *Phytophthora megakarya*. The particular role of those compounds in resistance process remains not well understood.

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