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Assessing relationship between phenolic compounds and resistance to *Phytophthora megakarya* using two cocoa (*Theobroma cacao*) families

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Black pod disease is an important fungal infection in cocoa (*Theobroma cacao* L.) which causes high production losses. In Cameroon, these losses reached 80% of cocoa production depending on ecological zones. In order to contribute to the efficiency of selection methods used in resistance or tolerance to black pod disease with the aim of improving on cocoa farming, the content of phenolic compounds was analyzed on the genotypes of two hybrid families (F79:♀$T_{79/467}$x♂SNK_{13} and F13:♀SNK_{13}x♂$T_{79/467}$) of cocoa which are different in productivity and vulnerability to black pod disease. After artificial inoculation of the pods by mycelium of *Phytophthora megakarya*, the content of the phenolic compounds significantly increased in all genotypes of the two families. The heterosis effect of each family revealed a higher variability within both families. These results alike showed that productive and tolerant genotypes (F1307, 1314, F7902 and F7928) have a high phenols content and positive heterosis meanwhile the less tolerant and productive genotypes (F1321, F1326, F7904 and F7911) have a weak content and negative heterosis.

Key words: Cocoa, disease, tolerance, heterosis effect, phenolic compounds, hybrid progenies.

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is a perennial crop of significant economic importance in producing countries of Africa, South America and South East Asia. Currently, over 50 countries are engaged in cocoa production and heavily rely on cocoa exportation for their economic development, as this commodity contributes significantly to foreign exchange earnings. However, the cultivation of this plant is faced with numerous problems such as

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ageing of plantations, parasitic constraints, black pod disease due to by *Phytophthora megakarya*, which causes significant loses worldwide. These losses globally attained 20 to 30% of production, but might reach 80% in some Central African countries (Pokou et al., 2008; Simo et al., 2011). In Cameroon, *P. megakarya* causes over 80% losses depending on the ecological zone (Djocgoue et al., 2010) which may reach 100% if no control measures are enforced (Ndoumbe-Nkeng et al., 2004). The predominant symptom of pods is a brownish or black lesion on the husk, leading to blackening and rotting. On stems, the symptoms appear as cankers (Opoku et al., 2007; Nyadanu et al., 2012). Even though damage due to cocoa pod and leaf infections by *P. megakarya* are difficult to estimate, their effect on the health and productivity of cocoa trees are significant.

The control of black pod disease is, therefore, a major challenge for world cocoa cultivation and selection of resistant materials is underway in many producing countries. According to Tan and Tan (1990), several methods have been adopted by famers to control disease caused by *Phytophthora* species in cocoa, of which use of copper-based fungicides is the most predominant. Although this is reasonably effective, the high cost of chemical control in Africa poses a serious challenge to peasant farmers who produce over 50% of world cocoa. Moreover, the chemical control is unattractive from commercial and environmental points of view and is not always effective. It is also toxic to animals including man and, therefore, poses a great danger to peasant farmers most of whom are illiterates (Opoku et al., 2000; Nyadanu et al., 2012).

The most practical and appropriate means to control cocoa black pod is by the use of resistant or tolerant genotypes, supported by further measures of an integrated control system (Adomako, 2006; Nyasse et al., 2007). Although complete resistance has not been detected, differences in susceptibility among clones or among hybrids derived from crosses have been observed in various countries, including Cameroon (Lockwood et al., 2007; Djocgoue et al., 2010). Host plant resistance in cocoa is described as polygenic and additively inherited (Tan and Tan, 1990).

A strong correlation exists between the production and the use of hybrids due to the heterosis effect. Heterosis is the heterozygotic manifestation and this manifestation is the hybrid vigour. On the phenotypic map, heterosis effect showed the performance of the hybrid genotypes which are higher than the one of the mean of the two parents. During the development of the necrosis, positive heterosis has negative values while in the productivity and phenolic compounds we have positives values.

One of the priority objectives for cocoa farming in Cameroon is the selection of genotypes, which are less vulnerable to black pod disease and productive (Djocgoue et al., 2010). Nevertheless, the rate of cocoa pods attack ranges from one to another. Nyasse et al., (2002) have proven the existence of a correlation between cocoa resistance vis-à-vis to *P. megakarya* and the development of the lesion on cocoa pods obtained through artificial inoculation of the pathogenic agent by the lesion surrounded by living cells which have become resistant to microorganisms. Those cells have their cell walls reinforced by a layer of cellulose, lignin and other phenolic compounds (El Hassni et al., 2004; Dogbo et al., 2008). Numerous biochemical pathways are stimulated and secondary metabolisms increased. All these reactions constitute the hypersensitive response. It occurs after the regeneration of the pathogenic agent in the resistant plant. The role of phenolic compounds in plant defense is well documented (Tan et al., 2004; Omokolo and Boudjeko, 2005; Mbouobda et al., 2010). 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 there is a higher accumulation of non-constitutive hydroxycinnamic acid derivatives in resistant cultivars (El Hadrami et al., 1997). Some plants can induce a large variety of phenolic phytoalexins. Kodama et al. (1992) listed some16 phytoalexins produced by rice (*Oryza sativa*) in response to pathogen attack.

In the present study, the assessment of heterosis effect of the phenolic compounds in the resistance of *T. cacao* vis-à-vis to *P. megakarya* was done. The quantitative analyses of the content of phenolic compounds in the healthy pods, scarified and inoculated pods with sterilized agar disc and scarified and in inoculated pods with *P. megakarya* mycelium were also realized in function of their productivity and necrosis surface. Finally, the study of the heterosis effect of phenolic compounds vis-à-vis to *P. megakarya* from parental clones to hybrid progenies was also performed.

MATERIALS AND METHODS

Plant material

The plant material derived from the experimental station of the Cocoa Development Corporation (SODECAO) was made up of 3-months-old cocoa pods that belong to two parental clones (the sensitive and productive SNK13, Trinitario group, a tolerant and less productive T79/467, Forastero group) as well as their hybrid progenies organized within the F13 (♀SNK13x♂T79/467) and F79 (♀T79/467x♂SNK13) populations. The fungal material was a local isolate strain of *P. megakarya* (TA121) obtained from the Institute of Agricultural Research for Development (IRAD) of Nkolbisson (Yaounde, Cameroon).

Cocoa pods inoculation

The apparently healthy pods were harvested, washed with tap

Table 1. Mean surface area of necrosis (cm^2) on cocoa pods 6 days after inoculation with *Phytophthora megakarya* and productivity of cocoa beans weight (g).

Genotype	Mean surface area of necrosis (cm^2)	Mean of 100 cocoa beans weight (g)
Parents		
SNK13	59.04±3.41^{i}	406.17±12.92^{hi}
T79/467	39.62±2.23^{g}	360.50±7.71^{ef}
F13		
F1307	6.16±1.46^{ab}	450.14±37.35^{lm}
F1314	17.85±0.43^{de}	581.23±12.79^{o}
F1315	16.62±1.35^{cde}	296.77±3.75^{ab}
F1313	20.98±1.23^{ef}	304.64±14.60^{ab}
F1324	69.44±2.95^{k}	519.01±5.15^{n}
F1308	42.05±4.61^{h}	441.94±33.55^{klm}
F1321	102.13±3.58^{m}	292.18±2.16^{a}
F1326	64.12±2.17^{jk}	304.07±2.48^{ab}
Parents		
SNK13	59.04±3.41^{hi}	406.17±12.92^{gh}
T79/467	39.62±2.23^{f}	360.50±7.71^{ef}
F79		
F7902	9.94±1.79^{a}	439.32±4.92^{hi}
F7928	11.88±3.09^{ab}	409.90±26.40^{gh}
F7926	13.73±2.66^{abc}	271.28±4.14^{a}
F7907	15.6±3.14^{abc}	249.87±20.30^{a}
F7915	74.86±6.12^{j}	550.36±8.46^{l}
F7919	50.46±3.22^{gh}	500.05±50.82^{k}
F7904	59.04±3.41^{hi}	252.80±21.79^{a}
F7911	57.47±4.70^{hi}	252.69±7.84^{a}

Values followed by the same letter within column for each family are not significantly different (P< 0.05).

water, sterilized with 70% alcohol and divided into three groups. The first group consisted of healthy pod (H), second group of pods scarified and inoculated with sterilized agar disk (S), third group of pods scarified and inoculated with an agar disc containing *P. megakarya* mycelium (I) obtained from 7-day-old PDA culture medium and incubated at 25 to 26°C in the dark in a humid chamber. The measurement of the necrosis surface area was done in days 3, 4, 5 and 6 after inoculation. The diameter of the circular necrotic spots was measured and the surfaces calculated using Blaha and Lotode's formula (1976).

Assessment of productivity

The productivity was assessed by measuring the weights of 100 fresh cocoa seeds in the different genotypes (Cilas, 1991).

Determination of heterosis

Heterosis or hybrid vigour is estimated by comparing the hybrid vigour of F_1 to the mean of those of the two parents (P_1 and P_2). This hybrid vigour (HF) is calculated according to Gallais (1990) and Zahour (1992) and is expressed in percentages (%).

Genotypic identification for phenolic compounds analysis

The parental and hybrid genotypes were identified for biochemical studies such as tolerant and productive (F1307, F1314, F7902, F7928), tolerant and less productive (F1315, F1313, F7926, F7907), less tolerant and productive (F1324, F1308, F7915, F7919) and less tolerant and less productive (F1321, F1326, F7904, F7911) (Djocgoue et al., 2010) (Table 1). The study of the necrosis and the productivity also permit obtaining heterosis effect. These different groups have been selected from the morphological study to use them in the study of phenolic compounds. This step will permit to see if there is a similitude between the morphological study and the study of phenolic compounds (Tables 2 and 3).

Phenolics analyses

For the analysis of phenolic compounds, samples were taken 6 days after inoculation from healthy tissue at approximately 2 cm outside of the lesion. Total phenolic compounds were extracted twice using methanol (80%). One gram of fresh tissue was ground in 5 ml of methanol (80%). After 30 min incubation at 4°C, the ground material was centrifuged at 6000 *g* for 20 min. The supernatant was collected and the precipitate re-suspended in 3 ml of methanol and incubated at room temperature for 15 min followed by another centrifugation. The supernatant was collected and mixed with the first to constitute the phenolic extract. The concentration of phenolic compounds was determined in the supernatant spectrophotometrically at 725 nm, according to Marigo (1973), using the Folin-Ciocalteu reagent. Phenolic contents were expressed in milligram equivalent of chlorogenic acid/gram of fresh

Table 2. Heterosis value (%) with respect to average parents of the necrosis of F13 family and F79 family.

Genotype	Time (day)			
	Day 3	Day 4	Day 5	Day 6
F 13				
F1307	-100	-88.53	-85.20	-87.51
F1314	-77.89	-63.29	-75.34	-63.81
F1315	-100	-45.43	-57.60	-66.30
F1313	-81.90	-84.33	-54.43	-57.47
F1324	+17.30	+1.20	+45.38	+40.76
F1308	-11.71	-30.07	-11.94	-14.75
F1321	-4.18	+21.42	+69.667	+107.03
F1326	-61.50	-54.90	+10.11	+29.98
F 79				
F7902	-100	-86.02	-81.42	-79.84
F7928	-100	-100	-90.00	-75.91
F7926	-100	-81.05	-68.92	-72.16
F7907	-100	-100	-92.50	-68.37
F7915	-100	+6.11	+87.23	+51.75
F7919	-68.06	+19.32	+26.76	+2.29
F7904	-100	+10.41	+33.96	+19.68
F7911	-63.32	-19.27	+4.77	+16.50

Table 3. Heterosis value (%) with respect to average parents during three years of the productivity of F13 family and F79 family.

Genotype	Time (year)		
	First year	Second year	Third year
F 13			
F1307	+26.77	+22.27	+5.57
F1314	+55.33	+50.04	+53.96
F1315	-22.01	-23.30	-24.08
F1313	-23.45	-23.33	-16.63
F1324	+41.51	+32.38	+35.73
F1308	+7.94	+17.14	+21.27
F1321	-24.25	-25.23	-23.69
F1326	-22.23	-22.00	-21.06
F 79			
F7902	+15.27	+14.36	+14.17
F7928	+0.84	+10.37	+9.40
F7926	-27.89	-31.15	-28.59
F7907	-39.61	-31.25	-33.71
F7915	+48.58	+41.54	+40.70
F7919	+48.28	23.13	+20.39
F7904	-37.23	-36.95	-28.03
F7911	-35.15	-34.41	-32.69

weight.

Statistical analysis

Data from this study are presented in the form of means ± SD, for at least three independent experiments during three successive trips. Three measurements were recorded for each campaign. Analysis of variance (ANOVA) and Duncan test were used to compare the susceptibility levels and phenolic contents of better progenies resulting from different crosses to access hybrid vigour, using SPSS 12 version for Windows P value less than 0.05 was considered significant. Principal component analysis (PCA) and hierarchical classification were performed with SPAD version 4.1 windows

software to have different groups of hybrids genotypes in function of the degree of their resistance.

RESULTS

Evaluation of the phenolic content and the heterosis effect

Phenolic content

In healthy condition, the accumulation of total phenolic content is more important in parental genotype SNK13. This accumulation, though important in situation of wound or inoculation, remains less than that observed in the tolerant genotypes T79/467.

In the hybrids of F13 family, in healthy conditions, the phenolic content was more abundant for F1321 (5.57 ± 0.46 $mg.g^{-1}$ of FW), followed by F1324 (5.42 ± 0.29 $mg.g^{-1}$ of FW), F1315 (5.40 ± 0.29 $mg.g^{-1}$ of FW) and F1308 (5.13 ± 0.43 $mg.g^{-1}$ of FW). In conditions of scarified and inoculated with sterilized agar disc (S), or scarified and inoculated with *P. megakarya* mycelium (I), there was an accumulation of total phenol content in both parents and all hybrids. This accumulation is more perceptible in F1313 (109%), F1307 (71%) and F1315 (58%) hybrids in healthy condition and in F1313 (202%), F1307 (186%), F1314 (109%) and F1315 (76%) for inoculation condition. Hybrids with lower necrosis surface had lower phenol content. However, the total phenols content was more abundant in inoculated conditions than in healthy conditions (Figure1a).

Under healthy conditions in F79 family, phenols contents are more abundant in hybrids F7904 (5.80 ± 0.54 $mg.g^{-1}$ of FW) and F7911 (5.25 ± 0.33 $mg.g^{-1}$ of FW). Under conditions of wound, 100% of individuals registered an accumulation of total phenols content. This accumulation was more significant in hybrids F7926 (171%), F7907 (123%), F7902 (122%) and F7928 (101%). Nonetheless, total phenol content is higher in (I) conditions than in (S) conditions (Figure 1b).

Heterosis effects

Heterosis of hybrid of the F13 and F79 families were estimated, with respect to the average parent; in the hybrid genotypes of F13 family, the manifestation of hybrid vigour was obtained in all treatment conditions. 63, 63 and 50% of hybrid genotypes had a positive heterosis effect under healthy, scarified and inoculated conditions (Table 4). The highest hybrids vigours were manifested by F1315 and F1314 genotypes under inoculated conditions with *P. megakarya* mycelium. For hybrids of F79 family, positive heterosis effects were also observed under all treatment conditions. This effect is respectively 25, 25 and 50% under healthy, scarified and inoculated conditions. These results show that the heterosis effect was more important in F13 family than F79 family (Table 5).

Hierarchical classification of the different genotypes

Total phenols content obtained under different treatment conditions permitted us to hierarchically classify the genotypes of different descendants. The various parental and hybrid groupings of genotypes were realized at 95% of homogeneity.

For F13 family

The direct hierarchical classification of individuals of F13 family permits to distinguish three groups when all treatment conditions are considered. The first group was made up of parent SNK13 and hybrids F1326, F1308, F1324 and F1321. The second was made up only of F1315 hybrid meanwhile the third group included parent T79/467 and hybrids F1314, F1313 and F1307 (Figure 2a). Under inoculated conditions with *P. megakarya* mycelium, two groups were distinguished. Group 1 is made up of parent SNK13 and hybrids F1326, F1321, F1324 and F1308 and the second includes parent T79/467 and hybrids F1313, F1315, F1314 and F1307 (Figure 2b).

For F79 family

The direct hierarchical classification of individuals of F79 family considering all treatment conditions permit distinguishing three groups. Group 1 was made up of hybrids F7911 and F7904. The second was made up of hybrids F7915 and F7919 meanwhile the third group was constituted by parents SNK13 and T79/467, and hybrids F7926, F7928, F7907 and F7902 (Figure 3a).

Under (I) condition only, the total phenols content permits distinguishing two groups. The first group was made up of hybrids F7915, F7904, F7919 and F7911 meanwhile the second was constituted by parents SNK13 and T79/467, and hybrids F7928, F7907, F7926 and F7902 (Figure 3b).

DISCUSSION

In an attempt to minimize the pathogens infection which is a major limiting factor in plant production, resistance parameter was conducted through the evaluation of total phenols content in the different cocoa genotypes based on the degree of tolerance of these genotypes. The main goal of the present study was to analyze the heterosis effect of the phenols content in the healthy pods, scarified and inoculated pods with sterilized agar disc and scarified

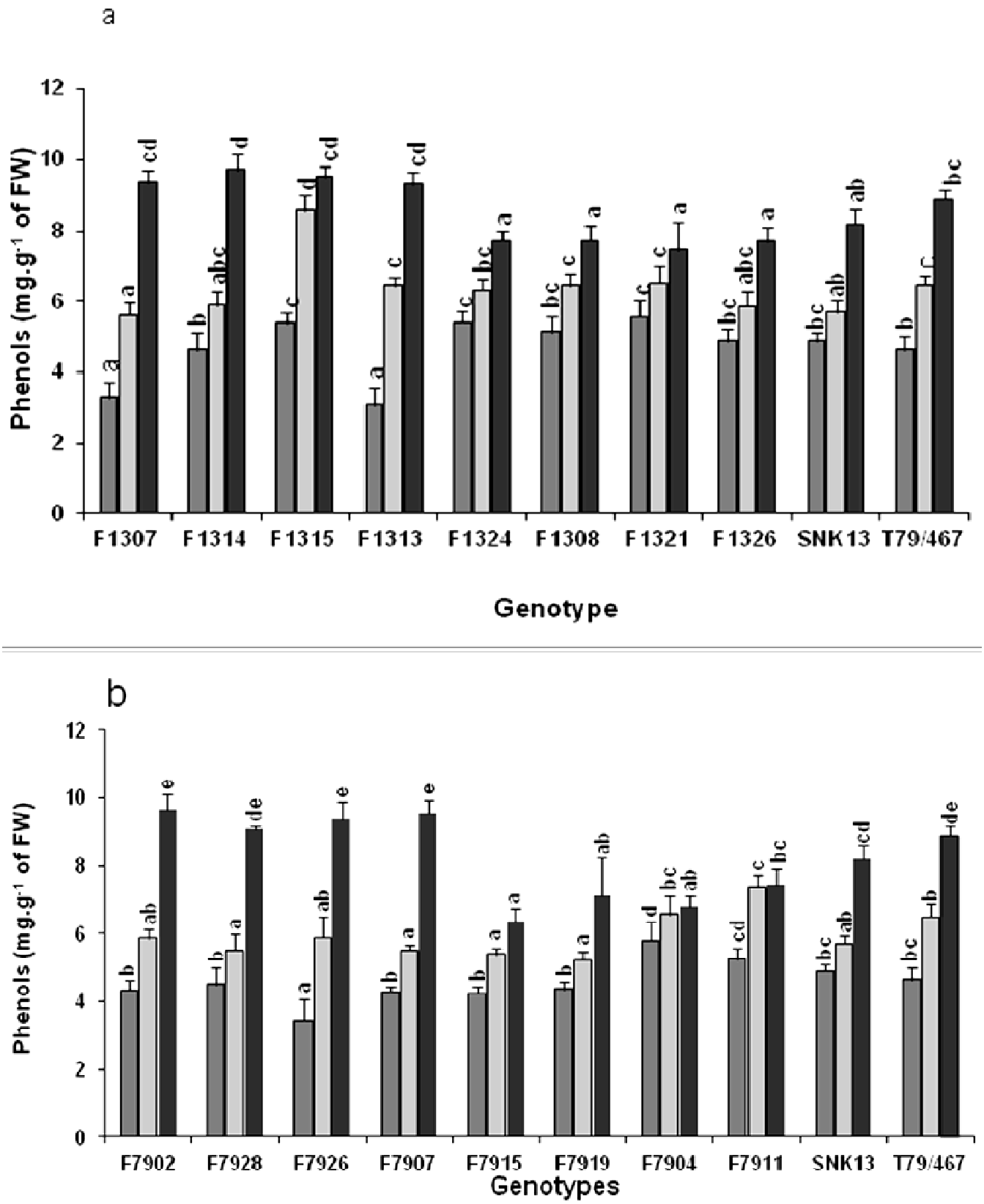

Figure 1. Average content in total soluble phenols in the pods of parental genotypes SNK13 and T79/467 and: (a) hybrids of family F13; (b) hybrids of family F79 under different treatment conditions. Means with the same colors following by the same letter are not significantly different (P< 0.05).

Table 4. Heterosis value (%) with respect to average parents of the phenolic compounds of extracts of F13 family under all conditions.

Genotype	Treatment		
	Healthy	Scarified	Inoculated
F1307	-31.29	-7.74	+9.77
F1314	-2.94	-3.43	+13.82
F1315	+13.29	+40.54	+11.51
F1313	-35.35	+5.97	+9.26
F1324	+13.71	+3.69	-9.46
F1308	+7.75	+5.59	-9.31
F1321	+16.89	+7.07	-12.68
F1326	+2.50	-3.73	-9.65

Table 5. Heterosis value (%) with respect to average parents of the phenolic compounds of extracts of F79 family under different treatment conditions.

Genotype	Treatment		
	Healthy	Scarified	Inoculated
F7902	-9.19	-3.83	+12.82
F7928	-5.42	-9.49	+6.46
F7926	-27.64	-3.41	+9.56
F7907	-10.11	-9.76	+12.12
F7915	-11.11	-11.64	-25.33
F7919	-8.41	-14.27	-16.61
F7904	+21.68	+7.72	-20.56
F7911	+10.21	+21.28	-12.75

and inoculated pods with *P. megakarya* mycelium of parental clones SNK13 and T79/467 and hybrids resulting from the reciprocal crossing of ♀SNK13 x ♂T79/467 in function of the productivity and the resistance of cocoa pod vis-à-vis to *P. megakarya*.

In the point of view of constitution, the pool of phenolic compounds in the tissues of *T. cacao* is a function of genotype. This observation suggests that these metabolic formed part of defense mechanisms. The implication of these phenolic compounds in the mechanism of resistance to pathogenic microorganisms of plants has been demonstrated by Temgo and Boyomo (2002). In this study, the increase of phenolic content under conditions of inoculation with *P. megakarya* mycelium was more abundant in the tolerant parent T79/467 and in 100% of hybrid genotypes. Similar rresults have already been reported by many authors on cocoa leaves inoculated by *P. megakarya* (Boudjeko et al., 2007). These results are also in conformity with those obtained by Baker et al. (2005) who observed an accumulation of phenolic compounds in the extracellular environment of vegetable cells suspensions few hours after interactions between these cells and the pathogenic agent.

Sensitive genotypes accumulate less phenolic compounds under conditions of inoculation compared to tolerant genotypes indicated that this increase is correlated to the degree of genotypes studied. These results are different from those of Boudjeko (2003) who instead found out that the increase in phenolic compounds in the roots of *Xanthosoma sagittifolium* after inoculation by *Pythium myriotylum* seems not to be correlated to the degree of resistance of this plant. In fact, the level of accumulation of phenol in response to inoculation can be correlated to the photosystem and the level of resistance of the host (Nicholson et al., 1992). According to Matern and Kneusal, (1988), the first stage of the defence mechanism of plants against parasitic attacks implies a rapid accumulation of phenolic compounds at the inoculation site. This stops or slows down the progression of the pathogenic agent. During this defence, phenolic compounds can play the role of phytoalexines. They can also contribute to the formation of lignine and contribute as such to the construction of structural barriers to stop the progression of the pathogenic agent. These phenols also have an antioxidant role to counteract prooxidant agents produced during stress caused by pathogenes or the environment (Dixon et Palva, 1995). The highest increases in phenolic compounds were observed in tolerant parent T79/467 and hybrid genotypes F1307, F1314, F1315, F1313,

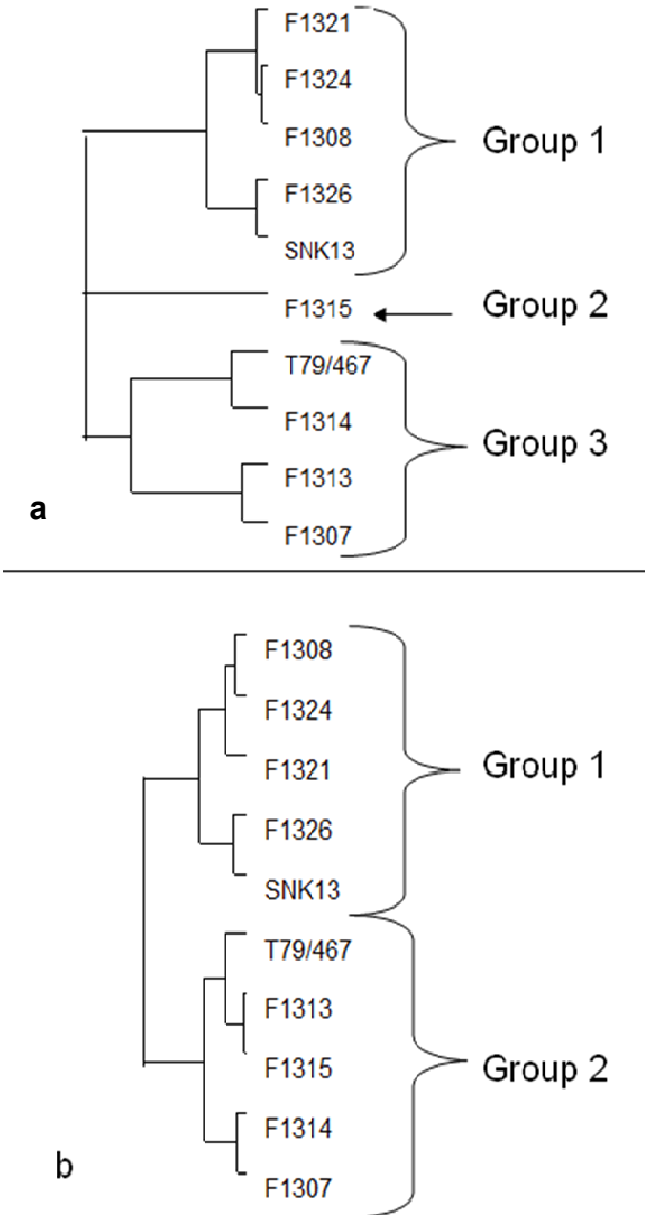

Figure 2. Direct hierarchical classification of the genotypes of F13 family using phenol content under all conditions (a) and under inoculated with *P. megakarya* mycelium alone (b).

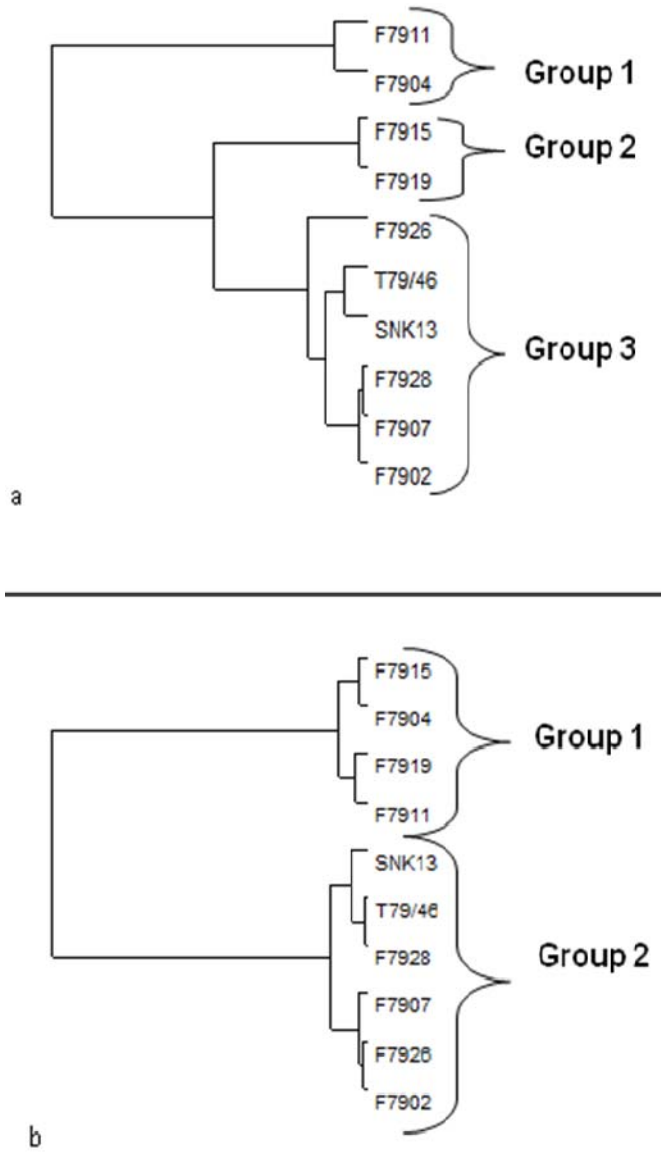

Figure 3. Direct hierarchical classification of the genotypes of F79 family using phenol content under all conditions (a) and under inoculated with *P. megakarya* mycelium alone (b).

F7902, F7928, F7926 and F7907. These genotypes also have the weakest necrosis surfaces. This result is closed to that obtained by Junqueira et al. (2004) who showed an accumulation of fatty acids and phenolic compounds during inoculation in hybrid genotypes of maize. Hierarchical classification of the different genotypes with respect to phenols content enabled 3 and 4 groups for F13 and F79 families at 95% homogeneity. This classification illustrates that about 50% of the progenies manifest hybrid vigour for this trait. This reveals the existence within the progenies of more efficient genotypes than the best parent and that this can be used as parents in future improvement programs (Lockwood et al., 2007). The heteosis effect of each F13 and F79 family when comparing the phenols content revealed a higher variability within both families. In fact, 50% of F13 hybrids and 50% of F79 hybrids presented a posisitive heterosis 6 days after inoculation. Hybrids that present a positive heterosis for a character might have genes containing additive effects in some situations that have an important implication in the transmission of that character.

Conclusion

The evaluation of heterosis effect of phenols in the pods of *Theobroma cacao* allowed the different hybrid genotypes of F13 and F79 families to be compared and classified. This physiological parameter has been analyzed in healthy pods, scarified and inoculated pods with sterilized agar disc (S) and in scarified and inoculated pods with an agar disk containing *P. megakarya* mycelium (I). The results showed that productive and tolerant genotypes (F1307, 1314, F7902 and F7928) have a high phenols content and positive heterosis effect after (I) meanwhile the less tolerant and productive genotypes (F1321, F1326, F7904 and F7911) have a weak content and negative heterosis effect after (I).

Conflict of Interests

The author(s) have not declared any conflict of interest.

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