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Heritability of the tolerance to *Phytophthora megakarya* Bras. and Grif. of *Theobroma cacao* L. in terms of their necrosis length, phenolic contents and activity of enzymes

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Abstract

Plant breeding through selected biochemical markers requires the genetic amelioration programs. Therefore, this study focused on the heritability of the necrosis length, phenolic contents and activity of enzymes during *Theobroma cacao*/*Phytophthora megakarya* interactions. Length of necrosis were measured after leaves infection by *Phytophthora megakarya* in 20 genotypes of *Theobroma cacao* L. derived from intercrossing of T79/467 and ICS40 parental clones. Total polyphenols (TPP), total flavonoids (TF) and variation in peroxidase (POX), polyphenoloxidase (PPO) and phenylalanine ammonialyase (PAL) activities in imparting tolerance to *P. megakarya* were estimated according to standard methods. The outcome showed heterosis linked to length of necrosis manifest a hybrid vigor (90% for 2013, 85% for 2014 and 80% for 2015). The F90.34, F90.35 and F95.36 genotypes showed a significant increase ($P<0.05$) of biochemical components correlated with tolerance rates and this increase was genotype-dependent. Principal component analysis (PCA) and hierarchical classification displayed two clusters where the first categorized 17 tolerant individuals and the second five susceptible ones. Heritability of the necrosis [narrow sense heritability (h^2: 0.231 for F90 and 0.243 for F95) and broad-sense heritability (H^2: 0.291 for F90 and 0.336 for F95)] and the metabolites studied from the two reciprocal crossings didn't show any significant difference implying the absence of maternal effect in the transmission of these characters.

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Introduction

Cocoa tree (*Theobroma cacao* L.) represents one of the major operating crops of most tropical countries by reason of its favorable ecology. It is mainly cultivated by small holders along with their subsistence crops, providing them with a cash crop of cocoa. Africa produces 72.5% of the world cocoa supply, with Cameroon 232,000 tons in 2015 (Anonymous, 2015). However, *Phytophthora megakarya* Bras. and Grif., is one of the *Phytophthora* species reported on *Theobroma cacao* and is the most virulent of the species, causing black pod disease. In Cameroon, approximately 50-80% of its production losses is orchestrated by this species (Mfegue, 2012). The disease can be controlled using pesticides, but the costs of this chemical control are often too expensive for African farmers. However, a multidisciplinary survey of pesticide use in Cameroon villages supported the conclusion that the current use of fungicide is not a successful disease management strategy for most farmers (Sonwa *et al.,* 2008). Thus, genetic control by breeding cacao cultivars tolerant to the disease and exploiting field tolerance factors could be an appropriate solution to enhance cocoa production.

To ensure sustainable production, breeding programs are increasingly focused on breeding hybrid by crossing the genotypes available. However Cameroon uses only 17.4% of hybrid genotypes, they show a strong production capacity and adaptability of the environment due to the effect of parental genes capacity. Thus, the field observation of the genotypes performances under natural or artificial infection conditions and leaf or pod inoculation has enabled the identification of tolerant genotypes (Simo *et al.,* 2011; Ondobo *et al.,* 2014). However, Ondobo *et al.* (2013) after crossing cocoa clones, obtained hybrid populations that had higher hybrid vigor than of the best parent of the development of necrosis in a nursery. Thus, hybrid varieties are characterized by high productivity and environmental adaptation capacity due to the additive effect of parental genes and can be used on a large scale (Tahi *et al.*, 2000; Djocgoue *et al.*, 2011). This purpose, the use of heritability is an effective genetic parameter of selection of quantitative traits (via the ability of parents to transmit their qualities to their progeny) in a determined reproductive system. Thus the additive component is the characteristic way and inheritable effect of a genotype (Gallais, 1990). Nyasse *et al.* (1995) and Djocgoue *et al.* (2011) reported that additive gene effects were important for transmission of character length of necrosis (tolerance gene at *P. megakarya*). Effa *et al.* (2015) showed that selection based on family performance or progeny test should be more effective. This selection can be achieved using biochemical markers such as phenols and antioxidant enzymes. Plants use inherent physical and chemical barriers to effectively stop a pathogen invasion, and their inducible defense reactions are activated by pathogen attacks (Aktaş and Guven, 2005).

Plants generate some biochemical and physiological reactions when they are facing biotic or abiotic stress factors, and several chemical compounds are consequently synthesized. Defense reactions may develop several hours or a few days after stimulation (Desender *et al.,* 2007). Recent studies on mechanisms of biological control by Plant Growth Promoting Rhizobacteria (PGPR) revealed that several strains protect the plants from pathogen attack by strengthening the epidermal and cortical cell walls with deposition of newly formed barriers beyond infection sites including callose, lignin and phenolic and by activating defense genes encoding chitinase, peroxidase (POX), phenylalanine ammonialyase (PAL), polyphenol oxidase (PPO) (Ngadze *et al.,* 2012). These differences can be attributed to the structure of the host tissue and the biochemical activity there in. Damodaran *et al.* (2009) and Ondobo *et al.,* (2014) reported that highly tolerant clones and hybrid of cacao contain high phenolic compounds.

The objective of this study was (1) to identify the tolerant hybrids of *T. cacao* after *P. megakarya* inoculation and (2) to estimate heritability values of some defense parameters as necrosis length, TPP, TF

and activities of POX, PPO and PAL during *T. cacao*/*P. megakarya* interaction.

Materials and methods

Plant and straminopilous isolate (Oomycete) materials

A total of 22 genotypes of *T. cacao* were derived from the nursery and field. Two parental clones T79/467 (tolerant to *P. megakarya* and less productive) of Forastero group, and ICS40 (susceptible to *P. megakarya* and high productive) of Trinitario group were used in this study. Their two population hybrids were come from the reciprocal crosses: F90 (♀T79/467 × ♂ICS40)/ F95: (♀ICS40 × ♂T79/467). The experiments were conducted in SODECAO (Cameroon Cocoa Development Corporation) station at Mengang, during three years season (2012/2013, 2013/2014 and 2014/2015).

P. megakarya used was the strain «lebdi» obtained from the Central laboratory of Phytopathology at IRAD (Research Institute for Agricultural Development). This Oomycete was maintained on V8 agar medium (200g V8 vegetable juice, 3g Ca_2CO_3, 15g agar and 1000 mL distilled water) and incubated in the dark at 25 ± 2 °C.

Leaf inoculation

An Artificial inoculation method was carried out in nursery upon the leaves were scarified along the midrib (semi ripened), during three-season period (2013, 2014 and 2015). They were then inner surface sterilized with ethanol 70%. Three treatments were realized on each parental genotype and hybrid: (i) healthy leaves, (ii) wounded leaves (iii) and infected leaves. Agar disks (6 mm diameter) cut from 5-day-old oomycete cultures were laid on the midrib after wounding by a razor sterilized. The scars were then covered with cotton that had been immersed in sterilized water. The necrosis length was measured at two days interval by a graduated ruler after inoculation. The samples were wrapped with aluminium foil 6 days after inoculation.

Estimate heterosis and heritability

The study of quantitative traits is based on statistical analysis of the measured performance of individuals such as: (i) the heterosis (Zahour, 1992) and (ii) heritability (Falconer and Mackay, 1996).

(i): Heterosis or hybrid vigor is usually the result of complementation between descendants.

$$HF(\%) = \frac{F1 - \frac{P1 + P2}{2}}{\frac{P1 + P2}{2}} \times 100$$

Where: HF (%) = Hybrid vigor in per cent; F1 = Hybrid genotype;
P1 and P2 = Parents

(ii): Heritability: is the proportion of phenotypic variance that is due to genetic variance. There are two types of expression of heritability:

Broad-sense heritability - It measures any genetic variance.

$$H^2 = \frac{V_G}{V_P} = \frac{V_G}{V_G + V_E}$$

Narrow sense heritability - It measures the variance of additive genetic.

$$h^2 = \frac{V_A}{V_P} = \frac{V_A}{V_G + V_E}$$

Where: V_G = Genetic variance; V_P = Phenotypic variance;
V_A = Additive genetic variance; V_E = Environmental variance

Biochemical essays

Phenolic compounds were extracted as described by Singleton and Rossi (1965) with minor modifications. Fifty milligrams of leaves (fresh weight) were extracted in 1 ml of 80% aqueous methanol and centrifuged (LABOFUGE 400R centrifuge) for 15 min at 1000 *g*. Two hundred µL of supernatant were mixed with 1.5 mL of Folin-Ciocalteu reagent and the mixture was shaken. After 5 min, 1.5 µL of Na_2CO_3 (0.566 M) solution was added. Total phenolic content was measured spectrophotometrically (JENWAY 6305) at 725 nm. Flavonoids were determined according to kramling and singleton (1969). All the

experiments were repeated three times and values were expressed as $\mu g.g^{-1}$.

Enzyme essays

Peroxidase (POX: EC 1.11.1.7) was extracted from the leaves. POX activity was assessed according to Rodriguez and Sanchez (1982). The reaction mixture contained 1ml of 0.05 M phosphate-citrate buffer (pH 4.6), 1 ml of 40 mM guaiacol and 0.5 ml of 26 mM H_2O_2. Absorbance at 470 nm were measured for 5 min using a JENWAY 6305 spectrophotometer. POX activity was expressed as $\Delta A470.min^{-1}.g^{-1}$ fresh weigh.

Polyphenoloxidase (PPO: EC 1.10.3.1) activity was assessed as described by Van Kemmenn and Broumer (1964). The reaction mixture contained 2.5 ml of 0.1 M acetate buffer (pH 6.0) and 0.5 ml of 10 mM catechol. The activity was expressed as change in absorbance ($\Delta A420.min^{-1}.g^{-1}$ fresh weigh) at 420 nm.

Phenylalanine ammonialyase (PAL: EC 4.3.1.5**)** assay was conducted according to the method described by Ross and Sederoff (1992) with minor modifications, using 500 µL of borate buffer and 600 µL of 12 mM L-phenylalanine along with 400 µL enzyme extract as reaction mixture. The PAL activity was measured at 290 nm and expressed in $nmol.min^{-1}.mL^{-1}$ fresh weigh.

Statistical analysis

Data presented are the means ± SE of three independent experiments. Triplicate samples were taken for each treatment and analyzed for biochemical parameters. Analysis of variance (ANOVA) and the Student Newman Keuls test were performed with the assistance of SPSS 20.0 for windows. Hierarchical classification and principal component analysis (PCA) were realized to describe the variability of length necrosis data and biochemical components respectively. This analysis was performed with SPAD 5.5 software package.

Results

Development of the lesion and heterosis

Pathogenicity tests of all parental and hybrid genotypes were tested by *P. megakarya* isolate in the nursery and the field (during three years). However, the rate of development of infection was genotypes-dependent. Six days after infection, the development of necrosis of the genotypes F 90.30 [7.43 cm (2013), 6.63 cm (2014) and 10.16 cm (2015)], F 95.37 [5.3 cm (2013), 4.73 cm (2014) and 10.26 cm (2015)] and ICS 40 [6.43 cm (2013), 6.77 cm (2014) and 8.06 cm (2015)] is very high compared to other genotypes in these periods (Fig.1). Contrariwise, the development of lesion was less important in F90.39 and F95.36 genotypes.

In individuals of F90 and F95 populations, 90%, 85% and 80% manifested a hybrid vigor during the period 2013, 2014 and 2015 respectively. All F95 population displayed a positive heterosis value during 2014 cocoa period. In contrast, negative heterosis value was observed continuously among F90.30 genotypes. However, there is a loss of vigor in individuals F 90.32 (+16.19) and F 95.33 (+8.63) during 2015 period (Table 1).

Table 1. Heterosis values (%) of lesion size of hybrids derived from F90 and F95.

Period (Year)							
Genotypes	2013	2014	2015	Genotypes	2013	2014	2015
F 90.30	+56.42	+34.76	+32.49	F 95.30	-43.79	-40.45	-16.28
F 90.31	-49.26	-24.80	-21.50	F 95.31	-60	-22.15	-7.80
F 90.32	-15.79	-15.65	+16.19	F 95.32	-16.42	-33.94	-4.41
F 90.33	-24.84	+17.28	-12.24	F 95.33	-5.26	-7.52	+8.63
F 90.34	-67.37	-38.21	-28.67	F 95.34	-44.21	-27.44	-28.80
F 90.35	-47.37	+8.54	-10.67	F 95.35	-53.68	-46.34	-34.67
F 90.36	-32.63	-24.80	-15.76	F 95.36	-76.21	-54.07	-49.14
F 90.37	-60.84	-20.33	-37.93	F 95.37	+11.58	-3.86	+33.67
F 90.38	-71.16	-45.73	-40.93	F 95.38	-16.42	-19.11	-20.45
F 90.39	-46.74	-52.44	-47.32	F 95.39	-53.68	-24.80	-26.06

Dendrogram of 22 genotypes obtained from development of infection during three years is presented in figure 2. The values enabled two groups to be distinguished at 90% homogeneity. Group 1, very polymorphic, contained 17 tolerant genotypes constituted of 16 hybrids (F90 and F95) and the best parent, T 79/467. The group 2 constituted of genotypes (ICS 40, F 95.33, F 90.32, F 90.30 and F 95.37 and) were susceptible (8.07, 8.33, 8.91, 10.16 and 10.25 cm, respectively).

Table 2. Average lesion size in the midrib (leaves) and biochemical activities of eleven genotypes of *T. cacao* in different treatments (H=Healthy, W=Wounded and WI= Wounded and infected).

Genotypes	Lesion size (cm) Period/2015	Total Polyphenols (µg.g-1 of fresh weight)			Total Flavonoids (µg.g-1 of fresh weight)		
		H	W	WI	H	W	WI
Parents							
T 79/467	7.27 ± 0.38b	104.39b	126.98b	169.88b	81.65b	90.26b	110.55b
ICS 40	8.07 ± 0.35c	80.29a	93.92b	110.66a	50.37a	66.24a	80.99a
F 90							
F 90.30	10.16 ± 0.68d	66.86a	78.74a	95.51a	55.20a	65.43a	78.25a
F 90.32	8.91 ± 0.26c	74.72a	81.27a	98.58a	52.65a	68.07a	76.17a
F 90.34	5.47 ± 0.38ab	136.95b	192.74c	250.29c	100.03b	125.71c	164.78b
F 90.35	6.85 ± 0.38ab	128.69b	155.46b	199.87b	74.59a	97.79b	114.11b
F 95							
F 95.31	7.07 ± 0.25b	113.84b	135.10b	163.53b	69.62a	89.92b	101.35b
F 95.32	7.33 ± 0.40b	98.51b	118.01b	140.23b	68.43a	78.66a	92.91a
F 95.33	8.33 ± 0.31c	82.36a	97.73b	100.63a	50.15a	81.29a	83.55a
F 95.36	3.90 ± 0.30a	275.02c	302.55d	360.91d	187.21c	211.33d	256.04c
F 95.37	10.25 ± 0.35d	57.03a	69.46a	93.10a	42.36a	65.86a	77.68a

*Values with the same letter in the same column and in the same family are not significant different (P < 0.05). Values are means of 3 replicates.

Variation of biochemical metabolites

Biochemical activities in the leaves of population F90 and F95 (parental and hybrids) healthy (H), wounded (W), wounded and inoculated (WI) varied significantly (*P<0.001*) for total polyphenol content (TPP) and total flavonoids (TF) between resistant and susceptible genotypes. In different treatment (H, W and WI), the PPT and FT in the leaves of F95.36, F90.34, F90.35, T79/467 and F95.31 were significantly higher than others genotypes (Table 2). The susceptible hybrids F95.37, F90.30, F 90.32 and ICS 40 had less accumulated of TPP and TF. Contrary to healthy and wounded, wounded and infected leave showed a significant (*P<0.001*) increase of TPP and TF content. In infection, the resistance genotypes F95.36 [360.91 $\mu g^{-1}.g^{-1}$ fresh weight (TPP), 256.04 $\mu g^{-1}.g^{-1}$ fresh weight (TF)] and F 90.34 [250.29 $\mu g^{-1}.g^{-1}$ fresh weight (TPP), 164.78 $\mu g^{-1}.g^{-1}$ fresh weight (TF)] displayed higher TPP and TF content when compared to susceptible genotypes (Table 2).

POX, PPO and PAL activities

Significant differences (*P*<0.05) in POX, PPO and PAL enzyme activities were recorded across F90 and F95 populations. POX activity increased significantly (*P*<0.05) in healthy, wounded, wounded and inoculated leaves of all genotypes (parental and hybrids).

In infection, the highest POX contents (tolerant genotypes) ranged from 18.6 to 14.7 $\Delta A470 \cdot min^{-1}.g^{-1}$ for F95.36 and F95.35 respectively when compared to susceptible genotypes where POX activity was less important (Table 3).

In healthy leaves of all genotypes (parental and hybrids), PPO activity showed significant difference (*P*<0.05). The tolerant genotypes had relatively higher PPO activity [(7.07 to 12.77) $\mu g^{-1} \cdot g^{-1}$ fresh weight] for wounded and [(8.72 to 14.02) $\mu g^{-1} \cdot g^{-1}$ fresh weight] for inoculated leaves when compared to the susceptible ones (Table 3).

Table 3. Enzyme activities in the midrib (leaves) of eleven genotypes of *T. cacao* in different treatments (H=Healthy, W=Wounded and WI= Wounded and infected).

Genotypes	POX activity			PPO activity			PAL activity		
	(Δ A470·min⁻¹.g⁻¹ fresh weight)			(Δ A420·min⁻¹.g⁻¹ fresh weight)			(nmol.min⁻¹·mL⁻¹ fresh weight)		
	H	W	WI	H	W	WI	H	W	WI
Parents									
T 79/467	7.39b	9.44c	10.21c	5.26bc	7.07c	8.72c	9.78c	10.75c	11.89c
ICS 40	5.90ab	7.01b	8.97bc	2.66ab	3.95b	5.70b	6.81b	7.39b	8.31b
F 90									
F 90.30	2.86a	3.05a	3.97a	1.29a	1.98a	2.39a	3.33a	4.08a	5.36a
F 90.32	4.95ab	6.53b	7.60b	3.08ab	4.19b	4.74b	6.92b	7.42b	8.00b
F 90.34	14.87d	15.31e	16.07e	8.75d	9.22cd	10.98d	14.51de	16.82e	18.21e
F 90.35	11.10c	13.20de	14.66d	6.56c	8.23c	8.98c	11.79cd	14.96d	16.07d
F95									
F 95.31	7.93b	10.00cd	10.87c	4.70b	5.67bc	8.88c	10.51c	11.66cd	11.85c
F 95.32	5.77ab	9.75c	10.18c	4.30b	5.85bc	7.35c	7.54b	10.87c	11.96c
F 95.33	5.11a	7.63b	8.99bc	3.24ab	4.56b	5.89b	8.81bc	9.53c	10.89c
F 95.36	16.82e	17.33f	18.60f	11.66e	12.77e	14.02e	15.67e	17.58e	20.76f
F 95.37	3.21a	4.02a	4.63a	1.22a	1.76a	2.25a	3.14a	4.84a	5.33a

POX=Peroxidase, PPO=Polyphenoloxidase and PAL=Phenylalanine ammonialyase.

*Values with the same letter in the same column and in the same family are not significant different ($P < 0.05$). Values are means of 3 replicates.

PAL activity in leaves of *T. cacao* showed significant variation ($P<0.05$) with time. The PAL activity increased in the genotypes F 90.34 (15.92%) and F 95.36 (12.18%) (Table 3). In the inoculated leaves, T 79/467 (tolerant parental clone) recorded the highest enzyme activity (11.89 nmol.min⁻¹.mL⁻¹ fresh weight), conversely, in susceptible clone (ISC 40) 8.31 nmol.min⁻¹.mL⁻¹ fresh weight (Table 3). However, the PAL activity of the higher susceptible genotypes F90.30, F95.37 and F90.32 was lower than that the ICS 40 clone.

Table 5. Correlation coefficients between tolerance tests and biochemical compounds (F90 and F95 populations).

	Nécrose	PPT	FT	POX	PPO	PAL
Nécrose	1					
PPT	-0,899**	1				
FT	-0,891**	0,971**	1			
POX	-0,923**	0,893**	0,944**	1		
PPO	-0,960**	0,914**	0,935**	0,967**	1	
PAL	-0,979**	0,842**	0,858**	0,926**	0,950**	1

PPT : Polyphénols totaux ; FT : Flavonoïdes totaux ; POX : Peroxydase ; PPO; Polyphénoloxydase, PAL : Phénylalanine ammonialyase

**. Correlation is significant at the 0.01 level (2-tailed);*. Correlation is significant at the 0.05 level (2-tailed).

Heritability and correlation coefficients

Narrow sense and broad-sense heritability estimates for necrosis character and biochemical parameters were obtained for the two populations from reciprocal crosses. The values obtained are the following: 0.231 for F90 (♀T79/467 × ♂ICS40) and 0.243 for F95: (♀ICS40 × ♂T79/467) concerning narrow sense heritability (Table 4). For broad-sense heritability, values obtained are 0.291 and 0.336, respectively, F90 and F95 (Table 4). However, heritability of the accumulated biochemical parameters were weak [PPO: (h^2 = 0.143 for F90 and h^2 = 0.214 for F95)

and PPT: (H^2 = 0.234 for F90 and H^2 = 0.266 for F30)] and high [FT: (h^2 = 0.533 for F90 and h^2 = 0.517 for F95) and POX: (H^2 = 0.859 for F90 and H^2 = 0.843 for F30)].

In our trials, a significant negative correlation is noticed between the necrosis length and polyphenol (r = -0.899, $P<0.01$), POX (r = –0.923, $P<0.01$), PPO (r = –0.960, $P<0.01$), and PAL (r = –0.979, $P<0.01$) (Table 5) in F90 and F95 populations. Besides, a significant positive correlation between all the studied biochemical parameters (F90 and F95 populations, $P<0.01$) were registered.

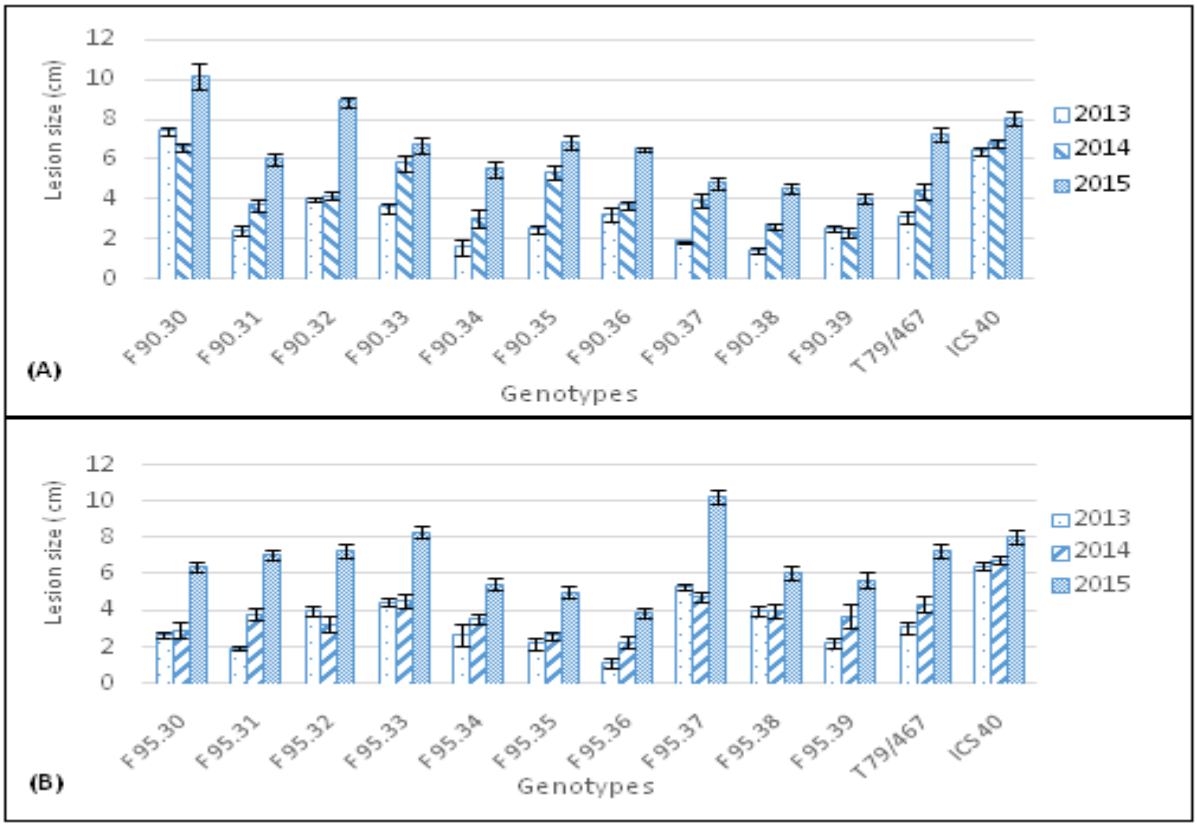

Fig. 1. Average lesion size (cm) on the midrib in two clones (T79/467 and ICS40) and their progenies F90 (A) and F95(B) of *T. cacao* L., 6 days after inoculation with mycelium of *P. megakarya* during 2012/2013, 2013/2014 and 2014/2015 cocoa seasons.

Principal component analysis (PCA)

A Principal component analysis (PCA) was used to visualize the variations in the genotypes during the development of necrosis and biochemical parameters as: TPP, TF, POX, PPO and PAL (Fig. 3). For the parental and hybrid genotypes the first two axes represented 96.68% of the total variability of the necrosis, total polyphenols, total flavonoids, peroxidase, polyphenoloxidase and phenylalanine ammonialyase. TPP, TF, POX, PPO and PAL were the dominant features in the first axis (92.52% of the total variability) while necrosis was the highest feature in the second axis (4.16% of the total variability). Examining a two-dimensional scores plot in the space defined by Principal components 1 and 2 showed that distribution of samples followed a pattern of four groups (Fig. 3). The first group composed of F 95.37 and F 90.30 was characterized by significant lesion size and the lowest amounts of the compounds analyzed.

The second group comprising four genotypes contained low amounts of POX, PPO and PAL, with the important lesion size. The third group embodied four genotypes (F 90.35, F90.34, F95.31 and T79/467) contained the lesion size less important

than that of the second [which comprised the susceptible parent (ISC40)]. Moreover, the fourth group was constituted only of tolerant genotype (F95.36) which differs from the others by their large tolerant to *P. megakarya* and their large amounts in biochemical parameters analyzed (TPP, TF, POX, PPO and PAL).

Discussion

In this study, we attempt to analyze heritability and to understand the role of metabolites in the *T. cacao* L./ *P. megakarya* interaction. For this purpose, the necrosis length and biochemical compounds (TPP, TF, POX, PPO and PAL) on leaves of the genotype (parental and hybrids) of *T. cacao* L. were determined.

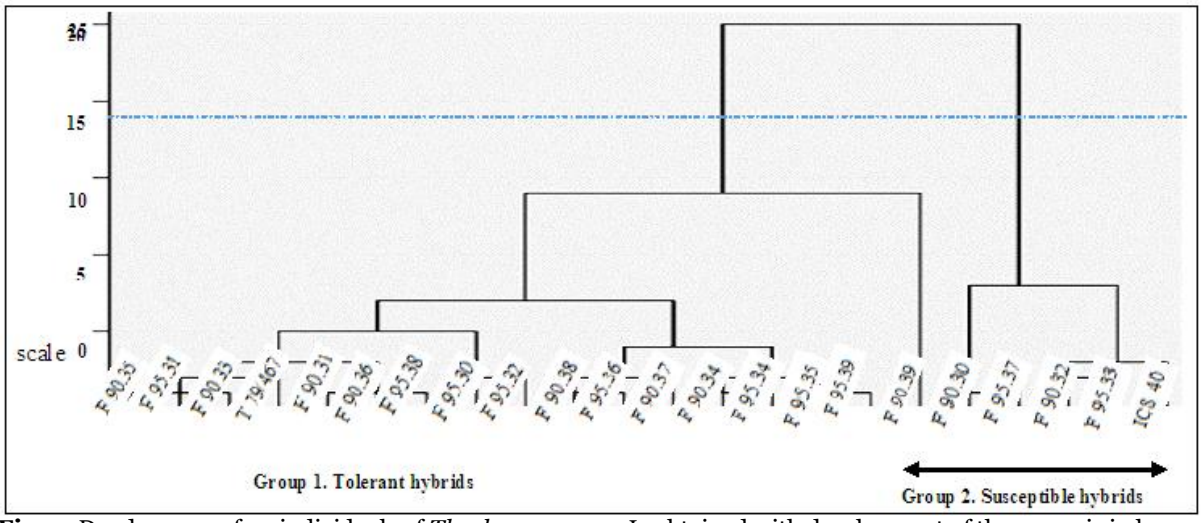

Fig. 2. Dendrogram of 22 individuals of *Theobroma cacao* L. obtained with development of the necrosis in leaves during three years cocoa season.

The production of parental and hybrid genotypes was achieved through hand pollination success (in 2012) and grafting (in 2012). The appearance of necrosis on the midrib of the leaves infected nursery and field, is due to the presence of the mycelium of *P. megakarya*. These results are in agreement with those obtained by Djocgoué *et al.* (2007) and Ondobo *et al.* (2014) on the leaves attached to the plant nursery. After three years of assessment in nursery and field, the necrotic development are less important in hybrid genotypes F90.34, F90.37, F90.38, F 90.39, F95.34, F95.35, F95.36 and F95.39. The hybrids produced from the two clones were more tolerant to *P. megakarya* than the best parent T79/467.

The development of necrosis (during the three cocoa season) in F90 and F95 hybrids displayed a positive heterosis for 80% and 90% populations respectively. So, this would suggest that these individuals demonstrate a manifestation of the hybrid vigor, which would imply the presence of the additive and dominant gene effect in the transmission of character (Cilas *et al.,* 1998; Djocgoue *et al.,* 2006).

Evaluation of biochemical markers of tolerance in the leaves (healthy, wounded, wounded and Inoculated) of *T. cacao* L., showed that the contents of total polyphenols and total flavonoids were found to differ significantly in tolerant and susceptible genotypes (genotype-dependence). Most plants synthesize toxic compounds such as phenols, proline and lignin during normal development, and their role in the resistance mechanism has been reported earlier by many authors (Ngadze *et al.,* 2012; Nyadanu *et al.,* 2013). These findings are in agreement with results reported by Koc and Ustun (2012) who showed an increase of phenolic content in leaves of susceptible and resistant Pepper (*Capsicum annuum* L.) plants on day 6 following infection. Also, others results indicate that the inhibitory effect of phenols on pathogen development depends on the level of these compounds in the plant tissue. Dogbo *et al.* (2008),

Simo *et al.* (2011), Djocgoue *et al.* (2011) and Ondobo *et al.* (2014) reported that highly susceptible clones of cocoa contain less phenolics. Among the total polyphenols groups found in cocoa, flavonoids represented about 70% (Di Mattia *et al.,* 2012). This class of secondary metabolite is known to be involved in plant-microbe interactions (Morandi, 1996). Some offsprings F 90.34, F 90.35 and F 95.37 showed an important increase in the amount of polyphenols and flavonoids in leaves at all different treatment (healthy, wounded, wounded and inoculated). Generally, the accumulation of phenolics following infection by a pathogen involves the neosynthesis of specific phenol compounds (Conceiçao *et al.,* 2006). Qualitative analysis of phenolics in leaves of cocoa showed a higher accumulation of some luteolin derivatives and apigenin derivatives (flavonoids) and some hydrocycinnamic acid derivatives (Djocgoue *et al.,* 2007). In the same way, the presence of unspecific fluorescence indicates the involvement of phenolic and flavonoids as host responses to infection (Shetty *et al.,* 2012). Hence, our study suggested that these compounds can be used as markers for selection of tolerant genotypes.

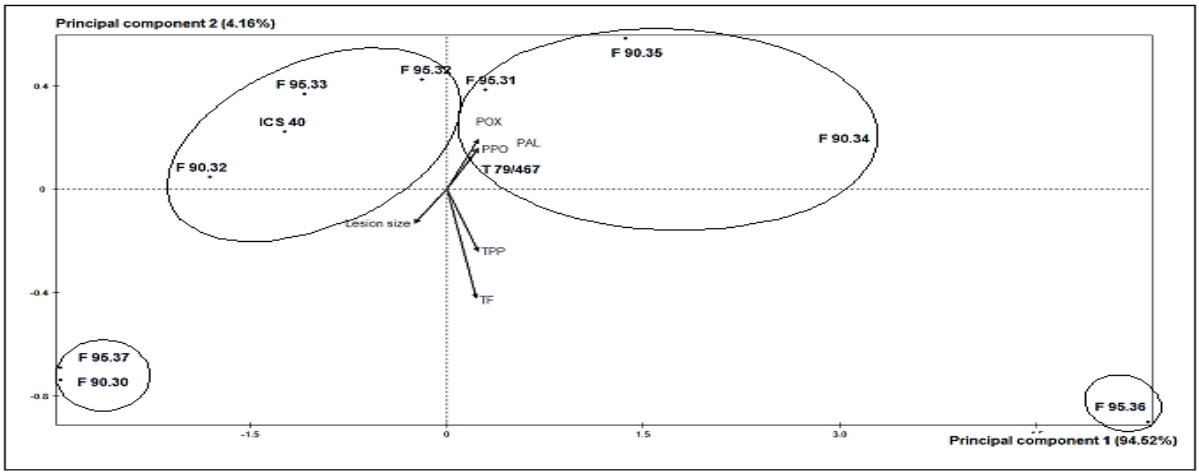

Fig. 3. Principal component analysis based on necrosis length, some secondary metabolites and activities of some oxidative enzyme from different cocoa genotypes (F90 and F95).

In this study, enzyme activities of POX, PPO and PAL were found to differ significantly in tolerant and susceptible genotypes. Activities of these three enzymes also increased significantly in wounded (W) and inoculated (WI) leaves. In our study, POX activity in the leaves of all genotypes was observed. Activity was notably higher in the genotypes F90.34, F90.35 and F95.36. The increase in POX activity indicates that it has a key role in local and systemic resistance (Delledone *et al.,* 2002). Also, the elevated activity of antioxidant enzymes could reduce membrane damage by eliminating reactive oxygen species (Unyayar *et al.,* 2010). The role of peroxidases has been cited in a number of defense mechanisms against the invading pathogens such as hypersensitive response (Levine *et al.,* 1994). Okey *et al.* (1997) reported significant higher enzyme activities in inoculated and wounded cocoa clones. These authors also reported that clones with higher enzyme activities exhibited resistance to *Phytophthora palmivora* infection. These compounds act as barriers against pathogen invasion and hence constitute part of host resistance mechanisms.

The PPO activity remains very important after infection of cocoa leaf with *P. megakarya*. High percentage of PPO activity was observed in tolerant genotypes as compared to the susceptible genotypes indicating their role in disease resistance. However, the PPO oxidizes phenols into highly toxic quinones and is hence considered to play an important role in disease resistance, particularly those affecting the tissues (Abbattista *et al.,* 1975). These results suggest the PPO appear to play a role in tolerance to soft rot since these compounds were present in considerably higher levels in tuber tissue of resistant varieties, as reported by Ngadze *et al.,* (2012). It was interesting to

note that PPO is involved in plant defense against pathogens by various actions. By their proteolytic activity, they accelerate cell apoptosis and restrict the growth of pathogens in infected organs (Kuwabara and Katoh, 1999). The activity of PAL in different leaves (wounded, and wounded and Inoculated) showed it implication in the development of systemic acquired reaction (SAR) in all genotypes. But, enhancement of PAL activity is genotype-dependent. Also, Oomycete infection results in *de novo* synthesis of PAL (Lawton *et al.,* 1983). The results confirm the findings of Ngadze *et al.* (2012) who stated that PAL catalyze the formation of cinnamic acid and therefore plays a role in plant defense systems by favoring the biosynthesis of polyphenols and lignin. For PAL to be effective, it must be available in potato tuber tissues in high concentrations at the initial stages of infection. Damodaran *et al.* (2002) reported that PAL is the first enzyme to signal the onset of Oomycete infection, even before the formation of the Oomycete hyphae of *Fusarium oxysporum*. This suggested the strong relation between the enzymes and resistance to this oomycete.

Narrow sense (h^2) and broad-sense (H^2) heritability of development necrosis (after three years) and biochemical factors were calculated between the parents and their progenies. Values of their heritability in the two reciprocal crossings were not significant.

Transmission of the character would thus seem to be governed by primarily additive heritability, which confirms previous studies (Nyasse *et al.,* 1995). Relationship was observed between the biochemical factors of parents and progenies. This findings suggested that the transmission of this character is not cytoplasmic but nuclear (Djocgoue *et al.,* 2011; Effa *et al.,* 2015). Nevertheless, selection based on family performance or progeny test should be more effective.

Spearman's correlation test showed that there is a significant negative correlation ($P<0.01$) between the character of development of necrosis and all the biochemical parameters. Nevertheless, a significant positive correlation ($P<0.01$) found between the PPT, FT, POX, PPO and PAL reflects the importance of the enzyme in resistance expression. However, these findings agree with of Ngadze *et al.* (2012), Ondobo *et al.* (2013) and Effa *et al.* (2015) observations who reported that tolerant genotype accumulates high amount of biochemical substances (PPT, FT, POX, PPO and PAL).

PCA based on the lesion size and biochemical parameters distinguished four groups in the F90 and F95 populations. With regard to the two reciprocal populations, PCA of genotypes revealed a diagonal opposition between the development of the necrosis and biochemical parameters in the tolerant and susceptible genotypes. This suggests that these compounds would be implicated in defense mechanism in *T. cacao*/*P. megakarya* interaction. Similar results were reported by Djocgoue *et al.* (2010) and Ondobo *et al.* (2013) after infecting cocoa pod and leaves.

Conclusion

In this study, necrosis length and biochemical parameters were found to differ significantly in tolerant and susceptible genotypes. Therefore, 16 hybrids and one parental clone (T79/467) were showed a tolerance to disease while four hybrids (F90.30; F90.32; F95.33 and F95.37) and ICS40 were susceptible to *P. megakarya*. Values of the heritability in the two reciprocal crossings were not significant. Transmission of the character would thus seem to be governed by primarily additive effect suggesting a nuclear origin of the transmission of these characters.

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