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Assessment of tolerance level to *Phytophthora megakarya* in four hybrid populations of *Theobroma cacao* L.

Pierre Effa Onomo[*1,3,4], Simon Perrez Akoa[1,3,4], Martine Louise Ondobo[1], Pierre François Djocgoue[1,2]

[1]*Department of Biological Sciences, University of Yaounde I, Cameroon*

[2]*Department of Plant Science, University of Yaounde I, Cameroon*

[3]*Department of Biochemistry, University of Yaounde I, Cameroon*

[4]*Biotechnology Center, University of Yaounde I, Cameroon*

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Abstract

In Cameroon, loss in cocoa seed production is mainly due to *Phytophthora megakarya* affections. The genetic improvement by controlled hybridisation is one of the methods attempted to minimize black pod disease caused by this fungus. The aim of this study is to generate resistant *P. megakarya* genotypes through the accumulation of resistant biomarkers. The fungi inoculations were done in the nursery on leaves of four hybrids and three parental clones. Necrosis length, total phenol content, flavonoids, flavan-3-ols, proteins, peroxidase and polyphenols oxidase activities were evaluated on the population under different treatment conditions (healthy, wounded ,wounded and inoculated). Necrosis length was more important in the F70 and F10 families, average in the F30 family and less significant in the F80 family six days after infection. The increasing sensitivity order of the parents based on the progression of the necrosis length is SCA12> T79/467> SNK413. Proteins and bioactive compounds content were genotype-dependent and a strong negative correlations between biochemical metabolites and the necrosis length ($P<0.05$) were found. Of the four reciprocal crossings realised, two (T79/467 x SCA12 and T79/467 x SNK413) showed a good suitability to the combinations. Also, some hybrids like F80.08, F80.05, F30.05, F30.01 F10.05, F10.06 F70.05 and F70.02 due to their low necrosis and high accumulation of fungicide compounds should be considered as tolerant. The heritability of traits to black pod disease doesn't show any significant difference suggesting the absence of maternal effect in the transmission of these traits.

* **Corresponding Author:** Pierre Effa Onomo ✉ peffafr@yahoo.fr.

Introduction

Cocoa is known since the Maya period in the South American continent and these people used cocoa in cultural and ritual ceremonies. It was also used as money. From the colonial period till now, cocoa is used in many industries. It is a major ingredient in the chocolate industry. According to its chemical composition, cocoa seeds have been reported important in agro alimentary, pharmacology, bioenergy and in cosmetics. Lass (2004) showed that over 6.000.000 tons of cocoa chocolate are produced a year for an average of four thousand cocoa seeds produced around the world. Africa participates up to 70% of the total world cocoa production. In Cameroon, cocoa represents the third exporting product behind banana and wood.

Over one million habitants of the forest zone gain their money by the commercialisation of cocoa seeds (Anonymous, 2002). The country produces over 232,000 tons of cocoa dried seeds from the year 2015 (Anonymous, 2015).

However, everywhere cocoa culture is done, the production encounters many difficulties among which the phythopathogenic attacks. In Cameroon, an oomycete named *Phytophthora megakarya* known as the very aggressive between the *Phytophthora* species can reduce the production by 80 or 100% of the total production if n measure is taken. Many authors have shown the implication of polyphenols after infection of cocoa leaves (Boudjeko, 2007; Ondobo and *al.*, 2014). Esnault and Chibbar (1997) had found a high concentration of proteins after tobacco leaves were attacked by the mosaic virus. On the other hand, cross breeding done between different cocoa parental clones had shown a hybrid population with a pronounced precocity and heterosis, reason why the use of hybrids had been generalised. The general objective of this study was therefore to cross cocoa clones between them and to select derived hybrids according to certain physiological and biochemical markers of resistance under different treatment conditions (healthy, wounded, wounded and inoculated).

Materials and methods

Plant and fungal material

Plant material was constituted of three different cocoa clones supplied by the SODECAO station of Mengang (Centre region of Cameroon). SCA12 tolerant and productive selected in Ecuador; SNK413 more tolerant and less productive selected at Nkoemvone (Cameroon) and T79/467 tolerant and very productive selected at Tafo (Ghana). Manual pollinisation was used to produce four reciprocal hybrid families: F10 (SCA12 x T79/467) and F80 (T79/467x SCA12); F70 (T79/467x SNK413) and F30 (SNK413x T79/467). Plantlets derived from hybrids were grown in the nursery while their parents were produced by grafting.

P. megakarya used was the strain « El_8» isolated at Eloumden-Mbankomo near Yaounde (capital of Cameroon). The fungi was purified and multiplied to improve aggressiveness using V8 medium (200g V8 vegetable juice, 3g Ca2CO3, 15g agar and 1000 mL distilled water) and incubated in the dark at 25 ± 2 °C at IRAD (Research Institute for Agricultural Development).

Leaf inoculation

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

Polyphenols essay

Phenolic compounds were extracted according to the method described by Hansen (1998) with some modifications.

Lyophilised leaves (150g) were ground in mortars with sand and incubated at 4°C with methyl alcohol 80° in mini spin eppendorf tubes for 20 min. After incubation, tubes were centrifuged twice at 10000g during 10minutes and the supernatant was collected each time. Mixture of the two supernatants constituted the soluble phenol compounds. Total phenol was quantified using the method described by Lachman *et al*. (1998). 100µl of crude extract was added to Folin-ciocalteu reagent (500µl), 3,5ml of distilled water and 1ml of sodium carbonate (20%). The mixture was incubated at 40°C for 20 minutes and the blue color was determined at 725nm. The content of soluble phenol was expressed in mg-equivalent of Gallic acid per lyophilised matter (LM).

Total flavonoids essay

The flavonoids were estimated as described by Roux *et al*. (1975) with some modifications. 100µl of methanol 80° extract was added to 1ml of formaldehyde. Both products were left to react for about 30 mins. The mixture was filtered and the supernatant obtained contained non-flavonoid compounds. 100µl of supernatant extract was added respectively to 0,5ml of Folin-ciocalteu reactant, 3,5ml of distilled water and 1ml of calcium carbonate 20%. The mixture was heated at 40° for 20 minutes, and optic densities were reported at 725nm after cooling. Gallic acid was used as standard and results were expressed in mg-equivalent of Gallic acid per lyophilised matter (LM).

Flavan-3-ol essay

The estimation of flavan-3-ol units was done as described by Di Stefano *et al*. (1989) with some modifications. 1ml of vanillin daily prepared was added to 100ml of methanol 80° cocoa leaves extract. After 15 min, 1.5ml of HCl (0.1N) was added. The mixture was reacted for 15 min in fresh water and optic densities (O.D) were shown at 500nm.

The flavan-3-ol units content was determined using this formula: (+)-catechin=$O.D_{sample}$-$O.D_{standard}$ and the result expressed in mg of catechin per gram of cocoa lyophilised leave powder.

Total protein essay

The total soluble proteins were extracted according to the method described by Lanaud (1986) with some modifications. Fresh leaves (150mg) were ground in mortars with 2ml of tris-maleate buffer (Tris base 0. 5 M, ascorbic acid 0.3M, EDTA (0.1% m/v), Triton x 100 (0.01% m/v), LiCl (17% m/v and pH = 7, 2) and Sand and polyvinyipolypyrridoline (PVP). After 40 min of incubation, the mixture was centrifuged at 6000g during 10 min at 0 to 4°C. The resulting supernatants were conserved at -20°C and were used as protein assays. Proteins of tissue extract (leaves) were quantified by the method described by Bradford (1976) using Bovine Serum Albumin (BSA) as the standard.

Assay of antioxidant enzyme activity

The Pox enzymatic activity was estimated spectrophotometrically using the method described by Rodriguez and Sanchez (1982), strongly modified. 1ml of phosphate-citrate buffer pH 7 was added to 1ml of guaiacol 40Mm and 0.5ml of H_2O_2 26mM. In this solution, 100µl of soluble proteins were mixed and the Pox activity was expressed at 420nm/min/g of lyophilised matter. Whereas PPO activity was estimated by the method described by Van kemmen and Broumer (1964) using cathechin as substrate. The PPO activity was expressed at 330 nm/min/g of lyophilised matter (LM).

Estimate heterosis and heritability

The hybrid vigor or heterosis was estimated using the formula shown by Zahour (1992). Expressed in percentage, Zahour's formula compares values of the hybrids (F1) with those of their two parents (P1 and P2).

$$HF_1(\%) = \frac{F_1 - \frac{P_1 + P_2}{2}}{\frac{P_1 + P_2}{2}} \times 100$$

Heritability is defined as the proportion or percentage of a character to be transmitted from parents to descendants, it is a quantitative parameter that can be used to select good yield varieties (Noubissie, 2002). Its formula was proposed by Falconer (1974) and values are found between 0 (no character transferred) and 1 (all characters transmitted).

Statistical analysis

The results were analysed by analysis of variance (ANOVA) followed by Tukey HSD at P=0.05 to compare means 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. Pearson's correlation tests between the different parameters were also undertaken.

Results

Physiological parameters

Hand-pollinisation and grafting

Table1 shows the results obtained and the percentage of success of manual pollinisation and grafting. F30 family displayed the lowest level of pollinisation success compared to other families while the parent SCA12 showed the lowest percentage of success of grafting.

Table 1. Average success of hand-pollinisation and grafting.

	Families	Crossing (or genotype)	Number	Success
Hand-pollinisation (hybrid families)	F80	T79/467(♀) x SCA12(♂)	150	8.66
	F10	SCA12(♀) xT79/467(♂)	150	10.66
	F70	T79/467(♀) x SNK413(♂)	172	11.04
	F30	SNK413(♀) x T76/467(♂)	175	1.71
	N°	Parents		
Grafting (parents)	1	SCA12	100	94
	2	T79/467	100	90
	3	SNK413	100	88

Evolution of the necrotic lesion size

The results have showed a response of parental and hybrid genotypes to *P. megakarya* 2 days after inoculation in the four families and three parents considered for the experiment.

The necrosis continued the same evolution till the sixth day. In a general view, the parents SCA12 and T79/467 and the families F10 and F70 had presented a high necrotic lesion size. The reverse effect was observed in the reciprocal hybrids F80 and F30. The parent SNK413 had presented a small necrotic lesion size compared to the two others. Six days after inoculation, F70.04 had shown the highest length compared to the other (6.73cm) hybrid families. On the other hand, the parent SCA12 showed 7.13cm necrotic lesion size (Table 2).

At 95% of homology, direct hierarchical classification of necrotic lesion size in inoculated leaves at days two, four and six differentiated into two groups for the F80 and four for F10 families respectively (Data not shown).

The first group of the F80 family contained only the hybrids of this family (8 individuals) characterised by small lesion sizes while the second group, was constituted by the two parents with high lesion sizes. In the F10 family, Group 1 was composed of three hybrids with small necrotic lesion size. Group 2 was composed of the two parents. Group 3 was constituted of two hybrids with average necrotic lesion size and the last also composed of two individuals which had shown a large necrotic lesion size.

Dendograms of F30 and F70 families presented 4 groups for each family. In F30, two groups were composed of one hybrid, F30.06 and F30.04 respectively for groups 2 and 4.

The first group was constituted only of hybrids whereas the third was composed only of parents. Group 1 and group 4 were constituted of hybrid genotypes, whereas group 3 was formed by one individual the parent T79/467. Group 3 was mixed between two hybrids and the parent SNK413 (Data not shown).

Table 2. Average necrotic lesion (cm) size in families (F80 and F10; F70 and F30) and the Heterosis (%) values.

	Day 2		Day 4		Day 6	
Genotypes	Necrosis(cm)	Heterosis (%)	Necrosis (cm)	Heterosis (%)	Necrosis(cm)	Heterosis (%)
F80						
F80.01	0.63±0.15^{a}	-52.63	2.33±0.15^{b}	+12.83	3.80±0.30^{c}	-43.53
F80.02	0.73±0.11^{ab}	-45.11	1.33±0.15^{a}	-35.59	2.53±0.35^{ab}	-62.40
F80.03	0.63±0.11^{a}	-52.63	1.26±0.20^{a}	-38.98	2.06±0.25^{a}	-69.39
F80.04	0.60±0.17^{a}	-54.88	1.36±0.11^{a}	-34.14	2.63±0.20^{ab}	-60.92
F80.05	1.13±0.15^{bcd}	-15.03	1.63±0.40^{a}	-21.06	2.90±0.17^{b}	-56.90
F80.06	0.80±0.00^{abc}	-39.85	1.63±0.28^{a}	-21.06	2.96±0.20^{b}	-56.01
F80.07	0.56±0.11^{a}	-57.89	1.46±0.20^{a}	-32.20	2.86±0.20^{b}	-57.50
F80.08	0.70±0.10^{ab}	-47.36	1.53±0.25^{a}	-25.90	3.06±0.15^{bc}	-54.23
F10						
F10.01	0.96 ± 0.20^{abcd}	-27.82	3.83±0.28^{abc}	+85.47	4.73±0.25^{ab}	-29.72
F10.02	1.26± 0.15^{bc}	-5.26	4.00±0.20^{c}	+93.70	4.96±0.20^{bcd}	-26.30
F10.03	0.53±0.05^{a}	-60.15	1.20±0.17^{a}	-41.89	2.36±0.20^{a}	-64.93
F10.04	2.06±0.20^{e}	+54.89	3.16±0.28^{bc}	+53.03	4.40±0.36^{abc}	-34.62
F10.05	2.10±0.20^{e}	+57.89	2.63±0.30^{abc}	+27.36	3.90±0.26^{ab}	-42.05
F10.06	0.83±0.15^{abc}	-37.59	1.60±0.36^{ab}	-22.52	2.96±0.20^{ab}	-56.02
F10.07	0.66±0.15^{ab}	-50.38	1.46±0.20^{a}	-29.30	2.66±0.30^{a}	-60.48
F70						
F70.01	0.63±0.15^{ab}	-47.93	2.43±0.30^{bc}	+23.66	6.63±0.37^{d}	+9.32
F70.02	1.16±0.30^{bcd}	-4.13	2.03±0.15^{abc}	+3.31	3.70±0.45^{a}	-38.99
F70.03	0.53±0.15^{a}	-56.20	1.230.25^{a}	-37.40	4.26±0.15^{ab}	-29.76
F70.04	0.50±0.17^{a}	-58.68	2.83±0.50^{c}	+44.02	6.76±0.37^{d}	+11.46
F70.05	0.80±0.17^{abc}	-33.88	2.70±0.20^{c}	+37.40	5.46±0.41^{cd}	-9.98
F70.06	1.33±0.25^{cd}	+7.44	2.46±0.35^{bc}	+25.19	5.33±0.30^{bc}	-12.12
F30						
F30.01	0.50±0.00^{ab}	-58.67	0.86±0.15^{a}	-56.23	2.26±0.25^{a}	-62.73
F30.02	0.50±0.20^{ab}	-58.67	2.73±0.25^{cd}	+38.93	3.70±0.20^{b}	-38.99
F30.03	0.30±0.10^{a}	-75.20	2.80±0.26^{d}	+42.49	3.56±0.11^{b}	-41.30
F30.04	0.66±0.20^{ab}	-145.45	2.93±0.20^{d}	-50.89	4.63±0.23^{c}	-123.66
F30.05	0.66±0.05^{ab}	-45.45	2.93±0.20^{d}	+49.10	4.70±0.26^{c}	-22.50
F30.06	0.80±0.17^{ab}	-33.88	1.73±0.25^{b}	-11.95	3.93±0.20^{bc}	-35.20
SCA12	1.20±0.20^{cd}	/	2.33±0.28^{b}	/	7.13±0.35^{e}	/
T79/467	1.46±0.30^{d}	/	1.80±0.20^{ab}	/	6.33±0.41^{d}	/
SNK413	0.96±0.20^{abcd}	/	213±0.23^{bc}	/	5.80±0.55^{cd}	/

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

Heterosis

The values of heterosis concerning biochemical parameters are presented in the corresponding table (Table 3). Three hybrids (F10.04, F10.05 and F70.06), displayed at day 2 a positive heterosis concerning the necrotic lesion size. This hybrid vigor went up to 4 and disappeared only at day 6. Some other genotypes displayed a positive heterosis at day 4.

At day 6, the entire values of heterosis became negative testifying the correlation between the necrotic surface and the resistance of plants.

Biochemical parameters

Variation of the phenol content

In more than 97% of genotypes, the wounding induced the accumulation of total phenol compounds in leaves.

The values altered between 12 and 36mg/g. The accumulation was very significant in F80 and F30, average in F10 and low in F70 and in parents except parent SNK413 (Fig. 1 A and B).

Variation of flavonoids content

Flavonoids content displayed variability of results according to the genotype and the family (Fig. 1 C and D).

The two reciprocal families F30.02 and F10.02 presented high amounts of flavonoid (5.72 and 9.88mg/g respectively). In the meantime, F30.01 displayed the least flavonoid content (1.08mg/g). Notably, the wounding and the inoculation went up with an accumulation of flavonoids in the cocoa leaves.

Table 3. Estimation of heterosis (%) of some biochemical parameters during the experiment.

	Hybrid vigor (Heterosis %) of biochemical markers								
	Day 2			Day 4			Day 6		
	TPP	TF	FL-3-ol	TPP	TF	FL-3-ol	TPP	TF	FL-3-ol
F80									
F80.01	+118.1	+206.67	+1594.83	+113.89	+220.99	+623.91	+102.00	+163.80	+515.66
F80.02	+117.9	+178.92	+1493.10	+128.24	+177.82	+821.74	+121.92	+198.94	+658.51
F80.03	+132.8	+173.33	+800.00	+122.50	+164.53	+673.91	+102.14	+141.27	+370.45
F80.04	+79.46	+143.42	+1281.03	+109.78	+128.98	+660.14	+114.41	+161.46	+561.45
F80.05	+74.92	+201.80	+1610.34	+117.77	+183.94	+837.68	+104.34	+179.98	+395.11
F80.06	+109.0	+211.89	+1755.17	+122.11	+182.15	+832.61	+133.64	+147.18	+390.02
F80.07	+111.3	+207.93	+1262.07	+134.37	+164.23	+712.32	+113.58	+127.66	+398.63
F80.08	+122.7	-24.32	+331.03	+127.15	+180.66	+978.99	+122.20	+164.58	+429.16
F10									
F10.01	+35.11	-9.01	+322.41	+38.71	-2.32	+160.14	+33.40	+26.27	+163.80
F10.02	+57.25	+109.19	+874.14	+40.50	+63.70	+331.88	+23.96	+17.68	+121.14
F10.03	+31.85	+47.57	+600.00	+39.10	+55.04	+306.52	+45.19	+54.16	+231.90
F10.04	+34.87	+58.92	+641.38	+48.80	+68.33	+341.30	+42.02	+58.73	+253.03
F10.05	+9.63	-43.06	+444.83	+47.48	+70.58	+108.70	+42.50	+46.35	+209.98
F10.06	+43.95	+11.71	+520.69	+43.21	+54.74	+308.70	+35.19	+64.86	+277.69
F10.07	+44.51	+16.94	+456.90	+44.69	+56.09	+247.10	+43.19	+54.16	+205.28
F70									
F70.01	+9.11	+39.48	+625.23	+76.03	+129.55	+399.53	+27.26	+27.28	+199.13
F70.02	+27.03	+86.07	+702.70	+81.82	+140.48	+263.85	+23.34	+46.31	+240.02
F70.03	+19.29	+10.29	+894.59	+44.50	++78.43	+156.81	+11.72	+31.94	+123.05
F70.04	+35.93	+72.76	+645.05	+77.79	+138.66	+296.71	+30.56	+50.77	+196.65
F70.05	+51.88	+113.42	+810.81	+50.81	+133.61	+283.57	+46.44	+65.00	+48.95
F70.06	+9.63	-9.68	+554.95	+64.44	+69.33	+348.83	+24.49	+63.74	+256.88
F30									
F30.01	+37.21	+30.67	+273.87	+97.07	+150.56	+229.11	+64.28	+70.46	+103.72
F30.02	+48.79	+35.38	+217.12	+101.98	-10.50	+246.48	+70.92	+87.76	1+18.09
F30.03	+46.41	-10.91	+109.01	+112.61	+70.03	+59.62	+69.34	+34.00	+9.85
F30.04	+48.68	-18.78	+82.88	+116.06	+99.02	+286.85	+51.03	+72.26	+124.54
F30.05	+73.41	+44.70	+114.41	+97.51	+129.55	+108.45	+44.38	+63.27	+165.18
F30.06	+52.17	+65.90	+384.68	+99.27	+161.90	+140.85	+42.85	+30.01	+188.97

TPP: Total Polyphenols, TF: Total Flavonoids, FL-3-ol: Flavan-3-ol.

Variation of flavan-3-ols content

Parents had shown very low content of flavan-3-ol units. F10.04 (9.02mg/g) presented the highest content of flavan-3-ol units in F10 hybrid. The accumulation of flavan-3-ol was highly observed in F80, reciprocal hybrids of F10. F80.02 showed the highest content of flavan-3-ols (19mg/g) four days after inoculation.

In the reciprocal families F30 and F70, F70.02 and F70.06 had presented the highest amount of flavan-3-ols (11.95 and 12.10mg/g respectively). Remarkably, F70 contained a higher amount of flavan-3-ol units than its reciprocal F30. These results were in contrast concerning the polyphenols and flavonoid contents (Fig. 1 E and F).

Variation of protein content

Among the four families chosen, the family F30 had good results according to the protein content six days after inoculation. On the contrary, in the healthy leaves F70 presented the highest protein content up to 4mg/g. As polyphenols or flavonoids, the wounding and the inoculation were accompanied by a high accumulation of proteins in all the genotypes experimented (Fig. 2 A).

Pox and PPO activities

The peroxidase activities varied according to the genotype as recently observed in proteins. Except for the F70.05 hybrid, abiotic and biotic stress are characterized by an increase in Pox activity for all hybrids. This increase reached 370% in F80.08 six days after inoculation.

Table 4. Correlations between the different parameters experimented.

F30	Necrosis	PPT	FT	Fla-3-ol	Pox	PPO
Necrosis	1					
PPT	-0,842**	1				
FT	-0,646	0,861**	1			
Fla-3-ol	-0,540	0,606	0,648	1		
Pox	-0,931**	0,802*	0,516	0,257	1	
PPO	-0,847**	0,757*	0,755*	0,415	0,750*	1
F70	Necrosis	PPT	FT	Fla-3-ol	Pox	PPO
Necrosis	1					
PPT	-0,075	1				
FT	-0,287	0,916**	1			
Fla-3-ol	-0,252	0,412	0,585	1		
Pox	-0,660	0,405	0,613	0,646	1	
PPO	-0,511	0,213	0,209	0,519	0,735*	1
F80	Necrosis	PPT	FT	Fla-3-ol	Pox	PPO
Necrosis	1					
PPT	-0,931**	1				
FT	-0,922**	0,931**	1			
Fla-3-ol	-0,858**	0,899**	0,944**	1		
Pox	-0,974**	0,900**	0,942**	0,891**	1	
PPO	-0,641*	0,704*	0,510	0,767	0,574	1
F10	Necosis	PPT	FT	Fla-3-ol	Pox	PPO
Necrosis	1					
PPT	-0,852**	1				
FT	-0,905**	0,816**	1			
Fla-3-ol	-0,867**	0,936**	0,919**	1		
Pox	-0,723*	0,663	0,600	0,694*	1	
PPO	-0,573	0,296	0,560	0,455	0,407	1

Concerning PPO activities, they had shown an evolution of effect considering the three states experimented. A small exception was observed in parent T79/467 where

PPO activity did not significantly changed after wounding and inoculation. F70.05 and F10.05 had shown the highest and least variation of polyphenol oxidase activities respectively (Fig. 2 B and C).

Table 5. Heritability values of physiological and biochemical parameters experimented.

Hybrid families	Heritability values (h^2)			
	Necrotic lesion size	Polyphenols content	Flavonoids content	Flavan3ol content
F80	0.46	0.75	0.79	0.81
F10	0.40	0.65	0.74	0.87
F30	0.80	0.35	0.46	0.84
F70	0.91	0.26	0.55	0.93

Correlations between the different studied parameters

In this part, we observed significant correlation in the four families studied.

The significant correlation values for physiological and biochemical parameter was observed in the family F70. A strong correlation between physiological and biochemical parameters is also observed in other families (Table 4).

Heritability

The values of the heritability (h^2) have been determined according to the size of lesion, the phenol content, the flavonoids content and the flavan-3-ol contents (Table 5).

Discussion

The general objective of this present work was to select cocoa hybrids according to their physiological (necrotic lesion size) and biochemical (proteins and polyphenols) parameters in the fight of the plant against black pod disease caused by an oomycete (*Phytophhora megakarya*).

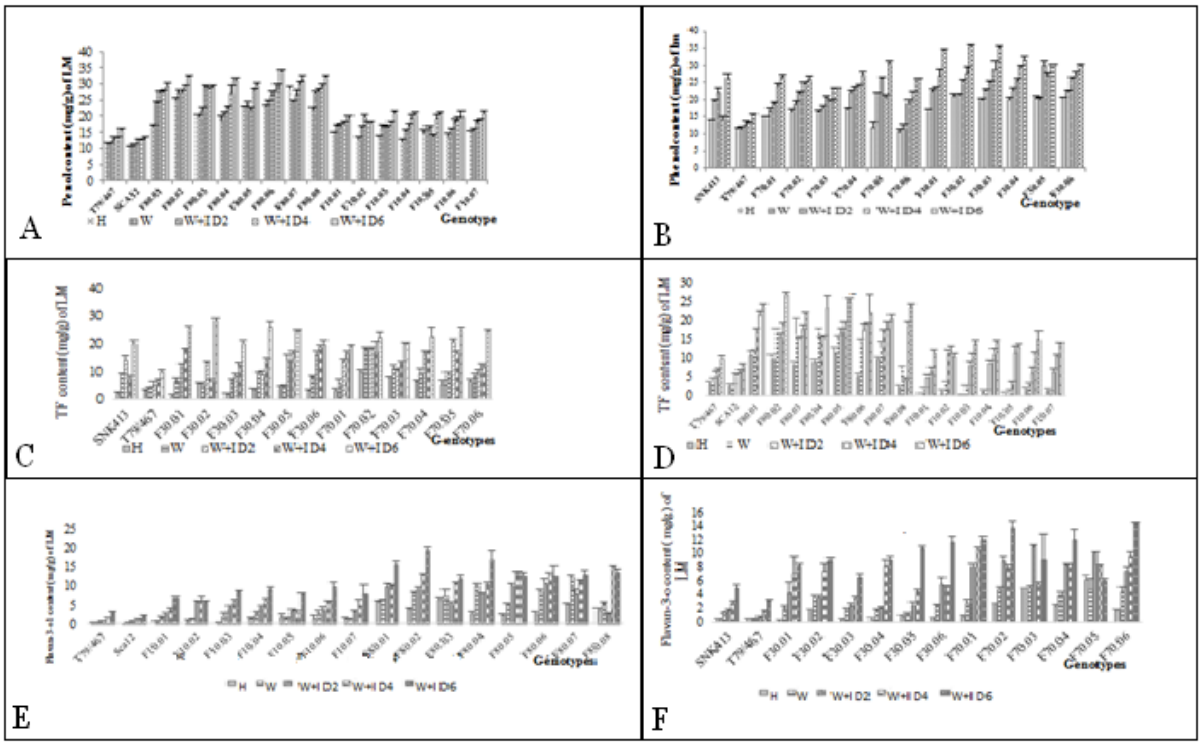

Fig. 1. Variations of phenol (A and B), Flavonoids (C and D) and flavan-3-ol content in three conditions of teatment.

The different cross breedings done between the three parents showed 5% success results. These values were analog with those obtained by Lachenaud and Mossu (1985). In fact, these authors demonstrated that one cocoa tree can produce over 125 flowers a year and surprisingly only 5% of these flowers grew into fruits. We obtained over 97% of cocoa seeds germinated. This great percentage was obtained by the maturity of seeds and the good quality of the soil used. Over 90% of grafting plants were obtained. This confirmed the interest and the advantages of lateral grafting over the others.

The brown coloration obtained two days after wounding of leaves may be caused by a structural reorganisation of plant tissues. Indeed, Djakou (2004) showed that wounding is followed by a reorganisation of plant tissues expressed by tuberisation and lignification effects. Necrotic lesion size had been observed 48 hours after inoculation of the fungal mycelia, confirming that this duration is necessary for the interaction between the two entities and the beginning of resistance of cocoa tissues.

The same results were being described by Nana (2011) in the evaluation of the necrotic lesion size in cocoa fruit (SNK10 and SNK413) cortex. The same results were reported by Ondobo *et al.* (2014) using leaves of cocoa plantlets in the nursery.

The accumulation of protein and polyphenol compounds in the wounding and moreover in the inoculation state described the important role played by this category of compounds in the resistance of cocoa against the fungal infection. The antifungal role of polyphenol compounds was reported by Fattouch *et al.* (2008). In fact, this author and his team illustrated the fungicide and/or microbicide action of the plant extracts caused by these compounds on the evolution of mackerel (scomber scombrus). The accumulation of proteins in case of plant attack was described by Esnault and Chibbar (1997).

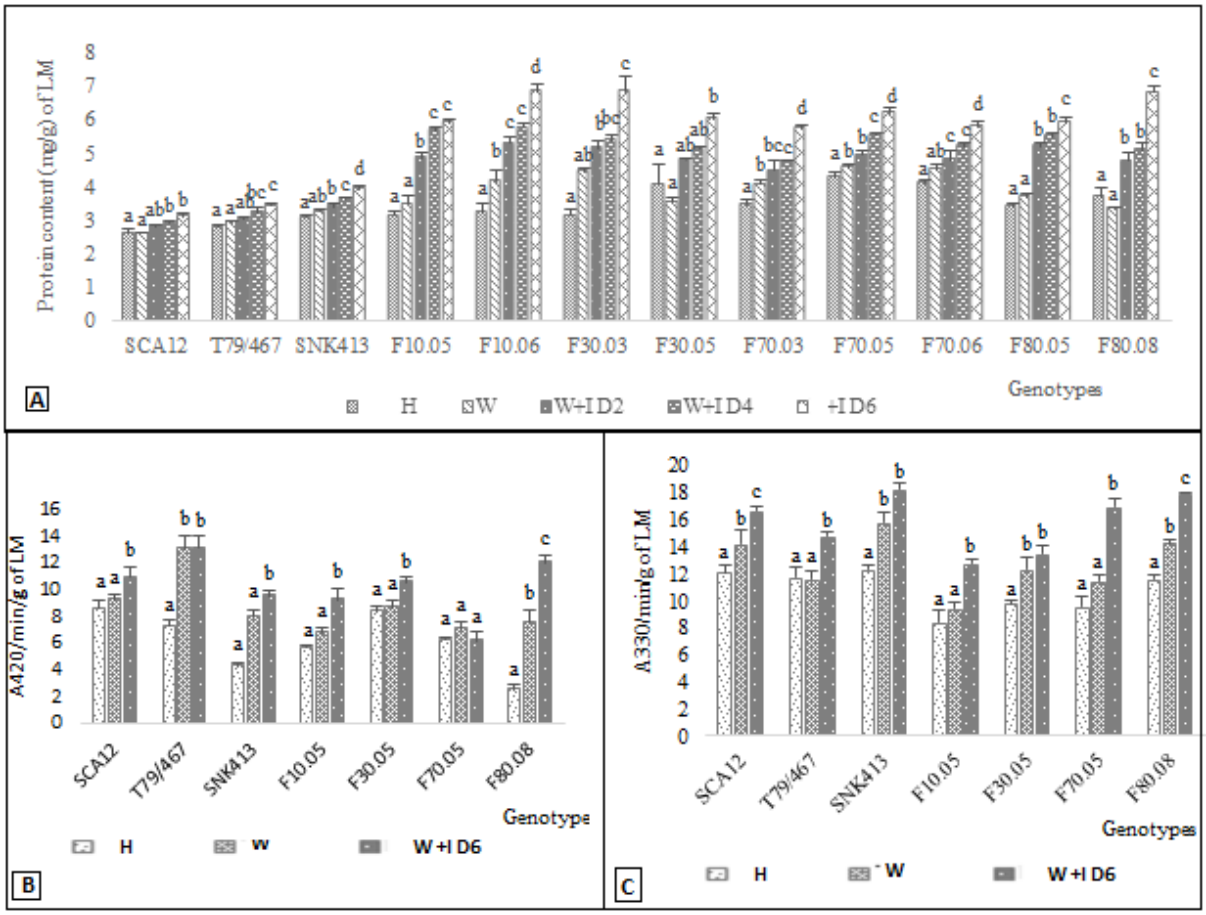

Fig. 2. Variation of protein content (A), Pox (B) and PPO (C) activities during the period and conditional state of cocoa leaves.

These authors showed the accumulation of proteins in tobacco leaf tissues affected by the mosaic virus. Proteins can be hydrolysed to form certain amino acids with antifungal role (proline, cysteine). The amino acids issued can be involve in the formation of others plant protective compounds (sugar, lipids). The polyphenols are present in a healthy plant (phytoanticipines) or are synthesized in case of infection (phytoalexines). Their accumulation in *Xanthosoma sangittifolium* tissues was also reported by Boudjeko and Omokolo (2005).

Other proteins act as activators of metabolic reactions in this point of view, are accumulated (polyphenols oxidase and peroxidase) in different tissues after abiotic and biotic stress. These different results were in harmony with Yao *et al.* (1995) hypothesis in which the modification in polyphenol content is an indicator of a susceptible infection or a stress state in plants. In this study, an important accumulation of polyphenols and proteins was observed in hybrid families compared to their parents.

This result agreed with those obtained by Cilas *et al.* (1991) which stipulated that hybrids may contain genes with additive effects in the transmission of a character.

Flavonoids and flavan-3-ols increased in the leaves till the sixth day. This accumulation of flavonoids supported the assertion that these compounds are a major class of polyphenols involved in the resistance of cocoa against *P. megakarya*. Among these flavonoids, flavan-3-ol units are a major class of them concerning the interaction of cocoa against stress.

In a global point of view, the highest content of biochemical markers appeared six days after wounding and inoculation. This duration is therefore considered as maximal to quantify the total compounds mobilised by plants to fight stress.

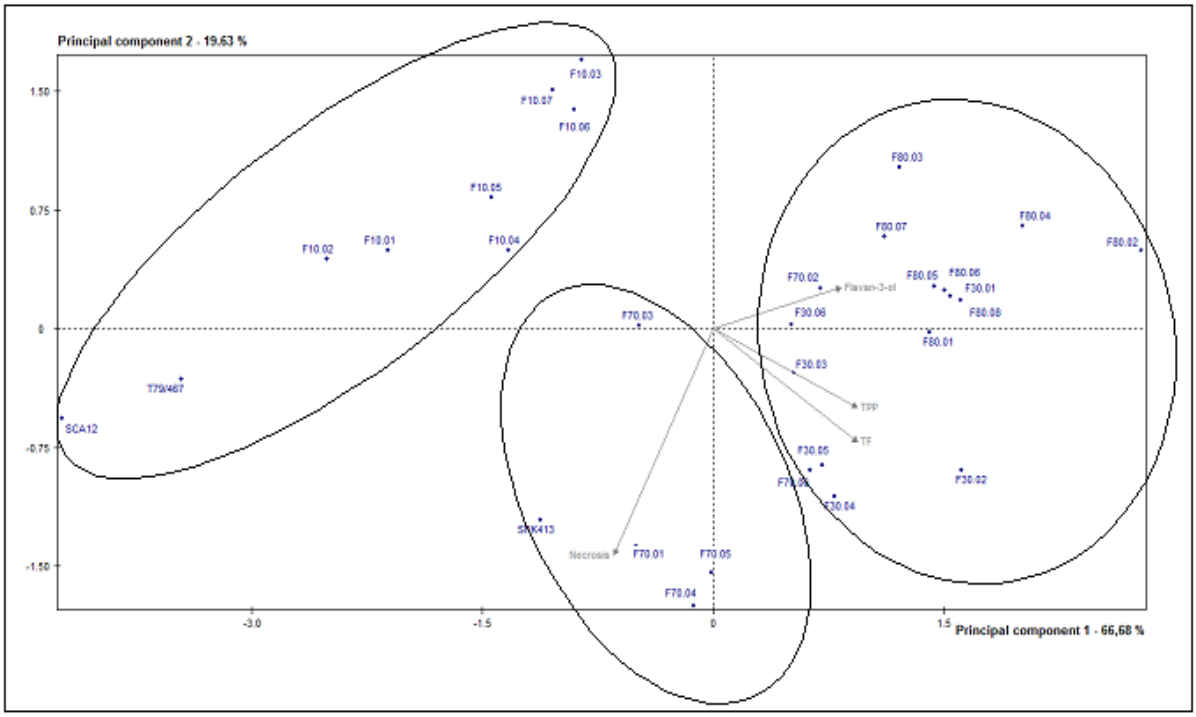

Fig. 3. Loading plot of necrosis, polyphenols, flavonoids and flavan-3-ols.

100% of hybrids of F80, F10 and F70 presented positive values of hybrid vigor; 83.33% of F30 hybrids presented a positive Heterosis of different parameters. This demonstrated the resistance of hybrids compared to their parents from which they originated. These results confirmed the hypothesis formulated by Djocgoue *et al.* (2006): the cross breeding done between parental clones in need of genes involved in the resistance against diseases is accompanied by hybrids characterised by a high vigor and a pronounced precocity.

According to classification of Lynch and Walsh (1998), heritability is considered as low ($h^2 < 0.1$), moderate ($0.1 < h^2 < 0.3$) or high ($h^2 > 0.3$).

Looking for this classification, 93.4% of our results showed a high heritability and 6.6% an average heritability. The same observations were described by Manga (2016) in the same plant. No significant difference was observed in reciprocal families for the parameters studied, suggesting a nuclear origin in the transmission of these characters.

Conclusion

The aim of this study was to generate resistant *P. megakarya* genotypes through the accumulation of resistant biomarkers. The increasing sensitivity order of the parents based on the progression of the necrosis length is SCA12> T79/467> SNK413

Of the four reciprocal crossings realised, two (T79/467 x SCA12 and T79/467 x SNK413) showed a good suitability to the combinations. Some hybrids like F80.08, F80.05, F30.05, F30.01 F10.05, F10.06 F70.05 and F70.02 due to their low necrosis and high accumulation of fungicide compounds should be considered as tolerant.

The heritability of traits to black pod disease doesn't show any significant difference suggesting the absence of maternal effect in the transmission of these traits.

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