Polyphenol oxidase and peroxidase analysis in newly selected clones of *Theobroma cacao* L. after inoculation with *Phytophthora megakarya* Bra and Grif.

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ABSTRACT

Cacao (*Theobroma cacao* L.) faces many pathogen attacks. The selection of tolerant clones is the major aim of mostcacao improvement programmes. Fifteen international clones were rated for their tolerance vis-à-vis *P. megakarya* vig: PA107, PlayaAlta$_2$, MXC67, SNK608, SNK607, SNK619, SNK602, NA33, AMAZ$_{15-15}$, UF676, UPA143, SPEC54-1, SCA24, IMC60, EQX3360. Based on the evolution of the necrosis area after inoculation of leaves with *P. megakarya*. The fifteen clones were classified as follows from high susceptibility to the less one: Playa Alta$_2$, MXC67, SNK608, AMAZ$_{15-15}$, NA33, SNK607, UF676, UPA143, SPEC54-1, SCA24, SNK619, IMC60, SNK602, EQX3360, PA107. The peroxidase and polyphenol oxidase activities were analysed in Playa Alta$_2$, UPA143 and PA107. Soluble peroxidase activity in inoculated leaves increase in the three clones. The increase was higher in the clone Playa Alta$_2$ than in clones UPA143 and PA107. At the same time, polyphenoloxidase activity, in 5-day old inoculated leaves increased by 92 % and 47 % in clones PA107 and UPA143 respectively, no change was observed in clone Playa Alta$_2$. Native polyacrylamide gel revealed the presence of five isoperoxidases (A_1, A_2, A_3, A_4, A_5), where A_4 was observed only in the clone UPA143. On the other hand polyphenoloxidase electrophoresis displayed three bands (B_1, B_2 and B_3) with B_2 specific to clone UPA143.

Key words: Peroxidase, Polyphenoloxidase, *Phytophthora megakarya*, *Theobroma cacao* L, tolerance,.

INTRODUCTION

Cacao, *Theobroma cacao* L. originated in the tropical rainforest of equatorial America. It is now cultivated in all tropical low lands of the world. Cacao production is affected by three main constraints: disease; cultivation of varieties and the age of plantation of which about 80 % are with low productive potential more than 40 years old (Lopez-Baez et al., 1998).

The increase of cacao production through breeding for yield and resistance to disease is dependent on the availability of suitable genetic variability in the germplasm (Crouzillat et al., 2000). Nevertheless different research institutes have developed programmes aimed at increasing yield, improving disease resistance and seed quality (Paulin and Eskes, 1995). Many genotypes of cacao have been selected and some are transfered to Cameroon for trials. So it is important to emphasize that the selection of cacao clones for black pod disease resistance must be directed to the areas where they will be planted and using the species of *Phytophthora* that occur there.

Many plant enzymes are involved in defence reactions against plants pathogens. Theses include oxidative enzymes such as peroxidase (POX) and polyphenol oxidase (PPO), which catalyse the formation of lignin and other oxidative phenols that contribute to the formation of defence barriers for reinforcing the cell structure (Avdiushko et al., 1993).

This study was conducted to evaluate the tolerance of newly selected clone to *P. megakarya* using detached leaves. The relationship between POX and PPO activities and cacao clone susceptibility vis-à-vis *P. megakarya* was also investigated.

MATERIALS AND METHODS

Cocoa and fungus materials. The fifteen international selected clones used in this study were obtained from a field plot at Institut de Recherche Agricole pour le Développement (I.R.A.D.). These clones were : PA107, Playa $Alta_2$, MXC67, SNK608, SNK607, SNK619, SNK602, AMAZ15-15, NA33, UF676, UPA143, SPEC54-1, SCA24, IMC60, IQX33-60. One month old leaves were used.

The isolate of *P. megakarya* used was the strain L_2C_2 isolated from the Yaoundé area and obtained from the Microbiology Laboratory at IRAD. The fungus was routinely cultured in a PDA (Potatoes Dextrose Agar) medium.

Artificial inoculation. Harvested leaves were washed thoroughly with tap water and blotted dry with Whatman N° 3 paper. They were then surface sterilized with ethanol 70° and conditioned for 24 hours in trays under saturating humidity. Leaves of each clone were scarified at the centre of the lower face along the midrib and inoculated by deposition of a mycelium disc of *P. megakarya* obtained from 7 day old culture. The leaves were labelled and place in trays containing humidified foam to maintain 100% relative humidity. They were sealed airtight and incubated in darkness at 25±1°C.

Assessment. Disease severity was rated by comparing necrotic lesion length and by the state of necrosis on leaf surface. Observation was made at day 5 after inoculation to describe and monitor symptom development on a basis of 0-5.

0 : No necrosis (NO)
1 : Spot at penetration point (PP)
2 : Point with some connections (PC)
3 : Reticulated necrosis (RN)
4 : Marbled necrosis (MN)
5 : True necrosis (TN)

Material For Biochemical Analysis. Out of our classification, three cocoa clones were chosen : Playa $Alta_2$ (high susceptibility); UPA 143 (susceptible); PA 107 (less susceptible), for enzymatic analysis.

For each clone, leaves were divided into three lots of ten leaves each. Lot I with intact leaves, Lot II with leaves inoculated with sterile agar (wounded), and Lot III with leaves inoculated with fungal cultures on agar (inoculated).

Preparation of crude enzyme extracts. At days 0, 1, 2, 3, 5 after inoculation, leave samples were collected at 1 cm beyond the necrosis for biochemical analyses. For soluble protein extract, 3 g of frozen tissue were ground in acetone, in a pre-chilled mortar, at 4°C and filtered under a reduced pressure. The resulting powder was homogenised in 3 ml of 50 mM Tris-maleate buffer pH 7 containing 0,5 M mannitol. The homogenate was centrifuged at 10000 g for 20 min and the supernatant was used for enzyme determination.

Enzyme assay and protein determination. Protein concentration were determined by the Coomassie Blue dye binding method of Bradford (1976) using Bovine Serum Albumin as standard. Guaiacol-peroxidase activity (incubation mixture : 7,8 cm^3 of 1/15 M phosphate buffer, pH 6,1 + 1 cm^3 H_2O_2 + 1 cm^3 1% guaiacol + 0,2 cm3 enzyme) was measured at

25°C by recording absorbance at 420 nm using a $°_{420}$ = 26.600 M^{-1} for oxidation products after 5 min. For catechin-polyphenol oxidase activity the reaction was initiated by addition of 20ìl of enzyme extract in 3 ml of reaction medium (0.1M Tris-acatate buffer pH 5.0 + 0.1 mM catechin). Catechin oxidation was monitored by increase in absorbance at 390nm using a $å_{390}$=4,600 M^{-1} cm^{-1} for the oxidation products after 30 seconds (Lopez-Serrano et Ros Barcelo, 1997).

PAGE gel staining. Isopolyphenol oxidase and isoperoxidase were analysed by non-denaturing gel electrophoresis according to Laemmli (1970). The resolving gel was 10 % and the stacking gel 5 % (w/v).

Following electrophoresis, the gel was rinsed twice in deionized water. For POX activity gel was placed in 500 ml of 25 mM citrate-phosphate buffer pH 5.3) containing 5 mM guaiacol and 0.01 % H_2O_2 (v/v). Staining PPO activity was done by placing gel in 500 ml of 0.1 M Tris-acetate buffer Ph 5.0 containing 0.1 mM catechin and 10mM 1,3-dihydroxyphenylalanine (DOPA). For both isoenzyme systems the bands will appeared 30 min after incubation.

RESULTS

Classification od Clones.

Leaves of 15-trail clones were use to evaluate disease severity caused by *P megakarya*. The external sings and symptoms of black pod disease appeared as black spots two days after inoculation . Length and percentage of necrosis of midrid, state of leaf and veins permitted to classify the clones in order of decreasing susceptibility: Playa , Alta2, MXC67, SNK608, AMAZ15-15, NA33,SNK607,UF676, UPA143, SPEC54-1, SCA24, SNK619, IMC60, SNK602, EQX3360, PA107 (Table I).

Soluble peroxidase activity.

Soluble POX specific activity was higher in the freshly haversted leaves of clones UPA143 followed by PA107 and Playa Alta2. Throughout the time-course analysis, POX activity was highest in clone Playa Alta2 . At day five, in inoculated leaves, POX specific activity was 52.82, 16.42 and 14.76 UE/mg of protein respectively in clones Playa Alta2, UPA143 and PA107. Moreover at day 5, the inoculation induced increase in POX activity only in the clone Playa Alta2 (Table II).

Native PAGE of soluble isoperoxidases revealed the presence of five isoperoxidases (A_1, A_2, A_3, A_4 A_5), where A_4 was observed only in the clone UPA134 (Fig.1).

Table 1. Whole leaf susceptibility of the 15 clones of *T cacao* from International selection clones inoculated with *P megakarya*.

N°	Midrib length (cm)	Length of necrotic rib (cm)	Necrosis of the midrib (%)	State of leaf and veins	Clone name
1	25,6	14	54,68	5	Playa $Alta_2$
2	21,6	11,8	54,63	5	MXC67
3	16,2	8,6	53,09	4	SNK608
4	29,5	12,3	41,69	4	$AMAZ_{15-15}$
5	21,2	9	42,45	5	NA33
6	19,7	7,9	40,10	2	SNK607
7	18,2	5,4	29,67	2	UF676
8	19,7	5,8	29,44	3	UPA143
9	16,2	4,4	27,16	3	SPEC54-1
10	12,3	2,7	21,95	2	SCA24
11	17,7	2,5	14,12	2	SNK619
12	32	4,4	13,73	1	IMC60
13	15,3	2,05	13,40	1	SNK602
14	22,6	3	13,27	1	EQX3360
15	23,6	3,1	13,14	1	PA107

Table II. Specific activity of soluble peroxidase in leaves of *T cacao* clones 0,1,3, and 5 days after inoculation with *P megakarya*. Enzyme activity was measured using guaiacol as substrate. The data are presented as means with standard error from three independent experiments with three replicas per treatment (UE/mg of proteins)

	Playa Alta$_2$			UPA143			PA107		
Treatment days	H	W	W+I	H	W	W+I	H	W	W+I
D0	1,41±0,1			9,22±1,7			5,77±0,5		
D1	12,75 ±1,1	17,92 ±1,1	17,27 ±1,6	17,11 ±1,3	16,54 ±1,2	14,75 ±1,6	9,14 ±0,07	11,69 ±0,9	8,63 ±0,1
D3	23,23 ±1,4	38,54 ±1,9	41,43 ±1,7	12,73 ±1,04	16,18 ±1,2	13,42 ±1,5	12,31 ±1,3	14,44 ±0,9	10,52 ±0,7
D5	18,73 ±1,8	34,10 ±1,2	52,82 ±1,5	19,84 ±0,9	22,81 ±1,1	16,42 ±0	15,28 ±0,5	15,93 ±1,3	14,76 ±1,1

Soluble polyphenol oxidase activity.

In freshly harvested leaves PPO specific activity was 5.96, 6.45 and 7.69 UE/mg of protein respectively in clones PA107, Playa Alta2 and UPA143. Increase in PPO specific activity was observed at day-3 in wounded and inoculated leaves of clones Playa Alta$_2$ and PA107. But when we compared soluble PPO specific activity in 5-day-old intact leaves significant increase was observe only in clone PA107. There was a 1.54 fold increase in soluble PPO specific activity in 5-day-old inoculated leaves of PA107. On the other hand and in the same condition a decrease in enzyme activity was recorded in clones Playa Alta$_2$ and UPA143.

Native PAGE analysis of soluble isopolyphenol oxidase revealed three bands (B_1, B_2 and B_3) with B_2 specific to clone UPA143 (Fig 2).

Table III: Specific activity of soluble polyphenol oxidase in leaves of *T cacao* clones 0,1,3, and 5 day after inoculation with *P megakarya*. Enzyme activity was measured using catechin as substrate. The data are presented as means with standard error from three independent experiments with three replicas per treatment (UE/mg of proteins)

	Playa Alta$_2$			UPA143			PA107		
Treatment days	H	W	W+I	H	W	W+I	H	W	W+I
D0	6,45±0,5			7,69±0,7			5,96±0,3		
D1	7,32 ±0,5	5,05 ±0,2	8,40 ±0,2	7,83 ±0,5	7,86 ±0,2	6,37 ±0,3	6,75 ±0,5	4,79 ±1,1	3,55 ±0,4
D3	3,1 ±0,4	7,64 ±0,4	13,99 ±1,4	4,83 ±0,8	6,20 ±0,1	4,61 ±0,2	11,63 ±0,6	19,13 ±0,2	8,83 ±0,3
D5	9,09 ±0,2	7,05 ±0,1	7,97 ±0,7	8,62 ±0,3	9,08 ±0	6,19 ±0,1	7,06 ±0,2	7,81 ±0,3	10,88 ±0,2

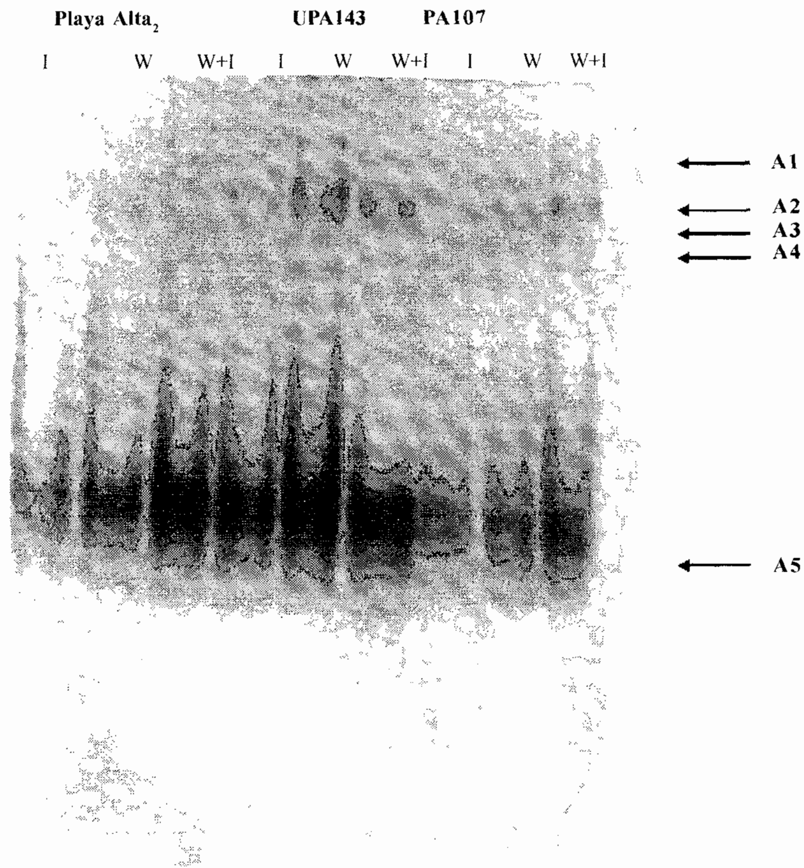

Figure 1: Isoperoxidase pattern in three *Theobroma cacao* clones run with anodic native gel electrophoresis. I: 5 day-old intact pods, w: 5 day-old wounded pods, W+I: 5 day-old inoculated pods.

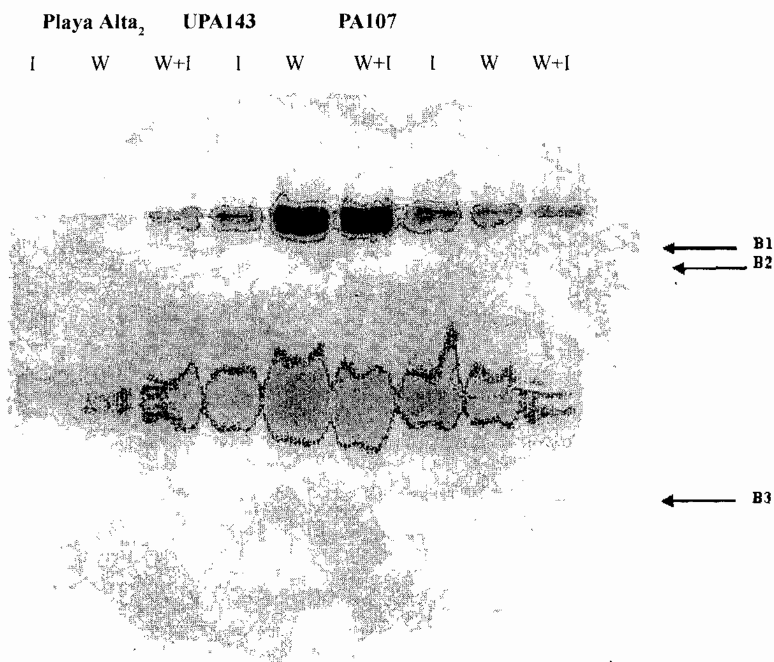

Figure 2: Isopolyphenol oxidase pattern in three *Theobroma cacao* clones run with anodic native gel electrophoresis. I: 5 day-old intact pods, w: 5 day-old wounded pods, W+I: 5 day-old inoculated pods.

DISCUSSION

The level of susceptibility to *P megakarya* using leaves inoculation was determined on fifteen cacao clones belonging to the International Cacao Germplasm collection. This evaluation permited us to classify them according to the disease severity. These results tends to support the general conclusion that to provide good intergroup hybrid performance selection must be done in different environmental condition and with a local pathogen. It is known that the rate of lesion development is the best indication of intrinsic resistance to black pod (Crouzillat et al., 2000).

In this study, we observed a significant increase in POX specific activity in the highest susceptible cacao clones following the inoculation with *P. megakarya*. Moreover POX activity was significantly greater in the higher susceptible clone than the less susceptible one. A similar result was obtained by Mohammidi and Kazemi (2002) in the interaction wheat/ *Fusarium graminearum*. Plant disease resistance has often been correlated with elevated POX activity.

PPO specific activity significantly increased in the less susceptible clones following the inoculation with *P. megakarya*. This activity was 1.4 times greater in less susceptible cacao clone than in the control. This may suggest that induced PPO activity in less susceptible cacao clone could be a defensive response against *P.megakarya* and seems to be related to disease tolerance. Similar results have previously been obtained in plant-pathogenic fungal interaction such as soybean/ *Phytophthora megasperma* and wheat/ *Fusarium graminearum* (Goodman et al., 1986; Mohammidi and Kazemi, 2002).

Neither isoperoxidase nor isopolyphenol oxidase was observe on electrophoretic analysis. But isoperoxidase A3 and isopolyphenol oxidase B2 were present only in UPA143 genotype. Omokolo at al (2003) also observed that UPA134 display a specific forms of PPO. These, Upper Amazonia selected clones have a short pod's length and short pod's cycle and seem to be less susceptible in the field than during artificial pod inoculation because their life cycle were shorter. It was recommended to use them in the breeding programs. The presence of these specific forms of POX and PPO can partly explain the behaviour of these genotypes.

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