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Virulence Test of Some *Phytophthora Megakarya* Isolates on Cocoa (*Theobroma Cacao* L.) Hybrid Pods

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Abstract: *Phytophthora megakarya is an Oomycete which has a negative impact on Cameroon cocoa production. An assessment of the level of virulence of different isolates of P. megakarya in mono or coinfection was performed on some hybrids of cocoa (Theobroma cacao L.) obtained from reciprocal crosses. The isolates tested on eight cocoa hybrids obtained by hand pollinisation were Bakoa, Le4 and Lebdi. Contents of soluble amino acids, sugars, polyphenols and proline were determined using standard methods in mono or coinfected cocoa pods. This study revealed that, the development of the necrotic area in the cortex of cocoa pods was genotype-dependent and isolate-dependent after infection. For this reason, the classification in terms of virulence of isolates was the following: Lebdi<Le4<Bakoa. However, SNK 413 x T 79/501 and T 79/501 × SNK 413 hybrids were the most tolerant while UPA 134 x ICS 40 and T 79/467 × SNK 13 hybrids proved to be the most sensitive to threeP. megakarya isolates. Bakoa + Le4 combination was the most aggressive among the other combinations.Groupings of samples were observed using principal component analysis (PCA) and hierarchical cluster analysis.Significant and negative correlations were found between necrosis and three biochemical metabolites (total polyphenols, soluble sugars, Proline andsolubleamino acids) in cocoa hybrids suggesting that these metabolites could be considered as markers of resistance in T. cacao/P. megakarya interactions.*

Keywords: *Biochemical metabolites, coinfection, cortex, markers of resistance, monoinfection, necrosis.*

I. Introduction

Cocoa is a tropical perennial plant native to the Amazon basin and this area also corresponds to its center of diversity [1]. Cocoa farming is an economic activity of several tropical countries. Africa is today the leading producer of cocoa with about 73.7% of world production.Five countries stand out for their active involvement in cocoa farming especially Côte d'Ivoire (40.7%), Ghana (20.2%), Indonesia (7.2%), Cameroon (5.5%) and Ecuador (5%) [2].

However, cocoa farming is facing many inconveniences, the most important are: ageing plantations and the high sensitivity of plant material to parasitic pressures [3]. The black pod disease (Bpd) is one of the most prevalent diseases in the genus *Theobroma*. The emergence of *P. megakarya* has had dramatic social and economic consequences in cocoa producing countries in West and Central Africa, clearly demonstrating the scale of damage that it may cause in case it spreads into other cocoa producing regions.It is caused by a fungus *Phytophthora* spp. belonging to the class Oomycetes. This fungus exists in several species [4], but in Cameroon, the predominance of the *P. megakarya* species has been noticed [5]. It causes damages that can reduce the production by 50 to 80% [6]and losses in fields up to 100% if no action of protection is taken [7].

To counter this disease, several control methods are envisaged.The first approach is chemical control, based on the use of fungicides [8][9]. Although effective, this technique is expensive, polluting, binding and not economically viable. Currently there are alternatives and / or additional chemical control methods such as genetic control that involves obtaining tolerant hybrids to this disease [10]. For Bpd resistance, there have been several tests based on leaf inoculation [11][12] and detached pod inoculation [13][14. In Cameroon, several studies were recently carried out to evaluate the resistance of the local and introduced cocoa germplasm. Clonal and progeny material were assessed using leaf disc [15][16] and pod tests [13].

It is in this context that the overall objective of our study was to evaluate, using laboratory testing, the virulence of three isolates of *P. megakarya* in some *T. cacao* hybrids.Quantitativecomparisons of polyphenols, soluble sugars, proline and amino acids contents in mono or coinfected cocoa podswere also investigated.

II. Materials And Methods

Cocoa plant material

The experimental material used included eight hybrids cocoa pods in experimental farms of SODECAO (Cameroon Cocoa Development Corporation) station in Mengang, Cameroon (Table 1). A total of 120 hybrids were obtained from crossing using hand-pollination techniques [17]. The healthy pods about 3-months old were harvested, washed thoroughly with sterile distilled water and then with ethanol 70%. Cocoa genotypes (hybrids) were tested by inoculating whole detached pods with 100 µL inoculum (concentrationof 2.5 $x10^4$ zoospores mL^{-1}) in this trial.

P. megakarya (Oomycete isolates) and cocoa pod inoculation

Three isolates of *P. megakarya* (Bakoa, Lebdi and Le4) were obtained from the Biotechnology Centre of the University of Yaounde I, Cameroon. For zoospore production, the isolates were cultured on V8 medium (20% V8, 0.3% Ca_2CO_3, 1.5% agar-agar and 1000 mL distilled water) and incubated in the dark at 25 ± 2 °C. *P. megakarya*sporangia were induced to liberate zoospores by adding sterile distilled water at 4 °C at room temperature for 1 hour. The zoospore concentration was then adjusted to 2.5 $x10^4$ zoospores per milliliter with a MALASSEZ hemocytometer.

Two conditions were tested: (i) monoinfection (single isolate treatment: Bakoa, Lebdi and Le4) and (ii) coinfection (combination of isolate treatment: Bakoa + Lebdi, Bakoa + Le4 and Lebdi + Le4). For each treatment, pods were inoculated by depositing 100 µL of zoospore suspensions into the cortex of pods after wounding. When two isolates were combined, 50 µL zoospore/mL of each suspension were added to obtain 100 µL of zoospores/ml of total solution. One hour after penetration of zoospore suspension, each scarification was then covered with cotton wool soaked in sterile distilled water. Inoculated pods were put into trays and incubated at 25-26 °C in a dark and humid (60%) room.

Evaluation of disease

The measurement of necrotic lesion size was done 2, 4 and 6 days after inoculation. The diameters of the more or less circular necrotic spots were measured and the surface calculated using [10]formula:

$$S = D \times d \times \pi$$

Where: S: necrotic area (cm^2);
D: necrotic diameter (cm);
d: small diameter (cm).

Total phenolic compound contents

Total phenolic content was determined following the method of [18]. A sample (50 mg) was extracted with 1 ml of 70% aqueous ethanol at room temperature. The mixture was centrifuged at 1000g for 15 min. The supernatant (200 µl) was mixed with 1.5 ml of Folin–Ciocalteu reagent, and allowed to stand at room temperature for 5 min; then 1.5 ml of sodium bicarbonate solution (0.566 M) was added to the mixture. After 60 min, absorbance was read at 725 nm. Results were expressed as gallic acid equivalents. The concentration used was in a range between 0.02 and 0.1 mg/ml.

Total soluble amino acid and total soluble sugar contents

Amino acid contents were determined by the ninhydrine method [19]with slight modifications. The incubation mixture containing 100 mL of the ethanol extract, 1 mL of 80% ethanol, 1 mL of 0.2 M citrate buffer (pH 5), and 2 mL of acetonicninhydrinsolution (1% ninhydrin and 0.006% KCN in acetone) was incubated for 15 min at 100°C. The mixture was cooled for 5 min under tap water before adding 8 mL of distilled water. The absorbance of the purple product was recorded at 570 nm (Hitachi spectrometer U-200). Glycine equivalents were calculated from a standard curve obtained with pure analytical grade glycine.

For carbohydrate determination, proteins were removed from the ethanolic extract after treatment with basic lead acetate. The carbohydrate extracts were then determined by the anthronmethod: one mL of the extract was incubated in 5 mL of anthronsolution (0.12 g anthron in 100 mL 6.5 M H2SO4) at 90°C for 10 min. The absorbance of the green product was measured at 630 nm. Results were expressed in µg eq. glucose by reference to the standard.

Proline contents

Proline contents were determined spectrophotometrically [20]. Acid-ninhydrin was prepared by heating 0.7 g ninhydrin in 15 mL glacial acetic acid and 10 mL 6 M phosphoric acid, with agitation till dissolution and stored at 4°C. Approximately 0.3mg of plant material was homogenised in 8 mL of 3% aqueous sulfosalicylic acid and the homogenate filtered through Whatman No2 filter paper. Two mL of filtrate was reacted with 2 mL acid-ninhydrin and 1.5 mL of glacial acetic acid in a test tube for 1 hour at 100°C, and the

reaction terminated in an ice bath. The reaction mixture was extracted with 4 mL toluene, mixed vigorously with a test tube stirrer for 20 sec. The chromophore containing toluene dissolved in the aqueous phase, warmed to room temperature and the absorbance read at 520 nm using toluene as a blank. The proline concentration was determined from a standard curve and calculated on a fresh weight basis as follows: [(µg proline/mL × mL toluene) / 115.5 µg/µmole]/[(g sample)/5] = µmoles proline/g of fresh weight material.

Statistical analysis

Data obtained was analysed using SPSS (version 20.0 for windows) to perform analysis of variance (ANOVA). The significance of differences was determined by Tukey's multiple comparison technique. Principal component analysis (PCA) andHierarchical classification of necrotic area were performed with SPAD 5.5 software package.

III. Results

Disease severity (monoinfection)

Reactions of *T. cacao* against *P. megakarya*took place under controlled conditions. The study showed significant differences ($p<0.05$) in the development of necrotic area in the cortex of cocoa pods after infection with 2.5 $x10^4$ zoospores mL^{-1} of *P. megakarya* isolateon days 2, 4 and 6(Table 2-4).

After infection of *P. megakarya*-Bakoa isolate, eight cocoa genotypes recorded a variation of necrotic lesion size between 5.51 (day 2) and 83.22 cm^2 (day 6).

After infection of *P. megakarya*-Lebdi isolate, the lowest necrotic lesion size was observed in thepodofSNK413×T79/501 and T79/501×SNK 413 genotypes on days 2, 4 and 6 (Table 3). Contrariwise, the greatest necrotic surface area was recorded in the UPA 134× ICS 40 genotypes at day 2, 4 and 6 following infection, and a significant difference was observed among the other genotypes ($p<0.01$) (Table 3).

On day 2following infection of *P. megakarya*-Le4isolate, the highest necrotic area was recorded in the T 79/467×SNK 13 genotype (9.01±0.60 cm^2) ($p<0.05$). Likewise, this hybrid also showed the greatest necrotic surface on days 4 and 6 after treatment (Table 4).

Principal component analysis (PCA) and Hierarchical cluster

Principal Component Analysis (PCA) shows the 8 pods genotypes distributed according to their tolerance levels to 3-isolates of *P. megakarya*. The first two components contributed to explain 88.26% of variability of the necrotic lesion size (Figure 1).The genotype UPA 134 × ICS 40 is showed great sensibility to*P. megakarya*-Bakoa and Lebdi isolates, and this is why Axis 1 clearly separates it from the other genotypes (Figure 1). However, only the genotype T 79/467 x SNK 13 recorded the highest necrotic lesion size after infection of *P. megakarya*-Le4isolate.

Dendrogram, at 95% similarity, is divided into 5 clusters according to tolerance and susceptibility to the three isolates(Figure 2). Genotypes T 79/476 × SNK 13 and UPA 134 x ICS 40 were grouped in cluster 1 and cluster 2 respectively, which is composed of susceptible genotypes. These genotypes recorded high necrotic surfaces and are characterized as susceptible to the three pathogens 6 days after inoculation. The third cluster constituted of four genotypes with necrotic surfaces less significant (intermediate tolerance). Clusters 4 and 5 contained genotypes (T 79 / 501 x SNK413 and SNK 413 x T 79 / 501) with highest tolerance to the three isolates. In order to study the coinfection effect of *P. megakarya* isolates, the most susceptible (T 79/476 × SNK 13) and the most tolerant (SNK 413 x T 79/501) hybrid to *P. megakarya* isolates were taking into account.

Attitude of coinfection

These two cocoa genotypes were infected at different treatment conditions [monoinfection and coinfection (Bakoa+Lebdi, Bakoa+Le4 and Lebdi+Le4)] and necrotic surfaces were obtained on days 2, 4 and 6. The differences in the necrotic area in the two cocoa genotypes were significant ($p<0.01$) (Figure 3).

The monoinfection showed the increasing level of virulence isolates of *P. megakarya* studied. To this effect, the combination of the isolates was carried out and the *P. megakarya*-Bakoa+Le4 isolates were observed for their high aggressiveness on days 2 and 4in tolerant genotype (SNK 413 x T 79/501).But, in the same genotype, *P. megakarya*-Bakoa+Lebdi isolates recorded the highest necrotic area on day 6. Also, on day 6, it was observed that coinfection was more virulent than all three mono infections(Figure 3A).

When the necrotic area on the cortex of cocoa pod (T 79/476 × SNK 13) infected with*P. megakarya* isolates (monoinfection and coinfection) was observed, the highest necrotic area was caused by the Bakoa isolate (monoinfection) and the Bakoa+Lebdi isolate (coinfection) on day 2 ($p<0.01$) (Figure 3B). On day 4 and 6 after infection, the genotype T79/476×SNK 13 was more susceptible toBakoa isolate.However, the coinfection (Bakoa+Lebdi) yielded a higher necrotic area of 39.97%, 443.33% and 431.14% ($p<0.01$) than the various monoinfections of Bakoa, Lebdi and Le4 respectively (Figure 3B). Likewise, the difference in necrotic

area among Bakoa+Lebdi, Bakoa+Le4 and Lebdi+Le4 combinations was significant ($p<0.01$), and the highest virulence was observed in the Bakoa+Lebdiisolate (Figure 3B).

Biochemical metabolites such as total polyphenols, soluble sugars, soluble amino acids and proline were evaluated in the most tolerant and the most susceptible cocoa hybrids obtained in this study (Figure 4). Results showed that SNK413 x T79/501 tolerant hybrid exhibited higher metabolite contents than the susceptible one, in all treatments.

For total polyphenols (TPP), pods infected with *P. megakarya-Lebdi* isolate recorded the highest amount of TPP in the two hybrids. These values are 10.3 and 7.8 mg.g-1FW for the tolerant and susceptible hybrids respectively. For SNK413 x T79/501 tolerant hybrid, pods coinfected by *P. megakarya-Lebdi+Le4* isolates displayed high amounts of TPP. This observation was also made in pods coinfected with *P. megakarya-Lebdi +Le4*and with *P. megakarya-Bakoa +Le4* in T79/467 x SNK13 susceptible clone (Figure 4A).

Contrary to TPP, cocoa pods infected with *P. megakarya-Bakoa + Lebdi* isolates recorded the highest amount (52.5 mg.g-1 FW) of soluble sugars both in tolerant and susceptible ones. The two other coinfections displayed lower sugar contents in T79/467 x SNK13 hybrid (Figure 4B).

The stress-related amino acid (proline) was mostly present in pods of tolerant hybrid in all treatments. However, *Lebdi*monoinfection and *Lebdi + Le4* coinfection yielded more proline content than other treatments in SNK413 x T79/501 tolerant hybrids (Figure 4C) with 248 $mg.g^{-1}$ FW for *Lebdi*monoinfection and 235 $mg.g^{-1}$ FW for *Lebdi + Le4* coinfection.

The amino acid contents varied from 4-7.2$mg.g^{-1}$ FW. Here, the effect of *Lebdi* and *Le4*monoinfection is accompanied by the increase of soluble amino acid contents in the two cocoa hybrids. *Lebdi + Le4* coinfection also gave high levels (7$mg.g^{-1}$ FW) of soluble amino acid contents in the tolerant clone (Figure 4D).

Except for soluble sugars where *Bakoa + Lebdi* coinfection was characterized by an increase of about 25% of this metabolite, the two other coinfections on cocoa pods yielded lower contents of other metabolites compared to monoinfection.

Spearman's correlations between parameters studied (Table 5) showed that there is a significant and negative correlation between necrosis and three biochemical metabolites (TPP, Proline and soluble amino acids) in T79/467 x SNK13 susceptible hybrid. Contrary to these three metabolites, soluble sugars displayed a significant positive correlation with necrosis and this correlation is more accentuated in tolerant hybrid ($p < 0.01$) than susceptible ones ($p < 0.05$).

IV. Discussion

To assess virulence of three different isolates of *P. megakarya*, pods of eight hybrids were infected. Data was collected in the second, fourth and sixth day after inoculation. The rate of spread of infection on the genotypes tested was the result of the presence of the pathogenic agent of Bpd of cocoa.

The study showed a considerable genetic variability among the hybrids of cocoa for tolerance to Bpd (Bakoa, Lebdi and Le4). The difference in the necrotic area was significant for all hybrids cocoa ($p<0.01$). We observed that SNK 413 x T 79/501 and T 79/501 x SNK 413 were hybrids that displayed the lowest necrotic area to all three isolates, whereas the disease progressed quickly in T 79/467 x SNK 13 and UPA 134 x ICS 40 and these two hybrids were assumed to be susceptible to the Bpd. However, the different responses of cocoa hybrids suggested that there could be an additive and dominant gene effect in the transmission of character [12] [21][22][23]. In the same way, a vast genetic variability for black pod resistance has also been reported in cocoa [9][14][24].In our studies, the levels of tolerance of cocoa could be identified using any *Phytophthora* species. These findings are in agreement with results reported bysome authors [14][25][26], who showed that the use of the most aggressive species of *P. megakarya* isolates could lead to the identification of useful levels of resistance against the pathogens.

The cluster analysis at 95% homogeneity performed on all tested variables grouped the varieties into three categories, namely tolerant, intermediate and susceptible, confirming field observations. These experiments clearly showed that two hybrids SNK 413 x T 79/501 and T 79/501 x SNK 413 considered as tolerant recorded lower necrotic lesion sizes following infection with all the *P. megakarya* isolates (Bakoa, Lebdi and Le4). The findings indicate that the tolerant hybrids could be used in future improvement programs [13][12].

The analysis of the evolution of the necrosis area on two hybrids [SNK 413 x T 79/501 (tolerant) and T 79/476 × SNK 13 (susceptible)] showed asignificant difference($p<0.05$) following infection with the three combinations of isolates. The variability of the necrosis area depended on the virulence of the three isolates of *P. megakarya*. Our study showed that, in the same circumstances, the *Bakoa* isolate was more aggressive than the *Ledbi* and *Le4*isolates. Coinfection also indicated that the spread of infection varied with time and was genotype-dependent. In both hybrids SNK 413 × T 79/501 and T 79/467 × SNK 13, an increase in necrotic surface area was observed from day four to daysix following coinfection (Bakoa+Lebdi). The rapid progression of necrotic area could be interpreted as a synergetic action between 2 pathogens involved in coinfection and the

flagging of necrotic area could be due to antagonistic effects of those same pathogens. The results of the present study support the previous results [27]who reported that the coexistence of other fungi with *P. palmivora*in cacao tissues could antagonize the development and virulence of *P. palmivora*. This agrees with findings of several authors[24][28][14][26].However, it has been shown that the host-specific resistance of cacao is often unstable due to the variability of the cacao population [29] while different species of black pod pathogens as well as isolates within the same species have been shown to vary in aggressiveness [30][31].

Quantitative determination of some metabolites revealed that, after *P. megakarya*-isolates treatment on cocoa pods, the tolerant SNK 413 × T 79/501 cocoa hybrid displayed high level of soluble amino acids, sugars, phenols and proline compared to the susceptible T 79/476 × SNK 13 one. In a study assessing relationship between phenolic compounds and resistance to *P. megakarya* using two cocoa families, some authors [32]found that productive and tolerant genotypes displayed high phenols content meanwhile less tolerant and productive ones had a weak content. The role of phenolic compounds in plant defense is well documented [33]. These metabolites accumulate at different levels in infected tissues in response to pathogen attack. For example, some 16 phytoalexins produced by rice (*Oryza sativa*) in response to pathogen invasionwere listed[34].

Amino acids content increased in leaves of cocoa infected with*P. megakarya* and proline appeared solely during conditions of infection in parental tolerant clone ICS84 suggesting its implication in the defence mechanism of *T. cacao* against *P. megakarya* ([35]. In fact, Amino acids might act directly to inhibit fungal development, or indirectly by their implication in the metabolic pathways associated with resistance to diseases [36].After infection of resistant genotypes of some host species, accumulation of certain specific amino acids such as glutamine, histidine, glycine and arginine were observed in tomatoes [37], tyrosine and alanine in wheat and asparagin, glutamic acid, proline, glycine and arginine in citrus [38].

In this study, monoinfection treatment of *P. megakarya*- Lebdi isolate yielded high amounts of phenols, amino acids and proline compared with the other isolates. The negative and significant correlation found between necrosis and three of the four metabolites namely phenols, amino acids and proline suggests that,*Lebdi* isolate is the less aggressive among the three isolates.

Except for soluble sugars where *Bakoa + Lebdi* coinfection was characterized by an increase of about 25% of soluble sugar content, the two other coinfections on cocoa pods yielded lower contents of metabolites compared to monoinfection. This result confirms in part the synergistic effect of *P. megakarya*-isolates observed on disease severity.

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Table 1. Identification and origin of cocoa clones used in this study.

Genotypes		**Origin**	**Collection**	**Group**	**Sensibility**
Clones	SNK 13	Cameroon	Nkoemvone	Trinitario	Moderately tolerant
	SNK 16				
	SNK 413				
	ICS 40	Trinidad	/	Trinitario	
	UPA 134	Ghana	Wacri	UA	Sensible
	T 79/467		Tafo		Tolerant
	T 79/501		Tafo		
	SCA 12	Ecuador	/		Moderately sensible

Hybrids	SCA 12 × SNK 16	Cameroon	Mengang	UA x T	Unknown
	SNK 16 × SCA 12			T x UA	
	T 79/501 × SNK 413				
	SNK 413× T 79/501				
	UPA 134 × ICS 40				
	ICS 40 × UPA 134				
	T 79/467 × SNK 13				
	SNK 13 × T 79/467				

SNK= selection Nkoemvone*;* ICS= Imperial college selection*;* UPA= Upper amazon*;* T= Tafo*;* SCA= Scavina. *Trinitario=T; Forastero=F; UA=Upper Amazon Forastero, LA=Lower Amazon Forastero*
(Blaha and Lotode, 1976), and (Ondobo et al. 2014).

Table 2. Necrotic area (cm^2) recorded from the inoculation of *P. megakarya*-Bakoa isolate on eight hybrids of*T. cacao*.

	Bakoa		
Hybrids	**Day 2**	**Day 4**	**Day 6**
SNK 413×T 79/501	5.51±0.29 **a**	7.27±0.14 **a**	15.40±0.02 **a**
T 79/501×SNK 413	12.18±0.13 **cd**	19.06±0.71 **c**	20.37±0.77 **b**
SNK 13×T 79/467	8.66±0.22 **b**	12.03±0.91 **b**	50.54±0.25 **c**
T 79/467×SNK 13	16.74±0.60 d**e**	28.31±0.96 **e**	83.22±0.23 **d**
SNK 16×SCA 12	15.27±0.16 **de**	22.04±0.56 **cd**	49.64±0.92 **c**
SCA 12×SNK 16	10.02±0.09 **c**	15.88±0.77 **bc**	46.20±0.09 **c**
ICS 40×UPA 134	6.70±0.39 **a**	11.98±0.68 **b**	24.68±0.23 **b**
UPA 134×ICS 40	11.46±0.62 **c**	26.41±0.22 **de**	78.97±0.05 **d**

Lowercase letters represent differences among days in the same genotypes. Mean symptom ratings within a day and followedby the same letter are not significantlydifferent according to the tukey test at 5%

Table 3. Necrotic area recorded from the inoculation of *P. megakarya*-Lebdi isolate on eight hybrids of T. cacao.

Hybrids	**Lebdi**		
	Day 2	**Day 4**	**Day 6**
SNK 413×T 79/501	2.25±0.24 **a**	3.59±0.63 **a**	9.75±0.24 **a**
T 79/501×SNK 413	4.81±0.13 **b**	6.72±0.51 **b**	19.66±0.44 **e**
SNK 13×T 79/467	7.63±0.07 **c**	8.30±0.76 **bc**	23.23±0.67 **c**
T 79/467×SNK 13	7.74±0.34 **c**	9.48±0.55 **c**	21.44±0.72 **c**
SNK 16×SCA 12	6.27±0.53 **bc**	9.75±0.16 **c**	17.50±0.61 **b**
SCA 12×SNK 16	5.92±0.86 **b**	8.92±0.91 **bc**	16.37±0.19 **b**
ICS 40×UPA 134	4.71±0.91 **b**	7.57±0.69 **b**	22.00±0.68 **c**
UPA 134×ICS 40	8.05±0.12 **cd**	10.45±0.15 **cd**	28.45±0.70 **d**

Lowercase letters represent differences among days in the same genotypes. Mean symptom ratings within a day and followed by the same letter are not significantlydifferent according to the tukey test at 5%

Table 4. Necrotic area recorded from the inoculation of *P. megakarya*-Le4 isolate on eight hybrids of *T. cacao.*

Hybrids	**Le4**		
	Day 2	**Day 4**	**Day 6**
SNK 413×T 79/501	1.52±0.51 **a**	6.86±0.26 **a**	13.49±0.23 **a**
T 79/501×SNK 413	4.22±0.19 **b**	6.96±0.89 **a**	19.00±0.09 **c**
SNK 13×T 79/467	3.08±0.74 **b**	7.88±0.31 **ab**	13.59±0.78 **a**
T 79/467×SNK 13	9.01±0.60 **d**	14.15±0.50 **c**	21.92±0.38 **d**
SNK 16×SCA 12	7.82±0.63 **c**	9.47±0.60 **b**	16.33±0.47 **b**
SCA 12×SNK 16	2.90±0.39 **a**	5.97±0.10 **a**	12.59±0.86 **a**
ICS 40×UPA 134	5.46±0.11 **bc**	8.45±0.94 **ab**	14.90±0.45 **ab**
UPA 134×ICS 40	5.47±0.22 **bc**	9.28±0.99 **b**	13.59±0.02 **a**

Lowercase letters represent differences among days in the same genotypes. Mean symptom ratings within a day and followed by the same letter are not significantlydifferent according to the tukey test at 5%

Table 5. Spearman's correlations between the different parameters studied.

SNK 413 × T 79/501	Necrosis	PPT	Carbohydrates	Proline	Amino acids
Necrosis	1				
PPT	-0,881*	1			
Carbohydrates	0,771**	-0,857*	1		
Proline	-0,903*	0,805	-0,991**	1	
Aminoacids	-0,912*	0,841	-0,815*	0,753	1
T 79/467 × SNK 13					
Necrosis	1				
PPT	-0,429	1			
Carbohydrates	0,829*	-0,486	1		
Proline	-0,426	0,952**	-0,486	1	
Aminoacids	-0,468	0,943**	-0,513	0,943**	1

**. Correlation is significant at the 0.05 level*
***. Correlation is significant at the 0.01 level*

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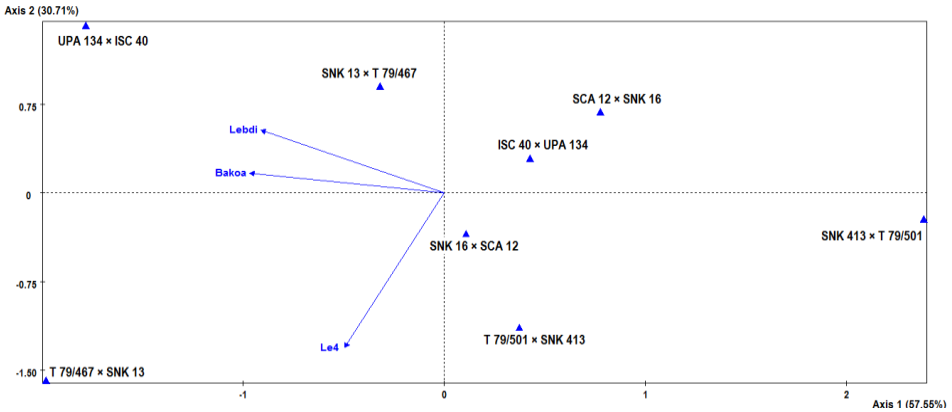


Fig.1. Loading plot of the first two axis based on infection of three *P. megakarya* isolates (Bakoa, Lebdi and Le4) on cocoa pods of different genotypes.

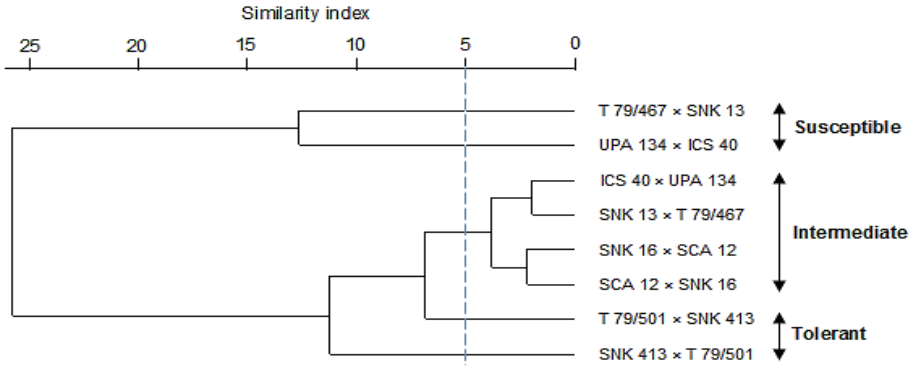
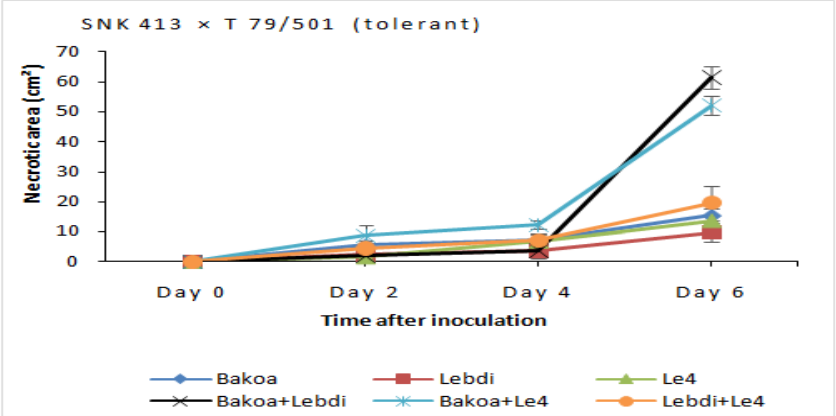


Fig. 2. Dendrogram of eight genotypes of *T. cacao* based on necrotic area after infection of three *P. megakarya* isolates (Bakoa, Lebdi and Le4).



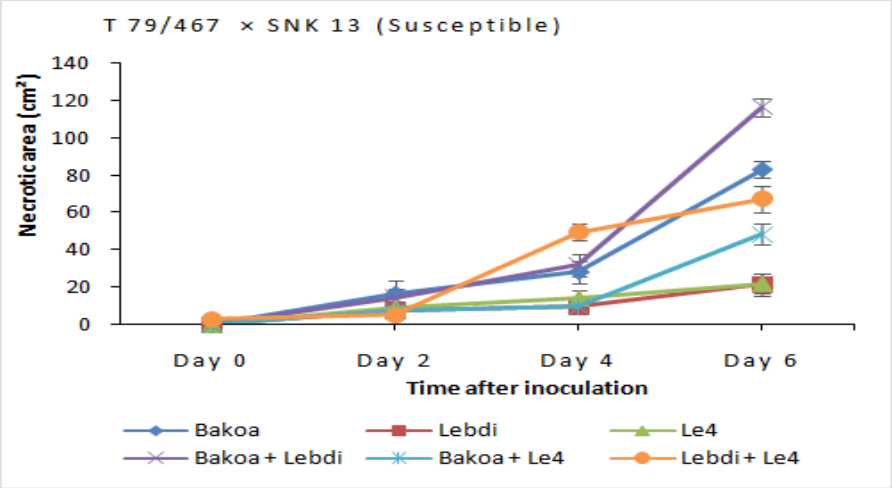


Fig. 3. Evolution of virulence level of *P. megakarya* isolates (Bakoa, Lebdi and Le4) and their three combinations (Bakoa+Lebdi, Bakoa+Le4 and Lebdi+Le4) on two genotypes [SNK413 x T79/501 (tolerant) and T79/467x SNK13 (susceptible)] after inoculation.

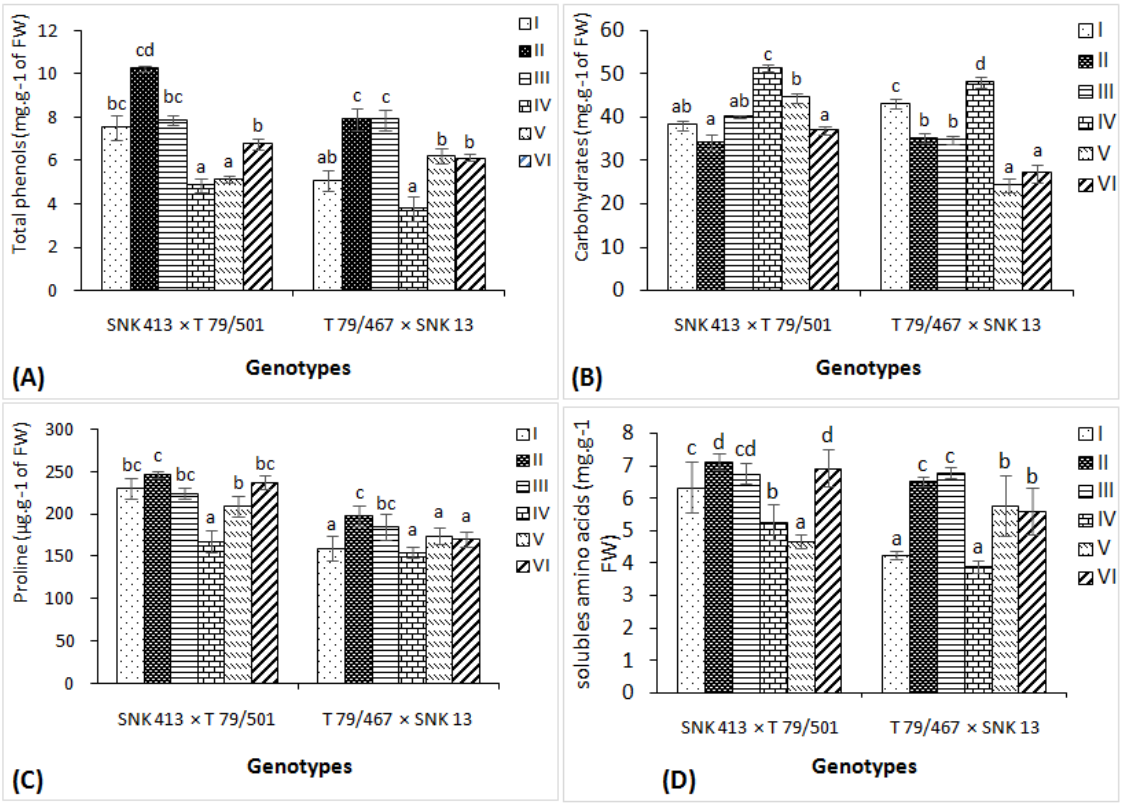


Fig. 4. Variation of some biochemical markers following infection of cocoa pods. (A): Total polyphenols; (B): Carbohydrates; (C): Proline; (D): Soluble amino acids. I: *P. megakarya*-Bakoa isolate, II: *P. megakarya*-Lebdi isolate, III: *P. megakarya*-Le4 isolate, IV: *P. megakarya*-Bakoa + Lebdi isolates, V: *P. megakarya*-Bakoa + Le4 isolates, VI: *P. megakarya*-Lebdi + Le4 isolates