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# Heterosis and Heritability Estimates of Amino Acids and Carbohydrates in the Theobroma Cacao/Phythophthora megakaryaInteraction

Pierre François Djocgoue[2,3],Pierre Effa Onomo[1,2*],Martine Louise Ondobo[2],Jules Christian Djoko Kouam[2], Annice Helene Mewouo[2], Jude Manga Ndjaga[2] and Nicolas Niemenak[2]

[1]*Department of Biochemistry, Faculty of Science, University of Yaounde I, P.O Box 812, Yaounde, Cameroon.*
[2]*Laboratory of Plant Physiology, Department of Biological Sciences, Higher Teacher's Training College, University of Yaounde I, PO Box 47, Yaoundé, Cameroon.*
[3]*Department of Plant Biology, Faculty of Science, P.O. Box 812 Yaounde, Cameroon.*
[*]*Corresponding author: Pierre Effa Onomo*

***Abstract:*** *Phythophthoramegakarya is the principal agent of the black pod disease of cocoa (Theobroma cacao L.) in Cameroon and is responsible for 80% loss of cocoa production. The use of hybrid clones displaying resistance to diseases is considered as one of the principal methods of ameliorating crop losses.To assess the T. cacao/P. megakarya interaction, comparative analysis of amino acids and sugars were conducted on healthy, wounded and infected leaves of SCA12, ICS40, SNK16 and T79/501 clones and their hybrids (families F12, F40, F79 and F16) derived from reciprocal crossings. Injury and infection were characterized by an increase in amino acids and a decrease in soluble sugars in F12-F40 families. Analysis of free amino acids revealed the absence of phenylalanine. ICS40 sensible clone lacked glycine, histidine and tyrosine while these amino acids were present in tolerant ones (SNK16 and T79/501) in wounded and infected conditions. Aspartate, glutamate, glutamine, proline and γ-amino butyric acid (GABA) were detected in leaves of all samples and these amino acids increased during injury and infection. This increase was more pronounced for proline and GABA. Low heterosis value was found for amino acids contrarily to soluble sugars where high hybrid vigor was found for F12, F40 and F16 families. No significant difference was observed between values of the broad-sense heritability in F79-F16 reciprocal crossings for amino acid contents and in F12-F40 reciprocal crossings for soluble sugar contents. The absence of significant difference between the heritability values portrays the absence of maternal heritability.*

***Keywords:****Black pod disease, cocoa clones, GABA, hybrid vigor,reciprocal crossings.*

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## I. Introduction

*Phytophthora* pod rot (Ppr), caused by *Phytophthora* spp., is one of the most important diseases of cocoa (*Theobroma cacao* L.)in Latin America, Caribbean islands, Asiaand the tropical African countries [1]. The disease is particularly important in Cameroon due to the existence of a single species, *P. megakarya*, which is the most aggressive [2]. *Phytophthora*pod rot disease causes almost 30% loss in world cocoa production[3] and in Cameroon, *P. megakarya* can be responsible, where it is present, for about 80% loss of cocoa production if no measure is taken [4]. Therefore, the methods available for controlling the rot of cocoa black pod are the use of resistant cultivars and other appropriate cultural practices or fungicide application. An increase in the effectiveness of control can be expected when these methods are combined [5]. However, chemical control is environmentally harmful, commercially non-viable and expensive.

Most of the cultivated cocoa trees in Cameroon were originally derived from old varieties introduced by German colonialists which were made of lines of the Trinitario group whose individuals are hybrids of the wild groups Forastero and Criollo and from second generation seeds obtained from new hybrid cultivars [2].
The selection of hybrids was also based on heterosis observed in crossing genetically distinct genotypes. In clonal banks, local and introduced clones available were often used as hybrid progenitors. These hybrids have been exploited on a large scale especially due to their yield capacity, environmental adaptation and vigor. Unfortunately, these hybrid varieties have also shown large phenotypic variation for many traits and are not readily accepted by farmers [6]. The development of high-yielding resistant crops has generally been the most effective and economical method for controlling the disease [7]. Progress in this direction has been slower,

probably due to the low levels of resistance in base parents, poor screening methods and narrow genetic base of most cocoa breeding programs [8].

The stagnation of cocoa production in Cameroon over several decades is partly due to high disease and pest incidence, and the lack of improved resistant and high-yielding varieties. In most fields, only about 21% of *T. cacao* cultivated today is of selected variety, the remaining 79% of trees are traditional ones [9]. In fact, several authors found a strong correlation between the production and the use of hybrids and they find that the main producing countries are those who use a high percentage of hybrids in their farms (Indonesia 76%, Ivory Coast 69.2%, Malaysia 69%, Ghana 63.2%, Cameroon 25.1% and Ecuador 17.4%). Some authors [10][11], after crossing cocoa clones, obtained hybrid populations that displayed higher values of hybrid vigor than that of the best parent based on the necrotic lesions.

When faced with biotic or abiotic stress factors, plants develop some biochemical and physiological reactions and several chemical compounds are synthesized as a consequence. According to [12], defense reactions may develop several hours or a few days after stimulation. Plants have developed appropriate defense mechanisms to recognize and resist inevitable pathogen attacks. Thus, to effectively stop a pathogen invasion, plants use inherent physical and chemical barriers, and their inducible defense reactions are activated by pathogen attacks [13]. For example, substantial changes in the carbohydrate and amino acid contents of host plants and metabolic alterations that may favor or inhibit fungal development can be induced by disease development [14].

Involvement of amino acids in the relationship between host and parasite has been established [15], indicating an accumulation during the development of the infection. Inhibition of fungal development can be directly acted upon by amino acids or indirectly by their implication in the metabolic ways associated with resistance to diseases [16].

Numerous studies have shown that the proline content in higher plants increases under different environmental stress [17]. *De novo* synthesis of defense proteins implies mobilization of amino acids. This is the case with the glycoprotein rich in hydroxyproline or proline which is involved in the hardening of cell walls [18] and the polygalacturonases rich in leucine [19].

As proline, numerous reports have shown that high levels of GABA accumulate rapidly in plant tissues exposed to a variety of different stress and it was suggested that GABA could function as an osmolyte and mitigate water stress [17]. Other important amino acids in plant/pathogen interactions are tyrosine and phenylalanine, which are substrates of secondary metabolite synthetic pathways, and are considered as possible nitrogen sources for the synthesis of antimicrobial compounds which are stored as glycosyl precursors (glycosides, cycasin, amygdalin, cyanogenic compounds) in vacuoles [20]. Aromatic amino acids are substrates of phenylpropanoid pathways which lead to the biosynthesis of phenols and the synthesis of various substrates involved in lignification processes [21].

As far as sugar level is concerned, it varies considerably between different plant-pathogen interactions. Infection of *Arabidopsis*with*A. candida*or tobacco with tobacco mosaic virus and *P. nicotianae*, of wheat with*Pucciniagraminis*results in an increase in the levels of soluble sugars [22]. In contrast, sugar levels in*Arabidopsis*are not altered by infection with *P. syringae*and decrease in tomato plants after inoculation with *B. cinereal* [23] as well as in sunflowers treated with*Sclerotiniasclerotiorum*[24]. The analysis of invertase activity and sugar levels in infected versus uninfected regions of an inoculated leaf showed only strong effects in the infected region [25].

Therefore, the objective of this research was to examine the biochemical interactions between *T. cacao* and *P. megakarya*by characterizing changes in the contents of ethanol-soluble carbohydrate, ethanol-soluble amino acid in leaves from four cocoa clones and their progenies derived from reciprocal crossings when unexposed and exposed to *P. megakarya*. Quantitative and qualitative comparisons of the free amino acid contents of cocoa leaves under abiotic and biotic stress were also realized.

## II. Materials and Methods

***Cocoa plant material***

Four cocoa clones with different susceptibility to *P. megakarya*available in gene banks of the Cameroon Cocoa Development Corporation (SODECAO) at Mengang Station (Southern Cameroon) were used to create four progenies. The four clones used were: One local Trinitario (SNK16, tolerant to *P. megakarya*), one Trinitario introduced from Trinidad (ICS40, high susceptibility to *P. megakarya*) and two Foresteros (SCA12 and T79/501, mildly and highly tolerant to *P. megakarya* respectively). Crossings were realized at the Mengang Station of SODECAO in May, June and July 2012 using hand-pollination techniques [26]. The four progenies obtained were:

F12: (♀) SCA12 × (♂) ICS40
F40: (♀) ICS40 × (♂) SCA12
F79 : (♀) SNK16 × (♂) T79/501

F16 : (♀) T79/501 × (♂) SNK16

***Production of seedlings and grafts***

Seeds from pods harvested in an experimental field were sown at the nursery of Cameroon Cocoa Development Corporation (SODECAO) at the Mengang Station (Cameroon) and 300 hybrids plants were obtained. Parental plantlets were obtained through top-grafting by using bud wood from the four clones listed above. This grafting was done on non-specific young cocoa plantlets.

***Leaf inoculation and analysis***

The experimental design consisted of four leaves per seedling. Leaves from three-month old plants were washed thoroughly with tap water and sterilized with 70% ethanol for 30 s. The isolate of *P. megakarya*used in this study was the « El8-» provided from the Institute of Agricultural Research for Development (IRAD), Nkolbisson research station. The fungus was routinely cultured on V8 juice agar at 25±1°C in a totally dark and humid chamber. The inner surface of the leaves was scarified along the midrib and inoculated by depositing a mycelium disc (6 mm) cut from 7-days-old fungal cultures. Control leaves were inoculated with sterile agar disc in the same conditions. Necrotic lesions appeared two days after inoculation and the size of these lesions was measured every two days until day 6.

***Extraction and determination of soluble amino acid and soluble sugar contents from leaf tissues***

Soluble carbohydrates and soluble amino acids were extracted from leaf tissues in 80% ethanol [27]. Two grams of leaves were crushed in 6 mL of 80% ethanol and boiled under reflux for 30 min. The ethanol-soluble extracts were filtered using filter paper (Whatmann No. 1) and concentrated under a vacuum at 50°C prior to analysis. The operation was repeated twice and the three extracts from each sample were mixed.

Total amino acid contents were determined by the ninhydrin method [28]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 acetonic ninhydrin solution (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 productwas 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 anthrone method of Yemm and Wills (1954): one mL of the extract was incubated in 5 mL of anthrone solution (0.12 g anthrone in 100 mL 6.5 M $H_2SO4$) 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.

***Analysis of free amino acids***

Free amino acid contents were analyzed[29]. Thirteen mg lyophilized cocoa leaves were crushed and stirred at 4°C for 1 h with 100 mg polyvinyl-polypyrrolidon (PVPP) and 5 ml distilled water. Immediately after adding water, the pH was adjusted to 2.5 with 10% aqueous trifluoroacetic acid. The homogenate was centrifuged for 10 min at 5,000 rpm. The clear supernatant solution was filtered through a 0.45 µm filter (Multoclear, CS-Chromatography). About 30 µL of each sample was lyophilized (1 h at -20°C, 0.05 mbar C) directly into the vial and kept at -20°C until analysis.

Free amino acids were derivatized with O-phthalaldehyde (OPA) prior to the HPLC analysis. Chromatographic separation was performed with a LiChroCART 250-4 (Merck) provided with precolumn Lichrospher 100 RP-18 (5 µm) (Merck, VWR international). Chromatographic analysis were made with a reverse phase binary gradient [A:1.6 L sodium acetate solution/glacial acetic acid (50 mmol.$L^{-1}$; pH 6.2), 50 mL MeOH (Lichrosolv ®, gradient grade); 20 mL tetrahydrofuran (Lichrosolv®, gradient grade); B: 200 mL sodium acetate solution/glacial acetic acid (50 mmol $L^{-1}$; pH 6.2), 800 ml MeOH (Lichrosolv ®, gradient grade)] at a flow rate of 1.3 mL.$min^{-1}$.The OPA derivatization procedure was performed [29]. Twenty µL of the derivative samples were injected into a column for separation and were subsequently detected with Hitachi F-1050 fluorescence spectrophotometer. The column temperature was 30°C. The autosampler was from Merck-Hitachi AS- 4000. The solution degasser consisted of the Degassex DG-4400 from Phenomenex. The quantity of amino acids was calculated via peak areas of chromatograms from standard mixture containing 1-10 pmol.$\mu L^{-1}$ of each amino acid. Total free amino acid concentrations were obtained by summation of the individual amino acid concentrations.

Proline content was determined spectrophotometrically [30]with some modifications. Acid-ninhydrin was prepared by warming 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 homogenized in 8 mL of 3% aqueous sulfosalicylic acid and the homogenate filtered through Whatman $N^o2$ 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 asa 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 was subjected to analysis of variance using SPSS 18.0 Software for Windows, followed by Tukey post hoc test. Pearson's correlation test was performed to establish correlations among variables. Cluster analysis, based on the severity of necrosis, amino acid and soluble sugar contents, using the unweighted pairwise group methods with an arithmetical average (UPGMA) on the basis ofNei's genetic distance, were performed with the assistance of SPAD 5.5.

***Heterosis***

The methods for calculating heterosis were as follows [31]:
Mid-parent heterosis (MPH) = [F1 -MP] x 100 / µ
Best-parent heterosis (BPH) = [F1 -BP] x 100/ µ
In above formula, F1 denotes hybrid value; MP denotes the mid-parent value of both parents; BP is the value of the best parent and µ denotes the average value of all parents and F1 combinations in the factorial mating design.

***Heritability***

Broad-sense heritability ($h^2_{bs}$) estimates of each cross were calculated for traits related to necrotic lesion size, soluble amino acid and sugar contents, using the relationships as follows [32]:
$Vp = V_G + V_E$
$h^2_{bs} = V_G / V_G + V_E$
$h^2_{bs} = [V_{F1} – (V_{P1} + V_{P2})/2]/ V_{F1}$
Where: $V_{F1}$ = the variance of any cross; $V_{P1}$ = the variance of female parent and $V_{P2}$ = the variance of male parent.
Estimation of environmental variance ($V_E$) for any cross was calculated as $(V_{P1} + V_{P2})/2$. In each cross, variances between F1 and parents were obtained from the analysis of variance following a completely randomized design, assigning replication as classes.

## III. Results

***Necrotic lesion size and variation of amino acids and soluble sugars***
***F12 and F40 families***

Six days after infection, the necrosis was less pronounced on hybrids than on parents (Table 1).

In healthy conditions, the content of amino acids was much greater in the leaves of F40, with respective values of 3.5 mg. g-1 dry weight. In wounded conditions, the content of amino acids increased in parents and 62.5% of hybrids (data not shown). Infection resulted ina significant increase in soluble amino acid contents for parents and 69% of hybrid genotypes.

For soluble sugar contents, the injury and the infection were characterized by an increase in parental genotypes and a decrease in hybrids.

***F79 and F16 families***

All hybrids displayed necrotic lesion size smaller than their two parents six days after *Phytophthora megakarya* infection (Table 1).

Here, the injury is accompanied by an increase in amino acids in parents, while infection doesn't have any significant effect on amino acid contents.

Likewise, wounded leaves provoked a decrease in soluble sugar contents in parents and in 50% of hybrids. The same results were also obtained in infected leaves.

Significant negative correlations were observed ($r = -0.78$, $P<0.05$) between amino acid contents, soluble sugar contents ($r = -0.73$ at $P<0.05$) and necrotic lesion size (Table 2). Genotypes with low amino acid and soluble sugar contents in infected conditions developed severe necrosis.

The value of heterosis (hybrid vigor) for amino acids and soluble sugars in F12-F40 and F79-F16 hybrids was estimated in six-day infected leaves (Table 3). For amino acids, F12.06 and F40.02 hybrids displayed in their respective families the highest MPH and BPH among other individuals while F12.09 and

F40.02 showed the lower hybrid vigor in their families. For soluble sugars, only 2/9 and 6/7 populations of F12 and F40 families respectively displayed both a positive MPH and BPH.

In F79-F16 families, all hybrids exhibited negative heterosis values for amino acids (Table 3).

Some specific hybrids (F12.06, F40.02, F79.04 and F16.02), determined in their family by a high heterosis value for amino acids would be used for qualitative analysis of free amino acids using HPLC method.

Determination of broad-sense heritability estimates in all crossingsshowed the same heritability value (0.45 in averages) for the severity of the disease (Table 4). The same observation was made in F79 and F16 families for amino acids and in F12 and F40 for soluble sugars.

Considering all the tested parameters (severity of necrosis, amino acid and soluble sugar contents), a dendrogram was built. The intercrossing between sensible parental clones (ICS40 and SCA12) generated a dendrogram where derived offspring's were divided into three groups irrespective of the family origin (Fig. 1A). However, crossing the tolerant ones (SNK 16 and T79/501) together, the dendrogram presented a clear discrimination between hybrids belonging to F16 and F79 families (Fig. 1B).

***Free amino acids***

The HPLC analysis of free amino acids of parents and hybrids displaying the highest heterosis values for amino acids from different families was performed (Table 5). Phenylalanine was not detected in leaves in different treatments. ICS40 sensible clone lacked glycine, histidine and tyrosine while these amino acids were present in tolerant ones (SNK16 and T79/501) in wounded and infected conditions.

In the same way, alanine was not detected in healthy leaves of sensible ICS40 and SCA12 clones, but appeared after injury and infection. Aspartate, glutamate, glutamine, proline and GABA were detected in leaves of all samples and these amino acids increased during abiotic (injury) and biotic (six days *Phytophthora* - Inoculated leaves) stress. The increase was more pronounced for proline and GABA (Fig. 2). For proline, the increase is in average 18 and 36% after injury and inoculation respectively. The increase of GABA is 30% in injury condition and 61% during infection (data not shown).

## IV. Discussion

The length of necrosis was evaluated in six-day infected leaves by *Phytophthora megakarya* from four cocoa clones and their offspring's derived from reciprocal crossings. Apart from T79/501 clone characterized by a low necrosis length (6.26 cm), there is no significant difference between the three other parental clones. This result confirmed the fact that T79/501 clone is characterized as tolerant to *P. megakarya*[33].

Like other nitrogen compounds, amino acids are important nutrients that are transferred from the plant to the fungus and, can be used as nitrogen sources by the fungus. Analysis of the total amino acid contents showed higher levels in the injury and inoculated material for all individuals. The increase in amino acid contents in infected leaves could account for a higher synthesis of disease-related molecules like phenols and hydroxyproline-rich glycoproteins which generally accumulate in fungus-infected plants [34]. Furthermore, in our study, a significant negative correlation ($r = -0.78$, $P<0.05$) was found between the level of amino acids and the lesion size.

In response to abiotic and biotic stress, cocoa plant gave a mitigate reaction according to amino acids. Some authors [34] found that total amino acids content increased during infection of cocoa pods and this metabolite was 745% higher in the less susceptible clone SNK413 than in the highly susceptible clone SNK10. On the other hand, in a study of comparative analysis of amino acidson leaves of parental genotypes, ICS95, ICS84 and hybrids,there is a significant positive relationship between amino acid contents and the severity of necrosis[9].

Quantification of proline colorimetrically showed that proline appeared in all leaf tissues irrespective of the treatment. But a drop in proline content was observed in wounded and infected leaves suggesting its implication in the defense mechanism in *T. cacao*/*P. megakarya*interaction. These results contrasted with those obtained byauthors [35][9] who found out that proline appears only in infected leaf tissues using thin layer chromatography.

HPLC analysis of free amino acids also displayed the presence of GABA in our samples. As proline, this specific non-protein amino acid increased during injury and infection especially for T79/501 tolerant cocoa clone for which injury and infection increased by 43% and 72% respectively. In fact, numerous reports have shown that high levels of GABA accumulate rapidly in plant tissues exposed to a variety of different stress. Some authors [17]suggested that GABA could function as an osmolyte and mitigate water stress. The cellular accumulation of GABA could balance the decrease in water potential that occurs during cellular dehydration and this opinion supports the biological protective role of GABA. This situation has been reported in heat-stressed cowpea cells [36]. Another role of GABA is to protect the plant from oxidative stress [37]. In fact, during colonization, the fungus induces the formation of GABAand in return, it appears to gain a nitrogen

source and this was justified by the finding that fungal GABA aminotransferase is induced *in vitro* in response to the addition of GABA.

When amino acids were individually analyzed, a clear change was observed in the patterns of amino acids in the infected tissues. Among the most abundant amino acids, significantly higher levels of aspartic acid (Asp), glutamic acid (Glu), and glutamine (Gln) were observed in the inoculated samples. In contrast, during the development of witches' broom (disease caused by *Crinipellisperniciosa*) in Brazilian cocoa, the levels of glutamine (Gln), serine (Ser), alanine (Ala), GABA, and glycine + threonine (Gly+Thr) were clearly reduced in infected plants [38].

The trend of variation in the total soluble sugar contents in plants after infection was quite varied from reciprocal crossing to another. For F40-F12 families, the main results showed an increase in soluble sugar contents of parental genotypes SCA12 and ICS40, and about 87% reduction in the hybrid genotypes under conditions of infection by *P. megakarya*. These results are in accordance with those of some authors [39]who demonstrated a reduction of hexoses in infected cucumber leaves 3 days after inoculation. In the *Theobroma grandiflorum*/*C. perniciosa* interaction, higher levels of soluble sugars (SS), starch, chlorophylls, phenolics, and tannins were observed in healthy leaves compared to infected leaves[40].

For the F16 family, soluble sugar contents increased in wounded and infected leaves. The same results were obtained by several authors. Accumulation in total soluble sugar after the infection has been reported in tobacco and papaya [41], cucumber [42] and potato [43]. Soluble carbohydrates may be involved in the synthesis of various defense chemicals such as phytoanticipin, lignin, phytoalexins, and phenolic compounds.

Nevertheless, considering all individuals studied here, a significant negative relationship was observed between soluble sugar contents and necrosis length ($r = -0.73$, $P<0.05$) confirming the assumption that this metabolite can be considered as a marker of resistance. In the same way, some authors [35]observed a negative relationship between the lesion size and carbohydrate contents in the cortex of nine clones of *T. cacao* with different degrees of susceptibility to *Phytophthora megakarya*.

Our findings suggest that the variations in amino acids and soluble carbohydrates in the *Theobroma cacao/Phytophthora megakarya* interaction may, at least to an extent, reflect the polygenic character of cacao resistance to black pod disease. This opinion joined assertion who suggested that, in the *T. cacao*/*P. megakarya*interaction, the induction of the defense mechanism is not characterized by a single metabolic signal, in spite of the modification of certain gene expressions[44].

The heterosis effect of each F12, F40, F79 and F16 families when comparing the amino acids and soluble sugars revealed high variability within both families. In fact, 44% of F12-F40 hybrids, and none for F79-F40 hybrids showed a positive heterosis for amino acids 6 days after inoculation. According to the low heterosis estimates for amino acid traits, these two combinations will provide a bad association for the regeneration of vigor hybrids. Except for the F79 family, the other three families showed a high level of heterosis estimates for soluble sugars. Hybrids belonging to these families might have genes containing additive effects in the transmission of that character [45].

Heritability of amino acid and total soluble sugar contents were estimated. Based on criteria for heritability [46], we can assume that apart from amino acids of the F79 and F16 families who presented low values of heritability, other variables displayed high heritability estimates since their values were superior to 0.3. Otherwise, no significant difference was observed between values of the broad-sense heritability in F79-F16 reciprocal crossings for amino acid contents and in F12-F40 reciprocal crossings for soluble sugar contents. The absence of a significant difference between the heritability values from reciprocal crossing generally portrays the absence of maternal heritability. For these reciprocal crossings, the heritability of biochemical compounds studied is nuclear rather than cytoplasmic. In the same way, the relationship between phenolic compounds, amino acids, carbohydrates and resistance to *T. cacao/ P. megakarya*interaction detected no maternal effect in the transmission of these characters [9][47].

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## List of tables

**Table 1.** Mean lesion size (cm) on the midribs of *Theobroma cacao*leaves, soluble amino acid and total soluble sugar contents (mg.g-1 dry weight) for parental clones (SCA12, ICS40, T79/501 and SNK16) and their reciprocal hybrids (F12, F40, F79 and F16), six days after inoculation with *Phytophthora megakarya*.

| Genotypes | Averagelesion size (cm) | Aminoacids | | | Soluble sugars | | |
|---|---|---|---|---|---|---|---|
| | | Healthy | Wounded | Inoculated | Healthy | Wounded | Inoculated |
| SCA12 | 7.1±1.2**c** | 2.0±0.1ab | 1.8±0.2a | 2.7±0.2b | 4.4±0.7c | 7.2±1.7e | 5.3±1.2d |
| ICS40 | 7.7±1.4**c** | 3.7±0.5a | 4.3±0.6b | 3.5±0.6a | 6.3±1.9c | 19.0±3.6e | 10.8±2.8d |
| F12 | 3.8±1.6**a** | 2.5± 0.9a | 3.1± 0.5ab | 3.0 ± 0.9ab | 20.5 ± 5.2e | 11.4 ± 5.5cd | 10.3 ± 5.5c |
| F40 | 3.6±0.8**a** | 3.5± 1.1a | 3.4± 0.4a | 4.4 ± 1.2b | 24.1 ± 8.6de | 14.9 ± 4.7c | 21.9 ± 12.5d |
| T79/501 | 6.2±1.1**b** | 2.9±0.2ab | 3.7±0.6b | 3.2±0.4b | 7.7±1.7d | 5.7±1.4c | 5.51.7c |
| SNK16 | 7.2±1.4**c** | 4.3±0.8a | 5.6±1.1b | 4.4±1.5a | 9.9±2.3c | 9.9±2.8c | 13.3±2.4d |
| F79 | 4.5±1.4**a** | 2.3±0.6a | 2.5±0.3a | 2.1±0.4a | 27.3±4.1c | 25.0±11.1c | 6.4±1.9b |
| F16 | 3.9±1.1**a** | 2.7±0.4a | 2.8±0.6a | 2.9±0.2a | 12.9±4.6b | 33.1±12.5c | 43.17±7.4d |

*Means with the same letter in the same line are not significantly different at the 0.05 probability level as calculated by the Tukey's test for amino acids and soluble sugars.
*Means with the same letter in the same column are not significantly different at the 0.05 probability level as calculated by the Tukey's test for lesion size.
N=3 replications x 9 individuals for F12; N=3 replications x 6 individuals for F40; N=3 replications x 8 individuals for F79 and N=3 replications x 9 individuals for F16.
SNK= selectionNkoemvone;ICS= Imperial collegeselection; T= Tafo; SCA= Scavina.

**Table 2.**Correlation coefficients among biochemical and resistant traits of four crosses of *T. cacao*.

| | Amino acids | Sugars | Necrosis |
|---|---|---|---|
| Amino acids | 1 | | |
| Sugars | 0.13 | 1 | |
| Necrosis | **-0.78*** | **-0.73*** | 1 |

* Correlation is significant at the 0.05 level.

**Table 3**. Mid-parent heterosis (MPH) and Best-Parent Heterosis (BPH) estimates of amino acids and soluble sugars of hybrids derived from crossing of SCA12 and ICS40 and from crossing of T79/501 and SNK16 cocoa clones.

| | Amino acids | Soluble sugars | | Amino acids | Soluble sugars |
|---|---|---|---|---|---|
| Genotypes | MPH(*BPH)* | MPH (*BPH)* | Genotypes | MPH(*BPH)* | MPH (*BPH)* |
| **F12** | | | **F79** | | |
| F12.01 | -26.07 (*-34.08)* | 3.95 (*-22.52)* | F79.01 | -50.59 (*-57.30)* | -12.68 (*-38.26)* |
| F12.02 | -10.58 (*-20.28)* | 188.53 (*115.07)* | F79.02 | -58.39 (*-64.04)* | -8.22 (*-35.11)* |
| F12.03 | 7.74 (*-3.94)* | 44.64 (*7.81)* | F79.03 | -45.64 (*-53.03)* | -15.97 (*-40.59)* |
| F12.04 | 8.69 (*-3.10)* | 11.47 (*-16.91)* | F79.04 | -25.10 (*-35.28)* | -54.59 (*-67.89)* |
| F12.05 | -9.00 (*-18.87)* | 22.81 (*-8.46)* | F79.05 | -34.72 (*-43.60)* | -31.46 (*-51.54)* |

| | | | | | |
|---|---|---|---|---|---|
| F12.06 | 20.70 (*7.61)* | -64.00 (*-73.16)* | F79.06 | -50.59 (*-57.30)* | -19.79 (*-43.29)* |
| F12.08 | 3.00 (*-8.17)* | -11.84 (*-34.28)* | F79.07 | -53.97 (*-60.22)* | -57.98 (*-70.29)* |
| F12.08 | -14.38 (*-23.66)* | 34.16 (*-0.74)* | F79.08 | -48.24 (*-55.28)* | -53.63 (*-67.22)* |
| F12.09 | -33.02 (*-40.28)* | 13.19 (*-15.63)* | - | - | - |
| **F40** | | | **F16** | | |
| F40.01 | -18.48 (*-27.32)* | 13.56 (*-15.35)* | F16.01 | -15.47 (*0.31)* | 333.00 (*639.31)* |
| F40.02 | 94.94 (*73.80)* | 218.00 (*137.04)* | F16.02 | -15.21 (*0.62)* | 323.24 (*622.64)* |
| F40.03 | -12.80 (*-22.25)* | 189.27 (*115.63)* | F16.03 | -15.73 (*0.00)* | 232.84 (*468.30)* |
| F40.04 | 32.70 (*18.31)* | 440.07 (*302.57)* | F16.04 | -30.56 (*-18.59)* | 243.87 (*487.14)* |
| F40.05 | 37.44 (*22.54)* | 42.17 (*5.97)* | F16.05 | -30.56 (*-17.59)* | 405.57 (*763.22)* |
| F40.06 | 44.71 (*29.01)* | 120.96 (*64.71)* | F16.06 | -27.96 (*-14.51)* | 435.49 (*814.31)* |
| F40.07 | -3.63 (*-14.08)* | 59.93 (*19.21)* | F16.07 | -18.08 (*-2.78)* | 402.49 (*757.97)* |
| - | - | - | F16.08 | -25.62 (*-35.73)* | 421.38 (*268.64)* |
| - | - | - | F16.09 | -23.54 (*-33.93)* | 429.34 (*274.27)* |

MPH = Mid-parent heterosis; *BPH= Best-parent heterosis*

**Table 4.**Broad-sense heritability estimates for 6-days necrosis, soluble amino acids and soluble sugars in *T. cacao/P. megakarya* interaction.

| | Crossings | 6-Days necrosis | Amino acids | Soluble sugars |
|---|---|---|---|---|
| F12 | (♀) SCA12 × (♂) ICS40 | 0.45 | 0.67 | 0.68 |
| F40 | (♀) ICS40 × (♂) SCA12 | 0.47 | 0.46 | 0.61 |
| F79 | (♀) SNK16 × (♂) T79/501 | 0.45 | 0.13 | 0.35 |
| F16 | (♀) T79/501 × (♂) SNK16 | 0.46 | 0.17 | 0.76 |

SNK= selectionNkoemvone;ICS= Imperial collegeselection; T= Tafo; SCA= Scavina.

**Table 5.**Summary of HPLC analysis of amino acids (µg.g-1 DW) in healthy (H), wounded (W) and 6- days *Phytophthora* -Inoculated leaves of F12, F40, F79 and F16 families.

| Genotype | Treatment | Asp | Glu | Gln | Gly | Arg | His | Ala | Tyr | Trp |
|---|---|---|---|---|---|---|---|---|---|---|
| SCA12 | H | 8.0 | 32.15 | 6.07 | nd | 8.75 | nd | 1.01 | nd | nd |
| | W | 11.3 | 38.17 | 9.41 | 1.12 | 12.05 | nd | 1.45 | 2.15 | 1.46 |
| | I | 15.6 | 44.56 | 12.05 | 2.25 | 17.4 | 1.56 | 2.16 | 3.05 | 2.52 |
| ICS40 | H | 4.1 | 25.15 | 4.58 | nd | 4.32 | nd | nd | nd | nd |
| | W | 7.0 | 30.58 | 7.18 | nd | 7.89 | nd | 0.75 | nd | 1.4 |
| | I | 12.1 | 36.18 | 9.47 | nd | 13.03 | nd | 1.09 | nd | 1.49 |
| SNK16 | H | 10.8 | 30.14 | 6.12 | nd | 10.44 | nd | 1.56 | nd | 1.7 |
| | W | 12.0 | 37.26 | 8.97 | 1.03 | 12.99 | 2.15 | 1.64 | 3.12 | 2.15 |
| | I | 16.1 | 49.56 | 11.49 | 2.31 | 15.75 | 3.18 | 1.96 | 3.8 | 3.05 |
| T79/501 | H | 11.8 | 40.44 | 7.12 | nd | 11.08 | 1.43 | 1.67 | 1.15 | 1.52 |
| | W | 14.5 | 42.18 | 10.53 | 1.35 | 15.18 | 3.16 | 2.19 | 3.16 | 2.45 |
| | I | 17.4 | 53.16 | 13.26 | 2.05 | 17.21 | 5.95 | 2.75 | 3.56 | 2.94 |
| F12.06 | H | 9.01 | 34 | 6.75 | nd | 9.7 | nd | 1.36 | nd | nd |
| | W | 12.3 | 39.06 | 10.41 | nd | 12.91 | 2.05 | 1.79 | 2.97 | 1.2 |
| | I | 16.1 | 41.81 | 12.36 | 2.45 | 16.92 | 4.95 | 3.05 | 3.56 | 3.05 |
| F40.02 | H | 9.8 | 37.46 | 8.52 | nd | 9.7 | 1.15 | 0.97 | 2.01 | nd |
| | W | 12.1 | 40.18 | 11.18 | nd | 12.35 | 3.19 | 1.56 | 3.51 | 1.1 |
| | I | 17.0 | 43.26 | 15.5 | nd | 17.43 | 5.78 | 2.98 | 3.97 | 2.94 |
| F79.04 | H | 12.5 | 38.51 | 7.52 | nd | 11.93 | 2.05 | 1.96 | nd | 1.9 |
| | W | 15.1 | 40.26 | 12.26 | 1.95 | 14.7 | 3.18 | 2.58 | 3.22 | 2.53 |
| | I | 18.1 | 45.13 | 15.18 | 2.15 | 18.52 | 4.07 | 2.91 | 4.05 | 3.18 |
| F16.04 | H | 8.4 | 30.18 | 5.14 | nd | 7.72 | nd | 0.71 | nd | nd |
| | W | 12.0 | 31.42 | 7.12 | nd | 12.98 | nd | 1.45 | 1.29 | 0.98 |
| | I | 14.6 | 34.5 | 10.41 | nd | 14.79 | nd | 1.79 | 2.31 | 1.19 |

*nd: not detected; DW: dry weight; Number of replicates = 3 assays

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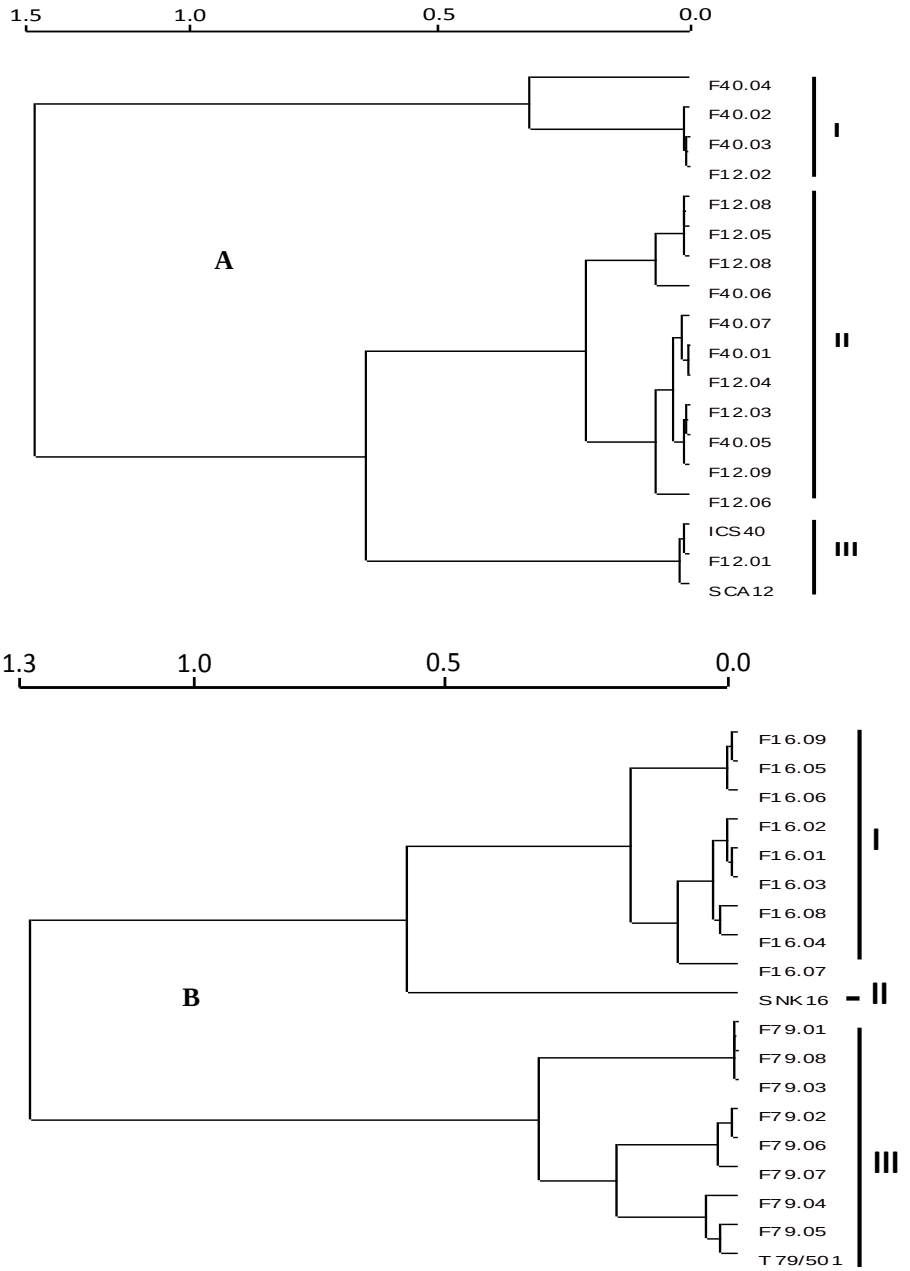


**Fig. 1**. Hierarchical classification obtained with severity of necrosis, amino acid and soluble sugar contents from F12 and F40 (A), F79 and F16 (B) families of *T. cacao*.

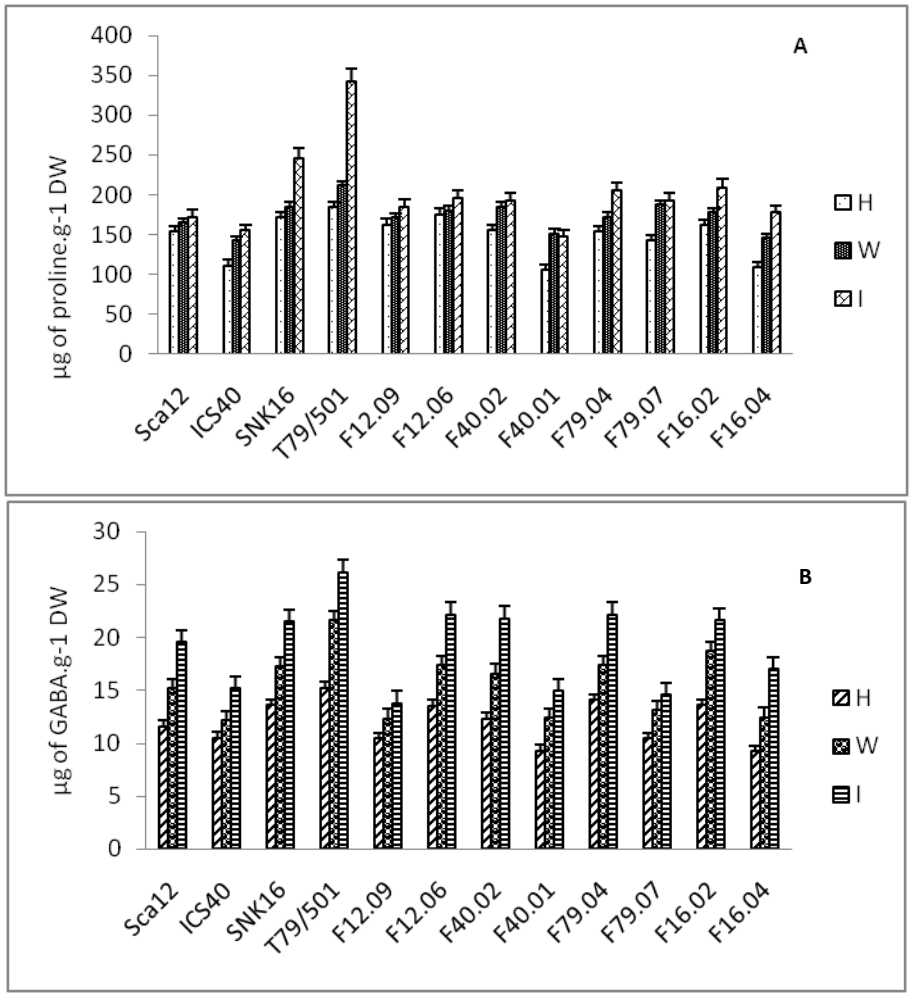


**Fig. 2.** Proline (A) and GABA (B) contents in healthy (H), wounded (W) and 6-days Phytophthora -Inoculated leaves (I) of cocoa clones and some hybrids.

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