

Caracterización bioquímica de hojas de clones de *Theobroma cacao* y su relación con los tricomas

Biochemical characterization of leaves in *Theobroma cacao* clones and its relation with trichomes

Janet Quiñones-Galvez^{*1}, Daynet Sosa^{**1,2}, JR. Demey^{***}, Silvia Alemán^{****}, Maryla Sosa^{****}; Dercy Parra^{*****}, Olga Móvil^{*****}, Reinaldo Trujillo^{*}, Yanelis Capdesuñer^{*}, Yemeys Quirós^{*}, Martha Hernández^{*3}, Diógenes Infante^{*****3}

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Resumen

Theobroma cacao L. es un cultivo de gran importancia por sus posibles aplicaciones en la farmacia y la agricultura. Contiene metabolitos que resultan de gran interés por sus funciones biológicas naturales en la respuesta de la planta a los diferentes tipos de estrés, tanto bióticos como abióticos. La presencia, tipo y concentración de los metabolitos (primarios y secundarios) pueden variar de acuerdo al genotipo analizado y se pueden acumular en estructuras asociadas a la arquitectura de la hoja como son los tricomas. El objetivo de este trabajo fue caracterizar 26 clones del banco de germoplasma de Padrón, INIA-Miranda, Estado Miranda, Venezuela y estudiar la relación existente entre los indicadores bioquímicos y el número de tricomas. Se determinó el contenido de compuestos fenólicos, proteínas solubles, prolina libre, carbohidratos totales, ligninas, el número de tricomas y la relación existente entre ellos. Los resultados mostraron que en el espacio bidimensional que explica el 46,68% de la variabilidad total, se definieron cinco grupos homogéneos: diez clones se asociaron con el máximo gradiente de prolina, carbohidratos totales y contenido de ligninas, nueve con alto contenido de proteínas y fenoles y siete con mayor número de tricomas en hojas. Se comprobó que el número de tricomas no se relacionó con la concentración de compuestos fenólicos. Los clones de cacao del tipo forastero mostraron mayor concentración de fenoles totales y carbohidratos, en tanto que los trinitarios presentaron mayor concentración de proteínas y prolina.

Key words: análisis de componentes principales; fenoles; prolina; proteínas; carbohidratos.

Abstract

Theobroma cacao L. is a crop of major importance for their potential applications in pharmacy and agriculture. It contains metabolites that are of great interest for its natural biological functions in plant response to different types of stress, both biotic and abiotic. The presence, type and concentration of both primary and secondary metabolites may vary depending on the genotype analyzed and these compounds can accumulate in the architecture associated structures such as leaf trichomes. The aim of this study was to characterize 26 clones of the germplasm bank of Padrón, INIA-Miranda, Miranda

* MSc. Janet Quiñones-Galvez, Dra. Martha Hernández, Dr. Reinaldo Trujillo, MSc. Yanelis Capdesuñer, Ing. Yemeys Quirós, Centro de Bioplantitas, Universidad de Ciego de Ávila, Carretera a Morón km 9 ½. Ciego de Ávila, Cuba. Email: jquinones@bioplantitas.cu, janet.quinonesgalvez@gmail.com.

** Dra. Daynet Sosa, Fundación Instituto de Estudios Avanzados IDEA, Carretera Nacional Hoyo de la Puerta. Valle Sartenejas. Baruta. Caracas, Estado Miranda, Venezuela. Universidad Estatal de Milagro, UNEMI, Milagro; Centro de Investigaciones Biotecnológicas del Ecuador, CIBE, Escuela Superior Politécnica del Litoral, ESPOL, Guayaquil; Guayas, Ecuador (Dirección actual).

*** Dr. JR. Demey, Fundación Instituto de Estudios Avanzados IDEA, Carretera Nacional Hoyo de la Puerta. Valle Sartenejas. Baruta. Caracas, Estado Miranda, Venezuela. Escuela Superior Politécnica del Litoral, ESPOL, Facultad de Ciencias Naturales y Matemáticas, Guayaquil, Ecuador (Dirección actual).

**** MSc. Silvia Alemán, MSc. Maryla Sosa. Universidad de Matanzas, Matanzas, Cuba.

***** MSc. Dercy Parra, MSc. Olga Móvil. Estación Experimental de Padrón, INIA-Miranda, Caucagua, Estado Miranda, Venezuela.

***** Dr. Diógenes Infante, Fundación Instituto de Estudios Avanzados IDEA, Carretera Nacional Hoyo de la Puerta. Valle Sartenejas. Baruta. Caracas, Estado Miranda, Venezuela.

¹ Ambas autoras tuvieron igual porcentaje de participación, por lo que se consideran autores principales.

² Codirectora del proyecto de investigación

³ Directores del proyecto de investigación

State, Venezuela and study the relationship between biochemical indicators assessed and the number of trichomes. The content of phenolic compounds, soluble protein, free proline, total carbohydrates, lignins, the number of trichomes and the relationship between all traits was determined. The results showed that in the two dimensional space explained 46.68% of the variability, five homogeneous groups were defined: 10 clones were associated with the maximum gradient of proline, total carbohydrate and lignin content. Nine clones were associated with high content of phenols and a group of seven clones with the highest number of trichomes on leaves. It was found that the number of trichomes was not related to the concentration of phenolic compounds. The clones of Forastero cocoa type showed higher concentration of total phenols and carbohydrates, meanwhile the Trinitario type presented higher concentration of proteins and proline.

Key words: principal components analysis; phenols; proline; proteins; carbohydrates.

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Introduction

Metabolites are responsible for many essential functions and phenomena for plant growth and development, as well as plants' interaction with their environment. They include compounds of great importance for the growth and survival of plants, such as phenols, proteins, proline and carbohydrates (Petti and Scully, 2009; Yooyongwech *et al.* 2012; Mulekar and Huq, 2014; Van den Ende, 2014).

Phenolic compounds are secondary metabolites with a complex nature in plants. They are related to the processes of maturation, defense mechanisms, and response to biotic and abiotic stress. In turn, they have a great influence on the organoleptic properties of food products derived from plants (Kubo and Matsumoto, 1984; Cimato *et al.* 1990; Niemenak *et al.* 2006). While proteins, proline and carbohydrates are primary metabolites that, like phenols, may be involved in resistance mechanisms to biotic and abiotic stress, as is the case of proteins related to pathogenesis (PR) (Martinez-Esteso *et al.* 2009), proline is related to the response to drought in some plants (Wagner *et al.* 2003). The carbohydrates on the leaf surface have an important role in the early colonization and growth of fungus, as well as sucrose which facilitates the formation of biofilms of bacteria in the culture medium (Lindow and Brandl, 2003; Tomihama *et al.* 2006).

Theobroma cacao L. (*T. cacao* L.) is cultivated throughout the tropical areas of the world (Elwers *et al.* 2009), although it is a native plant of the tropical region of the Americas (Rusconi and Conti, 2010). It is used in popular medicine as an antiseptic, diuretic and parasiticide (Edwards *et al.* 2005), and these properties are associated with the varied metabolites it contains. The same metabolites can be found in different plant organs (leaves, stems, roots, flowers, seeds and trichomes) according to their biosynthetic route and their biological function (Ávalos and Pérez-Urria, 2009). Currently, there are studies with the aim to analyze the quality and flavor of chocolate through the characterization of changes in the composition of proteins and phenols during the maturity

of the cacao fruits, the fermentation of the seeds and the key steps in the industrial production of chocolate (Pirovani *et al.* 2008; Guehi *et al.* 2010; Afoakwa *et al.* 2012a; Afoakwa *et al.* 2012b).

Plants with higher concentrations of proteins attract great interest because of their importance for the plants' development, as well as for human benefit. Phenols are also known for their benefits for human health in diverse activities, which include, regulation of fat absorption, anti-inflammatory effect, reduction of cardiovascular disease by reducing the synthesis of lipids, and as antioxidants (Guerrero *et al.* 2013; Gu *et al.* 2014; Sarria *et al.* 2014). They are also known for their antimicrobial activity against pathogens that affect plants and humans (Godard *et al.* 2009; Li *et al.* 2009; Vivot *et al.* 2009; Mishra *et al.* 2010; Pandey and Mishra 2010).

The leaf surface in the majority of plants is covered by modified cells of the epidermis that are called trichomes (Peter and Shanower, 1998). The compounds found in the trichomes of the leaf surface may be essentially involved in defense against different light conditions and the attack of pathogens and insects (Wagner, 1991; Peter and Shanower, 1998; Wagner *et al.* 2004; Ishida *et al.* 2008). In cacao plants, it was found that the glandular trichomes may provide the point of entry to *Trichoderma* species in the stem, contributing to systematic colonization of the tissue and establishment of an endophytic association (Bailey *et al.* 2009).

Therefore, and as far as we have knowledge, there is a lack of information in terms of the biochemical similarities and differences, as well as the relationship that exists between the trichomes and the chemical composition of the clones of *T. cacao* L. that are found in the germplasm bank of Padrón, INIA-Miranda, Venezuela. Therefore, the objective of this research was to study the existing relationship between the biochemical indicators (content of phenolic compounds, soluble proteins, free proline, total carbohydrates and lignins) and the number of trichomes to characterize the behavior in 26 clones of said bank.

Materials and Methods

Plant Material

The samples were collected in the Germplasm Bank of Estación Experimental de Padrón, INIA-Miranda, Miranda State, Venezuela (Table 1).

The samples of *T. cacao* were collected in the Germplasm Bank of Estación Experimental de Padrón, INIA-Miranda, Miranda State, Venezuela. Located at 41 m.a.s.l., at 10°13'14.1" N and 66°17'57.5" W, the sampled plants have been planted for 12 years in tropical humid forest in clay loam soil with pH 5.3, with flat topography and without irrigation. The density of the plantation was 1,600 plants per ha and it was fertilized with 100 g NPK fertilizer (12-12-17) + 50 g of urea. The humidity oscillated between 72-89% with an average rainfall of 2,441 mm.

For the biochemical analysis and count of trichomes, the four youngest green leaves were taken from plagiotropic branches of a medium age according to each evaluated clone of *T. cacao* L. They were immediately placed in liquid nitrogen and stored at -80 °C until the extractions were made.

Phenolic Compounds

The phenols were extracted and determined according to the method of Friend (1992) with modifications. To do this, the leaves (leaf blades without a petiole or central nerve) were macerated in liquid nitrogen into a fine powder (three replicas of 0.1 g of the mix of leaves from each plant material), and they were ex-

tracted three times with methanol for a final volume of 1 ml (*soluble phenols*). The residues were dried and hydrolyzed with 0.25 ml of 2 mol/l NaOH for 16 hours at 70 °C. Later, 0.25 ml of 2 mol/l HCl was added. The mixture was centrifugated at 15,000 g for 5 minutes and the supernatant was collected (*wall-bound phenols*). The residue was washed with 7 ml of distilled water and adjusted to pH 7.5 (with NaOH/HCl) and it was diluted to 10 ml with distilled water (*lignins*).

The soluble wall-bound phenols were determined with the Folin-Ciocalteu reagent. An aliquot of each extract was diluted with distilled water to 1 ml, and 0.1 ml of Folin-Ciocalteu reagent was added. After 5 minutes, 0.6 ml of saturated Na₂CO₃ was added to 1 mol/l NaOH was added. It was incubated for 60 minutes in the dark. The staining measurement used was in a spectrophotometer at 725 nm. The phenol concentration was expressed in milligrams per gram of fresh mass (FM) equivalent to a standard curve of chlorogenic acid. The total phenols were calculated with the sum of the soluble and wall-bound phenols.

For the quantification of lignins, 4 ml of 0.1 mol/l NaOH (pH 12) was added to 1 ml of each extract and 4 ml of phosphate buffer (pH 7) to another 1 ml. The absorbency was measured at 350 nm of extract at pH 12 against pH 7. The results were expressed as absorbance units (AU) for 0.1 g FM.

Soluble Proteins

The macerated leaves were used for the extraction of the soluble proteins just as was done for the phenols. One milliliter of extraction buffer was added (Urea:

Table 1. Classification of the 26 clones of *T. cacao* collected in the Germplasm Bank of Estación Experimental de Padrón, INIA-Miranda, Miranda State, Venezuela. Types: Forastero (F) and Trinitario (T).

No	Clone	Type	No	Clone	Type
1	Torno 3	T	14	PLA- 1	F
2	Concepción 164	T	15	PLA- 2	F
3	Cuira 32	T	16	PLA- 3	F
4	IMC-67	F	17	PLA- 4	F
5	Ocumare 61	T	18	PLA-1 x SC-10	T
6	Ocumare 67	T	19	EEM-003	T
7	Panaquirito 75	T	20	EEM-001	T
8	Peniche 158	T	21	Cumbo-177	T
9	UF 12	T	22	EET-250	T
10	SCA 6	F	23	PV-1 x IMC-11	T
11	SC- 6	T	24	OC-61 x PLA-159	T
12	SC- 8	T	25	IMC-67 x SC-10	T
13	SC- 10	T	26	IMC 67x Ocu 61	T

6 mmol/l, EDTA: 10 mmol/l, NaH_2PO_4 : 0.1 mol/l, β -mercaptoethanol: 14 mmol/l, Tween-20 (0.05%), Tris HCl: 10 mmol/l pH = 8) to 0.1 g of plant material (three replicas of the mix of leaves from each plant material). The mix was homogenized and centrifugated at a cold temperature of 4 °C for 10 minutes. The supernatant was collected for quantification. The concentration of TSP was determined according to Bradford, (1976) and the staining developed was measured in a spectrophotometer at 595 nm. The concentration was expressed in mg/g FM, equivalent to a standard curve of bovine blood serum albumin.

Total Carbohydrates and Free Proline

The extraction of carbohydrates and free proline was carried out on macerated leaves with the phenol procedure. Ten milliliters of methanol: 80% water (v:v) was added to 1 g of plant material (three replicas of the mix of leaves from each plant material). The mixture was shaken with a vortex and centrifugated at room temperature at 4,000 grams for 8 minutes. The extraction was repeated twice. The supernatants were collected, joined and filtered using filter paper. The ethanol phase was eliminated by vacuum in a rotary evaporator. The aqueous phase was collected for the quantifications.

The concentration of carbohydrates was determined according to the method of Dubois et al. (1956). To 1 ml of extract, 0.5 ml of phenol mixture: 5% water (v:v) and 2.5 ml of carefully concentrated H_2SO_4 , was added. It was incubated at room temperature for 30 minutes. The staining measurement used was in a spectrophotometer at 492 nm. The concentration of total carbohydrates was expressed in $\mu\text{g/g}$ FM, equivalent to a standard glucose curve.

The concentration of free proline was determined according to Bates et al. (1973). To 0.5 ml of plant extract, 0.5 ml of ninhydrin and 0.5 ml of acetic acid were added. Subsequently, it was shaken and placed in a bain-marie at 100 °C for 1 hour. Then, 1 ml of toluene was added and it was shaken strongly. The absorbency was determined in the organic phase at 520 nm. The concentration was expressed in $\mu\text{g/g}$ FM, equivalent to a standard L-proline curve.

Trichome Count

The number and type of trichomes per visual field was directly counted in an optical microscope from the epidermal sheet of the abaxial side of the 26 clones' leaves (six replicas of the total leaves of each plant material). The evaluations were made in a LEICA DM 3000 microscope with a 20x lens.

Statistical Analysis

The statistical processing of the results was carried out with the Statistical Package for Social Sciences (SPSS)

(Version 20 for Windows, SPSS Inc.). Parametric statistics were carried out (one-factor and two-factor ANOVA, and Tukey's range test). To study the relationship between the number of trichomes and the chemical composition of the leaves, the principal component analysis (PCA, principal component analysis) was used. This analysis was carried out with InfoStat (2011 Version, Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). The details of the statistical treatment appear in each figure or table of the results and discussion.

Results and Discussion

The concentration of soluble phenols, wall-bound phenols, lignin, soluble proteins and free proline in 26 clones of *T. cacao* is shown in Table 2.

For the soluble phenols, Clones 16 and 22 showed the highest values without significant differences with 9.92 and 9.71 mg/g FM, respectively, and Clone 17 showed the lowest value with 4.98 mg/g FM. While in Clones 9, 14 and 25, the greatest concentrations of wall-bound phenols was found with 13.71, 14.17 and 13.99 mg/g FM, respectively and without significant differences. The lowest value was found in Clone 22. When analyzing the total phenols, Clones 9 and 25 were the highest without significant differences between them and with concentrations of 22.69 and 23.37 mg/g FM, followed by Clones 2 and 16 with 21.87 and 21.27 mg/g FM, and subsequently, Clones 6, 14 and 18 with 19.73, 20.27 and 20.07, respectively.

It can also be appreciated that for lignins, Clone 1 with 0.35 AU had the highest content of lignins, and the lowest values were found in clones: 2, 3, 9, 20 and 26, with 0.10, 0.10, 0.10, 0.10 and 0.09 AU, respectively, and without significant differences. These results indicate a greater concentration of wall-bound phenolic compounds than the soluble phenols and do not coincide with the clones that have a greater concentration of both kinds of phenols. Phenolic compounds are an important part of plants' active defense. These compounds can be synthesized and mobilized in stress conditions and are sometimes esterified to form part of the cell wall and of lignin (Fry, 1987; Nicholson and Hammerschmidt, 1992; Cabrera-Soto et al. 2009). In this case, all the plants were exposed to similar cultivation conditions without symptoms of disease, and therefore, it cannot be confirmed that the differences in the composition of phenols are associated with the response to a specific kind of stress.

It can be appreciated that in the case of soluble proteins, Clones 3, 5 and 18 showed the highest concentrations with 5.84, 5.86 and 8.84 mg/g FM, and the lowest value was found in Clone 4 with 1.12 mg/g FM. In the total carbohydrates, the highest concentrations were found in Clones 7, 8 and 14 with 28.13, 30.73 and 31.17 $\mu\text{g/g}$ FM, respectively, while the lowest concentration was found in Clone 10 with 7.75 $\mu\text{g/g}$ FM.

Table 2. Concentration of soluble phenols (PH_s), wall-bound phenols (PH_{wB}), total phenols (PH_T), lignin (L_g), soluble proteins (P_s), total carbohydrates (C_T) and free proline (P_F) in 26 clones of *T. cacao* L.

Clone	PH _s (mg/g FM)	PH _{wB} (mg/g FM)	PH _T (mg/g FM)	L _g (AU)	P _s (mg/g FM)	C _T (µg/g FM)	P _F (µg/g FM)
1	7.30 fg	11.55 cde	18.84 ef	0.34 a	1.64 de	23.88 bc	20.55 abcde
2	8.68 de	13.19 b	21.87 b	0.10 i	4.24 bcde	19.11 def	19.94 abcde
3	7.25 fg	10.66 ghij	17.91 gh	0.10 i	5.84 ab	10.52 ijk	4.37 g
4	7.42 fg	10.42 hijk	17.85 h	0.15 efg	1.12 e	23.79 c	5.94 g
5	7.26 fg	11.49 cdef	18.75 ef	0.21 bc	5.86 ab	12.27 hij	10.59 cdefg
6	7.46 f	12.28 c	19.73 cd	0.12 ghi	1.78 de	11.59 ijk	14.98 abcdef
7	9.31 bc	10.09 jk	19.39 de	0.21 b	2.12 de	28.13 ab	15.17 abcdef
8	5.20 jk	8.84 l	14.04 o	0.16 def	4.29 bcde	30.73 a	14.12 bcdefg
9	8.98 cd	13.71 ab	22.69 a	0.10 i	3.48 bcde	14.19 ghi	22.74 abc
10	7.54 f	11.07 defgh	18.61 fg	0.16 def	2.76 bcde	7.75 k	7.32 efg
11	5.55 ij	10.25 ijk	15.80 kl	0.14 efgh	2.22 cde	17.62 efg	21.34 abcd
12	5.13 jk	7.49 m	12.62 p	0.12 ghi	4.74 bcd	17.92 efg	21.88 abc
13	6.95 g	8.33 l	15.28 lm	0.22 b	2.38 b	22.27 cd	21.33 abcd
14	6.10 h	14.17 a	20.27 c	0.13 fghi	2.05 fghi	31.17 a	13.74 bcdefg
15	6.16 h	9.87 k	16.04 jk	0.19 bcd	3.91 bcd	23.77 c	14.76 bcdefg
16	9.92 a	11.36 defg	21.27 b	0.14 fgh	1.62 fgh	21.45 cde	16.33 abcdef
17	4.98 k	11.64 cde	16.61 ij	0.11 ghi	4.51 ghi	21.52 cde	19.63 abcde
18	8.31 e	11.77 cd	20.07 cd	0.12 fghi	8.84 fghi	10.69 ijk	8.10 defg
19	5.83 hi	8.15 lm	13.98 o	0.18 cde	3.57 cde	18.71 def	28.21 a
20	5.82 hi	11.02 defgh	16.84 i	0.10 i	3.25 i	10.40 ijk	17.35 abcdefg
21	5.86 hi	8.56 l	14.41 no	0.11 hi	5.38 hi	19.44 def	23.49 abc
22	9.71 ab	5.13 n	14.83 mn	0.12 ghi	2.66 ghi	16.44 fgh	6.34 fg
23	5.97 hi	8.30 l	14.26 no	0.13 fghi	2.49 fghi	15.95 fgh	15.12 abccdefg
24	5.97 hi	10.79 fghij	16.77 ij	0.14 fgh	2.95 fgh	8.48 jk	26.30 ab
25	9.39 bc	13.99 a	23.37 a	0.14 fgh	3.26 fgh	8.88 jk	19.53 abcdef
26	5.80 hi	10.91 efghi	16.71 ij	0.09 i	2.90 i	24.82 bc	21.29 abcd
TE	0.13	0.20	0.19	0.01	0.84	1.11	3.42

* Values in each column represent the mean of each treatment. Means with different letters differ significantly according to parametric statistics of simple ANOVA, Tukey's test, $p \leq 0.05$, typical error (TE).

When analyzing the free proline, Clones 19 and 24 had higher concentrations with 28.22 and 26.29 µg/g FM, while Clones 3, 4 and 22 had the lowest concentrations with 4.37, 5.94 and 6.34 µg/g FM.

Regarding the number and type of trichomes, in Figure 1, it can be observed that glandular trichomes (indicated in Figure 1 a, g, h and i), non-glandular stellate trichomes (indicated in Figure 1 a, b, c and i) and non-glandular simple trichomes (indicated in Figure 1 d, e and f) were found.

There were no differences in the type of trichome for the 26 assessed clones. Similar behavior was observed in all cases with glandular trichomes, non-glandular stellate trichomes and non-glandular simple trichomes,

although the most frequent type was the non-glandular stellate trichome.

As can be observed in Figure 1 (a-i), the cacao trichomes showed diversity in terms of the number and type, as well as in the ramification of the non-glandular stellate trichomes. Additionally, the accumulation of colored compounds was found in them, which may be phenolic or other metabolites of interest, such as terpenes, among others (Tattini et al. 2000, McDowell et al. 2011). However, to corroborate the kind of compounds present, it is necessary to use other kinds of microscopy.

The number of trichomes varied depending on the clone, although the majority of the clones did not show

significant differences. The largest amount of trichomes was found in Clones 5, 23 and 26 with 45, 40 and 40 trichomes per square millimeter, without significant differences, and the smallest amount in Clone 10 with 15 trichomes per square millimeter. In potato and tomato leaves, the trichomes contain phenols and phenol oxidizing enzymes (polyphenol oxidases), which can kill insects, including aphids (Peter and Shanower, 1998). That is why it is of great interest to group the clones by their majority compounds, as well as assessing whether there is a relationship between the number of trichomes and the chemical compounds of the *T. cacao* leaves.

Figure 2 shows the analysis of the relationship between the 26 studied clones of *T. cacao* with their content of free proline, soluble proteins, total carbohydrates, lignins, soluble phenols and wall-bound phenols, and the number of trichomes.

The results show that in the bidimensional space, which explains 46.68% of the total variability, five

homogenous groups were defined. Group 1 (I) comprised of Clones 4, 16, 7, 10 and 1, associated with the maximum concentration of lignins. Group 2 (II) comprised of Clones 17, 12, 15, 26, 21, 23 and 24, associated with plants with leaves presenting the highest number of trichomes. Groups 3 (III) and 4 (IV) comprised of Clones 2, 5, 6, 9, 20, 22 and 25; and 3 and 18, respectively, associated with high contents of soluble proteins, soluble phenols and wall-bound phenols. Finally, Group 5 (V) comprised of Clones 14, 8, 13, 11 and 19, associated with the gradient of maximum content of free proline and total carbohydrates. When comparing these two kinds of cacao (Forastero and Trinitario) in terms of the concentration of total phenols, lignins, soluble proteins and free proline (Table 3), it was found that the Trinitario clones have a higher content of soluble proteins and free proline than the Forastero clones. While the latter exceed the Trinitario variety in total phenols (soluble phenols + wall-bound phenols) and carbohydrates.

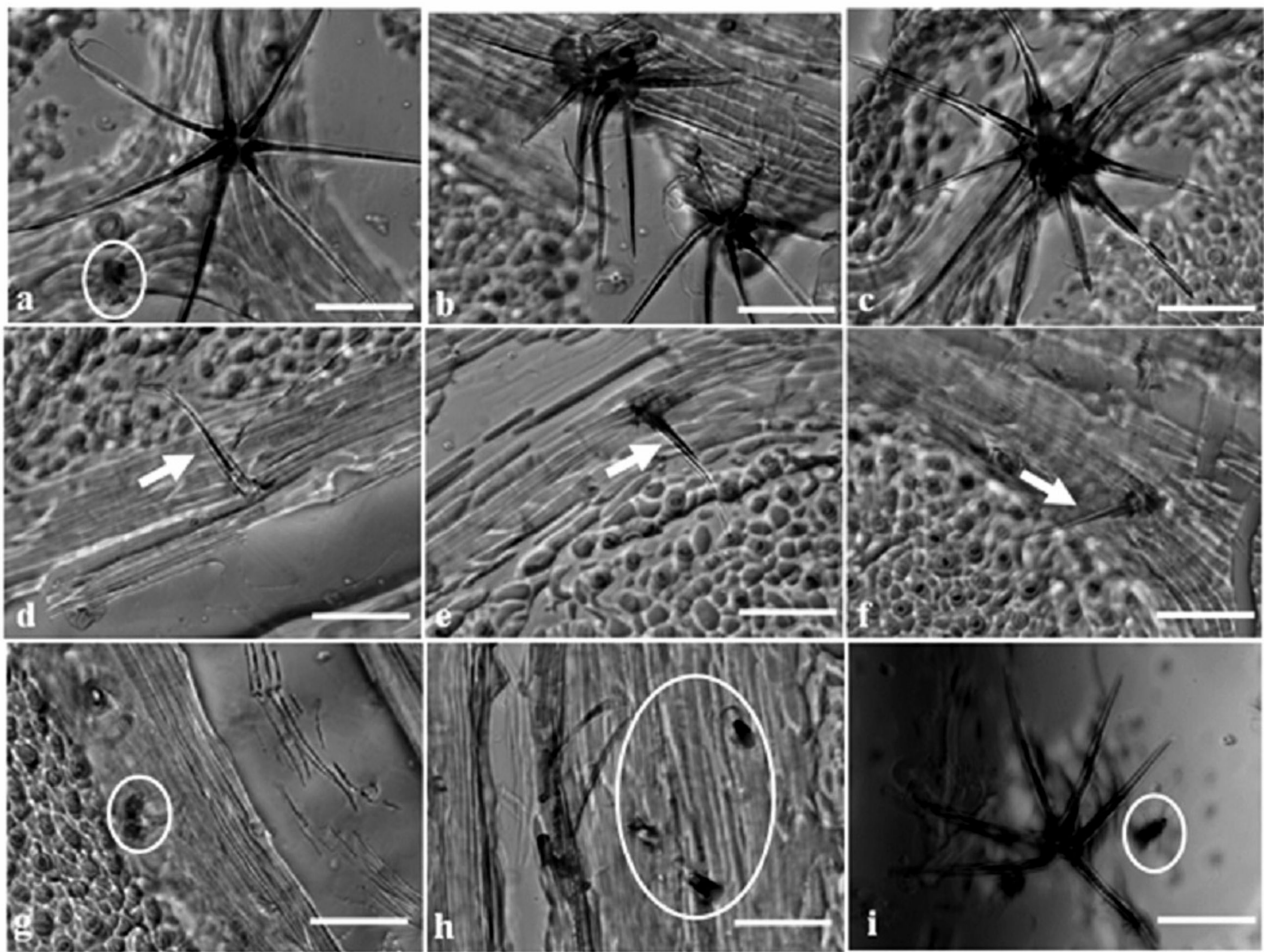


Figure 1. Types of trichome identified in the surface of adult plants of *T. cacao* L., Clone 5 (Ocumare 61). Complex, non-glandular stellate trichomes (a, b, c, i). Simple non-glandular trichomes (d, e, f). Glandular trichomes (a, g, h, i). Scale bar: 50 µm.

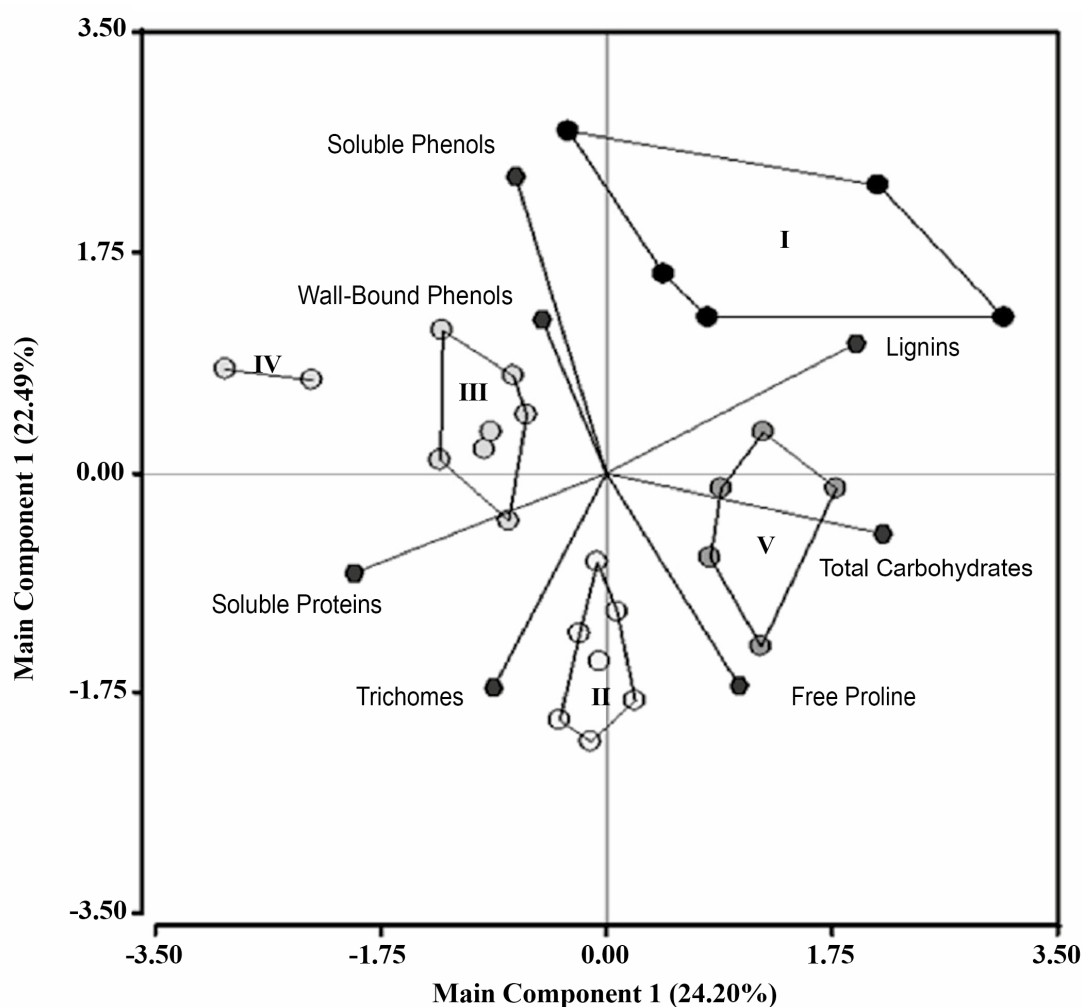


Figure 2. Relationship between the clones of *T. cacao* L. Groups comprised by Main Component 1 in free proline, soluble proteins, total carbohydrates, lignins, soluble phenols, wall-bound phenols and number of trichomes. Groups: I (Clones 4, 16, 7, 10 and 1), II (Clones 17, 12, 15, 26, 21, 23 and 24), III (Clones 2, 5, 6, 9, 20, 22 and 25), IV (Clones 3 and 18) and V (Clones 14, 8, 13, 11 and 19).

It is important to note that the substances produced by the aerial parts of the plants may be carbohydrates, organic amino acids, phenols and other compounds. The phenols include tannins, which are generally toxins because of their capacity to join to proteins, and they act as food repellents against many animals that could be harmful to the plants. However, they may have a positive effect on human health (Ávalos and Pérez-Urria, 2009). In the case of the total phenols (Table 3), their greater concentration in Forastero clones may be related to the fact that this kind of cacao is more resistant and of lower quality for the industry because of its high phenol content, which includes the tannins with a greater concentration in these clones (Figueira et al.1997).

The quantitative differences found in the phenol content (Table 2) between the clones are largely related

to the genotype, because the plants had the same age and were grown in similar conditions. This coincides with the proposal of Zapata et al. (2000), who demonstrated in tobacco that the phenols depend on the age, the stage of development and the genotype. Niemenak et al. (2006) found that the total phenols, catechin, epicatechin and anthocyanin in fresh and fermented seeds of *T. cacao* L. depended on the genotype, but qualitative differences were not observed in the content of polyphenols regarding their genetic origin. The differences were more closely related to the growth conditions. However, in this work, differences were found in the concentration of phenols, carbohydrates, proteins and proline related to the genotype and also to the genetic origin, because in the Forastero clones, the concentration of total phenols and total carbohydrates was higher than in the Trinitario clones, as long as the Trinitario clones were in their majority soluble

Table 3. Concentration of total phenols, lignin, soluble proteins, carbohydrates and free proline in Trinitario (T) and Forastero (F) clones of *T. cacao* L.

Type	Total Phenols (mg/g FM)	Lignin (AU)	Soluble Proteins (mg/g FM)	Total Carbohydrates (µg/g FM)	Free Proline (µg/g FM)
T	17.41 b	0.15 a	3.69 a	17.10 b	17.64 a
ET	0.40	0.01	0.25	0.83	0.92
F	18.44 a	0.14 a	2.66 b	21.58 a	12.95 b
ET	0.46	0.01	0.30	1.70	1.55

Values in each column represent the mean of each treatment. Means with different letters differ significantly according to parametric statistics of two-factor ANOVA, Tukey's test, $p \leq 0.05$, and typical error (TE).

proteins and free proline (Table 4). Furthermore, all the evaluated indicators varied between the different clones (Table 3).

Coinciding with Chen et al. (2005), they propose that the soluble proteins, ribulose-1.5-bisphosphate carboxylase/oxygenase (RuBisCo) and its activase content, as well as the activity of the sucrose-phosphate synthase, decrease significantly, while the content of soluble sugars, inorganic phosphate, chlorophyll and Complex II of the photosynthesis increased in rice leaves acclimatized with air enriched with CO₂. Currently, it is known that proteins are found in a low concentration in plant tissue compared to animals and bacteria. Furthermore, they are more difficult to extract in mature plants because of the thickness of the cell wall (Islam et al. 2004). In addition, the phenolic compounds combine with the proteins by covalent condensations (Carpentier et al. 2005), and proteins such as RuBisCo are very abundant, which may hinder the detection of those that are not so abundant (Neilson et al. 2010; Balbuena et al. 2011).

Matt et al. (2002) transformed tobacco plants with an antisense RuBisCo to study the consequences of damage to the metabolism of photosynthetic carbon on the metabolism of nitrogen and secondary metabolism. They demonstrated that the inhibition of photosynthesis and the decrease in the sugar content lead to the inhibition of the metabolism of nitrogen and dramatic changes in the composition of secondary metabolites of the leaves, such as chlorogenic acid and nicotine, which have a structure rich in carbon and nitrogen, respectively. They also demonstrated that the changes in the amino acid to sugar ratio were related to the nicotine to chlorogenic acid ratio. These results correspond with those presented in Table 4, where the group that presented the greatest concentration of phenols (Forastero clones), also had a greater concentration of carbohydrates.

On the other hand, proteins, carbohydrates and proline play very important roles in response to stress, and it has been demonstrated that in leaves infected by

C. pernicioso, the soluble proteins decrease (Frias et al. 1991). It has also been found that in the leaves of *T. grandiflorum* plants infected by fungus, the chlorophyll content is affected and therefore, photosynthesis and carbohydrate synthesis are also affected, as well as variations occurring in the phenol concentrations (Oliveira et al. 1997).

As can be observed, the groups that are formed with PCA are not related to the type of cacao, as the Forastero and Trinitario clones are mixed within the different groups (Figure 2). In Table 3, the group of Forastero clones showed a greater concentration of carbohydrates and phenols, the Trinitario clones showed a greater concentration of proline and proteins, and there were no differences between the two groups for lignins. However, in the PCA, the clones were grouped in a different way. Five clones were associated because of their high lignin content (three Forastero clones and two Trinitario clones), another five because of their high concentration of proline and total carbohydrates (two Forastero and three Trinitario clones), nine because of their high protein and phenol content (all Trinitario clones), and seven with a higher number of trichomes (one Forastero clone and six Trinitario clones). Unexpectedly, in the PCA, the greatest number of accumulation structures of metabolites (trichomes) was more closely related to the greater concentration of soluble proteins and the free proline than to the rest of the measured parameters.

In the case of the trichomes, it is known that the combination of glandular and non-glandular trichomes confers resistance to different pests, because the glandular trichomes contain or secrete mixtures of chemical compounds and the non-glandular trichomes serve as a mechanical barrier, above all against insects (Peter and Shanower, 1998). The clones with a larger number of phenols were not associated with the greater trichome content. This does not discard the fact there are kinds of phenols that are found in the trichomes or that are secreted by them and are of importance because of their biological activity, which makes them

interesting for pharmacy, pathogen control and obtaining pigments or aromatic substances. However, to select clones for the production of compounds in the trichomes, not only those of a greater number would have to be assessed, but also the profile of the metabolites present in the trichomes of each clone and the value of them because of their activity.

In the plants of the *Solanum* genus, there are six types of trichome, and in all of those of type 1, 4 and 6, genes were found for the biosynthesis of metabolites such as flavonoids, acetylated sugars and terpenoids (McDowell et al. 2011). To date, it has not been described what kind of compound may be in the trichomes in cacao, but it is known that there are glandular and non-glandular trichomes (Bailey et al. 2009), and that there is also a high phenol content. Therefore, there could also be synthesis and secretion of similar compounds to those described by *Solanum*.

Finally, the clones analyzed in this research were not visibly infected by any pathogen. However, within the 26 existing clones, there are some with a greater resistance to pathogens than others. Therefore, the concentrations of the analyzed compounds may indicate a greater amount of them in the resistant clones than in those susceptible to fungal diseases. For example, in the previous research carried out in the germplasm bank from which the evaluated material was selected, Clone 3 (high protein and phenol content) is resistant to *P. palmivora* and to *C. frimbriata*; Clone 8 (high carbohydrate and proline content) is moderately resistant to *P. palmivora*; Clone 4 (high lignin content) is resistant to *C. frimbriata* and has a low incidence of *M. roreri*; and Clone 24 (greater number of trichomes) is resistant to *P. palmivora* (Reyes et al. 1973; González et al. 2009; González et al. 2011; Morillo et al. 2012). Furthermore, when Clone 19 (high carbohydrate and proline content) was used as the mother clone in two crosses (19 x 4 and 19 x 22), the descendant of both showed a low incidence of *P. palmivora* in harvested fruits and a low incidence of *M. perniciosus* as plant brushes and in floral cushions. These crosses presented low rates of productivity, almond rate > 1.3, and corn rate < 22 (González et al. 2011). This corroborates that each group obtained offers the possibility of managing the clones depending on their chemical composition and future use.

Conclusions

This study demonstrated that there are five groups according to the existing relationship between the concentration of biochemical compounds and the trichomes of leaves of 26 clones of *T. cacao*. Five clones were associated because of their high lignin content; another five because of their high concentration of proline and total carbohydrates; nine because of their high protein and phenol content; and seven due to the greater number of trichomes according to the PCA,

which explains 46.68% of the total variability. Furthermore, the group of Forastero clones showed the greatest concentration of total phenols and carbohydrates, while the Trinitario clones presented a greater concentration of proteins and proline. The results obtained may serve as a basis for the selection of clones, taking into account the content of the different evaluated parameters, depending on the interest of future research.

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