

REGULAR ARTICLE

Evaluation of phenolic compounds, lignin, amino acids and carbohydrates in *Theobroma cacao* L. From three different climate regions in Venezuela

J.F. Alvarez-Barreto^{1,4*}, D. Quintero², M. Rodríguez², R. Rea³, D. Sosa^{4,5}

¹Instituto para el Desarrollo de Energías y Materiales Alternativos, IDEMA. Departamento de Ingeniería Química. Universidad San Francisco de Quito. Quito, Ecuador. jalvarezb@usfq.edu.ec, ² Escuela de Química. Universidad Central de Venezuela. Caracas, Venezuela, ³Area de Agricultura y Soberanía Alimentaria. Instituto de Estudios Avanzado, IDEA. Caracas, Venezuela, ⁴Grupo de Biotecnología. Universidad Estatal de Milagro, UNEMI. Milagro, Ecuador, ⁵Centro de Investigaciones Biotecnológicas del Ecuador, CIBE. Escuela Superior Politécnica del Litoral, ESPOL. Guayaquil, Ecuador

ABSTRACT

In Venezuela, Criollo cacao crops are distributed in different regions (eastern, central and western), and under different exploitation systems, and, in each of these regions, there is a uniqueness of materials with characteristics associated to an intrinsic, differential quality that could potentially affect chocolate quality. These regions have inherent climates that are significantly different; however, there is a lack of consistent technical information about the real effects of environmental factors on the organoleptic quality of cacao. Therefore, the present work aims to evaluate the content of important components of cacao, such as phenolic compounds, lignin, amino acids, and carbohydrate, in six *Theobroma cacao* L clones, cultivated in three different agro-climatic locations: humid, semi-humid and dry tropical forest. It was found that production of phenolic compounds and lignin vary in clones according to their location, with the highest values found in the humid forest and the lowest in the dry climate. Non-structural carbohydrates, on the other hand, were found in greater amounts in specimens from the dry forest. The largest production of proline was found in the humid forest for most of the clones, implying that high humidity levels promote the production of amino acids, a pattern followed by phenolic compound content as well. The results presented in this study indicate that there is a climate-dependent production of important metabolites, which play an important role in the organoleptic properties of cacao, and this could potentially translate into different levels of resistance to stress and diseases.

Keywords: Cacao; Climate effects; Metabolites; Organoleptic properties; Resistance

INTRODUCTION

In Venezuela, cacao crops are distributed in different regions (eastern, central and western), and under different exploitation systems, and, in each of these regions, there is a uniqueness of materials with characteristics associated to an intrinsic, differential quality. Therefore, a wide cultivar diversity exists, regarding disease tolerance and responses, related to variations or fluctuations of climatic parameters, similar to those found in the rest of the world (from Mexico to the Amazon basin). As a result, there is a great number of crop types that stem from processes of domestication and adaptation to particular climate conditions. Thus, the eastern region is rich in Forastero and hybrid cacao types, while the central and western regions are dominated by

Trinitario and Criollo, and pure Criollo types, respectively (S. Alvarez, Marsh, Schroeder, & Schachtman, 2008) (Rivas & Pavone, 2010).

Chocolate quality fundamentally depends on kernel characteristics, and, according to (Molina, Pérez-Martínez, Demey, Isturiz-Zapata, & Sosa, 2016), the latter is influenced by factors such as genotype (crop of cacao group), climate, soil, agronomic and phytosanitary management, and the technology involved.

There is a lack of consistent technical information about the real effects of environmental factors on organoleptic quality of cacao. Some researchers conclude that these effects are not significant when compared to those caused by

*Corresponding author:

J.F. Alvarez-Barreto, Campus Cumbaya, Edif Newton N104H. Diego de Robles y Via Interoceánica. Quito, Ecuador.
Tel: +593-2971700 ext. 1328. E-mail: jalvarezb@usfq.edu.ec

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genotype and post-harvest treatments. However, individual and/or synergistic effects of these factors could negatively or positively affect the kernel's organoleptic attributes (Quiñones Galvez et al., 2015) (Saltini, Akkerman, & Frosch, 2013).

Nevertheless, (Dominguez & Cirigliano, 1997) mention that the Ocumare 61 clone planted in the coastal valleys of Aragua state (tropical, dry forest) presented a lower kernel index than that found in the Barlovento region (humid tropical forest). On the other hand, (Saltini et al., 2013) mentioned that water and nutrient deficiency in the soil triggers a kernel size reduction, and thereby a decrease on pod size, translating into significant variations on the cotyledon's biochemical composition. Furthermore, other authors report that the plant's nitrogen metabolism is sensitive to the environment (Croteau, Kutchan, & Lewis, 2000) (Wollgast & Anklam, 2000) (Nygren & Leblanc, 2015).

Plants are subjected to different types of environmental stress that cause harvest losses and a decrease on product quality; these effects are often location or crop specific. The triggers are numerous, including increase in UV radiation, water deficit, high salinity, hypoxia, higher temperatures, and metal, herbicide and fungicide toxicity (Croteau et al., 2000) (Wang et al., 2016).

Regarding Venezuela, it is imperative to understand the biochemical, physiological and molecular differences as responses of different genotypes to varied agro-climatic conditions. Additionally, it is important to point out that C, N, lignin and polyphenol composition varies greatly at inter and intra-specific levels (Schroth & Sinclair, 2003) (Caprioli et al., 2016), depending on the species, part of the plant, soil fertility and water availability (Li et al., 2013).

The effect of shade on cacao, including clone diversities, has been analyzed (Costa, De Almeida, & Valle, 2001) (Gidoín, Avelino, Deheuvels, Cilas, & Bieng, 2014).

(Daymond & Hadley, 2004) studied cacao genotype susceptibility to temperature, finding that cacao's great variability is in part due to responses to thermal stress pertaining to temperature and light.

In many of Venezuela's cacao producing regions, there are short and prolonged periods with decreased precipitation that affect plant growth, photosynthetic performance, water usage efficiency (WUE), and productivity. Along this line, the work by (Daymond & Hadley, 2004) tackle differences in photosynthesis and WUE in response to the variation in water availability in soils from different climate regions in Venezuela. Similarly, (García Lozano & Moreno Fonseca, 2015) describe a high phenotypic and/or physiologic plasticity and differentiation among different crops in seasons with different water availability (rain and drought).

Despite these previous indications of phenotype-environment interactions, a profound study is yet to be conducted relating the environment with the organoleptic properties of Venezuelan Criollo cacao. Thus, the present study aims to evaluate the content of phenolic compounds, lignin, amino acids, and carbohydrate in six *Theobroma cacao* L clones, from three different agro-climatic localities from the Venezuelan territory. The locations were chosen within the state of Miranda, a cacao producer area, with diverse climate; these were: Padron (humid, tropical forest), Tesoro (semi-humid tropical forest), and Tacariguita (dry tropical forest).

MATERIALS AND METHODS

Experimental Design

Six to Eight *Theobroma cacao* L leaves were randomly collected from six cacao clones from three different locations, as shown in Table 1. All clones were donated by the Germplasm bank from Venezuela's National Institute of Agricultural Research (INIA- Miranda). Table 2 shows the sampling coordinates and date of seeding for each of the groups.

Table 1: Coding of *Theobroma cacao* L clones according to their climatic location

Genotypes	Location Name		
	Tacarigüita (Dry tropical forest)	Padron (Humid tropical forest)	Tesoro (Semi-humid tropical forest)
1***	EEM029	EET250	427
2*	EEM011	CUMBO177	421
3*	EEM012	SC10	SC10
4**	EEM010	PENICHE 158	PENICHE 158
5*	EEM028	PV1XIMC11	439
6*	EEM007	OC61XPLA159	443

* National materials. Some are derived from crosses among clones developed in Venezuela (Morillo et al., 2008).

*** (Ecuador). Turnbull, C.J. and Hadley, P. *International Cocoa Germplasm Database (ICGD)*. [Online Database]. NYSE Liffe/CRA Ltd./University of Reading, UK. Available: <http://www.icgd.reading.ac.uk> (16th April, 2012).

** (VENEZUELA) Turnbull, C.J. and Hadley, P. *International Cocoa Germplasm Database (ICGD)*. [Online Database]. NYSE Liffe/CRA Ltd./University of Reading, UK. Available: <http://www.icgd.reading.ac.uk> (16th April, 2012)

Table 2: Sampling coordinates, climatic conditions and sowing date of each group

Location	Climate Type	Height (m above sea level)	Coordinates
Tacarigüita, Brion Municipality, Miranda	Dry forest. Average Temperature: 27°C. Average Annual Precipitation: 979mm	52	N 10°26'37,2"x W 66°12'40,2"x
El Tesoro, Andres Bello Municipality, Miranda	Semi-humid tropical forest. Average Temperature: 22°C. Average Annual Precipitation: 1440mm.	12	N 10°15'16,7"x W 66°04'12,5"x
Padron, Acevedo Municipality, Miranda	Humid tropical forest. Average Temperature: 18°C. Average Annual Precipitation: 2000mm.	38	N 10°13'14"x W 66°17'57"x

The collected leaves were submerged in liquid nitrogen to stop their metabolism. The plant material was later washed with distilled water, dried with absorbent paper towels, and manually macerated in liquid nitrogen. For each clone, three (3) samples of macerated foliar material were weighed, and three extractions were carried out from them, in order to later quantify the metabolites.

Determination of phenolic compounds

500 µL of ethanol were added to 100 mg of macerated tissue, followed by agitation and centrifugation at 12000 rpm for 5 min, and finally collecting the supernatant. This procedure was repeated twice more but using 250 µL of ethanol. The supernatants were gathered in a microcentrifuge tube, and saved for the determination of soluble phenolic compounds. The resulting pellet was re-suspended in 250 µL of 2M NaOH, and incubated at 70°C for 16 h. Finally, the solution was neutralized, centrifuged at 12000 rpm for 5 min, and the supernatant was used to determine wall-linked phenolic compounds. The remaining solids were saved for lignin quantification.

To quantify total phenols, 100 µL of the ethanol extract from each foliar sample were mixed with 900 µL of distilled water and 100 µL of Folin-Ciocalteu reactant, and the mixture was incubated for 5 min. After that, 600 µL of 1M NaOH saturated with Na₂CO₃ were added, and the solution was incubated for 1 h at room temperature. Absorbance was then read at 725 nm in a Tecan Infinity M200 spectrophotometer. Phenol content in the sample was determined through a calibration curve made with known solutions of chlorogenic acid.

Determination of carbohydrates

Macerated tissue (250 mg) was mixed with 2.5 ml of 80% v/v ethanol, followed by homogenization and centrifugation at 4000 rpm for 8 min. This process was done three times, and the supernatants were collected. 50 µL of this ethanol extract were placed in a vial, and air dried, to later add 0.5 ml of 5% phenol and 2.5 ml of concentrated sulfuric acid. The resultant solution was incubated at room temperature for 15 min, and absorbance was read at 492 nm in quartz cells. Water was used as a control, and the carbohydrate concentration was determined through

the use of a standard curve with known concentration of glucose. Assays were done in triplicates.

Quantification of amino acids

For amino acid quantification, a free proline methodology was used. Briefly, 500 µL of the ethanol extract was mixed with an equal amount of ninhydrine reactant (anhydrous ninhydrine, glacial acetic acid, and 6M phosphoric acid), and 500 µL of glacial acetic acid. The mixture was vigorously agitated, and incubated in a water bath at 100°C for 1 h. 1 ml of toluene was added, followed by agitation. Absorbance of the organic phase was read at 520 nm, using toluene as the blank. Free proline content was expressed in µg/mg of fresh mass, using a proline standard curve.

Lignin Determination

The remaining pellet from wall-linked phenol determination was transferred to conical tubes, and 7 ml of distilled water were added, followed by vigorous shaking. The pH was adjusted to 7, and the solution was centrifuged at 4000 rpm. The resulting pellet was treated with 10 ml of 0.1M NaOH, vortex homogenized and then centrifuged. Two 1 ml aliquots of the supernatant were separated. One of them was mixed with 4 ml of 0.1M NaOH (pH 12), and the other was mixed with phosphate buffer at pH 7. Absorbance was determined at 280 nm for each extract at pH 12, against those at pH 7 (control). A calibration curve was prepared with commercial lignin, and results were expressed in µg lignin/mg of starting material.

Statistical analyses

Each metabolite quantification was done in triplicates, and a variance analysis, ANOVA, was applied, along with an analysis of media comparison, test of Di Rienzo, Guzman and Casanoves (DGC). ANOVA was carried out using PAST 2008, and DGC in INFOSTAT 2011 (Verslues & Sharma, 2010).

RESULTS AND DISCUSSION

Quantification of soluble phenolic compounds

Fig. 1 shows the concentration of soluble phenolic compounds for each of the clones in each of the climate regions. Clones 29, 12 and 28, from the humid

forest (Padron) presented the highest production of soluble phenolic compounds, while those from the dry tropical forest (Tacariguita) presented values that were 58.6%, 40.3% and 37.9% lower than the former, for each of those three clones, respectively. Clones from the semi-humid climate (Tesoro) showed intermediate values, or close to those from the humid forest. This could be, presumably, because of a stimulated phenolic compound production due to higher amounts of water present in Padron (Elwers, Zambrano, Rohsius, & Lieberei, 2009). On the other hand, clones 11, 10 and 07 had a larger content of phenolic compounds in the dry forest of Tacariguita (41.4%, 33.6%, and 30.2%, respectively) than in the humid forest of Padron, while phenol production in the clones from Tesoro were the lowest in all locations. This indicates that polyphenol, for these clones, is higher in hydric deficit. These results show the metabolic plasticity of *Theobroma cacao* L., and its adaptability to the environment in which it grows, a previously suggested by (Daymond & Hadley, 2008) (Niether et al., 2017), and even, resistance to diseases, such as black pod (Nyadanu et al., 2013) (Djocgoue et al., 2007) (McMahon et al., 2015).

Cell wall- linked phenolic compounds

The contents of cell wall-linked phenolic compounds are shown in Fig. 2 for all clones at the three different sampling locations. There were small differences between the amount of phenolic compounds linked to cell wall for each clone, according to its location of origin. Nevertheless, the proximity of these values indicate that the differences are not determinant, and, since they are random, cannot be attributed to environmental or genetic factors. In the case of clones 29, 12 and 07, polyphenols linked to cell wall are slightly higher at Tacariguita (dry tropical forest) than in those from the humid climate, as it happens with soluble polyphenols.

Total phenolic compounds

The concentrations of total phenols (the algebraic sum of soluble and wall-linked phenolic compounds for each clone) are displayed in Fig. 3. These compounds are considered to be the derived products of secondary metabolism with the largest influence and widest distribution in plants. They perform important roles in different physiological and ecological functions, especially those involved in resistance to different types of stress (Ayaz, Kadioglu, & Turgut, 2000) (Caprioli et al., 2016). Under stress conditions, the increase in the content of phenolic acids can be related to cell wall lignification and, in part, to the synthesis of particular amino acids that maintain the osmotic equilibrium of the cells (Delalonde, Barret, & Coumans, 1996) (Li et al., 2013) (Oracz, Zyzelewicz, & Nebesny, 2015).

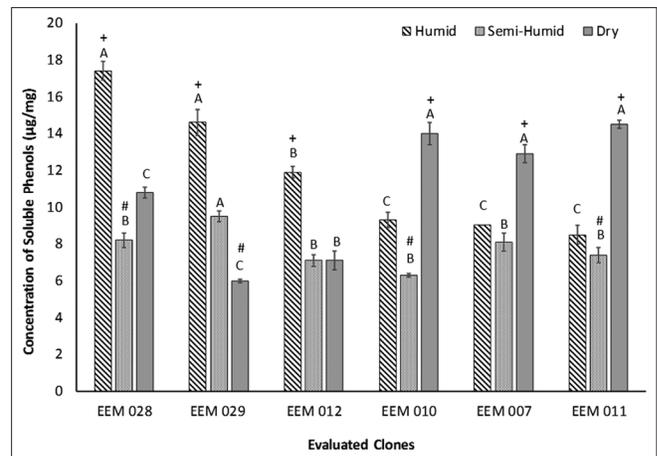


Fig 1. Concentration of soluble phenols ($\mu\text{g}/\text{mg}$) in leaf material from different clones in the three studied regions. Values from the same locality with a common letter are not significantly different. + and # represent the climatic region that yielded the highest and the lowest concentration for a determined clone, respectively.

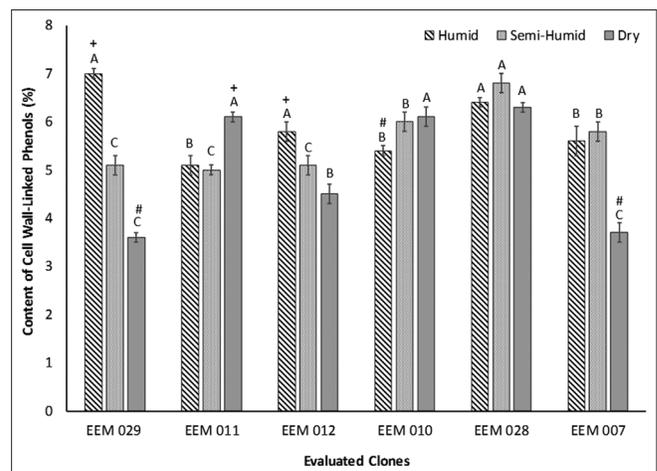


Fig 2. Concentration of cell wall-linked phenols ($\mu\text{g}/\text{mg}$) in the leaf material of different clones from the three studied climatic locations. Values from the same locality with a common letter are not significantly different. + and # represent the climatic region that yielded the highest and the lowest content for a determined clone, respectively.

In *T. cacao*, polyphenolic cells are found in the epidermis and sub-epidermis of leaves, roots, and the mesophyll of the seed's cotyledons. They play an important role in regulating plant growth and development, and in the plant's interactions with other organisms, as well as defense to different types of stress. They will also determine, along with other reserve elements, chocolate flavor and color (Martini, Figueira, Lenci, & Tavares, 2008) (Oracz et al., 2015). In spite of the fact that information about phenolic content and composition in cacao seeds is scarce, the available information of such parameters in leaves and roots is virtually non-existent.

The results on total phenolic compounds maintain a similar pattern to that observed for soluble phenols, where

clones 29, 12 and 28, presented higher concentrations in specimens cultivated at humid tropical forest conditions (Padron) than those found in the dry forest (Tacariguaita), while clones 11, 10 and 07 presented a reverse behavior. Nonetheless, according to the statistical analyses, the obtained results do not present a significant relation to the environmental factors. The difference on total phenolic compounds seem to be controlled rather by genetic differences among the clones.

Significant differences have been reported, however, on polyphenol content and composition in cacao seeds, both fermented and non-fermented, from different types of cacao, including forastero, trinitario and criollo, as a function of the genotype and geographic location (Carrillo, Londoño-Londoño, & Gil, 2014). (Elwers et al., 2009) further found that there are type-specific differences in phenolic compounds in non-fermented seeds, and that soil nutrient composition had a direct effect on polyphenol amount and composition. Likewise, (Djougoue et al., 2007) demonstrated that, in the presence of leaf infection with *P. megakarya*, the phenol content increased, thereby inducing a modification on polyphenol profiles, corroborating the role that these compounds play in plant susceptibility/ resistance.

In the future, it is of vital importance to differentiate the types of polyphenols present in each one of these clones, and all three climate regions, since, it is known, that not all phenols change their concentrations in under hydric stress. It has been previously shown that those of the non-flavonoid type (phenylpropanoids, derived from cinnamic, and ferulic acid, etc.) would be more sensible to hydric deficit (Santos et al., 2014).

Non-Structural carbohydrates

The method proposed by (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956) was used to quantify non-structural carbohydrates; this method is based on the formation of complexes between furfural derivatives of sugars and phenols, that absorb at 492 nm. Thus, the amounts of total carbohydrates are shown in Fig. 4. It can be said that, except for clone 29, there are higher amounts of non-structural carbohydrates for all clones in the dry tropical forest, while clones from Tesoro (semi-humid climate) had the lowest values of this parameter.

According to the statistical analyses, there are differences in one of the locations ($p < 0.01$). Specifically, it can be said that non-structural carbohydrate contents in clones from the semi-humid tropical forest, Tesoro, are significantly lower than those found in the other climate regions. Thus, a semi-humid tropical forest significantly has an effect on these parameter, causing its reduction.

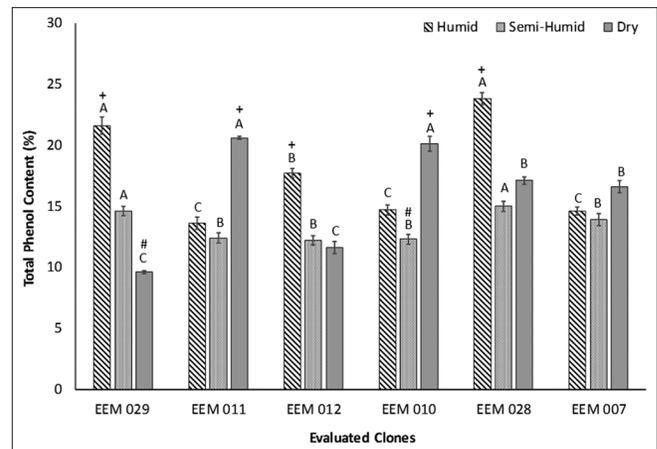


Fig 3. Concentration of total phenols ($\mu\text{g}/\text{mg}$) in leaf material from different clones in the three studied regions. Values from the same locality with a common letter are not significantly different. + and # represent the climatic region that yielded the highest and the lowest content for a determined clone, respectively.

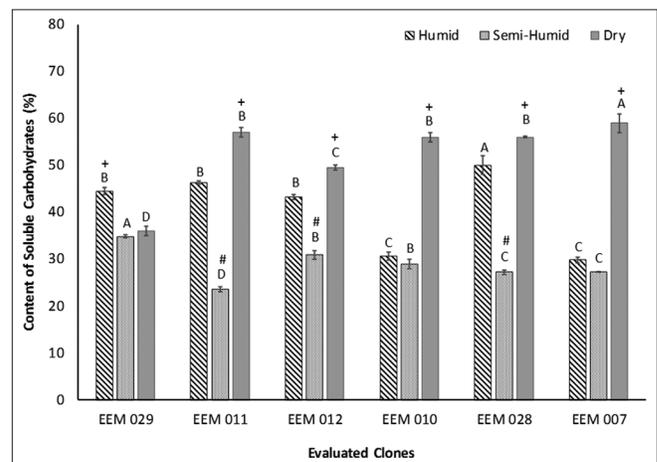


Fig 4. Concentration of soluble carbohydrates ($\mu\text{g}/\text{mg}$) in leaf material from different clones in the three studied regions. Values from the same locality with a common letter are not significantly different. + and # represent the climatic region that yielded the highest and the lowest content for a determined clone, respectively.

Carbohydrate production is directly related to photosynthesis, which, in turn, depends on three key factors: CO_2 concentration in the air, amount of light, and humidity. It is considered that CO_2 concentration is the same in the three climate regions studied. Therefore, the differences between the clones from the three regions can be attributed to cacao's metabolic plasticity to adapt to the environment where it grows (Scossa et al., 2016). From these observations, it can be inferred that if there is a greater amount of water available, the photosynthetic rate could increase, and this could explain why the amount of non-structural carbohydrates is greater in humid rainforest than in the semi-humid one (González, Gallardo, Hilal, & Prado, 2008). On the other hand, under conditions of hydric deficit, growth slows down,

implying an accumulation of carbohydrates and increasing, therefore, their concentrations in dry forests (Almeida & Valle, 2007)(Lahive, Hadley, & Daymond, 2018). At the same time, cacao's high adaptability causes that, under conditions of environmental stress, the plant modifies its metabolism to survive adversities. Consequently, carbohydrate concentrations would be, according to this fact, greater in humid and dry forests than in semi-humid tropical homologues (Scossa et al., 2016).

It has been reported that sucrose and reduced sugars are accumulated under stress, and that reducing water availability to plants under controlled conditions, leaf sugar contents increase (Delalonde et al., 1996)(Ayaz et al., 2000) (Zanetti et al., 2016). Other species, such as *Ctenanthe setosa*, under water deficit, tend to accumulate more low-molecular weight carbohydrates, and that these increase in sugars indicates metabolic adaptations of leaves to contribute to osmotic adjustment. A similar effect could perhaps take place in cacao.

Free proline

Values of free proline ($\mu\text{g}/\text{mg}$) for each clone in its respective climate region can be observed in Fig. 5. At a first glance it is notable that there was a greater amount of free proline in clones from Padron, which corresponds to humid tropical forest, than in the other climates. Through the statistical analyses, it is possible to conclude that there are no significant differences between the amounts of proline found in the different clones coming from Tesoro and Tacariguita (semi-humid and dry tropical forest, respectively). There is, however, a notorious difference between Padron (humid) and Tacariguita (dry), with the former accumulating greater amounts of free proline.

Stomas are epidermal structures distributed in regular patterns in the stem and underside of the leaves, that allow them to capture CO_2 to initiate photosynthesis. Moreover, through their opening and closing, plants avoid an excessive loss of water vapor, and this is regulated by external factors (i.e. light, temperature, humidity, etc.) and internal factors (amino acid concentration, abscisic acid, etc.). The closing of the stomas induces metabolic slowdown and, thereby, decreased growth. Amino acids such as proline stop abscisic and glutamic acid production, favoring the opening of stomas. Under drought, the presence of proline in plant cells diminishes to prevent the opening of stomas, and, at the same time, plant growth. The accumulation of these osmolytes (such as proline) contribute both to osmotic adjustment and the protection of proteins and plant cell membranes. It has previously been observed that this accumulation increases under stress conditions in plants, bacteria and fungi (Verslues & Sharma, 2010). This effect of genotype-environment interactions on the content

of essential amino acids in rice was studied by (Wu, Shi, Zhang, & Katsura, 2004), reporting that there is a genetic effect as well as an effect due to genotype-environment interactions on the content of certain amino acids. Similarly, it is important to point out that amino acid content can be directly related to resistance to pathogen in some crops (Aly A.A.H, et al, 2011), and could potentially be the case for cacao, although further research would be needed in this regard.

Lignin quantification

In Fig. 6, it is observed that, for clone EEM208, lignin production in the semi-humid forest was significantly greater than in the humid and dry forests (Padron and Tacariguita), whereas for the rest of the clones, lignin

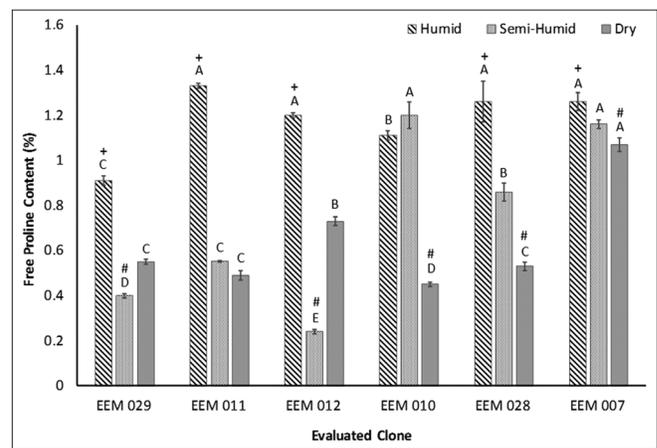


Fig 5. Concentration of free proline ($\mu\text{g}/\text{mg}$) in leaf material from different clones in the three studied regions. Values from the same locality with a common letter are not significantly different. + and # represent the climatic region that yielded the highest and the lowest content for a determined clone, respectively.

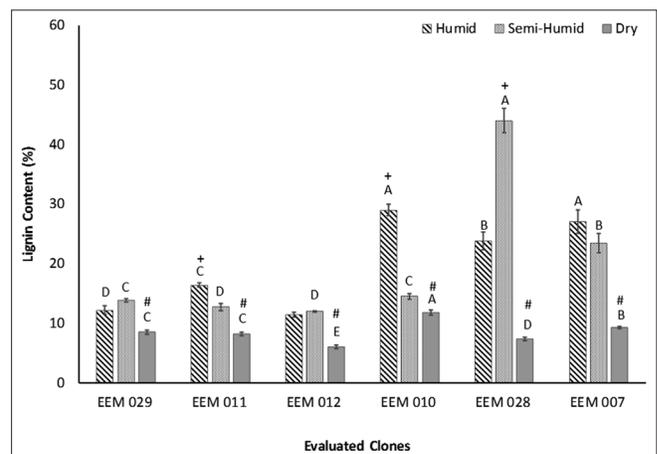


Fig 6. Lignin concentration ($\mu\text{g}/\text{mg}$) in leaf material from different clones in the three studied regions. Values from the same locality with a common letter are not significantly different. + and # represent the climatic region that yielded the highest and the lowest content for a determined clone, respectively.

concentration was similar in the humid and semi-humid climates. In general, for Tropical dry forest, lignin production was the lowest ($p < 0.05$), as corroborated by ANOVA analysis, but no significant differences were found between Padron and Tesoro, with the exception of EEM028.

There is very little known about the effects of drought conditions on lignin biosynthesis. (C. Alvarez, Perez, & Lares, 2007) observed a reduction on the amount of ferulic acid and increase on p-cumaric and caffeic acids in the sap of *Xilema maiç* after 10 days of increasing the water amount. According to these authors, the increase of free lignin precursors in this sap, as well as the activity of reduced anionic peroxidase, could be an indication that drought decreases lignin synthesis in corn. Different regions of *X. maiç* root could respond differently to drought, implying that lignin content could be higher in specific root areas or at different stress times (Fan et al., 2006)(Yang et al., 2006).

It has been demonstrated that, under hydric stress, the basal root section in corn plants demonstrate greater reduction on the growth of the apical region. This reduction was associated to an increase on the expression of cinnamoyl-CoA reductase 1 and 2 genes, which are involved in lignin biosynthesis. This phenomenon was also associated to an increase in lignin deposition that hardened cell wall extensibility, and decreased its expansion. In these plants, basal root growth reduction can improve the availability of water, minerals and sugars, which are necessary to maintain minimal growth, and the survival of young cells in the most apical portion, facilitating growth recovery after rehydration (Yang et al., 2006).

(Lee et al., 2007) studied lignin biosynthesis, and its functional importance during hydric stress in the leaves of *Trifolium repens*, subjected to 28 days of drought. Reduction of leaf growth simultaneously took place with an increase on lignin production. The involved enzymatic activities revealed that enzyme responses under drought conditions may vary, depending on the period in which plants are exposed to drought. Similar results have been found in soy (Song et al., 2016).

CONCLUSIONS

Production of phenolic compounds varies in clones according to their location. Clones EEM028 and EEM029 cultivated in Padron (humid tropical forest) presented the largest amount of total phenols. Lignin biosynthesis was greater in clones from Padron (tropical humid forest), while it was lowest in Tacariguita (dry forest). The clones that accumulated the greatest amount of lignins were EEM028 and EEM007, coming from Padron and Tesoro,

respectively. Tropical dry forest clones (Tacariguita) presented the largest accumulation of non-structural carbohydrates when compared to those from other regions. This can be due to a slowdown on plant growth under hydric stress conditions. Clones EEM011 and EEM007 specifically presented the greatest amount on this parameter, with important presence of glucose and fructose. Clones from humid tropical forest (Padron), specially EEM011, EEM028 and EEM007, presented the largest accumulation of free proline, making it clear that high humidity levels promote proline production, and the opening of stomas, accelerating plant growth.

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AUTHOR'S CONTRIBUTIONS

JFAB processed the data and prepared the manuscript. DQ performed the laboratory determinations. MR overlooked laboratory experiments and determinations. RR carried out the statistical analyses. DS is the director of the project and did all the field work and sample preparations.

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