SCREENING CACAO GERMPLASM FOR DROUGHT TOLERANCE

Mariana Rocha de Carvalho[1,3], Danilo Sampaio Novaes[2], Bruna Oliveira Santos[2], Karina Peres Gramacho[2], Uilson Vanderlei Lopes[2], Augusto Roberto Sena Gomes[2], Raúl René Meléndez Valle[1,2]*

[1]Department of Biological Sciences, State University of Santa Cruz, km 16, Highway BR 415, 45662-900, Ilhéus, BA, Brazil; [2]Cacao Research Center (CEPEC), km 22, Highway BR 415, 45600-919, Ilhéus, BA, Brazil; [3]Present address: Department of Biological Sciences, Federal University of Lavras, Aquenta Sol, 37200-900, Lavras, MG, Brazil.

*Corresponding author: raulrenevalle@hotmail.com

Thirty progenies of the Cacao Research Center's (Cepec) cacao breeding program were submitted to water restriction and evaluated for survival, height, stomatal density, relative water content (RWC) and parameters related to the pressure-volume curve under greenhouse conditions. Drought caused an average of 120% reduction in water potential, and a decrease in RWC and survival in some progenies. A high survival capacity was observed for progenies of clones CP-309 and CP-300 characterizing resistance to severe drought under greenhouse conditions. Furthermore, these progenies showed lower losses in RWC. In the progenies evaluated it was identified mechanisms compatible with osmotic and elastic adjustment that have a role in drought tolerance and some positive correlations with plant survival in severe drought in the greenhouse, like RWC at the turgor loss point, that was considered as the main predictor of severe drought survival under controlled conditions. Given the complexity of the plant's tolerance responses and the genetic variability of the plants in Cepec cacao germplasm, we highlight the importance of screenings like this, as a starting step for cacao breeding programs aiming drought tolerance.

Key words: Pressure-volume curves, relative water content, water potential.

Triagem de germoplasma de cacau para tolerância à seca. Trinta progênies do programa de melhoramento de cacau do Centro de Pesquisas do Cacau (Cepec) foram submetidas à restrição hídrica e avaliadas quanto à sobrevivência, altura, densidade estomática, teor relativo de água (TRA) e parâmetros relacionados à curva pressão-volume em casa de vegetação. A seca causou uma redução média de 120% no potencial hídrico, e uma diminuição no TRA e sobrevivência em algumas progênies. Uma alta capacidade de sobrevivência foi observada para as progênies dos clones CP-309 e CP-300, caracterizando resistência à seca severa em condições de casa de vegetação. Além disso, essas progênies apresentaram menores perdas em TRA. Nas progênies avaliadas foram identificados mecanismos compatíveis com ajustes osmóticos e elásticos que têm papel na tolerância à seca e algumas correlações positivas com a sobrevivência das plantas em seca severa em casa de vegetação, como TRA no ponto de perda de turgescência, que foi considerado como o principal preditor de sobrevivência à seca severa sob condições controladas. Dada a complexidade das respostas de tolerância da planta e a variabilidade genética das plantas no germoplasma de cacau Cepec, destacamos a importância de triagens como esta, como um passo inicial para programas de melhoramento de cacau visando tolerância à seca.

Palavras-chave: Curvas de pressão-volume, teor relativo de água, potencial hídrico.

Introduction

The cacao tree (*Theobroma cacao* L.) is a perennial crop, widely cultivated in the humid tropics to produce seeds, the raw material for chocolate production (Lahive, Hadley & Daymond, 2019). The crop is very sensitive to drought (Alvim and Kozlowsky, 1977; Moser et al., 2010; Ayegboyin and Akinrinde, 2016), therefore, its productivity is strongly affected by soil water deficit (Garcia Lozano and Moreno Fonseca, 2015; Abdulai et al., 2018). Climatic changes pose challenges to the cacao crop, and some weather events, such as El Niño Southern Oscillation (ENSO) (Niether et al., 2020), cause losses in cacao yield, suggesting that this phenomenon plays an important role in the world cacao production (Abdulai et al., 2018). Also, it has been reported ENSO-related droughts decreasing cacao production in the African, Asian and North and South American continents (Gateau-Rey et al., 2018).

Drought events may trigger many morphological and physiological plant responses, which are multiple, interconnected and reflect adaptation mechanisms (Peixoto et al., 2006; Lawor, 2013). Plant responses may vary between species, genotypes, geographic origins, and depend on environmental factors and the stage of development (Kosová et al., 2011); however, cell responses to abiotic stresses remain conserved in many species (Zhu, 2002).

In view of the challenges imposed by climate changes, the development of drought-tolerant cacao planting materials is relevant to cacao breeding programs. Concerning this, cacao has a high genetic diversity regarding morphological and physiological traits (Daymond et al., 2002), but their use for breeding drought tolerant genotypes is still recent. According to Mitra (2001), breeding genetic improvement aiming drought tolerance requires an efficient technique to identify truly tolerant germplasm. These techniques must be quick, allow the screening of a large number of genotypes, reliable and be able to assess the performance of the plant at critical stages of development.

Finding suitable screening techniques and tolerant germplasms is important for the success of developing new genotypes varieties with drought tolerance (Bayoumi, Eid and Metwali, 2008). Therefore, efforts have focused on looking for indirect ways of selecting plants for yield and other promising traits under water stress conditions. Morphophysiological traits can confer drought tolerance and may be used as indirect selection criteria to increase yield and plant tolerance when exposed to water stress (Fukai and Cooper, 1995). The use of these characteristics has demonstrated the improvement of performance in water limiting conditions in annual and perennial crops (Bayoumi, Eid and Metwali, 2008; Bernini, 2015; Fukai and Cooper, 1995; Cabral, Barbosa and Simabukuro, 2004; Peixoto et al., 2006; Zadraznik et al., 2017).

Among the morphophysiological traits, the alteration in growth rate is one of the first responses to water stress (Balasimha, 2016). In addition to growth, plant responses to stress may involve changes in stomatal arrangement (Grisi et al., 2008; Yu et al., 2008 and Ribeiro et al., 2012), and relative water content (Bayoumi, Eid and Metwali, 2008; Munns et al., 2010; Larkunthod et al., 2018). Other plant responses to water stress as osmotic adjustment (Wang et al., 2001; Almeida et al., 2002), cell wall elasticity (Bagherzadi et al., 2017; Wang et al., 2018), and biochemical and morphophysiological processes (Atkinson et al., 2000; Maes et al., 2009; Scoffoni et al., 2014; Neves et al., 2017) were reported.

In cacao, morphophysiological traits under water stress conditions have been used to evaluate drought tolerance of germplasms from Venezuela (Araque et al., 2012; Ávila-Lovera et al., 2016; De Almeida et al., 2016) and Nigeria (Ayegboyin and Akinrinde, 2016). Also, reports from Indonesia (Moser et al., 2010; Zakariyya, Setiyawan and Susilo, 2017), India (Balasimha and Daniel, 1988; Kacou et al., 2016) and Colombia (Garcia Lozano and Moreno Fonseca, 2015) are found. In Bahia, one of the main cacao producing areas in Brazil, relative water content (RWC), pressure-volume curves, growth and molecular techniques have been used to screen drought-tolerant cultivars in water deficit greenhouse experiments (Almeida et al., 2002; Santos et al., 2014, 2018). However, these studies used few genotypes or complex methodologies, making difficult their application in ranking large number of germplasms in practical breeding programs.

A quick and practical initial screening method for drought tolerance in seedlings is helpful to identify genotypes for further testing in the field (Lahive, Hadley and Daymond, 2019). Aiming to find screening

methods and drought-tolerant germplasms, seedlings of 30 cacao progenies, from a very diverse set of germplasms, were evaluated regarding their performance under water restriction in a greenhouse. In order to do that, it was assessed (i) resistance to water restriction, (ii) variability of morphophysiological responses to stress and their use for primary screening, and (iii) the relationship between evaluated traits and plant survival rate in those progenies.

Materials and Methods

Plant material and cultivation conditions

The experiment was carried out in a greenhouse at the Cacao Research Center (CEPEC), in Ilhéus, Bahia, Brazil ($14^0$47'S, $39^0$16'W, 55 m ASL). Seedlings were obtained from open-pollinated seeds collected from 30 cacao clonal germplasm accessions in CEPEC's Cacao Germplasm Bank (Table 1). Seeds of four open-pollinated pods of each genotype were mixed and a randomly composed sample were planted in 290 cm^3 polyethylene tubes containing soil as substrate. The fertilization occurred according to the crop requirements during the experiment. The seeds were sown in November/2016 and the water restriction was imposed between February 13 and March 15, 2017, totaling 32 days.

During the experimental period, temperature and relative humidity were monitored by the local climatological station. The relative humidity varied between 75% and 94.3% and the temperature between 23.2°C and 26.3°C. Initially, all plants were well-watered through sprinkler irrigation, and after 90 days of plant development, the plants were divided into two groups, the control (well-watered treatment - WW), which was irrigated daily, and the second group which was subjected to water restriction (drought-stressed treatment - DS) for 32 days. During this period, the water status of the plants was monitored by measuring midday leaf water potential (ψ_{leaf}). The water restriction experiment was established according to a split-plot design in a randomized block design, with four replications and nine plants per plot. The two treatments associated to water restriction were allocated to the main plot (WW and DS) and the 30 progenies in the subplots. In total, 240 subplots were established: 30 irrigated and 30 non-irrigated in each of the four blocks, totaling 2160 cacao seedlings.

Whole-plant responses

To assess the survival rate among progenies, an index was calculated by the ratio between the number of plants alive and the total number of plants in the plot, in two moments: at 21 days of water restriction. This period marked the beginning of the critical moment of dehydration, when the leaf senescence process started in most plants; and at 32 days, marking the end of the experiment. The relative growth rate (RGR) was calculated according to Hunt (2002), using measurements of plant height taken between the edge of the planting tube (beginning of the stem) and the apical shoot.

Table 1. List of 30 cacao genotypes used in this study and their geographical and genetic origin

Genotype	Genetic Origen	Geographical origin
BN-34	Primary germplasm*	Brazil
CCN-10	Primary germplasm*	Ecuador
CCN-51	(ICS-95 x IMC-67) x CCN-1	Ecuador
CEPEC-2002	Primary germplasm*	Brazil
CP-049	TSA-644 x CCN-51	Brazil
CP-055	CATONGO x VB-276	Brazil
CP-077	CCN-51 x TSH-565	Brazil
CP-102	Sca-6 x unknown	Brazil
CP-115	CCN-51 x EET-390	Brazil
CP-149	TSA-644 x CCN-51	Brazil
CP-176	TSA-644 x CCN-51	Brazil
CP-197	CCN-51 x TSH-1188	Brazil
CP-204	ICS-95 x TSH-516	Brazil
CP-300	(SIC-952 x Sca-12) x TSH-516	Brazil
CP-302	(SIC-952 x Sca-12) x TSH-516	Brazil
CP-305	(SIC-952 x Sca-12) x (EET-399 x RB-30)	Brazil
CP-307	(SIC-952 x Sca-12) x (EET-399 x RB-30)	Brazil
CP-309	(SIC-952 x Sca-12) x (EET-399 x RB-30)	Brazil
CP-312	(SIC-864 x Sca-12) x TSH-1188	Brazil
CP-331	TSH-1188 x TSH-1188	Brazil
CP-346	TSH-1188 x VB-514	Brazil
CSUL-04	Primary germplasm*	Brazil
IP-1	Primary germplasm*	Brazil
Ma-15	Primary germplasm*	Brazil
Pa-13	Primary germplasm*	Peru
PH-15	Primary germplasm*	Brazil
PH-16	Primary germplasm*	Brazil
PS-13.19	Primary germplasm*	Brazil
SIC-02	Primary germplasm*	Brazil
SJ-02	Primary germplasm*	Brazil

* Plant material originated from selections on farms or at the center of diversity.

Leaf water potential at midday (ψ_{leaf}, MPa) was measured in individual leaves from the second or third leaf flush, from the apex of the orthotropic axis using a pressure chamber (Scholander et al., 1965) between 12:00 and 14:00 h from a plant in each subplot.

For the estimation of the relative water content (RWC), one leaf per plant from five plants, chosen randomly out of the nine plants in the plot, for all subplots in the experiment, including well-watered and drought-stressed treatments and all progenies was taken. The leaves were collected between 12:00 and 14:00 h following the same collection protocol, and then it was measured the ψ_{leaf} and the fresh weight (FW) using a precision scale. Subsequently, the leaves were placed in a container with water to provide a constant relative humidity for overnight hydration to obtain the turgid weight value (TW). In order to obtain the dry weight (DW), the leaves were placed in an oven at 70°C for 48 h. After that, the RWC was computed by RWC = (FW - DW) / (TW - DW) x 100 (Turner, 1981).

To obtain the pressure-volume curves (p-v curves) an individual fully expanded leaf between the 3rd or 4th node of a plant in each subplot of both treatments (WW and DS) was collected at 10 days of water stress, and hydrated in a wet chamber overnight, following the same procedures reported for RWC and ψ_{leaf}. After the hydration period, the leaves were quickly weighed and their ψ_{leaf} measured using a Scholander pressure chamber. After that, they were left to dry out under laboratory conditions (22°C). This process was repeated seven times at intervals of approximately 30 min. Then, the leaves were dried at 70°C for 48h to obtain the dry mass. Linear regression equations were adjusted considering the inverse of the water potential ($-1/\psi_{leaf}$) as a function of the relative water content loss (100-RWC), using Microsoft Excel. The curves were adjusted according to Schulte and Hinckley (1985) and the parameters turgor loss point (π_{tlp}) and relative water content at the turgor loss point (RWC_{tlp}) were estimated based on the coefficients of the linear regression of the second part of the curve, using the Excel program described by Bartlett, Scoffoni and Sack (2012).

The bulk modulus of elasticity (ε) was calculated using the equation $\varepsilon = \Delta\psi / \Delta R^* \times R^*$ (Schulte and Hinckley, 1985; Cosgrove, 1988), where $\Delta\psi$ is the change in the cell water potential, ΔR^* is the change in RWC% and R^* is the water content at maximum turgor, assumed to be 100% here.

For stomatal density estimation, one fully expanded leaf of three plants per plot was sampled. For this determination, it was used the epidermal printing method with cyanocrilate ester (LOCTITE®), as described by Segatto, Bisognin and Benedetti (2014). Briefly, the method consists of putting a drop of universal instant adhesive (cyanocrylate ester) on a glass slide. A leaf section is then pressed onto the glass slide for approximately 10 s, long enough for the adhesive to spread and dry, allowing the leaf to separate from the glass slide and keep the impression of the epidermis. After this, the slides were observed directly in a binocular optical microscope, with 10x magnification, in an area of 1 mm^2, with a millimeter eyepiece. For each leaf section, five fields were counted.

Statistical analysis

The midday ψ_{leaf} data, RWC, p-v curve parameters, and stomatal density of the treatments (WW and DS) were subjected to paired Students t-test (P<0.05) for each progeny. Survival rate data were analyzed by Friedman's non-parametric test (P<0.05), for repeated samples in blocks, and subsequently, the data were subjected to paired comparisons using the Conover test at 0.05 probability. Ward's method based in significative variables grouped the genotypes in cluster analysis and Spearman's coefficients were calculated to determine globally the relationships between variables. All data were analyzed using the SAS (Statistical Analysis System) version 9.4.

Results

Water status

In all progenies, lower values of midday ψ_{leaf} were observed when compared with measurements in well-watered plants. Severe stress was confirmed by the values of midday ψ_{leaf}, which varied between -0.7 and -1.5 MPa in the well-watered treatment (WW) and -1.8 to -3.5 MPa in the drought-stressed treatment (DS) (Figure 1), with the greatest differences observed in progenies CP-049, CP-204, CP-307, CP-331, IP-1, PH-15 and SJ-02. However, no statistical differences were found among progenies within each
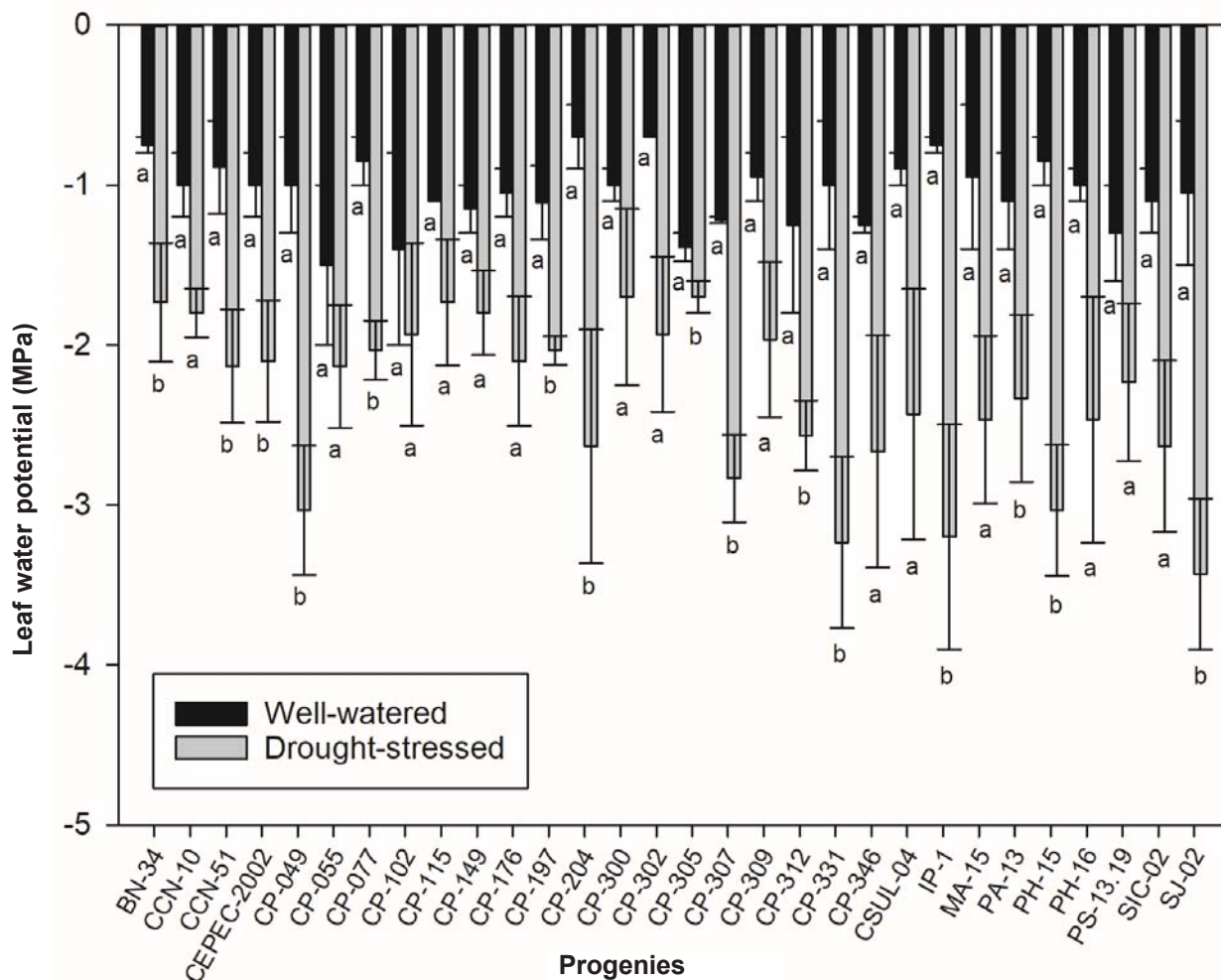
Figure 1. Midday leaf water potential for *T. cacao* progenies in well-watered (WW) and drought-stressed (DS) treatments. Average values of three replications (± se). The letters compare treatments (WW and DS) for each progeny. Means followed by the same letter showed no statistical difference by the Students *t*-test (p d ≤ 0.1).

treatment; however, differences were found when comparisons between WW and DS conditions were done (Figure 1).

Regarding RWC, in general all progenies had their values decreased under DS conditions; however, under the same conditions, CEPEC-2002, CP-149, CP-176, CP-305, PH-15 and PS-13.19 progenies maintained high RWC values. The CP-055 and CP-302 progenies showed the lowest values under DS conditions (Table 2).

p-v curves parameters

From the estimated p-v parameters, it was not observed an increase in turgor loss point (π_{tlp}) in any of the progenies. A significant rise in relative water

content at the turgor loss point (RWC_{tlp}) was shown in 14 of the 30 progenies studied, followed by reductions in π_{tlp}, probably due to the early plasmolysis caused by the drought stress (Table 2). Regarding cell wall elasticity (ε), nine of the 30 progenies showed significant differences between WW and DS conditions, with lower values under drought stress, except for CP-077 which increased the ε value over 100% (Table 2).

Progenies survival rate

The index of plants alive was evaluated in two moments: at 21 days of water restriction, when the senescence of most plants was observed, and at 32 days, the end of the experimental period (Figure 2).

Table 2. Mean values of relative water content (RWC%), turgor loss point (π_{tlp}, MPa), relative water content at the turgor loss point (RWC$_{tlp}$) and bulk modulus of elasticity (ε) of 30 cacao progenies under well-watered (WW) and drought-stressed (DS) conditions

Progenies	Water conditions	RWC %	π_{tlp} (MPa)	RWC$_{tlp}$ %	ε (MPa)
BN-34	WW	92.9 a	-1.70 a	80.4 a	5.36 a
	DS	66.0 b	-1.00 a	78.1 a	1.11 b
CCN-10	WW	92.6 a	-1.96 a	69.1 a	3.23 a
	DS	56.6 a	-0.76 b	89.2 b	3.28 a
CCN-51	WW	88.3 a	-1.50 a	87.2 a	1.02 a
	DS	64.4 b	-1.27 a	81.3 a	0.46 b
CEPEC-2002	WW	79.0 a	-1.40 a	48.5 a	2.94 a
	DS	83.7 a	-1.03 a	90.0 b	3.58 a
CP-049	WW	92.0 a	-2.50 a	62.0 a	2.91 a
	DS	73.4 a	-0.90 a	82.6 a	0.00 b
CP-055	WW	68.5 a	-1.75 a	75.0 a	1.24 a
	DS	46.3 b	-1.00 a	90.8 b	4.94 a
CP-077	WW	95.2 a	-2.00 a	68.9 a	3.63 a
	DS	52.6 b	-2.33 a	88.6 a	9.70 b
CP-102	WW	63.8 a	-2.00 a	54.9 a	2.91 a
	DS	55.1 a	-1.13 b	88.5 a	3.93 a
CP-115	WW	83.8 a	-1.30 a	79.9 a	1.76 a
	DS	80.0 a	-1.23 a	85.1 a	1.54 a
CP-149	WW	89.2 a	-2.10 a	66.7 a	3.03 a
	DS	84.9 a	-1.23 a	85.1 a	1.54 a
CP-176	WW	87.6 a	-1.98 a	61.0 a	4.10 a
	DS	87.6 a	-0.90 a	89.1 a	1.26 a
CP-197	WW	78.7 a	-2.20 a	67.3 a	5.04 a
	DS	63.7 a	-1.03 b	88.2 b	2.85 a
CP-204	WW	75.6 a	-1.50 a	73.7 a	2.44 a
	DS	72.1 a	-1.10 b	88.8 b	3.49 a
CP-300	WW	80.4 a	-2.00 a	72.4 a	4.36 a
	DS	59.0 a	-1.13 b	87.2 b	4.39 a
CP-302	WW	82.2 a	-1.60 a	66.5 a	2.12 a
	DS	40.3 a	-0.93 a	88.8 a	2.28 a
CP-305	WW	85.4 a	-1.80 a	80.0 a	2.89 a
	DS	88.5 a	-0.77 b	89.5 b	0.33 b
CP-307	WW	74.4 a	-1.30 a	68.6 a	2.25 a
	DS	82.3 a	-0.86 b	90.6 b	3.12 a
CP-309	WW	80.3 a	-1.90 a	70.7 a	3.34 a
	DS	69.4 a	-0.90 b	90.2 b	4.73 a
CP-312	WW	79.1 a	-2.0 a	61.8 a	2.53 a
	DS	67.5 a	-0.93 b	87.9 b	1.66 a
CP-331	WW	85.5 a	-2.60 a	55.4 a	4.84 a
	DS	68.9 a	-0.90 a	72.2 a	0.32 b
CP-346	WW	86.5 a	-1.71 a	91.5 a	4.30 a
	DS	79.6 a	-1.27 a	85.7 a	2.74 a
CSUL-04	WW	87.6 a	-2.00 a	52.0 a	2.18 a
	DS	65.4 a	-1.13 a	88.6 b	1.35 b

Continuation Table 2.

IP-1	WW	94.4 a	-2.30 a	66.6 a	5.55 a
	DS	70.3 a	-0.83 a	87.0 a	0.89 b
Ma-15	WW	78.8 a	-1.60 a	69.4 a	2.32 a
	DS	78.7 a	-0.83 a	88.7 a	0.75 b
Pa-13	WW	74.5 a	-1.51 a	80.7 a	2.46 a
	DS	60.6 a	-0.70 b	88.0 b	2.66 a
PH-15	WW	81.9 a	-1.70 a	73.3 a	4.85 a
	DS	87.7 a	-1.00 a	88.8 a	-1.03b
PH-16	WW	75.5 a	-1.80 a	76.4 a	4.17 a
	DS	72.1 a	-1.06 a	89.2 b	1.65 a
PS-13.19	WW	86.2 a	-1.75 a	82.2 a	2.74 a
	DS	84.3 a	-1.00 a	88.3 a	0.14 a
SIC-02	WW	83.0 a	-2.30 a	63.5 a	3.09 a
	DS	82.7 a	-0.73 a	87.0 b	0.32 a
SJ-02	WW	81.6 a	-1.57 a	87.0 a	4.04 a
	DS	75.7 a	-0.90 a	87.6 a	0.00 a

Means followed by same letter for each progeny within water conditions do not differ according to Student's t-test (P < 0.05).

However, significant differences between progenies were observed only at the 21 days measurements (Table 3).

The progenies that showed the higher survival rates were CP-309 and CP-300 (survival >75%); while the progenies of PH-16, CP-176, CP-302, CP-331, PS-13.19, BN-34, CEPEC-2002, CP-197, CP-204 and CP-102, had more than 50% survival after 21 days of water restriction in 290 cm³ containers (Table 3). At 32 days of water restriction, the progenies did not differ in relation to survival rates, showing mean values between 3% and 24%; however, CP-309 remained the progeny with the largest number of living plants, followed by PH-16, CP-302 and CP-115.

Stomatal density determination

The leaf epidermal printing technique, using universal adhesive, proved to be efficient in measuring the number of stomata per leaf area. The values varied between 443 and 816 stomata per mm² and were found only on the abaxial face (Table 4). The progeny PS-13.19 showed a 100% increase in stomatal density in DS, when compared to the WW conditions and BN-34, CCN-10, CP-055, CP-204 and CP-302 showed an opposite response.
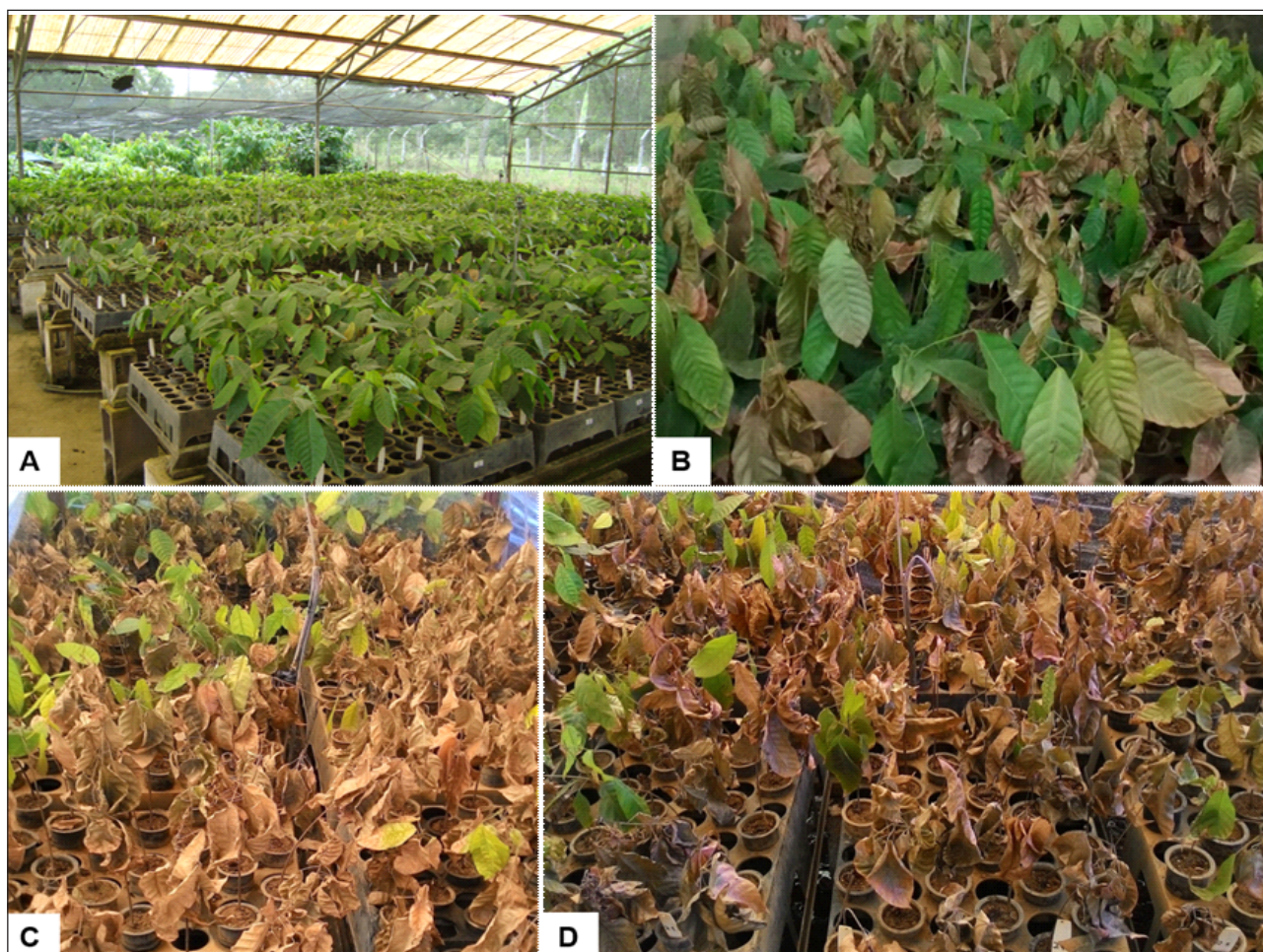
Figure 2. (A) Cacao seedlings in the acclimatization phase before irrigation interruption. (B) Symptoms of leaf wilt and senescence observed in plants at 14 days of water restriction. (C) and (D) Plants that resisted drought and remained alive after 32 days of water restriction.

Correlations

Progeny survival rate at 21 days of water restriction was negatively correlated with higher relative water content at the turgor loss point (RWC_{tlp}, WW, r = - 0.25* and DS, r = -0.18*). Survival rate at 32 days of water restriction was no significantly correlated with RWC_{tlp} (Table 5).

Leaf relative water content, in turn, was positively related to stomatal density (SD) measurements in both WW and DS treatments, with r = 0.27* and r = 0.29* respectively (Table 5). The values of ε were positively correlated with the survival at 21 days of water restriction in DS conditions (r = 0.23**) and RWC_{tlp} (r = 0.29**) and negatively correlated with RWC (r = - 0.22**) and the turgor loss point in stressed plants ($π_{tlp}$, r = -0.23*) and well watered plants (r = - 0.42***).

Identification of tolerant genotypes based on cluster analysis

A multivariate analysis was performed based on the similarity of the variables that showed differences under drought conditions. The results showed the formation of four distinct groups (Figure 3). The first group (I) was represented by 13 genotypes, the second (II) by five, the third (III) by two, and fourth (IV) by 10. The association was consistent with genetic similarities and drought tolerance responses. The break in the plots shows four well defined clusters because it shows a large break between branches of the tree. Thus, genotypes of groups I and III were grouped as being tolerant to water stress in this study, and genotypes of the group II were considered moderately tolerant, whereas, the more sensitive genotypes were grouped in group IV.

Table 3. Survival rate at 21 and 32 days of water restriction (SR 21, SR 32) in seedlings of 30 cacao progenies

Progenies	SR 21	SR 32
CP-309	77.8 a	23.6 a
CP-300	75.0 ab	8.33 a
PH-16	59.7 abc	22.6 a
CP-176	58.3 abc	8.33 a
CP-302	57.6 abcd	20.2 a
CP-331	57.5 abcd	8.33 a
PS-13.19	57.5 abcd	14.7 a
BN-34	55.9 abcd	5.90 a
CEPEC-2002	54.5 abcd	2.78 a
CP-197	52.8 abcd	11.8 a
CP-204	50.2 abcde	11.5 a
CP-102	50.0 abcde	17.0 a
CP-115	48.3 abcde	21.5 a
IP-1	46.6 abcde	14.3 a
CSUL-04	45.5 abcde	15.6 a
CCN-10	44.1 abcde	6.4 a
CP-307	42.0 abcde	12.2 a
PH-15	40.3 bcde	4.43 a
CP-149	40.3 bcde	12.5 a
SIC-02	36.5 cde	3.12 a
Pa-13	36.2 cde	13.8 a
CP-346	34.2 cde	5.55 a
CP-312	30.6 cde	2.78 a
CP-055	29.9 cde	5.90 a
CP-305	29.2 cde	6.35 a
CP-049	28.5 cde	8.33 a
CP-077	26.7 cde	4.17 a
SJ-02	22.5 cde	9.13 a
Ma-15	20.2 de	5.55 a
CCN-51	15.4 e	3.57 a

Means followed by same letter within each column do not differ according to Conover test (P < 0.05).

Table 4. Stomatal density (SD mm^2) of 22 progenies[1] of cacao plant in well-watered (WW) and drought-stressed (DS) conditions

Progenies	SD (mm^2) - WW	SD (mm^2) - DS
BN-34	721 a	507 b
CCN-10	747 a	603 b
CCN-51	671 a	816 b
CEPEC-2002	531 a	749 b
CP-055	725 a	541 b
CP-077	685 a	595 a
CP-115	613 a	645 a
CP-149	643 a	732 a
CP-176	504 a	681 b
CP-197	655 a	763 a
CP-204	780 a	671 b
CP-300	607 a	680 a
CP-302	593 a	489 b
CP-300	500 a	659 b
CP-309	523 a	576 a
CP-331	727 a	600 a
CP-346	591 a	723 a
CSUL-04	540 a	636 b
IP-1	635 a	675 a
PH-15	533 a	608 b
PH-16	443 a	704 b
PS-13.19	355 a	797 b

[1]Eight progenies did not have plants under drought conditions to compare with well-watered plants. Means followed by same letter within each line do not differ according to Students' *t*-test (P < 0.05).

Table 5. Correlation matrix for index (%) of survival rate at 21 and 32 days of water restriction (SR 21, SR 32), relative growth rate (RGR, cm cm^{-1} day^{-1}), relative water content (RWC), water potential (ψ_{leaf}, MPa), stomatal density (SD mm^2), bulk modulus of elasticity (ε, MPa), turgor loss point (π_{tlp}, MPa) and relative water content at the turgor loss point (RWC$_{tlp}$)

Variables	Condition	Live 32	RGR	RWC	ψ_{leaf}	SD	ε	π_{tlp}	RWC$_{tlp}$
SR 21	WW	0.23***	-0.02ns	-0.18ns	-0.07ns	0.07ns	-0.23*	-0.20ns	-0.25*
	DS	0.23***	0.18*	0.007ns	0.22**	-0.09ns	0.23**	-0.03ns	-0.18ns
SR 32	WW		0.10ns	0.003ns	-0.03ns	-0.13ns	-0.09ns	0.17ns	0.09ns
	DS		0.11ns	-0.18*	0.10ns	-0.17ns	-0.05ns	-0.07ns	-0.01ns
RGR	WW			0.09ns	-0.17ns	0.12ns	-0.04ns	0.17ns	-0.12ns
	DS			0.12ns	0.17ns	0.06ns	0.16ns	0.00ns	0.24*
RWC	WW				-0.04ns	0.27*	0.15ns	-0.15ns	0.13ns
	DS				-0.20*	0.29*	-0.22**	0.05ns	0.03ns
ψ_{leaf}	WW					0.13ns	0.13ns	0.09ns	0.19ns
	DS					-0.06ns	0.30***	-0.20ns	0.07ns
SD	WW						0.17ns	-0.08ns	0.10ns
	DS						-0.18ns	0.28ns	0.32ns
ε	WW							-0.42***	-0.36***
	DS							-0.23*	0.29**
π_{tlp}	WW								0.26*
	DS								0.21ns

WW = Well-watered treatment with ψ_{leaf} between -0.7 and -1.5 MPa; DS = Drought-stressed treatment with ψ_{leaf} between -1.8 and -3.5.0 MPa. Comparisons of the correlation coefficients were made using the Spearman test; (***) *p* <0.01; (**) *p* <0.05; (*) *p* <0.1; (ns) not significant.
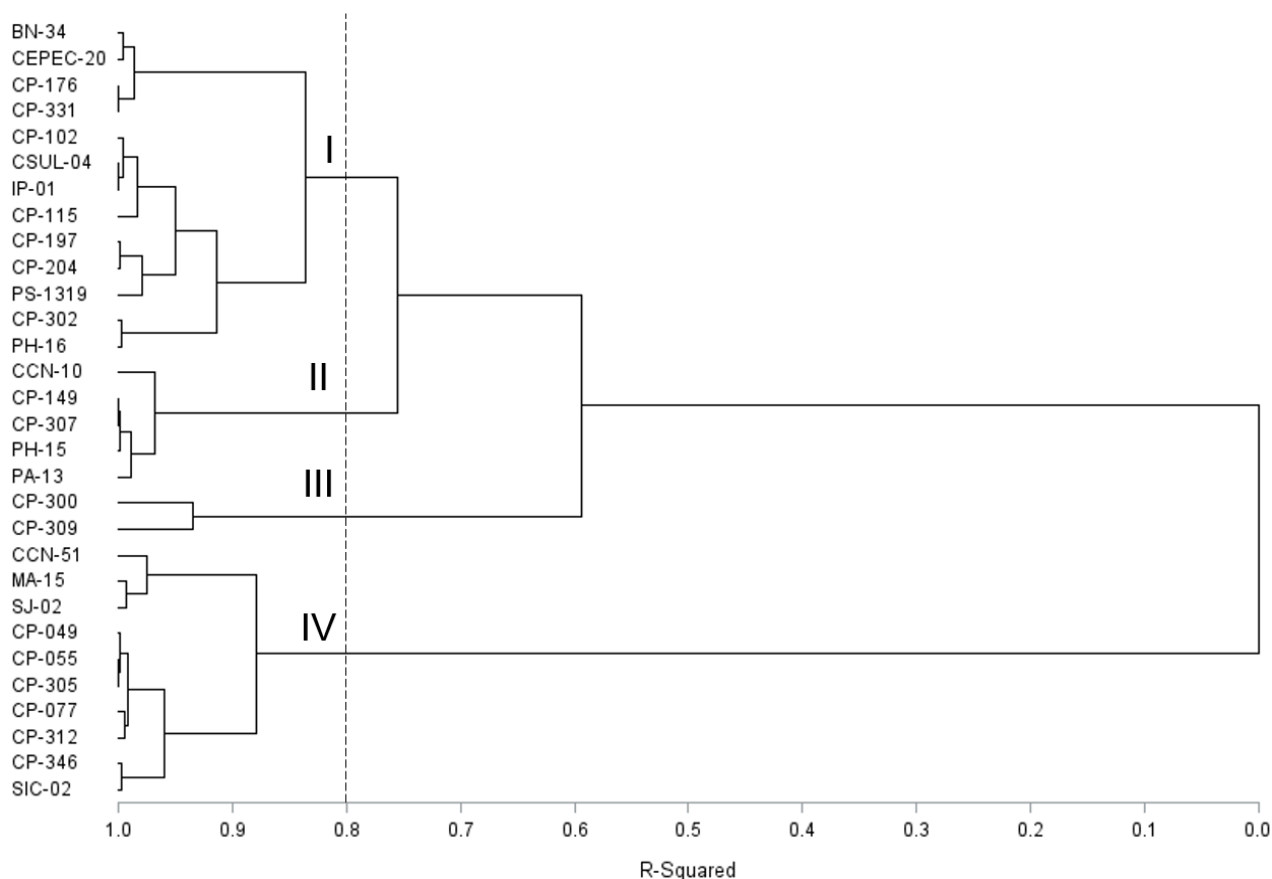
Figure 3. Cluster analysis of 30 genotypes of *Theobroma cacao* submitted to water restriction for 32 days, based in Euclidian distance from survival rate, relative water content, water potential, stomatal density, and p-v curve parameters under drought conditions, using the Ward's method.

Discussion

Plant death induced by water stress has often been observed, however, some plants are able to resist, while others succumb to drought (McDowell et al., 2008). This was corroborated by our study, that is, of the 30 cacao genotypes, sensitive progenies showed the greatest damage resulting in high mortality rates. Plants with leaves still turgid were found, while the majority had chlorosis, leaf senescence and permanent wilting point. Symptoms similar to those observed by other authors under severe water stress conditions were also observed in this study (Atkinson et al., 2000; Jeanneau et al., 2002; Bogeat-Triboulot et al., 2006). The cacao tree shows the potential of adaptive morphology under hydric limitations and rapid leaf fall occurs in some genotypes in response to water stress (Lahive, Hadley and Daymond, 2019).

In addition to senescence at the plant and leaf level, growth is one of the most sensitive responses to water stress. In our study, the surviving plants did not show changes in plant height due to drought, either when the RGR or the height difference was evaluated. Growth responses under water stress have been shown to be species-specific and different results have been reported for this parameter under drought conditions (Aguirrezabal et al., 2006; Pereyra-Irujo et al., 2008) featuring a dynamic and flexible balance between growth and survival of stressed plants (Claeys and Inze, 2013). In cacao, Araque et al. (2012) also did not observe responses in the growth of young plants in dry seasons. This can be explained if we consider that the plants were in the acclimatization phase, when the plants strategies are focused on staying alive and there is no investment of energy in other functions, such as growth. This phase is related to a plant strategy to

restore cellular homeostasis (Munns et al., 2010; Kosová et al., 2011, 2015).

Regarding the RWC, we observed a great variability among progenies when the difference under stress and well-watered conditions were considered. However, in general the RWC decreased during the first days of stress for all progenies. Considering that, with the interruption of irrigation, the rate of water removed from the soil becomes completely dependent on the loss of water to the atmosphere and this total water loss is in turn dependent on the size of the plant, leaf area, stomatal conductance, and environmental conditions; RWC allows inferring about the mechanisms to prevent dehydration.

Considering these factors, RWC is among the most frequently used criteria for differentiate tolerant germplasms in different crop species (Bayoumi, Eid and Metwali, 2008; Munns et al., 2010; Hopper, Ghan and Cramer, 2014; Anupama et al., 2018). In cacao, Balasimha and Daniel (1988) used this method to evaluate drought-tolerant and sensitive genotypes. They observed that drought-sensitive accessions exhibited more pronounced curves compared to tolerant clones (Balasimha and Daniel, 1988). Given this fact, we can infer that the RWC is a powerful measurement variable to assess the water status of the plant, but for a better interpretation of tolerance responses this measure must be complemented by other analysis at the plant and cell level, as for example parameters derived from the pressure-volume curve (p-v).

In our study, the parameters RWC_{tlp} (relative water content at turgor loss point) and ε (bulk modulus of elasticity) were related to survival in stress conditions and can be used as indirect measurements of plant resistance to water restriction. Similar correlations are described in the literature for a variety of species and biomes (Lenz, Wright and Westoby, 2006; Bartlett et al., 2014; Maréchaux et al., 2015). Furthermore, these two parameters were negatively correlated with each other, i.e., more elastic cell walls tend to lose cell turgor at higher water potential values, and even lower RWC_{tlp} values were related to more elastic walls, which indicates cell wall stiffness as a drought tolerance mechanism (Bartlett, Scoffoni and Sack, 2012; Bartlett, 2014). In this study, the progeny CP-077, which presented a discrepant response in ε, showing greater wall stiffness in DS conditions, also had a lower

survival rate, corroborating the idea that greater wall elasticity would be more advantageous under drought stress.

The turgor loss point (ψ_{tlp}) corresponds to the water potential in incipient plasmolysis and is considered the most determinant variable in the quantification of drought tolerance, among all parameters derived from the p-v curve. Its amplitude defines the range in which the leaf cells remain turgid and remain functional (McDowell, 2011; Sack et al., 2013; Scoffoni et al., 2012). In addition, this parameter is closely related to the permanent wilting point that had a limit of -1.5 MPa; however, it is known that this value varies between tropical species (Maréchaux et al., 2015). All p-v parameters evaluated here are cited as mechanisms that alleviate decreases in plant growth and yield during water stress (Merchant et al., 2007; Moore et al., 2008; Kozlowski and Pallardy, 2002).

The stomatal density is directly related to the processes of contraction and cellular expansion regulated by the elasticity/rigidity of the wall, and makes the stomata closer to each other, contributing to a better control of transpiration (Bosabalidis and Kofidis, 2002; Xu and Zhou, 2008). Stomatal density can vary in plants subjected to water stress and changes in this trait have been described in the literature as a mechanism for adapting to water stress in cacao (Zakariyya, Setiyawan and Susilo, 2017) and other species (Grisi et al., 2008; Yu et al., 2008; Maes et al., 2009), and may be result of new stomata or reduction of leaf area.

Stomatal density values found in our study agree with the average values obtained for cacao leaves (Gomes et al., 1988). The progenies BN-34, CCN-10, CP-055, CP-204 and CP-302 showed a reduction in stomatal density under water restriction; however, some showed an inverse behavior, such as CCN-51, CEPEC-2002, PH-16 and PS-13.19, which had higher values under this condition. Lowest stomatal density has been reported as drought tolerance trait for improvement water use efficient (Hughes et al., 2017), however, PS-13.19 considered a drought tolerant clone by Santos et al. (2014) based on growth variables, oxidative stress, and chemical composition, in this study had a 100% increase in stomatal density under drought conditions when compared to the control.

In the present work, the approach described by Lawor (2013) as a single drought cycle was used. A

method widely used to establish drought resistance due to its ease, simplicity, and clarity (Lawor, 2013). It is important to note that the drought imposition approach directly affects the dynamics of the stress phases. According to Kosová et al. (2015), our experiment covered the alarm and acclimatization phases; however, the resistance phase was not reached in senescent plants. In plants that resisted drought and recovered after irrigation was restored, we propose that this phase was reached, since they recovered when the stressor was removed. Another factor is the size of the pots used in the experiment, which over the days may have caused additional stress by limiting space and nutrients, accelerating this phase dynamics. Lastly, the control of water loss in drought tolerance tests is influenced by several factors inherent to the plant and the environment, and this may have interfered with the differentiation of progenies for some of the variables evaluated.

Conclusion

Progenies of CP-309 and CP-300 showed the highest levels of survival rate followed by PH-16, CP-176, CP-302, CP-331, PS-13.19, BN-34 and CEPEC-2002 characterizing resistance to severe drought. Therefore, these progenies should be studied in more detail as to their response mechanisms to water stress.

Among the progenies evaluated, a positive correlation between the parameters RWC, ε, RWC_{tlp} and survival under severe drought were found, with RWC_{tlp} being considered as the main predictor of survival to severe drought in the present work. These parameters were able to associate the studied genotypes in well-defined groups that in the future may support future studies on cocoa drought tolerance. These mechanisms may participate in responses to water stress, by limiting dehydration or maintaining greater water potential and/or processes that allow tolerance to lower water potential values, and therefore need to be further studied.

Acknowledgments

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