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RESEARCH ARTICLE

CHANGE IN LEAF EPICUTICULAR WAX AND BIOCHEMICALSECONDARY METABOLITES IN COCOA (*THEOBROMA CACAO L.*) UNDER HYDRIC STRESS

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ABSTRACT

Cocoa (*Theobroma cacao L.*) is continuously being exposed to frequent fluctuations in weather conditions during its crop cycle. Plants in general, adapt to these varying weather conditions by adopting different methods with morpho- physiological traits and with secondary metabolites. Biochemical constituents of cocoa leaves of different genotypes were studied in relation to two watering regimes represented by normal irrigation (100% of field capacity) and hydric deficit stress (20% of field capacity) conditions. Concentrations of leaf epicuticular wax, total soluble sugars, free amino acids, proline and proteinsshowed high genotypic variability due to the implementation of the watering regimes. Leaf epicuticular wax, cytosolic total soluble sugar and proline contents increased during hydric deficit stress whereas, cytosolic free amino acid and protein contents decreased during 20% FC level. The pattern of accumulation of these biochemical constituents varied among cocoa genotypesby the influence of hydric stress. Cocoa genotypes VTLCP-26, VTLCP-27, VTLCP-25, VTLCH-4 and VTLCP-22 with more accumulation of these compounds exhibited wider adaptability. The results indicated possible role of biochemical compounds in cultivar adaptability to hydric deficit stress, which can be exploited for screening of cocoa germplasm for drought.

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INTRODUCTION

In most of tropical plants, selection of drought tolerant genotypes was undertaken with different adaptation strategies. The methods of adaptation varied from one species to another and from one genotype to another within the same species. These strategies involve a wide range of combinations of anatomical, morphological, physiological, biochemical and molecular factors. Indeed, the study of drought adaptation mechanisms and identification of genes were taken up with the aim of obtaining capable plants to produce in moderate and severe water deficit situations. The induced water restrictions are accompanied by morphological, physiological and anatomical modifications that may result in changes in plant biochemical metabolism. Thus, at anatomical level, some plants induce accumulation of epicuticular waxes to limit the harmful effects of hydric deficit stress. Cameron *et al*. (2006), Kosoma *et al*. (2009) and Seo *et al*. (2011) reported that plants exposed to drought stress have higher levels of cuticular wax. In *Arabidopsis*, an alkane content of cuticular wax has recently been correlated with greater drought tolerance (Bourdenx *et al*., 2011). Morphologically, the reduction of the plant height and number of leaves are the methods to fight against drought stress. At cellular level, plant set up a water absorption mechanism and protection of the most sensitive structures against lethal dehydration, which is the osmotic adjustment (Zhang *et al*., 1999; El Mourid, 1988; Casals, 1996).This phenomenon induces accumulation of biochemical metabolites, the major compounds are amino acids (Proline, Alanine, Arginine, Citrulline), sugars (Sucrose, Fructose, Trehalose, Sorbitol), polyols (Mannitol, Pinitol), quaternary amines (Glycine, Betaine), organic acids (Malate, Glutamate, Citrate) or inorganic salts (K^+, Na^+, Cl^-).These osmolytes accumulated in the cytosol in high concentrations without disrupting the cellular metabolic activity on the one hand and other hand, protect cell vital structures.So they are qualified as compatible osmoticums or "osmoprotectants"(Yancey *et al*,. 1982; Bohnertand Jensen, 1996; Singer and Lindquist, 1998).

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The soluble sugars accumulated in response to stress showed significant negative correlations between biomass production and cytosolic sugar content in sunflower, beans and rice (El Midaoui *et al*., 1999; Rather, 1984). According to Jones *et al*. (1980), soluble sugars participate to over 60% in osmotic adjustment and almost all the cultivated plants accumulate free proline in leaf, when it was under stress (Wang *et al*., 2003; Monneuveuxand Memmar 1986). Proline through its amphiphilic nature had the interaction with hydrophobic structures of protein in the cytoplasm aqueous solution. High cytoplasmic concentration of proline is compatible with the metabolic activities of the cell due to its solubility and a lack of toxicity. The protein of stress play animportant role in the adaptation of plants to abiotic stresses and therefore many workers assess plant stress resistance by the isolation and study of these molecules (Campalans *et al*., 1999). Schulze *et al*. (2005) have written that a portion of the induced proteins have a direct function in increasing stress tolerance (functional protein) and others have a function in the transduction chain (regulatory proteins), which lead to the production of functional proteins. Most direct function of aquaporins and proteins are enzymatic, catalyzing the biosynthesis of osmolytes (carbohydrates and amino acids). High genotypic variability was observed in the accumulation of osmolytes in stress conditions in plants (Radhouane, 2011; El Midaoui *et al*., 2007). However, the accumulation of biochemical metabolites involved in tolerance mechanisms in cocoa under hydric deficit stress is little or unknown. This study aims to determine the effect of watering regime on two months old cocoa seedlings with epicuticular wax, total soluble sugars, free amino acids, proline and protein contents and to assess the existence of genotypic variability for the accumulation of osmolytes compounds.

MATERIALS AND METHODS

Plant material

Ten cocoa genotypes (VTLCP-22, VTLCP-11, VTLCP-24, VTLCP-25, VTLCP-26, VTLCP-29, VTLCP-27, VTLCP-28, VTLCH-4 and VTLCH-3) were used in this study.

Experimental condition

The study was carried out at the Regional Station of ICAR-Central Plantation Crops Research Institute (CPCRI), Vittal, Karnataka, India. The relative humidity, average temperature and evapotranspiration during the study period is presented in Fig. 1. The relative humidity ranged between 74.2 and 93.3%, temperature between 25.3 and 30°C and low evaporation indicate favorable conditions for cocoa farming.

Experimental design

The experiment in pots was laid out in a completely randomized design. Ten cocoa genotypes were used and two levels of watering treatments were practiced with three replicates. A control (100% of Field Capacity) group with daily watering (no stress), where the pot water content was kept wet corresponding to maximal soil field capacity (FC) and a stressed group (20%FC), in which soil moisture was kept at twenty percent with a pot watering with twenty percent of maximal soil field capacity (severe stress).

Determination of Epicuticular Wax (ECW)

The epicuticular wax content was estimated by the method of Ebercon *et al*. (1977), modified and adapted to our plant material. This approach has been the removal of twenty (20) leaf segments of 3 cm^2 (3 x 1 cm = 3 cm^2) on the third and fourth leaves of young cocoa. The leaf segments were immersed in 15 ml of chloroform. For 20 seconds of vigorous shake, chloroform was collected into vials and then evaporated at room temperature. To the residue 5 ml of potassium dichromate reagent was added and kept in a boiling water bath for 30 min. Thereafter, the obtained volume was made up to 17 ml with distilled water. The optical density of the diluted solution was read at 590 nm to the visible UV spectrophotometer (Shimadzu UV160A, Japan). The epicuticular wax content of cocoa tested was determined according to the regression line obtained from the concentrations and optical densities of the reference range varying from 0.4 to 2 mg/ ml ECW.

Determination of osmolytes compounds

Extraction of osmolytes compounds

1 g of fresh leaf tissue was ground in liquid nitrogen and then homogenized in 10 ml of ethanol (80%). The homogenate kept at room temperature, was filtered through WhatmanNo.1 filter paper. The ethanolic extract obtained was used for the quantitative estimation of total soluble sugars and free amino acids.

Determination of total soluble sugar content

The concentration of total soluble sugars was determined by the method of phenol sulfuric acid (Dubois *et al*., 1956). 1 ml of each collected ethanolic extract was adjusted to 25 ml with bidistilled water. 0.1 ml of the diluted extract was added with 1 ml of phenol reagent (5%) and 5 ml of 96% sulfuric acid. After 30 min. of incubation in boiling water, the optical density was read at 490 nm in a spectrophotometer. The soluble sugar content of leaf samples was determined from the regression between the concentrations and the optical densities of the reference range (0.02; 0.04; 0.06 mg/ ml) of a standard glucose solution 1mg/ ml.

Determination of free cytosolic amino acid

The free cytosolic amino acids were estimated by the method of Moor and Stain (1948). 0.1 ml ethanolic extract of leaf samples were added successively to 0.9 ml of distilled water and 3.8 ml of test solution (1%Ninhydrin, 2.4 ml of glycerol and 0.4 ml of citrate buffer). The reaction mixture was heated in a water bath at 100°C for 12 min. After cooling the solution to room temperature, the optical density of the solution was read at 570 nm with the UV-visible spectrophotometer. The concentration of cytosolic free amino acids of the leaf samples was determined according to the regression between the concentrations and the optical densities of the reference range

of concentration of leucine solution ranging from 0.01 to 0.06 mg/ml.

Determination of proline content

The protein concentration of the cocoa tree leaves was estimated by the method of Bates *et al*. (1973). 1 g of leaf tissue was ground in liquid nitrogen and then homogenized in 10 ml of sulfosalicylic acid solution (3%). The homogenate was filtered through WhatmanNo.1 filter paper. To 2 ml of the filtrate of each sample, 2 ml of ninhydrique acid and 2 ml of acetic acid were added. The mixture was brought to boiling water bath for one hour, then cooled in an ice bath. After cooling, 5 ml of toluene was added to the mixture and then it was mixed by vortexing for 10 min. After decantation, the toluene was aspirated and the optical density of the mixture was read at 520 nm UV visible spectrophotometer. Proline concentration of leaf samples was determined according to the regression line between the concentrations and the optical densities of the standard range of a proline standard solution ranging from 0.01 to 0.06 mg/ ml.

Determination of cytosolic soluble protein content

Bradford (1976) method was used for estimation of the total soluble proteins. It consisted of grinding 500 mg of leaf tissue in liquid nitrogen. The homogenate was homogenized in 15 ml extraction buffer (Tris buffer, pH 8) and centrifuged at 12,000 rpm at 4°C for 15 min. in a refrigerated centrifuge (Hareus, Germany). The supernatant (0.5 ml), the BBC reagent (0.5 ml) and distilled water (5 ml) was added. After stirring in the vortex, the optical density was read at 595 nm in the UVvisible spectrophotometer. Protein concentration of the leaf samples was determined according to the regression between the concentrations and the optical densities of the reference range of a standard solution of BSA.

Statistical analysis

The data analysis was performed with SAS9.3software to estimate the mean differences between cocoa hybrids under watering regime conditions for epicuticular wax and osmolytes compounds. The mean comparison of cocoa genotypes in water deficit regime compared to their respective unstressed controls was performed using the paired t-test. The comparison of each genotype from others in each watering regime was done using the GLM procedure of SAS.LSD test were used to compare the average at a significant level of 5% (LSD test). In this analysis, the residual variance E1 was used to test the influence of watering regimes (wr) also, where: E1 = rep x wr.

RESULTS

Epicuticular waxcontent

Epicuticular wax level in cocoa genotypes under hydric deficit stress 20% FC and normal irrigation 100% FC showed a greater dispersion in normal irrigation condition (min= 3.66 µg/cm^2; max= 29.94 µg/cm^2; Q3-Q1= 11.36 µg/cm^2) than under water stress 20% FC (min= 14.64 µg/cm^2; max= 31.87 µg/cm^2; Q3-Q1= 3.54 µg/cm^2) condition. Half of all genotypes tested scored higher wax content to 19.07 µg/cm^2 (median) under water stress and than 17.05 µg/cm^2 (median) in normal irrigation condition. The average content of wax under normal irrigation condition (18.97 µg/cm^2) increased under hydric deficit stress (20.59 µg/cm^2) (Fig. 2). Cocoa genotypes VTLCP-22, VTLCP-29 and VTLCP-27 obtained a low wax production under irrigation regime 20% FC compared to their respective controls. However, a comparison of cocoa genotypes under 20%FC regime to their control in 100% FC regimes showed significant differences for wax production except genotype VTLCH-4 (Table 1). In watering 100% FC, high levels of epicuticular wax were obtained by genotypes VTLCP-27 with 29.37 µg/cm^2, VTLCP-22 with 26.95 µg/cm^2 and VTLCP-29 with 24.94 µg/cm^2. However, genotypes VTLCP-11 with 10.93 µg/cm^2, VTLCH-3 with 11.33 µg/cm^2 and VTLCP-28 with 14.07 µg/cm^2 exhibited the lowest values. Similarly, under water stress 20% FC, the genotypes VTLCP-26 with 31.22 µg/cm^2, VTLCP-27 with 25.5 µg/cm^{2}and VTLCP-29 with 21.56 µg/cm^2 achieved the highest levels of epicuticular wax while, VTLCP-22 with 14.71 µg/cm^2, VTLCP-24 with 17.69 µg/cm^2 and VTLCH-4 with 18.66 µg/cm^2 showed the lowest levels of wax (Fig. 3).

Cytosolic total soluble sugar content

The box plot parameters indicated a greater dispersion of soluble sugars levels in water regime 20%FC (mini= 9.76 µg/g FM; max= 28.09 µg/g FM; Q1-Q3= 5.16 µg/g FM) than normal irrigation condition 100%FC (mini= 7.74 µg/g FM; max= 20.02 µg/g FM; Q1-Q3= 1.97 µg/g FM).Half of the genotypes tested for soluble sugar content showed more than 15.57 µg/g FM (median) in hydric stress to 10.65 µg/g FM (median) in normal irrigation conditions (Fig. 4).The effect of hydric deficit stress induced was marked by a strong accumulation of cytosolic soluble sugar deficit water stress condition 20%FC with an average of 16.86 ± 4.58 µg/g FM, representing a production of over 50% compared to unstressed control which obtained an average of 11.19 ± 2.29 µg/g FM. A comparative study of cocoa genotypes under irrigation regime 20%FC with their respective controls revealed two cocoa groups. The first group showed statistically significant differences between water regimes 100%FCand 20%FC.Thegenotypes are VTLCP-22 (Pr>| t |=0.0336), VTLCP-24 (Pr>| t |=0.013), VTLCP-25 (Pr>| t |=0.0093) VTLCP-28 (Pr>| t |=0.0095) and VTLCP-29 (Pr>| t |=0.0178). The genotypes of the second group, VTLCP-11 (Pr>| t |=0.1466), VTLCP-26 (Pr>| t |=0.3929), VTLCP-27 (Pr>| t |=0.1551), VTLCH-4 (Pr>| t |=0.187) and VTLCH-3 (Pr>| t |=0.9833) showed statistical homogeneity between water regime 20%FC and 100%FC (Table 2). Under deficit hydric stress 20%FC, genotypes VTLCP-25 (25.4 µg/g FM), VTLCP-28 (23.01 µg/g FM) and VTLCP-29 (18.81 µg/g FM) revealed high cytosolic soluble sugar concentrations while, the genotypes VTLCP-26 (11.88 µg/g FM) and VTLCP-27 (12.94 µg/g FM) showed lower contents of soluble sugar. In normal irrigation condition, the genotypes VTLCH-3with 15.89 µg/gFM, VTLCH-4 with 12.44 µg/g FM and VTLCP-29 with 12.33 µg/g FM presented the highest concentrations in soluble sugar while, VTLCP-22 with 9.69 µg/g MF, VTLCP-29 (9.69 µg/g FM) and VTLCP-24 (9.52 µg/g FM)presented the lower grades (Fig. 5).

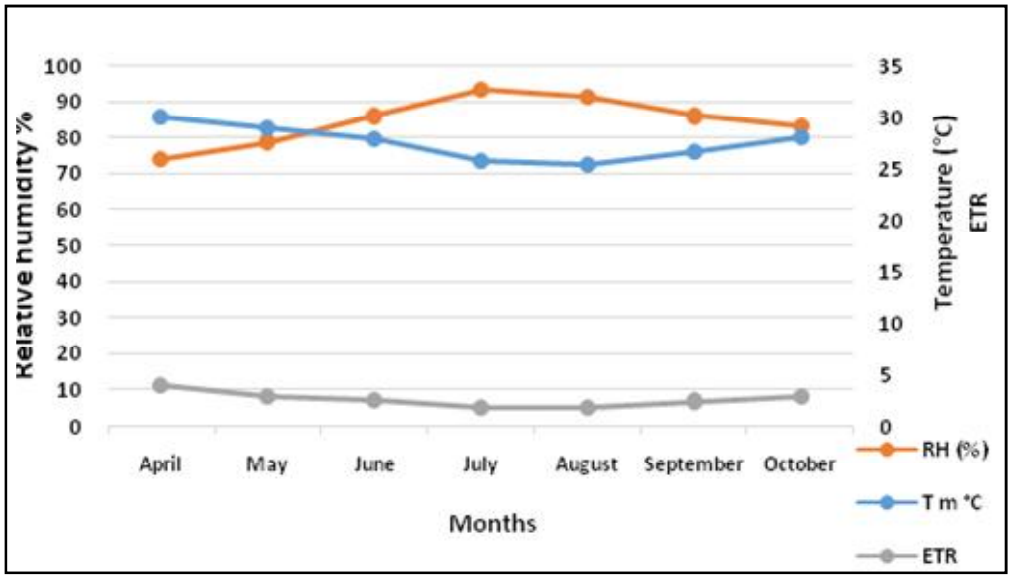


Fig.1. Change in monthly average of relative humidity (RH%) and temperature (T°C) at glass house and evapotranspiration ($ETR^{mmday-1}$) during experiment

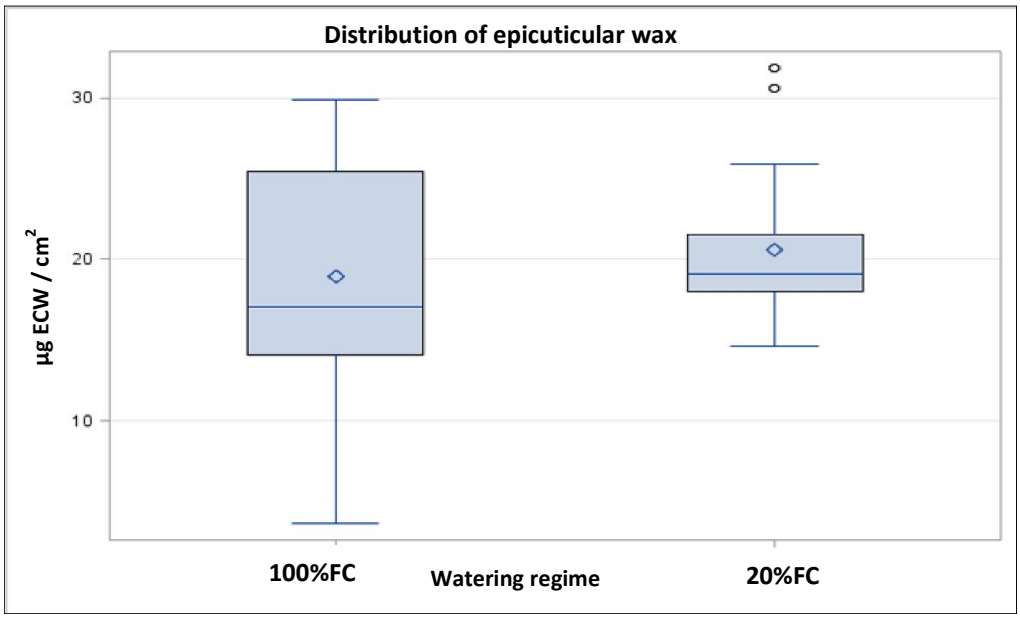
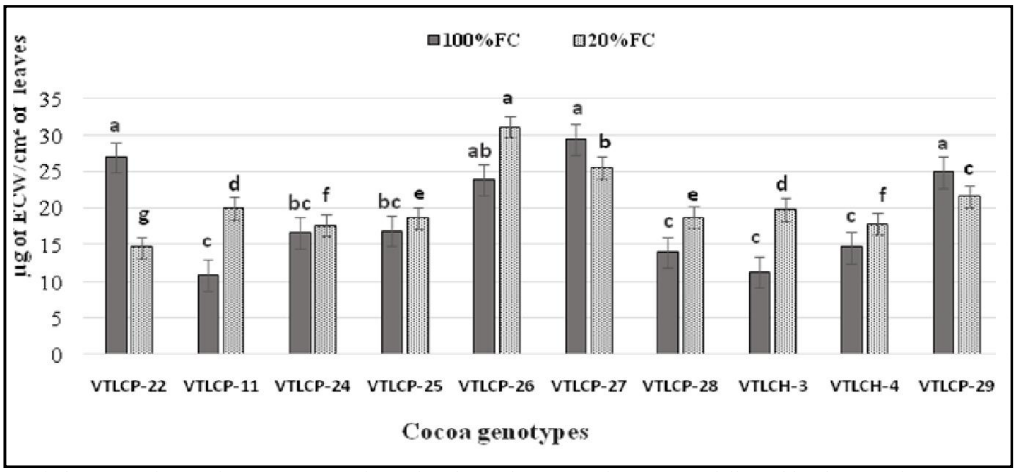


Fig. 2. Influence of watering regime on distribution of epicuticular wax in leaves of cocoa genotypes



*The comparisons of means were performed under each water regime. Means followed by the same letter are statistically identical at a significance level of 0.05 (LSD). LSD ($p \leq 0.05$); 100%FC, $p \leq 0.0001$; 100%FC,$p = 0.0027$

Fig. 3. Foliar epicuticular wax content in cocoa genotypes under water regimes

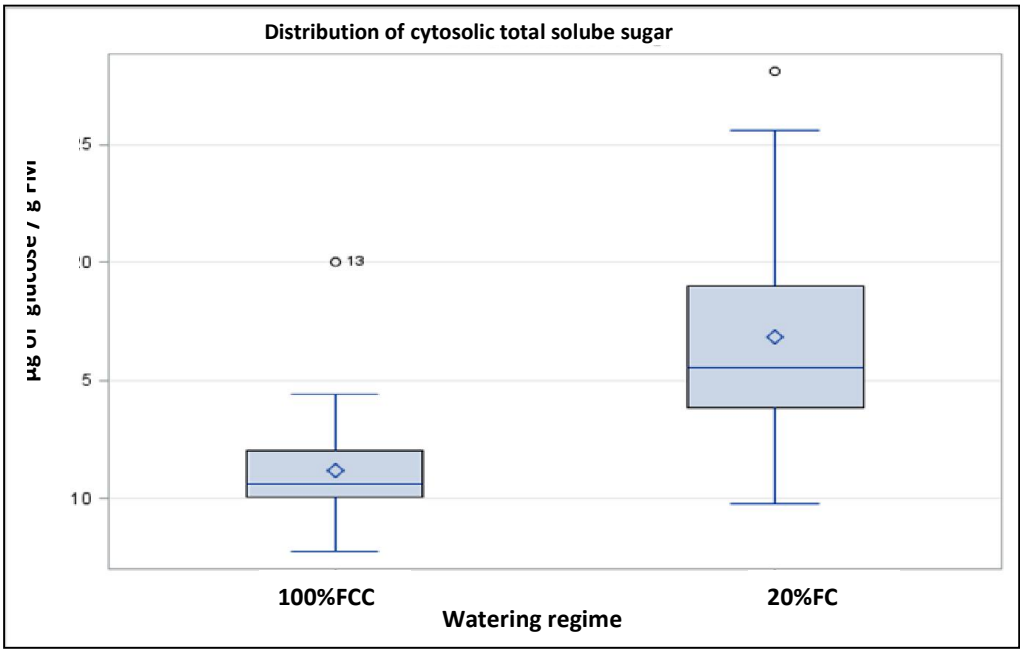
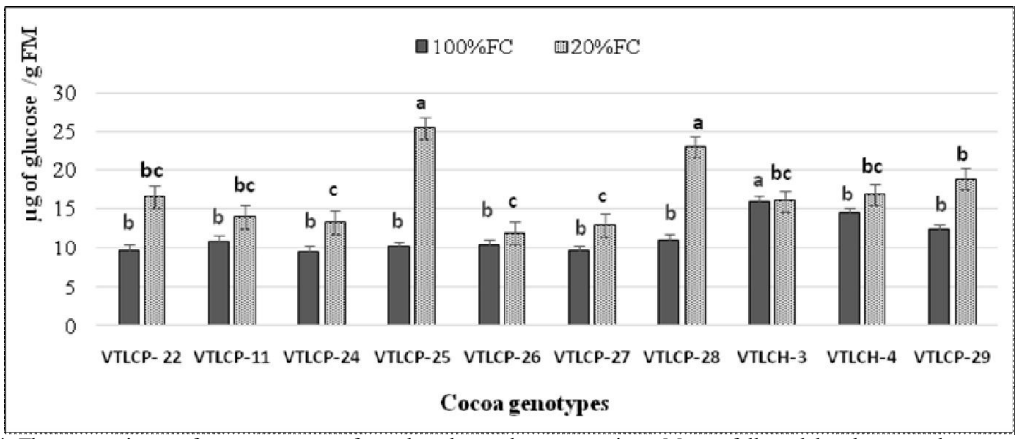


Fig. 4. Influence of watering regime on distribution of total soluble sugar content in leaves of cocoa genotypes



* The comparisons of means were performed under each water regime. Means followed by the same letter are statistically identical at a significance level of 0.05 (LSD). LSD ($p \leq 0.05$); 100%FC, $p \leq 0.0001$; 20%FC) $p = 0.0020$

Fig. 5. Cytosolic total soluble sugar content in cocoa genotypes under water regimes

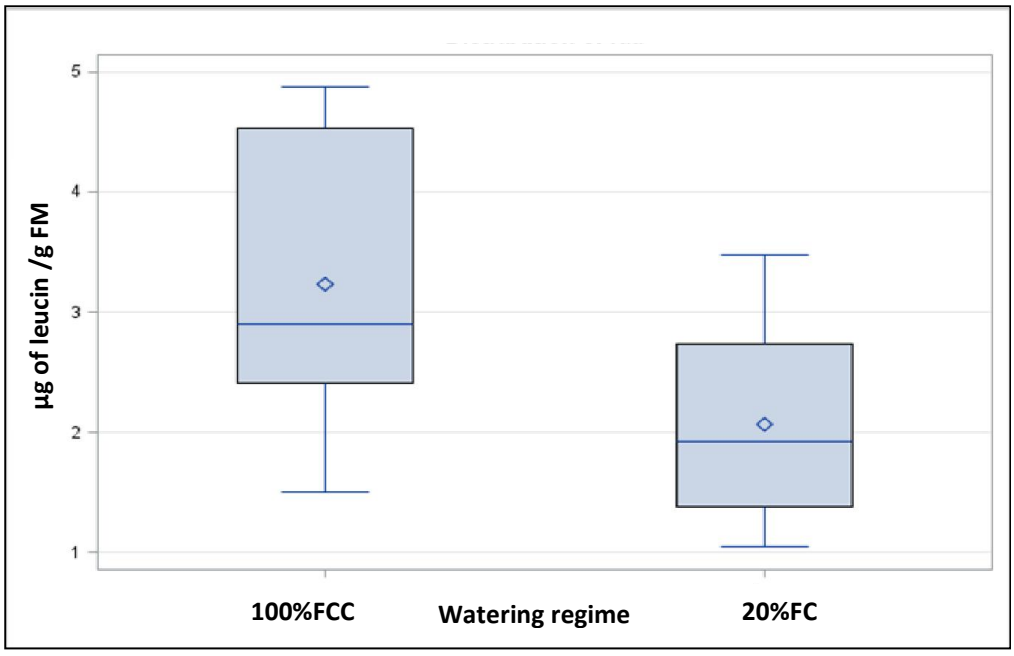
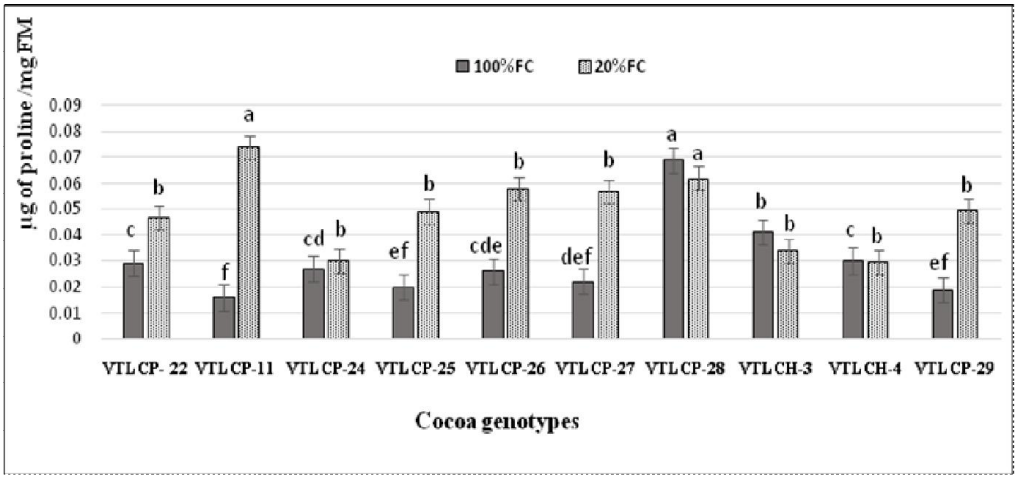


Fig. 6. Influence of watering regime on distribution of cytosolic free amino acid content in leaves of cocoa genotypes



* The comparisons of means were performed under each water regime. Means followed by the same letter are statistically identical at a significance level of 0.05 (LSD). LSD ($p \leq 0.05$); 100%FC, $p \leq 0.0001$; 20%FC, $p = 0.0001$

Fig. 7. Cytosolic free amino acid content in cocoa genotypes under water regimes

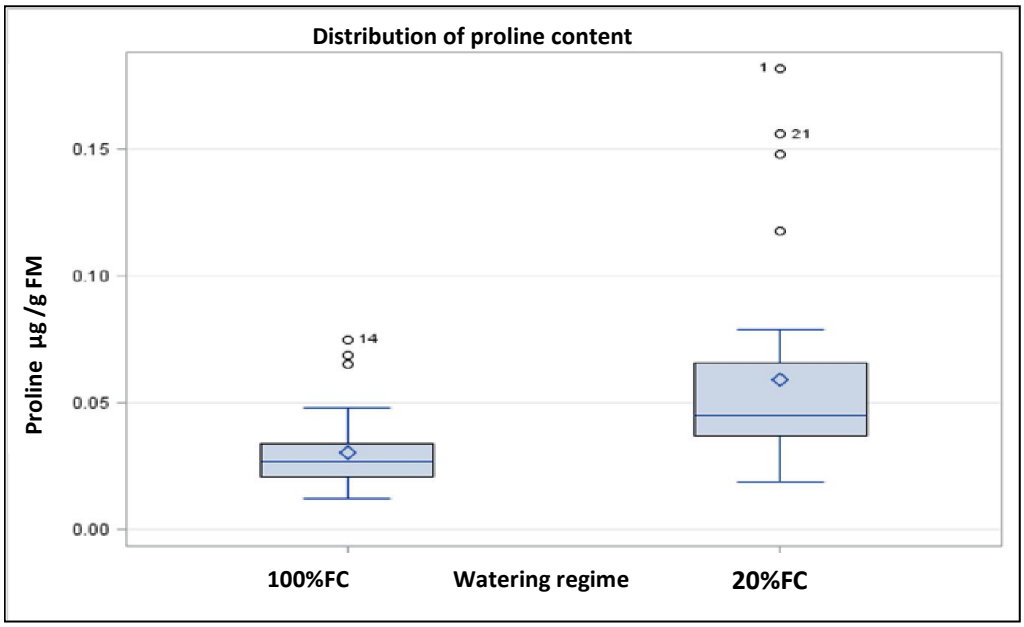
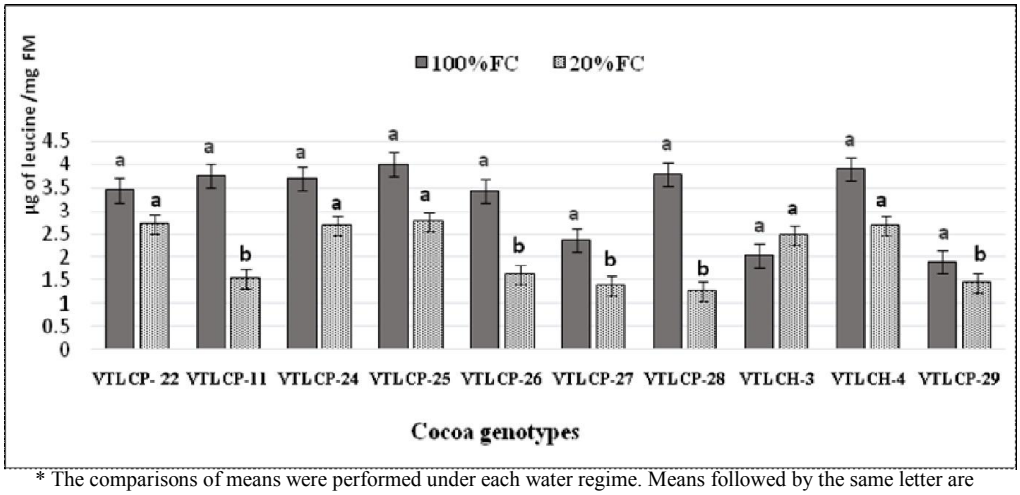


Fig. 8. Influence of watering regime on distribution of cytosolic proline content in leaves of cocoa genotypes



* The comparisons of means were performed under each water regime. Means followed by the same letter are statistically identical at a significance level of 0.05 (LSD). LSD ($p \leq 0.05$); 100%FC, $p \leq 0.0001$; 20%FC) $p = 0.0020$

Fig. 9. Cytosolic proline content in cocoa genotypes under water regimes

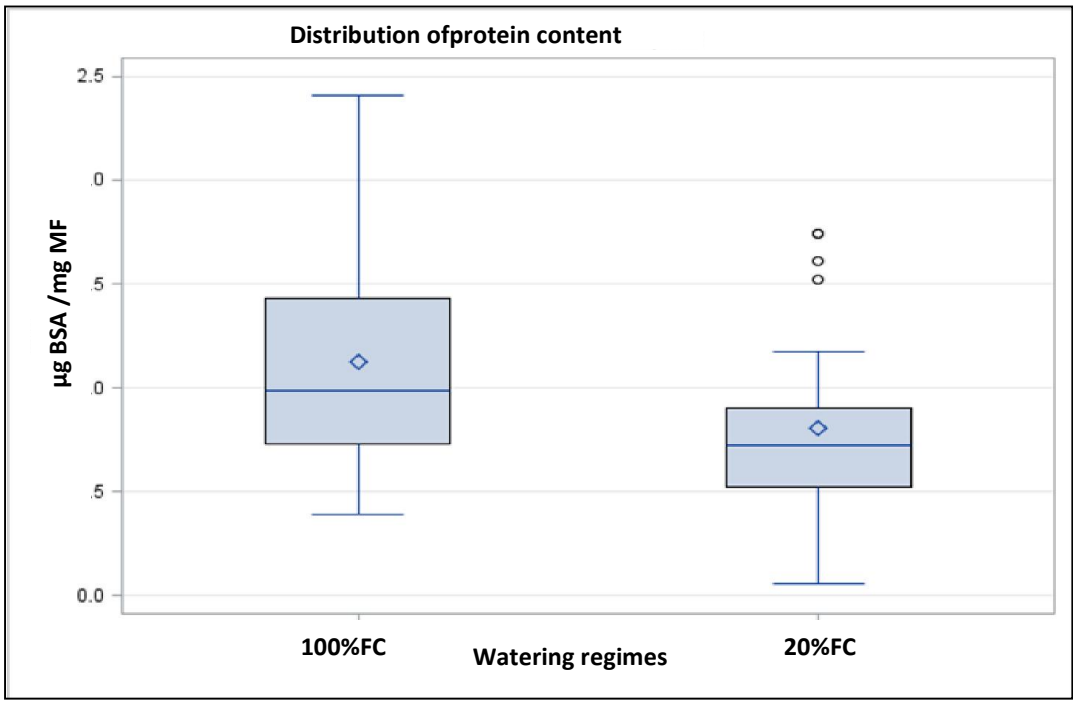
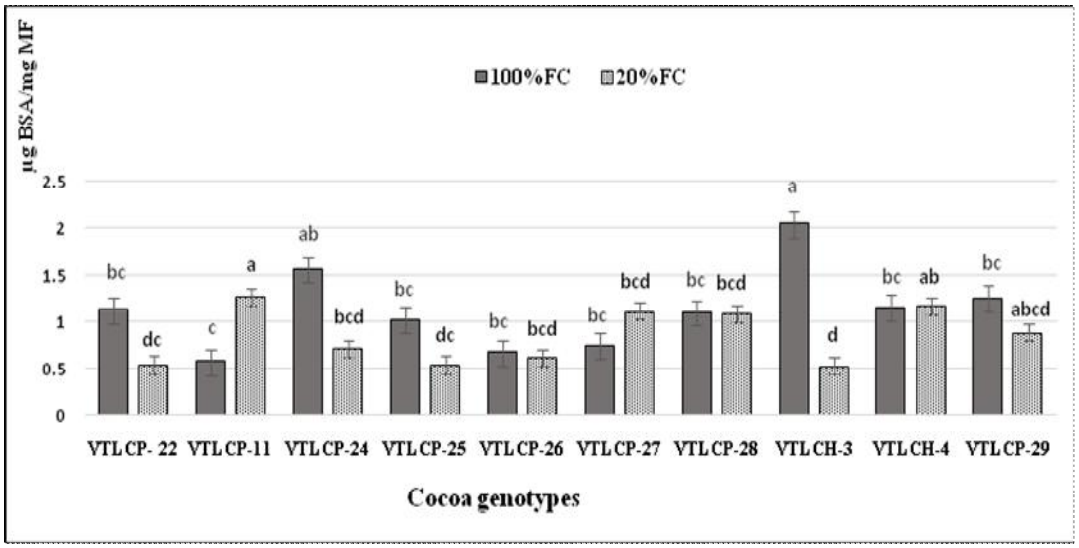


Fig.10. Influence of watering regime on distribution of cytosolic protein content in leaves of cocoa genotypes



* The comparisons of means were performed under each water regime. Means followed by the same letter are statistically identical at a significance level of 0.05 (LSD). LSD ($p \leq 0.05$); 100%FC, $p \leq 0.0012$; 20%FC, $p = 0.0001$

Fig. 11. Cytosolic protein content in cocoa genotypes under water regimes

Table 1. Comparison of cocoa genotypes under watering regime 100%FC and 20%FC for epicuticular wax

Cocoagenotypes	Difference between unstressed and stressed genotypes			
	µg/cm^2 of WAX	Error standard	t-Value	Pr > |t|
VTLCP-22	12.238	0.144	84.97	<.0001
VTLCP-11	-9.017	2.520	-3.58	0.0232
VTLCP-24	-1.046	0.036	-29.07	<.0001
VTLCP-25	-1.771	0.00024	-72315	<.0001
VTLCP-26	-7.407	0.360	-20.57	<.0001
VTLCP-27	3.864	0.072	53.66	<.0001
VTLCP-28	-4.750	0.108	-43.98	<.0001
VTLCH-3	-8.454	0.036	-234.69	<.0001
VTLCH-4	-3.236	4.904	-0.66	0.5453
VTLCP-29	3.381	0.072	46.95	<.0001

Comparisons of means were done between control (100%FC) and each stressed (20%FC) genotype of cocoa at a significance level of 0.05 (T-paired test)

Table 2. Comparison of cocoa genotypes under watering regime 100%FC and 20%FCfor total soluble sugar

Cocoagenotypes	Difference between unstressed and stressed genotypes			
	µg of glucose/gMF	Error standard	t-Value	Pr > |t|
VTLCP-22	-6.816	1.281	-5.32	0.0336
VTLCP-11	-3.056	1.320	-2.32	0.1466
VTLCP-24	-3.756	0.432	-8.68	0.0130
VTLCP-25	-15.313	1.489	-10.28	0.0093
VTLCP-26	-1.526	1.413	-1.08	0.3929
VTLCP-27	-3.323	1.487	-2.23	0.1551
VTLCP-28	-11.940	1.171	-10.19	0.0095
VTLCH-3	-0.066	2.830	-0.02	0.9833
VTLCH-4	-4.393	2.226	-1.97	0.1872
VTLCP-29	-6.483	0.877	-7.39	0.0178

Comparisons of means were done between control (100%FC) and each stressed (20%FC) genotype of cocoa at a significance level of 0.05 (T-paired test)

Table 3. Comparison of cocoa genotypes under watering regime 100%FC and 20%FC for free amino acid content

Cocoagenotypes	Difference between unstressed and stressed genotypes			
	µg of leucine/mgMF	Error standard	t-Value	Pr > |t|
VTLCP-22	0.75	0.1162755	6.45	0.0030
VTLCP-11	2.216	0.3689526	6.01	0.0039
VTLCP-24	1.012	0.1431223	7.07	0.0021
VTLCP-25	1.25	0.1341641	9.32	0.0007
VTLCP-26	1.826	0.1587640	11.50	0.0003
VTLCP-27	0.974	0.2347893	4.15	0.0143
VTLCP-28	2.516	0.3600083	6.99	0.0022
VTLCH-3	-0.45	0.0849706	-5.30	0.0061
VTLCH-4	1.2	0.1431084	8.39	0.0011
VTLCP-29	0.45	0.0983870	4.57	0.0102

Comparisons of means were done between control (100%FC) and each stressed (20%FC) genotype of cocoa at a significance level of 0.05 (T-paired test)

Table 4. Comparison of cocoa genotypes under watering regime 100%FC and 20%FC for proline content

Cocoagenotypes	Difference between unstressed and stressed genotypes			
	µg of proline/gMF	Error standard	t-Value	Pr > |t|
VTLCP-22	-0.017	0.008	-2.04	0.1784
VTLCP-11	-0.058	0.021	-2.73	0.1119
VTLCP-24	-0.003	0.007	-0.46	0.6896
VTLCP-25	-0.028	0.001	-23.85	0.0018
VTLCP-26	-0.032	0.008	-3.81	0.0624
VTLCP-27	-0.034	0.006	-5.43	0.0323
VTLCP-28	-0.092	0.007	-12.44	0.0064
VTLCH-3	0.0073	0.002	3.14	0.0881
VTLCH-4	0.001	0.005	0.18	0.8740
VTLCP-29	-0.029	0.009	-3.06	0.0921

Comparisons of means were done between control (100%FC) and each stressed (20%FC) genotype of cocoa at a significance level of 0.05 (T-paired test)

Table 5. Comparison of cocoa genotypes under watering regime 100%FC and 20%FC for cytosolic protein content

Cocoagenotypes	Difference between unstressed and stressed genotypes			
	µg of BSA/mgMF	Error standard	t-Value	Pr > |t|
VTLCP-22	0.600	0.350	1.71	0.2291
VTLCP-11	-0.701	0.230	-3.04	0.0931
VTLCP-24	0.846	0.358	2.36	0.1423
VTLCP-25	0.478	0.067	7.05	0.0195
VTLCP-26	0.049	0.043	1.15	0.3691
VTLCP-27	0.061	0.115	0.53	0.6476
VTLCP-28	0.016	0.304	0.05	0.9613
VTLCH-3	1.594	0.237	6.73	0.0214
VTLCH-4	-0.021	0.044	-0.48	0.6759
VTLCP-29	0.365	0.526	0.69	0.5598

Comparisons of means were done between control (100%FC) and each stressed

Cytosolic free amino acid content

The distribution of cytosolic free amino acids level in cocoa genotypes under water regime (Fig 6) showed a compression of inter quartile range and reduction in all parameters under 20% FC (min= 1.05 µg/g FM; max= 3.48 µg/g FM; Q3-Q1= 1.36 µg/g FM) compared to those in normal irrigation conditions, 100% FC (min= 1.50 µg/g FM; max= 4.87 µg/g FM; Q1-Q3= 2.12 µg/g FM).Half of the tested genotypes were obtained cytosolic content of free amino acids, greater than 1.93 µg/g FM (median) under water stress and greater than 2.90 µg/g FM (median) in normal irrigation conditions. A comparative study of cocoa genotypes under irrigation regime 20%FC compared to their respective controls indicated in all genotypes decreased production of free amino acids except in cocoa VTLCH-3. Differences in production of free amino acids between control and stress were observed (Table 3). Foliar levels of free amino acids in cocoa genotypes under normal irrigation condition (100%FC) not showed significant statistically differences. However, foliar amino acid levels ranged from 1.79 µg/g FM (VLCP-29) to 4.01 µg/g FM (VTLCP-26). Under induced deficit hydric stress (20%FC), genotypes VTLCP-25 (2.77 µg/g FM), VTLCP-22 (2.70 µg/g FM), VTLCH-4 (2.69 µg/g FM) and VTLCP-24 (2.68 µg/g FM) had high concentrations of cytosolic free amino acids. While low cytosolic levels of free amino acids was obtained with the genotypes VTLCP-28 (1.27 µg/g FM), VTLCP-27 (1.39 µg/g FM) and VTLCP-29 (1.44 µg/g FM) (Fig. 7).

Cytosolic proline content

The distribution of cytosolic proline content of cocoa genotypes under watering regime (Fig. 8) mentioned a decompression of the inter quartile range and an increase in all parameters at the cytosolic proline contents under deficit hydric stress, 20%FC (min= 0.02 µg/g FM; max= 0.18 µg/g FM; Q3-Q1= 0.03 µg/g FM) compared to those in normal irrigation condition (min= 0.01 µg/g FM; max= 0.07 µg/g FM; Q3- Q1= 0.01 µg/g FM). Half of the genotypes tested showed proline content greater than 0.04 µg/g MF (median) under water stress and greater than 0.03 µg/g FM (median) in normal irrigation conditions (Fig. 8). The study of genotypes of cocoa trees under watering regime 20%FC compared to their respective controls showed that the genotypes VTLCH-3 and VTLCH-4 had foliar proline levels below those of their controls. The levels of leaf proline of genotypes VTLCP-25 (Pr>| t |=0.0018), VTLCP-27 (Pr>| t |=0.0323) and VTLCP-28 (Pr>| t |=0.0064) were significantly different from those of their control under 100%FC (Table 4). Under 20%FC water regime, the high contents of proline were obtained by genotypes VTLCP-11 (0.074 µg/g FM), VTLCP-26 (0.058 µg/g FM) and VTLCP-28 (0.062 µg/g FM). While the genotype VTLCH-4 (0.016 µg/g FM) had lower foliar concentration of proline.

Similarly, in normal irrigation condition 100%FC, the genotypes VTLCP-28 with 0.068 µg/g FM and VTLCH-3 with 0.041 µg/g FM recorded strong levels of foliar proline. However, genotypes VTLCP-11 (0.016 µg/g FM) and VTLCP-29 (0.019 µg/g FM) had low levels of proline (Fig 9).

Cytosolic soluble protein content

The analysis of cytosolic protein content distribution in cocoa leaves under water regime (Fig 10) showed a compression of the inter quartile range and a reduction of all parameters in hydric stress 20% FC (mini= 0.05 µg/mg FM; max= 1.74 µg/mg FM; Q3-Q1= 0.38 µg/mg FM) compared to those in normal irrigation condition 100% FC (min= 0.39 µg/mg FM; max= 2.41 µg/mg FM; Q1-Q3= 0.7 µg/mg FM).Half of the genotypes tested showed protein content higher to 0.72 µg/mg FM (median) in water regime 20%FC and higher at 0.99 µg/mg FM (median) in normal irrigation condition. The genotypes VTLCP-11 and VTLCH-4 under watering regime 20%FC presented high levels of cytosolic protein than their control. The foliar protein content of all cocoa were statistically homogeneous as compared with those of their respective controls except genotypes VTLCP-25 (Pr>| T |=0.0195) and VTLCH-3 (Pr>| T |=0.0214) (Table 5).In normal irrigation condition (100%FC), the genotypes VTLCH-3 with 2.05 µg/mgFM VTLCP-24 (1.56 µg/mg FM) and VTLCP-29 (1.25 µg/mg FM) registered the higher levels of protein. However, the genotypes VTLCP-11 (0.57 µg/mg FM), VTLCP-26 (0.67 µg/mg FM) and VTLCP-27 (0.74 µg/mg FM) showed the lowest levels of foliar cytosolic proteins. Similarly, in watering regime 20%FC, genotypes VTLCP-11 (1.27 µg/mg FM), VTLCH-4 (1.17 µg/mg FM) and VTLCP-27 (1.12 µg/mg FM) got high protein levels the most. While genotypes VTLCH-3 (0.45 µg/mg FM) and VTLCP-22 (0.53 µg/mg FM) had the lower contents of protein (Fig 11).

DISCUSSION

Accumulations of secondary metabolites due to hydric deficit stress response varied according to the level of the water regimes and cocoa genotypes. From this study it was visible that water stress induced a significant accumulation of epicuticular wax. To reduce non-stomatal water loss, plants covering the external surface of the leaf with synthesized organic compounds (Li *et al*., 2007). According to Eglinton and Hamilton (1967), plant species with thick cuticle retain more moisture and maintain the state of leaf turgidity for long periods due to the reduced evapotranspiration. Cuticular waxes obtained in our cocoa genotypes are below the value of tolerance to drought which is 50 µg/cm^2 (Balasimha *et al*., 1999). These low values observed in our cocoa are justified by the fact that the leaf epicuticular wax concentration varies according to the season, part of the plant and the plant age (Eglinton and Hamilton, 1967). Plants under abiotic stress develop osmo-regulation phenomena which move through an accumulation of organic or inorganic solutes in cells (Salsac and Monneveux, 1989; Jones and Tuner, 1980). Our results revealed a significant increase in cytosolic soluble sugars, producing more than 50% compared to unstressed control. The accumulation of sugars due to lack of water in the leaf was reported by many workers in C4 plants like millet, wheat, corn etc. (Claassen and Shave, 1970; Golombek and Theek, 2001; Bousba *et al*., 2009; Bousba *et al*., 2011; Pirzad *et al.,* 2011). These authors also noted that it is mainly glucose and fructose which are accumulated in the young leaves in conditions of water stress. These high concentrations of soluble sugars under water stress are linked to activation of enzymes which

hydrolyze starch and also to an improvement in amylase activity which catalyzes the hydrolysis of starch into soluble sugars. Palta *et al*. (1994) and Bajji (1999) justify this accumulation of soluble sugars by remobilization of carbohydrates stored in different organs of plants. The sugars accumulated in response to water stress are the major part of the adjustment in osmotic solutes that contribute to maintaining the balance of osmotic force to maintain turgor and cytosolic volume as high as possible (Yancey *et al*., 1982; Bouzoubaa *et al*., 2001). They maintain membrane integrity in the dehydration bodies and protection of membrane proteins (Darbyshire, 1974).These cytosolic heavy accumulations of glucose that has been recognized by the authors, are consistent with ours. Thus, the high sugar accumulation capacity noted as cytosolic return in the genotypes VTLCP-25, VTLCP-28 VTLCP-29, VTLCH-4 and VTLCP-22.The study of plant nitrogen metabolism subject to abiotic stress shows an overall accumulation ofcytosolic amino acids and proteins (Ranieri *et al*., 1989; Belanger *et al*., 1990). However, in our study, among young cocoa subjected to hydric stress deficit, a significant reduction in cytosolic free amino acids was observed. Among the genotypes studied, strong changes of cytosolic free amino acids content were observed in the genotypes VTLCP-28 and VTLCP-11. Our results are contrary to those of Trotel *et al*. (2003), according to which the low water availability favor accumulation of low molecular weight compounds such as free amino acids. In presence of abiotic stress, El Midaoui *et al*. (2007) noted a strong accumulation of free amino acids in cultivated *Helianthus annus* L. In cocoa, the genotypes VTLCP-22 and VTLCP-24 which showed significant physiological traits of tolerance to water stress (Mbo *et al.*, 2015) had elevated cytosolic levels as well. This cytosolic accumulation is the cause of their alleged tolerance to water stress at early age.

A decrease of the cytosolic protein was registered under water stress in our study. Wilkins *et al*. (1994), Zhen *et al*. (2007) also showed lower soluble protein contents in abiotic stresses in *Glycine max*. However, Farshadfar *et al*. (2008) reported that during water stress durum leaves increase their levels of soluble proteins with low molecular weights than the high molecular weight proteins. Genotypes, VTLCP-11, VTLCP-27, VTLCH-4 and VTLCP-26 showed high levels of cytosolic protein in conditions of water stress application compared to other genotypes tested. These high levels are associated with an activation of genes for synthesis of specific proteins associated with stress such as "LEA" which provide protection of all vital cellular proteins and heat proteins that maintain protein and membrane structures of plant cells (Baker *et al*., 1988). The work of Greenway and Munns (1980) showed an accumulation of cytosolic free proline in plants under adverse conditions. These results are consistent with ours, which showed that conditions of water stress deficit, cocoa genotypes have accumulated high cytosolic concentrations of proline. The active accumulation of proline results in lower osmotic potential than those of water potential thereby enhancing the maintenance of turgor and allowing the opening of the stomatal and good physiological activity (Tallman, 1992; Silva *et al*., 1996). The role of proline remains controversial for some authors. However, its accumulation contributes to the acquisition of resistance due to the maintenance of cellular turgor by osmotic adjustment. In addition, proline accumulation and its increase exhibit significant differences among genotypes. Thus, certain cocoa under water stress, accumulated in cytosolic levels over 35% to those of their respective controls. This result is corroborated by Moulineau (1993) who showed that when the initial content of proline in millet ecotypes is low, more accumulation become important under stress. Furthermore, Singh *et al*. (1973) attributed the high accumulation of proline under water stress to resistance and/ or stability of the considered cultivar. Indeed, genotypes VTLCP-26, VTLCP-27, VTLCP-25 and VTLCP-22 with high concentration of cytosolic free proline presented remarkable physiological trait of tolerance (Mbo *et al*., 2015). The diversity of responses to water stress in proline accumulation leans toward a genetic difference in osmotic adjustment but any conclusion on tolerance or susceptibility to a lack of water by the sole criterion "accumulation of proline" remains derisory. Furthermore, this accumulation can be the result of damage to the plant and in this case, it does not serve as a reliable diagnosis for drought resistance as in the case of genotype VTLCP-11, which in view of these physiological traits seems to be induced by water deficit stress.

Conclusion

Under water stress, plants develop a series of mechanisms that can be morphological, physiological, biochemical or molecular. The processes that contribute to water stress tolerance and help the plant to endure periods of lack of water may act synergistically or additively. The mechanisms adopted in plants move through by selective solute accumulation and synthesis of compatible osmolytes. In our experiment, we studied the accumulation of epicuticular wax, total soluble sugars, free amino acids, protein and proline, which are compatible organic compounds or osmo-protectors. Indeed, in the case of water deficit stress, cocoa adjusts its osmotic potential between others by the accumulation of soluble sugars. However, we must not overlook the role of amino acids, cytosolic proteins and especially high cytoplasmic accumulation of proline in their tolerance to water stress deficit. The accumulation of osmolytes compounds during a severe constraint in the field could not be a selection tool for tolerant cocoa genotypes, but at the young age it could contribute to an effective method for drought selection. For cocoa genotypes, analysis of enzyme activity described in biochemical reactions will be necessary and it would be interesting to determine whether enzymatic changes occur for given stress intensities. To these biochemical indicators, if physiological criteria are involved it will help to draw reliable conclusion about the tolerance of cocoa genotypes to abiotic stress.

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