Research article

Screening for drought tolerance potential of nine cocoa (*Theobroma cacao* L.) genotypes from Ghana

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ABSTRACT

This study was conducted with a view to screen nine genotypes of Cacao from The Cocoa Research Institute of Ghana (CRIG) to test their abilities to withstand drought stress conditions using parameters such as leaf relative water content (RWC), proline accumulation in leaves and trichomes. The experimental design consisted of three replicates of the genotypes used and these were laid out in Complete Randomized Block Design (CRBD) to determine the drought tolerance potentials of the nine genotypes of cocoa at the seedling stage. Two water treatments were used which involved withholding water from one day after full saturation with water prior to the first appearance of drought symptoms (FADS) and watering every two days to the completion of the experiment. Results in this research revealed that proline was found to gather in water-stressed seedlings, and the differences in the mean proline amounts in the genotypes was found to be significant. Genotype T63/971 x Sca9 had the most elevated concentration of free proline at FADS (4 µg/g DW) followed by genotype T60 x Pound10 (3.5 µg/g DW) whereas genotype PA150 × 9006 had the smallest amount of accumulated proline in leaves. Genotype PA150 × 6020 had the highest RWC and SMC of 65% and 1.5% respectively at FADS whilst genotype PA7 x 6035 had the lowest RWC of 43%. There was a direct relationship between the amounts of free proline of genotypes T63/971 x SCA9 and T60 x POUND10 and their respective RWC of the leaves. Genotypes T63/971 x SCA9 and PA150 × 9006 had the highest and lowest numbers of trichomes respectively. Inference from this study revealed that T63/971 x SCA9 and T60 x POUND10 genotypes appear to be the most drought-tolerant genotypes in view of their relatively high values of free proline content, leaf RWC, trichomes and lower values of soil water use (SMC).

1. Introduction

Theobroma cacao L. (Cacao) is cultivated in tropical regions in West Africa, Latin America and the Caribbean and South-east Asia (Farrell et al., 2018; Lahive et al., 2019; Delgado-Ospina et al., 2021). Cacao grows well under canopies of large forest trees which provides the plant with shade (Jagoret et al., 2017). However, there has been recent attempts to have Cacao thrive under high light intensity (Lewis et al., 2021). Cacao is a species of great economic importance worldwide as its beans are the main raw material for the manufacture of chocolate (Krähmer et al., 2015). The crop is a major source of livelihood for the rural communities in some West African countries, especially Ghana and Cote d'Ivoire, and contributes actively to the economy of these countries (Ofori et al., 2017). Despite its importance, the production of cacao faces immense challenges that do not only cause yield reduction, but also raises concerns about sustainability of the crop. One of such challenges is climate change. The cacao plant is known to thrive best in the humid

tropics (including Ghana) as it is a delicate crop highly affected by changes in seasonal weather patterns (Anim-Kwapong and Frimpong, 2005; Gateau-Rey et al., 2018). To ensure growth and enable the production of ripen seeds, cacao requires specific weather conditions and as such, it is adversely affected by periodic drought (Bae et al., 2008). Climate change effect in Ghana, has been linked with global warming and has been identified as the cause of the gradual southwards shifting of the cacao growing belt and it is predicted to continue further southwards of the Brong-Ahafo, Bono East, Western North and Western regions by 2030 (CIAT, 2011), thereby causing a reduction of cacao growing areas and consequently, its production (Wessel and Quist-Wessel, 2015).

Drought stress has a tremendous impact on many aspects of plant morphogenesis. The loss of water under drought stress leads to growth inhibition as well as induces changes in the metabolism and physiology of a plant (Das and Kar, 2018; Khalvandi et al., 2021; Yang et al., 2021; Zhou et al., 2021). For example, photosynthetic capacity has been shown to be significantly obstructed when maize plants were exposed to prolong

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and severe drought conditions (Qi et al., 2021). Most plants that are drought-tolerant, including some genotypes of cacao, often use different methods in responding to drought stress (Fang and Xiong, 2015; Li et al., 2021). These methods can be used to identify drought-tolerant genotypes or species that ensures sustainable yield levels under drought conditions (Alban et al., 2015; Santos et al., 2014). These adaptive methods include structures which are typical of desert plants and these include smaller and thicker leaves, more epidermal trichomes, smaller and denser stomata, a thicker cuticle epidermis, thicker epidermis (Carr and Lockwood, 2011; Fang and Xiong, 2015; Wang et al., 2020), thicker palisade tissue (Karaba et al., 2007; Fang and Xiong, 2015), a higher ratio of palisade to spongy parenchyma thickness and a more developed vascular tissue (Abdulrahaman and Oladele, 2001; Sack and Holbrook, 2006; Fang and Xiong, 2015). Moreover, other varying approaches to reduce increasing drought stress levels in plants have been reported. For example, in some Crassulacean acid metabolism (CAM) species such as *Aptinia cordifolia*, alteration of the antenna of their photosystems ensure that they are able to withstand recurring periods of water deficit environments than when exposed to drought for the first time (Pintó-Marijuan et al., 2017). Some drought tolerant species, are able to gather lipids in the epidermis to form wax that enhances the reflection of sunlight to stop them from losing more water through transpiration (Pautou et al., 2016; Xue et al., 2017). Recent reports show that the application of K^+ (Potassium) to plants significantly ameliorated the effects of drought symptoms (Imakumbili et al., 2021; Nkrumah et al., 2021; Zhang et al., 2020). In some related findings, Salicylic acid has been shown to reduce the propensity of plants to environmental stresses thereby increasing the tolerance of such plants to abiotic stress (Hediji et al., 2021; Khalvandi et al., 2021). Plant growth promoting rhizobacteria (PGPR), has also been shown to increase tolerance of plants to drought stress (Pereira et al., 2020). Rabiei et al. (2020) reported similar mechanism, using PGPR, to increase plant tolerance to salinity in (*Coriandrum sativum L*). Plant also use physiological characteristics to adapt to water stress environments through maintaining high water status regardless of soil moisture content (Zhang et al., 2018). The Relative Water Content (RWC) provides a measurement of the water deficit of the leaf and may indicate a degree of stress, endured under conditions of drought and high temperatures (Torres et al., 2019). RWC has been shown to be a very effecttive way of measuring water status and to indicate how plants are able to tolerate water deficit in their growing environment (Barrs and Weatherly, 1962; Barrs, 1968; Yamasaki and Dillenburg, 1999; Xing et al., 2021).

When plants come into contact with water deficit environment, some of their metabolic reaction pathways may alter. For example, proline accumulation and metabolism are associated with mechanisms of abiotic stress avoidance in plants (Furlan et al., 2020; Semida et al., 2020.). It has further been shown that when proline is exogenously applied to maize seedlings, subjected to short term induced drought, it enhances photo system II (PSII) efficiency and chlorophyll metabolism, thereby slowing chlorophyll degradation (Altuntaş et al., 2020). Drought-induced accumulation of proline has been found in many plants including canola (Li et al., 2017; Din et al., 2011), cotton (Zhang et al., 2021), maize (Majeed et al., 2020; Voronin et al., 2019) and cacao (Bae et al., 2009).

Drought is a common occurrence in some regions of the world where water is in limiting supply (Jain et al., 2007; Rahman et al., 2021) but the effects of climate change may cause regions that previously enjoyed high amounts of rainfall to experience drought conditions. Recent research show trends of increasing temperatures and decreasing rainfall patterns across some ecological zones in Ghana which are also cacao growing areas (Hutchins et al., 2015; Schroth et al., 2016; Asare-Nuamah and Botchway, 2019) thereby raising concerns, as this change in weather patterns will have a profound impact on crop production (Lobell et al., 2008; Alam et al., 2011).

There is therefore, an urgent need in the search for the development of cocoa genotypes that can tolerate and survive the impact of the adverse effects of climate change in order to enhance successful cocoa cultivation to support the economy of Ghana. Consequently, efforts have

been made to screen different available cocoa genotypes for their efficiency to cope with drought either for direct cultivation, or the development of robust drought resistance genotypes. Unfortunately, physiological and biochemical methods such as determination of leaf relative water content and the build up of free proline content in leaves are not only laborious and time consuming but can also be expensive. It is precisely because of these constraints that efforts have been made in this study to elucidate the effectiveness of using anatomical techniques such as the presence and density of leaf epidermal trichomes in screening for drought tolerant genotypes of cacao. In addition, the methods of RWC and Proline contents of leaves of uniform chronological and physiological ages have been used as a rapid and cost effective criteria in screening for the drought-resistance potential of nine different genotypes of cocoa obtained from the Cocoa Research Institute of Ghana (CRIG). In this research, we hypothesise (Null; Ho) that variations are absent in morphological, physiological, and biochemical responses of cocoa genotypes to drought stress against the Alternate Hypothesis (H1) that variations exist in responses of cocoa genotypes to drought stress as may be revealed in morphological, physiological, and biochemical traits. We thus report the evaluation of the response of nine genotypes of Cacao to drought stress treatments, with the overall aim of identifying Cacao genotypes with the potential as drought tolerant hybrids.

2. Materials and methods

The study was carried out using nine selected cacao genotypes obtained from Cocoa Research Institute of Ghana (CRIG) in Tafo. These selected genotypes were screened for their tolerance to drought using the following criteria: Leaf RWC, SMC, presence of free proline content of uniformly-aged leaves, and trichomes presence as epidermal hairs. The experiment was performed between the months of April and May, 2016.

2.1. Location of study

The study was undertaken under a screen house at the premises of the Department of Plant and Environmental Biology, University of Ghana, Legon.

2.2. Planting materials

The genotypes used in this study were T60 x Pound10, PA7 x 6035, T85 x PA7, T63/971 x Sca9, PA150 × 6020, AMAZ X 9006, T79 × 9006, PA150 × 6020 and PA7 x MAN. Seedlings were obtained through Mendelian crosses with unknown drought tolerant genotypic parents. The seedlings, each in a 7 × 10 cm black plastic planting bag, were slowly made to acclimatize under shading conditions for two weeks before transplanting. The estimated ages of the Cacao seedlings used in this study were between 40-42 weeks old after planting.

2.3. Preparation of soil and repotting of seedlings

Cacao seedlings used for this experiment were transferred into plastic buckets (height = 17.5 cm; upper diameter = 21 cm, base diameter = 20 cm -Decorplast, Accra, Ghana), filled with sterilized sandy loam soil. The sandy loamy soil was to ensure that the water holding capacity of the root environment was high as well as providing a greater volume of rooting medium for increased nutrient availability for root extension and normal seedling growth. The seedlings were left to acclimatize for two weeks. The sandy loam soil had a pH of 7.3 and a soil moisture content of 13.3% and 13.8% at field capacity at depths of 5 cm and 10 cm respectively.

2.4. Experimental design

The experiment consisted of two treatments (Stressed – S and Control – C), nine blocks comprising nine cacao genotypes with three replicates each for the two treatments. Genotypes in each block were arranged in a

Randomized Complete Block Design (RCB). A total of 54 seedlings were used for the entire experiment.

2.5. Experimental set-up

Seedlings were randomly assigned to columns on all three benches with spacing of 18 cm across and 21 cm along the length of the bench. The soil surface in each bucket was covered with equal amount of scoops of finely chopped styrofoam (using a 180 ml cup) to minimize the rate of evaporation of water from the soil surface and ensure that all the water lost was due to uptake by each seedling.

2.6. Water stress application

At the start of the experiment, all seedlings were slowly saturated with water until water was seen dripping from the bottom of the bucket (assumed indicative of Full Saturation – FS) and allowed to drain over-night to field capacity. A set of plants were labeled *Stressed (S)*, comprising 27 seedlings from which water rehydration was stopped one day after full saturation. There was no further rehydration of seedlings whilst looking for the first emergence of visual drought symptoms (FADS). Control *(C)* treatments consisted of 27 seedlings (three seedlings per genotype) which received water every other day until FADS of the last genotype seedling that marked the end of the experiment.

2.7. Visual symptoms of drought stress

Plants were observed and monitored for possible appearance of drought symptoms such as yellowing of leaves, browning of leaf lamina, senescence of tender leaves, wilting, paling and drooping of tender leaves.

2.8. Data collection

Number of days for each seedling to exhibit signs of *first appearance of drought symptoms*-FADS (when seedlings first showed visual symptoms of drought) was recorded. The following parameters were also measured during the experiment; RWC, SMC, Anatomical studies on leaf and pro-line content of leaves.

2.9. Determination of leaf RWC of experimental seedlings

The Relative Turgidity Method of Barrs and Weatherley (1962) was used for the determination of the leaf RWC. Leaf RWC was measured on two sampling occasions; namely one day after water saturation (control) and at first appearance of drought symptoms (FADS) of each seedling. RWC determination was repeated four times on each sampling occasion and the mean RWC taken. Leaves were bored with a sharp 1 cm inner diameter cork-borer from each selected leaf and placed in a clean glass vial. Fresh weight (FW) of each set of three (3) leaf discs from each replicate of each genotype were weighed. Thereafter, each set of 3 leaf discs of each selected leaf was floated on 10 ml distilled water in a covered 2.5 cm diameter Petri dish at room temperature (25 °C) for 4 h. Leaf discs were weighed to attain the turgid weight (TW). The leaf discs were then dried in an oven at 60 °C for 24 h to determine the dry weight (DW). The leaf RWC for each set of three (3) leaf discs for each genotype were calculated using Eq. (1)

$$RWC = \frac{Fresh\ Weight(FW) - Dry\ Weight(DW)}{Turgid\ Weight(TW) - Dry\ Weight(DW)} \times 100 \qquad \text{Equation (1)}$$

where,

FW: Fresh weight of the three (3) 1cm inner diameter leaf discs
DW: Dry weight of the three (3) 1cm diameter leaf discs after oven drying at 60 °C for 24 h (hr)
TW: Turgid weight

2.10. Soil moisture content

Soil Moisture Content (SMC) was determined by the Gravimetric Method and expressed as a percentage of the initial weight. Soil samples were taken from each potting bucket (at 5 cm depth) on each RWC sampling occasion, along the sides of the bucket (in order to avoid breaking any roots), using a 1cm inner diameter stainless steel tube. The soil in the steel tube was emptied into a glass Petri dish, covered to determine the initial weight (IW) with a fine balance (AL 104; Mettler Toledo, Columbus, OH, USA). The soil was then dried in a hot-air oven at 60 °C for 24 h after which the dry weight (DW) was measured. The Soil Moisture Content was then calculated and expressed as a percentage of the initial weight of soil using Eq. (2):

$$SMC = \frac{Initial\ Weight(IW) - Dry\ Weight(DW)}{Initial\ Weight(IW)} \times 100 \qquad \text{Equation (2)}$$

2.11. Leaf anatomical study

Transverse sections (TS) of leaf, corresponding to each genotype, was made and observed under the low power (LP) and high power (HP) magnifications of a Leica binocular light microscope. For each sectioned leaf of each genotype, the following features were examined:

i. *Presence or absence and number of epidermal hairs or trichomes and*
ii. *Thickness of various layers of the leaf which included upper epidermis, mesophyll cells and lower epidermis.*

Photomicrographs of mounted sections for each selected genotype studied were taken using a Leica ICC50 camera attached to a Leica microscope; model DM 500/13613 210. Cellular measurements of upper epidermis, lower epidermis and the mesophyll layer were taken at a magnification of X400 and calibrated automatically using Leica LAS Z. The values obtained were recorded.

2.12. Determination of free proline content of leaves

Free proline content of the selected leaf of seedlings, corresponding to each of the nine selected genotypes were determined using leaf discs from the same leaf used for the determination of leaf RWC. Samples of leaf discs were taken at FADS and control seedlings for each seedling. The procedures of Troll and Lindsley (1955), as modified by Mukherjee (1974) and that of Bates et al. (1973), were modified and adapted for use in this study. In this procedure, an acid-ninhydrin reagent (Thermo Scientific Lot# MA154763) was prepared by heating 1.25 g of ninhydrin with 30 ml glacial acetic acid (Model number: 64-19-7) and 20 ml 6 M phosphoric acid over a water-bath with agitation. Two (2) ml of the aqueous solution obtained from the extraction procedure was mixed with 2 ml of acid-ninhydrin reagent and 2 ml glacial acetic acid (LAB ALLEY CAS No 44-19-7) in a covered test tube in a water-bath at 100 °C for 1 h. The reaction was stopped in an ice bath for 5 min. The colour produced was extracted by shaking with 4 ml benzene in a separatory funnel for 15 s. The benzene phase (upper layer with colour) was subsequently made to cool to room temperature (25 °C) in a test tube. This was followed by transferring the benzene phase into a glass cuvette and then the absorbance measured at 540 nm with a spectrophotometer (Jenway 6320D Spectrophotometer), using benzene as a blank. The exact proline content was quantified as μg proline per gram leaf dry weight using Eq. (3) below:

$$\text{Proline}(\mu g\,/\,g) = \frac{\text{Proline}(\mu g/ml) \times ml\ \text{benzene}}{\text{Weight of leaf discs}(g)} \qquad \text{Equation (3)}$$

2.13. Statistical analysis

All data obtained in this study were analyzed using the statistical software "Statgraphics Centurion XVI" version 16.1.11. The average

values of water-stressed seedlings were compared using analysis of variance (ANOVA) and Least Significance Difference (LSD) was used to determine differences in means at 5% level of probability.

3. Results

3.1. Days to First Appearance of Drought Symptoms (FADS) as a measure of drought tolerance potential

The seedlings used for the study exhibited varied symptoms of drought stress even within the same genotype. The first observable symptom of drought on leaves were chlorosis of leaves starting from the leaf margin and lamina. After leaf chlorosis, drooping and necrosis symptoms were seen on immature leaves. This was followed by wilting and senescence of leaves. Mean number of days for First Appearance of Drought Symptoms (FADS) for all the genotypes is presented in Figure 1. Genotypes T60 x Pound10 and T63/971 x Sca9 had the longest mean number of days to show FADS with 16.3, and 14.7 DAFS respectively. Genotypes that showed earliest FADS were AMAZ X 9006, PA150 × 9006 and PA7 X MAN with 11.3, 12.7.3 and 13.3 DAFS respectively (Figure 1). However, the differences observed in number of days for FADS between the genotypes were not statistically significant (p > 0.05).

3.2. Leaf RWC and SMC at 1 DAFS (control)

The initial mean leaf RWC and SMC of the soil (at Field Capacity one day after full saturation) were within the range of 80 ± 12–$92 \pm 4\%$ and $7 \pm -11 \pm 8\%$ respectively. However, the differences in mean leaf RWC and mean SMC were statistically not significant as expected ($p \leq 0.05$).

3.3. Mean leaf RWC and mean SMC at FADS

Although the differences in mean leaf RWC at FADS of the genotypes were not statistically significant, mean SMC of the genotypes were statistically significant (p < 0.05). Genotype PA150 × 6020 had the highest RWC of 65.55% (Table 1) followed by genotype PA7 x MAN (58.16%) and genotype T79 × 9006 (56.16%). SMC of genotype PA150 × 6020 was the highest (1.52%) followed by genotype T60 x Pound10 (0.81%). Genotype T79 × 9006 recorded the lowest SMC value (0.37%) (Table 1).

3.4. Leaf morphology and anatomical features

Mean width of leaf morphological and anatomical features such as upper and lower epidermis, mesophyll cells are presented in Table 2
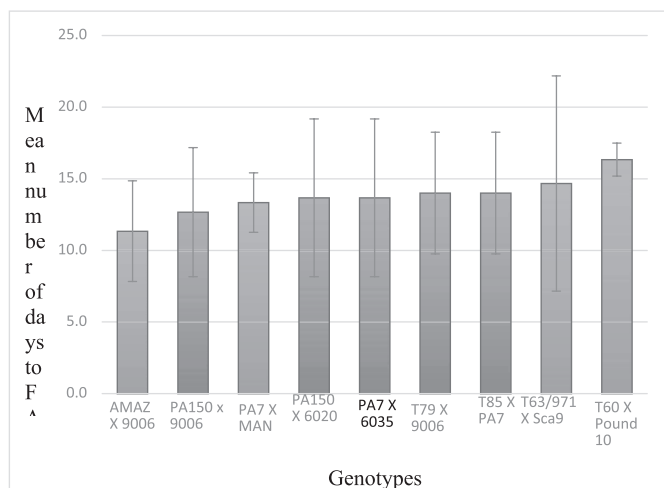

Figure 1. Mean number of Days After Full Saturation (DAFS) for First Appearance of Drought Symptoms for the nine different genotypes of Cocoa (Bars are means of 3 replicates with the standard error).

Table 1. Relative Water Content of leaves and Soil Moisture Content at Control and FADS.

Genotype	Control		FADS	
	RWC %	SMC %	RWC %	SMC %
T60 x Pound10	85 ± 1.0	8 ± 2.0	55 ± 11.0	0.8 ± 0.1
PA7 x 6035	87 ± 11.0	7.6 ± 1.0	43 ± 5.0	0.6 ± 0.1
T85 x PA7	88 ± 8.0	9.1 ± 4.0	51 ± 14.0	0.5 ± 0.0
T63/971 x Sca9	80 ± 12.0	8.8 ± 1.0	55.2 ± 6.0	0.6 ± 0.3
PA150 × 6020	90 ± 1.0	7.0 ± 0.0	65 ± 18.0	1.5 ± 0.2
AMAZ X 9006	87 ± 4.0	7.6 ± 2.0	47 ± 22.0	0.5 ± 0.2
T79 × 9006	92 ± 4.0	9.5 ± 1.0	56 ± .1.0	0.4 ± 0.4
PA7 x MAN	87 ± 4.0	9.1 ± 2.0	58 ± 5.0	0.7 ± 0.2
PA150 × 9006	86 ± 1.0	11 ± 8.0	51. ± 16.0	0.6 ± 0.1

whereas the mean number of modified hair outgrowths (trichomes) appearing on leaf epidermal surfaces are presented Figure 2.

3.4.1. Upper epidermis

Analysis of variance conducted (ANOVA) indicated that there were no statistically significant differences ($p \leq 0.05$) among the mean values of the mean upper epidermal widths of the various genotypes (Table 2).

3.4.2. Mesophyll

Genotype PA150 × 9006 had the largest mean mesophyll width (91.10 microns), closely followed by genotypes PA7 x 6035, T63/971 x Sca9 and PA150 × 6020 (with 83.02 microns, 83.05 microns and 82.88 microns respectively). However, genotype T85 x PA7 (11.80 microns) recorded the smallest mean mesophyll width. Mean mesophyll width of the rest of the genotypes were T60 x Pound10 (71.15 microns), AMAZ X 9006 (73.98 microns), *T79 x 9006* (47.60 microns) and PA7 x MAN (57.23 microns) (Table 2). ANOVA revealed that there were significant differences ($P \leq 0.05$) among the mean widths of the mesophyll of the various genotypes Table 2).

Results of the mean widths of the lower epidermis of leaves of the nine different genotypes showed that genotype PA7 x 6035 had the largest lower epidermal width (15.02 microns) compared to genotype T60 x Pound10 (9.52 microns) which recorded the least lower epidermal width (Table 2). Further, ANOVA revealed that mean widths of the lower epidermal cell layers of the various genotypes there were significantly different ($P \leq 0.05$).

Table 2. Mean numbers of width of upper epidermis, mesophyll, lower epidermis and trichomes of the nine cocoa genotypes used in this study.

Genotype	Mean Width			Mean Number of Trichomes
	Upper Epidermis (μm)	Mesophyll (μm)	Lower Epidermis (μm)	
T60 x Pound10	16 ± 4a	71 ± 17cde	10 ± 1a	3 ± 1a
PA7 x 6035	23 ± 1a	84 ± 5f	15 ± 2e	2 ±0a
T85 x PA7	22 ± 2a	85 ± 118f	12 ± 1bd	2 ± 1a
T63/971 x Sca9	24 ± 4a	83 ± 11f	11 ± 2bc	4 ± 2a
PA150 × 6020	19 ± 3a	83 ± 16f	12 ± 1bd	2 ± 1a
AMAZ X 9006	18 ± 4a	74 ± 3de	13 ± 2bd	1 ± 1a
T79 × 9006	16 ± 1a	47 ± 5a	11 ± 1c	0.50 ± 0.5a
PA7 x MAN	19 ± 3a	57 ± 15ab	12± 1bd	3 ±1a
PA150 × 9006	18 ± 3a	91 ± 10g	11 ± c	0.33 ± 0.5a

Note: Mean ± SE followed by different letters in the same column is significantly different at *P = 0*.05 by the Least significant difference method.
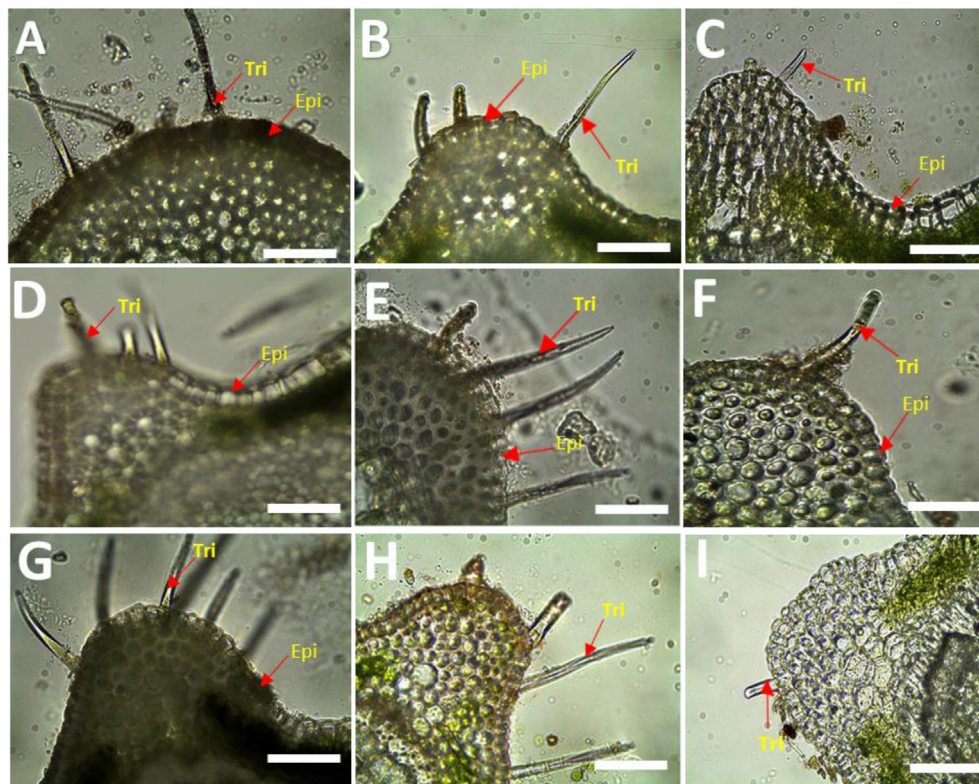
Figure 2. Cross section of leaves of Cacao genotypes indicating the presence and density of trichomes (Tri) on leaf epidermal (Epi) surfaces. **A** T60 x Pound10, **B** T85 x PA7, **C** AMAZ X 9006, **D** PA7 x MAN, **E** T63/971 x Sca9, **F** T79 × 9006, **G** PA7 x 6035, **H** PA150 × 6020, **I** PA150 × 9006. *Scale Bars* = 200 μm.

3.4.3. Presence and density of trichomes on epidermal surfaces

Mean number of trichomes on epidermal surfaces varied with genotypes of Cacao used in this study (Table 2). Mean number of three (3) trichomes were found in genotypes T60 x Pound10 (Figure 2A), T85 x PA7 (Figure 2B), PA7 x MAN (Figure 2D), PA7 x 6035 (Figure 2G) and PA150 × 6020 (Figure 2H). The mean highest number of trichomes was recorded in genotype T63/971 x Sca9 (Figure 2 E). The rest of the genotypes had a mean trichome number of one (Figure 2C, F and I).

3.5. Free proline

Free proline content in leaves of water-stressed seedlings of the selected cocoa genotypes at FADS were found to be significantly higher when compared to control seedlings. Further, the analysis of variance

Table 3. Mean free leaf proline accumulation values for nine (9) selected cocoa genotypes.

Genotype	Proline Concentration (μg/g DW)	
	Control±se	FADS±se
T60 x Pound10	0.24 ± 0.01a	3.5 ± 0.90c
PA7 x 6035	0.24 ± 0.04a	2.5 ± 0.39c
T85 x PA7	0.22 ± 0.02a	2.3 ± 0.15b
T63/971 x Sca9	0.22 ± 0.02a	4 ± 0.50c
PA150 × 6020	0.25 ± 0.06a	2.4 ± 0.05b
AMAZ x 9006	0.24 ± 0.04a	1.2 ± 0.36a
T79 × 9006	0.24 ± 0.05a	2 ± 0.22b
PA7 x MAN	0.22 ± 0.01a	1 ± 0.49a
PA150 × 9006	0.24 ± 0.04a	0. ± 0.27a

Note: Mean ± SE followed by different letters in the same column is significantly different at P = 0.05 by the Least significant difference by ANOVA method.

indicated that the differences in the mean accumulation of proline in the replicates of the various genotypes that were subjected to drought-stress were statistically significant ($P \leq 0.05$) (Table 3). Genotype T63/971 x Sca9 had the highest concentration of free proline at FADS (4 ± 0.05 μg/g DW) followed by genotype T60 x Pound10 (3.5 ± 0.09 μg/g DW) but were statistically not different from each other at FADS. However, they were statiscally different from the rest of the genotypes used in this study. Genotype PA150 × 9006 had the lowest amount of accumulated proline in leaves but were statistically not different from PA7 x MAN, PA150 × 9006 and AMAZ x 9006 (Table 3).

4. Discussion

4.1. Number of days to FADS

The observation in this study revealed that nine selected cocoa genotypes showed different morphological leaf changes at FADS after different durations (the number of days). This observation may be related to findings by Famuwagun et al. (2018), who reported differences in leaf development of cacao plants when these plants were subjected to 5 day and 10 day irrigation intervals.

4.2. Leaf anatomical features

Our findings in this research showed a corresponding increase in mesophyll cell sizes of cacao genotypes with high RWC at FADS. For example, genotype T63/971 x Sca9 had mesophyll size of 83 μm and 55% RWC and four trichomes. Genotype T85 x PA7 also had RWC of 51 % and 84 μm. Genotypes PA150 × 6020 also had high RWC of 65% and 82 μm. These indicators were at variance with other genotypes such as T60 x Pound10 and T79 × 9006 which had lower RWC and mesophyll cell sizes. We thus suggest that genotypes T63/971 x Sca9, T85 x PA7 and PA150 × 6020 may exhibit drought tolerance traits.

4.3. Relative water content

RWC has been shown to decrease with increasing water stress in plants (Boughalleb et al., 2016). In this study, we report the possible use of RWC as a physiological tool in distinguishing drought tolerance genotypes of Cacao at FADS. RWC in genotypes PA150 × 6020, PA7 x MAN and T79 × 9006 had high values (65.5%, 58.16% and 56.17% respectively) compared to genotype T85 x PA7 which showed one of the lowest values (51.12%). Comparable findings have been reported in the use of RWC to search for drought tolerance ability in seedlings of Cacao in prolonged dry season in Indonesia (Zakariyya et al., 2017). In another investigation, Janani et al. (2019), used RWC in distinguishing clones of cacao based on their tolerance potential to drought stress in India.

4.4. Soil moisture content

Results in this study revealed that genotypes with high SMC had both high RWC and Proline content which are all key drivers of drought tolerance in plants. High SMC values of genotypes PA150 × 6020, T60 x Pound10 and PA7 x MAN correlated with high values of RWC and free leaf proline content as compared to genotypes T79 × 9006 and AMAZ x 9006 which had lower SMC, RWC and proline contents. These observations seem to be consistent with cited literature that SMC could be a primary parameter of growth in plants (Stocker et al., 2019; Wurster et al., 2020). Results in this study further revealed the direct relationships between RWC, SMC and proline contents as criteria in screening for drought tolerance in cacao may depend on other factors as well. For example, Genotype T63/971 x SCA9 had a relatively high mean leaf RWC (55.2%) with the lowest mean SMC (0.67%). Nevertheless, genotype PA150 × 6020 had a high leaf RWC (65.55%) as well as the highest SMC of 1.52%. When free proline content is considered in here, genotype PA150 × 6020 may not be categorized as a high drought-tolerant potential genotype but one with medium drought-tolerance potential. These findings seem to implicate other factors such as temperature and vapour pressure deficit of the environment in screening for drought tolerance in plants (Grossiord et al., 2020; Novick et al., 2016). The interaction between plants and soil moisture is essential for carbon-water exchange and energy flow in the soil-plant atmosphere (Wang et al., 2018). Although, RWC and SMC at FADS were low, it thus seem that the breakpoints with respect to soil water availability between field capacity and wilting point in cacao may be enough to cause physiological and biochemical adaptations of cacao in a water deficit environment.

4.5. Free proline accumulation in the leaves

The findings in this study showed that free proline accumulation in the leaves of induced water-stressed genotypes were higher than that accumulated in their control counterparts. These were consistent with theories that increasing proline concentrations were related to increasing desiccation stress (Bae et al., 2009; Ayeh et al., 2021). From the results obtained in this study, free proline accumulation in leaves of the selected cocoa genotypes may be used as a criterion in the screening for drought-tolerance potential in cocoa. Generally, the observation that all the cocoa genotypes selected for this study appeared to have different drought-tolerance levels were not different to what was reported by Adu-Ampomah et al. (2001) and Adu-Ampomah and Frimpong (1993, 1994a, b, 2002).

4.6. Trichome presence

Plants exploit morphological characteristics of drought adaptation that ensure maximum water absorption during dry conditions (Sun et al., 2013). These morphological characteristics include trichomes which are epidermal appendages that have been found on the surfaces of the aerial organs of most terrestrial plants (Valletta et al., 2013). The trichomes may be produced on both the adaxial and abaxial surfaces of leaves.

Trichomes are able to sequestrate 1000 times more Na + than epidermal cells and assist plants as a second epidermis, reducing water loss and avoiding excessive damage of UV rays when exposed to salinity (Shabala and Mackay, 2011). However, there are reports of differences in response of the presence of trichomes on two sides of the same leaf associated with different environmental conditions (Lihavainen et al., 2017). Based on the presence of trichomes and their mean whole numbers at FADS, genotypes T63/971 x Sca9 and PA7 x MAN had the highest drought-tolerance potential with four trichomes each, whereas genotypes PA150 × 9006 and T79 × 9006 each had one. Our results also showed positive correlation between the mean number of trichomes observed in some of the genotypes studied and the leaf RWC. For example, genotype T63/971 x Sca9 which recorded the highest number of trichomes (4) had the second highest mean leaf RWC value of 55.20%. Genotypes T60 x Pound10 and PA7 x MAN had RWC of 55% and 58% respectively, both with trichome number of three (3). The influence of trichomes on maintenance of high RWC by these genotypes showed stable water status (turgor pressure). Such maintenance is also a result of reduction in water potential which ensures a favorable gradient of water absorption. Similar reports were reported on the influence of vesicular trichomes of *Atriplex nummularia* on osmotic adjustment and drought tolerance (Paulino et al., 2020).

5. Conclusion

Our investigations revealed that morphological, physiological and biochemical responses of cacao genotypes to water deficit stress, showed that tolerance to water stress was genotype specific. For instance, TA63/97 x SCA9 and T60 x POUND10 had accumulated the highest free leaf proline content of 3.5 μg/g DW and 4 μg/g DW respectively at FADS. Further, data from RWC, SMC, Trichomes and mesophyll width sizes indicated that TA63/97 x SCA9 and T60 x POUND10 were the most drought tolerant among the nine genotypes studied. From the study, the drought tolerant cacao genotypes selected might increase their survival in water deficit environments and to further boost yield of cacao to drive the economies of cacao producing countries.

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