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SEASONAL VARIATION IN GAS EXCHANGE AND WATER STATUS OF COCOA CLONES (*THEOBROMA CACAO* L.) FROM CONTRASTING AGROECOLOGICAL CONDITIONS

DANNES CRISTOBAL VARGAS ALMEIDA[1], RAMON JAIMEZ[2], VICTOR REYNEL[3], WILMER TEZARA[3,4,5*]

[1] Facultad de Posgrado, Universidad Técnica de Manabí, Portoviejo, Ecuador.
[2] Facultad de Ingeniería Agronómica, Universidad Técnica de Manabí, Portoviejo, Ecuador.
[3] Facultad de Ciencias Agropecuarias, Universidad Técnica Luis Vargas Torres, Estación Experimental Mutile, Esmeraldas, Ecuador.
[4] Instituto de Biología Experimental, Facultad de Ciencias, Universidad Central de Venezuela, Caracas, Venezuela.
[5] Universidad *Técnica Estatal de Quevedo*, Quevedo, Los Ríos, Ecuador.
*Corresponding author: Wilmer.tezara@utelvt.edu.ec

Abstract
Background: Esmeraldas province has distinct agroecological zones with a natural rainfall gradient, with higher rainfall in the north-than-south.
Hypotheses: Due to the high genetic variability in cocoa, clones from the south will be less affected by drought than those from the north.
Plant material: Nine cocoa clones belonging to the National × Criollo complex: five from the north and four from the south, all grown in the field.
Site and years of study: Esmeraldas province, Ecuador, during 2022.
Methods: Seasonal variation was evaluated for: water potential (Ψ), hydraulic conductance (K_L), photosynthetic rate (A), transpiration rate (E), stomatal conductance (g_s), intercellular CO_2 concentration (C_i), water use efficiency (WUE), leaf area index (LAI) and chlorophyll content (SPAD).
Results: There were significant differences in Ψ, K_L, A, E, g_s, WUE, LAI and SPAD among clones, between seasons and the interaction clone × season. Drought caused a reduction in Ψ, which was associated with a differential reduction of 40 % in A and WUE, depending on the clone. No correlation was found between A and g_s, suggesting that the reduction in A in drought was not due to the reduction in g_s but to metabolic factors. Southern clones, showed drought had a smaller effect on Ψ, did not affect g_s, E, LAI and SPAD and plants were more efficient in water transport due to higher rainfall K_L.
Conclusions: A differential physiological response to drought was found depending on the clone, supporting that genetic variability in the clones differently influenced physiological performance in drought.
Keywords: Cocoa, drought, photosynthesis, stomatal conductance, water use efficiency.

Resumen
Antecedentes: En la provincia de Esmeraldas existen diferentes zonas agroecológicas con un gradiente natural de precipitación, siendo el norte más lluvioso que el sur.
Hipótesis: Debido a la alta variabilidad genética en cacao, los clones provenientes del sur se verán menos afectados por la sequía que los del norte.
Especies de estudio: Nueve clones de cacao del complejo Nacional × Criollo: 5 provenientes del norte y 4 del sur cultivados en campo.
Sitio y años de estudio: Provincia de Esmeraldas, Ecuador, durante el año 2022.
Métodos: Se evaluó la variación estacional del potencial hídrico (Ψ), conductancia hidráulica (K_L), fotosíntesis (A), transpiración (E), conductancia estomática (g_s), concentración intercelular de CO_2 (C_i), eficiencia de uso de agua (EUA), índice de área foliar (IAF) y el contenido de clorofila (SPAD).
Resultados: Hubo diferencias significativas en: Ψ, K_L, A, E, g_s, EUA, IAF, SPAD entre clones, entre estaciones y clon × estación. La sequía causó una reducción diferencial del Ψ, A y EUA dependiendo del clon. La ausencia de correlación entre A y g_s, sugirió que la reducción de la A en sequía fue debida a factores metabólicos. En los clones del sur, la sequía tuvo un menor efecto en Ψ, no varió g_s, E, IAF y SPAD y fueron más eficientes transportando agua (mayor K_L en lluvia).
Conclusiones: Se encontró una respuesta fisiológica diferencial a la sequía dependiendo del clon, apoyando que la variabilidad genética en los clones influyó de manera diferente en el desempeño fisiológico en sequía.
Palabras clave: Cacao, conductancia estomática, fotosíntesis, eficiencia de uso de agua, sequía.

A current broad consensus is that climate change will increase the length and intensity of drought, which will affect the physiological processes of cocoa, *Theobroma cacao* L. (Medina & Laliberte 2017, Baligar *et al*. 2021, Ríos-Bolívar *et al*. 2022). In tropical regions, climate change will have a negative impact on agriculture and is a growing concern for cocoa farmers (Läderach *et al.* 2013, Lahive *et al*. 2018). Consequently, future climatic conditions can significantly influence production and its economy being especially affect small producers in the word, who are approximately 80 % (Baque Zambrano *et al*. 2024).

Advantage of the genetic variation in cocoa must be taken, which is essential to develop modern varieties capable of developing high yields under stress conditions (Lahive *et al.* 2019). Species-genotype-dependent interactions in cocoa-agroforestry systems can indirectly affect physiological processes, such as water use efficiency (WUE) and carbon storage of cocoa (Carvalho *et al.* 2023). In Ecuador, cocoa cultivation is carried out in full sunlight. The use of trees as shade for cocoa could be an indispensable drought mitigation strategy (Lahive *et al.* 2021); shade improved the physiology, growth and yield of cocoa trees; however, although shade benefits cocoa plants, it will not prevent them from suffering drought stress (Mensah *et al.* 2023).

Drought is considered a limiting factor in cocoa-producing regions around the world, significantly affecting growth and production and leading in severe cases to plant death (dos Santos *et al.* 2016, 2023). Jaimez *et al.* (2023) suggested rootstock selection as a key strategy to achieve greater resistance in water-scarce environments. The reduction in agricultural production caused by drought can be associated with a decrease in net photosynthetic rate (A), either by a reduction in stomatal conductance (g_s) or by metabolic damage (Tezara *et al.* 2020a, Osorio-Zambrano *et al*. 2021, dos Santos *et al*. 2023). In general, cocoa shows significant genetic and physiological variability in terms of physiological responses under different stress conditions, such as drought (Tezara *et al.* 2020a, b, dos Santos *et al.* 2023, Adet *et al.* 2024); in some cases, WUE decreased under conditions of water deficit (Tezara *et al.* 2020a, b, Osorio-Zambrano *et al.* 2021). Juby *et al.* (2021) reported different biochemical enzymes and osmolytes as a mechanism of drought tolerance, pointing at proline and nitrate reductase as the two biochemical markers to identify efficient drought-tolerant genotypes (Dzandu *et al.* 2021). Understanding some molecular mechanisms in cocoa in response to water deficit can be considered as a viable strategy to reduce agronomic losses of crops under the current environmental scenario (Montenegro *et al.* 2023).

Regulation of A and WUE through g_s in response to drought is not yet fully understood, and tolerance mechanisms that can provide functional details of cocoa growth and development remain to be studied (Lahive *et al*. 2019, Jaimez *et al*. 2023)

Recently, the physiological responses in Ecuadorian cocoa after a prolonged drought were reported; however, more research is needed to understand the differences in drought tolerance between clones for use in breeding programs (Jaimez *et al*. 2022a).

Due to the diversity of the environments where cocoa is produced in Ecuador and the variation in water regime existing in many of these areas, it is likely that there are genotypes with higher tolerance to drought in the province of Esmeraldas, there are different agroecological zones, with a marked natural gradient in rainfall, with higher rainfall- in the northern region compared to the southern zone (Tezara *et al.* 2015, 2024, Zambrano 2021). Therefore, it is hypothesized that due to the high genetic variability in cocoa, clones from the south will be less affected by drought than clones from the north.

In the last 20 years, many producers have replaced their National or Nacional × Criollo (N × C) cocoa plantations with CCN-51 cocoa, which is characterized by high production, medium resistance to diseases (moniliasis and *Phytophthora*) and great adaptability to different agroecological zones (Jaimez *et al*. 2022b).

It is important to select and physiologically characterize new promising cocoa materials with criollo characteristics, which belong to the N × C complex, to generate important information that can be used in the selection of new clones with good photosynthetic and productive performance. This research aims to understand the effect of seasonal variation in rainfall (rain-drought) on the physiological traits (leaf water potential, hydraulic conductance, gas exchange, leaf area index and chlorophyll content) of nine clones from parental trees of the "N × C" complex, selected from two contrasting rainfall zones in the northern and southern regions and stablished in an intermediate zone (750-1,000 mm) of the province of Esmeraldas, Ecuador.

Materials and methods

Location and duration of the study. The research was carried out at the Mutile Experimental Station of the Faculty of Agricultural Sciences of the Luis Vargas Torres Technical University of Esmeraldas, 0º 53’ 20.9” N and 79º 36’ 27.1’ W at an altitude of 32 m above sea level. The climatic characteristics of the location are average relative humidity (RH) of 86 %, average annual rainfall of 750-1,000 mm, average temperature (T_a) of 25.6 ºC, and a period of 779 hours of sunshine per year (INAMHI 2021). The most relevant agroclimatic difference where the parental trees were selected to obtain the cocoa clones was the existence of a natural gradient in the amount of contrasting rainfall in each area, with the northern region having the highest mean annual values (2,500-3,000 mm) compared to 1,550-1,750 mm in the southern area (Tezara *et al.* 2024, Zambrano 2021), without there being a difference in the duration of the dry and rainy period between areas (Tezara *et al.* 2024).

Plant material. Nine parental trees were selected in the north (Colon Eloy and Maldonado prefectures) and south (Atacames and Muisne prefectures) of the province of Esmeraldas (Table 1); the trees were chosen according to the following criteria: long lifespan, high yield, traits of N × C complex genotypes, and low incidence of pests and diseases. From the parent trees, scions were taken and grafted onto IMC 67 rootstocks from open-pollinated cocoa. The plants were maintained for one year in an area covered with neutral mesh across the experimental area, that reduced 65 % of the light intensity. They were irrigated frequently, every two days. The maximum radiation values (1,000 µmol m^{-2} s^{-1}) and maximum air temperature (30 ± 1 °C) were observed at noon. The maximum relative humidity (RH) (98 %) was found in the morning hours, while the minimum (55 %) was recorded in the midday hours. After one year, eight plants of each clone were planted in the field at a distance of 3 × 3 m. The soil was silty clay loam with a pH of 6.3, high levels of P, K, Ca, Mg; medium levels of S and Zn, Mn; and low levels of N, B, Cu, Fe. All plants were fertilized with YARAMila complex (Porsgrunn, Norway) twice a year (200 g per plant), a dose that was previously determined to supply the plant requirements and solve the nutritional deficiencies of the soil. Nine adult cocoa clones were evaluated at three years of age in the field: five clones from the north and four from the south (Table 1).

Table 1. Identification, clone codes, provenances and geographic coordinates of the selected parental trees. N: north; S: south, W: west.

Region number	Clone acronym	Code	Provenance	Geographic coordinates	
				N	W
N1	NEV 2	N2	Eloy Alfaro - Maldonado	01°01’50.5”	78°54’16.2”
N2	NEV 10	N10	Eloy Alfaro - Maldonado	01°01’38.4”	78°53’51.5”
N3	NEV 12	N12	Eloy Alfaro - Maldonado	01°01’38.4”	78°53’50.7”
N4	NEV 13	N13	Eloy Alfaro - Maldonado	01°01’44.8”	78°53’47.3”
N5	NEV 34	N34	Río Verde	0°55’40.32”	79°22’14.22”
S6	ARL 18	A18	Atacames - The Breezes	00°49’49”	79° 52’00”
S7	MOC 30	M30	Muisne - La Tola	00º 34’47.9”	79º 52’58.2”
S8	MOC 31	M31	Muisne - La Tola	00º 34’44.88”	79º 52’53.1”
S9	MOC 33	M33	Muisne - La Tola	00º 34’41.1”	79º 52’52.9”

Microclimatic variables. Microclimatic were evaluated under field conditions where the nine cocoa clones were grown. Daily changes in microclimatic variables were measured during 4 days rainy season and 5 days drought season (n = 9). Data were taken at hourly intervals from 7:00 to 17:00 h, of air temperature (T_a), leaf temperature (T_L), with YSI thermistors (Yellow Springs, Ohio, USA), photon flux density (PFD), with a LI-250I radiometer (Li-COR, Lincoln, USA). Relative humidity (RH) and T_a were recorded continuously for one month in the crop area using a HOBO U23 Pro v. 2 Datalogger (Onset, USA) data receiver - HOBO Waterproof Shuttle**.**

Physiological variables.- All physiological variables were evaluated in contrasting periods: rainy (March and April 2022) and dry (November 2022).

Measurements of physiological variables. Water potential (Ψ) and soil water content.- Measurements of leaf water potential (Ψ) were performed using a pressure chamber mod. PMS-600 (PMS Instrument Company, Oregon, USA) before dawn between 05:00 and 06:00 h (Ψ_{am}) and at noon (12:00-13:00 h, Ψ_m) in three different plants of each clone (n = 3). Soil water content (SWC) was assessed by taking a soil sample of approximately 1 kg in the experimental area, its wet weight (WW) recorded, then dried at 110 ºC for 72 h and the soil dry weight (DW) determined. The SWC was calculated as; SWC = 100 × (WW – DW)/DW.

Hydraulic conductance (K_L).- The determination of hydraulic conductance **(**K_L) was done after Sack *et al.* (2002) and Sack & Scoffoni (2012). When the steady state of transpiration (E, mmol m^{-2} s^{-1}) was reached, K_L was determined by dividing E by the driving force of the water potential, *i.e.,* the water potential difference ($\Delta\Psi = \Psi_{am} - \Psi_m$; MPa): $K_L = E/\Delta\Psi$ (mmol m^{-2} s^{-1} MPa^{-1}).

Instantaneous gas exchange.- Photosynthetic rate (A), transpiration rate (E), g_s, intercellular CO_2 concentration (C_i) and WUE = A/E were measured using a portable infrared gas analyzer model CIRAS 2 (PP Systems, Hitchin, UK). All measurements were made on fully expanded and healthy adult leaves of four different plants of each clone, chosen at random (n = 4). The measurements were made between 09:00 and 12:00 h under the following conditions: ambient CO_2 concentration (C_a) 415 ± 10 µmol mol^{-1}, at 21 % O_2, temperature 28 ± 1.3 °C, saturating photon flux density (PFD) 1,000 µmol m^{-2} s^{-1} and a leaf-air water vapor gradient (VPD) 1.5 ± 0.2 kPa.

Leaf area index.- Leaf area index (LAI) was measured using a Canopy Leaf Area Analyzer (LAI-2200C Li-COR, Lincoln, USA), in four plants (n = 4) of each clone.

Chlorophyll content.- Chlorophyll content was determined between 09:00 and 12:00 h using a SPAD-502 Plus chlorophyll meter (Minolta, Japan) in five different leaves of five plants per clone (n = 5), after having performed gas exchange measurements.

Statistical analysis. Statistica software version 10 was used to perform a two-way analysis of variance (ANOVA) of the physiological variables evaluated: factor 1 (northern and southern clones) and factor 2 (rainy and dry seasons). To check for normal distribution and homogeneity of variance we used Levene's test. Tukey 's a posteriori test was applied to determine the significance ($P < 0.05$) of differences observed in the variables studied.

A principal component analysis (PCA) was performed to test for coordination between multiple physiological traits using all data by the 'prcomp' a function in the stats package in R and 'ggplot2' visualization package in R software v. 4.1.3 (R Core Team 2022), setting the center and scale arguments to TRUE. SigmaPlot v. 11.0 (Systat Software, San Jose, CA, USA) was used to perform correlations, linear regressions and curve fitting.

Results

Microclimatic characteristics. Maximum PFD values were observed at 10:00 h, while maximum T_a and leaf temperature were observed at noon. The minimum T_a and the maximum RH were found in the morning hours. The minimum RH was recorded at noon (Figure 1). In the rainy season, leaf-to-air vapor pressure deficit (VPD) varied between 1.81 and 1.93 ± 0.03 KPa. Maximum VPD (2.03 ± 0.04 KPa) and minimum (1.75 ± 0.09 KPa) was found in drought (Figure 1).

Soil water content. The SWC in rain was 24.7 ± 1.9 % and was significantly reduced by 46 % in drought (13.3 ± 2 %). This supported that there was indeed lower water availability in the soil during the drought.

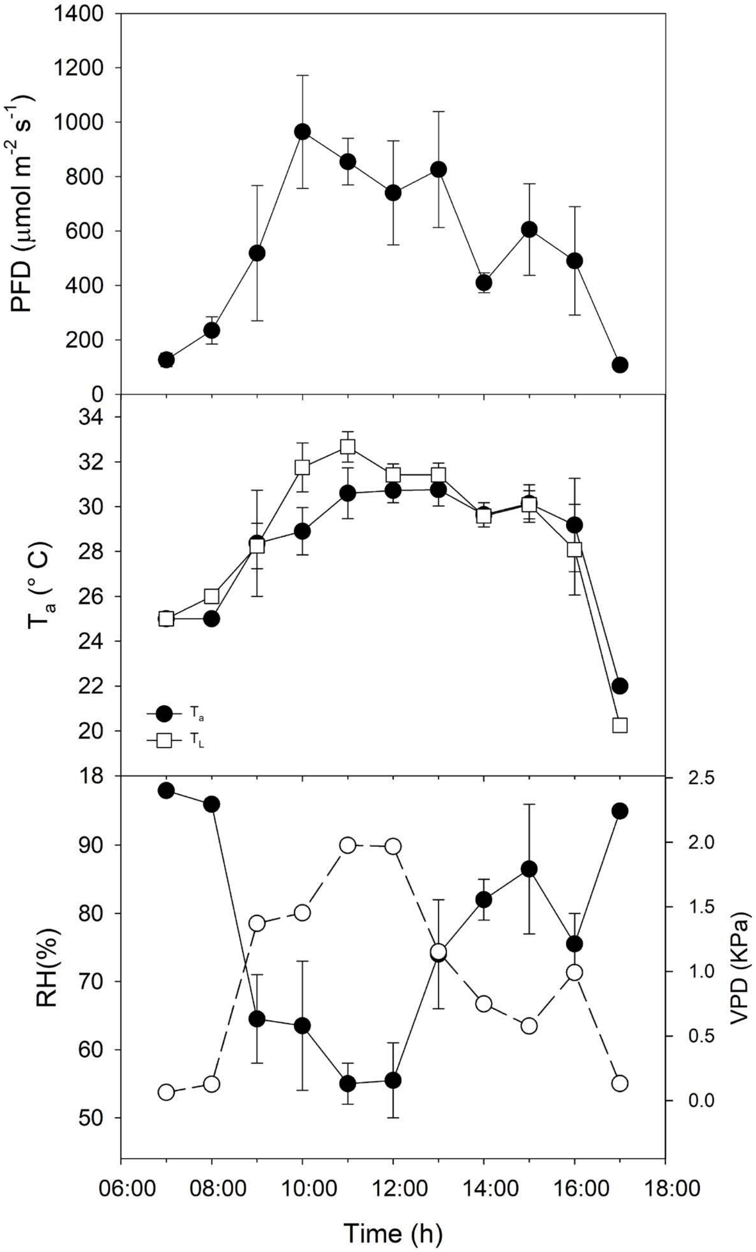

Figure 1. Time-course of daily changes in microclimatic variables: photon flux density, air temperature, average leaf temperature of nine clones, relative humidity and vapor pressure deficit (open circles), evaluated under field conditions where the nine cocoa clones were grown. Values represent the mean ± SE during the days of measurements (n = 9).

Water status. The variables Ψ_{am}, Ψ_m and $\Delta\Psi$ of plants in the rainy and dry seasons are shown in Figure 2. Average Ψ_{am} decreased from the rainy to the dry season, drought causing a significant 4.2-fold reduction in Ψ_{am}. The lowest value was recorded in clone N13. A significant reduction in Ψ_m was found, with the lowest value found in clones N12 and M33 ($P = 0.005$). There were significant differences among seasons in $\Delta\Psi$; the greatest variation of $\Delta\Psi$ was found in drought (0.67 and 0.80 MPa) compared to that obtained during the rainy season (0.43 and 0.42 MPa) in the southern and northern clones, respectively. The clones with the lowest variation in drought were A18 M30 and M31, while those with the greatest variation were M33, N12, N13, and N34.

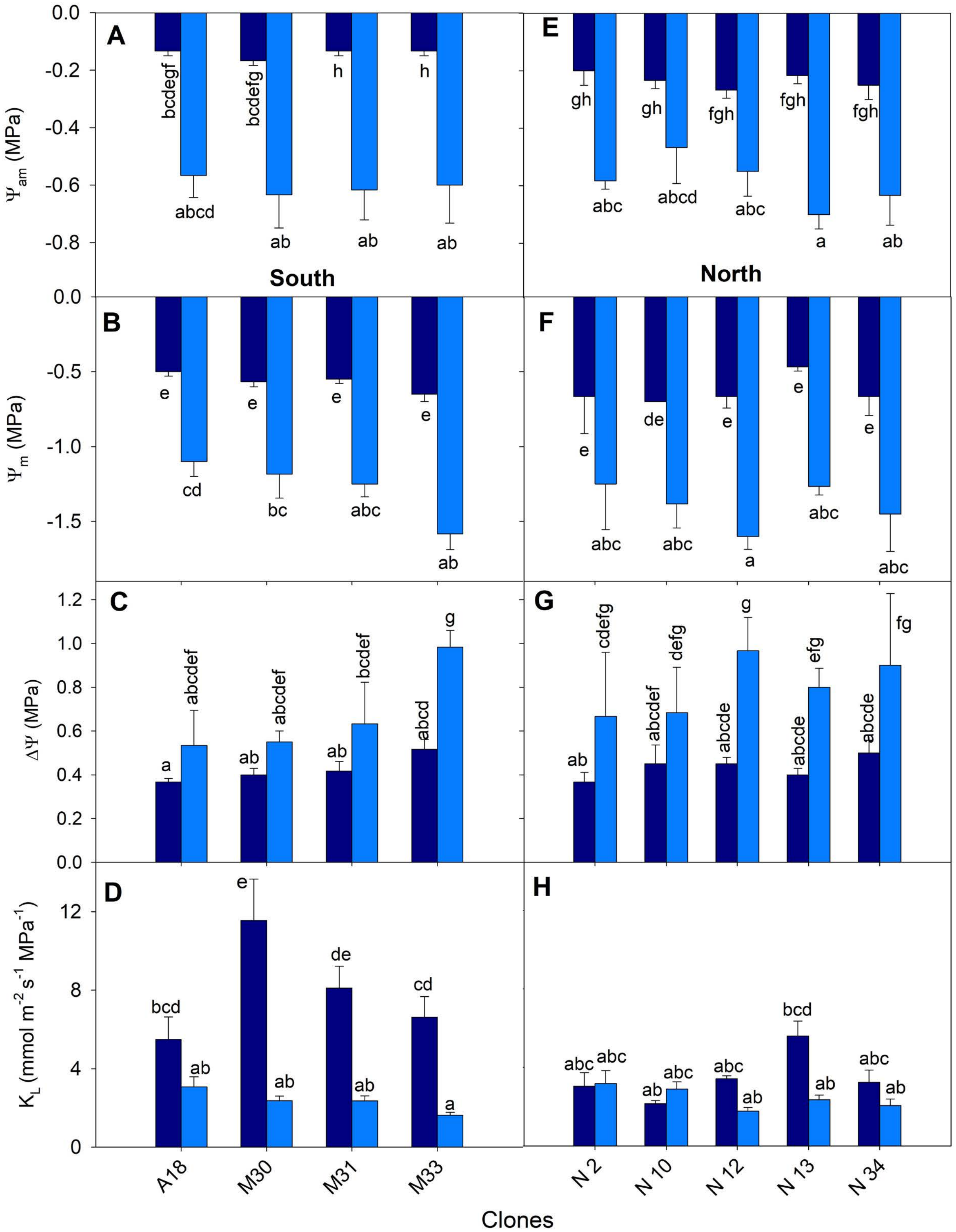

Figure 2. Seasonal changes in: water potential at dawn (A, E); minimum potential at noon (B, F); water potential difference (C, G) and hydraulic conductance (D, H) in plants grown in the field of nine cocoa clones from the south and north of Esmeraldas and measured during in the rainy season (dark blue bars) and the dry season (light blue bars). Values are means ± SE (n = 3). Different letters on each bar show significant differences ($P < 0.05$) according to Tukey's test of a two-way ANOVA: factor 1 (south and north clones) and factor 2 (rains and drought).

Hydraulic conductance. The highest K_L was observed in the rainy season, while the drought causing a 43 % reduction in K_L. Values in all the northern clones were higher in the rainy season compared to those in the south, with the highest value being observed in clone M30 during the rainy season, while in drought no significant differences were found between clones or areas (Figure 2). The K_L in the northern clones was less affected by drought than in the southern clones.

Instantaneous gas exchange. There was a significant effect per clone, per season and the interaction (clone × drought) on the gas exchange variables (Table 2). Drought caused a significant reduction in average A of all clones of 40.4 %, finding differences between clones ($P = 0.0000$; Figure 3A, F). The results showed a significant interaction ($P = 0.003$) indicating that there was a differential response of A to drought depending on the clone. Significant differences were found in A, g_s, E, C_i and WUE between the clones evaluated; in the rainy season clone N13 showed the highest A, and in drought the clones A18, N12, N13 and N34 showed the highest values.

Table 2. The *F* statistic and *P* of the Tukey post hoc tests of the two-way analysis of variance are shown: factor 1 (clones), factor 2 (season) and the interaction (clone × season) performed on all the physiological variables evaluated. Values that showed significant differences at $P < 0.05$ are shown in red.

	F			***P***		
	Clone	**Season**	**Interaction**	**Clone**	**Season**	**Interaction**
Water status						
Ψ_{am} (MPa)	4.09	230.6	2.8	0.0009	0.000	0.011
Ψ_m (MPa)	32.72	431.0	21.5	0.972	0.000	0.85
$\Delta\Psi$ (MPa)	4.11	130.0	2.16	0.0008	0.000	0.04
K_L (mmol m^{-2} s^{-1} MPa^{-1})	6.42	80.3	8.0	0.000	0.000	0.000
Gas exchange						
A (µmol m^{-2} s^{-1})	2.74	191.9	2.9	0.005	0.000	0.003
E (mmol m^{-2} s^{-1})	2.23	80.1	3.9	0.022	0.000	0.0001
g_s (mmol m^{-2} s^{-1})	2.34	36.1	5.1	0.016	0.000	0.000
C_i (µmol mol^{-1})	1.90	274.2	1.7	0.054	0.000	0.08
WUE (mmol mol^{-1})	0.95	450.9	2.1	0.481	0.000	0.029
Leaf area and chlorophyll index						
LAI	803.26	556.5	141.1	0.000	0.000	0.000
SPAD	2.312	7.8	1.8	0.033	0.008	0.09

The average g_s of all clones was significantly higher in drought than in rain; southern clones showed similar g_s values between seasons (in M30 g_s was higher in rains), while the highest g_s were found in northern clones in drought. The highest g_s was observed in clones N2 in drought, and the lowest in N10 in rain (Figure 3B, G).

The highest average values of E were found in drought and the lowest in rain. In drought, an increase of 29 % in E was observed; the highest E were observed in clones N2 and N10 in drought, and the lowest in N10 in rain (Figure 3C, H).

Ratio C_i/C_a. The C_i/C_a ratio in rain was on average lower (0.50) than in drought (0.73). Clone M31 showed the highest C_i/C_a, in drought, while it was lower in clones N2, N10, N12, N13, A18, and M30 in the rainy season (Figure 3D, I). In the cocoa clones evaluated, the average C_i in rain was 207.5 µmol mol^{-1} compared to 306.45 µmol mol^{-1} observed in drought, indicating a 1.47-fold increase in C_i.

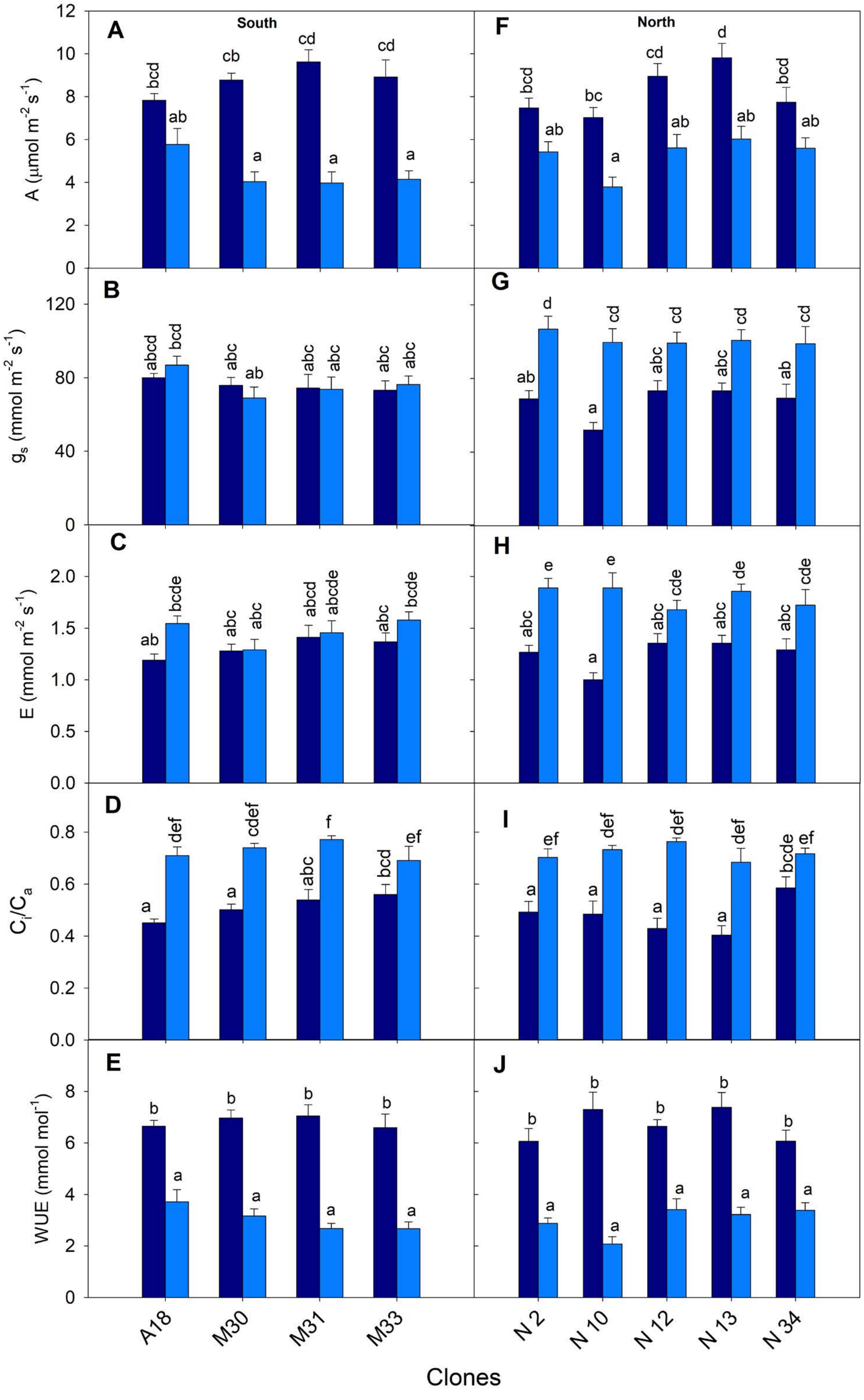

Figure 3. Seasonal changes in: photosynthetic rate (A, F); stomatal conductance (B, G); transpiration rate (C, H), relationship between intercellular and ambient CO_2 concentration (C_i/C_a) (D, I), and water use efficiency (F, J) in leaves of nine cocoa clones from the south and north of Esmeraldas in plants grown in the field, in the rainy season (dark blue bars) and in drought (light blue bars). Values are means ± standard error (n = 4). Different letters on each bar show significant differences ($P < 0.05$), according to Tukey's test from a two-way ANOVA: factor 1 (south and north clones) and factor 2 (rains and drought).

Water use efficiency. The WUE was significantly higher in rain than in drought, the latter being a result of a reduction in A and an increase in E, which translated into a significant reduction of 46.3 % in WUE (Figure 3F, J).

For the entire set of data obtained there was no correlation between A *vs.* g_s ($r = 0.016$, $P = 0.84$), indicating that A was not governed by g_s. However, a positive linear relationship between A and g_s for each season for the nine cocoa clones from each zone (north and south; Figure 4). Changes in g_s explained 33 % of the variation in A in rain and 39 % in drought, suggesting greater stomatal control in drought. This could be clearly seen in the slopes of the straight lines shown in Figure 4 representing the intrinsic water use efficiency (A/g_s), indicating a higher A/g_s in rains than in drought.

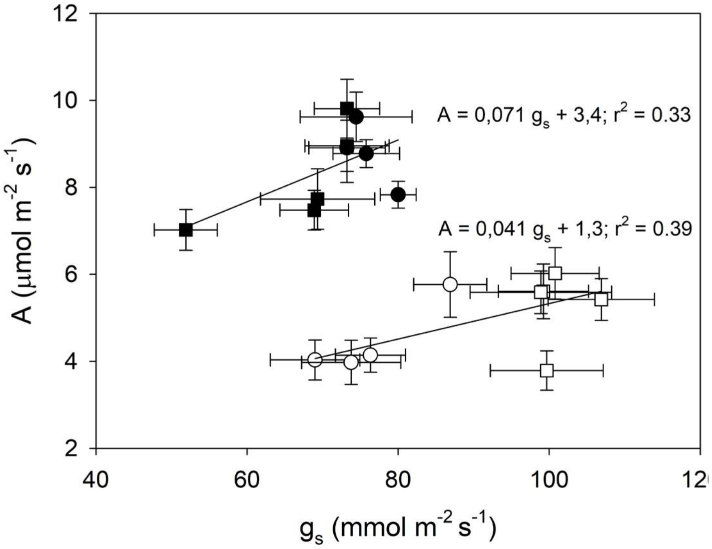

Figure 4. Relationship between net photosynthetic rate (A) and stomatal conductance (g_s) of intact leaves of nine cocoa clones from the north (square) and south (circles) of the province of Esmeraldas. The linear regression for each station is shown: rainfall (black symbols) and drought (white symbols), both significant at $P < 0.05$. The values represent the mean ± standard error. The regression equations for each station are shown.

A highly significant curvilinear relationship was observed between A and Ψ_{am} and Ψ_m in the nine clones evaluated. It was observed that as Ψ_{am} and Ψ_m decreased A was reduced in drought (Figure 5).

Leaf area index. The highest values of LAI were found in northern clones in rain, while the lowest were observed in southern clones. There was a significant reduction of the average LAI in drought. This reduction was marked in northern clones; in contrast, A18 showed an increase in LAI in drought (Figure 6).

Chlorophyll content. In six of the nine clones evaluated, there was a significant reduction with drought in the average values of SPAD units (Figure 6). The clones showed significant differences, with the most affected clones being M30 and M31 from the south and the unaffected N10 and N34 from the north; there was no significant effect in the interaction (Table 2).

In most of the physiological variables evaluated, highly significant differences were found between clones, between seasons and in the interaction; with the exception of Ψ_m, C_i and WUE, between clones, VPD (season) and Ψ_m, SPAD (interaction), where no significant differences were observed (Table 2).

Multivariate analysis. In the PCA results, the first two principal components explained 74.45 % of the data variation. The first principal component (PC1) was positively associated with g_s, E, C_i/C_a, $\Delta\Psi$ and negatively with A, WUE, A/g_s, Ψ_{am} and Ψ_{pm}. The second principal component (PC2) was positively associated with K_L, and negatively with LAI, SPAD and VPD (Figure 7). This PCA showed a clear functional separation of the cocoa clones in the two seasons

(Figure 7); in rains there was a clear separation, the northern clones were associated according to CP2 (LAI, SPAD, VPD and Ψ_{am}) while those in the south were connected according to CP1 (K_L, A and WUE). In drought the associating of clones N2, N10, and N12 was determined by CP1(g_s, E, C_i/C_a and $\Delta\Psi$).

Discussion

Regardless of the origin of the cocoa clones (north or south of Esmeraldas), it was found that in the majority of physiological variables evaluated (Ψ_{am}, Ψ_m, $\Delta\Psi$, K_L, gas exchange, LAI, SPAD), there were highly significant differences between the cocoa clones studied, between seasons and in the interaction (clone × season), with the exception of Ψ_m, C_i and WUE (between clones) and SPAD (interaction). These results indicate that there was a differential physiological response to drought depending on the clone, supporting the hypothesis that genetic variability in cocoa clones may also imply differences in physiological performance and its response to seasonal variation in rainfall. A generality is that in the clones that come from regions with less rainfall (south) the effect of drought was less on the water status (lower $\Delta\Psi$), and there were no differences in g_s, E and SPAD, in addition to proving more efficient in the transport of water in rain (higher K_L).

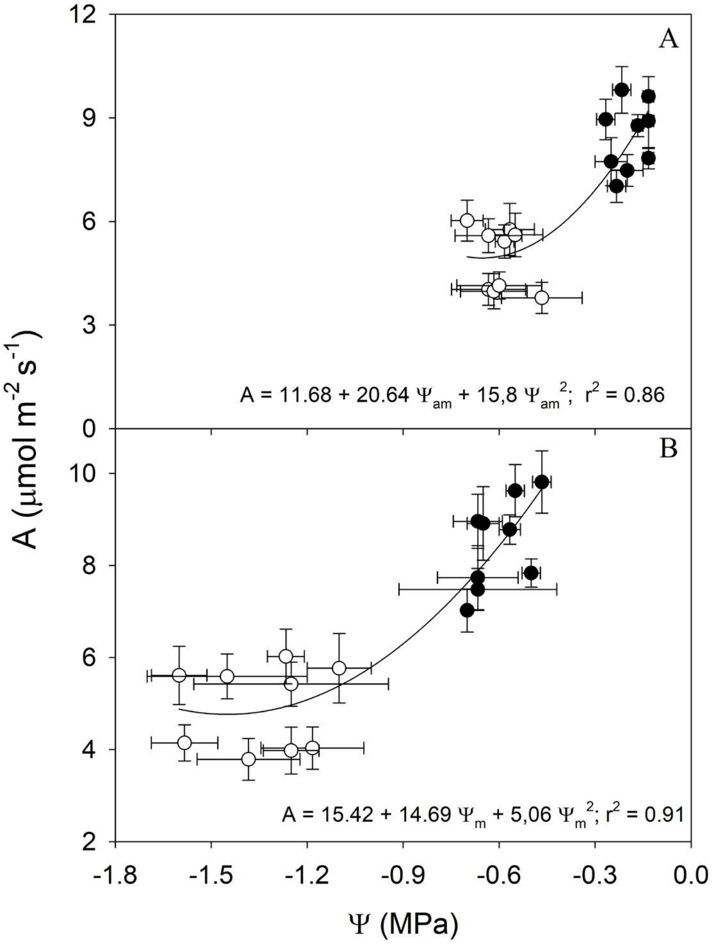

Figure 5. Relationship between net photosynthetic rate and morning (A) and midday (B) leaf water potential in nine cocoa clones from the north and south of the province of Esmeraldas. Rainfall (black symbols) and drought (white symbols), highly significant curvilinear correlations at $P < 0.05$ are shown. Values represent mean ± standard error. Equations for curvilinear fits are shown.

Water status. The lack of rainfall during the drought caused a significant reduction in the leaf water status (reduction of Ψ_{am} and Ψ_m) in all cocoa clones, due to a decrease in soil water availability. Similar trends have been reported in Criollo cocoa trees (Araque *et al.* 2012, De Almeida *et al.* 2017) and Trinitario clone seedlings subjected to water deficit in different greenhouse and/or germplasm bank experiments (Tezara *et al.* 2020a, dos Santos *et al.* 2023), and in different graft-rootstock combinations of Ecuadorian cocoa (Jaimez *et al.* 2023). In some Ecuadorian clones, a reduction in relative water content was observed in response to drought (Jaimez *et al.* 2022a, 2023). These results indicate that cocoa is sensitive to drought; and the clones with the least variation in $\Delta\Psi$ in drought were A18, M30 and M31 from the south, an area with less rainfall.

Hydraulic conductance. Drought also caused a 43 % reduction in K_L, suggesting that water flux through the xylem per unit of $\Delta\Psi$ was reduced in drought. Interestingly, despite showing the highest K_L values, the southern clones were much more affected than those from the north; these differences being probably due to intrinsic morpho-anatomical characteristics of the xylem that require more studies in this direction. Similar drought significantly reduced cocoa sap flux density, in cocoa clones grown under field conditions in central Côte d'Ivoire (Adet *et al.* 2024).

We propose that the lower amount of rainfall to which the southern clones have been subjected for ancestral periods of time in relation to those from the north has allowed them to develop a better adaptation mechanism such as a greater stomatal closure that allows them to maintain higher Ψ, especially at midday.

The highest K_L values were found in clone M30 (in rains); while the lowest was observed in M33 in drought. Similar K_L values have been reported for *Helianthus annuus* and *Rhaphiolepis indica* (Sack & Scoffoni 2012) and in four cocoa clones, in Venezuela (De Almeida *et al.* 2016). Our K_L results were several orders of magnitude higher than those previously reported in other cocoa studies. For example, the stem hydraulic conductivity measured by the pipette method reported in three Venezuelan cocoa clones (415, 443 and 445) was 0.07-0.19 (Kg m^{-1} s^{-1} MPa^{-1}) which corresponds to K_L values of 0.09-0.1138 (mmol m^{-2} s^{-1} MPa^{-1}, Ávila-Lovera *et al.* 2021), while in cocoa in an agroforestry system in Indonesia, values of 1.23 Kg m^{-1} s^{-1} MPa^{-1} (K_L of 0.09 mmol m^{-2} s^{-1} MPa^{-1}) were reported (Kotowska *et al.* 2015). Differences observed between hydraulic conductance (K_L) and stem hydraulic conductivity maybe due to the methodologies used for their assessments.

Gas exchange. The decrease in Ψ in drought caused a 40 % reduction in A; however, this reduction was not attributable to a reduction in g_s, since significantly higher g_s and C_i values were observed in drought. In both the northern and southern clones there were significant differences between seasons. Similar responses have been reported in cocoa seedlings under greenhouse conditions (De Almeida *et al.* 2016, Lozano & Fonseca 2016, dos Santos *et al.* 2023) and in cocoa trees in the field (Araque *et al.* 2012, Ávila-Lovera *et al.* 2016, Tezara *et al.* 2020b). In Ecuadorian clones under field conditions, a 33 % reduction in A has been presented, while g_s was higher in drought in three of the clones evaluated (T8, T23 and T24) whereas in four clones (T1, T14, EET 103 and CCN 51) did not vary with the drought (Jaimez *et al.* 2022a).

Decreases in A have also been reported in drought (Araque *et al.* 2012, Ávila-Lovera *et al.* 2016, Acheampong *et al.* 2019), indicating sensitivity of cocoa to water deficit that affects the photosynthetic apparatus, as reported by Carr & Lockwood (2011). In general terms, similar results in A and g_s have been previously reported in Venezuelan criollo-type and Ecuadorian national-type cocoa cultivars; as the plants are subjected to water deficit, an early stomatal closure occurs (Lozano & Fonseca 2016, Jaimez *et al.* 2023, dos Santos *et al.* 2023) and therefore E and A are reduced.

Avoiding dehydration becomes a strategy that involves increasing the water supply capacity and limiting its expenditure, increasing WUE, which increases the ability to remain and survive during periods of drought (Medrano *et al.* 2007). In this study, the Ecuadorian cocoas evaluated showed that WUE did not increase in drought. This coincides with other works on some cultivars under conditions of water deficit (Tezara *et al.* 2020a, b, Osorio-Zambrano *et al.* 2021, dos Santos *et al.* 2023). This supports the hypothesis proposed by Ávila-Lovera *et al.* (2016) that the selection of cultivars over time by producers and breeding programs for the different types of cocoa was carried out in high rainfall environments as corresponds to the tropical rainforest, the natural habitat of this species.

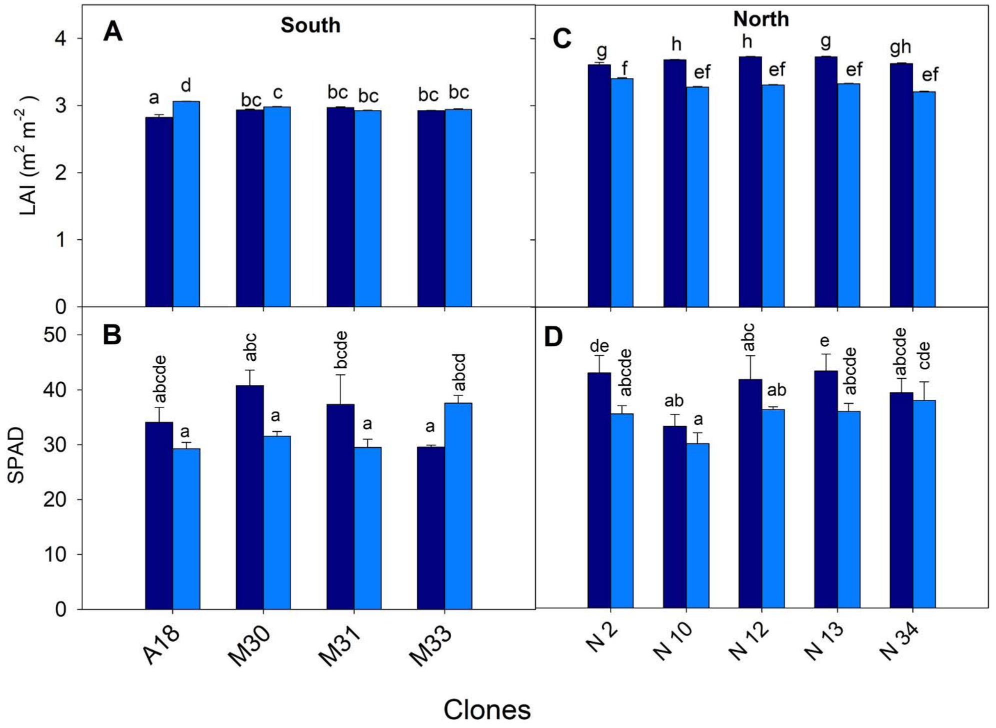

Figure 6. Seasonal changes in: leaf area (A, C) index and chlorophyll index (B, D) of nine cocoa clones from the south (left-hand panels) and north (right-side panels) of Esmeraldas in field-grown plants during the rainy season (blue bars) and the dry season (light blue bars). Values are means ± standard error (n = 3). Different letters on each bar show significant differences ($P < 0.05$) according to Tukey's test of a two-way ANOVA: factor 1 (south and clones) and factor 2 (rains and drought).

In the clones from the south of Esmeraldas, the little seasonal variation found in g_s suggests that drought had little effect on the stomatal regulation of these clones, these results agreeing with those obtained in National cocoa under field conditions (Jaimez *et al.* 2018, 2022a). Unlike in the northern clones, an increase in g_s was observed in drought, suggesting that the climatic conditions were not extreme enough to induce stomatal closure; on the contrary, this response represents a way of maintaining leaf temperatures close to their optimum through water loss through transpiration. This could be due to the fact that the climatic conditions on the coast of Ecuador, most of the year and especially during drought there is high cloudiness (low PFD) and low evaporative demand (high relative humidity) producing a low VPD (Jaimez *et al.* 2018). This environmental peculiarity could generate a less stressful environment in drought than other types of habitats and also leads to a greater possibility of growing cocoa in full sun exposure as referenced later in other studies (Tezara *et al*. 2020b, 2022).

In the study area, g_s was largely regulated by VPD; despite a lower SWC, there was a low VPD due to high humidity and cloudiness during the drought, which had little effect on g_s, previously reported by Jaimez *et al.* (2018, 2022a). The maximum values of VPD are between 1.7 and 2.0 KPa in the study area, suggesting that gas exchange in cocoa clones was not limited by stomatal closure. Contrary to what was expected, gs was significantly higher in drought in northern clones N2, N10, N12, N13 and N34. Southern clones showed similar g_s values between seasons and g_s was highest in M30 during rains.

The absence of correlation between A and g_s indicates that the decrease in A during drought was not attributed to stomatal closure (reduction in g_s), as previously reported in other studies (Tezara *et al*. 2020a, b, dos Santos *et al*. 2023). In these studies, the decrease in A is mainly attributed to a decrease in CO_2 diffusion that impacts carboxylation. Despite higher g_s in drought, the C_i/C_a ratio increased in drought, may suggesting an increase in cellular respiration and/or photorespiration (oxygenation of RuBP) process. The increase in C_i suggests that the reduction in A could be due to a decrease in metabolic factors such as ATP synthesis, RuBP content, amount and/or activity of RubisCO, among other factors (Lawlor & Tezara 2009).

At least in the northern clones the g_s in drought were higher than in rain, indicating that the stomata were open and therefore the diffusion of CO_2 to the carboxylation sites had little restriction. However, the C_i/C_a increased. This can only occur if processes that produce CO_2 are taking place, such as cellular respiration in non-photosynthetic tissues and leaf tissues, alterations in the rate of photosynthesis, increased photorespiration, and activation of alternative oxidation in the mitochondria (AOX) (Igamberdiev & Bykova 2023). Although these processes generate intracellular CO_2, their impact on photosynthesis will depend on the severity of the drought and the plant's ability to handle the stress (Chauhan *et al.* 2023). The interactions between photorespiration, cellular respiration, and gas transport are essential in determining the final concentration of CO_2 within leaves. Drought has significant effects on plant physiological processes, including respiration and photorespiration. However, its influence depends on several factors, such as the intensity and duration of water deficit, plant species or variety, and environmental conditions.

The WUE was significantly higher in rainfall than in drought due to a reduction in A and an increase in E, which translated into a significant 46 % reduction in WUE. Similar decreases in WUE with increases in Ψ have been reported in Forastero clones and Trinitario and Criollo cocoas (Joly & Hahn 1989, Tezara *et al*. 2020a). In contrast, in Trinitario clones, WUE increased with drought (Baligar *et al.* 2008).

Leaf area index. The highest values of LAI were found in northern clones in the rainy season, while the lowest were observed in southern clones; in drought, northern clones were more affected, showing a significant reduction in LAI. Similar to that reported on cocoa clones in the Ivory Coast (Adet *et al.* 2024). This decrease led to a smaller leaf area capable of absorbing a lower amount of light and to a lower rate of CO_2 assimilation per unit area and lower availability of photoassimilates, which would translate into a lower carbon balance and biomass accumulation in drought but

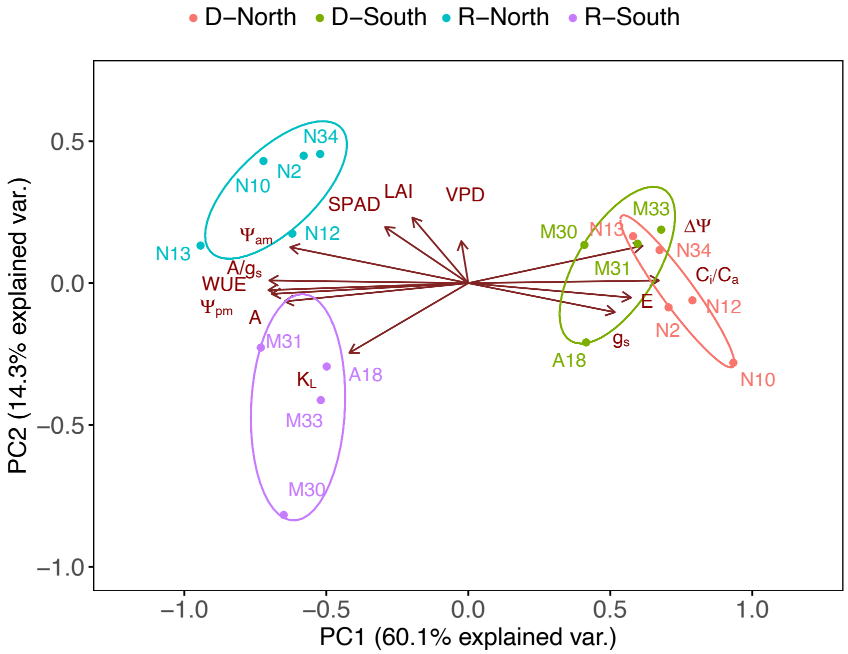

Figure 7. PC1 (60.1 %) and PC2 (14.3 %) from principal component analysis for the physiological characteristics evaluated (photosynthetic rate (A), transpiration rate (E), stomatal conductance (g_s), vapor pressure deficit (VPD), intrinsic water use efficiency (A/g_s), water use efficiency (WUE), intercellular and ambient CO_2 concentration ratio C_i/C_a, leaf area index (LAI), chlorophyll index (SPAD) units, hydraulic conductance (K_L), morning and noon water potential (Ψ_{am}, Ψ_{pm}) and their difference during the two seasons, in the nine cocoa clones. The colored ellipses represent the cocoa clones associated according to CP2 (LAI, SPAD, VPD and Ψ_{am}) and to CP1 (K_L, A and WUE).

it also indicates a smaller area to lose water through transpiration. Similar values of LAI were found in OC61 cocoa trees located in shade (3.38 ± 0.04 $m^2 m^{-2}$) and in full exposure (2.89 ± 0.16 $m^2 m^{-2}$; Tezara *et al.* 2022). In productive cocoa trees, the reported LAI values are very varied: from 1.5 up to 6 $m^2 m^{-2}$ in Ghana; between 3.7 and 5.7 $m^2 m^{-2}$ in Brazil and between 2.3 and 3.8 $m^2 m^{-2}$, and between 1.5 and 2.19 $m^2 m^{-2}$ in CCN51 and between 1.06 and 2.16 $m^2 m^{-2}$ ICS 95 in Colombia (Hutcheon 1976, Alvim 1977, Leiva-Rojas *et al.* 2019).

Chlorophyll content (SPAD). In most of the clones evaluated, a significant reduction in SPAD units of 9.2 % was observed, indicating a loss of chlorophylls due to drought, which would partially explain the reduction found in A. A decrease in the most important photosynthetic pigment would translate into a reduction in photochemical reactions, that is, a lower absorption and transduction of light energy, the final result of which would be a decrease in photosynthesis. Similarly, significant differences have been reported in the chlorophyll content (SPAD) of twelve-year-old cocoa clones in drought on the Ecuadorian coast, attributed to the greater adaptability of some clones to dry conditions and the low availability of water at this time (Héctor Ardisana *et al*. 2018).

Multivariate analysis. The PCA separated the northern and southern cocoa clones in rainfall, this separation being basically produced by K_L; in drought the separation was determined by g_s, E, C_i/C_a and $\Delta\Psi$, an overlap of clones occurring from the south and the north, where clones N13 and N34 were located within the south group. Clones N2, N12 and N10 showed a different response to drought than the rest of the clones. These results suggest that there is indeed a different physiological performance among the nine clones to drought.

There was a significant effect per clone, per season and the interaction on the water status variables evaluated. In general, a lower availability of water in the soil led to the reduction of the water potential at dawn and at noon in drought indicating that the clones were effectively subjected to water deficit, which translated into a reduction in K_L in the 9 cocoa clones, indicating a lower water flow per $\Delta\Psi$. Drought also caused a differential reduction in A depending on the cocoa clone that was associated with a decrease in chlorophyll rather than decreases in g_s.

Contrary to expectations, under drought, g_s and E did not vary in southern clones and were significantly higher in northern clones. The significant reduction in WUE under drought was due to a reduction in A and an increase in E during drought. There was no correlation between A and g_s, suggesting that the reduction in A under drought was not due to a reduction in g_s, but to metabolic factors that translated into an increase in C_i. Northern clones showed a reduction in LAI, while there was no change in southern clones. A significant decrease in LAI indicates a smaller leaf area that captures less light, negatively affecting photosynthesis, but also loses less water through transpiration in drought-affected clones.

It is concluded that there was a differential physiological response to drought depending on the clone, supporting the hypothesis that genetic variability in cocoa clones showed differences in physiological response to seasonal variation in rainfall. It was found that clones from the south (higher rainfall) were less affected by drought (lower $\Delta\Psi$), did not vary g_s, E, SPAD or LAI and were more efficient at moving water (higher K_L in rain).

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