Phyton-
International Journal of
Experimental Botany

Tech Science Press

http://doi.org/10.32604/phyton.2026.087160

ARTICLE

Progressive Water Deficit Modulates Growth, Photosynthetic Pigments, and Proline Accumulation in *Theobroma cacao* L. Seedlings

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Received: 11 June 2026; Accepted: 27 July 2026; Published: 28 August 2026

ABSTRACT: Water deficit is one of the major constraints to the establishment and early growth of cacao (*Theobroma cacao* L.), particularly under increasingly frequent and intense drought conditions. The objective of this study was to evaluate the morphological and physiological responses of cacao seedlings subjected to progressive water deficit under nursery conditions. Plants were evaluated over a 45-day period without irrigation and compared with well-watered plants maintained at 60% of substrate field capacity. Growth variables, photosynthetic pigment concentrations, and proline accumulation in leaves, stems, and roots were evaluated. Stem length showed no significant differences between treatments, whereas root length and fresh biomass decreased as the water deficit was prolonged. Dry biomass remained unchanged under moderate stress but was significantly reduced when the irrigation-free period was extended, indicating that stress duration was the primary factor influencing plant growth. Water-stressed seedlings maintained higher chlorophyll concentrations throughout the experimental period, whereas proline accumulation increased, reaching a maximum after 30 days of water deficit, with increases of approximately 119%, 71%, and 147% in leaves, stems, and roots, respectively, before declining under prolonged stress. Stress duration was identified as the main factor determining the magnitude of the physiological responses, revealing a distinct temporal- and organ-dependent pattern of proline accumulation. Overall, these findings demonstrate that the morphological and biochemical responses of cacao seedlings to water deficit occur in a coordinated manner and are primarily determined by stress duration, providing new insights into the temporal dynamics of proline accumulation and associated physiological responses during the early developmental stages of cacao.

KEYWORDS: Osmotic adjustment; abiotic stress; drought; tropical perennial species

1 Introduction

Cacao (*Theobroma cacao* L.) is a perennial crop of high economic, ecological, and sociocultural relevance in tropical regions, particularly in Mesoamerica, where it has been part of traditional production systems since pre-Hispanic times [1]. In Mexico, cacao cultivation is concentrated mainly in the southeastern part of the country, with Chiapas as one of the most important producing regions [2]. However, the productivity

and sustainability of cacao are strongly conditioned by climatic factors, making it a highly vulnerable species in the face of current climate change scenarios [3].

Cacao has strict environmental requirements, including optimal temperatures between 25 and 27°C, high relative humidity, and a uniform annual distribution of rainfall [4]. Alterations in these factors, particularly the reduction or irregularity of rainfall, directly affect vegetative growth, photosynthesis, and biomass accumulation [5]. In this context, the increasing frequency and intensity of prolonged droughts represents one of the main threats to seedling establishment and survival, a stage considered critical in the life cycle of perennial species [6].

Water deficit induces a series of morphological, physiological, and biochemical responses in plants, including growth reduction, limitation of cell expansion, stomatal closure, and alteration of carbon metabolism. In parallel, physiological mechanisms associated with osmotic adjustment are activated, including the accumulation of compatible solutes such as proline [7]. This amino acid plays key roles as an osmoprotectant, protein and membrane stabilizer, redox state regulator, and temporary reservoir of carbon and nitrogen [8].

Previous studies have shown that water deficit negatively affects the physiology of *Theobroma cacao*, reducing leaf water potential, stomatal conductance, photosynthetic performance, and biomass accumulation, although the magnitude of these responses varies according to genotype, stress intensity, and exposure time [9–11]. In addition to these physiological responses, osmotic adjustment constitutes one of the major adaptive responses activated under water deficit, in which proline accumulation contributes to maintaining cellular homeostasis during dehydration. However, information on the temporal dynamics of proline accumulation in different tissues of cacao seedlings subjected to progressive water deficit remains limited, thereby restricting its application as an early physiological indicator of drought stress during seedling establishment.

Additionally, water stress directly affects the photosynthetic machinery, resulting in changes in the content and proportion of photosynthetic pigments [12]. In sensitive species such as cacao, these changes may reflect both physiological damage processes and mechanisms of apparent chlorophyll concentration associated with tissue dehydration, without necessarily implying greater photosynthetic efficiency.

Despite the relevance of water deficit for cacao establishment, limited information is available regarding the coordinated responses of growth, photosynthetic pigments, and tissue-specific proline accumulation during progressive periods of water restriction in early developmental stages [13]. Improving the understanding of these responses may contribute to the identification of physiological indicators associated with drought stress during seedling establishment. Therefore, the present study aimed to evaluate growth responses, photosynthetic pigment dynamics, and tissue-specific proline accumulation in *Theobroma cacao* L. seedlings subjected to progressive water deficit under nursery conditions.

2 Materials and Methods

2.1 Experimental Site

The study was conducted in a shade house at the facilities of the Instituto Tecnológico de Tuxtla Gutiérrez, Chiapas, Mexico, during the dry season (March–April 2023).

The experimental structure was covered with a 25% shade net. During the experimental period, maximum and minimum air temperatures averaged 38 and 18°C, respectively, with an average relative humidity of approximately 70%. Plants were exposed to natural daylight under an average photoperiod of approximately 12 h. Throughout the experimental period, plants were maintained under natural ambient conditions.

2.2 Procurement and Establishment of Plant Material

Mature fruits of *Theobroma cacao* L. were collected in the municipality of Ángel Albino Corzo, Chiapas. Seeds were then extracted manually and sown in polystyrene forestry germination trays using commercial Peat Moss as substrate.

Thirty days after emergence, seedlings were transplanted into 1 kg nursery bags containing soil from a cacao plantation with silt-loam texture. From this point, plants were acclimated for one week before initiating the water treatments.

2.3 Experimental Design and Treatments

The experiment was established under a completely randomized design with factorial arrangement. Two factors were considered: (i) water condition with two levels: well-watered plants irrigated weekly with a volume equivalent to 60% of the substrate field capacity (306 mL), and water-deficit plants subjected to complete irrigation withdrawal.

Substrate field capacity was determined gravimetrically using the same nursery bags, substrate type, and substrate quantity employed throughout the experiment. Bags containing 1 kg of homogenized silt-loam soil were weighed, saturated with water, and allowed to drain freely for 24 h.

The amount of water retained after drainage, approximately 510 mL, was calculated as the difference between the drained saturated mass and the initial dry mass. Based on this value, a weekly irrigation volume of 306 mL, equivalent to 60% of the substrate field capacity, was established for the well-watered treatment. This irrigation level was selected because preliminary evaluations indicated that it maintained adequate substrate moisture without causing waterlogging or visible symptoms of water deficit under the experimental conditions. To assist irrigation management, additional nursery bags containing the same substrate but without plants were periodically weighed to monitor substrate water loss by evaporation under the prevailing environmental conditions. (ii) the evaluation time factor comprised three levels: 15, 30, and 45 days after irrigation withdrawal.

For each treatment–time combination, 15 plants were established, from which five plants per evaluation date were selected for destructive sampling. Each plant was considered an independent experimental unit.

For the proline content variable, the tissue type factor (leaf, stem, and root) was additionally considered, during sampling of each experimental unit to evaluate tissue-specific accumulation responses under the imposed water conditions.

2.4 Sampling and Experimental Unit

Prior to the initiation of water treatments, initial values (day 0) of all evaluated variables were recorded and considered the physiological reference for the initial plant status. At each evaluation date (15, 30, and 45 days), destructive sampling was performed by selecting five plants per treatment, which constituted the experimental units for the analysis of growth variables, chlorophyll content, and proline.

2.5 Growth Parameters

Plants were transferred to the laboratory, where the substrate adhering to the roots was carefully removed. Subsequently, shoot and root lengths were measured with a digital caliper. Once these variables were recorded, samples were weighed to determine fresh biomass and then placed in a drying oven (Thermo Scientific®) at 60°C until constant weight was reached, to obtain dry biomass.

2.6 Chlorophyll Determination

Chlorophyll content was determined from fully expanded leaves following the methodology described by Joya-Dávila et al. [14]. A total of 0.2 g of fresh leaf tissue was weighed and macerated in 80% (v/v) acetone. Samples were incubated at 4°C for 24 h in darkness.

Subsequently, the volume was adjusted to 6.25 mL with 80% acetone and samples were centrifuged at 4500 rpm for 10 min. Absorbances were recorded using a UV-Vis spectrophotometer (Beckman Coulter®, DU® 730) at 663 nm for chlorophyll a (Chl a) and 645 nm for chlorophyll b (Chl b).

Chlorophyll contents were calculated using the following equations:

$$\text{Total Chlorophyll} = \text{Chlorophyll } a + \text{Chlorophyll } b \tag{1}$$

$$\text{Chlorophyll } a = [(12.7 * A_{663}) - (2.5 * A_{645})](V)/(1000 * P)] \tag{2}$$

$$\text{Chlorophyll } b = [(22.9 * A_{645}) - (4.70 * A_{663})](V)/(1000 * P)] \tag{3}$$

where V corresponds to the final volume of the extract (mL) and P to the weight of plant tissue (g). The values obtained were expressed as mg g^{-1} fresh weight (FW) and used to evaluate temporal changes in photosynthetic pigment content under water stress and non-stress conditions.

2.7 Proline Content

For each experimental unit selected at each evaluation time, analysis was performed independently on different plant tissues (leaf, stem, and root) to evaluate differential proline accumulation responses associated with water stress, using the methodology of Bates et al. [15] with slight modifications. An acid ninhydrin solution was prepared by dissolving 1.25 g of ninhydrin in 30 mL of glacial acetic acid previously warmed to promote homogeneous dissolution; subsequently, 20 mL of phosphoric acid (6 M) was added under constant stirring until a uniform solution was obtained.

A total of 0.1 g of dry, finely ground plant material was weighed, to which 10 mL of boiling distilled water was added. Samples were centrifuged at 3740 rpm for 10 min at 5°C. From each supernatant, 1 mL was taken, to which 1 mL of acid ninhydrin and 1 mL of glacial acetic acid were added. Samples were vigorously vortexed and incubated in a water bath at 100°C for 1 h.

Subsequently, 2 mL of toluene was added for extraction of the chromophore complex, and the organic phase was recovered and read at 520 nm using a UV-Vis spectrophotometer (Beckman Coulter®, DU® 730). Quantification was performed using a proline standard curve with concentrations of 5, 10, 20, 25, 30, 40, and 60 ppm; results were expressed as µmol proline g^{-1} dry weight (DW).

2.8 Statistical Analysis

The data obtained were analyzed by analysis of variance (ANOVA) under a completely randomized factorial model. For growth variables and chlorophyll content, a two-way ANOVA was applied, considering water condition (with and without stress) and evaluation time (15, 30, and 45 days) as factors. For proline content, a three-way ANOVA was applied, incorporating evaluation time, water condition, and tissue type (leaf, stem, and root) as factors.

In all cases, the main effects of each factor and their interactions were evaluated. When statistically significant differences were detected ($p < 0.05$), means were compared using Tukey's test. Assumptions of normality and homogeneity of variances were verified prior to analysis.

The value recorded at the beginning of the experiment (day 0) was used solely as an initial physiological reference to describe the temporal dynamics of the evaluated variables and was not included in the statistical analysis. Analyses were performed using Statgraphics Centurion XVI software (Statgraphics Technologies, Inc.).

3 Results

3.1 Growth Parameters

Water deficit differentially affected the growth traits assessed in this study (Table 1). Stem length was not significantly affected by the watering regime, evaluation time, or their interaction ($p > 0.05$), remaining stable throughout the experimental period. In contrast, root length was significantly influenced by water deficit ($p = 0.0097$), decreasing by approximately 24% after 30 days of irrigation withdrawal. This reduction persisted until the end of the experiment.

Among all growth traits evaluated, fresh biomass was the most sensitive to water deficit. A significant interaction between watering regime and evaluation time was detected ($p = 0.0140$), with reductions exceeding 50% after 30 days of irrigation withdrawal and reaching approximately 62% by the end of the experimental period compared with well-watered plants (Table 1).

Dry biomass also exhibited a significant interaction between watering regime and evaluation time ($p = 0.0037$). Although no marked differences were observed during the first 30 days, water-stressed plants exhibited an approximately 52% reduction in dry biomass at the end of the experimental period relative to well-watered plants.

Table 1: Effect of water deficit and evaluation time on growth variables in *Theobroma cacao* L. seedlings.

Day	Stress Condition	Stem Length (cm)	Root Length (cm)	Fresh Biomass (g)	Dry Biomass (g)
D-15	NS	23.5 ± 0.7a	19.2 ± 1.2a	5.4 ± 0.5b	1.7 ± 0.4bc
D-15	S	23.0 ± 1.6a	18.7 ± 0.7a	5.6 ± 0.1b	1.8 ± 0.4bc
D-30	NS	25.3 ± 1.0a	19.6 ± 0.3a	7.2 ± 0.1a	2.4 ± 0.4ab
D-30	S	24.5 ± 0.8a	15.0 ± 1.0b	3.4 ± 0.5c	1.8 ± 0.2bc
D-45	NS	25.3 ± 0.6a	20.2 ± 0.2a	6.1 ± 0.2b	2.7 ± 0.3a
D-45	S	25.8 ± 1.2a	15.5 ± 0.5b	2.3 ± 0.5d	1.3 ± 0.1c
HSD		2.41	2.10	1.03	0.84
R^2		0.357	0.782	0.870	0.786
p-value	Treatment	0.3144	0.001	0.0001	0.0010
	Day (A)	0.3792	0.003	0.00001	0.0006
	Stress condition (B)	0.1311	0.0097	0.0232	0.1614
	Interaction A*B	0.6174	0.1841	0.0140	0.0037

Different lowercase letters indicate statistically significant differences between stress days ($p < 0.05$). Values correspond to mean ± standard error. NS: No stress; S: Stress.

3.2 Photosynthetic Pigments

Photosynthetic pigment content was significantly affected by water availability, whereas evaluation time had no significant effect on any of the variables evaluated. A significant interaction between both factors was detected only for total chlorophyll (Table 2).

Seedlings grown under well-watered conditions maintained relatively stable chlorophyll *a*, chlorophyll *b*, and total chlorophyll contents throughout the experimental period. Conversely, water-stressed seedlings consistently exhibited higher concentrations of all three pigments at each evaluation time. Maximum chlorophyll contents were observed after 45 days of water deficit, whereas the lowest values occurred in non-stressed seedlings (Table 2). At the end of the experimental period, total chlorophyll content in water-stressed seedlings was approximately 135% higher than that of non-stressed seedlings.

Table 2: Chlorophyll a, chlorophyll b, and total chlorophyll content in *Theobroma cacao* L. seedlings subjected to water deficit.

Day	Stress Condition	Chl a	Chl b	Chl T
			———mg g^{-1} Fresh Weight———	
D-15	NS	22.77 ± 4.37b	20.00 ± 0.86b	42.77 ± 4.82bc
D-15	S	31.93 ± 1.86ab	32.73 ± 1.51ab	64.67 ± 3.01ab
D-30	NS	19.23 ± 4.66b	15.71 ± 0.81b	34.93 ± 4.50c
D-30	S	38.90 ± 2.86a	33.02 ± 0.91ab	71.92 ± 3.05ab
D-45	NS	20.76 ± 2.38b	17.27 ± 2.42b	38.03 ± 4.78c
D-45	S	40.85 ± 0.87a	48.50 ± 8.76a	89.35 ± 8.92a
HSD		14.91	33.83	25.38
R^2		0.79	0.89	0.88
p-value	Day (A)	0.5608	0.1348	0.1244
	Stress condition (B)	0.0001	0.0001	0.0001
	Interaction A*B	0.1842	0.0607	0.0480

Values are mean ± standard error. Different lowercase letters within each pigment column indicate significant differences among treatment–time combinations according to Tukey's HSD test ($p < 0.05$). Chlorophyll *b* was analyzed after natural-log transformation; raw means are presented. NS: no stress; S: stress.

3.3 Proline Accumulation

The progression of water deficit was accompanied by gradual visual changes in *Theobroma cacao* L. seedlings, which became increasingly evident as the irrigation withholding period advanced. Symptoms of leaf wilting and reduced plant vigor were more pronounced after 30 and 45 days of water deficit than in well-watered seedlings (Fig. 1).

Figure 1: Visual progression of water stress in *Theobroma cacao* L. seedlings during the experimental period. Representative images showing the visual progression of water deficit symptoms in *Theobroma cacao* L. seedlings subjected to 15, 30, 45 and 60 days without irrigation, illustrating changes in leaf turgor and leaf coloration over the experimental period.

Proline accumulation was significantly affected by evaluation time and water availability, whereas tissue type showed no significant main effect. However, significant interactions were detected between evaluation time and water availability, evaluation time and tissue type, and water availability and tissue type, indicating that proline accumulation was jointly influenced by stress duration and the organ evaluated (Table 3).

Table 3: Effects of stress duration, water condition, and tissue type on proline content in *Theobroma cacao* L. seedlings.

Factor	Day (A)			Stress Condition (B)		Tissue (C)		
	15	30	45	NS	DS	Leaf	Stem	Root
µmol proline/g DW	15.22b	29.12a	10.01c	11.25b	25.02a	18.37a	18.3a	17.66[a]
p-value=		0.00001		0.0001			0.6821	
R^2				0.9597				
Interactions								
A*B				A*C			B*C	
0.00001				0.0061			0.0001	

Different letters indicate differences between groups ($p < 0.05$). NS: No stress; DS: Drought stress; DW: dry weight.

Water-stressed seedlings exhibited a marked increase in proline accumulation during the first 30 days of irrigation withholding, reaching maximum concentrations in leaves, stems, and roots. Compared with well-watered seedlings, proline accumulation at day 30 was approximately 119% higher in leaves, 71% higher in stems, and 147% higher in roots (Fig. 2).

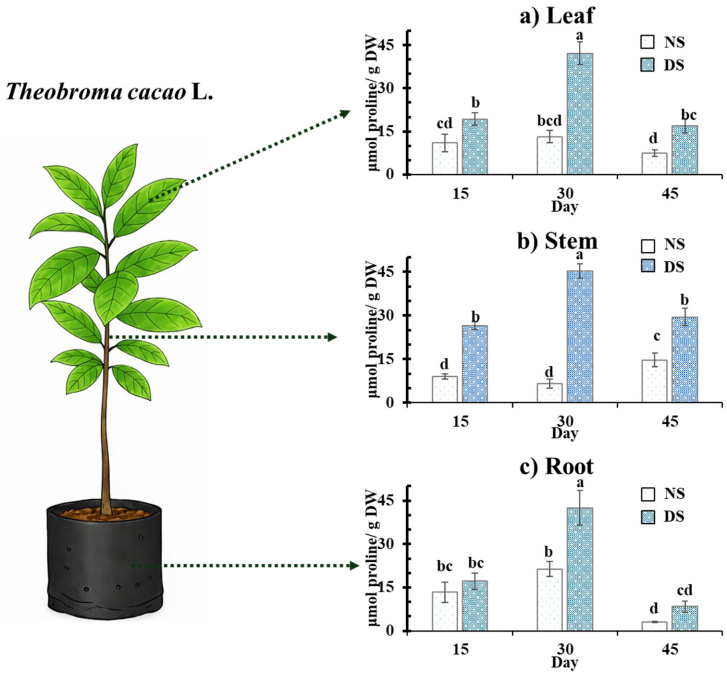

Figure 2: Proline content in leaf, stem, and root of *Theobroma cacao* L. seedlings during a water deficit period. Plants were evaluated at 15, 30, and 45 days after the onset of the water deficit treatment. Data represent mean ± standard error. The effects of time, water condition, tissue type, and their interactions were evaluated by three-way ANOVA (see Table 3). Different lowercase letters between columns indicate statistically significant differences between stress days ($p < 0.05$). NS: No stress; DS: Drought stress.

Although maximum proline accumulation was observed after 30 days of water deficit in all tissues, the subsequent response differed among organs. Proline content declined markedly in leaves and roots by day 45, whereas stems maintained higher proline concentrations than those recorded at the beginning of the experimental period, although remaining below the maximum values observed at day 30 (Fig. 2).

4 Discussion

Water deficit triggered a coordinated physiological response in *Theobroma cacao* L. seedlings, reflected by concurrent changes in plant growth, photosynthetic pigment content, and proline accumulation. These responses did not occur uniformly throughout the experimental period but progressively changed as irrigation withholding continued. Consequently, stress duration emerged as a key determinant of the magnitude of the morphological and biochemical adjustments exhibited by cacao seedlings.

Fresh biomass declined progressively under water deficit, reflecting a continuous loss of tissue water, whereas the reduction in dry biomass at the end of the experiment indicated that prolonged water limitation also impaired structural biomass accumulation. Under drought conditions, decreased cellular water potential and loss of turgor restrict cell expansion, promote stomatal closure, and limit carbon assimilation, ultimately reducing plant growth and biomass production [16,17]. Similar responses have been widely documented in perennial species, in which temporary growth inhibition and resource reallocation contribute to maintaining plant function during moderate water deficit; however, these compensatory responses become insufficient as stress persists, leading to a progressive decline in biomass accumulation [18,19]. In cacao, Lahive et al. [9] reported significant reductions in biomass production and photosynthetic performance under water deficit, whereas Baby et al. [11] demonstrated marked genotypic differences in the ability to sustain growth and plant water status during drought. Collectively, these studies support the view that growth reduction is determined not only by water availability itself but also by stress duration and the intrinsic physiological capacity of each genotype to cope with drought.

The reduction in root length observed after 30 days of water deficit contrasts with responses commonly reported in annual crops such as *Phaseolus vulgaris*, where enhanced root elongation may improve soil exploration and water uptake under limiting moisture conditions [20]. In perennial woody species, however, root responses vary considerably depending on genotype, developmental stage, stress intensity, and rooting volume. As drought progresses, reduced meristematic activity, limited photoassimilate supply, and the increasing metabolic costs associated with cellular maintenance may constrain root development [12,16]. Moreover, cultivation under nursery bag conditions inherently restricts root exploration and may intensify substrate drying, thereby exacerbating the effects of water deficit [21,22]. This response is not uniform among cacao genotypes. Dos Santos et al. [23] demonstrated marked genotypic variation in the physiological and morphological responses of cacao to water deficit, reporting that drought-tolerant genotypes maintained root growth at levels comparable to those of well-watered plants, whereas more sensitive materials exhibited greater growth restrictions. Therefore, the reduction in root length observed in the present study may reflect the specific response capacity of the evaluated plant material rather than a generalized response of the species.

Variations in chlorophyll content indicate that the photosynthetic response of cacao seedlings to water deficit depends on both stress intensity and duration. In contrast to studies reporting that prolonged drought promotes chlorophyll degradation as a consequence of photosynthetic apparatus deterioration and inhibition of chlorophyll biosynthesis [24–26], water-stressed seedlings in the present study maintained higher chlorophyll concentrations throughout the three evaluation periods. Suárez-Salazar et al. [27] reported that cacao seedlings exposed to prolonged moderate and severe drought exhibited reduced chlorophyll contents

while simultaneously increasing carotenoid and proline accumulation as photoprotective mechanisms against oxidative damage. These differences compared with our results most likely reflect variations in stress intensity, genotype, and the duration of water restriction, all of which are recognized as major determinants of the physiological response of cacao to drought.

The maintenance of relatively high chlorophyll concentrations throughout the experimental period coincided with the stage at which maximum proline accumulation was recorded. This response indicates that, during the initial stages of water deficit, cacao seedlings activate biochemical mechanisms that contribute to preserving the functionality of the photosynthetic apparatus, even though vegetative growth has already become constrained by reduced water availability [28–30]. Nevertheless, higher chlorophyll concentrations should not be interpreted as evidence of enhanced photosynthetic performance, since photosynthetic performance depends on additional physiological processes, including stomatal conductance, gas exchange, photochemical efficiency, and carbon assimilation, none of which were evaluated in the present study. A similar pattern has recently been described in cacao, where photosynthetic activity declined despite the activation of photoprotective and antioxidant mechanisms during prolonged drought [27].

When comparing these findings with those reported for other species, it should also be considered that chlorophyll responses to water deficit vary according to species, genotype, stress intensity, stress duration, and the procedures used to quantify photosynthetic pigments. In this regard, Joya et al. [14] pointed out that chlorophyll quantification based on a known amount of fresh biomass may lead to different interpretations than methodologies expressing pigment content per unit of known leaf area, where the photosynthetically active surface constitutes the basis of the estimation. Although methodological differences alone do not explain the physiological responses observed under drought, they should be considered when comparing results obtained under different experimental conditions.

Proline accumulation is one of the most extensively documented biochemical responses to water deficit because of its role in osmotic adjustment, stabilization of proteins and cellular membranes, preservation of photosynthetic function, and maintenance of cellular redox homeostasis [28–30]. Under drought conditions, increased proline accumulation contributes to maintaining cellular osmotic potential and mitigating oxidative damage, particularly during the early stages of water limitation [29,30]. Recent studies in cacao have likewise demonstrated that proline accumulation constitutes an integral component of the physiological mechanisms associated with drought tolerance, contributing to improved plant water status during prolonged periods of water deficit [11].

In the present study, proline content reached its maximum after 30 days of water deficit in leaves, stems, and roots, demonstrating that osmotic adjustment followed a well-defined temporal pattern. This response is consistent with observations in cacao and other woody species, where proline accumulation predominates during the early and intermediate phases of drought, whereas its concentration subsequently declines as water limitation persists, likely reflecting the high metabolic cost associated with proline biosynthesis and maintenance, together with the progressive advancement of stress-induced physiological impairment [11,31].

A particularly relevant finding of this study was that, although all three organs reached maximum proline accumulation after 30 days of water deficit, their subsequent responses differed markedly. Leaves and roots exhibited a pronounced decline by the end of the experimental period, whereas stems maintained proline concentrations above those recorded at the onset of the treatment. These findings indicate that proline accumulation is not uniformly distributed throughout the plant but instead depends on both the organ and the duration of water deficit. Although recent studies have identified proline accumulation as an important biochemical indicator of drought tolerance in cacao, most have focused on overall

physiological or genotype-dependent responses rather than on the temporal dynamics of organ-specific proline distribution [11,32]. Therefore, the present results provide additional evidence that the timing and tissue-specific distribution of proline should also be considered when evaluating drought responses in cacao seedlings.

The temporal pattern of proline accumulation observed in this study demonstrates that the biochemical response to water deficit evolves progressively as drought intensifies and occurs in parallel with the morphological and physiological adjustments previously discussed. Consequently, the duration of water limitation should be considered an essential factor when evaluating drought-response mechanisms in cacao seedlings.

The findings of the present study demonstrate that the response of *Theobroma cacao* seedlings to water deficit is not governed by a single physiological mechanism but rather by a coordinated sequence of morphological and biochemical adjustments that evolve progressively as water limitation intensifies. Growth inhibition represented one of the earliest responses to drought, whereas the transient maintenance of chlorophyll content and the subsequent accumulation of proline reflected the activation of complementary physiological mechanisms during the early and intermediate stages of stress. These observations highlight the importance of considering the temporal dynamics of physiological responses when evaluating drought responses in cacao, as the magnitude and behavior of each variable depend not only on the duration of water deficit but also on the specific plant organ being evaluated.

5 Conclusions

Theobroma cacao L. seedlings responded to water deficit through a coordinated sequence of morphological and biochemical adjustments, the magnitude of which was primarily determined by stress duration. The progressive reduction in growth and biomass accumulation demonstrated that prolonged water limitation restricts vegetative development from the earliest stages of seedling establishment, whereas the sustained maintenance of chlorophyll content throughout the experimental period, together with the transient accumulation of proline reflected the activation of physiological processes associated with responses to water deficit.

Proline accumulation exhibited a distinct temporal- and organ-dependent pattern, reaching maximum levels after 30 days of water deficit before declining as stress progressed. These findings demonstrate that the physiological response of cacao is strongly influenced by both the duration of water limitation and the plant organ evaluated, providing new evidence on the temporal dynamics of proline accumulation during the early developmental stages of the species. These findings also provide a physiological basis for future studies investigating drought responses in cacao.

Acknowledgement: Not applicable.

Funding Statement: The authors received no specific funding for this study.

Author Contributions: The authors confirm their contribution to the paper as follows: Conceptualization, José G. Joya Dávila and Daniel González Mendoza; methodology, José G. Joya Dávila, Leslie A. Serrano Gómez, Federico A. Gutiérrez Miceli and Daniel González Mendoza; validation, José G. Joya Dávila, Federico A. Gutiérrez Miceli, Daniel González Mendoza, Olivia Tzintzun Camacho and Dagoberto Durán Hernández; formal analysis, Leslie A. Serrano Gómez, José G. Joya Dávila and Daniel González Mendoza; investigation, Leslie A. Serrano Gómez; resources, José G. Joya Dávila, Federico A. Gutiérrez Miceli and Daniel González Mendoza; data curation, Leslie A. Serrano Gómez; writing—original draft preparation, Leslie A. Serrano Gómez; writing—review and editing, José G. Joya Dávila, Federico A. Gutiérrez Miceli, Olivia Tzintzun Camacho, Dagoberto Durán Hernández, Alexis Salazar Navarro, David Cervantes-García, Ulin Antobelli Basilio Cortes, Anayancy Lam Gutiérrez and Daniel González Mendoza;

visualization, Leslie A. Serrano Gómez; supervision, José G. Joya Dávila, Federico A. Gutiérrez Miceli and Daniel González Mendoza; project administration, José G. Joya Dávila; funding acquisition, not applicable. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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