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Posted Date: 23 March 2026

doi: 10.20944/preprints202603.1743.v1

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Dose-Dependent Effects of a Protein Hydrolysate Biostimulant on the Growth, Performance, and Quality of *Theobroma cacao* L. Seedlings

Fernando Gomes Hoste [1], Cristhiane Tatagiba Franco Brandão [2], Ana Júlia Câmara Jeveaux-Machado [1], Janyne Soares Braga Pires [3], Felipe Moro[2], Daniel Carvalho de Araújo [2], Bliane Morozini Bacheti [2], Maria Ester Lenzi de Souza[2], Danilo Balla Filho [2], Vinicius de Souza Oliveira [1,4,*], Enilton Nascimento de Santana [4], Karin Tesch Kuhlcamp [4], Lúcio de Oliveira Arantes [4] and Sara Dousseau-Arantes [1,2,3,4,*]

[1] Centro de Ciências Humanas e Naturais, Universidade Federal do Espírito Santo, Centro de Ciências Humanas e Naturais, Vitória 29075-910, Espírito Santo, Brazil
[2] Faculdades Integradas Espírito-Santenses, FAESA, Linhares 29900-070, Espírito Santo, Brazil
[3] Departamento de Ciências Agrárias e Biológicas, Universidade Federal do Espírito Santo, Centro Universitário Norte do Espírito Santo, São Mateus 29932-540, Espírito Santo, Brazil
[4] Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural—Centro Regional de Desenvolvimento Rural—Norte, Linhares 29901-443, ES, Brazil
* Correspondence: souzaoliveiravini@gmail.com (V.d.S.O.); saradousseau@gmail.com (S.D.-A.)

Abstract

Protein hydrolysate-based biostimulants have been widely investigated for their potential to enhance seedling growth; however, integrated interpretations of dose-dependent morphophysiological and photochemical responses remain limited for *Theobroma cacao* L., particularly during the nursery phase. This study aimed to evaluate dose-dependent physiological modulation in cacao seedlings of the Catongo and TSH 1188 genotypes under increasing concentrations of a protein hydrolysate-based biostimulant. The experiment was conducted in a randomized block design arranged in a 2 × 6 factorial scheme, corresponding to two genotypes and six biostimulant concentrations. Morphological traits, biomass accumulation, morphophysiological indices, chlorophyll indices, and chlorophyll *a* fluorescence parameters were assessed. The results revealed clear dose- and genotype-dependent responses, with predominantly quadratic patterns across most variables. Intermediate concentrations were associated with coordinated improvements in vegetative growth, root development, and photosystem II (PSII) functional performance, whereas higher concentrations were linked to reduced physiological balance. Overall, the findings indicate that protein hydrolysate-based formulations modulate cacao seedling performance through dose-dependent physiological adjustment, contributing to a more integrated understanding of biostimulant action during the nursery phase.

Keywords: chlorophyll fluorescence; dose-dependent modulation; nursery phase; protein hydrolysates; PSII functional performance; *Theobroma cacao*

1. Introduction

The cacao tree (*Theobroma cacao* L.) is a crop of high economic, social, and ecological relevance in tropical regions, playing a central role in income generation, biodiversity conservation, and the sustainability of agroforestry systems [1]. Despite advances in genetic improvement and crop management, the nursery phase remains a critical bottleneck in cacao production, as seedlings frequently exhibit low physiological vigor, limited photosynthetic performance, and high sensitivity

 doi:10.20944/preprints202603.1743.v1

to environmental stresses, which can compromise their establishment and performance in the field [2–4].

In recent years, plant biostimulants have gained increasing attention as sustainable tools to enhance plant growth and physiological efficiency, potentially reducing the reliance on conventional fertilizers [5–7]. These products act through complex mechanisms involving metabolic, hormonal, and redox regulation, influencing photosynthesis, nutrient uptake, and root system development [8,9]. Unlike mineral fertilizers, biostimulants do not act solely by nutrient supply, but also by biochemical signaling that modulates specific growth and stress-related pathways [10,11].

Among the different categories of biostimulants, protein hydrolysates stand out due to their broad applicability and reported physiological efficiency. These products are obtained through enzymatic or chemical hydrolysis of proteins of plant or animal origin, resulting in mixtures of free amino acids and short bioactive peptides [9,12]. Protein hydrolysates and amino acid-based biostimulants have been extensively investigated in recent years, with reported effects on plant growth, nutrient use efficiency, photosynthetic performance, and stress tolerance across different crops, as summarized in recent reviews [9,12,13]. Several studies have shown that these products can increase chlorophyll content, influence photosystem II (PSII) photochemical performance, and improve tolerance to abiotic stresses, effects that are commonly assessed through chlorophyll fluorescence parameters [9,14].

The organomineral biostimulant Terrativa®, formulated from animal-derived protein hydrolysate and containing complexed phosphorus and free amino acids (0.54%), represents an input with combined nutritional and physiological functions. In this formulation, amino acids may act as metabolic and signaling molecules, while phosphorus contributes to energy metabolism and cellular homeostasis. However, as with other protein hydrolysate-based products, the physiological responses to Terrativa® are expected to be strongly dose-dependent.

Despite the growing body of literature on protein hydrolysates, important gaps remain regarding their physiological mode of action in cacao seedlings. Most available studies focus on isolated growth parameters or general performance indicators, whereas integrated analyses linking dose-dependent morphological responses with PSII functional performance are still limited, particularly during the nursery phase and in seedlings used as rootstocks [4,15]. In addition, comparative evaluations among cacao genotypes with distinct physiological backgrounds are scarce, restricting a broader understanding of genotype-specific responses to this class of biostimulants.

Although protein hydrolysate-based biostimulants have been widely investigated and their dose-dependent effects on plant growth and photosynthetic traits are well documented, relevant uncertainties remain regarding the integrated physiological interpretation of these responses, particularly in perennial crops during the nursery phase. Most available studies focus on isolated growth parameters or general performance indicators, providing limited insight into how morphological development, root system architecture, and photosystem II (PSII) functional performance are coordinated across different biostimulant doses. This limitation is especially relevant for cacao seedlings used as rootstocks, where morphophysiological balance directly influences grafting success and early field establishment. Therefore, rather than aiming to demonstrate growth stimulation per se, this study seeks to advance current knowledge by providing an integrated morphophysiological–photochemical framework to interpret dose-dependent responses of two *T. cacao* genotypes to a protein hydrolysate-based biostimulant. By jointly analyzing growth traits, root development, chlorophyll indices, and PSII fluorescence parameters, we hypothesize that intermediate doses promote coordinated physiological adjustment, whereas higher concentrations may lead to functional imbalance during the nursery phase.

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2. Materials and Methods

2.1. Experimental Location and Plant Material

The experiment was conducted in a commercial nursery located in the municipality of Linhares, Espírito Santo, Brazil (19°27′28″ S; 39°52′44″ W; 38 m altitude). The altitude was determined based on the SRTM digital elevation model [16], with topographic validation from IBGE [17]. The area is located in a traditional cacao growing region in the state of Espírito Santo, with agroclimatic suitability for the development of *T. cacao* [18].

The local climate is classified as Aw, according to Köppen, with a rainy season in summer and a relatively dry period in winter [19]. The experimental period extended from September 15, 2025 to January 13, 2026. Meteorological data were obtained from the Linhares Automatic Meteorological Station (INMET, Code A614) [20]. The average air temperature during the period was 24.3 °C, with minimum and maximum values of 15.1 °C and 35.4 °C, respectively (Figure 1). The accumulated precipitation totaled 447.4 mm. The average relative humidity was estimated at approximately 84%, based on INMET climatological normals [21].

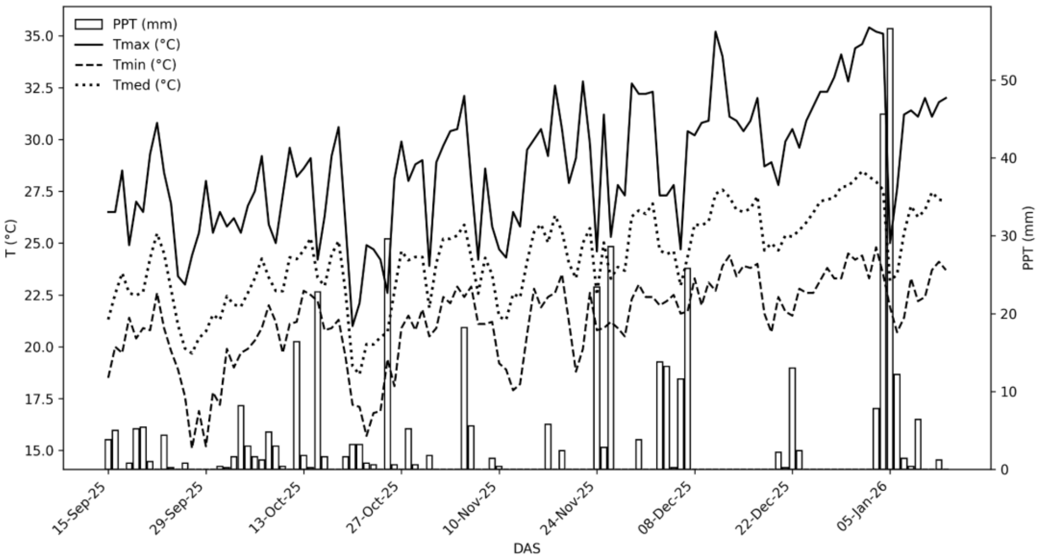

Figure 1. Maximum (Tmax), minimum (Tmin) and average (Tmed) air temperatures and accumulated precipitation (PPT) recorded daily during the experimental period (September 15, 2025 to January 13, 2026), based on data from the Linhares Automatic Weather Station (INMET, Code A614), Espírito Santo, Brazil.

The plant material consisted of seedlings of two *T. cacao* cultivars, Catongo and TSH 1188, used as rootstocks in clonal propagation systems. The Catongo cultivar belongs to the Forastero (Amazonian) genetic group and is recognized for having light-colored seeds, associated with spontaneous mutations in traditional cacao populations, as well as morphophysiological characteristics related to vegetative vigor and adaptation to tropical conditions [22]. While the TSH 1188 cultivar belongs to the Trinitario group, resulting from the cross between Forastero and Criollo genotypes, and presents morphophysiological characteristics associated with balanced growth and greater physiological stability, reflecting the genetic and adaptive diversity of the cacao tree [22,23].

2.2. Experimental Design and Treatments

The experiment was conducted in a randomized block design, in a 2 × 6 factorial scheme, with the first factor consisting of two cultivars of *T. cacao*, Catongo and TSH 1188, and the second factor consisting of six doses of the biostimulant Terrativa® (0, 1, 2, 3, 4 and 6 mL L^{-1}). Each treatment consisted of four replications, with 10 plants per plot, totaling 480 plants evaluated. The treatments were randomly distributed within each block, in order to minimize the effects of spatial variability in the growing environment.

Terrativa® biostimulant is a Class A organic organomineral fertilizer, based on hydrolyzed animal protein, containing macronutrients and free amino acids. The chemical composition and physicochemical characteristics of the biostimulant were obtained from the information declared on the product label [24], as shown in Table 1.

Table 1. Chemical composition and physicochemical characteristics of the Terrativa® biostimulant used in the experiment.

Parameter	**Specification**
Product type	Organic organomineral fertilizer, Class A
Origin	Hydrolyzed protein of animal origin
Nitrogen (N, %)	2.0
Phosphorus (P_2O_5, %)	4.0 – 4.29
Potassium (K_2O, %)	4.0 – 4.11
Organic carbon (%)	20
Hydrogen ion potential	8.0
Electrical conductivity (dS m^{-1})	1.0
Saline index (%)	8.0
Total free amino acids (%)	0.54

Data declared by the manufacturer on the product label (Terrativa®, 2025).

Laboratory analysis indicated a total free amino acid content of 0.54%, with a predominance of glutamic acid, valine, proline, aspartic acid, and leucine, as per an independent analytical report [25]. The detailed free amino acid profile is presented in Table 2.

Table 2. Free amino acid profile (%) of the biostimulant Terrativa®, according to laboratory report.

Amino acid	Content (%)
Glutamic acid	0.09
Valina	0.08
Proline	0.07
Aspartic acid	0.06
Leucine	0.06
Isoleucine	0.04
Phenylalanine	0.04
Tyrosine	0.03
Glycine	0.02

Alanine	0.02
Arginine	0.02
Lysine	0.02
Other amino acids	< 0.01
Total	0.54

Source: Campinas Institute for Soil and Fertilizer Analysis (ICASA), 2025.

The biostimulant doses corresponded to solution concentrations ranging from 0 to 6 mL of product per liter of water (0, 1, 2, 3, 4, and 6 mL L^{-1}). For each treatment, the solutions were prepared in advance by withdrawing the required aliquot of Terrativa® and subsequently adding distilled water to complete 1 L of final solution at the desired concentration. No surfactant or spreading agent was added to the spray solution. The pH determinations of the samples were performed at the Instituto Federal do Espírito Santo (IFES) using a pH meter (MPA-210 model, Tecnopon®). The instrument was previously calibrated with standard buffer solutions at pH 7.0 and pH 4.0, according to the manufacturer's recommendations, ensuring analytical reliability of the measurements. For each evaluated dosage, readings were carried out in triplicate (Table 3).

Table 3. pH values of the biostimulant solutions at different concentrations (0–6 mL L^{-1}), compared with the pure commercial product. Values are expressed as mean ± standard deviation (SD) of three independent measurements per treatment.

Concentration	pH
0 mL L^{-1}	6.52 ± 0.5139
1 mL L^{-1}	7.24 ± 0.2523
2 mL L^{-1}	7.58 ± 0.0351
3 mL L^{-1}	7.73 ± 0.1058
4 mL L^{-1}	7.85 ± 0.0305
6 mL L^{-1}	7.92 ± 0.0115
Pure	8.27 ± 0.0208

Authors, 2026.

The biostimulant applications were carried out via foliar application, in the morning, weekly during the first month and, subsequently, bi-weekly, starting 25 days after sowing (DAS). For each dose, 1 L of solution was prepared and applied uniformly to the plants using a manual pre-pressure sprayer. The volume of solution applied was approximately 25 mL per plant^{-1}, considering the application of 1 L for every 40 plants. Thus, although the spray volume per plant was constant (≈25 mL plant^{-1}), the effective amount of biostimulant delivered varied proportionally according to the solution concentration (0–6 mL L^{-1}).

2.3. Growing Conditions and Cultural Practices

The seedlings were kept in a nursery covered with 50% shade cloth, under micro-sprinkler irrigation, carried out three times a day, with two 30-minute irrigations and one supplementary 10 minute irrigation. Water management was conducted based on substrate moisture, with adjustments

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according to climatic conditions and the stage of seedling development, avoiding both water deficit and waterlogging.

The plants were grown in polyethylene plastic bags (nursery bags), measuring 15 × 28 × 10 cm and with an approximate useful volume between 2.8 and 3.5 L, filled with Produx Dreno commercial substrate (MAPA SP 81755), composed of pine bark, vermiculite, charcoal, and mineral amendments. The substrate had a pH of 6.2 (±0.5), an electrical conductivity of 0.6 ± 0.3 mS cm^{-1}, and an apparent density of 267 kg m^{-3}, characteristics favorable to the development of the root system of cacao seedlings.

Maintenance fertilization was carried out in a balanced and fractionated manner, using sources of macronutrients and micronutrients, according to technical recommendations for the production of cacao seedlings. No synthetic stimulants or seaweed extracts were applied to the experimental plot in order to avoid interference with the effects of the evaluated biostimulant.

Phytosanitary management was conducted in a preventive and corrective manner, with chemical and biological applications according to phytosanitary monitoring. Throughout the experimental period, no severe incidences of pests or diseases were observed, indicating the efficiency of the management adopted.

2.4. Evaluation of Growth and Development

Evaluations were carried out 120 days after sowing (DAS), considering representative plants from each experimental plot.

Shoot growth was characterized by the number of leaves (NL), total leaf area (LA), stem length (SL), and stem diameter (SD). Leaf area was determined using an LI-3100C electronic leaf area meter (LI-COR Biosciences, Lincoln, NE, USA) [26]. Stem length was measured from the collar to the apical bud with a graduated ruler, and stem diameter was measured at the collar region with a precision digital caliper.

The root system was evaluated by measuring the length of the longest root (LR) and the root volume (RV), determined by the water displacement method in a graduated cylinder [27,28].

From these variables, morphophysiological indices were calculated, including the robustness index, obtained by the ratio between the length and diameter of the stem (SL/SD) [28], the ratio between the length of the stem and the length of the longest root (SL/LR), used as an indicator of the morphophysiological balance of the seedlings.

2.5. Determination of Chlorophyll Indices and Fluorescence

Chlorophyll indices were determined 120 days after sowing (DAS) using the ClorofiLOG CFL 1030 electronic chlorophyll meter (Falker Automação Agrícola, Porto Alegre, Brazil). Measurements were taken on all plants in each experimental plot, evaluating two fully expanded leaves per plant, located in the middle portion of the aerial part. Relative indices of chlorophyll a, chlorophyll b, and total chlorophyll were obtained according to the methodology proposed by the manufacturer [29].

The transient fluorescence of chlorophyll a was evaluated using the Pocket PEA portable fluorometer (Hansatech Instruments, Norfolk, United Kingdom) [30]. Measurements were taken in the morning, between 08:00 and 11:00 h, on three randomly selected plants within each experimental plot, evaluating two fully expanded leaves per plant. The leaves were previously dark-adapted for 30 minutes using leaf clips to ensure complete opening of the photosystem II (PSII) reaction centers. Subsequently, a saturating light pulse of 3000 μmol m^{-2} s^{-1} photons was applied for a duration of 1 second.

The following fluorescence parameters were determined: initial fluorescence (Fo), variable fluorescence (Fv), maximum fluorescence (Fm), energy absorbed per reaction center (ABS/RC), energy transferred for electron transport (TRO/RC), and absorption-based photochemical performance index (PI abs). The chlorophyll a fluorescence transient (OJIP) was analyzed according to the JIP-test theory, which allows the evaluation of energy absorption, trapping, and electron transport within PSII reaction centers, as described by Strasser et al. [31].

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2.6. Statistical Analysis

The data were subjected to statistical analysis using R software (version 4.5.1) [32]. Initially, analysis of variance was performed in a 2 × 6 factorial scheme, considering the effects of cultivars, biostimulant doses, and the interaction between the factors, adopting a randomized block design.

When the interaction between the factors was not significant by the F test ($p \leq 0.05$), the main effects of cultivars and doses were evaluated. For the quantitative factor (biostimulant doses), the data were subjected to polynomial regression analysis, and the models were selected based on the significance of the coefficients. In cases where there was a significant fit to the quadratic model, the maximum or minimum points were estimated by the derivative of the function. For the qualitative factor (cultivars), the means were compared using Tukey's test at a 5% probability level ($p \leq 0.05$).

3. Results

Treatments with the biostimulant Terrativa® promoted significant changes in vegetative growth, root development, and physiological performance of *T. cacao* seedlings, with dose- and genotype-dependent responses. A predominance of quadratic adjustments was observed for most morphological and physiological variables, indicating greater efficiency at intermediate doses and reduced performance at higher concentrations.

Figure 2 summarizes the morphophysiological response pattern of *T. cacao* seedlings to Terrativa® doses, showing greater vegetative vigor and root development at intermediate doses (2–3 mL L^{-1}), as well as reduced performance at higher doses (4–6 mL L^{-1}), characterizing a typical dose-dependent response behavior.

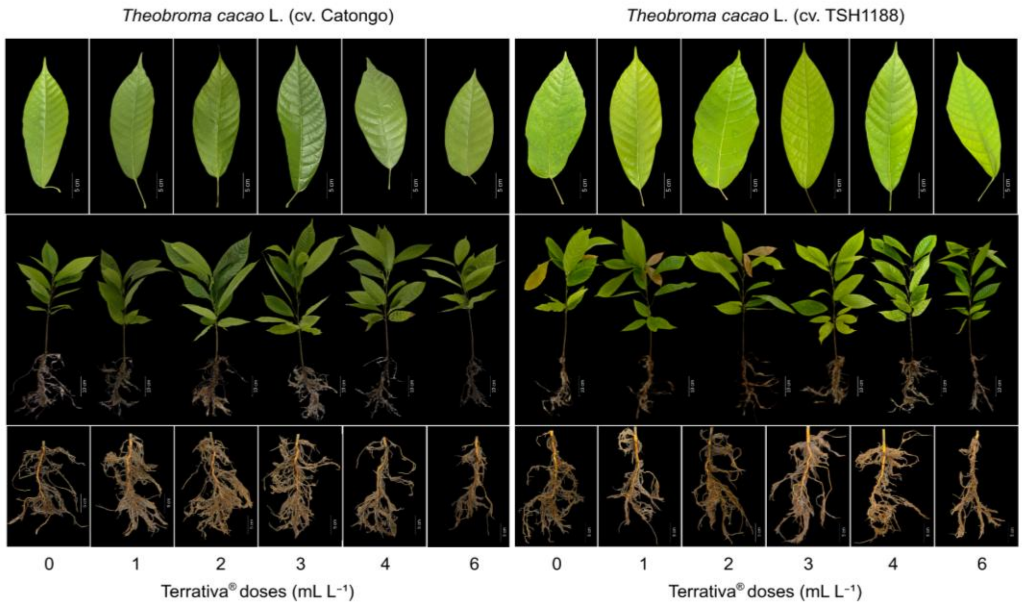

Figure 2. Leaf morphology, shoot development, and root architecture of *T. cacao* seedlings (cv. Catongo and cv. TSH 1188) subjected to different doses of the biostimulant Terrativa® (0, 1, 2, 3, 4, and 6 mL L^{-1}). A dose-dependent response is observed, with greater vegetative vigor, leaf expansion, and root branching at intermediate doses and reduced morphophysiological performance at higher doses.

For the growth and development characteristics of cacao seedlings (Table 4 and Figure 3). The number of leaves showed a quadratic behavior with a maximum point of 13.98 at a dosage of 2.54 mL L^{-1} of Terrativa®, with an R^2 of 0.9048 (Figure 3A). For leaf area, a quadratic effect was observed with a maximum point of 994.10 cm^2 at a dosage of 2.91 mL L^{-1} of Terrativa® and an R^2 of 0.8920 (Figure 3B). Stem length showed a quadratic adjustment with a maximum point of 42.79 cm at a Terrativa® dose of 3.44 mL L^{-1} and an R^2 of 0.9372 (Figure 3C). Stem diameter showed a quadratic

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behavior, with a maximum point of 7.78 mm at a dose of 2.66 mL L^{-1} of Terrativa® and an R^2 of 0.5278 (Figure 3D). Longest root showed a linear increasing effect in relation to increasing doses of Terrativa® (Figure 3E). For root volume, there was a quadratic fit with a maximum point of 9.21 at a dosage of 3.70 mL L^{-1} of Terrativa® and an R^2 of 0.6690 (Figure 3F).

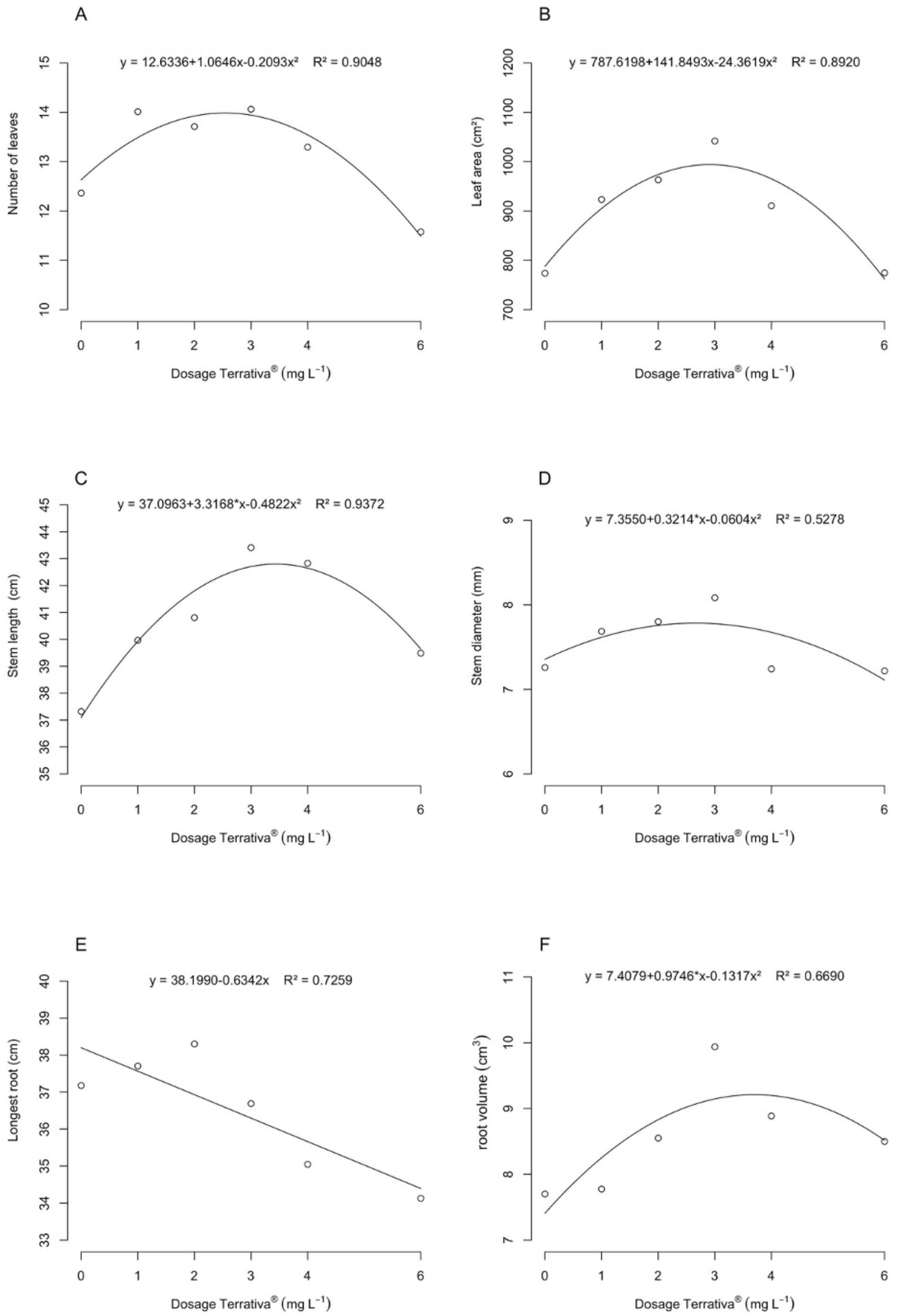

Figure 3. Effect of different dosages of Terrativa® on the number of leaves (A), leaf area (B), stem length (C), and stem diameter (D), longest root (E) and the root volume (F) in cacao seedlings of the Catango and THS1188 cultivars.

Table 4. Average values of number of leaves (NL), leaf area (LA), stem length (SL), and stem diameter (SD), longest root (LR) and root volume (RV) in cacao seedlings of the Catango and THS1188 cultivars.

Cultivars	NL	LA	SL	SD	LR	RV
Catango	12.32B	772.39B	35.34B	7.86A	34.81B	9.53A
THS1188	14.01A	1023.41A	45.92A	7.23B	38.19A	7.58B

Means followed by the same letter in columns do not differ from each other by Tukey's test at a 5% probability level.

Regarding quality characteristics (Table 5 and Figure 4), the stem length/longest root showed a significant interaction between the factors, with the Catango cultivar showing the highest average at a dosage of 3 mL L^{-1} and the TSH1188 cultivar showing the highest averages at Terrativa® dosages of 0, 1, 2, 4, and 6 mL L^{-1}. In the equation adjustments, the Catango cultivar presented the highest stem length/longest root values of 1.09 at a Terrativa® dosage of 3.73 mL L^{-1}. On the other hand, the highest stem length/longest root value of 1.33 in the TSH1188 cultivar was observed at a Terrativa® dosage of 6.13 mL L^{-1}. For stem length/stem diameter, there was no significant interaction between the factors, with the TSH1188 cultivar being statistically superior to the Catango cultivar. Regarding the regression analysis, a second-degree linear model was fitted with a maximum stem length/stem diameter value of 5.67 at a dosage of 5.06 mL L^{-1} of Terrativa® and an R^2 of 0.5252.

Table 5. Average values for stem length/longest root and stem length/stem diameter in cacao seedlings of the Catango and THS1188 cultivars.

Cultivars	Stem length/Longest root						stem length/stem diameter
	Dosage Terrativa®						
	0	1	2	3	4	6	
Catango	0.94B	0.97B	0.99B	1.15A	1.08B	1.02B	4.54B
TSH1188	1.08A	1.17A	1.16A	1.23B	1.37A	1.31A	6.41A

Means followed by the same letter in columns do not differ from each other by Tukey's test at a 5% probability level.

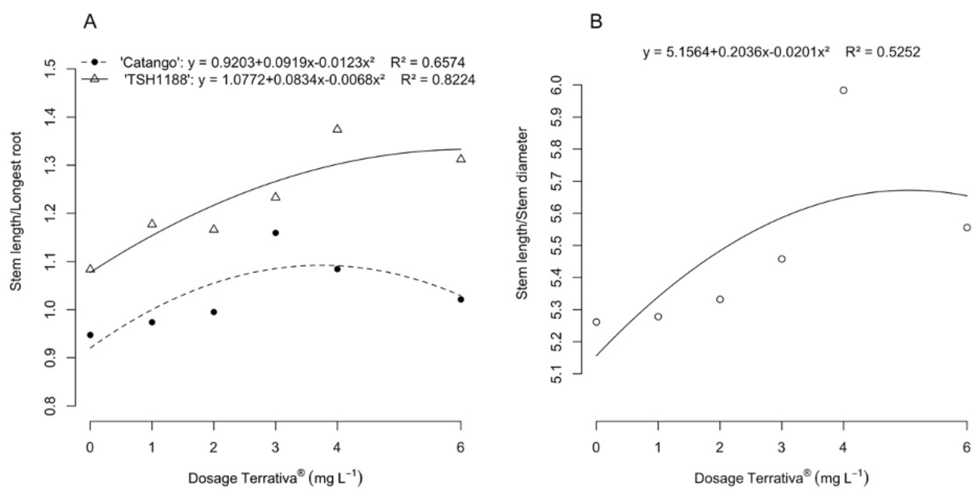

Figure 4. Effect of different dosages of Terrativa® on stem length/longest root (A) and stem length/stem diameter (B) in cacao seedlings of the Catango and THS1188 cultivars.

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For photosynthetic pigments (Table 6 and Figure 5), a significant interaction was observed between the cultivar and the dosage of the biostimulant Terrativa® for chlorophyll a, where the Catango cultivar was statistically superior at dosages of 1 and 2 mL L^{-1}. For chlorophyll b and total chlorophyll, no interaction was observed between the factors, with statistical superiority of the Catango cultivar for both characteristics. Chlorophyll a, in the Catango cultivar, showed quadratic behavior with a maximum point of 28.58 at a dosage of 2.45 mL L^{-1} of Terrativa® and an R^2 of 0.7059; however, for the TSH1188 cultivar, no statistical differences were observed between the dosages of Terrativa® applied for chlorophyll a, with an average of 26.20 in all dosages. The levels of chlorophyll b and total chlorophyll showed a quadratic effect with maximum points of 6.86 and 34.58, at Terrativa® biostimulant dosages of 3.24 and 3.08 mL L^{-1}, respectively, and R^2 values of 0.8526 and 0.9068.

Table 6. Average values for chlorophyll a, chlorophyll b, and total chlorophyll in cacao seedlings of the Catango and THS1188 cultivars.

Cultivars	chlorophyll a						chlorophyll b	Total chlorophyll
	Dosage Terrativa®							
	0	1	2	3	4	6		
Catango	26.57A	28.52A	28.71A	29.28A	26.59A	25.71A	6.82A	34.39A
THS1188	25.18A	25.16B	25.83B	27.05A	27.93A	26.02A	5.95B	32.15B

Means followed by the same letter in columns do not differ from each other by Tukey's test at a 5% probability level.

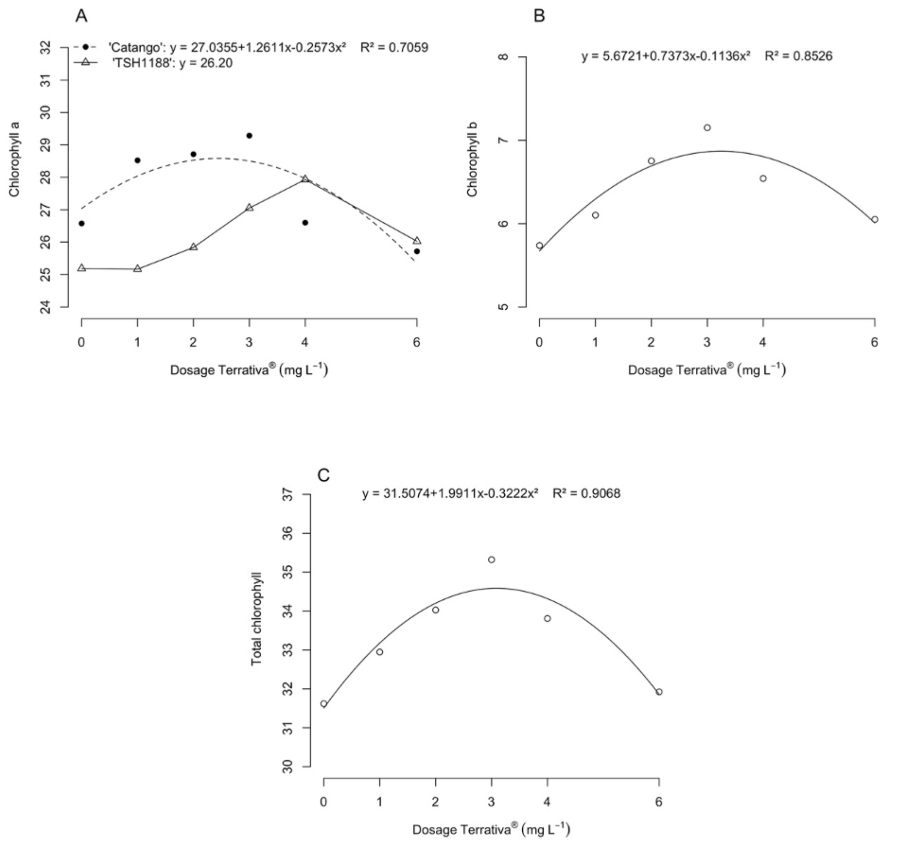

Figure 5. Effect of different dosages of Terrativa® on chlorophyll a (A), chlorophyll (B) and total chlorophyll (C) in cacao seedlings of the Catango and THS1188 cultivars.

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Regarding chlorophyll fluorescence characteristics (Table 7 and Figure 6), there was no significant interaction between the Catango and THS1188 cultivars and the dosage of the Terrativa® biostimulant applied. For Fo, Fm, and Fv, there were no differences between the cultivars. However, for the ABS/RC and TRO/RC characteristics, the THS1188 cultivar showed higher averages. On the other hand, for Pi abs, the Catango cultivar showed statistically higher averages. Regarding the regression curves of the characteristics as a function of the Terrativa® biostimulant dosage applied, a quadratic fit was observed in all cases. For Fo, a minimum point of 6536.37 was observed at a dosage of 2.85 mL L^{-1} and an R^2 of 0.3543. The minimum point of 28052.81 was found for Fv at a dosage of 3.29 mL L^{-1} with an R^2 of 0.7976. For Fm, the minimum point of 34595.88 was observed at a dosage of 3.24 mL L^{-1} of Terrativa® with an R^2 of 0.7861. Maximum points for ABS/RC and TRO/RC of 17395.01 and 14428.11 were observed at Terrativa® dosages of 4.42 and 5.39 mL L^{-1}, respectively, with R^2 of 0.5645 and 0.7039. For Pi Abs, the minimum point of 1961.13 was found at a dosage of 3.31 mL L^{-1} and an R^2 of 0.7006.

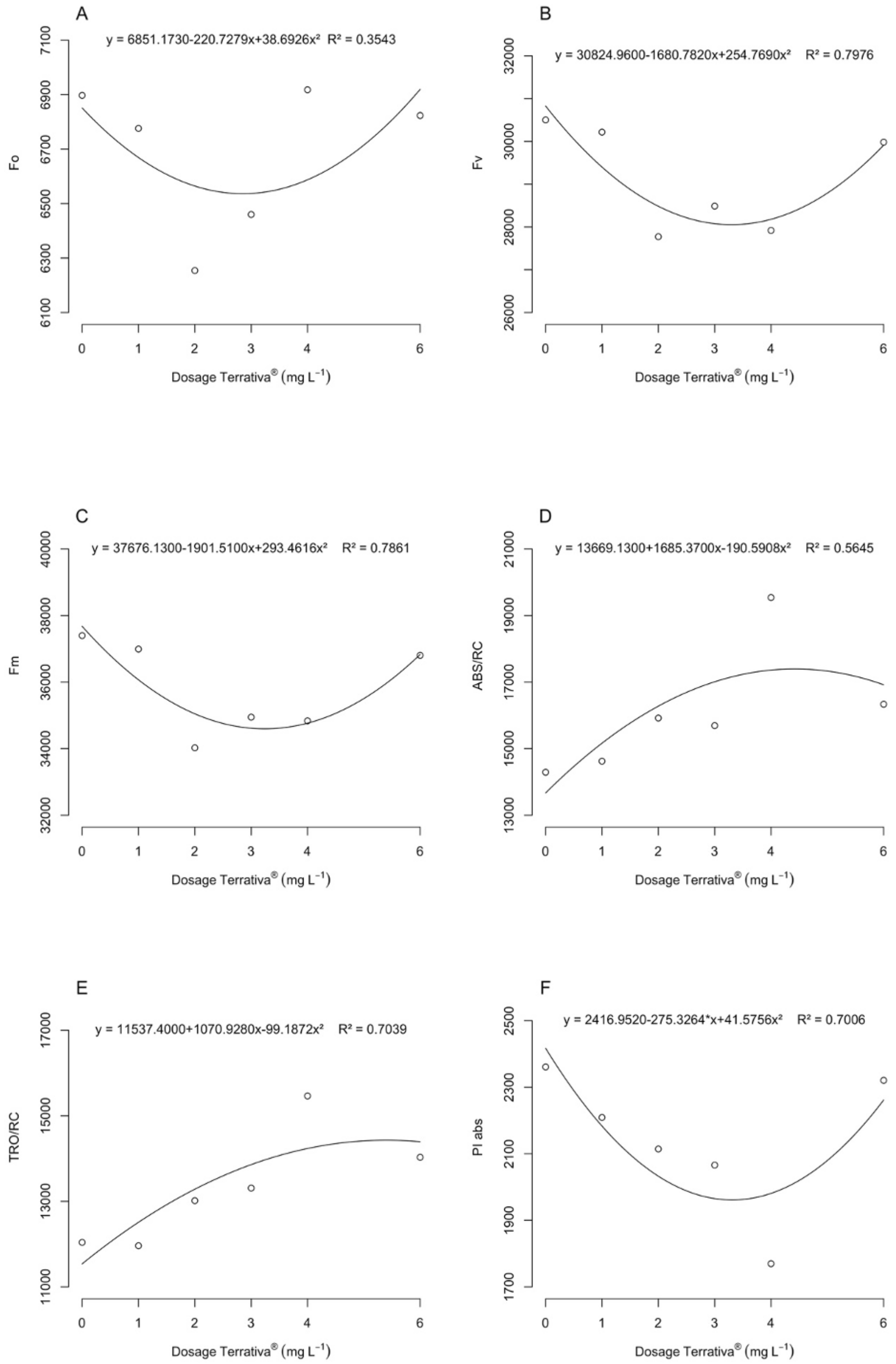

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Figure 6. Effect of different dosages of Terrativa® on initial fluorescence (Fo) (A), Variable Fluorescence (Fv) (B), maximum fluorescence (Fm) (C), energy absorbed per reaction center (ABS/RC) (D), energy transferred for electron transport (TRO/RC) (E), and absorption-based photochemical performance index (PIabs) (F) in cacao seedlings of the Catango and THS1188 cultivars.

Table 7. Average values for initial fluorescence (Fo), variable fluorescence (Fv), maximum fluorescence (Fm), energy absorbed per reaction center (ABS/RC), energy transferred for electron transport (TRO/RC), and absorption-based photochemical performance index (PIabs) in cacao seedlings of the Catango and THS1188 cultivars.

Cultivars	Fo	Fv	Fm	ABS/RC	TRO/RC	PI abs
Catango	6689.57A	29318.06A	36007.63A	15106.09B	12625.67B	1969.39B
THS1188	6686.79A	28972.61A	35659.40A	17027.82A	13978.62A	2310.772A

Means followed by the same letter in columns do not differ from each other by Tukey's test at a 5% probability level.

4. Discussion

Consistent with the proposed integrated morphophysiological–photochemical framework, the present results demonstrate that protein hydrolysate application modulates cacao seedling performance in a dose-dependent manner, characterized by coordinated adjustments between growth traits, root development, and photosystem II (PSII) functional parameters. Rather than indicating a simple growth-promoting effect, the observed responses reflect dose-dependent physiological modulation, a pattern widely described for plant biostimulants [5–8].

The improvement in vegetative growth observed at intermediate concentrations, particularly in leaf area, stem length, and root volume, indicates coordinated adjustments between shoot expansion and root system development. Protein hydrolysates are recognized not only as nutritional sources but also as metabolic signaling compounds capable of influencing nitrogen metabolism, hormonal balance, and carbon assimilation pathways [9–11]. The superior performance observed approximately between 2.5 and 3.5 mL L^{-1} may reflect a physiological condition in which amino acid availability supports biosynthetic pathways without exceeding the metabolic assimilation capacity of the plants. At higher concentrations, the reduction in some growth parameters may be associated with metabolic saturation or alterations in source–sink balance rather than direct phytotoxicity, as discussed for other protein hydrolysate-based products [9,11].

The responses of the root system reinforce this interpretation. The increase in root volume at intermediate doses suggests greater carbon allocation to belowground structures, potentially enhancing nutrient uptake efficiency and overall seedling robustness. However, since the formulation also contains phosphorus and organic carbon, the contribution of these components to the observed effects cannot be excluded. Phosphorus plays a central role in ATP synthesis and energy transfer, while organic carbon compounds may influence metabolic activity and rhizosphere interactions [10–12]. Therefore, the responses observed likely result from a combined nutritional and signaling influence rather than exclusively from amino acid action.

Chlorophyll indices exhibited a similar dose-dependent behavior, with maximum values recorded at intermediate concentrations. Increased chlorophyll content is generally associated with greater light-harvesting capacity and enhanced photosynthetic potential. Studies indicate that protein hydrolysates can stimulate chlorophyll biosynthesis and nitrogen assimilation pathways [9,12]. In the present study, the increase in chlorophyll *a*, chlorophyll *b*, and total chlorophyll suggests enhanced pigment synthesis or stability under moderate doses. However, these data should be interpreted as indicators of improved photosynthetic potential rather than direct measurements of carbon fixation rates.

Chlorophyll fluorescence parameters provided complementary information regarding the functional state of the photosynthetic apparatus. The quadratic adjustments observed for Fo, Fv, Fm,

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ABS/RC, TRO/RC, and PIabs indicate that PSII-associated performance was modulated by the applied doses. Variations in Fo may reflect changes in the proportion of active reaction centers, whereas changes in ABS/RC and TRO/RC suggest adjustments in energy absorption and electron transport efficiency. It is important to emphasize that chlorophyll fluorescence parameters represent functional indicators of PSII performance and energy distribution rather than direct measurements of biochemical activation or structural stability of the photosynthetic machinery [14,33]. Therefore, their interpretation requires caution, particularly under conditions of dose-dependent physiological modulation.

Although the dose–response curves observed resemble patterns described in hormesis models [34,35], the present study did not evaluate stress biomarkers, redox balance, or antioxidant enzyme activity that would allow confirmation of stress-induced adaptive responses. Accordingly, the results are more appropriately interpreted as dose-dependent physiological modulation rather than conclusive evidence of hormesis. The reduction in performance at higher doses may reflect metabolic imbalance, saturation effects, or alterations in energy dissipation mechanisms.

The genotypic differences observed between Catongo and TSH 1188 reinforce the importance of physiological background in modulating responses to biostimulants. Differences in chlorophyll indices and fluorescence parameters suggest distinct strategies of energy allocation and photochemical regulation between the genetic groups, consistent with the known ecophysiological variability of cacao [21,22]. These differences are particularly relevant in the context of rootstock production, where morphophysiological balance directly influences grafting success and early field establishment.

Overall, the integration of morphological traits and chlorophyll fluorescence parameters demonstrates that intermediate doses simultaneously optimize structural growth and photochemical functioning, supporting the concept of coordinated physiological adjustment under protein hydrolysate application [36]. However, the absence of molecular analyses, redox markers, or enzymatic activity measurements limits the mechanistic resolution of the interpretations. Future studies incorporating antioxidant responses, metabolic profiling, or transcriptomic approaches may further elucidate the biochemical pathways underlying these dose-dependent responses [8,34].

From an applied perspective, the results indicate that appropriate dose definition is critical for optimizing morphophysiological performance in nursery seedlings, avoiding suboptimal responses or inhibitory effects associated with excessive concentrations.

5. Conclusions

Within the proposed integrated morphophysiological–photochemical framework, the present study demonstrates that a protein hydrolysate-based organomineral biostimulant promotes dose- and genotype-dependent physiological modulation in *Theobroma cacao* seedlings, characterized by coordinated adjustments between morphological growth, root development, and photosystem II (PSII) functional performance. Intermediate dose ranges, corresponding to the highest morphophysiological efficiency observed under the experimental conditions, were associated with simultaneous optimization of vegetative growth and PSII functional performance, reinforcing the interpretation of dose-dependent physiological modulation rather than a simple growth stimulation effect.

The increases observed in chlorophyll indices and chlorophyll fluorescence parameters indicate improved photosynthetic potential and functional balance of PSII under moderate concentrations, whereas higher doses were associated with reduced performance, likely reflecting metabolic saturation or functional imbalance.

These findings contribute to a more physiologically grounded understanding of dose-dependent biostimulant action in cacao seedlings during the nursery phase. While the most responsive dose intervals were identified within the experimental framework, their interpretation should consider genotype-specific behavior and environmental conditions.

Future research should incorporate molecular, redox, and enzymatic analyses, as well as evaluate grafting performance and field establishment, to further elucidate the physiological mechanisms underlying dose-dependent responses to protein hydrolysate-based biostimulants.

Author Contributions: Conceptualization, F.G.H., C.T.F.B., A.J.C.J.-M. and J.S.B.P.; methodology, F.G.H, C.T.F.B., A.J.C.J.-M. and J.S.B.P.; software, V.d.S.O., L.d.O.A. and S.D.-A.; validation, V.d.S.O., L.d.O.A. and S.D.-A; formal analysis, V.d.S.O., L.d.O.A. and S.D.-A; investigation, F.G.H., F.M., D.C.d.A., M.E.L.d.S. and D.B.F.; resources, F.G.H., F.M., D.C.d.A., M.E.L.d.S. and D.B.F.; data curation, F.G.H., F.M., D.C.d.A., M.E.L.d.S. and D.B.F.; writing—original draft preparation, F.G.H., V.d.S.O. and S.D.-A; writing—review and editing, F.G.H., V.d.S.O. and S.D.-A; visualization, V.d.S.O. and S.D.-A; supervision, V.d.S.O. and S.D.-A; project administration, S.D.-A and E.N.d.S.; funding acquisition, S.D.-A, K.T.K., and E.N.d.S.; All authors have read and agreed to the published version of the manuscript.

Funding: Please add: This research was funded by Secretaria da Agricultura, Abastecimento, Aqui- cultura e Pesca (SEAG) and Fundação de Amparo a Pesquisa e Inovação do Espirito Santo - FAPES, grant number DI 004/2022 SEAG/FAPES - Banco de projetos Fase III.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Acknowledgments: The authors thank Terrativa® for supplying the biostimulant used in this study. The company had no role in the experimental design, data collection, analysis, interpretation of data, or manuscript preparation. The authors also thank the Cacau Aliança Nursery for providing the experimental area and technical support; the Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural (INCAPER) for laboratory infrastructure and technical assistance; and Fundação de Amparo à Pesquisa e Inovação do Espírito Santo (FAPES) for institutional support.

Conflicts of Interest: The authors declare no conflict of interest. Terrativa® supplied the biostimulant but had no involvement in the study design, data analysis, or publication decisions.

Abbreviations

The following abbreviations are used in this manuscript:

NL	number of leaves
LA	leaf area
SL	stem length
SD	stem diameter
LR	longest root
RV	root volume
Fo	initial fluorescence
Fv	variable fluorescence
Fm	maximum fluorescence
ABS/RC	energy absorbed per reaction center
TRO/RC	energy transferred for electron transport
PI abs	absorption-based photochemical performance index

References

1. Schmeling, T.I.; Velho, J.P.; Pinto, N.G.M.; Zecca, A.G.D. Cacau: Uma cadeia produtiva em busca da sustentabilidade. *Revista Vale* **2025**, *23*, 3. http://dx.doi.org/10.5892/ruvrd.v23i3.6896

2. Bustamante-González, C.A.; Ferras, Y.; Morán-Rodríguez, N.; Pérez-Almaguer, A.F.; Selva-Hernández, F.F.; Clappe-Borges, P. Effect of Enerplant® doses on the development and nutrient use by cacao (*Theobroma cacao* L.) seedlings. *Agro Productividad* **2022**, *15*, e2130. https://doi.org/10.32854/agrop.v15i4.2130
3. Freitas, J. L. de, Jardim, I. N.; Guedes, M. N. S. Desenvolvimento de mudas de cacau em função da concentração de biostimulante d'raz®. *Diversitas Journal* 2024, 9 (2), 660–671. https://doi.org/10.48017/dj.v9i2.2899
4. Rodriguez-Arrobo, T., Cajamarca-Crespo, K., Barrezueta-Unda, S., Luna-Romero, A., & Villaseñor-Ortiz, D. Efectos de bioestimulantes en el crecimiento morfológico de plántulas de cacao en etapa de vivero. *Manglar* **2023**, 20 (2), 117-122. http://doi.org/10.57188/manglar.2023.013
5. Calvo, P.; Nelson, L.; Kloepper, J.W. Agricultural uses of plant biostimulants. *Plant Soil* **2014**, *383*, 3–41. https://doi.org/10.1007/s11104-014-2131-8
6. Du Jardin, P. Plant biostimulants: Definition, concept, main categories and regulation. *Sci. Hortic.* **2015**, *196*, 3–14. https://doi.org/10.1016/j.scienta.2015.09.021
7. Yakhin, O.I.; Lubyanov, A.A.; Yakhin, I.A.; Brown, P.H. Biostimulants in plant science: A global perspective. *Front. Plant Sci.* **2017**, *7*, 2049. https://doi.org/10.3389/fpls.2016.02049
8. Rouphael, Y.; Colla, G.Editorial: Biostimulants in Agriculture. *Front. Plant Sci.* 2020, 11, 40. https://doi.org/10.3389/fpls.2020.00040
9. Colla, G.; Nardi, S.; Cardarelli, M.; Ertani, A.; Lucini, L.; Canaguier, R.; Rouphael, Y. Protein hydrolysates as biostimulants in horticulture. *Sci. Hortic.* **2015**, *196*, 28–38. https://doi.org/10.1016/j.scienta.2015.08.037
10. Ertani, A.; Pizzeghello, D.; Francioso, O.; Tinti, A.; Nardi, S. Spectroscopic-Chemical Fingerprint and Biostimulant Activity of a Protein Hydrolysate-Based Product in Maize Plants. *Molecules* **2018**, *23*, 1031. https://doi.org/10.3390/molecules23051031
11. Halpern, M.; Bar-Tal, A.; Ofek, M.; Minz, D.; Muller, T.; Yermiyahu, U. The use of biostimulants for enhancing nutrient uptake. *Adv. Agron.* **2015**, *130*, 141–174. https://doi.org/10.1016/bs.agron.2014.10.001
12. Lucini, L.; Rouphael, Y.; Cardarelli, M.; Canaguier, R.; Kumar, P.; Colla, G. The effect of a plant-derived protein hydrolysate on metabolic profiling and crop performance of lettuce grown under saline conditions. *Sci. Hortic.* **2015**, *182*, 124–133. https://doi.org/10.1016/j.scienta.2014.11.022
13. Sun, W., Shahrajabian, M. H., Kuang, Y., & Wang, N. Amino acids biostimulants and protein hydrolysates in agricultural sciences. Plants 2024, 13, 2, 210. https://doi.org/10.3390/plants13020210
14. Murchie, E.H.; Lawson, T. Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. *J. Exp. Bot.* **2013**, 64, 3983–3998. https://doi.org/10.1093/jxb/ert208
15. Valleser, V.C.; Protacio, C.M. Foliar sprays of biostimulants and PGRs improve the reproductive parameters of cacao under water stress. *Pertanika J. Trop. Agric. Sci.* **2025**, *48*, 17. https://doi.org/10.47836/pjtas.48.5.17
16. National Aeronautics and Space Administration (NASA); United States Geological Survey (USGS). Shuttle Radar Topography Mission (SRTM) 1 Arc-Second Global Dataset; USGS EROS Center: Sioux Falls, SD, USA, 2015. https://doi.org/10.5066/F7PR7TFT
17. Instituto Brasileiro de Geografia e Estatística (IBGE). *Modelo Digital de Elevação (MDE) do Brasil*; IBGE: Rio de Janeiro, Brazil, 2021. Available online: https://www.ibge.gov.br/geociencias/modelos-digitais-de-superficie/modelos-digitais-de-superficie/10856-mde-modelo-digital-de-elevacao.html (accessed on 15 January 2026).
18. Franco, L.B.; Almeida, C.D.G.C.; Freire, M.M.; Franco, G.B.; Silva, S.A. Rainfall zoning for cocoa growing in Bahia state (Brazil) using fuzzy logic. *Engenharia Agrícola* **2019**, *39*, 48-55. https://doi.org/10.1590/1809-4430-Eng.Agric.v39nep48-55/2019
19. Alvares, C.A.; Stape, J.L.; Sentelhas, P.C.; de Moraes Gonçalves, J.L.; Sparovek, G. Köppen's climate classification map for Brazil. *Meteorol. Z.* **2013**, *22*, 711–728. https://doi.org/10.1127/0941-2948/2013/0507
20. Instituto Nacional de Meteorologia (INMET). *Estação Meteorológica Automática de Linhares (Código A614)*. Available online: https://tempo.inmet.gov.br/GraficosDiarios/A614 (accessed on 15 January 2026).
21. Instituto Nacional de Meteorologia (INMET). Normais Climatológicas do Brasil (1991–2020); Ministério da Agricultura: Brasília, Brazil, 2020. Available online:

 doi:10.20944/preprints202603.1743.v1

https://portal.inmet.gov.br/uploads/normais/NORMAISCLIMATOLOGICAS.pdf (accessed on 15 January 2026).

22. Motamayor, J.C.; Lachenaud, P.; da Silva e Mota, J.W.; Loor, R.; Kuhn, D.N.; Brown, J.S.; Schnell, R.J. Geographic and genetic population differentiation of the Amazonian chocolate tree (*Theobroma cacao* L.). *PLoS ONE* **2008**, *3*, e3311. https://doi.org/10.1371/journal.pone.0003311
23. Almeida, A.A.F.; Valle, R.R. Ecophysiology of the cacao tree. *Brazilian Journal of Plant Physiology* **2007**, *19*, 425–448. https://doi.org/10.1590/S1677-04202007000400011
24. Terrativa®. *Terrativa® biostimulant: product technical information*. Brazil, **2025.** Available online: https://useterrativa.com.br/ (accessed on 15 January 2026).
25. Instituto Campineiro de Análise de Solo e Adubo (ICASA). Laboratory Analytical Report: Organic Fertilizer and Foliar Biofertilizer Analysis (Amino Acid Profile); ICASA: Campinas, Brazil, **2025**.
26. LI-COR Biosciences. LI-3100C Area Meter Instruction Manual; LI-COR Biosciences: Lincoln, NE, USA, **2013**.
27. Nunes, E.N.; et al. Crescimento de cultivares de mamona em diferentes níveis de água no solo. *Sci. Plena* **2013**, *9*, 104901.
28. Grossnickle, S.C.; MacDonald, J.E. Seedling quality: History, application, and plant attributes. *Forests* **2018**, *9*, 283. https://doi.org/10.3390/f9050283
29. Falker. ClorofiLOG CFL1030: Medidor Eletrônico de Teor de Clorofila; Falker Automação Agrícola: Porto Alegre, Brazil, **2009**.
30. Hansatech Instruments Ltd. Pocket PEA: Portable Chlorophyll Fluorimeter—Technical Manual; Hansatech Instruments: Norfolk, UK, **2018.**
31. Strasser, R.J.; Tsimilli-Michael, M.; Srivastava, A. Analysis of the chlorophyll *a* fluorescence transient. **In *Chlorophyll a*** *Fluorescence: A Signature of Photosynthesis***;** Papageorgiou, G.C., Govindjee, Eds.; Springer: Dordrecht, The Netherlands, **2004**; pp. 321–362. https://doi.org/10.1007/978-1-4020-3218-9_12
32. R Core Team. R: A Language and Environment for Statistical Computing; R Foundation for Statistical Computing: Vienna, Austria, **2023**. Available online: https://www.R-project.org/ (accessed on 15 January 2026).
33. Kalaji, H.M.; Schansker, G.; Brestic, M.; Bussotti, F.; Calatayud, A.; Ferroni, L.; Goltsev, V.; Guidi, L.; Jajoo, A.; Li, P.; et al. Frequently asked questions about chlorophyll fluorescence, the sequel. Photosynthesis Research **2017**, 132, 13–66. https://doi.org/10.1007/s11120-016-0318-y
34. Calabrese, E.J. Hormesis: A fundamental concept in biology. *Microb. Cell* **2014**, *1*, 145–149. https://doi.org/10.15698/mic2014.05.145
35. Agathokleous, E.; Kitao, M.; Calabrese, E.J. Hormesis: A compelling platform for sophisticated plant science. *Trends Plant Sci.* **2019**, *24*, 318–327. https://doi.org/10.1016/j.tplants.2019.01.004
36. Colla, G.; Hoagland, L.; Ruzzi, M.; Cardarelli, M.; Bonini, P.; Canaguier, R.; Rouphael, Y. Biostimulant Action of Protein Hydrolysates: Unraveling Their Effects on Plant Physiology and Microbiome. *Front. Plant Sci.* **2017**, *8*, 2202. https://doi.org/10.3389/fpls.2017.02202