CHITOSAN IMPROVES MORPHO-PHYSIOLOGICAL, ROOTING ATTRIBUTES AND PROFITABILITY OF TWO COCOA (*Theobroma cacao* L.) VARIETIES DURING VEGETATIVE PROPAGATION †

[QUITOSANO MEJORA LOS ATRIBUTOS MORFO-FISIOLÓGICOS, DE ENRAIZAMIENTO Y LA RENTABILIDAD DE DOS VARIEDADES DE CACAO (*Theobroma cacao* L.) DURANTE LA PROPAGACIÓN VEGETATIVA]

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SUMMARY

Background: The sexual propagation of Cocoa (*Theobroma cacao* L.) has some limitations to preserve some desirable agronomic characteristics in successive generations. **Objective:** To evaluate the effect of a chitosan based-formulation (Quitomax®) on morpho-physiological, rooting attributes and benefit-cost ratio of two cocoa varieties during vegetative propagation. **Methodology:** The experimental design was completely randomized with a factorial arrangement (A × B), where A represented the two clones and B the three concentrations of chitosan based formulation used (0, 100, 500 and 1000 mg L^{-1}), with three repetitions. per treatment. The survival (%), the stem diameter (mm), the number of leaves, the number and length (cm) of roots, the biomass (g), the gas exchange (A, gs, Ci, E), and an economic analysis of the two cocoa plant varieties were evaluated at 120 days after starting the trial. **Results:** The clone CCN-51 treated with 500 mg L^{-1} had significantly the highest results on survival (80%), stem diameter (6.83 mm), number of leaves per plant (8.2), number and length of roots (6.21 and 35.74 cm), aerial and root biomass (4.07 g and 1.64 g) parameters. In gas exchange, the highest values of Water use efficiency (WUE) were observed at 500 mg L^{-1} in CCN-51 (5.36 mmol mol^{-1}) and 1000 mg L^{-1} in INIAP-EETP-801 (7.62 mmol μmol^{-1}). In both clones, higher profitability was obtained when applying the chitosan dose of 500 mg L^{-1}, reaching profitability of 40.65 and 50.00% for clones INIAP-EETP-801 and CCN-51, respectively. **Implications:** The cocoa clone CCN-51 showed plants that exhibited greater development of both the aerial part and the root part of the cocoa seedlings than INIP-EETP-801 coca clones. **Conclusion:** The chitosan based formulation at 500 mg L^{-1} is a promissory alternative to improve the evaluated parameters after 120 days of cocoa vegetative propagation.

Keywords: asexually propagation; biostimulants; Quitomax®; Cocoa; stem cuttings.

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RESUMEN

Antecedentes: La propagación sexual del Cacao (*Theobroma cacao* L.) tiene algunas limitaciones para conservar algunas características agronómicas deseables en generaciones sucesivas. **Objetivo:** Evaluar el efecto de una formulación a base de quitosano (Quitomax®) sobre los atributos morfo-fisiológicos, de enraizamiento y relación costo-beneficio de dos variedades de cacao durante la propagación vegetativa. **Metodología:** El diseño experimental fue completamente al azar con arreglo factorial (A × B), donde A representó los dos clones y B las tres concentraciones de formulación a base de quitosano utilizadas (0, 100, 500 y 1000 mg L^{-1}), con tres repeticiones por tratamiento. Se evaluaron la supervivencia (%), el diámetro del tallo (mm), el número de hojas, el número y longitud (cm) de raíces, la biomasa (g), el intercambio gaseoso (A, gs, Ci, E), y un análisis económico en las dos variedades vegetales de cacao a los 120 días de iniciado el ensayo. **Resultados:** El clon CCN-51 tratado con 500 mg L^{-1} tuvo resultados significativamente más altos en supervivencia (80%), diámetro de tallo (6.83 mm), número de hojas por planta (8.2), número y longitud de raíces (6.21 y 35.74 cm), parámetros de biomasa aérea y radicular (4,07 g y 1,64 g). En intercambio gaseoso, los mayores valores de eficiencia en el uso del agua (EUA) se observaron a 500 mg L^{-1} en CCN-51 (5.36 mmol mol^{-1}) y 1000 mg L^{-1} en INIAP-EETP-801 (7.62 mmol µmol^{-1}). En ambos clones se obtuvo mayor rentabilidad al aplicar la dosis de quitosano de 500 mg L^{-1}, alcanzando una rentabilidad de 40.65 y 50.00% para los clones INIAP-EETP-801 y CCN-51, respectivamente. **Implicaciones:** El clon de cacao CCN-51 mostró plantas que exhibieron mayor desarrollo tanto de la parte aérea como de la parte de la raíz de las plántulas que los clones de cacao INIP-EETP-801. **Conclusión:** La formulación a base de quitosano a 500 mg L-1 es una alternativa promisoria para mejorar los parámetros evaluados luego de 120 días de propagación vegetativa del cacao.

Palabras clave: propagación asexual; bioestimulantes; Quitomax®; Cacao; esquejes de tallo.

INTRODUCTION

Cocoa (*Theobroma cacao* L.) is an important worldwide crop, and it grows in tropical areas from Africa, Asia and America (Nair, 2021). Cocoa is cultivated by approximately 6 million farmers in countries such as Brazil, Cameroon, Colombia, Dominican Republic, Ecuador, Ghana, Indonesia, and Peru with a world production in 2020-2021 of 5.2 million tons (Wainaina *et al.,* 2021). In Ecuador, its production is located in 23 of the 24 provinces and is produced as a monoculture or associated with other species (León-Bravo and Jaramillo-Villacrés, 2021). Cacao beans are the fundamental ingredient for chocolate food products, and additionally, as a formulation ingredient for pharmaceutical and cosmetic industries (Predan *et al.,* 2019).

Cacao plants can be sexually (seed) or asexually propagated (grafting, cuttings, air layering, and tissue culture) (Garcia *et al.,* 2018). Nonetheless, sexual propagation by botanical seeds has some limitations due to cross-pollination or allogamy which difficult to preserve some desirable agronomic characteristics in successive generations (Hernandez and Granados, 2021). Thus, asexual vegetative propagation is a better alternative to obtain identical and desirable characteristics from their predecessors such as resistance to pests and diseases and high yield (Vásquez-Zamora *et al.,* 2022). This propagation is done through meristematic tissue that contains undifferentiated cells that can give rise to the organs required to form a new plant (Garate-Navarro *et al.,* 2017). Many countries in South America such as Brazil, Colombia, Costa Rica, and Ecuador have cultivated cocoa by air layering and grafting propagation techniques, however, these are time-consuming and can lead to tissue incompatibility (Laliberté and End, 2015). As consecuence, the search for alternative methodologies that guarantee the successful propagation of selected cocoa plants is still ongoing (Essola-Essola *et al.,* 2017). Stem cuttings are a promissory propagation technique because of their quick an efficient performance (Reyes-Pérez *et al.,* 2021). The propagation of cocoa clones by stem cuttings depends on the development of adventitious roots from the pericycle region at the stem base just above the cut and treating them with phytoregulators to promote root initiation (Junior *et al.,* 2017).

Biostimulants are natural preparations that increase the efficiency of nutrient utilization and tolerance to abiotic stress and improve the quality of crops (Drobek *et al.,* 2019). Chitosan is a biopolymer with confirmed bio-stimulatory activity in several crops (Shahrajabian *et al.,* 2021). Chitosan is chemically structured by multiple sub-units of D-glucosamine and N-acetyl-D-glucosamine with 1,4-glycosidic bonds (Kou *et al.,* 2021). Since it is classified as GRAS (generally recognized as safe), easy plant absorbed, and inexpensive has gained great relevance to agronomic purpose (Hidangmayum *et al.,* 2019). The initial application of chitosan in agriculture is related to its elicitor effect by stimulating the biosynthesis of molecules against phytopathogens (Chouhan and Mandal, 2021). It increased photosynthetic activity, tolerance to abiotic stressors (high temperatures, salinity, and drought) and the overexpression of defensive genes and antioxidant enzymes (Jogaiah *et al.,* 2020). Chitosan also is a plant growth and root length promoter by increasing nutrient uptake and serving as secondary carbon source (Chouhan and Mandal, 2021).

There are many proved applications of chitosan effectiveness in agriculture; however, it is important to remark that the obtained results are not similar because different factors such as methodological approaches and chitosan chemical composition. For all the mentioned above, the main objective of this research was to evaluate the effect of a chitosan based formulation on morpho-physiological and rooting attributes of CCN-51 and INIAP-EET-801 cocoa varieties during vegetative propagation. Also, the economic feasibility of the chitosan-based formulation treatments were evaluated.

MATERIALS AND METHODS

Experimental site and general conditions

This research was established at the Faculty of Agricultural Sciences of the State Technical University of Quevedo, located between the geographic coordinates of 01º 06' S and 79º 27' W, at an altitude of 73 meters. The soil has an irregular topography with little slope, loamy-clayey texture, with a pH of 5.5-7.5 and regular drainage (Reyes-Pérez *et al*., 2021). The ecological zone where the experiment was established is classified as tropical humid forest (INAMHI, 2021). The average temperature during the period of the experiment was 24.8 °C; average relative humidity of 84%, total precipitation of 1587.5 mm/year and a total heliophany of 894.4 hours/light/year (INAMHI, 2021).

Material collection and preparation of cuttings

Cocoa cuttings of 35 days old were used, from clones CCN-51 and INIAP-EETP-801 from the germplasm bank of the National Institute of Agricultural Research (INIAP). Shoots were collected in the morning (before 8 am) from the midle area of cacao tree crowns (Vázquez-Zamora *et al.,* 2022). Prior to harvesting cuttings, hands and tools (pruning shears and razor) were disinfected with a 1% sodium hypochlorite aqueous solution. The cuttings were obtained from shoots originating in the 4th or 5th lateral stem, cutting 15 ± 2 cm from it. To obtain it, a cut was made in the stem just below each node, in addition to making a cross-shaped cut at the base of each cutting. All collected shoots had active axillary buds and were transported to the propagation greenhouse in an icebox, to minimize the physiological stress of the tissues (Vázquez-Zamora *et al.,* 2022).. Before the application of the chitosan based formulation, cuttings were immersed in a 1% NaClO solution for 1 minute. Immediately, after rinsing with distilled water, they were placed on a sterile tray to allow the evaporation of excess water (Vázquez-Zamora *et al.,* 2022)..

Chitosan based formulation

As a source of chitosan, the biostimulant Quitomax® (RFC 010/17, central registry of fertilizers of Cuba) was used, which is a mixture of chitosan biopolymers and chemical salts with a broad spectrum of application in crops, obtained by the group of products bioactive products from the National Institute of Agricultural Sciences of Cuba (INCA) in 2017. The chitosan in this product has a molar mass of 100 kDa and an acetylation degree of 13.7%.

Treatments and experimental design

The experimental design was completely randomized with a factorial arrangement (A × B), where A represented the two clones (CCN-51 and INIAP-EETP-801) and B the three concentrations of chitosan based formulation used (0, 100, 500 and 1000 mg L-1), with three repetitions. per treatment. The 0 treatment was used as control. Ten cuttings per replicate were used for a total of 240 cuttings in the experiment. The plants were treated by immersing the base of the cuttings for 30 minutes in Quitomax® concentrations, and subsequently transferred to 1 kg polyethylene bags with 25% sterile sand and 75% Sogemix commercial substrate (75% Sphagnum spp., 25% vermiculite and limestone) (Vázquez-Zamora *et al.,* 2022). The plants were stored in a grow house and covered with plastic at 80% relative humidity and temperatures of 26-34 °C during the day throughout the rooting phase. Daily irrigations were carried out up to field capacity, thus avoiding water stress in the plants.

The survival (%), the stem diameter (mm), the number of leaves, the number and length (cm) of roots, the biomass (gr), the gas exchange parameters (A = net photosynthetic rate; E = transpiration rate; gs = stomatal conductance; Ci= intercellular concentration of CO_2; WUE = water use efficiency), and economic analysis (Benefit-cost ratio) of the two cocoa plant varieties were evaluated at 120 days after starting the trial. The stem diameter and root length were measured using a digital caliper. The number of leaves and roots per plant was determined by simple counting performing. The biomass of the total plant was determined with an analytical balance. Once the fresh weight of the plants was obtained, each type of biomass was placed in paper bags and taken to a drying oven at a temperature of 80 °C for 72 hours until completely dehydrated. Subsequently, it was weighed on the analytical balance (USS-DBS83, USA) and the weight was expressed in grams of dry plant matter. The

Gas exchange parameters were measured in six individuals in clones of CCN-51 and INIAP-EETP-801, subjected to different concentrations of chitosan (n = 6); by use of a portable infrared gas analyzer (CIRAS-II, PP Systems Inc., Amesbury, MA). All measurements were made on fully expanded and healthy adult leaves (third leaf from the apex), under the following conditions: CO2 concentration of 400 ± 10 μmol mol-1, leaf temperature of 28.0 ± 1 °C, a ΔW of 2.4 ± 0.5 KPa and a photosynthetic photon flux density (PFD) of 1000 ± 50 μmol m-2 s^{-1} (light was provided by an LED-based light unit from the same manufacturer). Instantaneous water use efficiency (WUE) was estimated as:

$$WUE = \frac{A}{E} \quad (1)$$

Where:
A: Net photosynthesis rate
E: Water use efficiency of two cocoa clones subjected to different concentrations of chitosan.

Measurements were made randomly between 0800 and 1200 h in all plants. Also, an Economic analysis (USD) was performed considering the costs of each of the treatments under study, and the income generated by the sale of 1000 seedlings, to then find the benefit/cost ratio using the formula:

$$\frac{B}{C} = \frac{I.B}{C.T.P} \quad (2)$$

Where:
B/C: Benefit-cost ratio
I.B.: Gross Income
C.T.P.: Total cost of production

Statistical analysis

The data were subjected to the Kolmogorov-Smirnov normality test (Massey, 1951) and the homogeneity of variances according to the Bartlett criterion (Bartlett, 1937). Analysis of variance and multiple comparison of means were performed using Tukey's test with a significance level of 5% (α = 0.05). Statistical data were processed using the statistical program Statistica v.12.0 (Statsoft).

RESULTS

Survival percentage

The survival of seedlings of the two cocoa clones in response to the application of different doses of chitosan is presented in Table 1. The analysis of variance determined that the varieties and doses of chitosan reached high statistical significance, while they did not obtain statistical significance for the interactions.

Among the cocoa clones, CCN-51 registered the highest percentage of survival with 61.67%, statistically superior to the INIAP-EETP-801 clone, which presented 55.83% of live plants. When applied in doses of 500 mg L^{-1}, Chitosan obtained a higher percentage of seedling survival with 76.67%, showing significant differences with respect to the other doses that registered values between 38.33 and 65.00%. The comparison of the interactions showed that when the clone CCN-51 is combined with the dose of 500 mg L^{-1}, a higher percentage of survival is obtained with 80.00%, without statistically differing from the interactions INIAP-EETP-801 + 500 mg L^{-1} and INIAP-EETP-801 + 1000 mg L^{-1}, which presented values of 73.33 and 66.67%, respectively. The mentioned interactions statistically surpassed the others that showed percentages of seedling survival between 33.33 and 63.33%, being the interaction INIAP-EETP-801 + 0 mg L^{-1}, the one that presented the lowest survival.

Stem diameter

In Table 1, the diameter of the stem of cocoa seedlings from the planting of cuttings is presented. In the following evaluations at120 days, it was observed that statistical significance was recorded at 99.00%. At 120 days of age of the seedlings, the clones reached significant differences, the highest value corresponding to clone CCN-51 (6.41 mm). The dose of chitosan, 500 mg L^{-1}, showed a larger diameter with 6.62 mm, without differing from the dose of 1000 mg L^{-1} (6.46 mm), statistically superior to the two remaining doses that showed values below 6.11 mm. In the case of interactions, CCN-51 + 500 mg L^{-1} presented a higher average with 6.83 mm, without differing from the interactions CCN-51 + 1000 mg L^{-1} (6.60 mm) and INIAP-EETP-801 (6.41 mm), statistically superior to the other interactions that presented values between 5.28 and 6.32 mm, respectively.

Number of leaves

The averages of the number of leaves per plant at 120 days are presented in Table 1. The clone CCN-51 had a higher number of leaves per plant (7.32), without statistically differing from clone INIAP-EET-801 (6.94). The comparison between the doses of chitosan; reflected that the dose of 500 mg L^{-1} registered the highest number of leaves per plant (8.11), and statistically surpassed the other doses that presented values between 6.02 and 7.72 leaves per seedling. For the interactions, CCN-51 + 500 mg L^{-1} registered a

Table 1. Parameters evaluated in cocoa seedlings in response to the application of chitosan based formulation in the vegetative propagation of clones CCN-51 and EETP-801.

Treatments	Survival (%)	Stem diameter (cm)	Leaves per seedling	Roots per seedling	Root lenght (cm)	Aerial biomass (g)	Root biomass (g)
Clones							
C_1	55.83 ± 1.31 b*	5.98 ± 0.08 b	6.94 ± 0.16 b	4.97 ± 0.52 a	29.19 ± 3.51 b	3.13 ± 0.14 b	1.24 ± 0.08 a
C_2	61.67 ± 1.50 a	6.41 ± 0.09 a	7.32 ± 0.21 a	5.25 ± 0.93 b	31.16 ± 2.12 a	3.47 ± 0.10 a	1.40 ± 0.11 b
Dose							
D_0	38.33 ± 2.87 d	5.60 ± 0.11 c	6.02 ± 0.32 d	4.19 ± 1.32 d	24.82 ± 1.95 d	2.59 ± 0.12 d	1.11 ± 0.10 c
D_1	55.00 ± 3.25 c	6.11 ± 0.07 b	6.68 ± 1.20 c	4.66 ± 0.84 c	25.66 ± 3.54 c	2.98 ± 0.13 c	1.13 ± 0.13 c
D_2	76.67 ± 4.84 a	6.62 ± 0.12 a	8.11 ± 1.50 a	6.08 ± 1.22 a	35.74 ± 2.23 a	4.00 ± 0.21 a	1.57 ± 0.09 a
D_3	65.00 ± 2.92 b	6.46 ± 0.13 a	7.72 ± 0.90 b	5.51 ± 1.53 bd	34.49 ± 2.97 bd	3.65 ± 0.16 b	1.47 ± 0.08 b
Interactions							
C_1D_0	33.33 ± 3.01 e	5.28 ± 0.19 d	6.00 ± 1.7 c	4.00 ± 1.98 c	24.23 ± 3.23 c	2.41 ± 0.23 d	1.03 ± 0.12 d
C_1D_1	53.33 ± 2.43 cd	5.93 ± 0.16 c	6.13 ± 2.3 c	4.50 ± 0.93 c	25.41 ± 2.53 c	2.57 ± 0.08 d	1.03 ± 0.11 d
C_1D_2	73.33 ± 4.32 ab	6.40 ± 0.09 abc	8.01 ± 0.42 a	5.95 ± 1.13 b	35.86 ± 3.45 b	3.92 ± 0.16 ab	1.50 ± 0.09 ab
C_1D_3	63.33 ± 1.27 abc	6.32 ± 0.12 bc	7.63 ± 0.58 ab	5.42 ± 1.57 bc	33.12 ± 1.98 bc	3.63 ± 0.11 bc	1.41 ± 0.12 b
C_2D_0	43.33 ± 1.92 de	5.92 ± 0.08 c	6.03 ± 2.60 c	4.38 ± 0.99 c	25.25 ± 2.54 c	2.76 ± 0.21 d	1.19 ± 0.09 cd
C_2D_1	56.67 ± 3.28 cd	6.29 ± 0.11 bc	7.23 ± 0.97 b	4.82 ± 1.12 c	26.07 ± 2.73 c	3.40 ± 0.09 c	1.23 ± 0.13 c
C_2D_2	80.00 ± 2.37 a	6.83 ± 0.09 a	8.21 ± 1.55 a	6.21 ± 0.93 a	37.30 ± 3.38 a	4.07 ± 0.22 a	1.64 ± 0.17 a
C_2D_3	66.67 ± 2.53 bc	6.60 ± 0.17 ab	7.80 ± 2.76 ab	5.60 ± 0.11 bc	34.18 ± 4.20 bc	3.67 ± 0.12 bc	1.52 ± 0.14 ab
Average	58.75	6.20	7.13	5.11	30.18	3.30	1.32
CV	9.19	2.85	3.13	2.68	2.48	4.05	4.66

* Means with the same letter in each data group do not differ statistically according to Tukey's test ($p>0.05$) ± SE. C_1 = INIAP-EETP-801; C_2 = CCN-51; D_0 = 0 mg L^{-1}; D_1 = 100 mg L^{-1}; D_2 = 500 mg L^{-1}; D_3 = 1000 mg L^{-1} ; CV = Coefficient of variation (%).

higher number of leaves per plant (8.21), without statistically differing from the interactions: INIAP-EETP-801 + 500 mg L^{-1}, CCN-51 + 1000 mg L^{-1} and INIAP-EETP-801 + 1000 mg L^{-1}, which registered values between 7.64 and 8.01 leaves per plant. The aforementioned interactions statistically outperformed the other interactions that presented values that fluctuated between 6.00 and 7.23. The lowest average was recorded in the interaction INIAP-EETP-801 + 0 mg L^{-1} of chitosan.

Number and length of roots

The averages of the number of roots and the root length of seedlings of the two cocoa clones in response to the application of different doses of chitosan are presented in Table 1. The analysis of variance determined that the varieties and doses of chitosan reached high significance. statistics for the variable number of roots, while the interactions did not register statistical significance. For the length of the root, the clones and the doses of chitosan reached high statistical significance, and the interactions presented statistical significance at 95%.

Cocoa clones showed significant differences, being clone CCN-51 the one with the highest number of roots, with 5.26 roots. The application of chitosan in doses of 500 mg L^{-1}, presented a greater number of roots, with 6.08 roots, statistically surpassing the other doses that registered values between 4.19 and 5.51 roots. In the absence of chitosan application, the lowest number of roots was presented. The comparison of the interactions showed that when the clone CCN-51 was combined with the dose of 500 mg L^{-1}, the highest number of roots (6.21) was obtained in the absence of significant differences with respect to INIAP-EETP-801 + 500 mg L^{-1} (5.85). These interactions statistically outperformed the other interactions that showed averages between 4.00 and 5.60 roots. The lowest average was obtained in the interaction INIAP-EETP-801 + 0 mg L^{-1}.

For the root length variable, the CCN-51 clone had the greatest root length (31.16 cm), showing a significant difference over the INIAP-EETP-801 clone (29.19 cm). With the application of the chitosan dose of 500 cc of chitosan per liter of water, longer roots (35.74 cm) were produced, statistically surpassing the other doses of chitosan that showed averages between 24.82 and 34.49 cm of root length. At the level of interactions, the combination CCN-51 + 500 mg L^{-1} showed greater root length, with 37.30 cm, in statistical equality with CCN-51 + 1000 mg L^{-1}, which registered an average of 35.86 cm, statistically superior to the other interactions that registered values between 24.23 and 34.18 cm of root length. The shortest root length was found in the INIAP-EETP-801 + 0 mg L^{-1} interaction.

Biomass

The averages of the weight biomass of the aerial and the root zone part of the cocoa seedlings in response to the treatments under study are presented in table 1. For the dry aerial and root biomass, it was observed that the clones and the doses of chitosan reached high statistical significance, while for the interactions, no statistical significance was observed for both variables.

In the case of the dry weight of the biomass of the aerial part of the seedlings, the existence of significant differences could be observed among the clones, being clone CCN-51 the one that presented the highest average (3.47 g). The application of 500 mg L^{-1} of chitosan registered a higher dry weight of the biomass of the aerial part (4.00g), statistically surpassing the other doses. The lowest weight was recorded in the absence of chitosan. The interactions: CCN-51 + 500 mg L^{-1} (4.07 g) and INIAP-EETP-801 + 500 mg L^{-1} (3.92 g), registered the highest weights, without statistically differing from each other, but in turn; exceeded the other interactions that showed values that ranged between 2.41 and 3.67 g. The lowest dry weight of the biomass of the aerial part of the seedlings took place in the interaction INIAP-EETP-801 + 0 mg L^{-1}.

For the dry weight variable of root biomass, clone CCN-51 had the highest weight (1.39 g), showing a significant difference over clone INIAP-EETP-801 (1.24 g). With the application of the dose of 500 mg L^{-1} of chitosan per liter of water, a higher dry weight of the root biomass (1.57 g) was presented, statistically surpassing the other doses of chitosan that showed averages between 1.11 and 1.47 g. The lowest average was recorded when chitosan was not applied. At the level of interactions, the combination CCN-51 + 500 mg L^{-1} showed: the higher dry weight of root biomass, with 1.64 g, in statistical equality with CCN-51 + 1000 mg L^{-1} and INIAP-EETP-801 + 5000 mg L^{-1}, which registered averages of 1.52 and 1.50 g, respectively. These interactions statistically outperformed the others, which registered values between 1.03 and 1.41 g dry weight of root biomass. The lowest average was obtained in the INIAP-EETP-801 + 100 mg L^{-1} and INIAP-EETP-801 + 0 mg L^{-1} interactions, which each had a weight of 1.03 g.

Gas exchange

No significant differences in A were observed between clones or chitosan treatment, with A values between

2.7-4.0 µmol m-2 s^{-1} (Figure 1). Significant changes in E were observed in both cocoa clones due to chitosan treatment (p = 0.05) and between clones (p = 0.004), with the highest E observed in clone CCN-51 (1.4-1.7 mmol m -2 s^{-1}) (Figure 1). Low gs values were found (19.8 and 36.5 mmol m-2 s^{-1}), with significant differences between clones (p = 0.08). Ci ranged from 163 and 208 µmol mol^{-1} in CCN 51 and INIAP-EETP-801, respectively, showing significant differences between clones (p = 0.01). The WUE showed significant changes due to chitosan treatment (p = 0.045) and among clones (p = 0.01), the highest values of WUE were observed at 500 mg L^{-1} in CCN-51 (5.36 mmol mol^{-1}) and 1000 mg L^{-1} in INIAP-EETP-801 (7.62 mmol µmol^{-1}) (Figure 1).

Economyc analysis

In Table 2, the economic analysis (USD) of the treatments under study is presented based on the production of 1000 cocoa seedlings. In both clones, higher profitability was obtained when applying the chitosan dose of 500 mg L-1, reaching profitability of 40.65 and 50.00% for clones INIAP-EETP-801 and CCN-51, respectively. For the CCN-51 clone, a profit of $0.50 was obtained for each dollar invested, while for the clone INIAP-EETP-801: a profit of $0.41 was recorded for each dollar invested. The treatment cost for the mentioned dose was $403.15. When chitosan was not applied, economic losses were recorded for each clone, with 20.82 and 1.31%, for INIAP-EETP-801 and CCN-51, respectively.

DISCUSSION

Chitosan and chitin sources have emerged as promising groups of biological substances that can induce plant growth, disease resistance, and postharvest quality preservation (Paul *et al.*, 2017; González-Estrada *et al.*, 2023). In the present study, it was possible to verify different benefits of the application of chitosan in cocoa seedlings, among which it stands out that it promoted the survival of cocoa seedlings of both clones, the dose being a determining factor for obtaining more live plants. in the nursery phase, so that the dose of 500 mg of chitosan per liter of water improved the survival conditions of the seedlings, observing a possible negative effect when increasing the dose up to 1000 mg of chitosan per liter of water. This agrees with (Rivas-García *et al.*, 2021; Reyes-Perez *et al.*, 2022), who, at the tomato and cocoa nursery level, maintains that the application of 3000 mg L^{-1} and 1000 mg L^{-1} doses respectively of foliar Quitosan based products (Quitomax®) should be considered as one of the most important to guarantee success in obtaining seedlings, since that a higher dose could generate negative effects, as well as economic losses due to a waste of inputs.

Another point to take into account is that clone CCN-51 showed a higher percentage of survival, which can be attributed to greater resistance to environmental conditions, such as possible pathogens and factors associated with the microclimate inside the greenhouse. Reyes-Pérez *et al.* (2021), mentions that genetic materials with a higher degree of resistance to both diseases and environmental factors, could present greater survival in controlled conditions, as a result of a greater degree of adaptability, which is also verified when observing that when interacting the mentioned clone with the dose of chitosan of 500 mg L^{-1}, the percentage of survival increased up to 80.00%. Chakraborty *et al.* (2020) mentioned that plants can survive on a larger scale in comparison to the environmental survival if a common growing material is added with an ideal source of fertilization.

The development of the CCN-51 seedlings was notably greater, so that after 120 days, they exhibited a growth of 1.05 mm, compared to 0.77 mm of the seedlings of the clone INIAP- EETP-801, which can be attributed to a greater adaptability of the seedlings of the first clone, as well as a greater development and production potential (Junior *et al.*, 2017). In addition, this is supported by the foliar emission, which, at the age of 120 days, the seedlings of clone CCN-51, registered an emission of 4.64 leaves, compared to the increase of 4.26 in the INIAP-EETP-801 clone, being able to point out that the CCN-51 clone presents a greater development than the INIAP clone, however, these results are opposed to those obtained by (Reyes-Pérez *et al.*, 2021), who observed that clone CCN-51 was characterized by plants of lower height, but with high productive potential at the field level. This difference can be attributed to the fact that the lighting conditions, as well as other edaphoclimatic factors, differ notably under cover, agreeing with (Leiva-Rojas *et al.*, 2019), who maintain that, under controlled conditions, the response of cocoa clones may differ directly depending on the conditions in which they grow in the open field.

When analyzing the variables mentioned above, depending on the doses of chitosan, a significant effect could be identified when increasing the dose of this biopolymer, however, when excessively increasing its dose, a contradictory effect is produced, restricting the thickening of the stem, as well as the foliar emission of cocoa seedlings (Vázquez-Zamora *et al.*, 2022). This is reflected when analyzing the interactions, in which it can be seen that in both clones the dose of 500 mg L^{-1}

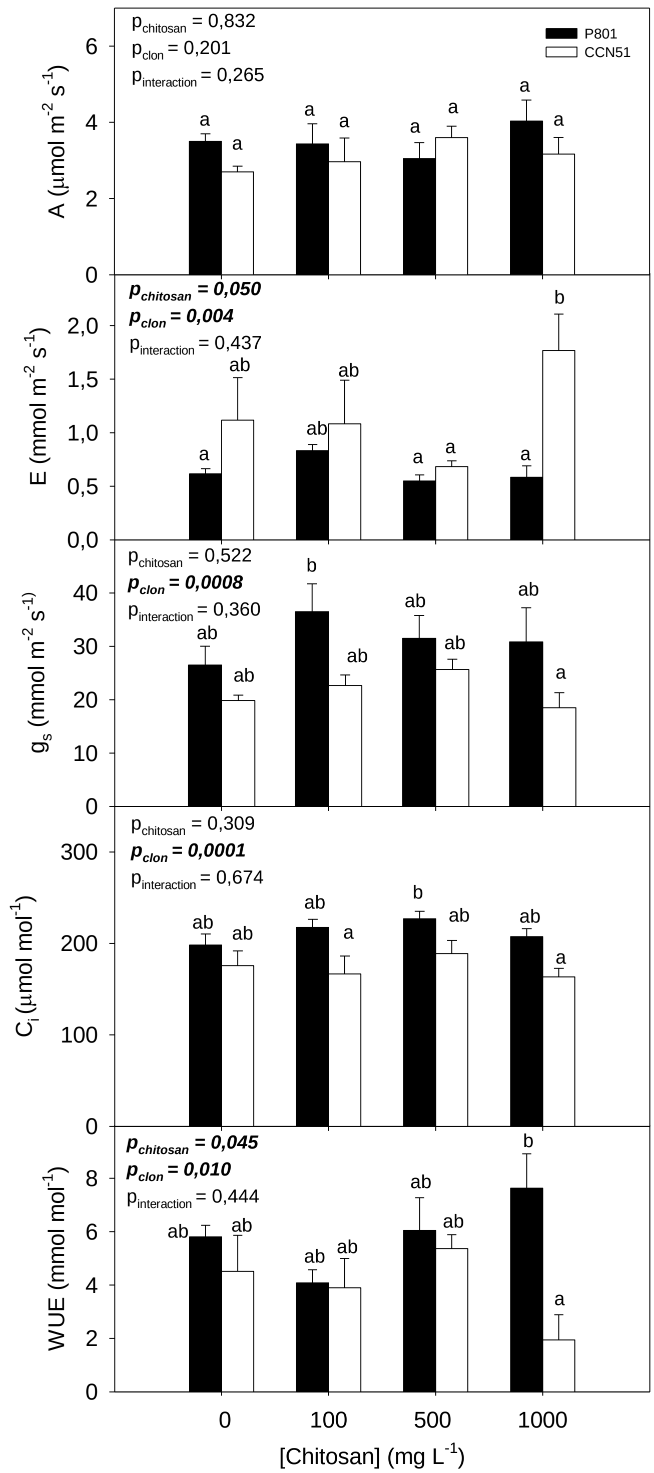

Figure 1. Gas exchange parameters: A. Net photosynthesis rate, E. Transpiration rate, gs. Stomatal conductance, Ci. Intercellular concentration of CO_2, WUE. Water use efficiency of two cocoa clones subjected to different chitosan concentrations. Both cacao clones: INIAP-EETP-801 (black bars) and CCN-51 (white bars). Each bar shows the average of six different seedlings ± SE (n = 6). Significant differences from the two-way ANOVA (chitosan × clone) are indicated by different letters above the bars. P-values for the significance of the effect of each factor on the response variables are indicated within each panel.

Table 2. Economic analysis (USD) of the production of 1000 cocoa seedlings in response to the application of chitosan in the vegetative propagation of clones CCN-51 and EETP-801.

Treatments	Survival (%)	Produced plants	Gross income ($)	Variable cost ($)	Treatment cost ($)	Total cost ($)	Net profit ($)	B/C	Profitability (%)
C_1D_0: INIAP-EETP-801 + 0 mg L^{-1}	33.33	333	183.15	33.30	70.00	231.30	-48.15	0.79	-20.82
C_1D_1: INIAP-EETP-801 + 100 mg L^{-1}	53.33	533	293.15	53.30	74.18	255.48	37.67	1.15	14.74
C_1D_2: INIAP-EETP-801 + 500 mg L^{-1}	73.33	733	403.15	73.30	85.34	286.64	116.51	1.41	40.65
C_1D_3: INIAP-EETP-801 + 1000 mg L^{-1}	63.33	633	348.15	63.30	93.00	284.30	63.85	1.22	22.46
C_2D_0: CCN-51 + 0 mg L^{-1}	43.33	433	238.15	43.30	70.00	241.30	-3.15	0.99	-1.31
C_2D_1: CCN-51 + 100 mg L^{-1}	56.67	566	311.30	56.60	74.18	258.78	52.52	1.20	20.30
C_2D_2: CCN-51 + 500 mg L^{-1}	80.00	800	440.00	80.00	85.34	293.34	146.66	1.50	50.00
C_2D_3: CCN-51 + 1000 mg L^{-1}	66.67	666	366.30	66.60	93.00	287.60	78.70	1.27	27.36

Currency: United State Dollar

Cocoa seedling cost:	0.55
Variable cost:	$ 0.10
Fixed cost	$ 128.00/1000 plants
Chitosan cost	$9.20
Seedling cost	$ 0.07

produces better results, agreeing with (Peña *et al.,* 2014), who maintain that the selection of a dose in a certain crop in specific phenological stages that presents the best response, helps to obtain better results, which are reflected both in the development and in the productivity of the crops. Regarding this, (Reyes-Pérez *et al.*, 2020), reported similar results for the Floradade and Amalia tomato varieties, showing that the ideal dose under cover is 2 g L^{-1}, so that by increasing the dose to 3000 mg L^{-1}, it generates a negative effect on the growth of seedlings in the nursery stage.

The parameters of the number of roots, length of the main root, as well as the dry biomass of both the aerial and root parts, showed higher averages in clone CCN-51, it being possible to speculate that this genetic material, possibly in the initial stage of the crop presents greater development, allowing to allude that the INIAP-EETP-801 clone, when it passes to open field conditions, presents a greater development, with taller plants (Garate-Navarro *et al.,* 2017). Regarding this, (Vásquez-Zamora *et al.,* 2022) maintain that in the San Martin area from Peru, an important region for the commercial production of cocoa, the highest yields of the yield variables and a high incidence of fungal diseases in the pods were obtained after indole-3-butyric acid (IBA) treatment in mini-tunnel conditions.

Regarding the doses of chitosan, a significant effect could be identified when applying the dose of 500 mg L^{-1}, so that, after 120 days of study, it produced 36.15 and 33.50% more leaves in the clones CCN-51 and INIP-EETP-801, with respect to their respective controls, correspondingly. On the other hand, it significantly increased the number of roots, as well as their length in both clones, and despite the fact that the dose was increased to 1000 mg L^{-1}, it was observed that it produces a negative effect on the seedlings. which may be due to the fact that phytotoxicity is produced, since due to the early age of the seedlings they do not support high doses of inputs. This becomes more important when analyzing the fresh and dry biomass of both the aerial part and the root zone, which was boosted with the dose of 500 mg L^{-1}, setting the guidelines for a similar effect to foliar emission and root development. This corroborates what is maintained by (Peña *et al.,* 2014) who observed that in most cases, the application of chitosan had a positive effect on the length. These authors showed that, with concentrations of 100, 1000, and 2500 mg L^{-1}, values higher than the control were reached, however, when using 10 000 mg L^{-1} there was an inhibitory effect on growth, perhaps because these levels are negative for certain events. Finally, Peña *et al*. (2014) showed that, in general, it was found that chitosan applications influenced the growth variables in tomato seedlings. The response was modulated by the concentrations of chitosan. polymer, although the results also varied with when the determinations were made.

In gas exchange parameters, there were observed low values of stomatal, transpiration, and photosynthesis conductance. This results indicates that a low CO_2 assimilation rate per water loosed through transpiration was performed. Almeida *et al.* (2022) reported low photosynthesis rates. It is important to remark that gas exchange parameters were recorded on a specific period of time and, thus, those parameters are not directly correlated with growth parameters. It has been widely reported that cocoa is a shady species that grows in understory environments of tropical forests with high rainfall and reduced light availability (Reyes-Perez *et al.*, 2022). As reported by (Reyes-Perez *et al.,* 2022), the values of A, gi, and E observed in this study are lower than those reported in National cocoa clones in San Agustín, in Pichilingue, and TCS and CCN 51 evaluated in Antioquia, Colombia, respectively (Tezara *et al.*, 2020; González-Ceballos *et al.,* 2021).

Finally, the economic analysis showed the effect generated by the increase in the dose in obtaining profitability when producing 1000 cocoa plants. The economic benefit was derived from the survival of seedlings, so that the dose of 500 mg L^{-1}, by allowing more live seedlings to be obtained, helps to obtain a greater profit, which agrees with (Solís-Bonilla *et al.*, 2022), who maintains that the application dose of biostimulant products plays a determining role for the survival of seedlings, and therefore for the generation of profitability, since a dose that allows obtaining a greater number of seedlings will contribute to a greater economic benefit.

CONCLUSIONS

The treatment of chitosan based formulation at 500 mg L^{-1}, is an effective treatment in order to improve the survival percentage, the stem diameter, the number of leaves, the number and length of roots, the biomass, the gas exchange, and the economic profitability of the CCN-51 and INIAP-EETP-801 cocoa plant varieties at 120 days after starting the trial during vegetative propagation. The cocoa clone CCN-51 showed plants that exhibited greater development of both the aerial part and the root part of the cocoa seedlings than INIAP-EETP-801 coca clones, and it also showed an increase in stem diameter, foliar emission, number and length of roots.

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Data availability. All data is presented in the presented the present paper.

Conflict of interests. The authors declare that they have no competing interests.

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