Role of foliar application of natural compounds in modulating vegetative parameters and enzyme activities of stevia plants exposed to salinity.

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Abstract

An experiment was conducted at a research station affiliated with the Department of Horticulture and Landscape Engineering, College of Agriculture, Tikrit University, during the 2024 spring growing season. The objective was to evaluate the effectiveness of foliar spraying with various natural substances obtained in enhancing the production of active compounds and promoting the growth of stevia plants under saline conditions. The study involved two factors: the first factor comprised five foliar treatments with natural substances, designated as factor "A." In contrast, the second factor included two levels of sodium chloride (NaCl) salinity plus a control (no salt), designated as factor "S." The experiment was arranged in a randomized complete block design (RCBD) with three replicates. Statistical analysis was performed using GenStat software. Results indicated that among the foliar treatments, treatment A3 significantly increased plant height70.43cm, while treatment A0 improved leaf dry matter percentage45.34% and peroxidase enzyme activity6.831Unit ml-1. Treatment A4 enhanced chlorophyll 1.99 mg g-1content and catalase enzyme activity3.613Uint ml-1. Regarding the effects of salinity, treatment S2 increased the leaf dry matter percentage43.67% ,PAL enzyme activity, and increased catalase activity 3.594 Uint ml-1. The interaction between the two factors was significant, with treatment A3S1 showing superiority in plant height72.38cm, A1S2 in branch number7.93, A0S1 in dry leaf matter percentage, A2S0 in PAL enzyme activity, and A0S0 in peroxidase and catalase enzyme activities.

Keywords: Stevia, Amino acids, Sugar alcohols, Activities enzyme, Salinity stress

Introduction

Stevia plants have been farmed and made commercial in a number of nations, including Canada, lndonesis, Korea, Malaysis, lndia, and the United States. It has also been successfully cultivated in Iraq. [1] This plant has economic, medicinal, and nutritional importance. [2] Stevioside and rebaudioside glycosides (Reb A, B, C, etc.) are the main components of stevia sweeteners, which are used as natural Stevia rebaudiana Bertoni, also called sugar bush. The plant belongs to the Asteraceae family. [3] The genus Stevia comprises more than 230 species, but only two of them contain steviol glycosides: Stevia rebaudiana and Stevia filipendulina. [4] The plant is native to the forests of Paraguay, Mexico, and Brazil in South America.[5] It grows in tropical and subtropical regions.[6] Due to its health acids and sugar alcohols, given their prominent role in supporting vital processes and stress resistance. Numerous studies have demonstrated that amino acids play a beneficial role in enhancing plant productivity and quality. They contribute to mitigating the effects of drought and salinity stress through

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various physiological activities by altering the osmotic capacity of plant tissues. They also significantly reduce injuries caused by biological stresses, stimulate physiological and biochemical processes, and participate in protein and carbohydrate synthesis. Amino acids are also believed to be responsible for cell division and the production of certain natural growth hormones, such as IAA and GA3, which enhance productivity and improve quality. [7] Sugar alcohols are simple organic compounds produced naturally within plants.[8] They were first discovered in plants in 1996 within tree bark, and are rich in boron and other elements. Called polyols, they are alcoholic compounds containing a polyhydroxyl group and are characterized by a low molecular weight and high solubility, up to 30% of the total elemental carbon.[9.[

sweeteners in the pharmaceutical, food, and beverage industries. Stevia glycosides are beneficial in treating insulin resistance, preventing atherosclerosis, regulating blood pressure, lowering blood sugar levels in individuals with diabetes, and potentially preventing certain types of malignant tumours.[10] Stevia is sensitive to salinity, and plant growth, as well as its secondary metabolites, can be affected by salinity toxicity.[11] Salinity is one of the most widespread abiotic stresses, leading to significant losses in agricultural crop production, particularly in arid and semi-arid regions.[12] The area of land affected by salinity was recently estimated at 1.125 million hectares worldwide.[13] This area is expected to increase due to the effects of climate change, human activities, and freshwater scarcity [14,15]. Salinity can cause various molecular, physiological, and biochemical abnormalities [16,17,18]. It also inhibits plant growth and development by affecting water relations, cell turgor pressure, and disrupting plant growth regulators, which restricts cell division and hyperplasia[19]. The objective was to evaluate the effectiveness of foliar the spraying in enhancing the production of active compounds and promoting the growth of stevia plants under saline Conditions

Material and Methods

Prepare samples:

To help for proper drainage andexperiment al site preparation, a 5 cm layer of small gravael was applied to the surface[20].Treatments were then allocated according to the experimental design. The study involved two main factors: The first factor (A) consisted of foliar spraying treatments with amino acids and sugar alcohols as follows: , A0 (Control): No spraying with active compounds; plants were sprayed with distilled water only. , A1: Foliar spraying The experiment was conducted during the 2024 growing season at the wooden canopy facility of the Department of Horticulture and Landscape Engineering, College of Agriculture, Tikrit University. A designated area within the canopy was prepared for the study. All weeds and residues from previous crops were removed, and the surface was levelled. The area was then covered with transparent polyethene film prior to the placement of planting trays to suppress weed growthwith of 2 g/L. Factor 2 (S): Salinity levels applied by adding sodium chloride (NaCl) to the culture medium at the following concentrations: S0 (Control): No salt added. S1: 0.5 g/L NaCl added., S2: 1.0 g/L NaCl added.
the amino acid arginine at a concentration of 1 g/L. , A2: Foliar spraying with the amino acid cysteine at a concentration of 1 g/L. , A3: Foliar spraying with sorbitol at a concentration of 2 g/L.,A4: Foliar spraying with mannitol at a concentration of 2g L .

Studied traits:

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equation: Total chlorophyll (mg/l) = 20.2 × optical density at wavelength 654 + 8.02 × optical density at wavelength 663. The result was then converted from (mg/100 g fresh weight .(

Enzyme Measurements and Activity

The activity of the enzymes Phenylalanine ammonia-lyase (PAL), Peroxidase, and Catalase was measured after collecting the tender plant leaves in clean polyethene bags according to the instructions. The leaves were placed in a refrigerated box due to the sensitivity of the enzymes to heat. The leaves were then transferred to the Biotoxins and Enzyme Purification Laboratory, Food Contamination Research Department, Scientific Research Authority. They were washed with water to remove dust, then washed with sterile water and cut into small pieces using sterile scissors to measure the following enzymes

Phenylalanine ammonia-lyase (PAL) enzyme

The activity of the PAL enzyme was measured after extraction according to the method [22] and Kallati: - 0.5 g of leaves were crushed in a chilled ceramic mortar with the addition of 10 ml of sodium borate buffer (pH = 7.5). The crushed material was finely ground and placed in sterile plastic tubes. - The extract was centrifuged at 8000 rpm for 20 minutes at 4°C. - 0.2 ml of the filtrate, which is the source of the enzyme, was taken into a glass tube, and 1 ml of 0.1 mM phenylalanine solution and 2.5 ml of sodium borate buffer (pH = 8.7) were added. The tubes were incubated at 38°C for one hour. - 0.5 ml of 1 M

Plant Height (cm(

Plant height was measured from the soil surface to the top of each labelled plant within the experimental units using a measuring tape. The average height per treatment was then calculated .

Number of Branches per Plant

The total number of branches was counted on each labelled plant within the experimental units, and the average number of branches per plant was determined .

Percentage of Dry Matter in Leaves(%)

Fresh (wet) leaf weight was measured using an electronic balance. The leaves were then airdried until a constant weight was achieved, and the dry weight was recorded. The percentage of dry matter was calculated using the formula: Percentage of dry matter= (Dry weightWet weight)×100\text{Percentage of dry matter} = \left(\frac{\text{Dry weight}}{\text{Wet weight}} \right) \times 100Percentage of dry matter=(Wet weightDry weight)×100

Total chlorophyll content (mg/l00g (

The total chlorophyll content of the fresh leaves was estimated using the method described in [21]. Full-width leaves were selected from the experimental unit plants by weighing 0.2 g of fresh leaves. These were washed with water to remove dust, dried, cut into small pieces, and soaked in 80% acetone for 24 hours. The samples were then placed in a dark, sealed place. The light absorbance of the sample was then read using a spectrophotometer at two wavelengths, 645 and 663 nm. Chlorophyll was then estimated using the following

mixture was taken and placed in a spectrophotometer cuvette, to which 0.2 ml of the plant extract was added. The absorbance of light was measured directly using a spectrophotometer at a wavelength of 240 nm every 30 seconds (at least six readings were taken) [26]. The change in absorbance was calculated according to the following equation: Enzyme activity = ΔA/(g(0.25) sample weight × (0.5)ΔT) ΔA = change in absorbance. Δt = change in time/minute .

Catalase Enzyme

Method [27] was followed to assess the activity of the catalase enzyme, which involved monitoring the change in light absorption at a wavelength of 240 nm using a spectrophotometer, resulting from the oxidation of hydrogen peroxide as a substrate for the enzyme. To assess the activity of the catalase enzyme, the following components were prepared: 0.5 g of leaf fragments was crushed with a chilled ceramic mortar. 10 cm3 of sodium phosphate buffer (pH = 7) was

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added. The mixture was finely crushed and placed in glass tubes. The extract was then chilled and centrifuged at 3000 rpm for 20 minutes. A 2 M hydrogen peroxide (H2O2) solution was added. A 0.1 M sodium phosphate buffer (pH = 7) solution was added. The reaction mixture was prepared by mixing the three solutions in volumes of 0.1 cm^3, 1 cm^3, and 2 cm^3, respectively. The activity of the catalase enzyme was measured using a spectrophotometer by placing the reaction mixture in the device's cell and monitoring the change in light absorbance at a wavelength of 240 nm. The device was calibrated using the reaction mixture without the addition of the leaf extract. Enzyme activity was calculated from the following equation: Enzyme activity = $\Delta b/(\Delta T \times (0.5)$ sample weight) Δb = change in absorbance. ΔT = time (1 minute.(
trichloroacetic acid (TCA) C2HCI3O2 was added to stop the reaction. - The absorbance of the samples to ultraviolet (UV) light was measured using a spectrophotometer at a wavelength of 290 nm. The control sample consisted of the previous solutions except for phenylalanine. - A standard curve was drawn for a series of known concentrations of cinnamic acid (0, 1, 2, 3, 4, 5, 10 µg mL-1) in sodium borate buffer at pH 8.7, and absorbance readings were taken at a wavelength of 290 nm. - Enzyme activity was measured based on micrograms of cinnamic acid/hour/gram fresh weight [23] according to the equation. $Y = 0.0974x + 0.005$ Where Y = absorption intensity X = cinnamic acid concentration in the sample

Peroxidase (PO) activity

Peroxidase activity was measured after extraction, according to the method described previously [24, 25]. This was summarized as follows: 0.5 g of chopped leaves was taken and crushed as described in the previous paragraph. 2 ml of 0.2 M phosphate buffer (pH 7.2-7.5) was added to the mixture in a sterile ceramic mortar and transferred to a sterile glass tube. The extract was centrifuged using a centrifuge cooled to 4°C for 10 minutes at 6,000 rpm. The reaction mixture consists of a 0.05 molar guaiacol solution prepared by adding 1.36 ml of 30% hydrogen peroxide to 250 ml of distilled water (prepared online), a 0.02 molar hydrogen peroxide solution prepared by adding 0.56 ml of 30% hydrogen peroxide to 250 ml of distilled water (prepared online), and a 0.04 molar Tris base solution containing NaCl (1 molar) = pH 7, prepared by dissolving 1.211 g of Tris base and 14.16 g of NaCl in distilled water. The pH is adjusted to 7 using 1 M hydrochloric acid to bring the volume up to 250 mL of distilled water. The above solutions are mixed in a 1:1:1 ratio with seven parts distilled water before immediate use. 3 ml of the reaction

Results and Discussion

Trait due to the salinity factor. From the results of the interaction between natural additives and salinity, we observe significant differences. Treatment 1A3S significantly outperformed this trait, yielding the highest value of 72.38 cm, while treatment A0S0 recorded the lowest value of 59.47 cm.

Plant Height (cm) The results in Table 1 indicate that certain experimental factors significantly influenced plant height. We note that natural additives A3 significantly outperformed this trait, reaching 70.43 cm, compared to treatment A0, which recorded the lowest value of 63.73 cm. However, there were no significant differences in this

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Table 1. Effect of natural additives, salinity and their interactions on plant height (cm(

One Factor / Two Factor	A0	A1	A2	A3	A4	Average S
S0	59.47 b	62.77 ab	69.53 ab	70.30 a	63.60 ab	65.13 A
S1	67.03 ab	67.73 ab	67.63 ab	72.38 a	63.30 ab	67.62 A
S2	64.70 ab	66.87 ab	69.13 ab	68.60 Ab	70.17 a	67.89 A
Average A	63.73 b	65.79 ab	68.77 ab	70.43 a	65.69 ab	

Number of branches (plant-1 branch (

According to the results in Table 2, we found no significant differences between natural additives and salinity. According to the results in the same table, we find that the two-way interaction between the first and second factors was significant. That treatment A1S2 yielded the highest value for this trait, reaching 7.93 plant-1 branches, which is significantly superior to the other treatments. Treatment A1S1, on the other hand, recorded the lowest value, reaching3.25plant-1

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Table 2. Effect of natural additives, salinity, and their interactions on the number of branches per plant (plant-1 branch(

One Factor / Two Factor	A0	A1	A2	A3	A4	Average S
S0	4.25 bc	6.15 Ab	5.25 abc	5.63 abc	6.30 abc	5.52 a
S1	4.25 bc	3.25 C	6.78 ab	5.50 abc	5.87 abc	5.13 a
S2	5.07 abc	7.93 a	5.98 abc	6.55 ab	3.68 bc	5.84 a
Average A	4.52 a	5.78 a	6.00 a	5.89 a	5.28 a	

Percentage of Dry Matter in Leaves :(%)

The results of Table 3 show that the trait, leaf dry matter percentage, was affected by the study factors. Natural additive treatment A1 outperformed, yielding a 45.34% improvement. The salinity treatment significantly affected this trait, with treatment S2 outperforming treatment S0, yielding a value of 43.67%, compared to the lowest value recorded for treatment S0, at 39.36%. The two-way interaction between the first and second factors resulted in treatment A0S1 outperforming the treatment, yielding a value of 55.09%, compared to the lowest value recorded in treatment A4S1, at 32.14% .

Table 3. Effect of natural additives, salinity and their interactions on the percentage of dry matter in leaves(%)

One Factor / Two Factor	A0	A1	A2	A3	A4	Average S
S0	36.99 def	47.26 bc	33.27 F	42.52 bcde	36.75 Ef	39.36 b
S1	55.09 a	47.26 bc	36.24 Ef	42.59 bcde	32.14 F	42.66 a
S2	43.95 bcd	40.86 cde	42.41 Bcde	49.43 ab	41.72 Cde	43.67 a
Average A	45.34 a	45.13 a	37.30 B	44.85 a	36.87 B	

Total chlorophyll (mg g-1 (

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The results in Table 4 show that chlorophyll content was affected by the study factors. Natural additives A4 significantly outperformed, recording 1.99 mg g-1 , compared to treatment A0 ,
which recorded 1.14 mg g-1 . Salinity treatment S1 significantly influenced the treatment, producing the highest chlorophyll content, recording 1.72 mg g-1 , compared to treatment S2, which recorded 1.60 mg g-1 . The results in the table show that the interaction between the first and second factors was significant, with treatment A4S2 outperforming, recording 2.03 mg g-1,while treatment A0S0 recorded the lowest value, at 1.03 mg g-1.

Table 4. Effect of natural additives, salinity and their interactions on the Total chlorophyll (mg g-1(

One Factor / Two Factor	A0	A1	A2	A3	A4	Average S
S0	1.03 g	1.60 d	1.70 cd	2.02 a	2.00 a	1.67 a
S1	1.21 f	1.69 cd	1.81 bc	1.95 ab	1.93 ab	1.72 a
S2	1.17 fg	1.84 bc	1.57 d	1.39 e	2.03 a	1.60 a
Average A	1.14 d	1.71 bc	1.69 c	1.79 b	1.99 a	

PAL Enzyme (U/mL (

The results in Figure 1 indicate that the PAL enzyme was affected by the study factors in the experiment. Natural additives did not cause significant differences, and the salinity factor S0 produced the highest

value, 13.90 (U/mL), significantly superior to the S2 treatment, which produced the lowest value, 13.38 (U/mL). The two-way interaction between the first and second factors resulted in significant differences,
with the A2S0 treatment significantly superior to the other treatments, producing a value of 14.45 (U/mL), while the A3S2 treatment recorded the lowest value, 12.87 (U/mL.(

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Figure 1. Effect of natural additives, salinity, and their interactions on the PAL enzyme

Peroxidase Enzyme (U/mL (

As shown in Figure 2, the peroxidase enzyme was affected by the study factors. We find that the natural additive treatment A0 significantly outperformed, yielding the highest value of 6.831 (mL/U), compared to the A3 treatment, which yielded the lowest value of 5.164 (mL/U). There were no significant differences in the salinity factor. From the results of the two-way interaction between the first and second factors, we find that the A0S0 treatment significantly outperformed, yielding the highest value of 6.060 (U/mL), compared to the A3S2 treatment, which yielded the lowest value of 4.720 (U/mL.(

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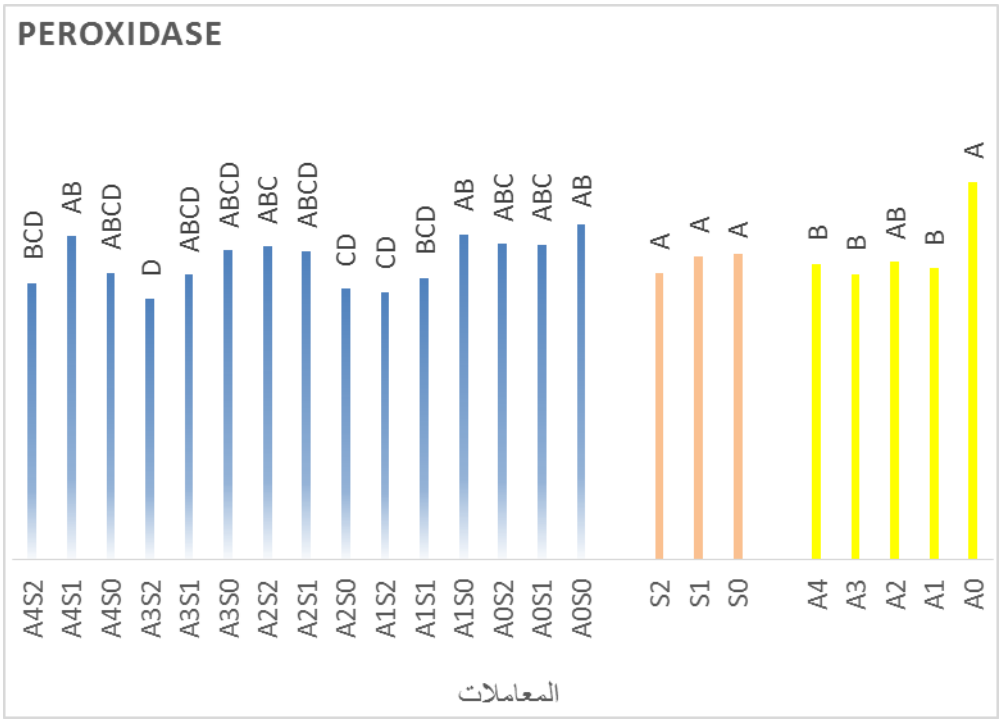

Figure 2. Effect of natural additives, salinity, and their interactions on the Peroxidase enzyme

Catalase Enzyme (U/mL (

The results in Figure 3 show that natural additives caused significant differences in catalase enzyme levels, with treatment A4 outperforming the treatment with a ratio of 3.613 (U/mL), compared to treatment A3, which produced the lowest ratio of 3.309 (U/mL). From the results of the two-factor interaction, we find that the interaction between the first and second factors was significant, with treatment A0S0 outperforming and recording the highest value for this trait, 4.016 (U/mL), while treatment A3S1 recorded the lowest value for this trait, 3.158 (U/mL.(

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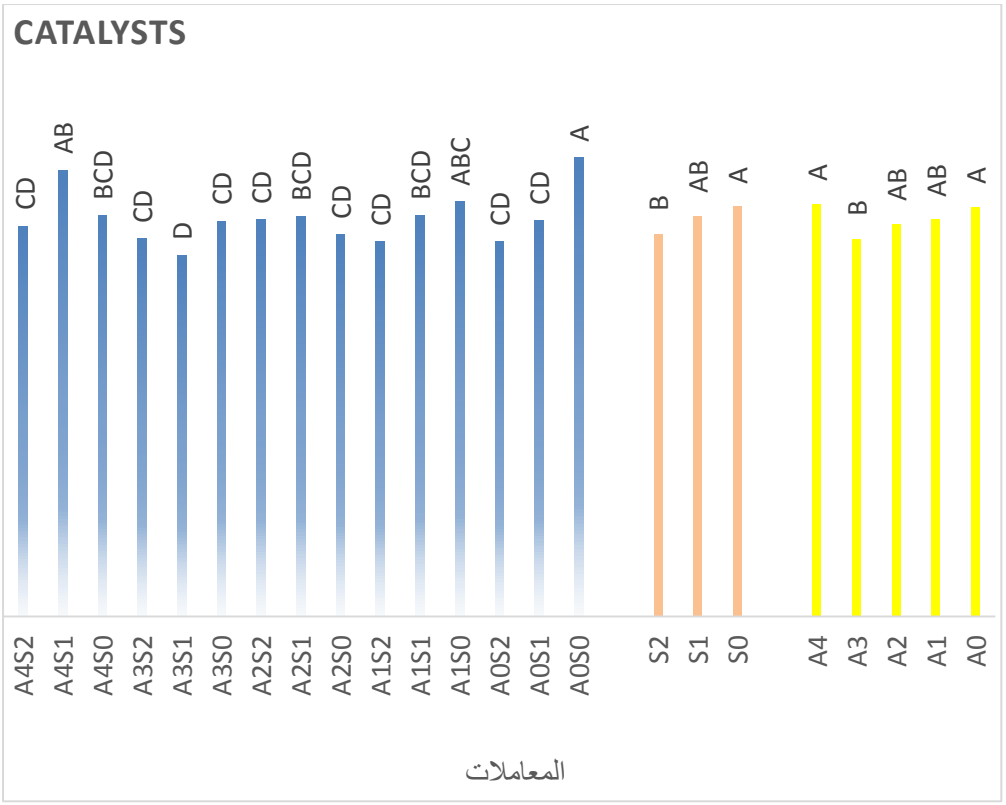

Figure 3. Effect of natural additives, salinity, and their interactions on the catalase enzyme

The observed increase in several vegetative traits can be attributed to the role of amino acids accumulated within the plants in regulating ion transport, modulating stomatal opening and closing, and detoxifying heavy metals. Additionally, amino acids influence the synthesis and activity of certain enzymes, gene expression, and the balance of metabolic and redox reactions [28]. Recognized as biostimulants, amino acids provide plants with energy necessary to compensate for losses due to respiration and metabolic breakdown [29]. Their application has been widely reported to positively impact plant growth and significantly mitigate abiotic stresses. For instance, amino acid treatments have been shown to increase chlorophyll a and b content in leaves, thereby enhancing shoot growth rates and fresh biomass accumulation. Moreover, they significantly elevate total nitrogen, phosphorus, and potassium levels in mleaves, alongside improvements in total yield, dry weight, total soluble solids, vitamin C, and total sugar content compared to untreated controls [30]. The supplementation of polyamines, either through exogenous application or genetic engineering, similarly promotes growth and productivity in medicinal plants; however, the magnitude of these effects depends on various factors [31]. Conversely, salt stress induces multiple physiological and molecular alterations that hinder plant growth by inhibiting cell division in meristematic tissues, as well as cell expansion and elongation [32]. Structural changes in leaf thickness and spongy tissue have also been reported under saline conditions [33]. Furthermore, osmotic pressure resulting from salt accumulation around roots reduces water availability to leaf cells [34]. Salt stress also restricts carbon dioxide assimilation by causing stomatal closure, thereby disrupting electron flow during the Calvin cycle and reducing dry biomass synthesis [35]. In line with these findings, treatment with arginine increased the

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number of branches, corroborating the results of [36], who studied Calendula officinalis L. and observed a 13.9% increase in branching with increasing arginine concentrations (0, 3, 6 mM). Similarly, [37] investigated the effect of foliar-applied arginine on Stevia rebaudiana Bertoni using three concentrations (0, 50, 100, and 150 mg/L). Their results showed a significant increase in branch number at 50 mg/L, reaching an average of 2.2 branches per plant.

conclusion

This study aimed to investigate the effect of foliar spraying with some natural compounds on improving vegetative characteristics and enzyme activity in stevia plants under salt stress conditions. Results showed that the sorbitol treatment with 0.5 g L-1 salinity resulted in the highest increase in plant height, while the highest number of branches was recorded when cysteine was treated with 1 g L-1 salt. The percentage of dry matter also outperformed.In leaves, arginine was treated with 0.5 g L-1 salt. Chlorophyll content was higher when mannitol was treated with 1 g L-1 salt. The highest activity of the PAL enzyme was observed when cysteine was treated without salinity, while the activity of peroxidase and catalase was higher in untreated plants. These results demonstrate the importance of foliar spraying with natural compounds, which is effective in enhancing the tolerance of stevia plants to salt stress .
And improve its physiological and

enzymatic performance

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