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Investigating the effect of foliar spraying of zinc nanoparticles and biostimulants on modulating the effect of water deficit stress in sugar beet by using Integrated Biomarker Response Version 2

Shahnaz Nikzad[1], Soran Sharafi[1]✉, Hamze Hamze[2], Toraj Mir Mahmoodi[1] & Saman Yazdan Seta[1]

The effects of foliar applications of zinc oxide nanoparticles and various biostimulants were studied to alleviate water stress in sugar beet. The experiment used a split-split-plot layout based on a Randomized Complete Block Design (RCBD) with three replications over two growing seasons (2022–2024). The main plot consisted of two irrigation levels: Irrigation after 60 and 120 mm of evaporation was considered normal irrigation (WW) and water deficit stress (WD). Zinc nanoparticle (ZnO-NPs) levels (control, 0.2, and 0.4 mg L^{-1}) were assigned to subplots, and biostimulants (control, chitosan, proline, and chitosan + proline) were assigned to sub-subplots. The results shoewd under WD conditions foliar spraying of 4 mg L^{-1} of ZnO-NPs increased the chlorophyll *b* content (22.85%), carotenoid (9.58%), proline content (18.42%), beta-glycine (13.20%), stomatal conductance (33.53%), gibberellin (GA) (9.09%), cytokinin (CK) (13.07%), catalase enzyme activity (CAT) (7.86%), superoxide dismutase (SOD) (25.56%), ascorbate peroxidase (APX) (5.34%), and root yield (RY) (10.48%) and decreased abscisic acid (ABA) (11.24%), malondialdehyde (MAD) (17.33%), and hydrogen peroxide (17.18%) compared to the control. Among biostimulants treatments, application of chitosan + proline under WD conditions increased the content of chlorophyll *a* (37.44%), chlorophyll *b* (16.23%), proline (4.87%), beta-glycine (18.09%), GA (7.00%), auxin (IAA) (35.40%), CK (18.03%), CAT (11.42%), APX (19.6%), and RY (11.46%) compared to control, and decreased the content of ABA (24.16%), MAD (9.03%), and hydrogen peroxide (11.50%). In this experiment, the combination of 2 mg L^{-1} ZnO-NPs with chitosan and proline exhibited a synergistic effect, increasing the content of chlorophyll a and b, relative water content (RWC), SOD, and RY, while reducing ABA. The lowest IBRv2 values were recorded for control + proline, control + chitosan, 2 mg L^{-1} ZnO-NPs + Control, 4 mg L^{-1} ZnO-NPs + Control, and 4 mg L^{-1} ZnO-NPs + Proline treatments. 4 mg L^{-1} ZnO-NPs + chitosan + proline (I = 0.803) and 4 mg L^{-1} ZnO-NPs + proline (I = 0.809) showed the smallest increases in MAD content. In terms of RY, the least decrease was observed in the treatments of 4 mg L^{-1} ZnO-NPs + Chitosan and Proline (I = − 0.492) and 4 mg L^{-1} ZnO-NPs + Proline (I = − 1.014).

Keywords Antioxidant enzymes, Beta vulgaris, Integrated Biomarker Response version 2 (IBRv2), Water deficit stress, Zinc oxide nanoparticle

[1]Institute of Agriculture, Water, Food, and Nutraceuticals, Mah.C., Islamic Azad University, Mahabad, Iran. [2]Sugar Beet Research Department, Agricultural and Natural Resources Research Center of Hamedan, Agricultural Research, Education, and Extension Organization (AREEO), Hamedan, Iran. ✉email: S.sharafi@iau-mahabad.ac.ir

Intrudution

Sugar beet (*Beta vulgaris* L.) is a vital crop with various applications and nutritional benefits. It is the second most widely cultivated source of white sugar globally, after sugarcane, and is crucial for meeting the growing needs of the population[1], Multiple factors frequently influence the production of sugar beet, with drought being regarded as the most significant. Drought stress can lead to a reduction in sugar beet yield of up to 30%, resulting in substantial economic and nutritional impacts[2]. Sugar beet demonstrates distinctive physiological adaptations to drought, such as extensive root systems that enhance water extraction and osmotic regulation through the accumulation of betaine. Nevertheless, extended periods of water shortage hinder sucrose transport and root biomass[3].

Water shortages affect around one-third of the world's agricultural land, significantly impacting crop yields[4,5].

Climate change's expected rise in global temperatures of 2 to 5 °C by 2050 is likely to intensify these challenges, especially in tropical, semi-arid, and coastal areas, potentially jeopardizing global food security[6,7].

This situation has made drought stress a critical global challenge, disrupting plant growth by altering biochemical and metabolic processes, which leads to significant reductions in agricultural productivity. Drought stress leads to the overproduction of reactive oxygen species (ROS), resulting in oxidative stress that damages cellular membranes and disrupts vital processes, such as photosynthesis and mineral absorption,[8–10]. To reduce the damage caused by ROS, plants have evolved various adaptive strategies, which involve the activation of antioxidant enzymes and the accumulation of osmoprotectants, such as glycine betaine and proline[11–13]. Tus Finding effective technological solutions is essential for mitigating the adverse impacts of severe drought stress on plant yield and quality in regions with scarce water supplies. Various approaches have been explored to enhance plants' ability to withstand drought. One solution is the use of compounds that promote abiotic stress tolerance.

Proline is an essential amino acid that serves as an osmoregulator, helping to mitigate the effects of stressors such as drought. It improves growth and physiological traits such as chlorophyll concentration, relative water content, and yield production[14,15]. The external application of proline promoted plant growth under stress conditions in calendula and barley plants and reduced oxidative damage by mitigating the detrimental effects of ROS[15,16]. It has been suggested that when faced with abiotic stress, the use of proline leads to changes at both the structural and ultrastructural levels in the affected plants, such as enhancing the root surface to cope with deficiencies in water and nutrients[17]. Interestingly, proline had a beneficial impact by improving the number of roots and causing structural changes in the stems and leaves of rice plants subjected to salt stress. Furthermore, the use of proline enhanced water management and the production of sugar beets in conditions of drought[18].

Lately, there has been significant interest in using natural compounds externally to enhance a plant's resistance to drought. Biostimulants, including chitosan, are among these compounds[19,20]. Chitosan is a biodegradable material that is a natural biopolymer and an amino polysaccharide commercially obtained from aquatic crustaceans' exoskeletons[20]. It is recognized that chitosan can enhance plant protection against oxidative stress[13]. The use of chitosan at lower concentrations (0.2 mg L^{-1}) can mitigate the impacts of drought stress and enhance lettuce growth[21]. Research has shown that chitosan can function as an elicitor of signaling pathways related to the control of stomatal opening and closing, which are crucial processes for plants experiencing water limitations[22,23]. Conversely, this polymer triggers defensive actions against oxidative stress through both enzymes and non-enzymatic antioxidants, like ascorbate, while also minimizing lipid peroxidation and the production of H_2O_2[24] . The use of chitosan has been assessed in various crops that are important for agriculture[25–27]. Recent studies indicate that the use of biostimulants (such as chitosan and proline combined) could lead to enhanced stress tolerance through synergistic effects. For example, applying both treatments to wheat increased ROS scavenging by 40% when compared to using them separately[28]. Nevertheless, these synergistic interactions have yet to be investigated in sugar beet under actual field situations.

Excessive chemical use harms nutrient efficiency and causes environmental issues. To address this, optimizing fertilization practices and exploring alternatives like biofertilizers and nanotechnology are essential.

Nanoparticles, due to their unique physical and chemical properties, including nanometric size and high reactivity, have been recognized as effective tools for improving nutrient uptake in plants and reducing environmental pollution compared to conventional chemical fertilizers[4,29]. Among them, zinc oxide nanoparticles (ZnO–NPs) play a key role in facilitating zinc uptake and improving the nutritional status of plants under various environmental stresses, such as drought and salinity, due to their highly active surface and nanoscale size[30]. In addition to enhancing zinc absorption, these nanoparticles strengthen antioxidant enzyme activities, improve photosynthesis, and increase plant resilience against abiotic stresses, which ultimately leads to better plant performance and enhanced agricultural product quality[29,31]. Given that ZnO–NPs improve nutrient uptake and plant performance, their significance extends beyond agriculture, offering a promising solution to address one of the major global challenges: micronutrient deficiency and hidden hunger, particularly in developing countries, which have far-reaching adverse effects on human health and agricultural productivity[32–35]. In this context, numerous studies have demonstrated that the use of ZnO–NPs under abiotic stress conditions leads to increased plant performance and enhanced nutrient uptake. For example, Dimkpa et al.[36] examined the effect of ZnO–NPs at concentrations of 1, 3, and 5 mg kg^{-1} soil on sorghum plants under drought stress and reported that the application of these nanoparticles resulted in a 22–183% increase in seed yield, 16–30% increase in nitrogen and potassium uptake, and a 94% increase in zinc uptake. Similarly, Shirvani-Naghani et al.[37] investigated the effects of ZnO–NPs at concentrations of 50, 100, and 200 mg L^{-1} and zinc sulfate ($ZnSO_4$) at 400 mg L^{-1} on soybean plants under two different moisture levels, showing that the application of ZnO–NPs led to a 33.1% increase in chlorophyll a, 20.7% in chlorophyll b, enhanced antioxidant enzyme activity, a 25% increase in seed yield, and a 39% decrease in proline content. Research on ZnO-NPs in sugar beet highlights their potential to improve drought tolerance by enhancing root growth and reducing oxidative stress[3]. They are also more effective in addressing zinc deficiency compared to conventional fertilizers[38]. However, the optimal application rates and long-term effects on sugar beet physiology are still unclear.

Months	Average temperature (C°)		Total evaporation (mm)		Total precipitation (mm)	
	2023	2024	2023	2024	2023	2024
March	10.2	9.2	1	0	22.2	20
April	15.4	14.5	113	112	5.4	2.1
May	15.2	17	190	190	7.22	9.1
June	22.1	21	295	280	3.4	2.2
July	25.2	24.8	280	273	1.2	3.8
August	24. 3	23.5	270	270	0	0.1
September	20.2	22	210	210	3.7	5.5
October	12.6	14.4	12.0	143	2.2	0.1
November	11.4	12.5	87	90	60.2	52

Table 1. Meteorological information in the experimental site in 2023–2024 growing seasons.

Parameters	Sp %	EC (ds/m)	F.C 1/3 A+	W.P	B.D	pH	T. N. V %	O.C%	N%	P (ppm)	K (ppm)	Sand %	Silt %	Clay %	Soil texture
Value	43	1.38	27.1	12.6	1.3	8.15	4.72	1.21	0.15	14.9	442	16	56	28	Silt clay loam

Table 2. Physical and chemical analysis of the experimental soil.

In recent years, the use of IBR and IBRv2 indices has gained attention in various studies focused on environmental and ecological aspects, particularly concerning a wide array of biotic and abiotic stressors. Although these indices are gaining popularity, research on their application in assessing the impact of biotic and abiotic stressors on specific crops, such as sugar beet, remains limited. Only a few studies have been conducted on other plant species[39–41]. The absence of research highlights the need for further investigation and understanding of the potential advantages and limitations of IBR/IBRv2 indexes in assessing the effects of drought stress on agricultural crops, particularly in the case of sugar beet plants. Although IBRv2 has previously been utilized to evaluate water deficit stress in sugar beet[42], its use particularly with combinations of nanoparticles and biostimulants has not been explored before. This index may serve as a consistent measure for assessing the effectiveness of different treatments.

Our current research aimed to investigate the possible impacts of applying various levels of ZnO NPs and biostimulants—specifically chitosan, proline, and a combination of chitosan and proline—on the widely grown Iranian sugar beet variety, "Shokofa," under water deficit stress over two years (2022–2023 and 2023–2024). By focusing on 17 distinct morpho-physiological and yield-related characteristics, we aim to comprehend and measure the effects of these treatments on sugar beet plants. Our research indicated that the IBRv2 index might function as a dependable measure, where a higher index signifies a greater level of oxidative stress. In comparison, a lower index reflects effective management of drought stress. By employing IBRv2, we aim to highlight the potential of these treatments as viable solutions to combat the detrimental impacts of water deficit stress in sugar beet plants.

Many studies on ZnO-NPs and biostimulants are conducted in controlled environments[38] , which limits their practical application. Our two-year field trial fills this gap by assessing the real-world scalability of sugar beet production.

Materials and methods

Plant material and experimental design

This two-year research was conducted at the Faculty of Agriculture of Mahabad Azad University field in Northwestern Iran during the growing seasons from 2022 to 2024. The study was conducted at an elevation of 1320 m above sea level, with coordinates 36°10'N and 45°43'E. This research location experiences 360 mm of annual precipitation and maintains an average temperature of 11 °C, with highs reaching 16.5 °C and lows dropping to 4.3 °C (Table 1). Table 2 presents the physicochemical properties of soil samples collected from the experimental site at a depth of 0–30 cm. The cultivar used in this experiment was Shokofa. The Shokofa variety, a monogram resistant to rhizomania and nematodes, is the most widely used sugar beet cultivar in Iran. It was sourced from the Sugar Beet Seed Institute (SBSI) in Karaj, Iran.

The planting took place in the first half of April in both years. Annually, the experimental field was provided with 150 kg ha^{-1} of P_2O_5, 100 kg ha^{-1} of K_2SO_4, and 300 kg ha^{-1} of nitrogen in the form of urea. Each plot consisted of four rows of plants, each 5 m long, with 0.50 m between the rows and 0.2 m between the individual plants in each row. During the 4–6 leaf stage, the plants were spaced 0.20 m apart. We managed weed control through manual weeding as needed.

Experimental design and application of treatments

The experiment used a split-split plot approach grounded in a Randomized Complete Block Design (RCBD) with three replications. The main plot was assigned Irrigation conditions at two levels (Normal (WW) and water

deficit stress (WD)). Zinc nanoparticle (ZnO-NPs) levels (control, 2, and 4 mg L^{-1}) were assigned to subplots, and biostimulants (control, chitosan, proline, and chitosan + proline) were assigned to sub-subplots.

All plots were watered simultaneously, immediately after planting.

All plots were irrigated simultaneously immediately after planting. Irrigation after 60 and 120 mm of evaporation was considered as well-watered (WW) and water-deficient (WD)[18]. After the plants were fully established, water stress was applied starting from the 8-leaf stage. Under normal irrigation conditions, the amount of water consumed was 10,200 m^3, while under water stress conditions, it was only 5,900 m^3.

Foliar application of ZnO-NPs, at a concentration of 2 and 4 mg L^{-1} (On the recommendation of its manufacturer, RochChemi Company (Tehran, Iran), and chitosan and proline, at a concentration of 0.5% and 150 ppm, respectively was done at two stages after the water defect stress and at the 12-leaf and 20-leaf stages[43]. The control treatments received applications of distilled water for both the ZnO-NPs and biostimulant treatments.

To assess the traits, samples were gathered from the fourth and fifth leaves of each experimental treatment in October, just before harvesting. The samples from each treatment were wrapped in aluminum foil, labeled, and frozen in liquid nitrogen at − 196 °C before being transferred to a − 80 °C freezer.

Measurements

Relative water content (RWC) assay

The RWC was evaluated in completely matured upper leaves. The fresh leaf weight was measured after cutting to determine the fresh weight (FW). All samples were immersed in distilled water and maintained at 4 °C for 24 h. Afterward, the turgid weight[44] was measured, and the leaf samples were then dried to determine the dry leaf mass[45]. The relative water content was calculated using a particular formula[46].

$$\mathrm{RWC} = \left(\frac{FW - DW}{TW - DW}\right) \times 100 \quad (1)$$

Stomatal conductivity assay

The SC-1 Leaf Porometer (Decagon Devices, Pullman, WA) was used to measure stomatal conductance[47], a crucial element in plant development and water management. This approach to measurement was utilized from 10 a.m. to 1 p.m. on the top surface of the leaf, selected for its ideal sunlight exposure. To guarantee precision and reduce the possibility of bias, each plant was measured three times, resulting in four plants per plot, with each growing season repeated only once[48].

Proline and beta-glycine content assay

Proline concentration is vital for understanding plant stress responses. Bates et al.[49] established the method for accurately measuring proline levels in sugar beet leaf samples. In this approach, 25 mg of the frozen leaf samples was first homogenized in 10 ml of 3% sulfosalicylic acid, then centrifuged for 15 min at 14,000 × g, and the supernatant was collected separately.

For each sample, 2.0 ml of the resulting supernatant was combined with an equal volume of acid ninhydrin and glacial acetic acid, then incubated in an oven at 100 °C for 1 h. After completing the reaction by cooling in an ice bath, the reaction mixture was extracted with 4 ml of toluene. The toluene layer was then eliminated, the upper chromophore layer was gathered, and its absorbance was recorded at 520 nm using a UV–Vis spectrophotometer (UV-1800 240 V, Shimadzu Corporation, Kyoto, Japan). Finally, the concentration of proline was measured using a proline standard curve, with the results expressed in milligrams per gram of fresh weight (mg g FW^{-1}).

The concentrations of glycine betaine in leaf tissue samples were assessed using the original periodide technique described by Shaw et al.[50], with modifications made by Chołuj et al.[51].To start, 1.0 g of fresh leaf tissue was blended with 25 ml of 80% ethanol in a 50 ml Falcon tube. The ethanol fraction obtained was evaporated, and the homogenate was then mixed with deionized water. Afterward, 200 µl of the chilled KI-I2 solution was combined with 500 µl of the homogenate, and the resulting mixture was well-mixed before being placed in storage overnight at 4 °C.

Following 15 min of centrifugation in a microcentrifuge, the supernatant was gently discarded, ensuring the betaine periodide complex remained adhered to the walls and base of the tube. The resultant residue was subsequently re-dispersed in 1,2-dichloroethane and moved into a 10 ml graduated tube. The samples were kept in dark conditions at room temperature for 2 h, and the absorbance was recorded at 365 nm. Finally, the concentrations of glycine betaine in the samples were measured using a glycine betaine standard curve, with the results expressed in milligrams per gram of fresh weight (mg g FW^{-1}).

Measurement of photosynthetic pigments

The Lichtenthaler[52] method was employed to assess chlorophyll a, chlorophyll b, and carotenoid levels by utilizing a spectrophotometer to evaluate absorbance at wavelengths of 663, 646, and 470 nm. In the end, the formulas provided below were used to measure each pigment Islam et al.[53]:

Cholorophyll a(mg g^{-1} FW) = 12.25(A663.2) – 2.79(A646.8).

Cholorophyll b(mg g^{-1} FW) = 21.50(A646.2) – 5.10(A663.2).

Carotenoid (mg g^{-1} FW) = (1000(A470) – 1.8(Chl a) – 85.02(Chl b))/198.

Determination of antioxidant enzyme activities

A 500 mg portion was combined with 5 mL of 50 mM sodium phosphate buffer solution (pH 7.8) using a pre-chilled mortar and pestle. The solution was centrifuged at 15,000 × g for a duration of 20 min at a temperature of

4 °C. Following the collection of the supernatant, the enzyme extract was stored at 4 °C for subsequent analysis. The activities of antioxidant enzymes were assessed using the following methods.

The activity of superoxide dismutase (SOD; EC 1.15.1.1) was assessed by evaluating the reduction of nitroblue tetrazolium (NBT) at 560 nm, where one unit of SOD is defined as the enzyme amount necessary to achieve 50% inhibition of NBT reduction[54].

The activity of ascorbate peroxidase (APX; EC 1.11.1.11) was measured by observing the oxidation of ascorbate at 290 nm ($\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$) in a reaction mixture that included 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.1 mM H_2O_2, and enzyme extract[55].

Catalase (CAT; EC 1.11.1.6) activity was assessed by monitoring the breakdown of H_2O_2 at 240 nm ($\varepsilon = 39.4\ M^{-1}\ cm^{-1}$) in a 50 mM potassium phosphate buffer (pH 7.0) containing 15 mM H_2O_2[56]. All spectrophotometric analyses were conducted at a temperature of 25 °C utilizing a UV–visible spectrophotometer, with enzyme activities reported as units per milligram of protein (U mg^{-1} protein).

Determination of malondialdehyde (MAD) and hydrogen peroxide (H_2O_2)

To determine the MAD, 250 mg of leaf samples were ground in 5 mL of 0.1% trichloroacetic acid, after which they were centrifuged at 12,000 × g for 10 min at 4˚C and kept at 4˚C for further analysis. Malondialdehyde (MDA) concentration in the sugar beet leaves was utilized to indicate lipid peroxidation[53]. The concentration of hydrogen peroxide (H2O2) was evaluated following the method described by. After adding 0.5 mL of supernatant to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide, the combination was allowed to sit in the dark for 20 min. The combination's absorbance was measured at 390 nm, and the concentration of H2O2 was determined using a calibration curve derived from various concentrations of H_2O_2.

Measurement of plant hormones

To quantify cytokinins (CK), gibberellic acid (GA3), indole-3-acetic acid (IAA), and abscisic acid (ABA), fresh leaf tissue weighing 0.1 to 0.5 g was snap-frozen in liquid nitrogen and stored at -80 °C. Hormones were extracted using cold 80% methanol (v/v) with 1% acetic acid. This was followed by homogenization, sonication for 15 min, and centrifugation at 12,000 × g for 15 min at 4 °C. The supernatant was purified using solid-phase extraction with C18 columns, and the eluate was concentrated under nitrogen gas. Quantification is carried out using high-performance liquid chromatography-tandem mass spectrometry (LC–MS/MS), chosen for its high sensitivity and capability to detect multiple hormones simultaneously[57,58]. To enhance recovery, internal standards should be added prior to extraction. This approach allows accurate detection of hormonal changes under different physiological conditions, particularly in stress-related studies where ABA and CK are recognized as essential regulatory components[59].

Measurement of root yield

The last harvest occurred on October 20th for both years. Before harvesting, the two outer rows, 0.5 m from the beginning and end of each row, were eliminated. Four square meters were sampled from every experimental unit, and the roots were counted by hand. They were later weighed and classified as the root yield (RY).

Statistical analysis

The experimental setup was conducted as split-split plots using a Randomized Complete Block Design (RCBD) with three replications. The analysis of variance (ANOVA) and mean comparison (least significant differences, LSD; $p < 0.05$) were conducted using SAS version 9.4 (SAS Institute Inc., USA). Principal component analysis (PCA) was conducted using the FactoMineR package (v.1.34) in R Studio (v. 3.4.4)[60]. The IBRv2 index, which combines responses from multiple biomarkers by assessing their log-transformed values against a mean reference dataset, was computed utilizing the innovative IBRtools R package[61]. The IBRv2 assessment included measuring 17 distinct biomarkers in sugar beet plants cultivated in diverse treatments. The reference (Ref) site for this calculation was the "Control + Control" treatment, which involved growing sugar beet plants under standard conditions and spraying only distilled water. All treatments involving ZnO-NPs and biostimulants administered under WW conditions were excluded from the IBRv2 calculation. The values of IBRv2 for each biomarker were computed, where positive values signify an increase compared to the reference site and negative values denote a decrease.

Results

The combined analysis of variance revealed significant effects of irrigation levels and ZnO-NPs on all studied traits. Additionally, a significant interaction between year and irrigation was observed for carotenoid content, RWC, beta-glycine, ABA, SOD, and MAD. The interaction between ZnO-NPs and year significantly influenced MAD and hydrogen peroxide content. Biostimulant treatments exhibited significant differences for all traits except chlorophyll b. The interaction between year and biostimulants had a significant effect on chlorophyll b and hydrogen peroxide levels. Furthermore, the two-way interaction of irrigation and ZnO nanoparticles significantly influenced chlorophyll a, chlorophyll b, carotenoids, proline, beta-glycine, stomatal conductance, ABA, GA, IAA, CK, CAT, SOD, APX, MAD, hydrogen peroxide, and RY. Similarly, irrigation combined with biostimulants significantly affected chlorophyll a, chlorophyll b, carotenoid content, RWC, proline, beta-glycine, ABA, GA, IAA, CK, CAT, APX, MAD, hydrogen peroxide, and RY. Finally, the interaction between ZnO-NPs and biostimulants had a significant impact on chlorophyll *a*, chlorophyll *b*, RWC, proline, beta-glycine, stomatal conductance, ABA, IAA, GA, CK, CAT, SOD, MAD, and RY (The table is not included in the article text.).

Irrigation	ZnO-NPs	Chlorophyll a (mg g/ FW)	Chlorophyll b (mg g/ FW)	Carotenoid (mg g /FW)	Proline (mg g/FW)	Beta-glycine (mg g/ FW)	stomatal conductance(mol $m-^2/s$)	Abscisic acid (ppm)	Gibberellin (ppm)
Normal	Control	4.90 ± 0.02^{b}	2.08 ± 0.01^{c}	2.10 ± 0.012^{b}	0.23 ± 0.001^{e}	83.99 ± 0.180^{d}	14.74 ± 0.039^{b}	35.69 ± 0.056^{b}	198.58 ± 0.445^{bc}
	2 g/L	5.29 ± 0.01^{a}	2.99 ± 0.01^{a}	2.55 ± 0.011^{a}	0.24 ± 0.001^{e}	88.47 ± 0.221^{c}	15.72 ± 0.034^{a}	26.98 ± 0.090^{d}	211.44 ± 0.364^{a}
	4 g/L	4.35 ± 0.01c	2.53 ± 0.009^{b}	2.02 ± 0.010b	0.28 ± 0.001^{d}	88.94 ± 0.235^{c}	15.99 ± 0.042^{a}	31.37 ± 0.123^{c}	195.62 ± 0.547^{c}
Water deficit	Control	2.48 ± 0.01e	1.40 ± 0.01^{e}	1.67 ± 0.005^{d}	0.38 ± 0.001^{c}	90.47 ± 0.244^{c}	10.08 ± 0.039^{e}	35.75 ± 0.118^{b}	186.75 ± 0.258^{d}
	2 g/L	3.28 ± 0.02d	1.90 ± 0.01^{d}	2.01 ± 0.11^{bc}	0.41 ± 0.002^{b}	98.24 ± 0.285^{b}	11.09 ± 0.036^{d}	40.13 ± 0.083^{a}	185.70 ± 0.306^{d}
	4 g/L	2.69 ± 0.01e	1.72 ± 0.01^{d}	1.83 ± 0.009^{cd}	0.45 ± 0.001^{a}	102.42 ± 0.349^{a}	13.46 ± 0.039^{c}	31.58 ± 0.98^{c}	203.74 ± 0.351^{b}

Irrigation	ZnO-NPs	Auxin (ppm)	Cytokinin (ppm)	CAT (U mg/ protein)	SOD (U mg/ protein)	APX (mM mg/protein)	MAD (µM $mg_{/}FW$)	Hydrogen peroxide (µM mg/ FW)	Root yield (ton/ ha)
Normal	Control	31.26 ± 0.106^{b}	41.39 ± 0.123^{d}	2.44 ± 0.010^{d}	3.12 ± 0.012^{d}	3.91 ± 0.020^{d}	7.19 ± 0.016^{d}	2.73 ± 0.009d	67.12 ± 0.167b
	2 g/L	36.97 ± 0.120^{a}	50.46 ± 0.077^{a}	2.93 ± 0.007^{bc}	3.90 ± 0.012^{b}	5.02 ± 0.012^{b}	6.93 ± 0.015^{d}	2.62 ± 0.009d	74.55 ± 0.224a
	4 g/L	35.79 ± 0.106^{a}	47.44 ± 0.058^{b}	2.69 ± 0.011^{cd}	3.43 ± 0.017^{c}	4.62 ± 0.020c	7.05 ± 0.016^{d}	2.68 ± 0.009d	66.65 ± 0.158b
Water deficit	Control	28.22 ± 0.119^{c}	39.62 ± 0.089^{e}	3.18 ± 0.0092^{ab}	3.56 ± 0.013^{c}	5.05 ± 0.016^{b}	10.56 ± 0.079^{a}	5.18 ± 0.024a	52.44 ± 0.144d
	2 g/L	29.36 ± 0.117^{bc}	42.19 ± 0.069^{d}	3.25 ± 0.010^{a}	4.51 ± 0.012^{a}	5.16 ± 0.012^{ab}	9.38 ± 0.052^{b}	4.77 ± 0.027b	56.46 ± 0.162 cd
	4 g/L	30.08 ± 0.087^{bc}	44.80 ± 0.074^{c}	3.43 ± 0.0091^{a}	4.47 ± 0.013^{a}	5.32 ± 0.017^{a}	8.73 ± 0.045^{c}	4.29 ± 0.018c	57.94 ± 0.190c

Table 3. Mean comparison effect of irrigation with ZnO-NPs application interaction treatments on the studied traits of sugar beet cultivar Shokofa. Different superscript letters in the same column indicate significant differences ($p < 0.05$).

Irrigation	Biostimulants	Chlorophyll a (mg g/ FW)	Chlorophyll b (mg g/ FW)	Carotenoid (mg g/ FW)	Relative Water Content (%)	Proline (mg g/ FW)	Beta-glycine (mg g/ FW)	Abscisic acid (ppm)	Gibberellin (ppm)
Normal	Control	3.87 ± 0.02^{c}	2.04 ± 0.01^{b}	1.87 ± 0.010^{c}	66.45 ± 0.221^{c}	0.26 ± 0.001^{d}	87.67 ± 0.263^{cde}	37.50 ± 0.094^{b}	188.76 ± 0.480^{b}
	Chitosan (Ch)	4.63 ± 0.02^{b}	2.70 ± 0.02^{a}	2.15 ± 0.011^{b}	73.41 ± 0.267^{b}	0.25 ± 0.001^{e}	89.70 ± 0.380^{cd}	29.8 ± 0.1556^{e}	209.28 ± 0.600^{a}
	Proline (Pr)	5.42 ± 0.03^{a}	2.73 ± 0.01^{a}	2.65 ± 0.019^{a}	81.83 ± 0.380^{a}	0.23 ± 0.001f.	84.80 ± 0.227^{e}	29.38 ± 0.139^{ef}	216.01 ± 0.516^{a}
	Ch + Pr	5.46 ± 0.03^{a}	2.66 ± 0.02^{a}	2.22 ± 0.015^{b}	73.52 ± 0.297^{b}	0.25 ± 0.001^{e}	86.37 ± 0.260^{de}	28.65 ± 0.103f.	216.80 ± 0.628^{a}
Water deficit	Control	2.27 ± 0.02^{e}	1.54 ± 0.01^{d}	1.71 ± 0.011^{c}	57.92 ± 0.289^{e}	0.41 ± 0.002bc	90.97 ± 0.361^{bc}	42.09 ± 0.111^{a}	180.07 ± 0.414^{c}
	Chitosan (Ch)	2.86 ± 0.02^{d}	1.68 ± 0.02^{cd}	1.89 ± 0.013^{c}	62.65 ± 0.260^{cd}	0.42 ± 0.001^{ab}	94.37 ± 0.274^{b}	34.15 ± 0.153^{c}	194.56 ± 0.553^{b}
	Proline (Pr)	3.02 ± 0.03^{d}	1.68 ± 0.02^{cd}	1.91 ± 0.021^{c}	65.88 ± 0.360^{c}	0.40 ± 0.001^{c}	95.40 ± 0.434^{b}	35.11 ± 0.119^{c}	177.62 ± 0.369^{c}
	Ch + Pr	3.12 ± 0.03^{d}	1.79 ± 0.02^{c}	1.84 ± 0.014^{c}	59.75 ± 0.283^{de}	0.43 ± 0.002^{a}	107.43 ± 0.437^{a}	31.92 ± 0.124^{d}	192.68 ± 0.290^{b}

Irrigation	Biostimulants	Auxin (ppm)	Cytokinin (ppm)	CAT (U mg/ protein)	APX (mM mg/ protein)	MAD (µM $mg_{/}FW$)	Hydrogen peroxide (µM $mg_{/}FW$)	Root yield (ton/ ha)
Normal	Control	31.02 ± 0.151^{c}	42.53 ± 0.167^{c}	2.47 ± 0.011^{d}	4.18 ± 0.021f.	7.27 ± 0.023^{c}	2.82 ± 0.012^{d}	64.89 ± 0.131^{b}
	Chitosan (Ch)	31.38 ± 0.151^{c}	46.69 ± 0.143^{b}	2.65 ± 0.015^{cd}	4.55 ± 0.023^{e}	7.17 ± 0.020^{cd}	2.74 ± 0.013^{d}	71.52 ± 0.273^{a}
	Proline (Pr)	37.63 ± 0.163^{ab}	46.27 ± 0.150^{b}	2.85 ± 0.014^{c}	4.46 ± 0.022^{ef}	6.79 ± 0.025^{d}	2.39 ± 0.009^{e}	69.67 ± 0.378^{a}
	Ch + Pr	38.66 ± 0.096^{a}	50.23 ± 0.125^{a}	2.78 ± 0.015^{c}	4.88 ± 0.027^{cd}	7.00 ± 0.013^{cd}	2.75 ± 0.011^{d}	71.68 ± 0.229^{a}
Water deficit	Control	26.41 ± 0.121^{d}	39.09 ± 0.118^{d}	3.15 ± 0.014^{b}	4.69 ± 0.017^{de}	10.18 ± 0.101^{a}	5.04 ± 0.036^{a}	53.73 ± 0.176^{d}
	Chitosan (Ch)	27.81 ± 0.060^{d}	43.56 ± 0.098^{c}	3.20 ± 0.012^{b}	5.11 ± 0.023^{bc}	9.63 ± 0.069^{b}	4.72 ± 0.027^{b}	52.58 ± 0.260^{d}
	Proline (Pr)	26.89 ± 0.064^{d}	40.03 ± 0.154^{d}	3.28 ± 0.010^{ab}	5.30 ± 0.019^{b}	9.15 ± 0.071^{b}	4.75 ± 0.037^{b}	56.25 ± 0.209^{cd}
	Ch + Pr	35.76 ± 0.099^{b}	46.14 ± 0.100^{b}	3.51 ± 0.010^{a}	5.61 ± 0.021^{a}	9.26 ± 0.0884^{b}	4.46 ± 0.030^{c}	59.89 ± 0.222^{c}

Table 4. Mean comparison effect of irrigation with Biostimulants application interaction treatments on the studied traits of sugar beet cultivar Shokofa. Different superscript letters in the same column indicate significant differences ($p < 0.05$).

Chlorophyll *a*

The mean comparison results indicated that applying 2 mg L^{-1} ZnO-NPs under WW conditions resulted in the highest chlorophyll *a* content, averaging 5.29 mg g FW^{-1}. The lowest chlorophyll *a* content was also attributed to the control under WD conditions, with an average of 2.48 mg g FW^{-1} (Table 3).

In this experiment, foliar spraying of proline and chitosan + proline under WW conditions resulted in the maximum chlorophyll *a*, with averages of 5.42 and 5.46 mg g FW^{-1}, respectively. The lowest chlorophyll *a* content, with an average of 2.27 mg g FW^{-1}, was also associated with the biostimulants control under WD conditions. The results of the present experiment showed that foliar spraying of chitosan, proline, and chitosan + proline

increased chlorophyll *a* content by 19.63%, 40.05%, and 41.08% under WW conditions, and by 25.99%, 33.03%, and 37.44% under WD conditions compared to the corresponding control, respectively (Table 4).

Comparing the mean of ZnO-NPs with biostimulants interaction treatments showed that the application of 2 mg L^{-1} ZnO-NPs along with chitosan + proline foliar spray achieved the highest chlorophyll *a* content with an average of 4.89 mg g FW^{-1}. The difference between the mentioned treatment with 2 mg L^{-1} ZnO-NPs with proline foliar spray and the Zno-NPS control with proline was insignificant. Plots not treated with ZnO-NPs or biostimulants exhibited the lowest levels of chlorophyll *a*, averaging 2.72 mg g FW^{-1}. In this experiment, the simultaneous application of ZnO-NPs and biostimulants did not demonstrate significant superiority compared to their separate application (Table 5).

Chlorophyll *b*

A Comparison of the mean treatments revealed that applying 2 mg L^{-1} Zno-NPs in WW conditions led to the highest chlorophyll *b* content, averaging 2.99 mg g FW^{-1}. Plants that did not receive ZnO-NPs in WD conditions exhibited the lowest accumulation of this pigment, averaging 1.40 mg g FW^{-1}. In this study, the application of both levels of ZnO-NPs under the different irrigation conditions notably enhanced the chlorophyll *b* content in comparison to the respective control (Table 3).

Among the irrigation-biostimulant interaction treatments, foliar spraying of chitosan, proline, and the combination of both achieved the maximum chlorophyll *b* content under WW conditions, with averages of 2.70, 2.73, and 2.66 mg g FW^{-1}, respectively. The plants that were not treated with sprays under WD conditions showed the lowest chlorophyll *b* content, averaging 1.54 mg g FW^{-1}. The variation between this treatment and the chitosan and proline foliar spray treatments under WD conditions was not statistically significant. The findings of the current research indicated that applying chitosan + proline enhanced chlorophyll *b* levels by 30.39% and 16.23% in comparison to the control group under WW and WD conditions, respectively (Table 4).

In this experiment, foliar spraying of chitosan, proline, and chitosan + proline with 2 mg L^{-1} ZnO-NPs showed the highest chlorophyll *b* content with an average of 2.47, 2.59, and 2.65 mg g FW^{-1}, respectively. The

ZnO-NPs	Biostimulants	Chlorophyll a (mg g/ FW)	Chlorophyll b (mg g/ FW)	Relative Water Content (%)	Proline (mg g/ FW)	Beta-glycine (mg g/ FW)	Stomatal conductance(mol $m-^2$ /s)	Abscisic acid (ppm)
Control	Control	2.72 ± 0.07^{d}	1.54 ± 0.03f.	58.93 ± 0.787f.	0.31 ± 0.005f.	93.87 ± 0.890^{bc}	11.09 ± 0.258f.	41.84 ± 0.281^{a}
	Chitosan (Ch)	3.54 ± 0.08^{c}	1.76 ± 0.06^{ef}	63.47 ± 0.600^{def}	0.31 ± 0.007f.	87.07 ± 0.769^{d}	12.49 ± 0.261^{de}	38.15 ± 0.260^{b}
	Proline (Pr)	4.54 ± 0.12^{ab}	1.73 ± 0.06^{ef}	71.90 ± 0.812^{bc}	0.28 ± 0.007^{g}	79.12 ± 0.742^{e}	11.82 ± 0.216^{ef}	36.70 ± 0.286^{c}
	Ch + Pr	4.37 ± 0.11^{b}	1.94 ± 0.05^{de}	59.81 ± 0.781^{ef}	0.32 ± 0.009^{def}	88.87 ± 0.776^{cd}	14.23 ± 0.210^{abc}	34.93 ± 0.238^{d}
2 g/L	Control	3.49 ± 0.08^{c}	2.06 ± 0.04^{cd}	66.60 ± 0.684^{d}	0.33 ± 0.008^{cde}	80.77 ± 0.584^{e}	12.46 ± 0.239^{de}	38.64 ± 0.505^{b}
	Chitosan (Ch)	4.18 ± 0.08^{b}	2.47 ± 0.06^{ab}	76.72 ± 0.710^{b}	0.33 ± 0.10^{cd}	98.02 ± 0.766^{ab}	13.03 ± 0.244^{cde}	29.3`5 ± 0.332f.
	Proline (Pr)	4.58 ± 0.12^{ab}	2.59 ± 0.06^{a}	84.80 ± 1.390^{a}	0.33 ± 0.10^{cd}	93.22 ± 1.134^{bc}	14.11 ± 0.276^{abc}	29.18 ± 0.555f.
	Ch + Pr	4.89 ± 0.12^{a}	2.65 ± 0.05^{a}	73.15 ± 1.262^{b}	0.31 ± 0.007f.	93.32 ± 0.998^{bc}	14.54 ± 0.223^{ab}	28.30 ± 0.317^{fg}
4 g/L	Control	3.01 ± 0.07^{d}	1.98 ± 0.03^{de}	61.02 ± 0.964^{ef}	0.37 ± 0.008^{ab}	91.02 ± 0.936^{cd}	13.71 ± 0.174^{bcd}	38.91 ± 0.175^{b}
	Chitosan (Ch)	3.51 ± 0.08^{c}	2.13 ± 0.05^{cd}	63.90 ± 0.931^{def}	0.36 ± 0.007^{b}	97.97 ± 1.240^{ab}	14.43 ± 0.175^{ab}	28.51 ± 0.390^{fg}
	Proline (Pr)	3.53 ± 0.10^{c}	2.30 ± 0.04^{bc}	64.85 ± 1.054^{de}	0.34 ± 0.008^{c}	100.42 ± 0.827^{a}	15.07 ± 0.190^{ab}	30.85 ± 0.161^{e}
	Ch + Pr	3.61 ± 0.11^{c}	2.09 ± 0.05^{cd}	66.94 ± 0.737^{cd}	0.38 ± 0.010^{a}	101.42 ± 1.944^{a}	15.15 ± 0.206^{a}	27.63 ± 0.129^{g}

ZnO-NPs	Biostimulants	Auxin (ppm)	Gibberellin (ppm)	Cytokinin (ppm)	CAT (U mg/ protein)	SOD (U mg/ protein)	MAD (µM $mg_{/}$FW)	Root yield (ton/ ha)
Control	Control	26.15 ± 0.503^{g}	179.42 ± 1.57^{e}	35.88 ± 400f.	2.59 ± 0.049^{e}	3.35 ± 0.046^{e}	9.49 ± 0.319^{a}	58.74 ± 0.890^{d}
	Chitosan (Ch)	28.01 ± 0.229^{fg}	183.37 ± 1.030^{de}	41.48 ± 0.207^{de}	2.83 ± 0.058d^{e}	3.34 ± 0.051^{e}	8.48 ± 0.242^{bc}	57.96 ± 0.781^{d}
	Proline (Pr)	29.17 ± 0.455^{ef}	188.93 ± 1.305^{cde}	39.13 ± 0.380^{ef}	2.99 ± 0.032^{bcd}	2.93 ± 0.042f.	8.71 ± 0.236^{b}	59.48 ± 0.751^{d}
	Ch + Pr	35.63 ± 0.145^{bc}	208.71 ± 1.987^{a}	45.54 ± 0.277^{bc}	2.85 ± 0.054^{cde}	3.82 ± 0.039^{d}	8.82 ± 0.288^{b}	62.95 ± 1.086^{bcd}
2 g/L	Control	29.74 ± 0.356^{ef}	189.72 ± 1.068^{cd}	43.27 ± 0.330^{cd}	3.02 ± 0.040^{bcd}	3.87 ± 0.048^{d}	8.58 ± 0.220^{bc}	58.34 ± 0.605^{d}
	Chitosan (Ch)	29.68 ± 0.476^{ef}	208.66 ± 2.050^{a}	47.37 ± 0.456^{ab}	2.98 ± 0.026^{bcd}	4.26 ± 0.041^{abc}	8.44 ± 0.182^{bc}	67.66 ± 1.190^{ab}
	Proline (Pr)	33.91 ± 0.714^{c}	195.15 ± 2.507^{c}	45.12 ± 0.492^{bc}	$3.24 \pm 0.027_{ab}$	4.32 ± 0.057^{a}	7.61 ± 0.153^{d}	67.24 ± 1.433^{abc}
	Ch + Pr	30.26 ± 0.220^{ef}	208.17 ± 1.602^{a}	43.29 ± 0.384^{cd}	3.13 ± 0.043^{bc}	4.30 ± 0.039^{ab}	8.00 ± 0.186^{cd}	68.77 ± 0.821^{a}
4 g/L	Control	31.10 ± 0.185^{de}	184.11 ± 1.408^{de}	46.53 ± 0.207^{abc}	2.83 ± 0.048^{de}	3.95 ± 0.028^{cd}	8.12 ± 0.197^{cd}	60.84 ± 0.441^{d}
	Chitosan (Ch)	33.70 ± 0.391^{cd}	213.72 ± 1.104^{a}	45.19 ± 0.129^{bc}	2.97 ± 0.049^{bcd}	3.97 ± 0.053^{bcd}	8.27 ± 0.124^{bc}	59.41 ± 1.211^{d}
	Proline (Pr)	36.68 ± 0.653^{ab}	206.35 ± 2.191^{ab}	49.48 ± 0.277^{a}	2.97 ± 0.057^{bcd}	$4.05 \pm 0.076^{a-d}$	7.59 ± 0.160^{d}	61.75 ± 0.652^{cd}
	Ch + Pr	39.32 ± 0.279^{a}	197.34 ± 1.680^{bc}	49.54 ± 0.245^{a}	3.47 ± 0.033^{a}	$4.00.0.079^{a-d}$	7.57 ± 0.122^{d}	67.17 ± 0.515^{abc}

Table 5. Mean comparison effect of ZnO-NPs with Biostimulants application interaction treatments on the studied traits of sugar beet cultivar Shokofa. Different superscript letters in the same column indicate significant differences ($p < 0.05$).

ZnO-NPs and biostimulants control exhibited the lowest chlorophyll *b* content, averaging 1.54 mg g FW^{-1}; the difference between this treatment and the treatments using chitosan and proline foliar sprays, as well as the ZnO-NPs control, was not significant. In this study, the combined use of chitosan, proline, and chitosan + proline at a concentration of 2 mg L^{-1} ZnO-NPs showed a notable benefit compared to the individual application of each biostimulant treatment and the levels of ZnO-NPs (Table 5).

Carotenoid

The findings from the mean comparison indicated that applying 2 mg L^{-1} of ZnO-NPs under WW conditions resulted in the highest carotenoid content, averaging 2.55 mg g FW^{-1}. Conversely, the control subjected to WD conditions exhibited the lowest carotenoid content, with an average of 1.67 mg g FW^{-1}. In this study, the application of ZnO-NPS to the leaves under both WW and WD conditions led to a rise in carotenoid content by 19.04% and 25.00%, respectively, when compared to the control (Table 3).

The results showed that foliar spraying of proline under WW irrigation conditions yielded maximum carotenoids with an average of 2.56 mg g FW^{-1}. The lowest carotenoid content was associated with the biostimulants control under water stress conditions, with an average of 1.71 mg g FW^{-1}. The difference between this treatment and the chitosan, proline, and chitosan + proline foliar spray under WD and the control under WW conditions was not significant. It should be noted that under WW conditions, foliar spraying of chitosan, proline, and chitosan + proline increased the carotenoid content by 14.97%, 41.71%, and 18.71% , respectively, compared to the control. Under WD conditions, the difference between the control and biostimulant foliar spraying treatments was not significant (Table 4).

Relative water content (RWC)

Comparing the means of different irrigation and biostimulant interaction treatments revealed that applying proline under WW conditions resulted in the highest RWC, with an average of 81.83%. The biostimulant control exhibited the lowest RWC during WD conditions, averaging 57.92%. There was no significant difference between this treatment and the foliar spray of chitosan + proline when subjected to WD conditions (Table 4).

Under WW conditions, the application of chitosan, proline, and chitosan + proline, and under WD conditions, foliar application of chitosan and proline had significant superiority over the control. Foliar spraying with proline, combined with a concentration of 2 mg L^{-1} ZnO-NPS, resulted in the highest average RWC of 84.80%. This treatment demonstrated a significant benefit compared to applying each component separately. The plots that did not receive biostimulants and ZnO-NPs showed the lowest average RWC content, measuring 58.93%. The difference between this treatment and both the control and the chitosan foliar spray treatment with 2 mg L^{-1} ZnO-NPs was not statistically significant (Table 5).

Proline

In this study, plots treated with 4 mg L^{-1} ZnO-NPs and subjected to WD exhibited the greatest proline concentration, averaging 0.45 mg g FW^{-1}**.** The control and foliar spray treatments at 2 mg L^{-1} of ZnO-NPs under WW conditions resulted in the lowest proline content, with average values of 0.23 and 0.24 mg g FW^{-1}, respectively. In this research, the application of 4 mg L^{-1} ZnO-NPs elevated the proline levels by 21.73% and 18.42%, respectively, in comparison to the corresponding control (Table 3).

The results of the treatment interaction indicated that chitosan and chitosan + proline foliar sprays under WD conditions achieved the maximum proline content with an average of 0.42 and 0.43 mg g FW^{-1}, respectively. Plants treated with proline under WW conditions had the lowest proline content, averaging 0.23 mg g FW^{-1}. In our research, using all three biostimulant treatments under WW conditions and foliar application of chitosan and proline under WD resulted in elevated proline levels (Table 4).

An analysis of the average from the interaction treatments revealed that plots treated with 2 mg L^{-1} ZnO-NPs and sprayed with a combination of chitosan and proline exhibited the highest proline content, averaging 0.39 mg g FW^{-1}. The control plots and plots sprayed with chitosan, along with ZnO-NPs control, as well as plants sprayed with chitosan + proline and receiving 2 mg L^{-1} ZnO-NPs, had the lowest proline content, with an average of 0.31 mg g FW^{-1} (Table 5).

Beta-glycine

In the current research, the application of 4 mg L^{-1} ZnO-NPs under WD conditions resulted in the highest beta-glycine.

content, averaging 102.42 mg g FW^{-1}. The minimum level of this compound, averaging 83.99 mg g FW^{-1}, was linked to the ZnO-NPs control under WW conditions. In this study, the application of 2 and 4 mg L^{-1} of ZnO-NPs raised the beta-glycine content by 5.33% and 5.89% under WW conditions, respectively, while under WD conditions, it increased by 8.58% and 13.20%, respectively (Table 3).

The current study's results indicate that foliar application of chitosan + proline under WD conditions resulted in the highest beta-glycine content, averaging 107.43 mg g FW^{-1}. The lowest beta-glycine content, averaging 84.80 mg g FW^{-1}, was attributed to the proline treatment under WW conditions. The difference between the aforementioned treatment with chitosan + proline foliar spray and the control under WW conditions was insignificant. Under WW conditions, the difference between the control and foliar spray treatments regarding beta-glycine content was insignificant, while under WD conditions, foliar spray of chitosan + proline increased beta-glycine content by 17.66% compared to the corresponding control (Table 4).

In this study, applying chitosan combined with proline, proline alone, and 4 mg L^{-1} ZnO-NPs led to the highest beta-glycine content, averaging 101.42 and 100.42 mg g FW^{-1}, respectively. The difference between the aforementioned treatments with chitosan spraying combined with 2 and 4 mg L^{-1} ZnO-NPs was insignificant. The lowest beta-glycine content was attributed to the proline foliar spray treatment with ZnO-NPs control and

the biostimulants control with 2 mg L^{-1} ZnO-NPs level, averaging 79.12 and 80.77 mg g FW^{-1}, respectively (Table 5).

Leaf stomatal conductance coefficient

The findings of this study indicated that the application of 2 and 4 mg L^{-1} ZnO-NPs under WW conditions resulted in the highest coefficients of stomatal conductance, averaging 15.72 and 15.92 mol m^{-2} s^{-1}, respectively. In contrast, the control with ZnO-NPs under WD conditions recorded the lowest average at 10.08 mol m^{-2} s^{-1}(Table 3). It should be noted that the application of 2 and 4 mg L^{-1} ZnO-NPs increased stomatal conductance by 6.64% and 8.48% under WW conditions, respectively, and by 10.01% and 8.84% under WD conditions, respectively.

In this study, while the foliar application of chitosan + proline with 4 mg L^{-1} ZnO-NPs resulted in the highest stomatal conductance coefficient of 15.15 mol m^{-2} s^{-1}, the difference between this treatment and the treatments with proline and chitosan at both 2 and 4 mg L^{-1} ZnO-NPs was not statistically significant. The biostimulants control and proline spraying, with ZnO-NPs control achieved the index's lowest values, with an average of 11.09 and 11.82 mol m^{-2} s^{-1}, respectively (Table 5).

Plant hormones

Abscisic acid (ABA)

The findings from the average comparison indicated that plants exposed to 2 mg L^{-1} ZnO-NPs during WD exhibited the highest level of ABA, averaging 40.13 ppm. In contrast, plants that received 2 mg L^{-1} ZnO-NPs while under WW showed the lowest ABA content, averaging 26.98 ppm. In this research, levels 2 and 4 mg L^{-1} of ZnO-NPs decreased ABA content by 24.40% and 12.10% under WW conditions, respectively. Under water WD conditions, this reduction was limited to only 4 mg L^{-1} ZnO-NPs (21.30%) (Table 3).

In this experiment, the biostimulant control under WD conditions had the highest ABA content with an average of 28.65 ppm. The lowest ABA content, with an average of 28.65 and 29.38 ppm, was attributed to the chitosan + proline and proline foliar spray treatments under WW conditions. All three biostimulants foliar spray treatments significantly increased ABA content in this experiment compared to the corresponding control under both irrigation conditions (Table 4).

This study's findings revealed that plots that did not receive both ZnO-NPs and biostimulants reached the highest average ABA content of 41.84 ppm. The lowest ABA content, with an average of 27.63 and 28.51 ppm, was assigned to the chitosan + proline and proline treatment with a level of 4 mg L^{-1} ZnO-NPs. The difference between the aforementioned treatment and the foliar spraying treatment of chitosan + proline and proline treatment with a level of 2 mg L^{-1} ZnO-NPs was not significant. It is important to highlight that using ZnO-NPs in conjunction with biostimulants significantly reduced ABA content, showing a clear benefit over applying each treatment separately (Table 5).

Gibberellin (GA)

The findings indicated that utilising 2 mg L^{-1} ZnO-NPs under WW conditions resulted in the highest GA content, averaging 211.44 ppm. The control and application of 2 mg L^{-1} ZnO-NPs exhibited the lowest GA content, with average values of 486.75 and 185.70 ppm, respectively. In this research, the use of 2 mg L^{-1} ZnO-NPs under WW conditions and 4 mg L^{-1} ZnO-NPs under WD conditions notably enhanced the GA content in comparison to the respective control (Table 3).

The findings indicated that applying chitosan, proline, and chitosan + proline under WW conditions resulted in the highest GA, averaging 209.28, 216.01, and 216.8 ppm, respectively. The control and proline foliar sprays under WD conditions had the lowest GA content, averaging 180.07 and 177.62 ppm, respectively. The results showed that the GA content in plants treated with proline and chitosan + proline was significantly higher than that of the corresponding control in both environmental conditions (Table 4).

The findings indicated that the greatest GA content, averaging 213.72 ppm, was linked to the treatment of chitosan foliar spray used in conjunction with applying 4 mg L^{-1} ZnO-NPs. There was no significant difference between the aforementioned treatment and the treatments of chitosan and chitosan + proline application, along with the application of 2 mg L^{-1} ZnO-NPs and foliar spraying of 4 mg L^{-1} ZnO-NPs, along with proline foliar spraying and the control ZnO-NPs, along with chitosan + proline foliar spraying. The lowest GA content, with an average of 179.42 ppm, was assigned to the control of both treatments. There was no significant difference between the aforementioned treatments, with chitosan foliar spraying, proline foliar spraying with ZnO-NPs control, and biostimulants control, with a 4 mg L^{-1} ZnO-NPs level (Table 5).

Auxins (IAA)

The current study's findings indicated that using 2 and 4 mg L^{-1} of ZnO-NPs resulted in the highest IAA levels under WW conditions, averaging 36.97 and 35.79 ppm, respectively. In contrast, the lowest IAA level, averaging 28.22 ppm, was observed in the control of ZnO-NPs under conditions of WD. Plots treated with 2 and 4 mg L^{-1} levels of ZnO-NPs significantly increased IAA content compared to the control. In contrast, the difference between the control and the ZnO-NPs treatments under WD conditions was insignificant (Table 3).

In this study, the highest IAA were observed with the foliar application of proline and chitosan + proline under WW conditions, with averages of 37.63 and 38.66 ppm, respectively. Control of biostimulants and foliar applications of chitosan and proline under WD conditions resulted in the lowest IAA content, averaging 26.41, 27.81, and 26.89 ppm, respectively (Table 4).

The findings demonstrated that applying of proline and chitosan + proline under WW conditions, as well as all three foliar spray treatments during WD, notably enhanced auxin levels when compared to the respective control.

A comparison of the mean of the interaction treatments showed that foliar spraying of proline and chitosan + proline with a level of 4 mg L^{-1} ZnO-NPs had the maximum auxin content, with an average of 36.68 and 39.32 ppm, respectively.

The control of ZnO-NPs and biostimulants had the lowest IAA content with an average of 26.15 ppm. It should be noted that foliar spraying of proline, chitosan + proline, and 4 mg L^{-1} ZnO-NPs, significantly increased auxin content compared to the separate application of each treatment (Table 5).

Cytokinins

In this experiment, the application of 2 mg L^{-1} ZnO-NPs under WW conditions resulted in the highest CK content, averaging 50.46 ppm. The minimum level of this hormone, averaging 39.62 ppm, was linked to the ZnO-NPs control when subjected to WD conditions. The findings revealed that the use of 2 and 4 mg L^{-1} ZnO-NPs boosted the CK levels by 21.91% and 14.61% under WW conditions, and by 6.48% and 13.07% under WD conditions, respectively, when compared to the relevant control (Table 3).

The comparison results for the interaction between irrigation and biostimulants revealed that the foliar application of chitosan + proline under WW conditions had the highest average CK content, measuring 50.23 ppm. In contrast, the foliar application of proline and control under WD conditions produced the lowest average CK content, which was 40.03 and 39.09 ppm. In WW conditions, the use of all three growth stimulants, as well as the foliar application of chitosan and chitosan combined with proline under WD stress conditions, notably enhanced CK levels in comparison to the relevant control (Table 4).

This experiment demonstrated that foliar spraying of proline and chitosan + proline combined with 4 mg L^{-1} ZnO-NPs resulted in the highest CK content, averaging 49.48 ppm and 49.54 ppm, respectively. However, the ZnO-NPs and biostimulants control, proline foliar spray treatment, along with ZnO-NPs control, and biostimulants control with 4 mg L^{-1} ZnO-NPs, achieved the lowest CK content with an average of 43.27, 39.13, and 46.53 ppm, respectively (Table 5).

Antioxidant enzyme activity

Catalase (CAT)

In this experiment, the control of ZnO-NPs and the 2 mg L^{-1} and 4 mg L^{-1} ZnO-NPs exhibited the highest CAT enzyme activity under WD conditions, with average values of 3.18, 3.25, and 3.43 U mg $protein^{-1}$, respectively. Plants not treated with ZnO-NPs under WW conditions exhibited the lowest CAT activity, averaging 2.44 U mg $protein^{-1}$. The difference between the aforementioned treatment and the 4 mg L^{-1} ZnO-NPs treatment under WW conditions was insignificant. In this experiment, applying 2 mg L^{-1} ZnO-NPs significantly increased cat activity compared to the corresponding control. In this study, using 2 mg L^{-1} ZnO-NPs under WW conditions considerably enhanced the CAT activity compared to the relevant control (Table 3).

The findings from the mean comparisons indicated that under WD conditions, foliar application of proline and chitosan + proline resulted in the highest CAT enzyme activity, with average values of 3.28 and 3.51 U mg $protein^{-1}$, respectively. The biostimulants control and chitosan foliar application under WW conditions had the lowest catalase activity with an average of 2.47 and 2.65 U mg $protein^{-1}$, respectively. The results indicated that foliar spraying of proline and chitosan + proline under WW conditions and application of chitosan + proline under WD conditions increased CAT enzyme activity compared to the corresponding control (Table 4).

In this study, foliar spraying of chitosan + proline with 4 mg L^{-1} ZnO-NPs achieved the highest catalase enzyme activity, with an average of 3.47 U mg $protein^{-1}$. The difference between the aforementioned treatment and the chitosan + proline treatment with 2 mg L^{-1} ZnO-NPs was not significant. The lowest CAT activity was associated with the control of both treatments, with an average of 2.59 U mg $protein^{-1}$. The difference between the aforementioned treatment, with the chitosan and chitosan + proline treatments with ZnO-NPs control, and the biostimulants control with the application of 4 mg L^{-1} ZnO-NPs was not significant (Table 5).

Superoxide dismutase (SOD)

The study found that applying 2 and 4 mg L^{-1} of ZnO-NPs under WD resulted in the highest SOD content, with averages of 4.51 and 4.47 U mg $protein^{-1}$, respectively. In contrast, the control of ZnO-NPs under WW conditions showed the lowest SOD activity with an average of 3.12 U mg $protein^{-1}$. The results indicated that applying 2 and 4 mg L^{-1} increased SOD activity compared to the control by 25.00% and 9.93% under WW conditions, and by 26.68% and 25.56% under WD conditions (Table 3).

Among the irrigation with biostimulants interaction treatments, although the maximum SOD activity was achieved by foliar spraying of proline with a level of 2 mg L^{-1} with an average of 4.32 U mg $protein^{-1}$, However, the difference between the aforementioned treatment and the foliar spraying of chitosan and chitosan + proline with a level of 2 mg L^{-1} ZnO-NPs and the foliar spraying of proline and chitosan + proline under the treatment of 4 mg L^{-1} ZnO-NPs was not significant. The prolamin foliar spray treatment and the ZnO-NPs control exhibited the lowest SOD enzyme activity, averaging 2.93 U mg $protein^{-1}$ (Table 5).

Ascorbate peroxidase (APX)

In this study, the highest Apx activity was observed at 2 mg L^{-1} and 4 mg L^{-1} ZnO-NPs levels, under WD conditions with average values of 5.16 and 5.32 U mg $protein^{-1}$, respectively. The lowest amount of activity of this enzyme with an average of 3.91 U mg $protein^{-1}$ was attributed to the ZnO-NPs control under WW conditions. It should be noted that levels of 2 and 4 mg L^{-1} ZnO-NPs under WW and applying 4 mg L^{-1} ZnO-NPs under WD significantly increased APX enzyme activity compared to the related control (Table 3).

The findings indicated that applying a chitosan + proline under WD conditions resulted in the highest activity of APX, averaging 5.61 U mg $protein^{-1}$. The lowest APX activity was also assigned to the biostimulants control under WW conditions, with an average of 4.18 U mg $protein^{-1}$. The difference between the aforementioned

treatment and the prolamin foliar spray treatment under WW conditions was insignificant. In this research, the use of proline and chitosan + proline under WW conditions, as well as the use of all three biostimulants treatments during WD conditions, notably enhanced the level of APX activity (Table 4).

Malondialdehyde (MDA)

The findings from the current research indicated that under WD conditions, the ZnO-NPs treatment demonstrated the highest average MAD content, with an average of 10.56 µM mg FW^{-1}. The control and the application of 2 and 4 mg L^{-1} ZnO-NPs under WW conditions exhibited the lowest MAD content, averaging 7.19, 6.93, and 7.05 µM mg FW^{-1}, respectively. The results indicated that applying 2 mg L^{-1} and 4 mg/L mg L^{-1} ZnO-NPs under WD conditions reduced MAD content by 11.17% and 17.33%, respectively. There was no significant difference between treatments under WW conditions.

We found that the biostimulant control under WD conditions showed the highest MAD content, averaging 10.18 µM mg FW^{-1} (Table 3).

The proline foliar spray treatment had the lowest MAD content under WW conditions, averaging 6.79 µM mg FW^{-1}. The distinction between the previously mentioned treatment and the chitosan and chitosan + prolamin treatments in standard irrigation conditions was insignificant. In this study, the use of chitosan, proline, and the combination of chitosan and proline markedly decreased MAD levels when subjected to WD; however, under WW conditions, only the foliar treatment of proline led to a significant reduction in MAD levels compared to the control (Table 4).

The mean comparisons of ZnO-NPs and biostimulants treatment interactions showed that the control of both treatments had the highest MAD content, with an average of 9.49 µM mg FW^{-1}. The biostimulants control, proline, and chitosan + proline foliar sprays containing 4 mg L^{-1} of ZnO-NPs had the lowest MAD content, averaging 8.12, 7.59, and 7.57 µM mg FW^{-1}, respectively. The difference between the above treatments and the foliar spray treatment of proline and proline + chitosan with a level of 2 mg L^{-1} of ZnO-NPs was insignificant (Table 5).

Hydrogen peroxide

The findings indicated that the control ZnO-NPs achieved the highest hydrogen peroxide levels, averaging 5.18 µM $mg_{/}$FW under WD conditions. The lowest hydrogen peroxide content was attributed to the control, and 2 and 4 mg L^{-1} ZnO-NPs under WW conditions, with an average of 2.73, 2.62, and 2.68 µM mg FW^{-1}, respectively. It is important to mention that applying 2 and 4 mg/L ZnO-NPs in WD conditions decreased the hydrogen peroxide levels by 7.91% and 17.18%, respectively, compared to the control group (Table 3).

The results of the present study showed that the biostimulants control under WD conditions achieved the highest hydrogen peroxide content with an average of 5.04 µM mg FW^{-1}. The lowest average hydrogen peroxide content was 2.39 µM mg FW^{-1}, attributed to proline foliar spraying under WW conditions. In this experiment, foliar spraying of proline under WW conditions, along with the application of all three biostimulants treatments chitosan, proline, and the chitosan + proline under WD conditions, significantly reduced hydrogen peroxide content compared to the control (Table 4).

Root yield (RY)

In our study, using 2 mg L^{-1} ZnO-NPs under WW conditions resulted in the maximum RY, averaging 74.55 ton ha^{-1}. Under WD conditions, the ZnO-NPs control produced the lowest RY, averaging 52.44 ton ha^{-1}. There was no significant difference between this treatment and the 2 mg L^{-1} ZnO-NPs. In this study, the application of 2 mg L^{-1} ZnO-NPs under WW conditions increased the RY by 11.07%. Additionally, the use of 4 mg L^{-1} ZnO-NPs under WD conditions resulted in a 10.48% increase compared to the respective control (Table 3).

The results of the mean comparison of irrigation with biostimulants interaction showed that foliar spraying of chitosan, proline and chitosan + proline produced the maximum RY under WW conditions with an average of 71.52, 69.67 and 71.68 ton ha^{-1}, respectively. The lowest root yields were associated with the control of biostimulants and the application of proline foliar treatments under WD conditions, averaging 53.73 and 52.58 ton ha^{-1}, respectively. In this experiment, foliar spraying of all three biostimulant treatments under WW conditions and chitosan + proline under WD conditions significantly increased RY compared to the corresponding control (Table 4).

In this experiment, foliar spraying of chitosan + proline combined with 2 mg/L of ZnO-NPs resulted in the highest RY, averaging 68.77 ton ha^{-1}. The difference between the treatment above, the foliar spraying of proline and chitosan with a level of 2 mg L^{-1} ZnO-NPs, and the foliar spraying of chitosan + proline with a level of 4 mg L^{-1} ZnO-NPs was not significant. The lowest RY, averaging 58.74 ton ha^{-1}, was observed in plots that did not receive biostimulants or ZnO-NPs. The difference between the treatment above and the foliar spraying of chitosan, proline, and chitosan + proline, along with the ZnO-NPs control, the biostimulants control, and the foliar spraying of chitosan and proline, along with the level of 2 mg L^{-1} ZnO-NPs, and the biostimulants control and foliar spraying of chitosan and proline, along with the level of 4 mg L^{-1} ZnO-NPs, was not significant (Table 5).

Biplot analysis

In this experiment, the first two factors accounted for 65.5% of the total variance in the data, with contributions of 44.4% and 22.1%, respectively (Fig. 1).

In the first area of the biplot (top left), the treatments WW-Control + Control, WW-control + chitosan, WW-control + proline, WW-control + chitosan + proline, WW-2 mg L^{-1} ZnO-NPs + control, WW-4 mg L^{-1} ZnO-NPs + control, WW-4 mg L^{-1} ZnO-NPs + Proline, WW-4 mg L^{-1} ZnO-NPs + Chitosan, and WW-4 mg L^{-1} ZnO-NPs Chitosan + Proline were located. The chlorophyll a and b and carotenoid traits were adjacent to the treatments

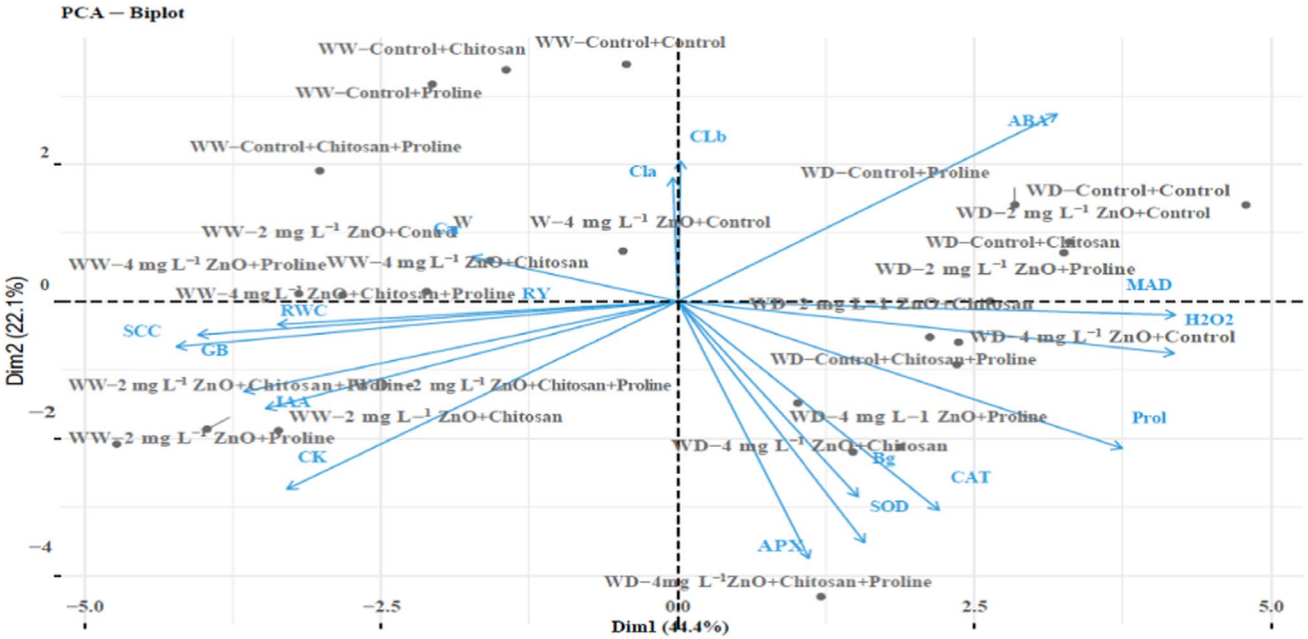

Fig. 1. Principal component analysis (PCA) elucidates the variable treatment relationships across 24 treatments. *Note:* Cha- Chlorophyll a, Chb- Chlorophyll b, Car-Carotenoid, Prol-Proline, Bg-Beta-glycine, SCC, stomatal conductance, ABA- Abscisic acid, GA- Gibberellin, AII-Auxin, CK- Cytokinin, CAT-Catalase, Superoxide dismutase -SOD, APX-ascorbate peroxidase, MAD-Malondialdehyde, H_2O_2-Hydrogen peroxide, RY- Root yield. Figure 1 presents a principal component analysis (PCA) biplot illustrating the relationships among 24 different treatments based on measured variables. PCA reduces multivariate data dimensionality by transforming original variables into principal components (PCs), with PC1 and PC2 typically explaining the highest variance. The spatial distribution of treatment points reflects their similarities or differences, where closely clustered treatments exhibit comparable responses. If variable vectors are included, their direction and magnitude indicate the influence of each variable on the treatments, with treatments aligned along a vector being strongly associated with that variable. The percentage of variance explained by each PC is often indicated on the axes (PC1: 44.4%, PC2: 22.1%). This analysis helps identify key treatment patterns, outliers, and the dominant variables.

above. In the opposite direction of the traits above were the traits WD-2 mg L^{-1} ZnO-NPs + chitosan, WD-4 mg L^{-1} ZnO-NPs + control, WD-control + chitosan + proline, WD-2 mg L^{-1} ZnO-NPs + chitosan + proline, WD-4 mg L^{-1} ZnO-NPs + proline, WD-4 mg L^{-1} ZnO-NPs + chitosan, and WW-4 mg L^{-1} ZnO-NPs chitosan + proline (Fig. 1).

In the second area of the biplot (top right), the treatments WD-control + control, WD-Control + proline, WD-2 mg L^{-1} ZnO-NPs + control, WD-control + chitosan, and WD-2 mg L^{-1} ZnO-NPs + proline were positioned close to the ABA trait. The ABA content was in the opposite direction of the WW-2 mg L^{-1} ZnO-NPs chitosan + proline, WW-2 mg L^{-1} ZnO-NPs + proline, and WW-2 mg L^{-1} ZnO-NPs + chitosan treatments (Fig. 1).

In the third area of the biplot (bottom left) were the treatments WW-2 mg L^{-1} ZnO-NPs + chitosan + proline, WW-2 mg L^{-1} ZnO-NPs + proline, and WW-2 mg L^{-1} ZnO-NPs + chitosan. The mentioned treatments were consistent with the traits RWC, RY, SCC, GB, IAA, and CK. It should be noted that for the aforementioned traits, the treatments WD-control + control, WD-control + proline, WD-2 mg L^{-1}ZnO-NPs + control, WD-control + chitosan, and WD-2 mg L^{-1} ZnO-NPs + proline were opposite (Fig. 1).

In the fourth area of the biplot (bottom right), the treatments including WW-2 mg L^{-1} ZnO-NPs chitosan + proline, WW-2 mg L^{-1} ZnO-NPs + proline, and WW-2 mg L^{-1} ZnO-NPs + chitosan were positioned near the traits MAD, hydrogen peroxide, proline, beta-glycine, CAT, SOD, and APX. It should be noted that the traits above were located in the opposite direction of the vector of the treatments WW-control + control, WW-control + chitosan, WW-control + proline, WW-control + chitosan + proline, WW-2 mg L^{-1} ZnO-NPs + control, WW-4 mg L^{-1} ZnO-NPs + control, WW-4 mg L^{-1} ZnO-NPs + proline, WW-4 mg L^{-1} ZnO-NPs + chitosan, and WW-4 mg L^{-1} ZnO-NPs chitosan + proline (Fig. 1).

IBRv2

Sugar beet plants grown under WS conditions and without foliar application of ZnO-NPs and biostimulant (WD-Control + Control) (IBRv2 30.26) showed a significant reduction in the values of RWC, carotenoids, chlorophyll *a*, *b,* and RY, stomatal conductance, GA, IAA, and CK compared to Ref treatment (i.e., WW + control + control) (Fig. 2A).

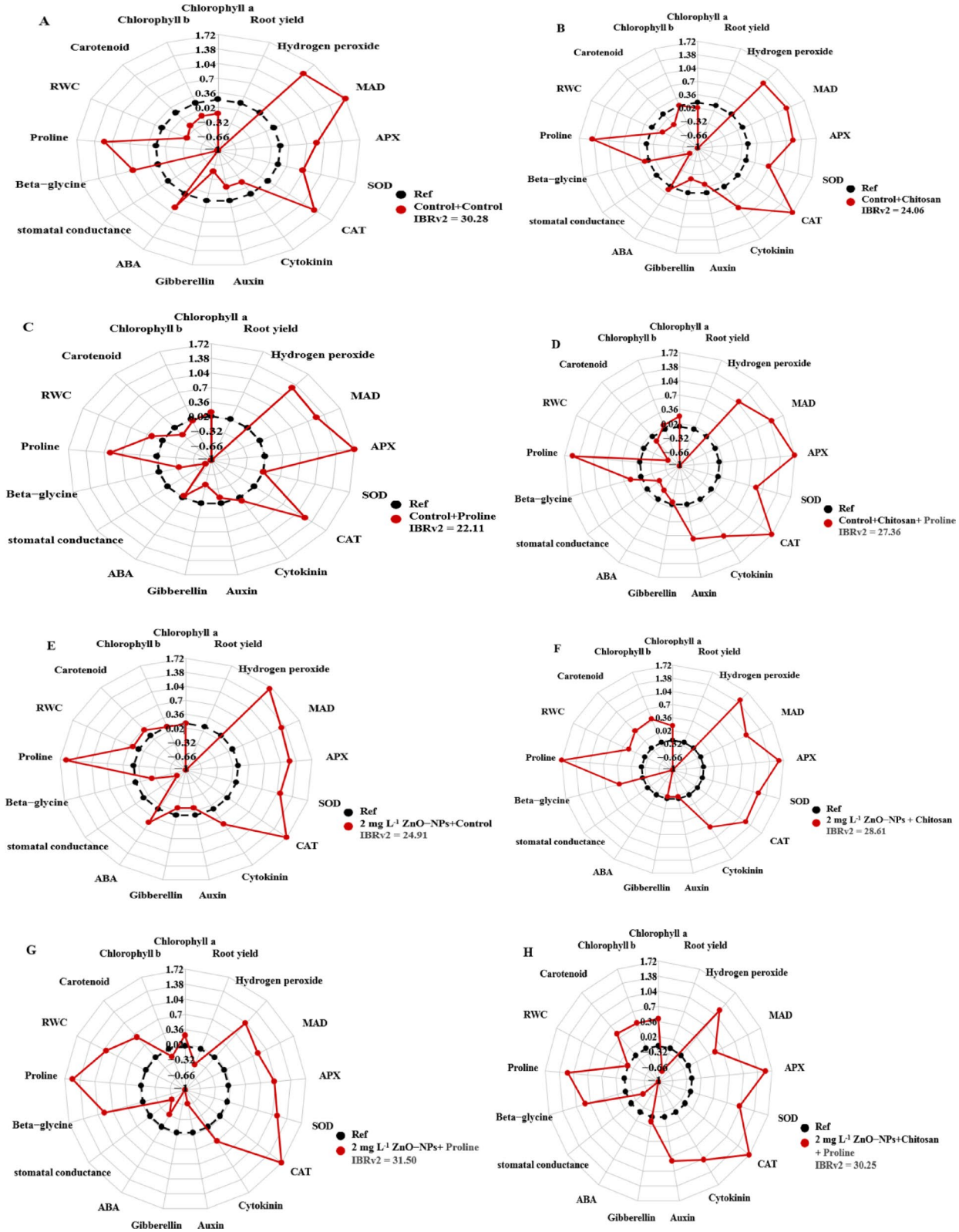

Fig. 2. The radar charts were performed as a graphic representation of the IBRv2 index from the sugar beet plants grown under different irrigation shortages as well as different foliar treatments. To calculate IBRv2, 14 different biomarkers were measured in the corresponding sugar beet plants grown in different environments (sites). In this sense, only the "WW+Control" treatment (i.e., sugar beet plants grown under normal conditions sprayed only with distilled water) was considered as the reference (Ref), while treatments applying ZnO-NPs and biostimulants to the surfaces were omitted under WW conditions. Then, the IBRv2 calculation was computed, the first IBRv2 was performed for the sugar plants grown under water deficit and ZnO-NPs with Biostimulants application.

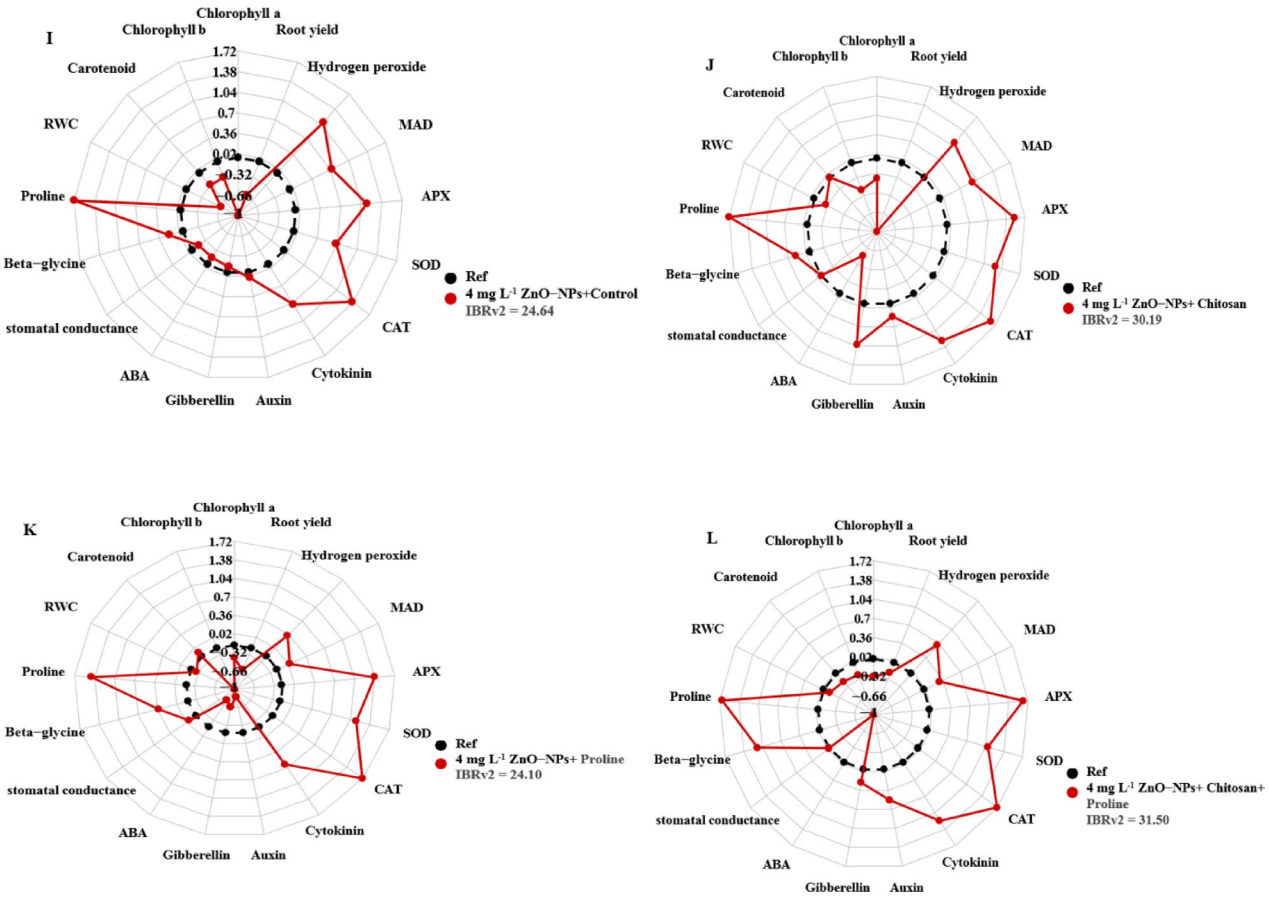

Fig. 2. (continued)

At the same time, other traits, such as hydrogen peroxide, MAD, APX, SOD, CAT, beta-glycine, and proline, showed an increase. Plants grown under WD-Control + chitosan treatment (IBRv2 = 24.06) showed a significant reduction in the values of RWC, carotenoid, chlorophyll a, GA, and IAA content, stomatal conductance coefficient, and RY compared to the control treatment. The values of hydrogen peroxide, MAD, APX, SOD, CAT, CK, ABA, beta-glycine, and proline increased in this treatment compared to the reference treatment (Fig. 2B).

In the case of WD-control + proline, it was observed that compared to the Ref treatment (IBRv2 22.11), the values of carotenoids, RY, IAA, GA, stomatal conductance coefficient, and beta-glycine decreased. At the same time, other traits, such as RWC, chlorophyll a, hydrogen peroxide, MAD, APX, CAT, and CK, also increased (Fig. 2C).

In the case of the WD-control + chitosan + proline treatment (IBRv2 = 27.36), a decrease in the values of RWC, carotenoids, GA, ABA, and stomatal conductance coefficient was evident compared to the Ref treatment, and an increase in the values of proline, chlorophyll *a* and *b*, hydrogen peroxide, MAD, APX, SOD, CAT, CK, and IAA (Fig. 2D).

In the case of WD-2 mg L^{-1} ZnO-NPs + control treatment (IBRv2 = 24.91), a decrease in the values of RY, IAA, GA, stomatal conductance, beta-glycine, and an increase in the values of RWC, carotenoids, chlorophyll *a* and *b*, hydrogen peroxide, MAD, APX, SOD, CAT, CK, ABA, and proline were observed compared to the Ref treatment (Fig. 2E).

In the case of WD-2 mg L^{-1} ZnO-NPs + chitosan treatment (IBRv2 31.50), stomatal conductance, ABA, GA, IAA, and RY decreased compared to Ref treatment. While the content of RWC, carotenoids, chlorophyll *a* and *b*, hydrogen peroxide, MAD, APX, SOD, CAT, CK, beta-glycine, and proline were higher than Ref treatment (Fig. 2F).

A decrease in stomatal conductance, ABA, and RY was observed in the WD-2 mg L^{-1} ZnO-NPs + proline treatment (IBRv2 = 31.50) compared to the Ref treatment. Other investigated indices, such as carotenoids, chlorophyll a and b, hydrogen peroxide, MAD, APX, SOD, CAT, CK, IAA, GA, beta-glycine, and proline, were significantly superior to the Ref" treatment (Fig. 2G).

In the WD-2 mg L^{-1} ZnO-NPs + chitosan + proline treatment (IBRv2 = 30.25), a decrease in RY, ABA, and stomatal conductance coefficient and an increase in the amounts of hydrogen peroxide, MAD, APX, SOD, CAT, CK, IAA, GA, beta-glycine, and proline were observed compared to the Ref treatment (Fig. 2H).

In the case of WD-4 mg L^{-1} ZnO-NPs + control treatment (IBRv2 = 24.64), a significant decrease in the values of RWC, carotenoids, chlorophyll *a* and *b*, root yield, GA, ABA, stomatal conductance coefficient and a

considerable increase in the values of hydrogen peroxide, MAD, APX, SOD, CAT, CK, IAA, beta-glycine and proline were detected compared to the Ref" treatment (Fig. 2I).

Compared to Ref treatment, in the WD-4 mg L^{-1} ZnO-NPs + chitosan treatment (IBRv2 = 30.19), a significant decrease in RWC, chlorophyll a and b, RY, and ABA traits and a substantial increase in the amounts of hydrogen peroxide, MAD, APX, SOD, CAT, CK, IAA, GA, beta-glycine, and proline were observed (Fig. 2J).

In the case of WD-4 mg L^{-1} ZnO-NPs + proline treatment (IBRv2 = 24.10), RWC, chlorophyll a and b, root yield, gibberellin, ABA showed a significant decrease, and the values of hydrogen peroxide, MAD, APX, SOD, CAT, CK, beta-glycine and proline showed a considerable increase compared to Ref" treatment (Fig. 2K).

A decrease in RWC, carotenoid, chlorophyll a and b content, root yield, ABA, and an increase in the values of hydrogen peroxide, MAD, APX, SOD, CAT, CK, IAA, GA, beta-glycine, and proline were observed in WW-4 mg L^{-1} ZnO-NPs + chitosan + proline (IBRv2 = 31.50) compared to the Ref treatment (Fig. 2L).

The spider plot serves as a visual representation of the biomarker deviation index (I), with regions above zero.

indicating biomarker induction and regions below zero indicating biomarker inhibition[61]. The results of IBRv2 calculations showed that drought stress activated the antioxidant defense system in sugar beet plants, regardless of whether foliar stimulators were applied. However, the foliar application of both stimulators resulted in a higher biomarker deviation index (I) for all scavenging enzymes compared to the control plants, under WD conditions (Fig. 2A–L).

The impact of WD on RY, as a critical characteristic in sugar beet breeding programs, was evident in the decrease of its I value. However, the application of 4 mg L^{-1} ZnO-NPs + Chitosan + Proline showed a lower decrement (I = − 0.492) compared to the 4 mg L^{-1} ZnO-NPs + control (I = − 1.365), control + chitosan + prolin (I = − 1.993) and control + control treatments. These findings suggest that applying 4 mg L^{-1} ZnO-NPs + chitosan + proline may have a beneficial effect on mitigating the negative impact of severe drought stress on sugar beet plants and their fundamental characteristics.

Discussion

Photosynthetic pigments and carotenoid content

The water deficit decreased the levels of photosynthetic pigments; however, adding 2 mg L^{-1} ZnO-NPs significantly increased the levels of chlorophyll a, b, and carotenoids under both environmental conditions compared to the respective controls. The reduction in chlorophyll concentration during drought is linked to the buildup of reactive oxygen species (ROS), which cause chlorophyll breakdown through the action of the chlorophyllase enzyme, leading to chloroplast damage. Additionally, drought conditions decrease photosystem II activity and lower the rate of CO_2 assimilation in sugar beets[62].

Hsu and Kao[63] found that chlorophyll (Chl) levels decrease during drought conditions. This decline is linked to osmotic stress, reduced water retention, and altered stomatal movement, which limits CO_2 entry into leaves, ultimately reducing photosynthesis and lowering chlorophyll a and b concentrations.

As noted, applying ZnO-NPs (Especially 2 mg L^{-1}) positively influenced the enhancement of photosynthetic pigments in conditions of water scarcity. Research shows that ZnO nanoparticles can improve leaf membrane stability, enhance water absorption, and increase nutrient uptake. These effects help maintain and stabilize chlorophyll levels[64,65]. Recent studies by Mir Mahmoudi et al.[66] and Hamze et al.[42] indicate that treating sugar beet with ZnO nanoparticles during water stress conditions results in increased levels of chlorophyll a, chlorophyll b, and carotenoids.

Chitosan, proline, and a combination of both improved chlorophyll a and b pigments under WW and WD conditions, with proline showing the most significant effect. However, there was no notable difference in carotenoid content between controls and biostimulant treatments under WD conditions. Plants that experienced drought stress exhibited the least amount of chlorophyll in comparison to control plants[45,67], leading to a decrease in photosynthesis efficiency and overall plant growth[6,68,69]. Chitosan applications mitigate these adverse effects by enhancing chlorophyll content in plants experiencing water stress and boosting photosynthesis[70,71]. The foliar application of chitosan to maize plants cultivated under conditions of water deficit has shown beneficial impacts on photosynthesis, chlorophyll fluorescence, and water-use efficiency[72].

As mentioned above, in this study, proline foliar application had a significant advantage over the control in photosynthetic pigments. Farooq et al.[73] found that utilizing proline as an osmotic safeguard against water scarcity resulted in increased levels of chlorophyll, proline, glycine betaine, and total soluble phenols in wheat plants.

Merwad et al.[74] found that applying proline to cowpea foliage under drought stress improved growth and yield traits, as well as increased levels of leaf chlorophylls a and b, and total carotenoids.

The experiment found that applying 2 mg L^{-1} ZnO-NPs alongside proline and a combination of chitosan + proline led to the highest chlorophyll a and b levels. Additionally, the combination of these treatments had a synergistic effect on chlorophyll b content. The application of silicon (Si), proline, or both significantly increased the concentrations of chlorophyll a, chlorophyll b, and relative water content (RWC) in drought-stressed sugar beet plants compared to untreated stressed plants[75]. The application of salicylic acid (SA) and proline, whether used individually or in combination, mitigated the negative effects of drought and enhanced the levels of chlorophyll and carotenoids in Rice[76].

Physiological characteristics

In the experiment, water deficit reduced the RWC and stomatal conductance. The adverse effects of drought on the RWC could stem from decreased water flow. This decline in RWC may also induce oxidative stress, leading to the production of ROS in plant tissues and ultimately causing lipid peroxidation and cellular harm. Fugate et al.[77] reported a decrease in relative water content and stomatal conductance in sugar beet plants subjected to drought conditions. Drought reduces soil water absorption, leading to stomata closure and decreased stomatal

conductance, as noted in research by Raza et al.[78] Drought stress leads to a significant reduction in leaf area index and chlorophyll content. This is attributed to the increased production of reactive oxygen species, which cause lipid peroxidation and result in chlorophyll degradation. In our research, we found that foliar spraying with chitosan and proline increased RWC under both conditions compared to the control treatment. It has been noted that the use of proline, either alone or in conjunction with SA, enhanced RWC in rice varieties[76]. Proline is said to assist in preserving the levels of chlorophyll and carotenoids, contributing to leaf turgor and improving stomatal conductance, which are factors related to drought tolerance[79]. They enhance membrane stability, leaf area, and relative water content, which improves the rate of photosynthesis and confers drought tolerance to crops.

Results showed that applying 2 mg L^{-1} of ZnO-NPs with proline led to the highest RWC, significantly improving this measure compared to the control group and individual treatments. Earlier studies have shown that ZnO nanoparticles can enhance RWC, overall yield, and the absorption of key nutrients like nitrogen and potassium in sorghum[36]. In a different study, it has also been noted that using ZnO nanoparticles may alleviate the adverse effects of drought on cell membrane stability and relative water content in corn[80].

We observed the application of both levels of ZnO-NPs increased stomatal conductance compared to the corresponding control under both condition. The study found that combining proline with level 2 mg L^{-1} ZnO-NPs significantly improved stomatal conductance compared to using each treatment separately. This growth might be due to better absorption of water, greater photosynthetic effectiveness, increased production of phytohormones, and the accumulation of osmolytes, providing improved resilience[43,81].

It has been observed that ZnO-NPs (150 ppm) enhanced stomatal conductance and leaf area index[77]. ZnO-NPs boost the production of cytokinins, which enhance metabolic activity and promote cell growth, thereby significantly increasing the LAI and chlorophyll levels[82]. It has been found that ZnO nanoparticles enhanced stomatal conductance in Coriandrum sativum L. and maize when exposed to drought and salt stress, respectively[31,81]. Hamze et al.[42] reported that the application of ZnO NPs had a positive effect on RWC and the stomatal conductivity coefficient in sugar beet under water deficit conditions.

Osmotic adjustment

The study found that water deficit increased the production of proline and beta-glycin in leaves. Osmolytes maintain turgor pressure and enhance water uptake by increasing cytoplasmic osmotic pressure[83]. Additionally, they function as ROS and MDA scavengers, reducing oxidative stress through enhanced enzyme activity[84]. In conditions of water stress, an increased accumulation of proline serves as one of the most efficient methods for osmotic regulation and for reducing the effects of drought stress[84].

The water status of plants can become unbalanced during drought conditions, disrupting osmotic regulation and resulting in increased levels of compatible osmolytes in crops. Proline possesses antioxidant properties that decrease lipid peroxidation and support cell stability by safeguarding the redox balance[85].

Plants are said to reduce damage caused by drought by increasing their accumulation of soluble sugars[47], In addition, studies on sugar beet seedlings have indicated that levels of proline and betaine rise in response to water stress, highlighting their role in alleviating the impact of drought[77].

Our research showed that the application of ZnO-NPs, especially at a concentration of 4 mg L^{-1}, significantly increased the levels of proline and soluble sugars in both conditions, In addition, the combination of 4 mg L^{-1} ZnO-NPs with proline and chitosan + proline effectively increased the content of compatible osmolytes, demonstrating a synergistic effect that resulted in the highest levels of soluble sugars. In this framework, the combined application of ZnO–NPs and biostimulants enhances plants' adaptive mechanisms, which markedly increases the levels of proline and soluble carbohydrates. Additionally, the elevated concentration of proline in stressed plants, triggered by the application of chitosan and glycine betaine, could signify improved tolerance of these plants to water stress[86,87]. According to reports, the use of chitosan and glycine betaine has improved and increased the levels of soluble sugars and proline in okra plants that are stressed by drought[70].

The rise in proline concentration resulting from chitosan application might be due to a reduction in the oxidation of proline to glutamate and a heightened synthesis of prolin[86].

External applications of proline notably enhanced the levels of proline and soluble sugars when the plants experienced water stress in Rice, suggesting that they contribute to replenishing water in plant tissues and provide osmotic protection[76]. Yasmin et al.[88] demonstrate that the combined application of ZnO–NPs and AMF synergistically enhances nutrient uptake, alleviates drought stress, and promotes the accumulation of proline and soluble carbohydrates in safflower (*Carthamus tinctorius* L.). Previous research has demonstrated that the joint use of ZnO–NPs and AMF diminishes oxidative stress, elevates proline and soluble carbohydrate levels, and ultimately improves drought resistance[66,89].

Plant hormone content

In this study, WD significantly increased the content of ABA hormones. In response to drought stress, plants manage their growth and development by adjusting the levels of different endogenous hormones, thereby reducing the negative impacts of drought conditions[90].

ABA is the primary plant hormone that initiates immediate responses (like controlling the opening and closing of stomata) as well as long-term responses (such as altering root structure) during drought conditions. It is essential for managing plant growth and development[91]. Bhusal et al.[92] indicate that drought stress causes a notable rise in ABA levels, with higher drought conditions leading to increased ABA concentrations. In drought circumstances, ABA notably lowers the net photosynthetic rate by decreasing the plants' stomatal conductance, which helps mitigate the harm caused by drought stress. However, Certain studies indicated that stomatal closure and reduced transpiration rates are influenced not only by ABA content but also by the combined action of AB[93].

In this experiment, the use of ZnO-NPs under WW environmental conditions decreased ABA content. Under water deficit WD conditions, the response of ABA to foliar application of ZnO-NPs was different. The level of 2 mg L^{-1} of ZnO-NPs increased the ABA content, and the level of 4 mg L^{-1} of ZnO-NPs decreased the content of this hormone. A previous study found that ZnO nanoparticles reduce ABA gene expression in strawberries (*Fragaria ananassa*), supporting current findings[47]. The findings from Ahmed et al.[31] indicated that stomatal conductance improved in plants treated with ZnO nanoparticles, which also corroborated the decrease in ABA levels that are crucial for stomatal function during drought conditions[94]. In our experiment, all three biostimulant treatments significantly reduced ABA content under both environmental conditions, with the maximum reduction associated with applying chitosan + proline. The lowest ABA content was observed with a combination of 4 mg L^{-1} ZnO-NPs and chitosan + proline foliar application. This treatment had a synergistic effect, reducing ABA content more effectively than applying each treatment separately.

Our research findings showed that water deficit decreased the levels of the hormones GA, IAA, and CK. A reduction in GA content may slow plant growth rates to mitigate the effects of water scarcity on their normal physiological functions, thereby enhancing drought resistance[95].

The study found that a concentration of 2 mg L^{-1} ZnO-NPs under WW conditions increased levels of GA, IAA, and CK hormones. Under WD conditions, a concentration of 4 mg L^{-1} ZnO-NPs benefited only GA and CK. Additionally, combining 4 mg L^{-1} ZnO-NPs with proline foliar spray yielded the highest levels of GA and IAA, significantly boosting hormone levels compared to using each treatment alone.

The application of zinc (Zn) has a significant impact on the level of indole-3-acetic acid (IAA), and zinc is recognized as a co-enzyme in the synthesis of tryptophan, which is a precursor in the production of IAA[96,97].

Applying Zn during periods of drought stress elevates IAA levels, which enhances root development, aids in water absorption, and consequently boosts drought resistance[91]. Water deficiency greatly decreases the levels of IAA and GA, while the application of Zn significantly enhances these levels, leading to improved plant performance[98]. The use of Zn significantly enhances the metabolic routes of tryptophan under drought conditions. As a result, since tryptophan is a precursor to IAA and melatonin, elevated levels of tryptophan help mitigate the impacts of drought by decreasing oxidative damage and maintaining osmotic balance[99]. Inoculating wheat with PGPR and ZnO-NPs also enhanced the levels of phytohormones, specifically IAA, when exposed to drought stress[100]. Elewa et al.[101] found that water shortage stress in Chenopodium quinoa plants resulted in a notable reduction in IAA hormone levels. On the other hand, the addition of proline in these circumstances elevated the IAA hormone content.

Antioxidant properties, MDA, and hydrogen peroxide concentration

In this experiment, WD considerably enhanced the activity of CAT, SOD, and APX enzymes. Drought stress leads to the buildup of ROS, which subsequently triggers the activation of antioxidant enzymes, which are a component of the plant's defense mechanism against oxidative harm[12]. Nonetheless, during extended and intense drought periods, the effectiveness of the antioxidant defense system is undermined by the reduction of vital cofactors, decreased ability to regenerate, and interruption of ROS-related signaling pathways[102].

Azzouz-Olden et al.[103] found that the differential expression of genes involved in the defense against oxidative stress, particularly those related to producing antioxidant enzymes, is a key strategy for S. bicolor to withstand drought conditions. Haghaninia et al.[13] noted a preliminary rise in the activity of antioxidant enzymes in response to moderate drought stress in lavander (*Lavandula angustifolia* L.). This supportive interaction enhances the plant's defensive capabilities by boosting water retention, triggering protective signaling pathways, and promoting the production of essential protective compounds[44,104]. It has been reported that CAT and SOD activities increased significantly in sugar beet under drought conditions compared to controls. This rise is attributed to their role as antioxidant enzymes that help stress tolerance. SOD acts as the first line of defense against oxidative damage by converting superoxide radicals into hydrogen peroxide and oxygen[105].

CAT is involved in the transformation of H_2O_2 into water and oxygen, playing a crucial role in plant metabolism and in the recognition of signals. These findings align with the results reported by[14,15,106].

The results of our experiment indicated that treatment with ZnO-NPs significantly increased antioxidant enzyme activities at levels 2 and 4 mg L^{-1} under both environmental conditions. Importantly, ZnO-NPs play a role in stabilizing cell membranes, triggering essential antioxidant pathways, and together enhancing the plant's resilience to stress caused by drought[107,108]. In line with our results, Mir-Mahmoudi et al.[66]. showed that the simultaneous application of ZnO-NPs not only promotes nutrient absorption but also modulates ROS-dependent signaling pathways, thus boosting the expression of antioxidant genes and strengthening the plant's overall defense mechanisms[109].

In this experiment, biostimulant treatments, particularly chitosan + proline, enhanced the activity of CAT, SOD, and APX enzymes under both environmental conditions. In addition, applying 4 mg L^{-1} of ZnO-NPs with chitosan and proline enhances CAT enzyme activity through a synergistic effect. Additionally, 2 mg L^{-1} of ZnO-NPs combined with proline positively influences SOD enzyme activity.

The use of chitosan derivatives, either alone or in combination, has been shown to enhance the activity of antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, and guaiacol peroxidase during the early stages of stress. Furthermore, it contributes to reducing lipid peroxidation[110]. Furthermore, chitosan and its derivatives have demonstrated efficacy in improving crop resilience to water shortages by alleviating the harmful impacts of water deficit on yield[111,112].

As mentioned, proline foliar application had a positive effect on increasing the content of antioxidant enzymes. Furthermore, proline's critical function may be attributed to its beneficial impact as an osmoprotectant, safeguarding plant cells from oxidative stress through osmotic regulation, protein stabilization, and maintaining the balance of antioxidant enzymes. AlKahtani et al.[75] found that silicon (Si), proline, and their combined application significantly benefited drought-stressed sugar beet plants compared to untreated stressed plants.

The content of hydrogen peroxide and MAD increased significantly under WD conditions. The results of this research clearly show that drought stress substantially improves the levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), which are key indicators of membrane lipid peroxidation and cellular harm caused by oxidative stress[12,113]. Under drought conditions, the heightened production of ROS results in cellular damage, which in turn elevates the levels of MDA and H_2O_2[5]. In line with these findings, Mazhar et al.[7] stated that drought stress activates lipid peroxidation and causes oxidative imbalance in canola. The elevated levels of MDA and proline are a reaction to drought conditions. Our results align with those of several researchers who have observed significant increases in proline and MDA under stressful conditions in various plants[15,18,114].

The results showed that applying both levels of ZnO-NPs significantly decreased the content of hydrogen peroxide and MAD. However, applying ZnO-NPs under WW conditions did not significantly affect the reduction of these two substances. In contrast, the use of ZnO–NPs significantly alleviated oxidative stress, as indicated by the decrease in levels of MDA and H_2O_2. This combined treatment bolstered the plant's resistance to oxidative stress by enhancing its nutrient levels and promoting the activity of antioxidant enzymes[115,116]. ZnO-NPs specifically aimed at enzymatic antioxidant mechanisms, which consequently reduced ROS production.
[104,117,118]. Prior research has highlighted the effect of ZnO–NPs in diminishing oxidative stress and improving membrane stability in a range of crops, such as wheat and maize[44,104]. Research has indicated that drought stress results in an excessive generation of ROS, which causes damage to cell membranes, accumulates malondialdehyde (MDA), and ultimately leads to cell death. The application of zinc (Zn) during drought stress decreases MDA levels, highlighting the essential function of Zn in safeguarding cell membranes from ROS under drought conditions[119].

In this experiment, foliar spraying of proline under WW conditions, as well as the foliar spraying of all three growth stimulant treatments under WD conditions, significantly reduced the levels of hydrogen peroxide and MAD. Other scientists reported comparable results, discovering that the use of proline markedly decreased the concentrations of H_2O_2 and MDA in various plants experiencing stress[34,120]. In the study conducted by AlKahtani et al.[75] a significant reduction in H_2O_2 was observed due to the foliar application of proline. This treatment was particularly effective in mitigating the adverse effects of drought.

Root yield (RY)

WD significantly increased RY compared to the corresponding control. Notably, applying both levels of ZnO-NPs under WW conditions significantly enhanced RY compared to the control. The increase in RY under WD conditions was limited to only 4 mg L^{-1} of ZnO-NPs.

The increase in root yield under water deficit conditions under 4 mg L^{-1} of ZnO-NPs treatment can be attributed to the positive effect of this treatment on increasing the activity of antioxidant enzymes, increasing the content of compatible osmolytes, and consequently reducing the content of hydrogen peroxide and MAD. The sum of these processes paves the way for improving physiological characteristics, photosynthetic pigments' content, and consequently, RY.

Drought stress significantly diminishes soybean seed yield by affecting soil moisture levels, impairing the absorption of water, and obstructing the transport of nutrients[121]. Consequently, the reduction in photosynthetic ability, along with metabolic impairment, significantly affects seed production[29,122].

Previous studies on wheat and maize have demonstrated that AMF and ZnO–NPs treatments more effectively promote plant growth, strengthen stress tolerance, and improve yield outcomes compared to individual treatments[118]. It has been observed that ZnO–NPs support the stability of photosystem structures and boost the activity of antioxidant enzymes, which improves the efficiency of light energy conversion and decreases oxidative damag[33,123]. These results are in agreement with the obtained results with AlKahtani et al.[75], Hamze et al.[42], and Molavi et al.[124], who reported that drought led to inhibiting the assimilation of CO_2 and reducing the assimilate supply in sugar beet, consequently decreasing sucrose%, root, and sugar yield. In the study by[36] on sorghum and hirvani[37] on soybean, the foliar application of ZnO–NPs significantly improved the economic yield of the crops compared to the control group.

On the other hand, applying all three biostimulant treatments under WW conditions increased RY compared to the control. The increase in RY under WS conditions was attributed solely to the chitosan + proline treatment. The treatment above positively increased photosynthetic pigments, compatible osmolytes, growth hormones, antioxidant enzyme activity, and reduced MAD content. These processes can significantly improve root yield under water deficit conditions. In this experiment, chitosan + proline spraying with both levels of 2 and 4 mg L^{-1} ZnO-NPS produced maximum RY. The foliar application of chitosan + proline, combined with 2 mg L^{-1} ZnO-NPs, had a synergistic effect on enhancing RY compared to the separate application of each.

Chitosan has been found to activate signaling pathways that regulate stomatal opening and closing, which are essential for plants under water stress conditions[22,23]. Furthermore, Chitosan can enhance the plant's defenses against oxidative stress by promoting certain enzymes and non-enzymatic antioxidants, such as ascorbate, while reducing lipid peroxidation and hydrogen peroxide production[24]. Research has shown that applying chitosan to the leaves of plants during periods of water deficiency significantly enhances their economic yield. This has been observed in various crops, including marigold (*Calendula officinalis* L.)[25], rice[26], and sunflower (*Helianthus annuus* L.)[27]. Our findings align with these studies. It has been reported that proline application has been shown to enhance plant growth and reduce oxidative damage under stress conditions in calendula and barley plants by alleviating the negative effects of ROS[15,16]. Ghaffari et al.[18] discovered that employing proline improved water management and boosted sugar beet production under drought conditions. The superior effect of proline might be due to the role of proline in improving sugar beet as a storage sink for nutrient elements, for example, carbon and nitrogen, and as a scavenger for free radicals; consequently, decreased TSS%[125].

Relationships between traits and treatments

Based on the results, root yield in the vicinity of the treatments WW-2 mg L^{-1} ZnO-NPs + Chitosan + Proline, WW-2 mg L^{-1} ZnO-NPs + Proline, and WW-2 mg L^{-1} ZnO-NPs + Chitosan, and the traits RWC, RY, SCC, GB, IAA, and CK were located in one area of the biplot. It can be concluded that in this study, the use of 2 mg L^{-1} ZnO-NPs treatment along with all three biostimulant foliar spray treatments resulted in maximum RWC, RY, SCC, GB, IAA, and CK traits and ultimately root yield. In the opposite direction to the root yield axis, the treatments Control + Control, Control + Proline, Control + Chitosan, 2 mg L^{-1} ZnO-NPs + Control, and 2 mg/L ZnO-NPs + Proline ABA under water deficit were placed. It can be stated that the lowest values of RWC, RY, stomatal conductance, GB, IAA, and CK, and finally root yield were related to the aforementioned treatments.

IBRv2

We recorded the lowest IBRv2 values of 22.11, 24.06, 24.91, 24.64, and 24.10 for Control + Proline, "Control + Chitosan, 2 mg L^{-1} ZnO-NPs + Control, 2 mg L^{-1} ZnO-NPs + Control, and 4 mg L^{-1} ZnO-NPs + Proline treatments. The maximum IBRv2 with a value of 31.50 was assigned to the 2 mg/L ZnO-NPs + Proline treatments. Since lower IBRv2 values indicated lower levels of toxicity and/or oxidative stress, it could be concluded that foliar application of Proline under drought stress could be accompanied by better improvements toward diminishing adverse consequences of severe drought stress. The influence of drought stress on MAD content, a fundamental characteristic for osmotic tension, revealed that 4 mg L^{-1} ZnO-NPs + Chitosan + Proline (I = 0.803) and 4 mg L^{-1} ZnO-NPs + Proline (I = 0.809) had the lowest increases in MAD content. The highest increase in MAD index was assigned to Control + Control (I = 3.601). In terms of root yield as an economic trait in sugar beet, the least decrease in this trait was observed in the treatments of 4 mg/L ZnO-NPs + Chitosan and Proline (I = − 0.492) and 4 mg/L ZnO-NPs + Proline (I = − 1.014).

These findings suggested that applying 4 mg L^{-1} ZnO-NPs combined with Chitosan and Proline and 4 mg L^{-1} ZnO-NPs with Proline might have a beneficial effect on mitigating the negative impact.

of severe drought stress on sugar beet plants and their fundamental characteristics. Since a higher value of the IBRv2 index corresponds to increased oxidative stress reflected in decreasing growth rate[126], foliar spraying of 4 mg L^{-1} ZnO-NPs combined with Chitosan and Proline, and 4 mg L^{-1} ZnO-NPs with Proline seemed to have a positive effect on improving cell health by regulating the activity of antioxidant enzymes and betaglycine proline in the foliar treatments. This is because of lower values of IBRv2 for the sugar beet plants exposed to ZnO-NPs combined with chitosan and proline, under water deficit stress conditions. Similarly, in soybean, lower values of IBRv2 index were also observed for the inoculated strain DNS10, which was able to destroy the atrazine in the soil and moderate the phytotoxicity caused by atrazine compared to the other treatments[127].

Conclusion

According to the findings from the comparison of treatments under water deficit (WD) conditions, applying a foliar spray of 4 mg L^{-1} of ZnO-NPs enhanced the levels of chlorophyll b, carotenoids, proline, beta-glycine, stomatal conductance, GA, CK, CAT, SOD, APX, and RY. At the same time, it reduced the amounts of ABA, MAD, and hydrogen peroxide compared to the control. Among the biostimulant treatments, the combination of chitosan and proline applied under WD conditions increased chlorophyll *a*, chlorophyll *b*, proline, beta-glycine, GA, IAA, CK, CAT, APX, and RY, and decreased ABA, MAD, and hydrogen peroxide levels. The application of 4 mg L^{-1} of ZnO-NPs and foliar spraying of chitosan + proline under water-deficit conditions increased the plant's defense capability against water-deficit stress by increasing the content of antioxidant enzymes. Reducing MAD and hydrogen peroxide content can prove this improvement in plant defense properties. By enhancing defensive characteristics, the plant upholds ideal quantities of growth hormones, which help preserve the arrangement and stability of photosynthetic pigments, ultimately boosting photosynthetic output and enhancing root yield. In this research, under both environmental conditions, the combined use of 4 mg L^{-1} of ZnO-NPs along with the foliar application of chitosan and proline exhibited a synergistic effect on enhancing RY, which is a crucial economic trait in sugar beet, when compared to the individual application of each treatment. Even though the control + proline treatment received the lowest IBRv2 index in this study, the 4 mg L^{-1} ZnO-NPs + chitosan + proline treatment showed the smallest RY decline and the smallest rise in MAD content. It can be inferred that the treatment of 4 mg L^{-1} ZnO-NPs + chitosan + proline likely alleviated the negative impact of water deficit stress on RY by triggering different defense mechanisms, including enhancing the activity of antioxidant enzymes and boosting the levels of compatible osmolytes.

To summarize, our findings indicated that the application of ZnO nanoparticles (NPs) in conjunction with biostimulants, especially the mixture of 4 mg L^{-1} of ZnO-NPs with foliar treatments of chitosan and proline, notably improved RY. In addition, this combination demonstrated the potential to activate antioxidant enzymes and promote growth in the Shokofa variety of sugar beet when subjected to water stress conditions.This study suggested that using zinc nanoparticles and biostimulants could present promising opportunities for future research in cultivating sugar beet and various crops in arid and semi-arid regions. This is particularly relevant in light of the current trends in global warming, which have resulted in a progressively warmer planet and have considerably impacted the accessibility of water resources around the globe considering the consequences of current global warming trends, employing such stimulators could be a reliable method to mitigate the negative impacts of water scarcity on the growth, yield, and quality of sugar beets in the future. This is especially important for farmers whose sugar beet crops are negatively impacted by water shortages.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

SH. N and H. H. collected and organized the experimental data, performed data analysis, and wrote the manuscript. T. M. collected and organized the experimental data and edited the manuscript. S.Sh. rewrote the manuscript. S. Y. S., H. H., and SH. N conducted experiments and assisted in collecting and organizing the experimental data.

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Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to S.S.

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