



Chitosan Induces Sorghum Tolerance to Water Deficits by Positively Regulating Photosynthesis and the Production of Primary Metabolites, Osmoregulators, and Antioxidants

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Abstract

Current climate change scenarios require strategies that mitigate the effects of water deficits. Given that chitosan stimulates induced resistance responses, we tested the hypothesis that foliar application of this polysaccharide can have positive effects on the primary metabolite, antioxidant, and osmoregulatory metabolisms of sorghum, mitigating the effects of water deficit on grain yield. Sorghum plants were cultivated under either irrigated or water-deficit conditions and were either treated or untreated with chitosan, and we assessed the following responses: water potential, gas exchange, nutritional status, biosynthesis of primary and osmoregulatory metabolites, the activity of the antioxidant system constituents, and hydrogen peroxide and malondialdehyde contents. An active methodology was used to impose water stress on plants grown in pots with 20 kg of soil, 06 replications per treatment. Sorghum plants subjected to water deficit that were treated with chitosan exhibited higher stomatal conductance and transpiration than the untreated plants. Water restriction reduced the nutrient contents of leaves, but in the irrigated plants, chitosan treatment increased the tissue contents of certain nutrients and leaf concentrations of total proteins and total amino acids. In the plants under water-deficit conditions that were treated with chitosan, an elevated photosynthetic rate contributed to an increase in carbohydrate accumulation and a higher production of panicle and grain dry biomass compared with the untreated plants. The activity of catalase and ascorbate peroxidase also increased in response to chitosan. Overall, chitosan was effective in enhancing the water deficit tolerance of sorghum plants cultivated under water-deficit conditions by 57%. With this work, we generate perspectives for studies focusing on the development of water-deficit-resistant sorghum lineages, by means of chitosan elicitation.

Keywords Grain sorghum · Water stress · Antioxidant enzymes · Photosynthetic performance · Osmoregulation

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1 Introduction

Originating from tropical Africa, sorghum [*Sorghum bicolor* (Moench) L.] is a source of food for over 500 million people, being cultivated on more than 44.96 m ha in about 111 countries worldwide (Vanamala et al. 2018). Additionally, this cereal is used as raw material for animal feed formulation and the manufacture of ethanol (Dutra et al. 2013). Its small genome (~ 720 bp) has been completely sequenced and made available in public databases. The complete annotation of each transcription unit was a major breakthrough in understanding the biology of this cereal and unraveling the mechanisms associated with its tolerance to water deficit (Pennisi 2009; Thanmalagan et al. 2022). This is because the water deficit induces changes in the transcript profile. Studies have shown that in *S. bicolor*, the expression of gene coding for

the formation of osmoprotective molecules or molecules related to water transport and stomatal opening and closing is affected by water deficit (Abdel-Ghany et al. 2020; Azzouz-Olden et al. 2020; Johnson et al. 2014).

In this context, as an environmental stressor, water deficit can severely compromise the growth and production of different agricultural crops (El-Bially et al. 2018; Saady et al. 2021). We say “can,” because reductions in the parameters of plant growth and production are potentially influenced by multiple factors, including the intensity and duration of drought conditions, phenological stage, soils condition, species, climate, and genetic material (Araus et al. 2012; Magalhães and Durães 2008; El-Metwally and Saady 2021; Abd El-Mageed et al. 2022). However, extremely intense water restriction during the reproductive stage can reduce grain yields by more than 55% (Assefa et al. 2010; Avila et al. 2021; Mubarak et al. 2021; Salem et al. 2021).

Water-deficit commences with a reduction in the water potential of plant tissues (Avila et al. 2020; Lavinsky et al. 2015; El-Bially et al. 2022; Saady et al. 2022a), which generates metabolic disturbances that in turn negatively influence plant growth and development (El-Metwally et al. 2021). Among these changes, the most notable are the reductions in hydraulic conductivity (Liu et al. 2015) and photosynthetic rate (Avila et al. 2016, 2020); an increase in the production of reactive oxygen species (ROS) (Avila et al. 2021; Gill and Tuteja 2010); reduced nutrient uptake (Garg 2003; Saady and El-Metwally 2019); perturbation of the balance between the synthesis and degradation of photosynthetic pigments; accelerated leaf senescence; and, consequently, a reduction in photosynthetically active leaf area, reduced growth, and lower grain yield (Farooq et al. 2009; Avila et al. 2020; Saady et al. 2022b).

In an attempt to mitigate the deleterious effects of water deficit, plants modify their morphophysiology, thereby conferring a degree of tolerance to periods of stress. Thus, the typical responses of reduced stomatal conductance and transpiration are observed as strategies designed to enhance water-use efficiency (Avila et al. 2020; Yang et al. 2021). Furthermore, as protective measures, plants also increase the activity of antioxidant system enzymes and production of non-enzymatic antioxidants in response to elevated ROS generation (Ali et al. 2022; Fujita and Hasanuzzaman 2022; Irato and Santovito 2021). In addition, alterations in osmoprotective and osmoregulatory systems are detected, which contribute to water uptake and the protection of cellular molecules during periods of tissue desiccation (Sharma et al. 2011; Abd-Elrahman et al. 2022; Da Silva et al. 2022).

A knowledge of the effects of water deficit on plant metabolism and the responses of plants to these stress conditions can contribute to the development of technologies that will potentially enhance the performance of crops

subjected to water deficit, which is essential in view of current climate change scenarios. Among these technologies is the application of chitosan, an organic compound obtained industrially via the alkaline deacetylation of chitin, a polysaccharide found in the cell walls of filamentous and yeast-like fungi (Crognale et al. 2022). Studies have shown that chitosan can act as an elicitor of signaling systems associated with the regulation of stomatal opening and closure (Agurla et al. 2014; Czékus et al. 2021), which are processes that play extremely important roles in plants subjected to water restriction. On the other hand, this polymer stimulates defense responses to oxidative stress mediated by enzymes or non-enzymatic antioxidants such as ascorbate, in addition to reducing lipid peroxidation and H₂O₂ formation (Almeida et al. 2020). Although chitosan application has been evaluated in many crops of agronomic importance (Akhtar et al. 2022; Moolphuerk et al. 2021; Sohail et al. 2021), studies that tested its elicitor effect in minimizing damage caused by abiotic stresses in sorghum plants are rare.

Foliar application of chitosan, and its derivatives *N*-succinyl and *N,O*-dicarboxymethyl chitosan, to maize plants grown under water-deficit conditions has been found to have positive effects on photosynthesis, chlorophyll fluorescence, and water-use efficiency (Dos Reis et al. 2019). Furthermore, the application of the aforementioned chitosan derivatives, or a mixture of the two, has been demonstrated to enhance the activity of the antioxidant enzymes superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, and guaiacol peroxidase during the initial stages of the stress period and also contributes to reducing lipid peroxidation. In addition, chitosan and its derivatives have been shown to be effective in enhancing the crop tolerance to water deficit by mitigating the deleterious effects of water deficit on harvest index and yield (Rabêlo et al. 2019; Makhoul et al. 2022).

However, there have been no studies that have examined the application of chitosan to sorghum with a view toward assessing the effects of this polymer on plant tolerance to water deficit at the pre-flowering stage. Thus, we used plants from this stage to test the hypothesis that foliar application of chitosan would enhance the primary, antioxidant, and osmoregulatory metabolisms of sorghum and thereby mitigate the deleterious effects of water deficit on grain yield.

2 Materials and Methods

2.1 Plant Material, Experimental Design, and Growth Conditions

The experiment was conducted in a greenhouse at the Maize and Sorghum National Research Center (19° 28' S, 44° 15' 08" W, 732 m above sea level) using sorghum plants of the

cultivar BRS 332, which is characterized as being sensitive to water deficit during the pre-flowering stage. We adopted a completely randomized experimental design with four treatments and six replications, giving a total of 24 experimental units. Each unit consisted of a plastic pot with one plant. We used an active method to impose water deficit and applied the following treatments: irrigating soil at field capacity (irrigated), water deficit (water deficit), irrigating soil at field capacity + chitosan application (irrigated + chitosan), and water deficit + chitosan application (water deficit + chitosan).

In the irrigated and irrigated + chitosan treatments, the soil was irrigated, so as to maintain a soil water tension of approximately -18 kPa throughout the entire crop cycle. In the water deficit and water deficit + chitosan treatments, the soil water tension was reduced from -18 to -138 kPa when plants had reached the pre-flowering stage (60 days after emergency) and was maintained at this level for a period of 12 days. Thereafter, the plants were re-irrigated, so as to maintain a soil water tension of approximately -18 kPa until the end of the crop cycle. Soil water tension in plants subjected to the water-deficit treatments corresponded to 50% of the available water in the soil of the irrigated plants (Souza et al. 2013).

Chitosan was applied directly to the leaves of sorghum plants on day zero of water deficit, using a chitosan solution prepared in accordance with the methodology described by Korsangruang et al. (2010), with some modifications. The solid chitosan was dissolved in acetic acid (0.25% v/v) with constant stirring, adjusted to a pH value of 6.0 using sodium hydroxide, and applied directly to the leaves at a concentration of 80 mg L^{-1} on day zero of water deficit using a CO_2 -pressurized sprayer, calibrated for an output of 200 L ha^{-1} .

The plants were grown in plastic pots of 20 L, containing 20 kg of a typical dystrophic red clayey latosol, with particle size distribution of 19.7% sand, 22.5% silt, and 57.8% clay and the following chemical parameters in the 0–20-cm layer: $\text{pH (H}_2\text{O)} = 5.0$, $\text{pH (CaCl}_2) = 5.5$, $\text{H} + \text{Al} = 10.77 \text{ cmolc kg}^{-1}$, $\text{P} = 134 \text{ mg kg}^{-1}$, $\text{K} = 145.9 \text{ mg kg}^{-1}$, $\text{Ca} = 3.1 \text{ cmolc kg}^{-1}$, $\text{Mg} = 0.7 \text{ cmolc kg}^{-1}$, cation exchange capacity = $14.9 \text{ cmolc kg}^{-1}$, $\text{Cu} = 1.1 \text{ mg kg}^{-1}$, $\text{Fe} = 34.8 \text{ mg kg}^{-1}$, $\text{Mn} = 10.6 \text{ mg kg}^{-1}$, $\text{Zn} = 21.9 \text{ mg kg}^{-1}$, and $\text{Si} = 9.5 \text{ mg kg}^{-1}$. Only one plant was grown per pot, and the cultivation was extended for 120 days after planting, until the harvest period. Soil water content was monitored daily between 09:00 and 15:00 at a depth of 20 cm using GB Reader N1535 moisture sensors (Measurement Engineering, Australia) installed in the center of each pot and a spiral auger. These sensors, which were connected to digital meters, detect soil water tension based on electrical resistance. Water levels were restored via irrigation based on the readings obtained using the sensor and according to the requirements of the respective treatments. The calculations for restoring water levels were determined as a function of a soil water retention curve. We made the requisite adjustments and applied base and

top-dressing fertilization based on chemical analyses of the soil and plant requirements. Thus, liming was done with dolomitic limestone (1.5 kg T^{-1} of soil), the base fertilization was carried out with NPK 8–28–16 (200 g T^{-1} of soil), and the topdressing was carried out with urea (3 g pot^{-1}). In addition, temperature and humidity data were monitored throughout the experiment, with an average temperature of 24°C and an average humidity of 65%.

2.2 Measurements

Having subjected plants to water deficit or normal irrigation for 12 days, we performed ecophysiological evaluations, determined leaf areas, and collected leaves for nutritional and biochemical analyses. Agronomic characteristics were assessed only at the time of harvest.

2.3 Leaf Water Potential

Leaf water potentials (Ψ_{md}) were determined at 12:00 using a Scholander pressure bomb (model 1000; PMS Instrument Company, Albany, OR, USA). Measurements taken from the first leaf located below the flag leaf (the last leaf to emerge from the stem) were performed following the methodology described by Scholander et al. (1964).

2.4 Ecophysiological, Leaf Area, and Nutritional Analyses

We examined the maximum quantum yield of photosystem II (Fv/Fm) and leaf gas exchange in the first leaf located below the flag leaf. The PSII photochemical efficiency was determined on a FluorPen FP 100 portable fluorometer (Photon Systems Instruments; Drasov, Czech Republic) and leaf gas exchange was measured using an LI-6400 infrared gas analyzer (LI-COR Inc., Lincoln, NE, USA), equipped with a LI-6400–40 fluorometer. Measurements were performed at leaf level between 09:00 and 11:00 under an artificial photosynthetically active radiation of $1500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ in an atmosphere of 21% O_2 and $400 \mu\text{mol CO}_2 \text{ mol}^{-1}$. Information on net photosynthetic rate (A), stomatal conductance (g_{sw}), transpiration rate (E), intercellular carbon concentration (C_i), ratio between the intercellular and atmospheric carbon concentrations (C_i/C_a), and instantaneous transpiration efficiency (ITE) were obtained.

Leaf area measurements were evaluated for all leaves of the plants using a leaf area meter (Li-Cor Inc.) in conjunction with a WinRhizo computerized system (WinRhizo Pro; Regent Inc., Quebec, Canada). We also determined the macronutrient (N, P, K, Mg, Ca, and S) and micronutrient (Cu, Fe, Mn, and Zn) contents of the leaves of plants subjected to the different treatments, according to the methodology described by Silva (2009). For this, the content of K, Ca,

Mg, Fe, Mn, Zn, and Cu was obtained by atomic absorption spectrophotometry; P and S by optical spectrophotometry; and N by Kjeldahl distiller.

2.5 Extraction and Quantification of Primary Metabolites and Proline

For the extraction of primary metabolites and proline, we collected the third and fourth leaves below the flag leaf, which were dried in a forced-air oven at $\pm 65^{\circ}\text{C}$ until obtaining a constant mass, after which the dried samples were ground in a mill. Subsequently, 200 mg of the processed dry leaf tissue was homogenized in a 0.1 M potassium phosphate buffer (pH 7.0), and the homogenate thus obtained was incubated in a water bath at 40°C for 30 min. Following incubation, the homogenate was centrifuged at $1000\times g$ for 20 min, and the resulting supernatant was collected. This process was subsequently repeated, and the two supernatants were pooled and stored at -20°C . The final homogenate pellet was used to extract starch. Aliquots of the supernatants were used for analyses of the following constituents: total soluble sugars (TSS), using the anthrone method (Yemm and Willis 1954); reducing sugars (RA), using the dinitrosalicylic acid method (Miller 1959); total proteins, using the Bradford method (Bradford 1976); and amino acids, using the ninhydrin method (Yemm et al. 1955). Leaf sucrose contents were determined based on the difference between the concentrations of TSS and RA. Starch was extracted by resuspension of the pellet in potassium acetate buffer (pH 4.8), followed by the addition of the enzyme amyloglucosidase (1 mg mL^{-1}) and subsequent incubation in a water bath at 40°C for 2 h. Thereafter, the samples were centrifuged at $5000\times g$ for 20 min, and the resulting supernatants were collected, the volumes of which were made up to 15 mL with water. Starch was quantified using the anthrone method (Yemm and Willis 1954). A standard curve used for the spectrophotometric measurement of carbohydrates was prepared using D-glucose.

Quantification of leaf proline content was based on the method proposed by Bates et al. (1973), in which 100 mg of dried leaf tissue was macerated in 3% sulfosalicylic acid. The macerated tissues were homogenized at room temperature for 60 min and then filtered through filter paper. Aliquots of the filtrate thus obtained were reacted in a test tube with the addition of a mixture of 2 mL of acid ninhydrin and 2 mL of glacial acetic acid for 1 h at 100°C . Proline concentrations were determined from the absorbance values of samples at 520 nm extrapolated using a standard curve prepared with known concentrations of proline. Proline/amino acid ratios were determined based on the values obtained for total amino acids and total proline as follows:

$$\text{Proline : Amino acid ratio} = \frac{\text{Total proline concentration}}{\text{Total amino acid concentration}} \times 1000$$

2.6 Extraction and Quantification of Antioxidant Enzyme Activities

For the determination of the activity of antioxidant system enzymes, ascorbate and hydrogen peroxide contents, and lipid peroxidation, we used the second leaf below the flag leaf of sorghum plants, which were immediately frozen in liquid nitrogen and stored in an ultra-low temperature freezer at -80°C until used for analyses.

The enzymes were extracted by maceration of 200 mg of leaf tissue in liquid nitrogen containing 50% PVPP following the extraction protocol proposed by Biemelt et al. (1998). We used the extract thus obtained to evaluate the activities of catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POD), superoxide dismutase (SOD), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR). Protein concentrations of the extract were determined using the Bradford method (1976).

SOD activity was determined using the methodology of Giannopolitis and Ries (1977), which is based on the capacity of the enzyme to inhibit the photoreduction of nitro-blue tetrazolium (NBT). Readings were obtained spectrophotometrically at 560 nm, and SOD activity was expressed in terms of U mg^{-1} protein, where 1 U corresponds to the amount of enzyme needed to inhibit 50% of the photoreduction of nitro-blue tetrazolium. CAT activity was assessed by following the methodology proposed by Havir and McHale (1987), which is based on the consumption of H_2O_2 at 15-s intervals for 3 min measured spectrophotometrically at 240 nm. The molar extinction coefficient was $36\text{ mM}^{-1}\text{ cm}^{-1}$ and CAT activity was quantified in terms of $\mu\text{mol H}_2\text{O}_2\text{ min}^{-1}\text{ mg}^{-1}$ protein. APX activity was determined using the methodology of Nakano and Asada (1981) based on the consumption of ascorbate at 15-s intervals for 3 min measured spectrophotometrically at 280 nm. The molar extinction coefficient was $2.8\text{ mM}^{-1}\text{ cm}^{-1}$ and APX activity was expressed as $\mu\text{mol AsA min}^{-1}\text{ mg}^{-1}$ protein. We followed the methodology of Fang and Kao (2000) to determine POD activity, wherein the formation of tetraguaiacol is determined by an increase in absorbance. The molar extinction coefficient was $26.6\text{ mM}^{-1}\text{ cm}^{-1}$ and POD activity was expressed as $\mu\text{mol H}_2\text{O}_2\text{ min}^{-1}\text{ mg}^{-1}$ protein. DHAR activity was measured using the method described by Nakano and Asada (1981) based on the formation of ascorbate determined by an increase in absorbance at 265 nm and expressed as $\mu\text{mol AsA min}^{-1}\text{ mg}^{-1}$ protein. The methodology proposed by Hossain et al. (1984) was used to quantify the activity of monodehydroascorbate reductase (MDHAR) based on the oxidation of NADH measured by a reduction in absorbance at 340 nm and expressed as $\mu\text{mol NADH min}^{-1}\text{ mg}^{-1}$ protein.

2.7 Extraction and Quantification of Ascorbate, Hydrogen Peroxide, and Malondialdehyde Contents

To quantify the concentration of ascorbate, we applied the protocol established by Arakawa et al. (1981). Leaf ascorbate concentrations were based on the absorbance of the samples at 534 nm with reference to a standard curve prepared using known concentrations of ascorbate.

For the quantification of H_2O_2 and malondialdehyde (MDA), 200-mg samples of leaf tissue were macerated in liquid nitrogen and PVPP, followed by homogenization in 0.1% (w/v) trichloroacetic acid and centrifugation at $10,000\times g$ for 15 min at 4 °C. Values for H_2O_2 and MDA concentrations were determined based on the methods described by Velikova et al. (2000) and Buege and Aust (1978), respectively. For this, the samples used for the determination of H_2O_2 were read at 390 nm. For the determination of MDA, absorbance values obtained by the difference between readings at 535 and 600 nm were considered.

2.8 Agronomic Analyses and Tolerance Index

To analyze plant dry biomass, the panicles and vegetative parts of sorghum plants were dried in a forced-air oven at 70 °C for 72 h, and using the dried material, we determined the dry masses of the panicles, grains, and vegetative parts (leaves, stems, and roots). Using the total dry weight of grains and plants, we calculated the harvest index (Durães et al. 2002). To express the water deficit tolerance of sorghum plants induced by chitosan, we also calculated the tolerance index, which was determined by dividing the weight of the grain dry biomass obtained from plants grown under water-deficit conditions by the weight of the grains of plants grown in soil irrigated at field capacity (Souza et al. 2013).

2.9 Statistical Analyses

Having obtained the respective data, where necessary, we applied preliminary statistical tests to adjust the results for the purposes of analysis of variance (ANOVA). For all variables analyzed, we used Tukey's test at the 0.05 ($p < 0.05^*$) significance level to determine differences between the means values obtained in different treatments; the exception being for the tolerance index, as this was determined only for stressed plants, the mean values of which were compared using Student's t -test at the 0.05 ($p < 0.05^*$) significance level. We also evaluated correlations among all variables based on principal component analysis (PCA). Given that variables had different units of measurement, we initially standardized the data to present a mean of zero and a standard deviation of 1. The number of components was selected as a function of eigenvalues

(> 1.0) and the variance explained (> 80%). All statistical analyses were performed and values were plotted using R version 4.1.1 (R Core Team 2021).

3 Results

3.1 Leaf Water Potential

We found that water restriction had a notable effect on $\Psi_{at\ noon}$ in the leaves of *Sorghum bicolor* plants, with similar lowest average values being obtained for plants subjected to the water deficit (− 2.32 MPa) and water deficit + chitosan (− 2.28 MPa) treatments (Fig. 1S).

3.2 Ecophysiological, Leaf Area, and Nutritional Analyses

The assessed photosynthetic parameters of *S. bicolor* plants were found to be negatively affected by water deficit (Fig. 1). Plants grown under water-deficit conditions (water deficit and water deficit + chitosan) were characterized by a lower maximum quantum yield (F_v/F_m) than the irrigated plants (Fig. 1B) and were also demonstrated to have a lower net photosynthetic rate (A), stomatal conductance (g_{sw}), and transpiration rate (E) than the irrigated plants (Fig. 1B–D). In contrast, however, compared with the irrigated plants, the water-deficit treatments did not significantly affect the intercellular carbon concentration (C_i) or the ratio between intercellular and atmospheric CO_2 concentrations (C_i/C_a) (Fig. 1E, F). Notably, compared with the untreated plants (water deficit), the application of chitosan (water deficit + chitosan) was observed to attenuate the effects of water deficit on A , g_{sw} , E , C_i , and C_i/C_a . Furthermore, compared with the irrigated plants, the irrigated + chitosan treatment was found to promote reductions in A , g_{sw} , and E .

Compared with the irrigated plants, water restriction was also found to promote reductions in leaf area (Fig. 2B) and instantaneous transpiration efficiency (ITE — Fig. 2B) in the plants subjected to the Water deficit and Water deficit + Chitosan treatments.

With respect to leaf macronutrient contents, we found that only the concentration of S was influenced by water deficit (Fig. 2S). The imposition of water deficit reduced the concentration of S relative to that detected in the irrigated plants (Fig. 2SF). Similarly, compared with the untreated plants (irrigated) plants, the application of chitosan also reduced the concentration of S in the irrigated plants (irrigated + chitosan) (Fig. 2SF). Conversely, however, the application of chitosan in irrigated plants (irrigated + chitosan) was observed to promote increases in leaf N, P, Ca, and Mg concentrations compared with those in plants subjected to

Fig. 1 Maximum quantum yield of photosystem II (Fv/Fm) (A), net photosynthetic rate (A, B), stomatal conductance (g_{sw} , C), transpiration rate (E, D), intercellular carbon concentration (Ci, E), and ratio between the intercellular and atmospheric carbon concentrations (Ci/Ca, F) of the leaves of *Sorghum bicolor* plants subjected to water deficit and treated with chitosan. The plots represent the dispersion of the quartiles relative to the median. Plots followed by the same letter do not differ statistically at the 0.05 ($p < 0.05^*$) significance level based on Tukey's test

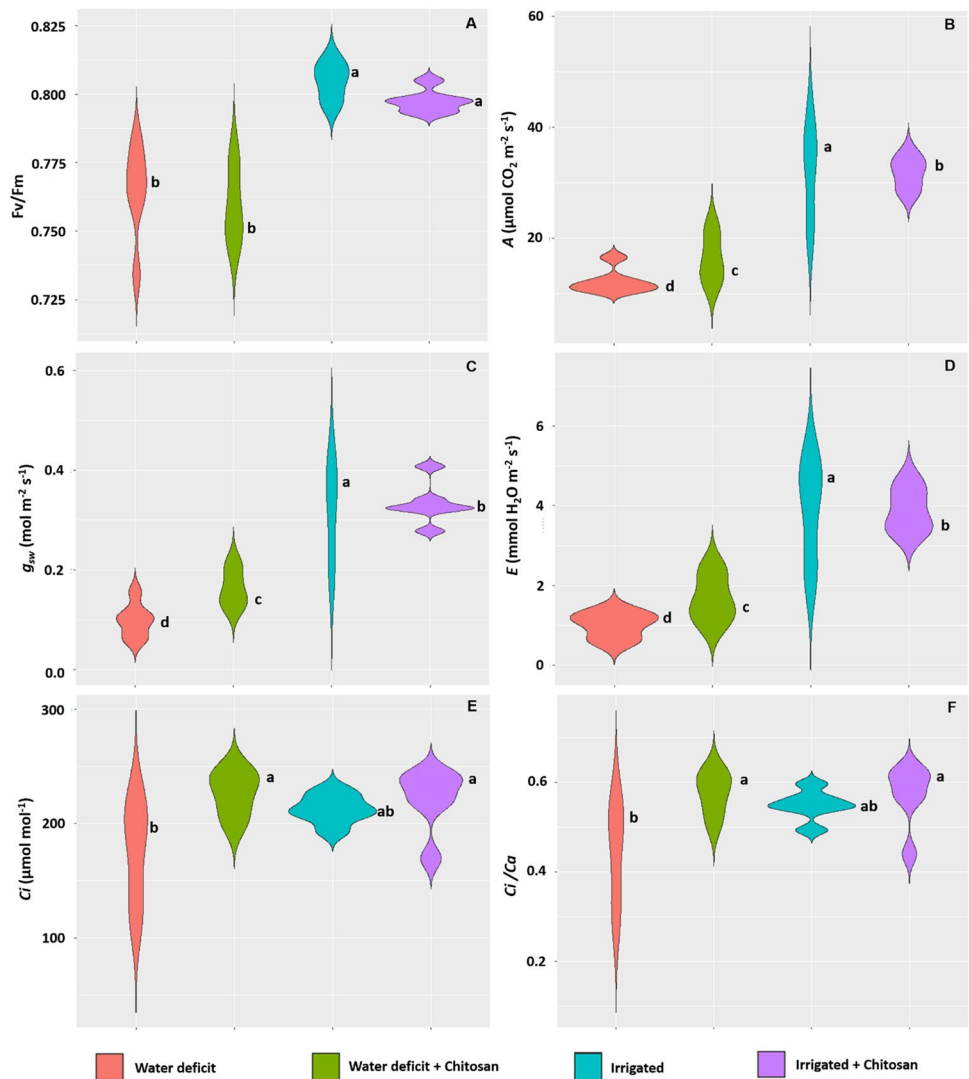
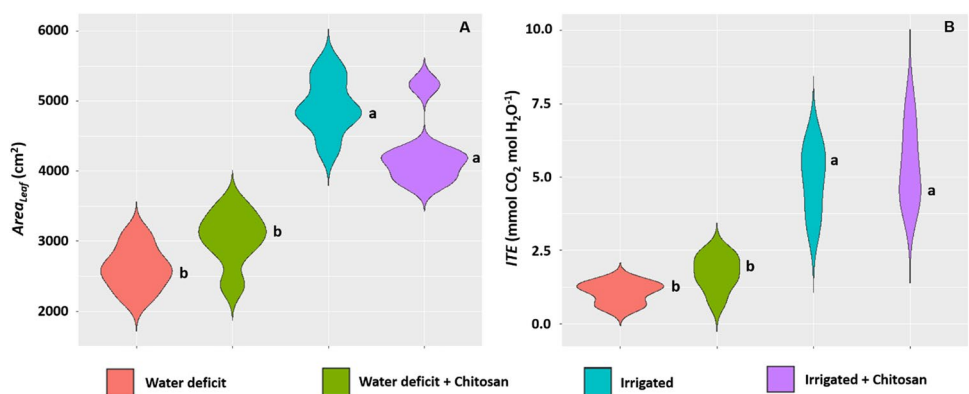


Fig. 2 Leaf area (A) and instantaneous transpiration efficiency (ITE, B) of the leaves of *Sorghum bicolor* plants subjected to water deficit and treated with chitosan. The plots represent the dispersion of the quartiles relative to the median. Plots followed by the same letter do not differ statistically at the 0.05 ($p < 0.05^*$) significance level based on Tukey's test



water-deficit conditions (water deficit + chitosan) (Fig. 2SA, B, D, E).

Compared with the irrigated plants, subjecting plants to water deficit only had the effect of reducing leaf Cu content in the water-deficit plants (Fig. 3S), whereas the application

of chitosan to irrigated plants (irrigated + chitosan) was observed to elevate leaf Cu content relative to the levels recorded in the untreated plants (Irrigated) (Fig. 3SA). In addition, the application of chitosan to irrigated plants (irrigated + chitosan) promoted increases in the Cu and

Zn contents and reductions in Fe contents relative to those detected in the water-deficit plants (water deficit + chitosan) (Fig. 3SA, B, D). The Mn content, however, was not affected by water deficit or the addition of chitosan (Fig. 3SC).

3.3 Primary Metabolites and Proline

With regard to the carbohydrate profiles of treated sorghum plants, we established that subjecting plants to water-deficit conditions has the effect of inducing the accumulation of reducing sugars and total soluble sugars in both the untreated and chitosan-treated plants (Fig. 3B, C). Specifically, the application of chitosan was found to promote the accumulation of TSS in irrigated plants (irrigated + chitosan) and that of starch in the water-deficit plants (water deficit + chitosan) compared with the untreated plants subjected to the same water regimes (Fig. 3C, D). Moreover, compared with irrigated plants (irrigated + chitosan), the application of chitosan under water-deficit conditions (water deficit + chitosan) was observed to promote increases in the accumulation of reducing sugars, sucrose, total soluble sugars, and starch (Fig. 3B–D).

Compared with the irrigated plants, water deficit also had the effects of reducing total amino acid content (Fig. 4B) and promoting increases in proline content (Fig. 4C) and the ratio between proline and total amino acids (Fig. 4D). Application of chitosan to irrigated plants (Irrigated + Chitosan) also promoted increases in total protein and total amino acid contents compared with levels recorded in the untreated plants (Irrigated) (Fig. 4B, B),

whereas under water-deficit conditions, the application of chitosan (water deficit + chitosan) contributed to a reduction in the proline content of plants compared with that in untreated plants in the water-deficit treatment. Similarly, applying chitosan to irrigated plants (irrigated + chitosan) resulted in higher accumulations of total proteins and total amino acids than in plants exposed to a water deficit (water deficit + chitosan).

3.4 Antioxidant Enzyme Activity

Neither exposure to water deficit nor the application of chitosan had any significant effects on the activities of the enzymes SOD and MDHAR, nor did we detect any appreciable differences in the activities of these enzymes among plants subjected to the different treatments (Fig. 5B, E). Contrastingly, however, exposing plants to water deficit was observed to stimulate an increase in the activities of APX and DHAR compared with those detected in irrigated plants (Fig. 5C, F). Compared with the untreated plants, the application of chitosan was also found to stimulate CAT activity in plants subjected to water deficit (Fig. 5B) and elevated APX activity in the irrigated (irrigated + chitosan) and water-deficit (water deficit + chitosan) plants (Fig. 5C). However, compared with the untreated plants, the application of chitosan was observed to reduce the activity of POD under water-deficit conditions (water deficit + chitosan) and that of DHAR in both irrigated (irrigated + chitosan) and water-deficit (water deficit + chitosan) plants (Fig. 5D, F).

Fig. 3 Reducing sugars (A), sucrose (B), total soluble sugars (C), and starch (D) contents in the leaves of *Sorghum bicolor* plants subjected to water deficit and treated with chitosan. The plots represent the dispersion of the quartiles relative to the median and indicate the variation between the maximum and minimum values observed. The black line in each plot is the median, and the red line is the mean. Plots followed by the same letter do not differ statistically at the 0.05 ($p < 0.05^*$) significance level based on Tukey's test

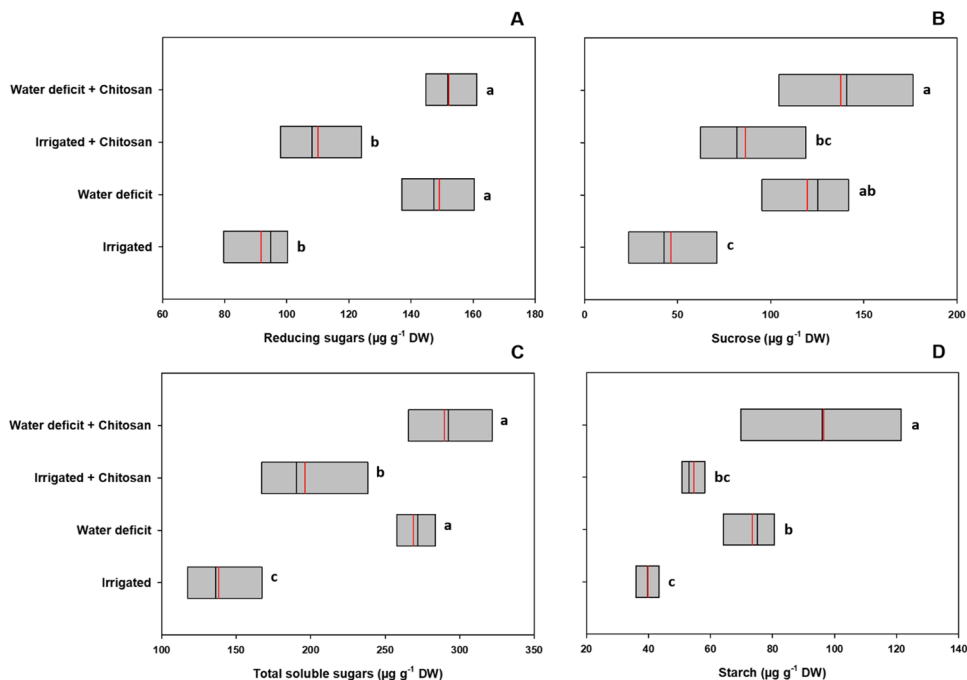


Fig. 4 Total proteins (A), total amino acids (B), proline (C), and proline/total amino acids ratio (D) in the leaves of *Sorghum bicolor* plants subjected to water deficit and treated with chitosan. The plots represent the dispersion of the quartiles relative to the median. The plots represent the variation between the maximum and minimum values observed. The black line in each plot is the median, and the red line is the mean. Plots followed by the same letter do not differ statistically at the 0.05 ($p < 0.05^*$) significance level based on Tukey's test

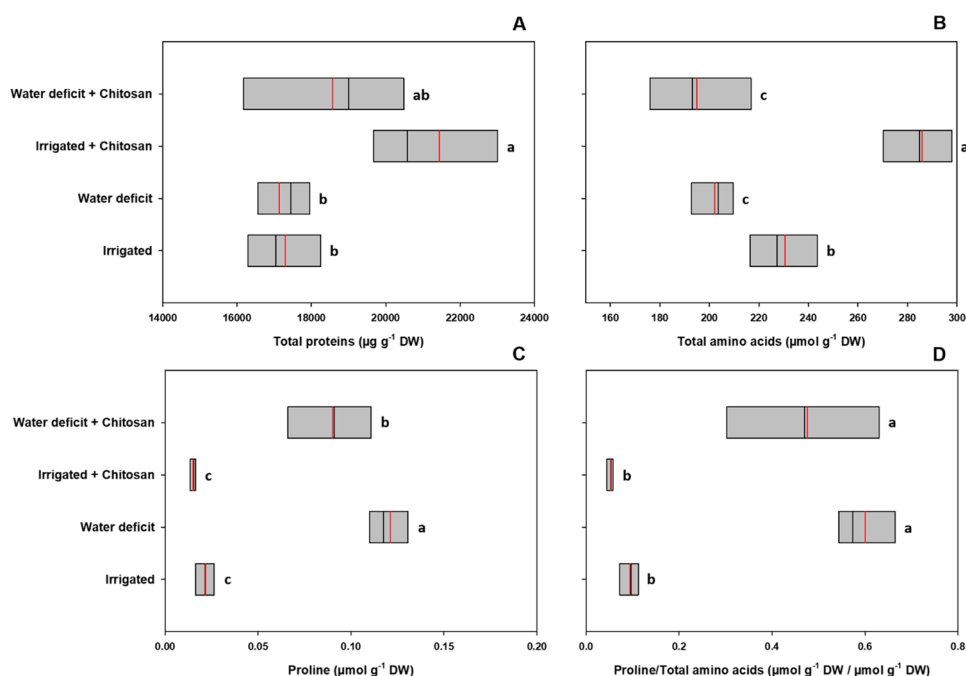
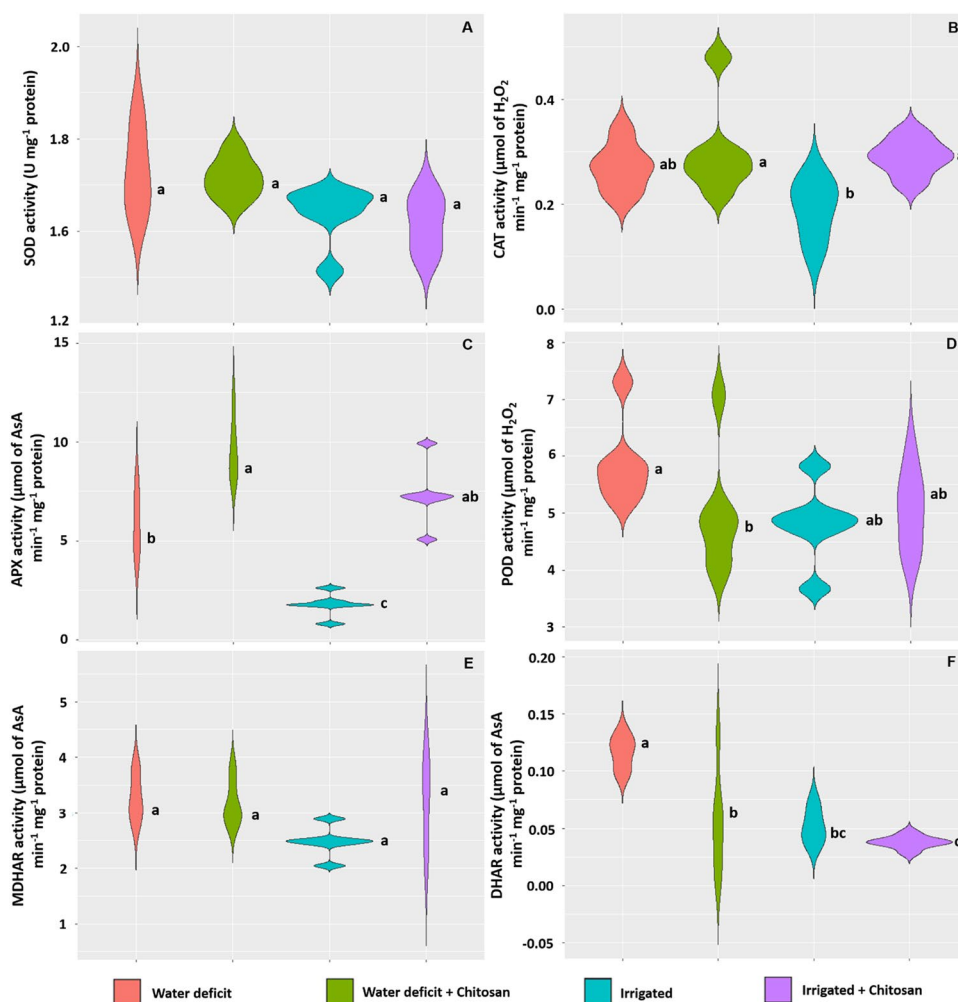


Fig. 5 Activity of the enzymes superoxide dismutase (SOD, A), catalase (CAT, B), ascorbate peroxidase (APX, C), guaiacol peroxidase (POD, D), monodehydroascorbate reductase (MDHAR, E), and dehydroascorbate reductase (DHAR, F) in the leaves of *Sorghum bicolor* plants subjected to water deficit and treated with chitosan. The plots represent the dispersion of the quartiles relative to the median. Plots followed by the same letter do not differ statistically at the 0.05 ($p < 0.05^*$) significance level based on Tukey's test



3.5 Ascorbate, Hydrogen Peroxide, and Malondialdehyde Contents

Compared with the irrigated plants, subjecting plants to water deficit had the effects of promoting increases in the leaf concentrations of ascorbate, H_2O_2 , and MDA (Fig. 6). However, whereas the application of chitosan had no appreciable effect on ascorbate concentrations in either irrigated or water-deficit plants (Fig. 6B), under water-deficit conditions, plants treated with chitosan were observed to generate less H_2O_2 and undergo lower levels of lipid peroxidation (as indicated by lower MDA contents) than untreated plant (Fig. 6B, C). Indeed, the amounts of H_2O_2 and MDA generated in plants treated with chitosan under water-deficit conditions (water deficit + chitosan) were observed to be similar to those detected in irrigated plants (Fig. 6B, C).

3.6 Agronomic Productivity and Tolerance Index

Compared with the irrigated plants, exposing sorghum plants to water deficit during the pre-flowering stage was found to lead to a shorter panicle length and lower panicle dry mass (Figs. 7B, B and 8B–D), as well as a reduction in grain dry mass and lower harvest index (Fig. 7C, D). However, we found that the application of chitosan contributed to mitigating the effects of water deficit on the assessed agronomic parameters of sorghum, resulting in greater panicle dry mass, grain dry mass, and harvest index compared with those recorded in the untreated plants (water deficit).

Under water-deficit conditions, the application of chitosan was also found to increase the tolerance index of the sorghum plants by 57% (Fig. 4S).

In Fig. 9, we present the results of our principal component analyses. The two-dimensional principal component plot depicts the separation of the four different treatments in the four quadrants of the biplot. The plot reveals that the water deficit + chitosan treatment was associated with greater activities of the enzymes CAT, APX, and MDHAR and concentrations of sucrose, starch, TSS, and RS, whereas the water-deficit treatment appeared to have more pronounced effects on the concentrations of H_2O_2 and MDA and activities of the enzymes DHAR, POD, and SOD. In contrast, the variables relating to macronutrient and micronutrient content, photosynthetic performance, and productivity were found to be affected to a greater extent by the irrigated treatment, and responses to the irrigated + chitosan treatment were more marked with regard to the accumulation of proteins and amino acids and Ci and Ci/Ca concentrations (Fig. 9).

4 Discussion

Given that it substantially reduces the growth, development, and productivity of agricultural crops, drought-induced water stress is considered among the most concerning environmental stressors in terms of plant production (Ding et al. 2018; El-Metwally et al. 2022; Salem et al. 2022; Saudy and El-Metwally 2022). In this study, we detected marked reductions in the panicle length and dry masses of panicles and grains in sorghum plants subjected to water deficit for

Fig. 6 Ascorbate (A), hydrogen peroxide (H_2O_2) (B), and malondialdehyde (MDA) (C) contents in the leaves of *Sorghum bicolor* plants subjected to water deficit and treated with chitosan. The plots represent the dispersion of the quartiles relative to the median. Plots followed by the same letter do not differ statistically at the 0.05 ($p < 0.05^*$) significance level based on Tukey's test

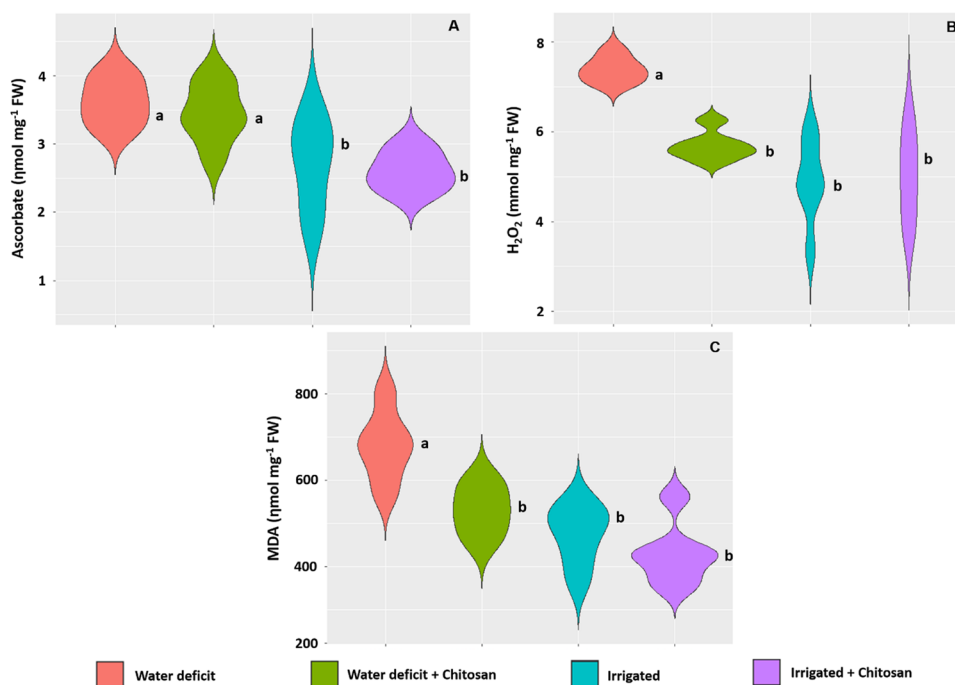


Fig. 7 Panicle length (A), panicle dry mass (B), grain dry mass (C), and harvest index (D) of *Sorghum bicolor* plants subjected to water deficit and treated with chitosan. The plots represent the variation between the maximum and minimum values observed. The black line in each plot is the median, and the red line is the mean. Plots followed by the same letter do not differ statistically at the 0.05 ($p < 0.05^*$) significance level based on Tukey's test

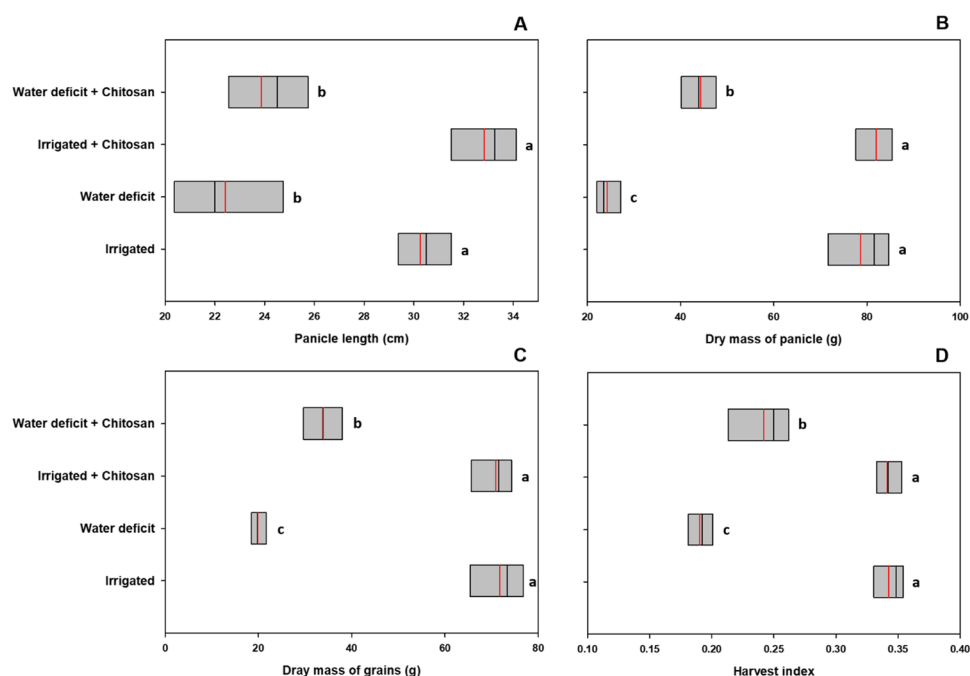
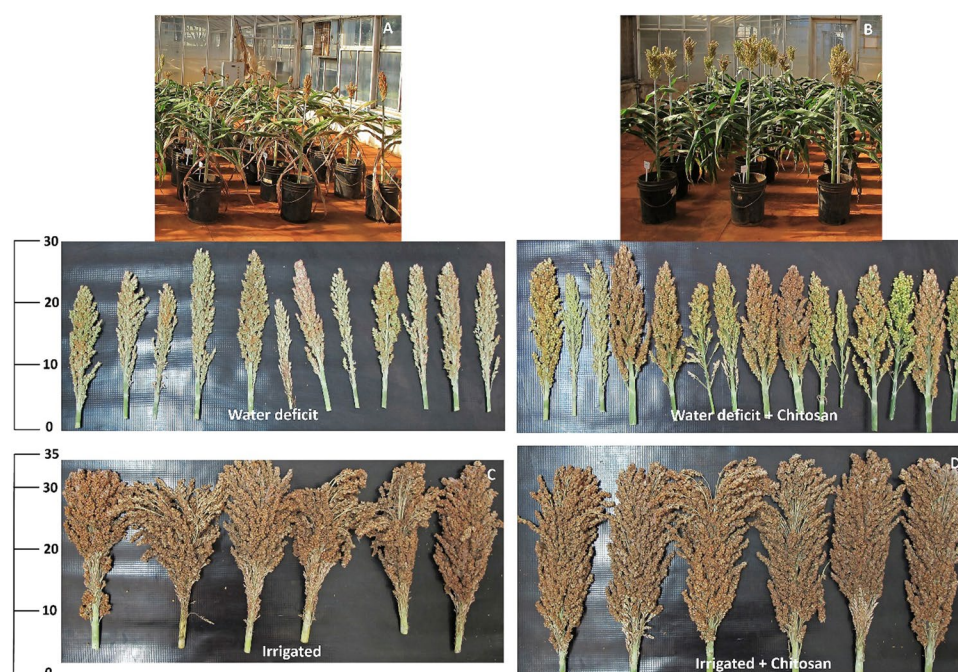


Fig. 8 Development and panicle length of *Sorghum bicolor* plants subjected to water deficit (A), water deficit + chitosan (B), irrigated (C), and irrigated + chitosan (D)



12 days during the pre-flowering stage; however, the foliar application of chitosan is an effective approach for enhancing the water deficit tolerance of the sorghum genotype BRS 322, which is known to be sensitive to this abiotic stressor. Furthermore, compared with the untreated plants, we also established that the application of chitosan increased the tolerance index of sorghum under water-deficit conditions. This happened, in part, because chitosan improved stomatal conduction and transpiration in plants under water deficit.

During periods of low rainfall, low humidity, and high temperatures, the vapor pressure deficit of the atmosphere increases, thereby inducing high rates of transpiration water from plant surfaces, mainly the leaves, via stomatal conductance (Avila et al. 2020). Consequently, plants begin to lose water through the aerial part at a higher rate than roots can absorb water from the soil, wherein water availability is limiting.

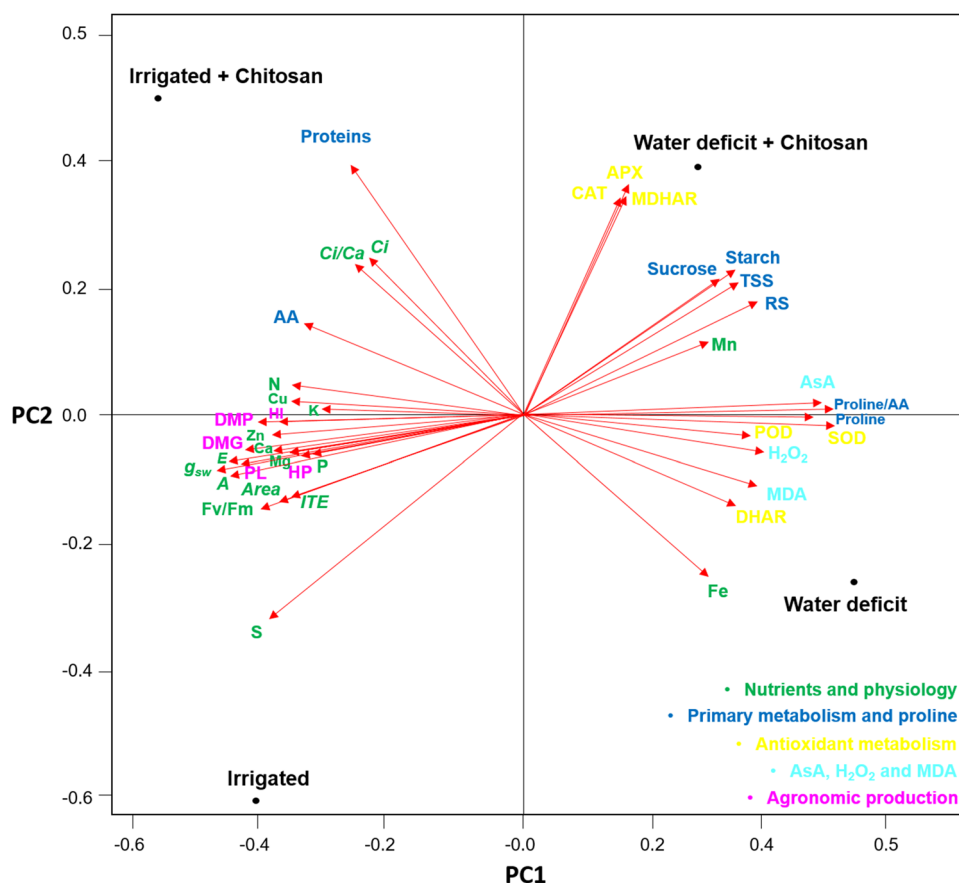


Fig. 9 Principal component analysis of macronutrient and micro-nutrient contents; photosynthetic characteristics; concentrations of primary metabolites and proline; antioxidant metabolism enzyme activities; ascorbate (AsA), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA) contents; and agronomic production of *Sorghum bicolor* plants subjected to a water deficit and treated with chitosan. A, photosynthetic rate; g_{sw} , stomatal conductance; C_i , internal CO_2 concentration; E, transpiration rate; Area, leaf area; ITE, instantaneous

transpiration efficiency; Fv/Fm, maximum quantum yield of photosystem II; AA, total amino acids; RS, total reducing sugars; TSS, total soluble sugars; CAT, catalase; APX, ascorbate peroxidase; POD, guaiacol peroxidase; SOD, superoxide dismutase; DHAR, dehydroascorbate reductase; MDHAR, monodehydroascorbate reductase; WP, water potential at noon; PL, panicle length; GDM, grain dry mass; PDM, panicle dry mass; and HI, harvest index

Chitosan is a polysaccharide derived from chitin that is typically extracted from fungal cell walls (Huq et al. 2022) and is obtained industrially as a product of the alkaline deacetylation of chitin. When absorbed and metabolized by plants, it triggers a signaling cascade, which is assumed to represent a response mechanism to pathogen attack. The fine response system thus activated involves important signaling molecules such as Ca^{2+} , nitric oxide, H_2O_2 , and ethylene that play roles in regulating stomatal opening and closure during periods of water deficit (Agurla et al. 2014; Czékus et al. 2021; Hann et al. 2014). It has also been proposed that the influence of chitosan on stomatal control may be closely associated with the metabolic conditions of leaf tissues during and after treatment. Indeed, Dos Reis et al. (2019) found that after 15 days under water-deficit conditions, plants receiving the chitosan derivatives *N*-succinyl and *N,O*-dicarboxymethyl chitosan were characterized by higher rates of stomatal

conductance than untreated control plants. This response was interpreted to be an effect of induced systemic resistance, given that these effects were not detected in plants at the initial stage (day 1) of treatment. Notably, this chitosan-induced resistance was verified only in sorghum plants that had been subjected to water deficit, thereby indicating the efficacy of chitosan in preventing water loss in plants.

Although reducing stomatal conductance and transpiration is a mechanism of paramount importance for plant survival during periods of water deficit, this strategy can exact a high metabolic cost on plants. However, in limiting water lost through transpiration by reducing stomatal conductance, this also limits the absorption of carbon dioxide, the substrate for the Calvin cycle. In parallel and dynamically, low transpiration reduces the transport of nutrients to the aerial parts, as this process is governed by mass flow (Avila et al. 2020). Consistently in this regard, we found

that plants experiencing water deficit are generally characterized by reduced concentrations of N, P, Mg, Ca, S, Cu, and Zn. Conversely, the tissue contents of N, P, Ca, Mg, Cu, and Zn were generally found to be increased in plants treated with chitosan under normal irrigation conditions. In this context, protonated chitosan, which is rich in positive charges, has a particularly high affinity for plant cell membranes, thereby resulting in greater reactivity in the plant system, and plausibly a greater uptake of available nutrients. Indeed, Wang et al. (2015) have demonstrated that chitosan nanoparticles can enhance the uptake of macronutrients such as N (by 9.8–27.4%), P (17.3–30.4%), and K (30–45%) in routinely irrigated *Coffea canephora* Piere var Robusta plants.

Similarly, Sami et al. (2016) found that chitosan can promote plant growth by increasing nutrient uptake and biomass accumulation in irrigated *Phaseolus vulgaris* plants. In *Sorghum bicolor* plants, water appears to play an important role in enhancing the effects of this polysaccharide on nutrient acquisition, which may be associated with chitosan-induced changes in ion fluxes through the membrane (Li et al. 2020). In this regard, Magalhães and Durães (2008) have shown that chitosan can dose-dependently trigger a rapid transient depolarization of *Mimosa pudica* cell membranes and that this modification is accompanied by a transient increase in pH. Given that modifications of rhizosphere pH may induce changes in macro- and micronutrient uptake (Yu et al. 2021), and taking into consideration the fact that water is essential for soil mineral solubilization, it can be speculated that the nutritional effects of chitosan may be more pronounced under irrigation conditions. Alternatively, it is conceivable that the nutritional constitution of chitosan per se (9–10% nitrogen) may be nutritionally beneficial to treated plants.

In terms of gas exchange, it is assumed that in plants subjected to water-deficit treatment, photosynthesis would be limited by stomatal conductance, as evidenced by a reduction in internal carbon concentrations compared with other treatments. However, a reduction in photosynthesis may also be linked to a perturbation of biochemical processes, given that although plants treated with chitosan under water-deficit conditions are characterized by a lower photosynthetic rate, their internal carbon concentrations were similar to those of irrigated plants. As evidence in support of this hypothesis, we detected a reduction in the quantum efficiency of photosystem II (Fv/Fm) in plants experiencing water deficit, regardless of the application of chitosan. Nonetheless, it is plausible that in plants subjected to water deficit, compared with untreated plants, the higher photosynthetic rate of those treated with chitosan may be associated with the higher carboxylation efficiency exhibited by these plants, which would corroborate the findings of (Dos Reis et al. 2019).

The elevated photosynthetic rate of chitosan-treated plants under water-deficit conditions compared with that of

the untreated water-stressed plants would go some way in explaining the higher panicle and grain dry mass production of these plants, since photosynthesis is the source of all carbon-sustaining plant growth and development. A similar increase in photosynthetic rate and stomatal conductance has also been reported by Khan et al. (2002) and Dos Reis et al. (2019) following the application of chitosan to soybean and maize. This in turn indicates how chitosan treatment can be used to induce water deficit tolerance. Consistently, based on the phenotyping of different maize materials for assessing water deficit tolerance, Souza et al. (2013) and Lavinsky et al. (2015) established that maintaining a higher photosynthetic rate during the water restriction period is an important mechanism enabling plants to tolerate this stress. Moreover, Avila et al. (2017) found that in addition to maintaining a higher rate of carbon assimilation, drought-tolerant maize genotypes are also more efficient in partitioning carbon from the source to sink organs of commercial interest. In this regard, although in the present study we detected a reduction in harvest index in plants experiencing water deficit, the extent was less pronounced in those plants receiving the water deficit + chitosan treatment, thereby tending to indicate a more efficient partitioning of carbon.

In this scenario, both water-deficit treatments gave rise to an increase in leaf carbohydrate accumulation, as indicated by our observations of higher levels of reducing sugars, sucrose, total soluble sugars, and starch in plants subjected to water deficit. Moreover, this accumulation was generally more pronounced in the chitosan-treated plants than in the controls. We suspect that this drought-induced accumulation of carbohydrates could be linked to the reduced activity of saccharolytic enzymes, such as sucrose-phosphate synthase and cell wall invertase, the activities of which significantly alter the pattern of carbon partitioning between source and sink (Xu et al. 2015). Moreover, during a period of water deficit, the low water content in plants compromises the water exchange between the xylem and phloem and thereby diminishes the forces between sources and sinks. This in turn results in a lower or even cessation of transport via the phloem (Sevanto 2018), thereby compromising carbon partitioning efficiency, and as a consequence, reducing grain yield (Avila et al. 2021). Thus, among plants cultivated under water-deficit conditions, the greater accumulation of these carbohydrates in the leaves of chitosan-treated plants was directly associated with a heightened photosynthetic performance.

On the basis of our principal component analyses, however, we detected a negative correlation between photosynthesis and carbohydrates, which explains the lower photosynthetic rate exhibited by the irrigated plants treated with chitosan in relation to the untreated irrigated plants. In this case, it can be speculated that under these conditions, there was negative feedback by the product, given that synthesis

of the final product can exert short-term metabolic feedback control via Pi recycling. Furthermore, under circumstances in which there is an imbalance between source and sink at the whole plant level, the accumulation of carbohydrates in leaves can promote the downregulated expression of photosynthesis-related genes and an acceleration in leaf senescence (Du et al. 2020; Pennisi 2009).

Although it may appear somewhat contradictory, an accumulation of sugars coinciding with a drought-induced energy deficit is a vital mechanism enabling plants to tolerate this stress, and an understanding of this mechanism involves leaving the Cartesian realm and entering the holistic realm. In response to water deficit, as a strategy to maintain the rate of water uptake, cells alter the vacuolar levels of ionic solutes, such as potassium, chloride, and calcium. However, for water balance to occur between the vacuole and the cytosol, it is necessary to produce and accumulate compatible organic compounds, such as proline and soluble sugars, in the cytosol. The accumulation of sugars in the leaves may also have a protective effect against the generation of ROS, primarily $^1\text{O}_2$ and OH^- , produced during electron transfer processes in leaf and root tissues, as these ROS are not eliminated enzymatically (Siddiqui et al. 2020).

Proline and the proline/amino acid ratio (the latter of which is a measure of the proportion of total amino acids allocated to the synthesis of proline) increase in stressed plants. However, we found that the untreated stressed plants were characterized by the highest proline values, thereby indicating the potential efficacy of chitosan in alleviating the stressful effects of water deficits, as proline is recognized as a physiological marker of osmotic stress (Avila et al. 2021; Silva et al. 2011). By acting as an inert compatible osmotic agent, proline contributes to osmotic adjustments in tissue and also plays the role of a molecular chaperone by stabilizing proteins and preventing these from misfolding and aggregating (Ghosh et al. 2022; Hayat et al. 2012).

We also noted that the application of chitosan promoted increases in the concentration of total proteins and total amino acids in irrigated plants. We suspect that this effect may also reflect the previously mentioned property of chitosan as an agent triggering signaling cascade interpreted as a pathogen attack, which in turn activates a MAP kinase cascade, and thereby alters the expression of genes inherent in the synthesis of proteins and amino acids associated with the induced resistance of plants (Agurla et al. 2014; Lehmann et al. 2015). Chitosan nanoparticles induced the innate immune response in plants through the upregulation of defense-related genes, including those of several antioxidant enzymes (Chandra et al. 2015).

Regardless of chitosan treatment, the enzymes of the antioxidant system were grouped as a function of water deficit in our principal component analysis. This would seem a reasonable association, given that we detected increases

in the activities of APX and CAT in the plants treated with chitosan. Similarly, we observed increases in the activities of POD and MDHAR in response to the water deficit, although these activities did not appear to be influenced by chitosan. APX and CAT are key enzymes in redox homeostasis that contribute to controlling the levels of H_2O_2 , which, despite being a key signaling molecule, can be toxic at high concentrations, and thus reduce the growth and productivity of agricultural crops cultivated under stressful conditions (Khan et al. 2018). Azzouz-Olden et al. (2020) demonstrated that the differential expression of genes that act in the defense against oxidative stress, including those related to the synthesis of antioxidant enzymes, may constitute an important strategy for *S. Bicolor* to resist drought. This fact corroborates the results verified in this study. Consistent with this activity, we found that stressed plants treated with chitosan were characterized by lower levels of H_2O_2 and lipid peroxidation (MDA levels), thereby highlighting the efficacy of chitosan in reducing drought-induced oxidative stress.

Having subjected the data obtained in this study to univariate and multivariate analyses, our overall findings indicated that chitosan has the effect of enhancing water deficit tolerance in sorghum. Compared with the untreated plants under water-deficit conditions, the application of chitosan to drought-stressed plants promoted higher rates of photosynthetic, transpiration, and stomatal conductance; enhanced carboxylation efficiency; gave rise to a higher harvest index; and induced increase in APX and CAT activities and the leaf contents of starch and total soluble sugars. Furthermore, the principal component analysis revealed APX, CAT, MDHR, DHR, POD, sucrose, starch, reducing sugars, total soluble sugars, proline, Mn, and Fe to be clustered as a function of water deficit. Consequently, irrespective of chitosan treatment, these variables are identified as contributing to mechanisms underlying the response to water deficit, thereby ensuring the “normal” functioning of the primary metabolic machinery and mitigating sorghum productivity losses in the face of water-deficit events. These variables can accordingly provide a focus of further studies that will seek to enhance the water deficit tolerance of grain sorghum, either by elicitation or genetic improvement.

5 Conclusion

In this study, we obtained evidence in support of the hypothesis that foliar application of the polysaccharide chitosan enhances the water deficit tolerance of grain sorghum plants. Plants cultivated under water-deficit conditions that were treated with chitosan had a final grain yield that was 19.19% higher than that of plants that were not treated with this biopolymer. Moreover, we established that this increase in productivity in response to chitosan was associated with

higher rates of photosynthesis, transpiration, and stomatal conductance; enhanced carboxylation efficiency; a higher harvest index; and increases in activities of the antioxidant enzymes ascorbate peroxidase and catalase. Therefore, chitosan is indicated to improve sorghum performance under water-deficit conditions and can be better investigated with the objective of prospecting water-deficit-resistant sorghum lineages, by means of elicitation with this polysaccharide.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s42729-022-01111-4>.

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Declarations

Conflict of Interest The authors declare no competing interests.

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