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Foliar application of gamma radiation processed chitosan triggered distinctive biological responses in sugarcane under water deficit stress conditions

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ABSTRACT

Chitosan, being one of the most promising biological macromolecules, has an immense scope in agriculture to boost crop growth and defense responses. In this study, chitosan was exposed to gamma rays in order to obtain a low molecular weight derivative. Viscometric characterization showed a sharp decrease in molecular weight and FTIR based analysis confirmed retention of structural integrity of the polymer upon gamma irradiation. Assessments of various physiological and biochemical attributes were carried out on sugarcane plantlets that were subjected to progressive water deficit stress. The irradiated chitosan was found to differentially ameliorate water deficit stress tolerance against that of normal chitosan through positive modulation of various gas exchange parameters alongside significant improvement in relative tissue water content, SOD activity, soluble sugars and adenine energetics. Furthermore, application of irradiated chitosan significantly reduced cell membrane damage, lipid peroxidation, H_2O_2 and free-proline accumulations. This is the first report on the use of gamma irradiated chitosan to alleviate water deficit stress tolerance in sugarcane. Overall comparative assessments showed that differential plant responses were triggered upon foliar application of normal and gamma irradiated chitosan in sugarcane plants grown under water deficit stress conditions.

1. Introduction

Abiotic stress factors have become an important and serious challenge to plant growth and productivity and these negatively impact average crop yield by >50% [1]. Globally, climate-change has shown a decisive impact on availability and unpredictability of water resources for agricultural use [2]. Water deficit stress occurs as a result of depletion of soil moisture and rise in global temperature and, hence there is a need to develop appropriate breeding, agronomic and genomic strategies to alleviate drought resistance in crop plants with higher productivity and water-use efficiency [3]. While conventional breeding and transgenic methods are time consuming, use of bioregulators for alleviating stress and enhancing plant productivity has attracted much attention. Various chemical and hormonal based bioregulators are exogenously applied to boost the plant signaling to enhance growth and crop yield [4].

Sugarcane (*Saccharum* spp.) is the world's highest biomass producing crop with immense industrial importance mainly due to its major (70–80%) share in the global sugar production [5,6]. Commercial cultivation of sugarcane is challenged by adverse environmental calamities (such as scarcity of water, excess of salts and toxic metal in soils, extreme low and high temperatures) that limit sugarcane productivity [5,7]. Since sugarcane crop is grown in the tropical and subtropical regions, the crop experiences sudden and erratic changes in climatic milieu for some or other time during the cropping cycle [8,9]. Moreover, sugarcane is considered as water guzzler, water intensive crop as its cultivation demands frequent and large quantity of irrigation water [10]. Among the four developmental growth stages, the tillering and grand growth phases are considered to be the most critical contributing to >80% of sugarcane yield and it is at these stages, the crop is most vulnerable to water scarcity [8,11,12].

Drought is one of the major limiting factors to sugarcane yield and sugar productivity [13] and is much emphasized over salinity and osmotic stresses, due to its huge economic impact. Furthermore, the occurrence, intensity and duration of drought are difficult to predict but crop losses due to water deficit are not unusual. Almost every year, one or other sugarcane growing region suffers mild to acute water shortages [9]. Under severe water deficit conditions, yield losses in

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sugarcane can be up to 60% or more [9,12,14,15]. Therefore, drought can cause major economic losses for cane growers and thereby sugarcane-based industries. Various abiotic stress conditions directly affect physiological and metabolic status of plants [16]. Drought stress induces oxidative stress in sugarcane that shows acute increase in lipid peroxidation and H_2O_2 content [17].

Chitosan, derived from chitin which is one of the most abundant biopolymers, next to cellulose, has non-toxic, non-allergenic, biodegradable and biocompatible properties and imparts multiple stress tolerance in plants [18]. Therefore, it has been immensely exploited as a versatile bioactive substance with superior material and functional properties. Much of the chitosan is derived from different waste materials, chiefly wastes of fishery and sea food industries which have abundant renewable resource and alterative for waste management. Being a potential bioactive substance, chitosan and its derivatives have found immense applications in diverse fields that include cosmetics, pharmaceuticals to agriculture [19,20]. Biological effects of chitosan include antimicrobial (against bacteria, fungi and viruses) and antioxidant (to encounter oxidative damages caused due to adverse conditions) activities beside its growth promoting properties [21]. Exogenous application of chitosan has potential to alleviate adverse effects of salinity [22–24] and drought stresses [25–32]. Despite earlier reports on the use of chitosan against biotic and abiotic stresses, studies on the use of gamma irradiated chitosan in crop plant are limited.

Plant cellular responses to exogenously supplied chitosan differ based on the type of chitosan (high/low MW), degree of acetylation, availability of functional group etc. Different approaches to obtain low molecular weight chitosan derivatives are shown to increase biological potential (in food, medicine/pharmaceuticals, agriculture, biotechnology, material science) over the native chitosan [33]. Chitosan nanoparticles were shown to induce innate immune response in plants through up-regulation of defense related genes including that of several antioxidant enzymes as well as elevation of total phenolics [34]. Low molecular weight chitosan can be obtained by various ways but these processes, although effective for desired recovery of the final product, are associated with flaws such as lengthy treatment time, low productivity and selectivity, high processing cost (enzymatic method) and formation of toxic chemical by-products [35]. However, irradiation of chitosan with gamma rays is very efficient and without any of these drawbacks and the product formed can be directly used for downstream applications without any further processing. In this study, we explored if normal chitosan and its gamma irradiated- low molecular weight derivative could differentially modulate water deficit stress in sugarcane, which is a commercial crop grown in many parts of the world. To the best of our knowledge, exogenous foliar application of gamma irradiated chitosan to alleviate water deficit stress tolerance has not yet been reported in sugarcane. With this aim, a comparative study was carried out to assess the effects of normal CSN (NL-CSN) with gamma irradiated CSN (IR-CSN) on physiological and biochemical attributes of sugarcane plants grown under water deficit stress.

2. Materials and methods

2.1. Sugarcane plant husbandry and growth conditions

Sugarcane settlings (of popular commercial cultivar Co-86032) derived from single eye buds were raised at the nursery of the Vasantdada Sugar Institute (VSI), Manjari (Bk.), Pune, India (18° 31′ 38.9244″ N, 73° 58′ 20.568″ E, 549 m above MSL). For this purpose, individual eye buds setts were excised out and pre-treated with fungicide and insecticide as per the recommended package of practices. The single eye bud setts were planted in nursery trays filled with coco-peat: vermi-compost mixture (1:1 ratio) and grown under ambient conditions in a shade-net house. After 30 days, healthy uniformly grown settlings were transplanted in polybags (12 cm × 15 cm) containing soil: vermi-compost: coco-peat mixture (2:1:1 ratio). Plants were then after grown into a climate-controlled plant growth chamber (with temperature 30 ± 2 °C and RH > 60%). All plants were received equal quantity of irrigation at regular intervals (200 ml per bag every alternate day) till imposition of respective treatments.

2.2. Preparation of chitosan and gamma irradiation

Two percent solution of chitosan (extracted from shrimp shells having degree of deacetylation 85%) was prepared in 1% (v/v) glacial acetic acid in distilled water and continuously stirred for couple of hours. This homogenous, highly viscous solution was then packaged in polythene bags of appropriate dimension for gamma radiation treatment. Gamma irradiation (^{60}Co) was carried out using gamma irradiation facility (dose rate of 35 Gy/min) at the Food Technology Division, Bhabha Atomic Research Centre (BARC), Mumbai, India. The gamma irradiation dose of 100 kGy was chosen based on earlier reports [35,36] and worked out to be optimal based on our optimization studies (data not shown).

2.3. Characterization of normal and gamma irradiated chitosan

2.3.1. Determination molecular weight (MW)

A capillary Ubbelohde viscometry (Model 1 Type B J-Sil) was used at 25 ± 1 °C to determine the average viscometric molecular weight ($\overline{M}v$) of normal (non-irradiated, NL-CSN) and gamma radiation degraded chitosan (IR-CSN).

2.3.2. Determination of chemical changes in polymer chain

Changes in functional groups of the polymer were confirmed by Fourier Transform Infrared Spectroscopy (FTIR) analysis. For this purpose chitosan solutions were vacuum dried to obtain dry crystalline powder. An aliquot of the respective chitosan was then homogenized with potassium bromide (KBr) and a pellet of chitosan-KBr was made. FTIR spectra were recorded in the range of 4000–400 cm^{-1} using Jasco FTIR Spectrometer (Jasco Model FTIR-660 plus).

2.4. Drought imposition and chitosan foliar spray to sugarcane plants

After thirty days of transplanting, these two months old sugarcane settlings were grouped in different sets for foliar application of respective chitosans at different concentrations. Details of foliar treatments and imposition of water deficit conditions are described in Table 1. The water deficit condition was imposed by withholding irrigation, except to the control plants that received irrigation at regular interval. For foliar spray, chitosan solutions of respective concentrations (50, 100 and 200 ppm) were prepared in distilled water containing 0.2 ml/l Tween-20 as wetting agent. Sprays of respective solutions were carried out thrice at one week interval during early hours on the day using a handheld mist sprayer to get fine mist of solution on both the abaxial and adaxial surfaces of the leaves. Both sets of control (irrigated and water deficit) plants were sprayed with equal volume of distilled water (containing 0.2 ml/l Tween-20) alone instead of chitosan solutions. With the commencement of first foliar spray, water withholding was followed for water deficit control and all chitosan sprayed plants till third spray.

2.5. Assessments of gas-exchange indices

The gas exchange measurements were performed 24 h after third foliar spray of respective chitosan concentrations. For this purpose, three settlings of each treatment group were randomly selected and measurements were performed on 3rd leaf from the top. The parameters such as net photosynthesis (A), stomatal conductance (g_s), internal CO_2 concentration (Ci), and net transpiration (E) were measured using a LICOR-6400 Portable Photosynthesis System (LI-COR Inc., Lincoln, NE, USA) during bright sunlight from 11:00 am to 12:30 pm. Water use efficiency

(ratio between *A* and *E*) and stomatal resistance (ratio between *A* and *Ci*) [37] and intrinsic water use efficiency (iWUE, ratio of *A* and g_s) were also determined.

2.6. Assessments of biochemical attributes

2.6.1. Determination tissue relative water content (RWC)

The effects of water deficit and chitosan treatment on plant growth were evaluated in terms of relative water content (RWC) [38]. Briefly, the leaf pieces (~1 cm^2) from middle portion of actively growing 3rd leaf were harvested 24 h after the third foliar spray, fresh weight (FW) was recorded immediately after harvesting and placed in screw cap tubes containing 10 ml of distilled water and incubated on shaking incubator (180 rpm) at 25 ± 2 °C for 24 h in dark. Turgor weigh (TW) was recorded after blotting the leaf pieces on paper towel and tissue was oven dried at 80 °C for 48 h to measure dry weight (DW). Based on this, RWC was calculated as RWC (%) = [(FW − DW) / (TW − DW)] × 100.

2.6.2. Determination of relative electrolyte leakages (REL)

Relative electrolyte leakage (REL) was measured by incubating leaf pieces (~1 cm^2) in 10 ml of distilled water on shaking incubator (180 rpm) at 25 ± 2 °C for 24 h. Electrical conductivity (EC1) of the water was recorded using an electrical conductivity meter (Equitron) and the tubes were autoclaved for 20 min to release all the electrolytes in the solution. Conductivity (EC2) of the solution was recorded after cooling the content to room temperature. The REL was calculated as REL (%) = (EC1 / EC2) × 100.

2.6.3. Determination of lipid peroxidation

Lipid peroxidation was determined by the estimation of malondialdehyde (MDA) content following the protocol of Heath and Packer [39] as described previously by Srivastava et al. [40]. Leaf tissue was ground in liquid nitrogen using mortar and pestle and 1 ml of freshly prepared 0.5% thiobarbituric acid (TBA) in 20% tri-chloroacetic acid (TCA) was added. The mixture was boiled in water bath for 30 min and centrifuged after cooling to room temperature. The supernatant was used to read absorbance at 532 nm and 600 nm using spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Japan). The amount of MDA (extinction coefficient of 155 mM^{-1} cm^{-1}) was calculated by subtracting non-specific absorbance at 600 nm from absorbance at 532 nm.

2.6.4. Determination of free proline

The level of proline was measured following the method given by Bates et al. [41]. Leaf tissue was weighed (approx. 100 mg), homogenized in 3% (w/v) aqueous sulfosalicylic acid and centrifuged at 12,000 rpm for 10 min. One ml of supernatant was mixed with one ml acid ninhydrin (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M orthophosphoric acid) and one ml glacial acetic acid and was boiled at 100 °C for 1 h. The reaction was terminated by cooling on an ice-bath. The red coloured-water insoluble product was extracted with 2 ml toluene, and the absorbance was read at 520 nm using spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Japan). Proline content was calculated from the standard curve prepared using known concentrations of L-proline and was expressed as μM g^{-1} FW.

2.6.5. Determination of reactive oxygen species (H_2O_2)

For the estimation of hydrogen peroxide (H_2O_2) levels, plant samples were homogenized in 0.5% (w/v) trichloroacetic acid (TCA) in an ice-bath and centrifuged at 14,000 ×*g* for 15 min at 4 °C [42,43]. Equal volumes of the supernatant and 100 mM potassium phosphate buffer (pH 7.0) were mixed with double volume of freshly prepared 1 M potassium iodide. Reaction was allowed to occur for 1 h in dark and the absorbance was measured at 390 nm using spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Japan). The amount of H_2O_2 was calculated from a standard curve prepared using the known concentrations of H_2O_2.

2.6.6. Estimation of superoxide dismutase (SOD) activity

Cellular proteins were extracted in 100 mM Sodium phosphate buffer (pH 7.0) containing 0.1 mM ethylene diamine tetra acetic acid (EDTA). One ml ice cold buffer was added in each tube with finely ground tissue samples and vortexed vigorously. The tubes were incubated for 1 h on ice and subjected to centrifugation at 13,000 rpm for 20 min at 4 °C. The supernatant containing crude proteins was collected separately in microcentrifuge tubes and frozen at −80 °C for further use in enzyme activity assays. Protein concentration was determined by Bradford reagent using bovine serum albumin (BSA) as a standard [44]. Assay of SOD activity was performed as given by Beauchamp & Fridovich [45] with some modifications. Assay mixture comprised of 50 mM sodium phosphate buffer (pH 7.8), 0.1 M EDTA, 14.3 mM methionine, 82.5 μM nitro blue tetrazolium (NBT). Independent sets for light and dark incubation were prepared and light and dark blank reactions were kept separately. Assay mixture comprised of suitable aliquot of sample extract and Riboflavin (2.2 μM) were sequentially added and mixed by inversion. Respective sets of tubes were then immediately transferred to dark and light for 30 min incubation. After completion of incubation period, absorbance of the reaction content was read at 560 nm using spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Japan). The specific activity of SOD was empirically determined and expressed in SOD activity mg^{-1} protein.

2.6.7. Estimation of chlorophyll pigments and chlorophyll stability index (CSI)

For estimation of plant pigments (chlorophyll *a* and b), finely ground leaf tissues were extracted in 1 ml of ice cold 80% (v/v) acetone. Mixture was vortexed to ensure dispersion of tissue clumps and allowed to stand at room temperature for about 15 min followed by centrifugation at 13000 rpm for 10 min. The supernatant was collected in fresh tubes and tissue pellet was re-extracted using ice cold 80% (v/v) acetone, centrifuged and supernatants were pooled together. The absorbance was read at 645 nm and 663 nm using spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Japan). Estimation of individual pigment was done following formulae given by Arnon [46],

$$\text{Chlorophyll a } (\text{mg gm}^{-1}) = [(12.7 \times A_{663}) - (2.69 \times A_{645})] \times [V/(1000 \times W)]$$

$$\text{Chlorophyll b } (\text{mg gm}^{-1}) = [(22.9 \times A_{645}) - (4.68 \times A_{663})] \times [V/(1000 \times W)]$$

where,

A = absorbance at respective wavelength of light (nm)
V = final volume of chlorophyll extract in 80% acetone
W = fresh weight of the tissue in grams.

Table 1
Details of experimental setup for foliar application of chitosan on sugarcane settlings grown under water deficit conditions.

Treatment code	Treatment condition	Chitosan type	Chitosan concentration
WC	Water control	No	No
DC	Water deficit control	No	No
NL50	Water deficit + NL-CSN	NL-CSN	50 ppm
NL100	Water deficit + NL-CSN	NL-CSN	100 ppm
NL200	Water deficit + NL-CSN	NL-CSN	200 ppm
IR50	Water deficit + IR-CSN	IR-CSN	50 ppm
IR100	Water deficit + IR-CSN	IR-CSN	100 ppm
IR200	Water deficit + IR-CSN	IR-CSN	200 ppm

NL-CSN: normal chitosan; IR-CSN: gamma irradiated chitosan.

The chlorophyll stability indices (CSI) were measured using the formula [38,47]

$$CSI = (\text{Chlorophyll content under stress condition}/\text{Chlorophyll content under control condition}) \times 100$$

2.6.8. Determination of adenine energetics status (ATP/ADP)

The analysis of adenine nucleotide energetics (ATP/ADP) was performed by High Performance Liquid Chromatography (HPLC) as per method followed by Lokhande et al. [43]. Leaf tissue samples (approximately 100 mg) were extracted using 0.6 M perchloric acid and supernatant aliquots were collected after centrifugation at 14,000 ×*g* at 4 °C for 10 min. The supernatants were neutralized and centrifuged at 14,000 ×*g* at 4 °C for 10 min to remove the precipitate. Supernatant was then passed through 0.22 μm filters. HPLC conditions (Waters) were consisted of mobile phase of 0.1 M potassium phosphate buffer (pH 6.0) with mobile phase flow rate of 1 ml/min and a photodiode array (PDA, Waters 996) detector at 254 nm. Separation was performed on a 10 μm C18 analytical column (250 × 4.6 mm) equipped with a guard column. The peaks were identified using individual standards and data was analyzed using Empower® software.

2.7. Statistical analysis

All the analyses were carried out in a randomized block design. One-way analysis of variance (ANOVA) was performed to confirm the variability and the significance of differences between treatments was validated by performing the Duncan's multiple range test (DMRT). All data analyses were performed using the SPSS software tool package (SPSS 16.0) for Windows and represented as treatment mean ± SE (n = 3). Data followed by same letter are not significantly different by DMRT test at $p < 0.05$.

3. Results

3.1. Effects of gamma irradiation on characteristics of chitosan

An aqueous solution of normal chitosan (NL-CSN) prepared in 1% (v/v) acetic acid was gamma irradiated (100 kGy) to obtain degraded low molecular weight chitosan (IR-CSN). Both NL-CSN and IR-CSN were characterised for their viscometric average molecular weight and FTIR spectroscopic analyses.

3.1.1. Gamma irradiation drastically reduced molecular weight ($\overline{M}v$) of NL-CSN

The change in Ubbelohde capillary viscometric measurements (intrinsic viscosity ηsp/C) of individual dilutions of NL-CSN and IR-CSN were plotted against the dilution concentrations of respective chitosan solutions (Supplementary Fig. S1). The viscometric average molecular weight ($\overline{M}v$) was calculated as per the Mark-Houwink equation, which confirmed that NL-CSN had high $\overline{M}v$ of about 235 kDa. Upon gamma (γ) irradiation (100 kGy), the viscometric properties of the polymer were drastically reduced and the $\overline{M}v$ of IR-CSN was dropped down to 25 kDa (reduced about 9.4 folds).

3.1.2. FTIR based analysis confirmed retention of structural integrity of chitosan upon gamma irradiation

The FTIR based analysis was performed to identify changes in polymer backbone structure after gamma irradiation of chitosan polymer. Spectral bands of the amide group (N—H bending and NH_3 bending vibrations) were visible at 1546 cm^{-1} and 1558 cm^{-1}, however the band corresponding to amino group (NH_2) was observed at 1560 cm^{-1} in both the polymers (Fig. 1). The characteristic transmittance band at 1410 cm^{-1} that corresponds to the hydroxyl groups (OH bending vibrations) and the transmittance bands between 1074 and 1080 cm^{-1} that usually attributes to the C—O and C—N stretching vibrations were present also in both NL-CSN and IR-CSN spectra. Therefore from the analysis of FTIR spectra of NL-CSN and IR-CSN; it was confirmed that there were not many alterations in polymer ring structure caused upon gamma irradiation.

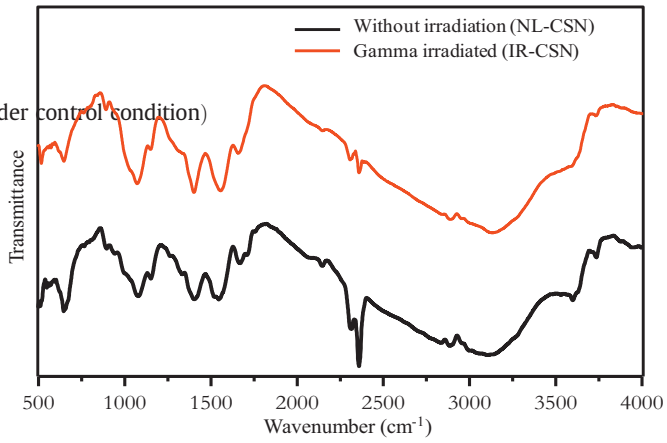

Fig. 1. FTIR spectroscopy of normal and gamma irradiated chitosan polymers.

3.2. Differential physio-biochemical attributes of water-stressed sugarcane plants upon NL-CSN and IR-CSN application

Physiological attributes, such as gas exchange indices, relative water content (RWC) and relative electrolyte leakage (REL) were markedly affected in response to imposed water deficit conditions. In the present study exogenous foliar application of chitosan (either NL-CSN or IR-CSN) showed improvement in physiological status over that of water deficit (DC) control plants (Figs. 2-i–vi and 3-i and ii).

3.2.1. NL-CSN and IR-CSN improved net photosynthetic (A) and transpiration (E) rate

Leaf net photosynthetic rate (*A*) was drastically reduced (−67.9%) in DC plants due to imposition of water deficit stress compared to that of WC plants those received watering at regular intervals (Fig. 2-i). This means that photosynthetic machinery was severely affected due to imposed water deficit conditions. Apparently, foliar application of chitosan significantly improved the net photosynthetic rate (*A*), irrespective of concentration and type of chitosan derivative applied (either NL-CSN or IR-CSN). However, the photosynthetic rate (*A*) was highest (8.1 μM CO_2 m^{-2} s^{-1}) in NL200 among all water deficit imposed-chitosan-sprayed treatments (NL50 to NL200, IR50 to IR200). Furthermore, foliar application of NL-CSN showed concomitant increase in photosynthetic rate (*A*) with increase in concentration from 50 to 200 ppm (NL50 to NL200), being highest in NL200 (131% increase over DC). Intriguingly, photosynthetic rate (*A*) significantly decreased with increase in concentration in case of IR-CSN from 50 to 200 ppm concentration (IR50 to IR200) (Fig. 2-i), although being higher in IR50 (109% higher than DC).

Transpiration rate (*E*) was highest in WC plants that received irrigation at regular intervals. This confirms that the WC plants expected to have their stomata open to maintain canopy temperature through active transpiration because of availability of sufficient moisture in the rhizosphere (Fig. 2-ii). On the other hand, DC plants and all the drought imposed-chitosan-sprayed plants (NL50 to NL200, IR50 to IR200) showed significantly lower transpiration rate (*E*). Transpiration rate (*E*) was recorded lowest in treatments NL50 (12.7% less) and NL100 (14.8% less). However, transpiration rate was increased in IR50 (13.9%) and IR200 (8.3%) treatments while it was at par with DC plants in case of NL 200 and IR100.

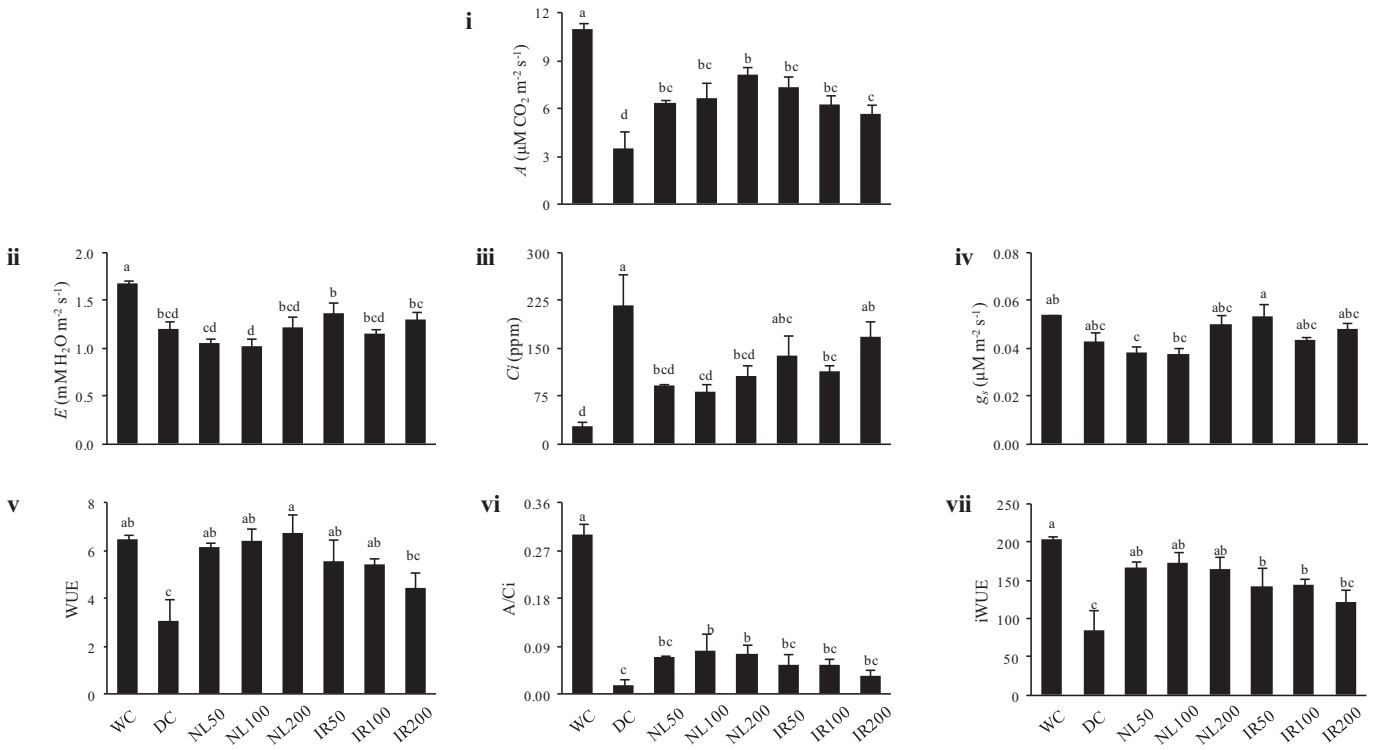

Fig. 2. Effects of foliar application of CSN on i) net photosynthetic rate (*A*), ii) transpiration (*E*), iii) internal CO_2 (*Ci*), iv) stomatal conductance (g_s), v) water use efficiency (WUE), vi) stomatal resistance (*A*/*Ci*) and vii) intrinsic water use efficiency (iWUE) of sugarcane plants grown under water deficit conditions. The bar indicates mean ± SE of biological replicates. Different letters in superscript indicate significance of differences using DMRT at $p \leq 0.05$.

3.2.2. NL-CSN and IR-CSN differentially modulate stomatal conductance (g_s) and water use efficiency (WUE)

Stomatal conductance (g_s) was decreased (−20%) in DC plants compared to WC plants (Fig. 2-iv). Spraying of NL50 and NL100 led to further decrease in g_s (−10.5 and −11.9%, respectively) over DC plants. Conversely, it was surprising that NL200 resulted in 16.8% increase in g_s over DC plants. Furthermore, IR50 showed higher g_s among all treatments, wherein NL200, IR100 and IR200 were at par to DC. Similarly, WUE was drastically reduced in DC plants (−53.5%), however the water deficit imposed-chitosan sprayed plants showed significantly higher WUE over DC plants (Fig. 2-v). The WUE of NL-CSN treatments (NL50 to NL200) showed increasing trend, however, IR-CSN treatments (IR50 to IR200) showed decreasing trend with increase in chitosan concentration. The iWUE also showed similar trend as that of WUE (Fig. 2-vii).

3.2.3. Higher internal CO_2 concentration (Ci) and stomatal resistance (A/Ci) in drought stressed plants exposed to NL-CSN and IR-CSN

Internal CO_2 (*Ci*) concentration was significantly higher in all water deficit imposed plants, wherein it was found lowest WC plants (Fig. 2-iii). Foliar application of chitosan showed significantly lower *Ci* compared to DC plants. *Ci* values and corresponding net photosynthetic rate (*A*) indicated possible influence of chitosan on protecting carboxylation efficiency under drought stress. However, *Ci* was significantly higher in case of IR-CSN treatments (IR50 to IR200) compared to NL-CSN treatments. On the other hand, the *A*/*Ci* ratio was highest for WC plants that inferred to have minimum impact on photosynthetic efficiency and maximum assimilation of absorbed CO_2 (Fig. 2-vi). However, DC plants showed lowest *A*/*Ci* that indicated the photosynthetic machinery has been adversely affected due to imposition of water deficit and very less amount of absorbed CO_2 being fixed. All water deficit imposed chitosan sprayed treatments showed significant increase in *A*/*Ci* over that of DC plants. Wherein increase in *A*/*Ci* was higher in case of NL-CSN treatments compared to IR-CSN treatments (Fig. 2-vi).

3.2.4. NL-CSN and IR-CSN modulate relative water content (RWC) and relative electrolyte leakage (REL)

The water control (WC) plants had RWC of 86.5% and that of DC plants had reduced RWC to 67.1% (Fig. 3-i). Both WC and DC plants were sprayed with water alone, and therefore this reduction in RWC

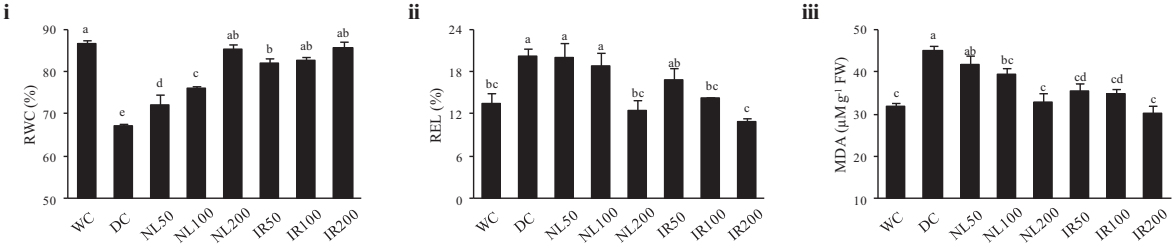

Fig. 3. Effects of foliar application of CSN on i) relative water content percent (RWC), ii) relative electrolyte leakage percent (REL), iii) malondialdehyde content (MDA) of sugarcane plants grown under water deficit conditions. The bar indicates mean ± SE of biological replicates. Different letters in superscript indicate significance of differences using DMRT at $p \leq 0.05$.

was only because of water deficit condition imposed upon water withholding in case of DC plants. In contrast, all the water deficit imposed-CSN sprayed (either NL-CSN or IR-CSN) treatments showed higher RWC compared to DC plants. The RWC was increased with increase in CSN concentration, being significantly high at 200 ppm concentration (85%) as compared to DC plants. Furthermore, the IR-CSN treatments (IR50 to IR200) showed significantly high RWC compared to same concentration of NL-CSN treatments (NL50 to NL200). The application of NL200, IR100 and IR200 treatments were at par of the water control plants (WC). This showed that foliar applications of CSN (either NL-CSN or IR-CSN) at 200 ppm are equally efficient to refurbish tissue water content under water deficit conditions (Fig. 3-i). It is notable that the IR-CSN even at lower concentration (IR50) gave significantly higher recovery in the RWC compared to DC plants. Spraying of IR50 and IR100 increased RWC to about 82% compared to that of DC plants (67.1%).

On the other side, relative electrolyte leakage (REL) was increased in DC plants (48.8% higher) compared to WC plants (Fig. 3-ii). Foliar application of chitosan (either NL-CSN or IR-CSN) significantly reduced the REL caused upon imposition water deficit stress. The IR-CSN showed significantly lower REL at all the three treatment concentrations (IR50, IR100 and IR200). However, NL-CSN could significantly reduce the REL values only at 200 ppm concentration (NL200). Notably, the application of IR-CSN showed significantly lower REL even at lowest concentration (IR50), wherein treatments of NL50 and NL100 showed non-significant decrease.

3.2.5. NL-CSN and IR-CSN ameliorate lipid peroxidation (MDA) but increase free-proline

Lipid peroxidation is a direct measure of membrane damage resulted due to stress imposed production of malondialdehyde (MDA). In the present study, DC plants showed highest MDA content (42% more) than control (WC) plants (Fig. 3-iii). However, foliar application of chitosan to plants under water deficit conditions showed reduction in MDA content with increase in chitosan concentrations compared to DC plants. Foliar application of NL200 and IR200 showed least MDA accumulation. Furthermore, IR-CSN treatments showed rapid reduction in MDA content compared to NL-CSN. Treatment of NL50 gave 8% reduction in MDA content, wherein IR50 resulted in 21.7% reduction in MDA content (nearly three folds more reduction). However it is interesting to note that MDA content upon NL200 and IR200 treatments was at par to WC plants.

The trigger in accumulation of free-proline due to water deficit stress was evident with a sharp rise in case of DC plants compared to WC plants (Fig. 4-i). However, there was a significant decrease in proline content upon foliar spraying with chitosans at different concentrations. Compared to DC plants, NL-CSN sprayed plants (NL50, NL100 and NL200) showed decreased proline accumulation (17%, 40% and 78% decrease, respectively) (Fig. 4-i). Irradiated chitosan sprayed plants, IR50, IR100 and IR200 showed 65%, 71% and 83% decrease in proline accumulation, respectively. However, this decreased proline accumulation was at par in case of NL200 and IR200 with WC plants. This reduction in proline accumulation was due to reduced impact of water deficit conditions upon foliar chitosan applications. Among all treatments, spraying with IR50 was most significant because there was 65% decrease compared to DC plants.

3.2.6. Increased H_2O_2 generation and superoxide dismutase (SOD) activity under NL-CSN and IR-CSN treatment

Stress induced generation of reactive oxygen species (ROS) is well known in plants. In the present study, water deficit induced accumulation of H_2O_2 showed decrease with increase in chitosan concentration (Fig. 4-iii). Accumulation of H_2O_2 was significantly high (65.4% higher) in DC plants than control (WC) plants. Application of foliar spray of chitosan significantly reduced accumulation of H_2O_2, which was least in IR200 (34% less compared to DC plants). NL-CSN showed 16.9%, 23.3% and 31% reduced accumulation of H_2O_2 at NL50, NL100 and NL200 concentrations, respectively compared to DC plants. On the other side, IR-CSN showed 22%, 30.8% and 34.1% reduction at IR50, IR100 and IR200 concentrations, respectively.

In the present study, water deficit (DC) plants showed enhancement in SOD activity (23.6% increase) over control plants (WC) (Fig. 4-iv). Foliar spray of NL-CSN resulted into reduction in SOD activity (43.9%, 22.1% and 4.1% reduction at NL50, NL100 and NL200 concentrations, respectively). In contrary, IR50 resulted in 35% reduction in SOD activity, which further resulted in increased SOD at IR100 and IR200 concentrations (20.6% and 52.5% increase, respectively).

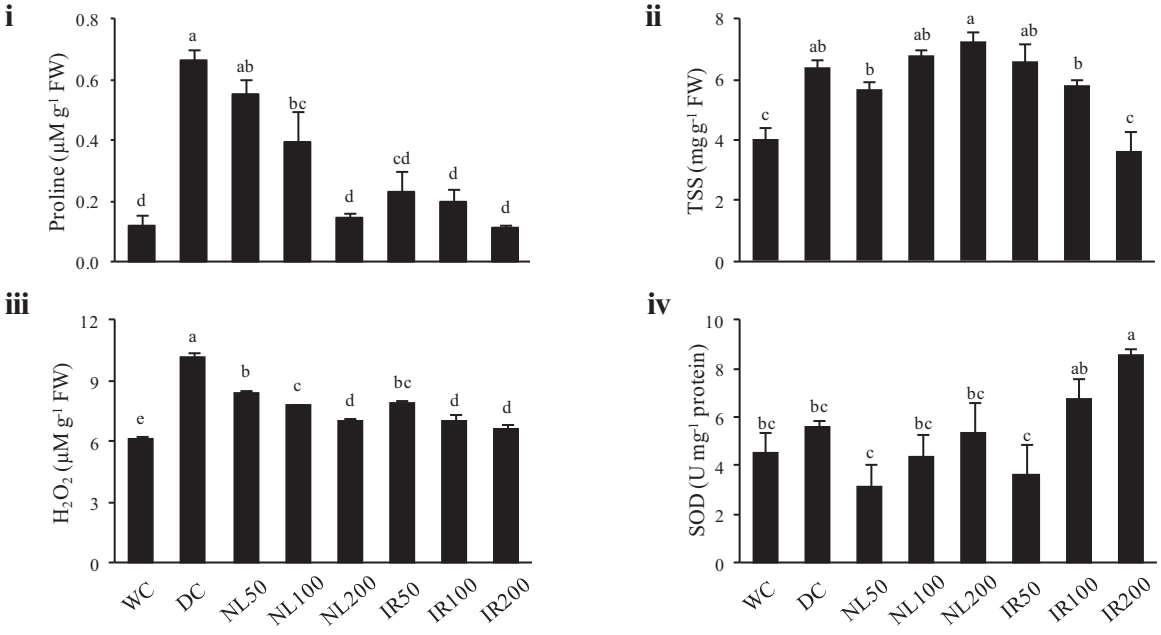

Fig. 4. Effects of foliar application of CSN on i) proline content, ii) total soluble sugars (TSS), iii) H_2O_2 content, iv) SOD activity of sugarcane plants grown under water deficit conditions. The bar indicates mean ± SE of biological replicates. Different letters in superscript indicate significance of differences using DMRT at $p \leq 0.05$.

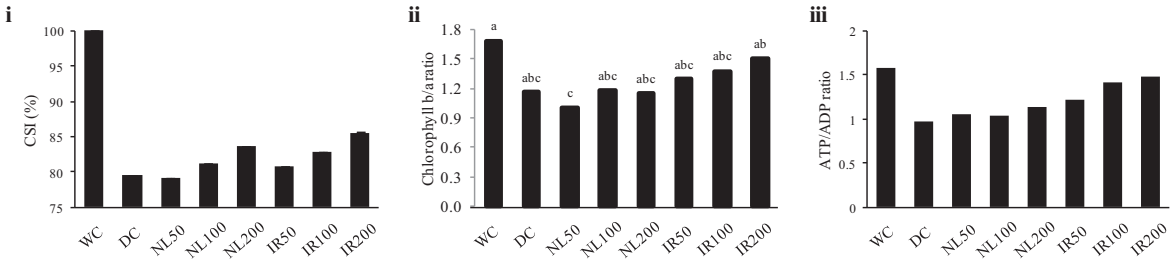

Fig. 5. Effects of foliar application of CSN on i) chlorophyll stability index (CSI), ii) chlorophyll *b*/*a* ratio, iii) ATP/ADP ratio of sugarcane plants grown under water deficit conditions. The bar indicates mean ± SE of biological replicates. Different letters in superscript indicate significance of differences using DMRT at $p \leq 0.05$.

3.2.7. NL-CSN and IR-CSN positively affect chlorophyll pigments and chlorophyll stability index (CSI)

Water deficit conditions cause influence on photosynthetic pigments (chlorophylls). In the present study, water deficit (DC) plants exhibited drastic reduction in chlorophyll *a*:chlorophyll *b* (Chl *b*/*a*) ratio (31.2% decrease) compared to WC plants (Fig. 5-ii). Foliar application of chitosan resulted in concentration dependent increase in chlorophyll *b*/*a* ratio. IR-CSN exhibited 11.4%, 18.7% and 29.5% increase in chlorophyll *b*/*a* ratio at IR50, IR100 and IR200 concentrations, respectively; wherein NL-CSN could not significantly alter chlorophyll *b*/*a* ratio.

On the other hand, CSI index of water deficit (DC) plants was 20.5% less compared to control (WC) plants (Fig. 5-i). Exogenous application of chitosan helped to refurbish photosynthetic pigments thereby improved photosynthesis. NL-CSN spray application at NL50 concentration failed to improve the CSI, but has resulted in significant increase at NL100 and NL200 (2.1% and 5.2%, respectively). In comparison, IR-CSN showed significant increase in CSI index at IR50, IR100 and IR200 concentration by 1.5%, 4.3% and 7.6%, respectively.

3.2.8. Better osmotic and energetic status under NL-CSN and IR-CSN treatment

Stress induced accumulation of sugars for cellular maintenance of osmoticum was evident with increased TSS content (57% increase) in DC plants compared to WC plants (Fig. 4-ii). Foliar spray of NL-CSN showed concentration dependent gradual increase in TSS content with significantly high TSS in NL200 treatment (14% increase) compared to DC plants. Contrastingly, spraying of IR-CSN showed concentration dependent decrease in TSS content being least in IR200 treatment (43% less) compared to DC plants and was at par to the WC plants.

Cellular energetics in plants is known to directly affect due to adverse environmental conditions. In the present study, water deficit stress caused decrease in ATP/ADP ratio of DC plants (38.2% decrease) compared to WC plants (Fig. 5-iii). However, exogenous chitosan application improved ATP/ADP ratio of NL-CSN and IR-CSN sprayed plants. NL-CSN spray resulted in marginal increase (7 to 16% increases) in ATP/ADP ratio, wherein IR-CSN application showed significant increase (26 to 52%) at all the three concentrations.

4. Discussion

In both conventional and advanced perspectives, radiation processing is an area of active research having diverse applications (from agriculture to pharmaceuticals). In particular, radiation processing of naturally occurring polymers such as chitosan has immense implications [36,48,49]. Radiation processing has become a method of choice for modification of polysaccharides for being safer, environmental friendly and easier method to modify polymers. Considering the recent advancements in this area to utilize chitosans for diverse agricultural applications, ranging from resistance against diseases to protection from abiotic stresses, it is imperative to study plant responses to exogenously applied CSN under adverse environmental conditions.

With this view, the present study was aimed to attempt a comparative assessment of two different kinds of chitosans for their influences on various physiological and biochemical attributes of water deficit stressed sugarcane plants. For this purpose, two kinds of chitosans were taken; gamma irradiated (IR-CSN) and normal or non-irradiated (NL-CSN) chitosans. The very first noticeable observation upon gamma ray treatment was the drastic reduction in viscosity of the CSN polymer. Reduction in the viscosity upon gamma irradiation has been proven to have direct relation with the molecular weight of chitosan polymer. Similar observations were already reported by Bano et al. [50] and Garcia et al. [51] wherein lobster shell chitosan showed drastic reduction in viscometric molecular weight ($\overline{M}v$) upon gamma irradiation. Lately, Muley et al. reported gamma irradiation (100 kGy) mediated degradation of crab shell chitosan that eventually brought down the molecular weight to 82.2 kDa from 337.73 kDa [35]. These studies found inverse proportion between absorbed radiation and viscometric molecular weight ($\overline{M}v$) of the chitosan polymer. The reduction in viscosity is brought about predominantly due to breakage and depolymerisation of the long polymer chains upon exposure to the gamma rays [51–53]. Exposure of chitosan to gamma radiation generates radical sites at position 1 and 4 of the 2-deoxy-D-glucose residue of the backbone and results in scission of the 1,4-glycosidic bond, ultimately causing depolymerisation of the chitosan chain [54–56]. However, from the practical point of view, radiation processing of chitosan to obtain degraded low molecular weight (oligo) chitosan, γ-irradiation dose of 100 kGy is considered to be sufficient and effective [36]. Recently, the suitability of 100 kGy gamma irradiation dose has been reported for significant influence in corn [57] and potato [35]. However, higher radiation (>100 kGy) doses does not lead to further significant reduction in $\overline{M}v$. Furthermore, with the objective to investigate impact of gamma radiation on CSN polymer, we performed FTIR analysis of both the polymers. Comparison of FTIR spectral data of NL-CSN and IR-CSN showed lack of any global changes caused upon exposure to gamma rays (Fig. 1). The FTIR band vibrations at 2920, 2872, 1410, 1265 cm^{-1} corresponding to symmetric and asymmetric CH_2 groups of the D-glucopyranose ring were not altered which indicated that the ring structure has not been affected upon irradiation [58]. Moreover, there was no band at 1730 cm^{-1} that corresponds to carbonyl groups of a transient product of glucopyranose ring cleavage, which further confirmed retention of backbone of the polymer structure [58]. The chain scission mechanism was known to occur upon gamma irradiation of chitosan that induces breaks in polymer chain [52,53] and this cause depolymerisation, leading to shorter chitosan chains [51]. Similar previous studies also reported that gamma irradiation exposure does not affect the chemical structure leading to alteration in functional groups of the polymer [35,53,59]. Apparently, Gryczka et al. reported that exposure to ionizing radiations (including gamma and electron beam) induces chain breakage, cross linking and ring opening, ultimately leads to irreversible chemical changes in chitosan polymer chain [60]. But we did not find any evidence of ring cleavage in our IR-CSN upon gamma irradiation that was used for the present study.

Plants respond to environmental cues and therefore orchestrate numerous physiological, biochemical and metabolic adjustments (synthesis

and accumulation of osmolytes, antioxidants compounds or modulation of sugar metabolism, alterations in photosynthetic activity etc.) to cope up with the adverse conditions [61]. Drought is regarded as a 'multidimensional stress' as it affects an array of cellular processes in crop plants, thereby resulting in considerable yield losses [62]. Upon exposure to water deficit conditions, plants close their stomata that are primarily triggered by combined effects of the water status of adjacent guard cells, photon flux intensity [63] and the water deficit sense-signals received from roots [64]. Stomatal closure proceeds to reduce transpiration that in turn reduces photosynthetic rate and increases leaf temperature [65]. Other characteristic changes in the physiological status include high solute concentration due to accumulation of ions inside the cell, which subsequently results in osmotic imbalance. Plants usually mitigate abiotic stresses like drought and salinity by accumulating free-proline, sugars, betaine, and other compatible solutes collectively termed as osmoprotectants [66].

Sugarcane, being a typical C_4 plant display acute sensitivity to water deficit stress conditions by sudden decrease in stomatal conductance (g_s), transpiration rate (E), internal CO_2 concentration (Ci), and photosynthetic rate (A) as a result of increased stomatal regulation [8,67,68]. In the present study, we observed rapid and significant alteration in above all the parameters in case of water deficit control (DC) plants. But it is intriguingly evident that plants sprayed with chitosan (either NL-CSN or IR-CSN) showed improvement and retention of optimal values of these sensitive parameters (Fig. 2-i, ii, iii, iv). Besides this, water stress-induced non-stomatal limitations have also been reported that cause photosynthetic inhibition in sugarcane [69]. But this usually occurs under severe stress or under prolonged moderate water deficit conditions [68].

Elicitation of plant innate responses and physio-biochemical properties upon treatment of CSN has been proved to confer enhanced plant growth, biotic and abiotic stress tolerance [18,28,35,70–72]. Implications of exogenously applied chitosan on regulation of stomata and thereby reduction in transpirational water loss have been reported [73–79].

Until now, the experimental evidences showed that chitosan induces stomatal closure triggered upon signaling pathway mediated by ABA, H_2O_2 and/or nitric oxide [73,76–78]. However, Iriti et al. showed that the ABA-mediated stomatal closure being predominant in bean plants that also confirmed anti-transpirant activity of chitosan [77]. Similar evidences on anti-transpirant properties of chitosan that reduces transpiration by stomatal closure were also reported in other plants such as pepper [74], bean (*Phaseolus vulgaris*) [77] and barley (*Hordeum vulgare*) [79]. But these evidences were based on observation recorded under optimal plant growth conditions (in absence of drought stress). As speculated by Iriti et al. under drought stress stomatal regulation may differentially affect responses to exogenously applied chitosan [77]. Mainly for the reason that, under drought stress, the endogenous ABA levels rise due to root-to-shoot translocation of ABA synthesized in roots [80]. This rise in ABA may lead to reduction in photosynthesis and that negatively affect other cellular metabolic processes. Although stomatal closure under water deficit condition has great significance in context to conserve excessive water loss but it should not be at the cost of reduced carbon fixation and damage to metabolic processes. Furthermore, Iriti et al. showed that upon CSN application there was no significant improvement in WUE but photosynthetic activity was enhanced compared to that of the treatment of commercial anti-transpirant VapourGuard [77]. Therefore in the present study we considered the above points as an imperative to be addressed to investigate effects of exogenously applied chitosan on sugarcane plants grown under water deficit stress.

Among other physiological parameters, RWC is a direct indicator and easiest measure to assess physiological status [67,81] and water balance as it represents the absolute water content of the tissue [82]. Moisture or water deficit stress severely reduces the cellular water content thereby leads to cell injuries and hinder cell division and expansion that causes decreased plant growth. In practice, RWC indicates cellular and tissues hydration level which is required to maintain physiological status and metabolism of plant [67]. RWC directly corresponds to physiological functioning and any change in RWC directly affects vital processes including photosynthesis and respiration in sugarcane plants [65]. In sugarcane a decrease of 10 to 20% in RWC cause reduction in photosynthetic efficiency of tolerant and sensitive sugarcane plants submitted to water deficit [65]. In the present study RWC was decreased nearly 20% between water control (WC) plants and deficit imposed control (DC) plants which mean that the water deficit was severe enough to cause subsequent damage to photosynthetic machinery (Fig. 3-i). However, foliar application of NL200, IR50, IR100 and IR200 showed significant retention of RWC under water deficit conditions (Fig. 3-i). Typically, RWC values below 80% increases accumulation of osmolytes (like free-proline) and reactive oxygen species (ROS) at the cost of cessation of photosynthesis and respiration, and inhibition of other metabolic activities [83]. In the present study, both free-proline and H_2O_2 accumulation showed concomitantly decreased with increase in RWC values upon chitosan application (Figs. 3-i and 4-i, iii). In addition, increased RWC also showed correlating with decrease in REL and MDA accumulation (Fig. 3-i, ii and iii). On comparative accounts, IR-CSN was much more efficient in retaining higher RWC with less REL and MDA accumulation than that of NL-CSN (Fig. 3-i, ii and iii).

The plant growth stimulating effects of chitosan have been attributed to an increase in the availability and uptake of water and essential nutrients through adjusting cell osmotic pressure, and increased ROS scavenging enzyme activities [84]. In the present study, CSN application showed significant improvement in RWC, SOD activity and with decreased REL, MDA, proline, TSS and H_2O_2 levels (Figs. 3 and 4). Similarly, in white clover (*Trifolium repens*) chitosan treatment helped to elevate dehydration stress tolerance by increased production of stress protective metabolites [31]. In the same way, foliar spray of chitosan on *Thymus daenensis* Celak plants during different flowering stages helped to nullify the negative effects of drought condition on the oil yield and dry matter production [32]. Furthermore, similar studies on apple [26], sweet basil [29], coffee [30], cowpea [85] and rice [86] plants also showed induced drought tolerance conferred with improvement in plant growth, biochemical and yield attributes.

Upon onset of water deficit, plants experience loss of tissue water that decreases RWC thereby decrease the cell turgor. This triggers stomatal closure which put limits on to the CO_2 uptake rate [87]. In the present study, net photosynthetic rate (A) and WUE were found correlating wherein foliar application of NL-CSN and IR-CSN significantly improved net photosynthetic rate (A) and WUE (Fig. 2-i, v and vii). However, net photosynthesis (A), WUE and iWUE showed non-significant decrease at higher concentrations of IR-CSN compared to NL-CSN (Fig. 2-v and vii). This might be due to slightly higher transpiration rate (E) (Fig. 2-ii); elevated free-radical scavenging antioxidant activity (Fig. 4-iv) and/or involvement of other stress tolerance metabolic processes as a result of imposed water deficit. Recent report on application of semi-synthetic (*N*-succinyl and *N*,*O*-dicarboxymethyl) chitosan derivatives also showed to confer water deficit stress tolerance in a drought sensitive maize [88,89]. These semi-synthetic chitosan derivatives significantly elevated photosynthesis and stomatal conductance [88,89].

Water deficit stress triggers endogenous accumulation of free-proline that cause to reduce water loss by lowering the leaf water potential, favors the water transport to leaves and increases their turgor. Such rise in proline accumulation was evident upon chitosan application to white clover [31] and thyme [32] plants subjected to drought stress. Furthermore, the amplitude of proline accumulation has been reported to be either concentration dependents [90] or may differ among plant species [71,89]. Contrastingly, in the present study, we observed concentration dependent (50, 100, 200 ppm CSN) decrease in proline accumulation wherein the decrease was much more prominent in case of IR50 and IR100 treatments compared NL-CSN treatments (Fig. 4-i). Proline content of both NL-200 and IR-200 were at par of

WC plants. This indicated that the negative effects of water deficit were relieved upon chitosan application.

Earlier reports on effects of chitosan on stomatal aperture suggest that it would be a valuable anti-transpirant with useful agricultural applications [29,77]. Certainly in the present study it was evident that there was non-significant variations in transpiration rate (E) and stomatal conductance (g_s) of chitosan-sprayed and non-sprayed plants and among different concentration gradients of chitosans. But this observation is obvious enough to underline property of chitosan to regulate stomatal movements by acting as an anti-transpirant film-forming material [77]. This property confers the plants to utilize available soil moisture thereby has implication in obtaining elevated WUE under water deficit stress conditions.

Chitosan treatment known to enhance production and accumulation of H_2O_2 and nitric oxide (NO) in epidermal cells of tobacco [91], rapeseed (*Brassica napus*) [92], pea leaves [75], pearl millet seedlings [93], rice cell suspensions [94] and drought sensitive maize hybrid [89]. But in case of sugarcane, we found exogenous application of CSN concomitantly decreased water deficit stress-induced accumulation of H_2O_2 (Fig. 4-iii) and significantly increased antioxidant scavenging (SOD) activity (Fig. 4-iv). SOD activity is very crucial in order to regulate detoxification of sudden rise in ROS upon stress exposure. SOD is a most effective intracellular enzymatic antioxidant that serves as the first line of defense against elevated levels of intracellular ROS [95].

Decrease in lipid peroxidation along with enhanced membrane stability and ROS scavenging activities were previous reports upon pretreatment with chitosan in bean, potato, thyme, *Hydrilla verticillata* and apple seedlings [26,27,32,96,97]. ROS scavenging activity of chitosan possibly attributed due to its structural property of predominant occurrence of hydroxyl and amino groups that reacts with ROS to form a stable, non-toxic macromolecular radicals [98–100]. Chitosan induced SOD and catalase antioxidant activities promoted scavenging of superoxide anion and reduced lipid peroxidation in plants subjected to drought stress [26,100].

In the present study both MDA and H_2O_2 levels were increased upon water deficit stress (Figs. 3-iii and 4-iii). However, MDA and H_2O_2 levels decreased in case of chitosan sprayed plants with increasing chitosan concentration, which indicates reduction in lipid peroxidation and thereby less membrane damage and reduced generation of toxic free-radicals along with enhanced SOD activity (Figs. 3-iii, 4-iii and iv). Similar response was observed in a previous study wherein chitosan induced resistance to osmotic stress has been reported in rice [101]. Both H_2O_2 and MDA contents were increased under osmotic stress that were observed to be decreased after chitosan treatment through chitosan induced activation of ROS scavenging enzymes [101,102]. A previous report on spray application of CSN on potato shown to decrease membrane permeability and MDA content along with increase in proline, soluble protein contents, SOD and peroxidase activities [27]. Recent report on metabolic and transcriptomic profiling of white clover subjected to dehydration stress showed that supplementation of CSN greatly increased accumulation of sugars, sugar alcohols, amino acids, organic acids and related metabolites [103].

Drought stress induces accumulation of total soluble sugar due to the breakdown of polysaccharides which help in the maintenance of turgor [104] and this contributes to drought-resistance through signal transduction to modulate plant growth, development and stress responses [105]. Plants treated with chitosan showed increased accumulation of carbohydrates such as glucose, fructose, mannose, trehalose, sorbitol, myoinositol etc. and they caused up-regulation of many genes involved in carbohydrate transport and metabolism in leaves of white clover [103]. Foliar application of chitosan significantly increased RWC, leaf carbohydrates and protein contents of sour orange seedlings grown for under different irrigation levels [106]. Similarly such increase in TSS levels was also observed upon foliar application of semi-synthetic chitosan derivatives on drought sensitive maize hybrid [89]. Corroboratively, we found significant rise in TSS levels upon imposition of water deficit stress, but apparently IR-CSN application showed concentration dependent decrease in TSS levels which was at par with WC plants at 200 ppm concentration (Fig. 4-ii).

Water deficit induced intracellular ROS generation induces disturbance in cellular energetics and ensuing ATP synthase activity that leads to decreased ATP content which progresses with intensity of water deficit [107]. This decreased ATP level limits RuBP production by the Calvin cycle and thus photosynthetic potential. Effect of chitosan on cellular energetics has not been reported. In the present study we attempted to investigate the effects of chitosan application on ATP energetics under water deficit conditions. ATP/ADP ratio was found drastically reduced under water deficit stress condition (Fig. 5-iii). However, in this study, application of chitosan concomitantly restored the ATP/ADP ratio with gradual increase in chitosan concentration, wherein IR-CSN found to be more efficient over that of NL-CSN treatments (Fig. 5-iii).

Exogenous application of chitosan and chitosan oligomer solutions on coffee plants and grapevine have reported to confer drought tolerance by significant improvement in maintenance of chlorophyll

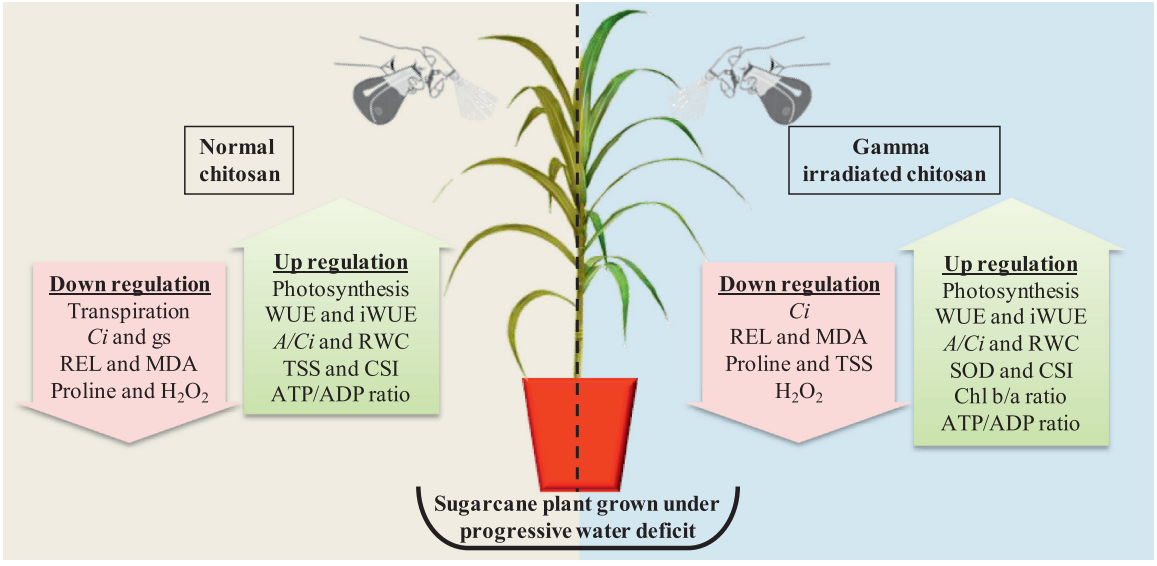

Fig. 6. Relative dynamic regulation of physiological and biochemical attributes upon foliar application of normal and gamma irradiated chitosan on sugarcane plants subjected to progressive water deficit stress. Attributes shown to either up or down regulate are against its corresponding drought imposed-CSN non-sprayed (DC) treatment.

pigments [28,30]. The higher CSI value is considered as an indicator for the ability of plant to withstand under adverse environmental conditions [47]. Similarly, in the present study we found significantly higher CSI and maintenance of optimal chlorophyll *b*/*a* ratio (Fig. 5-i and ii). Comparative assessment showed that IR-CSN has more efficient to maintain higher CSI and optimum chlorophyll *b*/*a* ratio. The increase in levels of chlorophyll pigments and Chl *b*/*a* ratio upon chitosan treatments were previously reported in rice [102] and *Dendrobium* [108].

The biosynthesis of photosynthetic pigments and proline share and compete for their common precursor/substrate inside a living plant cell. Hence sudden rise in either of them cause negative impact on the other. Therefore, under drought stress increase in the synthesis of proline antagonistically decrease synthesis of photosynthetic pigments [109]. Such phenomenon of reduction in photosynthetic pigments with concomitant increase in proline content in response drought stress was reported in basil plants [29]. In the similar manner, such antagonistic effect between CSI and free-proline accumulation was evident in the present study under influence of water deficit and chitosan treatments (Figs. 5-i and 4-i).

In essence, taking into account the comparison of NL-CSN and IR-CSN treatments to sugarcane plants under progressive water deficit conditions, application of IR-CSN showed positive regulation of RWC, SOD, CSI, Chl *b*/*a* ratio and ATP/ADP ratio along with least REL, MDA, free-proline, TSS and H_2O_2 accumulation. In order to summarize the key findings we propose a model of comparison between NL-CSN and IR-CSN modulated regulation of photosynthesis, antioxidant defense and adenine energetics of sugarcane challenged by water deficit stress (Fig. 6).

5. Conclusion

Foliar application of gamma irradiated chitosan (IR-CSN) was found to differentially regulate photosynthetic gas exchange, osmo-protectants, antioxidant activities, chlorophyll stability and adenine energetics against that of normal chitosan (NL-CSN). The IR-CSN has shown have potential to modulate biochemical attributes conferring water deficit stress tolerance in sugarcane. This potential was incurred upon gamma irradiation mediated degradation of normal CSN. Uniquely, the present study reported dynamics between physiological indices and biochemical attributes under water deficit stress in sugarcane under influence of foliar application of chitosan. All together the present study was carried out to demonstrate the potential of normal and gamma irradiated CSN to improve ability of sugarcane crop to withstand under water deficit stress conditions. Elucidation of molecular mechanisms underlying plant responses to NL-CSN and IR-CSN needs further attention. It would be worth to study actual consequences and impact on crop yield and productivity by conducting field trial in near future under naturally occurring adverse environmental conditions.

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Author's contribution

S.J.M., S.G.D. and P.S. conceived, planned and conducted the experiments. S.D.R. provided help in gas exchange analyses. S.J.M., S.G.D. and P.S. analyzed the data and wrote the manuscript.

Declaration of competing interest

The authors express no conflict of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.08.093.

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