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Plant water status, ethylene evolution, N_2-fixing efficiency, antioxidant activity and lipid peroxidation in *Cicer arietinum* L. nodules as affected by short-term salinization and desalinization

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Summary

Salinity induced changes in ethylene evolution, antioxidant defense system, N_2-fixing efficiency and membrane integrity in relation to water and mineral status in chickpea (*Cicer arietinum* L.) nodules were studied under screen house conditions. At vegetative stage (55–65 DAS) plants were exposed to single saline irrigation (Cl^- dominated) of levels 0, 2.5, 5.0 and 10.0 dS m^{-1} and sampled after 3 d. The other set of treated plants was desalinized by flooding and the plants were sampled after further 3 d. Water potential (Ψw) of leaf and osmotic potential (Ψs) of leaf and nodules significantly decreased from –0.44 to –0.56 MPa and from –0.65 to –1.15 MPa and from –0.75 to –1.77 MPa, respectively upon salinization. RWC of leaf and nodules also reduced from 86.05% to 73.30% and 94.70% to 89.98%, respectively. The decline in Ψs of nodules was due to accumulation of proline and total soluble sugar. In comparison to control, the increase in ethylene (C_2H_4) production was 35–108% higher and correspondingly increase in 1-aminocycloprane-1-carboxylic acid (ACC) content (37–126%) and ACC oxidase activity (31–118%) was also noticed. Similarly, marked increase in H_2O_2 (25–139%) and thiobarbituric acid substances (TBRAS, 11–133%) contents was seen. N_2-fixing efficiency i.e. N_2-ase activity, leghemoglobin and N contents of nodules declined significantly after saline irrigation. The induction in specific activity of antioxidant enzymes was confirmed by the increase in activity of superoxide dismutase, peroxidase, ascorbate peroxidase, glutathione reductase and glutathione transferase, whereas reverse

Abbreviations: AA, ascorbic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; ARA, acetylene reduction assay; LHb, leghemoblobin; RWC, relative water content; TBARS, thiobarbituric acid reactive substances; Ψ_s, osmotic potential; Ψ_w, water potential

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was true for catalase. These activated enzymes could not overcome the accumulation of H_2O_2 in nodules. Ascorbic acid content also declined from 20 to 38%, whereas Na^+/K^+ ratio and Cl^- content were significantly enhanced. Upon desalinization, a partial recovery in all above metabolic processes and water relations parameters was noticed. It is suggested that ethylene in relation to water status and lipid peroxidation and along with other metabolic processes has an important role in induced nodules senescence under salinity.

Introduction

In arid and semi-arid regions, salinity (both soil and water) is one of the major factors responsible for deterioration of soil and making it unfit for agriculture. Salinity caused reduction in growth is the consequence of alterations in several physiological processes (Flowers and Yeo, 1986; Kukreja et al., 2005; Nandwal et al., 2000a, b; Sharma, 1996, 1997; Zhu, 2001) including N_2 fixation (Cordovilla et al., 1994; Farnandez-Pascual et al., 1996; Nandwal et al., 2000a, b) etc. As a result of these primary effects secondary stress such as oxidative damage often occurs (Bartels, 2001; Becana et al., 2000; Kukreja et al., 2005; Sairam et al., 2002) under salinity.

Legumes root nodules are especially at risk from oxidative damage by reactive oxygen species (ROS) because they contain an abundance of oxygen labile protein such as leghemoglobin (LHb) and Fe potentially available for catalysing free radical production (Becana et al., 1998). Dalton et al. (1986) and Gogorcena et al. (1995) reported that H_2O_2 and O_2^- radical can be generated by oxidation of nitrogenase, hydrogenase and by autoxidation of oxygenated LHb. Interaction of LHb with H_2O_2 also gives rise to highly toxic hydroxyl ions (OH^-)) which are likely to damage the membrane structure and one or more component of N_2 fixing machinery. In addition to enzymes, super oxide dismutase catalase (CAT) and peroxidase (POX), a major antioxidant mechanism operating in nodule cytosol is the ascorbate-glutathione cycle which results ultimately in detoxification of H_2O_2 at the expense of NAD (P) H (Becana et al., 2000; Dalton et al., 1986).

Lipid peroxidation which leads to impairment of membrane function is the system most easily ascribed to oxidative damage and also most frequently measured (Sairam et al., 2002).

The induction of senescence has also been correlated with augmentation in ethylene evolution under various environmental stress conditions (Abeles et al., 1992; John, 1997). Chickpea is an important crop in semi-arid and arid regions of the world and the mechanism by which salinity affects its nodule metabolism is still not completely understood especially the ethylene-correlated processes. Hence, the present investigations on an indeterminate type of nodules are confined to antioxidant defense system, ethylene evolution and membrane integrity in relation to changes in plant water and mineral status under single saline irrigation of three ECe levels and subsequently on their desalinization.

Methods and materials

Growth conditions

Chickpea (*Cicer arietinum* L.) cv. H 96-99 was raised in earthenpots (30 cm dia) filled with 5.5 kg of dune sand (Typic torrispmments) under screen house conditions in the Department of Botany and Plant Physiology, CCS Haryana Agricultural University, Hisar-125 004, India. The seeds before sowing were surface sterilized and inoculated with effective *Rhizobium* culture (Ca 181). The crop was supplied with an equal quantity of nitrogen free nutrient solution at regular interval of 15 d. After thinning two plants were retained in each pot. The chloride (Cl) dominated salinity was prepared by using a mixture of different salts such as NaCl, $MgCl_2$, $MgSO_4$ and $CaCl_2$ where Na:Ca+Mg was in the ratio of 1:1 and Ca:Mg in the ratio of 1:3, the $Cl:SO_4$ ratio was 7:3 on a meq basis. At vegetative stage i.e. 55–65 d after sowing, the desired salinity was applied to saturate each pot so as to maintain four levels [0 (control), 2.5 (S_1), 5.0 (S_2) and 10.0 (S_3) dS m^{-1}] of Cl dominated salinity. The sampling was done at 3 d after treatments. Half of the treated plants were revived by flooding and were sampled after 3 d to see their revival and were designated as S_1R, S_2R and S_3R, respectively.

Water potential

The third fully expanded leaf from the top was used to measure water potential (Ψ_w) with the help

of a Pressure Chamber (Model-3005, Soil Moisture Equipment Corporation, USA) and was expressed in '-MPa'.

Osmotic potential

The osmotic potential (Ψ_s) of leaf of the same position and nodules was determined separately using psychrometric technique with a Vapor Pressure Osmometer (Model-5100, Wescor, Logan, USA) and was expressed in '-MPa'.

Relative water content

The relative water content (RWC%) of leaf and nodules was calculated as described by Weatherley (1950).

The plants were sampled and nodules were detached from the root. Sand was removed with the help of a soft brush and a light washing was given with distilled water and weighed immediately to take their fresh weight after removing excess of water by using filter papers. After that the nodules were oven dried at 85 °C till a constant weight. The calculations on dry weight (DW) were done on the basis of conversion factor from fresh weight to DW under different stress levels.

These measurements were made between 10.00 and 12.00 h (local time) during a sunny day having the mean temperature of 20 ± 2 °C.

Proline

The proline content of nodules was estimated by the methods of Trotel et al. (1996) and calculated as mg g^{-1} DW.

Total soluble sugar

The total soluble sugar (TSS) of nodules were determined with the method of Dubois et al. (1956). The values of TSS were calculated as mg g^{-1} DW.

Ascorbic acid

Ascorbic acid (AA) content of nodules was measured by using the method of Schopfer (1966). The AA content was calculated as mg g^{-1} DW.

1-Aminocyclopropane-1-carboxylic acid content

Free 1-Aminocyclopropane-1-carboxylic acid (ACC) content of nodules was assayed following the method of Miller and Pengelly (1984). One gram of fresh nodules was ground in 2 mL of 5% (w/v) 5-sulfosalicyclic acid with a mortar and pestle and the extract was centrifuged at $30{,}000 \times g$ for 30 min at 4 °C. Then 0.4 mL of supernatant and 0.2 mL of 50 mM $HgCl_2$ was added to 0.6 mL of 5% (w/v) 5-sulfosalicylic acid in 15 cm^3 reaction vials. The vials were made airtight with subaseal and 0.1 mL of 2.6% NaOCl in 5 N NaOH was injected into the vials. The vials were then vortexed for 5 s and incubated in ice for 50 min. The ethylene produced was estimated by injecting 2 mL of gas sample in to a steel column (2 m × 2 m) filled with Porapak-R (Water Assoc. Inc) and fitted in a gas chromatograph (Nucon, 5700) using a flame ionization detector (FID). Standard ethylene (110 vpm) was used for quantification of data. The ACC content was calculated in terms of *n* moles C_2H_4 g^{-1} DW h^{-1}.

ACC oxidase

The activity of ACC oxidase was measured by the method described by Fearn and La Rue (1991). One gram of fresh nodules was incubated in 15 cm^3 reaction vials containing 2 mL of 20 mM ACC. The vials were made airtight with subaseal and kept in dark for 4 h at 25 °C. Then 2 mL of gaseous sample was taken from each vial and analyzed for ethylene using Gas Chromatograph (Nucon, 5700). The activity of ACC oxidase was calculated as *n* moles C_2H_4 g^{-1} DW h^{-1}.

Ethylene evolution

The fresh nodules after detaching from roots were placed within 50 mL reaction vials containing wet cotton pad. The vials were made airtight with subaseal and kept in dark for 20 min at 25 °C as described by Fearn and La Rue (1991). Then 2 mL of gas sample was taken from each vial and assayed for ethylene production on Gas Chromatograph (Nucon 5700). The DW of each sample was recorded and the amount of ethylene evolved was calculated as *n* moles C_2H_4 h^{-1} g^{-1} DW.

Nitrogenase activity

Nitrogenase (N_2-ase) activity of nodules was measured using acetylene reduction assay (ARA) described by Hardy et al. (1968) and expressed as μmol C_2H_4 produced g^{-1} DW h^{-1}.

Leghemoglobin

Immediately after uprooting the plants of each treatment, the nodules of the whole plants were detached and washed. Estimation of LHb content of nodules was done according to the method of Hartree (1955) and the pigment content was expressed as mg g^{-1} DW of nodules.

Extraction of protein for enzymes assay

One g of fresh nodules were washed in chilled distilled water and homogenized with a chilled pestle and mortar in 5 mL of extraction buffer (0.1 M phosphate buffer, pH 7.0), containing 10 mM KCl, 1 mM $MgCl_2$ and 10 mM EDTA and centrifuged at 10,000 × *g* at 4 °C for 20 min. The supernatant was used for the following enzymes assay. The enzymatic protein was determined by the method of Lowry et al. (1951).

Catalase

CAT (EC 1.11.1.6) activity was estimated by the UV method of Aebi (1983). The reaction mixture contained 300 μL of enzyme extract, 0.5 mL of 10 mM H_2O_2 and 600 μL of 30 mM potassium phosphate buffer and the decrease in absorbance was recorded at 240 nm for 30 s. The enzyme activity was calculated as μmol H_2O_2 decomposed mg^{-1} protein min^{-1} by using the H_2O_2 extinction coefficient 36 μM^{-1} cm^{-1}.

Peroxidase

POX (EC 1.11.1.7) was assayed by using guaicol as substrate. 0.1 mL of the enzyme extract was taken and to this 3.0 mL of 0.1 M phosphate buffer (pH 7.0), 0.05 mL of guaicol solution (20 mM) and 0.03 mL of 12.3 mM H_2O_2 were added. Change in absorbance at 436 nm was recorded for 2 min at an interval of 15 s. The enzyme activity was expressed as units mg^{-1} protein min^{-1} using extinction coefficient 6.39 μM^{-1}.

Glutathione reductase

Glutathione reductase (GR) activity (EC 1.11.1.9) was estimated by the method of Goldberg and Spooner (1983). To 0.1 mL of enzyme extract, 2.5 mL of 120 mM K-phosphate buffer (pH 7.2), 0.1 mL of 0.015 mM EDTA and 0.1 mL of 0.065 mM oxidised glutathione were added. After 5 min 0.05 mL of 9.6 mM NADH was added and mixed thoroughly. The absorbance was recorded at 340 nm at an interval of 5 s. The enzyme activity was calculated as units mg^{-1} protein min^{-1} using the molar extinction coefficient of NADH 6.23 μM^{-1} cm^{-1}.

Glutathione transferase

Glutathione transferase (GTase, EC 2.5.1.18) activity was measured by thiolysis method of Habig and Jakoby (1981). To 0.3 mL enzyme extract, 1.0 mL of phosphate buffer (pH 7.0) containing 0.2 mM *p*-nitrophenylacetate was added. Then 0.1 mL of GSH (50 mM) solution was added at the time of taking absorbance. Change in absorbance was recorded at 400 nM at time interval of 15 s for 5 min. The extinction coefficient for *p*-nitrophenol at pH 7.0 was taken as 8.79 mM^{-1} cm^{-1}. The enzyme activity was calculated as μmol *p*-nitrophenol mg^{-1} protein min^{-1}.

Ascorbate peroxidase

Ascorbate peroxidase (ASC-POX) activity (EC 1.11.1.11) was measured by the method of Nakano and Asada (1981). To 0.1 mL of enzyme extract, 0.3 mL of 50 mM K-phosphate buffer (pH 7.0) containing 0.5 mM AA was added. Total reaction volume was made to 1 mL. The reaction started with addition of 0.06 mL of 1 mM H_2O_2 and the absorbance decrease was recorded after 10 s at 290 nm. Under the assay conditions a decrease in 0.01 absorbance corresponds to 3.6 mmol ascorbate oxidised. The enzyme activity was calculated as μmol ascrobate decomposed min^{-1}.

Superoxide dismutase

The activity of superoxide dismutase (SOD, EC 1.15.1.1) was estimated by the method of Giannopolitis and Ries (1977). The reaction mixture contained 0, 0.2, 0.3, 0.5 and 1.0 mL of enzyme extract in different sets. To each set 1 mL of 390 mM methionine, 1 mL of 2.25 mM nitroblue tetrazolium, 1 mL of 3 mM EDTA and 1.5 M Na_2CO_3 were added. Total reaction volume was made to 5 mL adjusting the pH to 10.2. At the end 1 mL of 60 μM riboflavin was added. The tubes were shaken and placed 30 cm from light source consisting of two 15 W fluorescent lamps. The reaction was allowed to run for 10 min and then stopped by switching off the light. The tubes were immediately covered with black cloth. The absorbance was recorded at 560 nm. A non-irradiated reaction mixture which did not develop a color, served as the control. However, in the presence of SOD, the

reaction was inhibited and the amount of inhibition was used to quantify the enzyme. Log A_{560} was plotted as a function of volume of enzyme extract used in reaction mixture. From the resultant graph, volume of enzyme extract corresponding to 50% inhibition of the photochemical reaction was obtained and considered as one enzyme unit. The enzyme activity was calculated as units mg^{-1} protein min^{-1}.

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) content of nodules was determined by a modified Patterson et al. (1984). Five hundred milligram of fresh nodules were homogenized with 0.2 g of activated charcoal and 10 mL of 5% TCA. The homogenate was filtered through Whatman no. 1 filter paper and used directly for assay. A 200 μL of the extract was brought to 4 mL with 100 mM K-phosphate buffer (pH 8.4). Then 2 mL of colorimetric reagent was prepared by mixing 0.6 mM M_4-(2-pyridylazo) resorcinol and potassium titanium oxalalate 1/1, v/v (the mixture was kept on ice until use). H_2O_2 was determined from the difference in absorbance at 508 nm between sample and blank. H_2O_2 content was calculated using its molar extinction coefficient of 36 $\mu M^{-1} cm^{-1}$ and calculated as mol g^{-1} DW.

Lipid peroxidation

The level of lipid peroxidation in nodules was measured in terms of thiobarbituric acid reactive substances (TBARS) contents (Heath and Packer, 1968). Nodules samples of 500 mg were homogenised with 5 mL of 0.1% TCA. The homogenate was centrifuged at 8000 × *g* for 15 min. One milliliter of supernatant was precipitated by 4 mL of 20% TCA containing TBA. The mixture was heated in a water bath shaker at 95 °C for 30 min and quickly cooled in an ice-bath. The absorbance was read at 532 nm after centrifugation at 8000 × *g* for 10 min and the value for non-specific absorption at 600 nm was subtracted. The TBARS content was calculated using its extinction coefficient of 155 $mM^{-1} cm^{-1}$ as μmol TBARS g^{-1} DW.

Minerals

The sodium (Na), potassium (K) and chloride (Cl) contents of nodules were determined from oven dried ground material. Fifty milligram material was digested in 5 mL of a diacid mixture of H_2SO_4 and $HClO_4$ (9:1) and diluted to the desired volume. Na and K contents were estimated using the Flame Photometer (Elico, India) and further expressed on Na/K ratio. The Cl content in the digested material was determined by EIL mV meter from Caltex instruments (Model CM2400A, UK) using Calomel Chloride electrode and expressed as mmol g^{-1} DW. The total nitrogen (N) was estimated by the MicroKjeldahl method using Kjeltac Auto 1030 analyzer (Sweden) and expressed as mg g^{-1} DW of nodules.

Statistical analysis

Three replicates consist of three pots and each pot containing two plants were used for each observation under each treatment. The data were analyzed statistically using complete randomized design and the significance was tested at 5% level of critical difference using the table 'ANOVA'. Vertical bars in figures indicate value of standard error (SE ±) mean.

Results and discussion

Water relations

With raising the level of saline irrigation from 2.5 to 10 dS m^{-1} a significant decrease in Ψ_w of leaf from –0.44 to –0.56 MPa was noticed. Similarly, Ψ_s of leaf and nodules also declined from –0.65 to –1.15 MPa and from –0.75 to –1.77 MPa, respectively (Table 1). The decrease observed in relation to RWC of leaf and nodules was from 86.05% to 73.30% and from 94.70% to 89.95%, respectively (Table 1). Substantial variations in Ψ_s and RWC of leaf and nodules were seen. Nodules showed more negative value of Ψ_s but higher RWC than leaf. The prepared mechanism for decreasing Ψ_s is that plants adjust under stress conditions to maintain turgor (Nandwal et al., 2000a; Sairam et al., 2002).

The proline and TSS contents of nodules increased significantly from 9% to 78% and from 7% to 33%, respectively, under salinity in comparison to control (Table 1). The concentration of these metabolites usually increases in response to salt stress (Nandwal et al., 2000b; Sairam et al., 2002), and have mainly recognized as osmotic adjustment agents. Decline in Ψ_s can be a result of solute accumulation like proline and TSS. After desalinization proline and TSS sharply decreased due to their utilization. Ψ_w of leaf and Ψ_s of leaf nodules became less negative due to reduced level of these metabolites. These effects were also facilitated by

Table 1. Changes in ψ_w, ψ_s and RWC of leaf and nodules and TSS and Proline content of nodules in chickpea upon salinization and desalinization

Parameters	C	S_1	S_1R	S_2	S_2R	S_3	S_3R	CD at 5%
ψ_w leaf (-MPa)	0.44	0.47	0.43	0.52	0.47	0.56	0.55	0.03
ψ_s leaf (-MPa)	0.65	0.75	0.65	0.88	0.74	1.15	0.83	0.05
RWC leaf (%)	86.05	79.10	82.10	76.60	80.75	73.30	77.45	3.01
ψ_s nodules (-MPa)	0.75	0.85	0.75	1.15	0.90	1.77	1.15	0.07
RWC nodules (%)	94.70	92.05	94.50	88.10	92.12	86.95	88.85	2.96
TSS nodules (mg g^{-1} DW)	37.70	39.28	37.17	42.15	38.33	50.01	42.46	3.62
Proline nodules (mg g^{-1} DW)	0.71	0.77	0.74	1.02	0.86	1.26	1.00	0.05

the simultaneous increase in RWC of leaf and nodules.

Salinity stressed nodules showed over production of ACC from 37% to 126% over control (Fig. 1). Upon disalinization it decreased by 23–17% depending upon stress levels. Similarly, activity of ACC oxidase increased by 31%, 73% and 118% at 2.5, 5.0 and 10.0 dS m^{-1} levels of salinity, respectively. Upon revival these values declined from 11% to 16%. The increase in ACC content and ACC oxidase activity led to more production of C_2H_4 under said conditions. Hence, on exposure to saline irrigation, chickpea nodules showed 35–108% increase in ethylene evolution in comparison to control (Fig. 1). A positive correlation between the levels of salinity and the amount of ACC content, ACC oxidase activity and ethylene production in chickpea nodules was noticed. Kacperska and Kubaca-Zebalska (1993) also showed that stress promoted ethylene evolution was due to stress promoted synthesis of ACC and ACC oxidase activity. In the present investigation it is clear that salinity stress promoted ethylene evolution in chickpea nodules through the disturbance in water status as a result of decrease in Ψ_w of leaf, Ψ_s and RWC of leaf and nodules in addition to increase in lipid peroxidation, ACC content and ACC oxidase activity. Upon desalinization, decrease in the level of C_2H_4, ACC content and ACC oxidase activity was noticed along with the increase in Ψ_w of leaf and Ψ_s and RWC of leaf and nodules, suggesting that salinity plays a very important role in ethylene production in chickpea nodules.

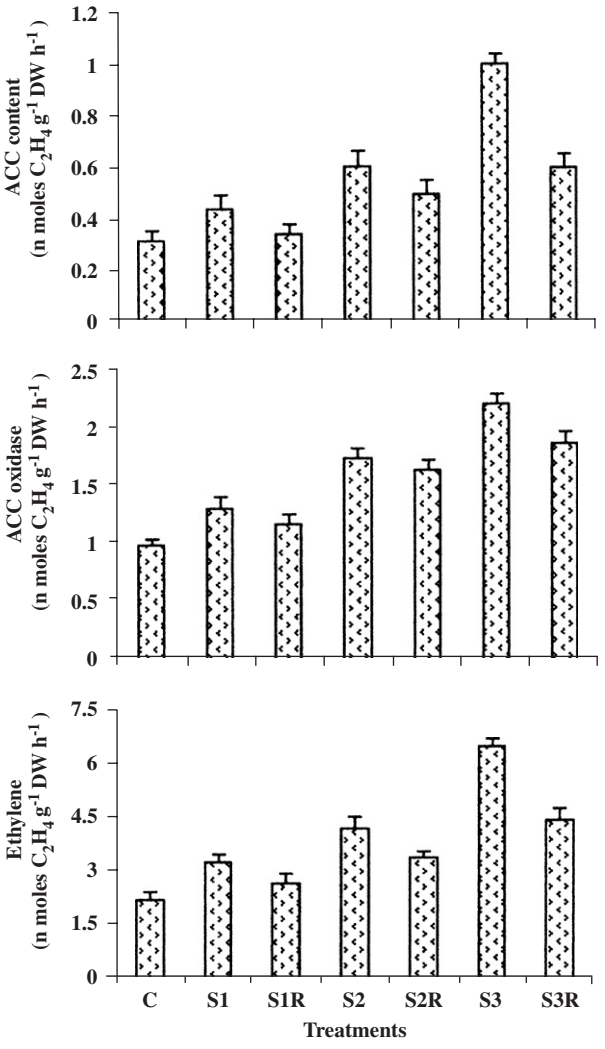

Figure 1. ACC content, ACC oxidase activity and ethylene evolution in chickpea nodules as affected by salinization and desalinzation. Vertical bars indicate value of SE (±) mean.

Nitrogen fixing efficiency

The N_2-ase activity of nodules sharply declined from 17% to 68% under different levels of saline irrigation (Fig. 2). Similarly increasing level of salinity markedly reduced (39–56%) LHb and N (12–58%) contents of nodules (Fig. 2). Salinity stressed induced decrease in water status and LHb content of nodules therefore can at least partially explain the inhibition of N_2-ase activity. The possible reasons for decline in LHb content and

thus ARA under salinity can be categorized as (i) due to degradation of LHb and (ii) due to decrease in nodule permeability (high lipid peroxidation, Table 3) for O_2 diffusion resulting in decreasing of respiration thus inhibiting ARA (Nandwal et al., 2000a, b). In chickpea, it is reported that in addition to the decrease in LHb content and ARA of nodules under salinity there was an osmotic regulation in nodules because of increase in proline and TSS contents. These findings suggest the possibility of the existence of a compensatory mechanism that enables this salinity affected nodules to partially mitigate the imbalance in N_2 fixation, perhaps by osmoregulation as discussed earlier.

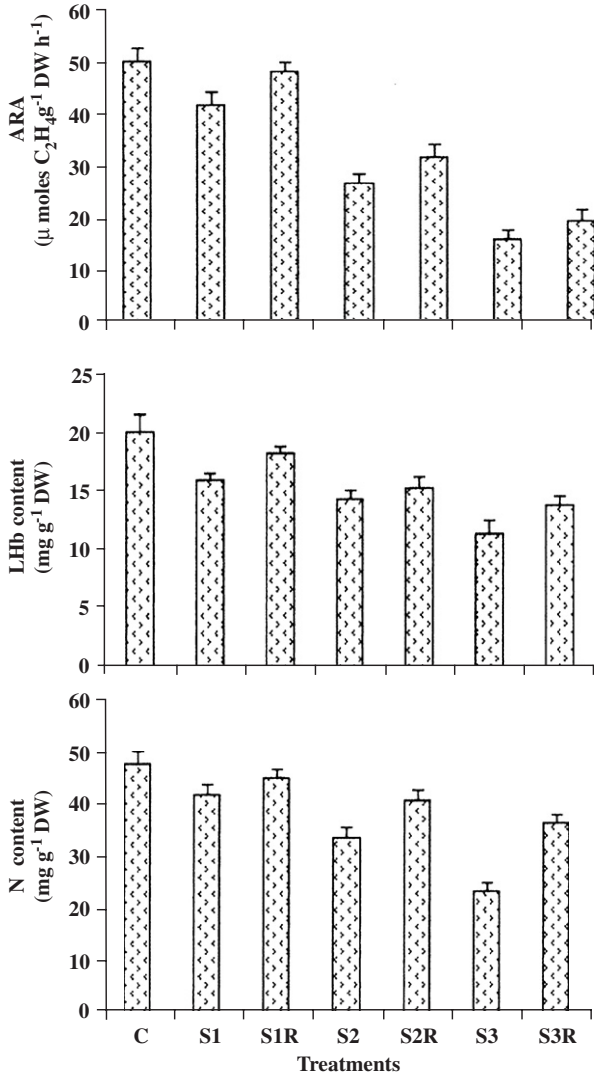

Figure 2. N_2-fixing efficiency in chickpea nodules as affected by salinization and desalinzation. Vertical bars indicate value of SE (±) mean.

Moreover high ethylene production in nodules under salinity may have a direct/indirect role to decrease nodulation and ARA. LHb is probably the most important source of ROS in nodules and at the same time, it is also damaged by these toxic species with the impairment of its fundamental role as O_2 carrier (Becana et al., 2000). The decrease in ARA, LHb and change in the permeability of nodules under salinity were the reasons for decrease in N content.

With the rise of saline irrigation level, Na^+/K^+ ratio in nodules increased significantly (Table 2). About five times increase in Na^+/K^+ ratio was noticed at 10 dS m^{-1} level of salinity. The changes in Na^+/K^+ ratio also reflected in the changes in Cl^- content (Table 2) of nodules. Hence, the Cl^- content increased from 1.54 to 3.07 mmol. Upon desalinization, the ratio of Na^+/K^+ and Cl^- content were decreased, however, the values were still higher than control.

In the present investigation, attempts were also made to establish a correlation between antioxidative defense system and salinity induced changes in chickpea nodules. Upon salinization, the defense mechanism was activated which was apparent from increased activity of various antioxidant enzymes. The specific activity of SOD increased from 8.4% to 51.3% under salinity, whereas reverse was true for of CAT i.e. it decreased from 24% to 73% (Table 3). The specific activity of ASC-POX and GTase were increased from 18% to 88% and 37% to 52%, respectively. The increase in the activity of POX and GR was from 1.5 to 3 folds and 2 to 2.5 folds, respectively (Table 3). Thus, the effect of saline irrigation on the defense mechanism may be characterized by its activation in chickpea nodules. However, activation of these enzymes could not overcome the accumulation of H_2O_2. Upon desalinization a partial reversibility was observed in the activity of these enzymes. Beneficial effect of higher osmolyte concentration i.e., soluble sugars and proline was affected in maintenance of higher RWC in nodules and stabilization of essential protective enzymes proteins, resulting

Table 2. Na^+/K^+ ratio and Cl^- content in chickpea nodules as affected by salinization and desalinization

Parameters	C	S_1	S_1R	S_2	S_2R	S_3	S_3R	CD at 5%
Na^+/K^+ ratio	28.80	35.20	32.20	56.65	44.40	82.05	65.07	3.27
Cl^- Content (mmol g^{-1} DW)	1.54	1.90	1.58	2.25	2.17	3.07	2.60	0.15

Table 3. The specific activity of antioxidant enzymes and AA, H_2O_2 and TBARS contents in chickpea nodules as affected by salinization and desalinization

Parameters	C	S_1	S_1R	S_2	S_2R	S_3	S_3R	CD at 5%
Superoxide dismutase (Units mg^{-1} protein min^{-1})	4.15	4.50	4.23	5.03	4.36	6.27	4.88	0.02
Catalase (μmol H_2O_2 decomposed mg^{-1} protein min^{-1})	52.32	39.76	46.80	20.30	27.30	14.18	21.30	5.20
Peroxidase [(Units mg^{-1} protein min^{-1}) $\times 10^{-3}$]	4.41	6.54	5.00	9.94	7.59	13.70	7.81	0.76
Ascorbate peroxidase (μmol ascorbate decomposed min^{-1})	2.43	2.87	2.70	4.45	3.00	4.56	3.50	0.26
Glutathione reductase (Units mg^{-1} protein min^{-1})	33.74	66.43	46.84	82.80	54.31	85.68	64.45	4.20
Glutathione transferase (μmol *p*-nitrophenol mg^{-1} protein min^{-1})	1.67	2.28	1.84	2.52	2.20	3.29	2.59	0.21
AA content (mg g^{-1} DW)	2.83	2.26	2.41	1.99	2.16	1.75	2.00	0.09
H_2O_2 Content [(mol g^{-1} DW) $\times 10^{-4}$]	54.29	67.62	62.12	109.47	85.05	129.88	97.02	6.40
TBARS content (μmol g^{-1} DW)	1.98	2.21	2.11	3.13	2.66	4.63	3.23	0.08

in their higher activity under salinity stress than control. However, this induced antioxidant enzymes activity was not sufficient to scavenge the oxidative damage of nodules, as is clear from increased lipid peroxidation and H_2O_2 content, LHb damage and decreased ARA under salinity as compared to noticed in control nodules.

H_2O_2 is most stable ROS and most of the H_2O_2 present in the plant cell is produced by the action of SOD. The enhanced its activity led to the accumulation of H_2O_2 from 25% to 139% under salinity (Table 3). H_2O_2 is also known to produce to many other non-enzymatic and enzymatic processes in plants. Upon reirrigation of stressed plants, the H_2O_2 content in nodules declined with simultaneous decrease in the activity of SOD and POX. H_2O_2 is a relatively stable metabolite that may act as a second messenger, since it could diffuse from the site of production and is known to induce several other genes and proteins involved in stress defenses like CAT (Prasad et al., 1994a), POX (Prasad et al., 1994b) and ASC-POX (Morita et al., 1999).

In nodules, the AA content decreased from 20% to 38% upon salinization (Table 3) in comparison to control. The failure to maintain AA content under said conditions may be an indication of oxidative damage of nodules. AA participate in the removal of H_2O_2 as a substrate of ASC-POX enzyme and directly reduced O_2 and regenerate reduced α-tocopherol (Foyer, 1989), thus affording protection to the integrity of cellular membrane along with α-tocopherol (Menconi et al., 1995). The decrease in AA content could be due to the rapid oxidation or its slow rate of synthesis (Gogorcena et al., 1995). A complete recovery in AA content of nodules was not seen upon desalinization of the plants.

The lipid peroxidation (in terms of TBARS) in nodules increased significantly from 11% to 133% over control upon salinization (Table 3). A possible cause of increased TBARS under salinity was due to the accumulation of H_2O_2 and as a result of induced nodule senescence. It has been reported that free iron ions (LHb) directly or indirectly participate in lipid peroxidation (Becana et al., 2000). The level of TBARS was brought down upon desalinization but values were still higher than that of control. The results here proposed that lipid peroxidation goes along with ethylene formation. It is suggested that correlation processes i.e., a sharp rise in lipid peroxidation, ethylene evolution and H_2O_2 content and decrease in ARA, LHb and AA contents and water status are the characteristics of induced senescence in nodules under salinity. However, these changes were reversible to certain extent after 3 d of desalinization.

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