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Nodule structure and functioning in chickpea (*Cicer arietinum*) as affected by salt stress

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Abstract

Cicer arietinum L. plants raised in sand culture under natural light were subjected to salinity stress induced by mixture of NaCl, $CaCl_2$, $MgCl_2$ and $MgSO_4$ (40, 60 or 80 meq dm^{-3}). Acetylene reduction activity (ARA) of nodules, leghemoglobin content and nodule structure were followed 55, 75 and 85 d after sowing. ARA declined significantly under salt treatments and the lowest ARA was observed at day 85 after sowing. Decrease in ARA was consistent with decreased nodule leghemoglobin content. The leghemoglobin content of control plants decreased by 50 % at day 85 indicating senescence of nodules. This senescence was further accelerated by salt treatment after which the leghemoglobin content fell to negligible levels. The structural changes associated with salt stress were mainly reduction in size of the nodules, decreased meristematic zone, reduced number and degradation of symbiosomes, reduced intercellular spaces and deposition of electron dense material in the intercellular spaces in the cortex of nodules.

Additional key words: acetylene reductase activity, leghemoglobin, nodule senescence.

Introduction

The marked influence of environment on symbiotic nitrogen fixation has been known for a long time. The delicate balance between the host plant and the symbiont is disturbed even by mildly adverse conditions which have otherwise no effect on plant growth. Salt stress is one of the major environmental stresses adversely affecting legume production in arid and semiarid regions (Bernstein and Ogata 1966, Tu 1981). High soil salinity can deleteriously affect symbiotic association between legume and *Rhizobium* by osmotic stress and ionic toxicity and imbalance (Sprent 1972, Sánchez-Díaz *et al.* 1982). Treatment of plants with NaCl accelerated greening of the nodules accompanied by decrease in leghemoglobin (Lb) content (Wilson 1970, Yousef and Sprent 1983) which is considered to be an index of nodule senescence.

There are only few studies where the structural changes in nodules from salt stressed plants has been related to the decrease in N_2-fixation (Zahran 1986, James *et al.* 1993). Therefore; the present investigations were conducted to investigate further the structural and functional changes in chickpea nodules under salt stress.

Materials and methods

Plants: Chickpea (*Cicer arietinum* L. cv. C-235) plants were raised in sand culture in earthenware pots (22.5 × 22.5 cm) each containing 6 kg of river sand. Before sowing, seeds were surface sterilized and inoculated with *Rhizobium* culture (strain Ca 181) obtained from Microbiology Department, Chaudhary Charan Singh Haryana Agricultural University, Hisar. Plants were grown under natural light and temperature and supplied with non-limiting amounts of nitrogen-free nutrient solution comprising of KH_2PO_4 and K_2SO_4 (2 mM), $CaSO_4.2\ H_2O$ (3 mM), $MgSO_4$ (1 mM), $MnCl_2O$ (5 μM), $ZnSO_4.7\ H_2O$ (5 μM), H_3BO_3 (25 μM), $CuSO_4.5\ H_2O$ (0.5 μM), Na_2MoO_4 (0.5μM), $CoCl_2.6\ H_2O$ (0.17 μM), $CaCO_3$ (10 mM), and ferric citrate (4 μM) (Wilson and Reisenauer 1963). Pots were divided into four lots. One served as control and the other three were subjected

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to salinity stress. The desired salinity levels of 0, 40, 60, 80 meq dm^{-3} were created with a mixture of NaCl, $CaCl_2$, $MgCl_2$ and $MgSO_4$ (using Na:Ca+Mg in 1:1, Ca:Mg in 1:3 and Cl:SO_4 in 7:3 on milliequivalent basis) added to the N-free nutrient solution. The control plants were raised in dune sand with nitrogen free nutrient solution. From every treatment four pots were sampled at 55, 75 and 85 d after sowing (DAS).

Nitrogen fixation: The rate of N_2-fixation was determined by incubating isolated roots bearing nodules in 10 % C_2H_2 for 10 min in 100-cm^{-3} bottles fitted with subaseals. The acetylene reduction assay was earned out according to Hardy *et al.* (1973).

Leghemoglobin content: The detached nodules were thoroughly mixed and their leghemoglobin content was estimated by the method of Hartee (1955).

Light microscopy: The roots and nodules were fixed in FAA (formaldehyde, acetic acid, alcohol and water in the ratio of 2:1:10:7), passed through ethanol-xylene series for dehydration, infiltration and embedded in wax. Sections (8 - 10 μm thick) were stained with methylene blue and toluidine blue combination. Roots were stained in safranin and light green combination. Stained sections were mounted in DPX resin and observed under light microscope.

Electron microscopy: Nodules cut into smaller blocks were fixed in glutaraldehyde overnight at 4 °C. The blocks were washed thrice with 0.1 M phosphate buffer and postfixed in 2 % OsO_4 at 4 °C and washed again thrice with phosphate buffer. After dehydration in acetone series, clearing was done by placing the sections in toluene. Infiltration was done in vacuum for 48 h in three parts of toluene and one part of embedding medium (*Araldite*). The sections were then transferred into mixture of two parts of embedding medium and 2 parts of toluene and kept for 48 h in vacuum. Subsequently, the sections were transferred to three parts of embedding medium and two parts of toluene for 48 h in vacuum. Ultimately, toluene was completely eliminated and the blocks were transferred to pure *Araldite*-twice to make sure that no trace of toluene was left. Ultra thin sections (10 nm) were cut and observed under the electron microscope at desired magnification.

Results and discussion

Acetylene reduction activity (ARA) of nodules declined significantly under salt treatments (Table 1). At 55 DAS, ARA showed a decrease of 40, 57 and 78 % at salinity 40, 60 and 80 meq dm^{-3} as compared to control. A similar trend was observed 70 and 85 DAS. This result is consistent with considerable inhibition of nodulation and N_2-fixation under saline conditions as reported by earlier workers (Yousef and Sprent 1983, Bekki *et al.* 1987, Hafeez *et al.* 1988, James and Sprent 1988, Elsheikh and Wood 1990, Fougere *et al.* 1991, Sheokand *et al.* 1995). Serraj *et al.* (1994) observed under short term NaCl salinity stress that decrease in ARA of soybean nodules was accompanied by decrease in nodule respiration, decrease in permeability to O_2 diffusion and increase in fermentative metabolism.

Table 1. Acetylene reduction assay [mmol(C_2H_4 evolved) g^{-1}(f.m) s^{-1}] under salinity in chickpea (*Cicer arietinum*) cv. C-235.

Salinity [meq dm^{-3}]	55 DAS	70 DAS	85 DAS
Control	36.0 ± 3.6	32.4 ± 7.2	27.0 ± 4.5
40	21.6 ± 9.0	23.4 ± 9.0	14.4 ± 3.6
60	15.3 ± 1.8	25.2 ± 1.8	14.4 ± 7.2
80	7.7 ± 3.6	16.2 ± 1.8	7.2 ± 0.5

Decrease in ARA probably reflected decreased nodule leghemoglobin content (Table 2). At vegetative stage, *i.e.*, at 55 DAS, reduction in leghemoglobin content by 27, 42 and 57 % was observed with 40, 60 and 80 meq dm^{-3} salinity, respectively. This decline further increased at 75 DAS and maximum decline was observed at 85 DAS where the Lb content of the control plant nodules had also come down by 50 % indicating senescence. This senescence was further accelerated by salt treatment and the leghemoglobin content fell to negligible levels. Pladys and Rigaud (1985) suggested from their observations on the ratio of Lb to total soluble protein that Lb is more prone to proteolysis under salt stress. For this reason Lb was chosen as a substrate to assay proleolytic activity.

Table 2. Leghaemoglobin content [mM] under saline conditions in symbiotic nodules of chickpea (*Cicer arietinum*) cv. C-235.

Salinity [meq dm^{-3}]	55 DAS	70 DAS	85 DAS
Control	41 ± 0	34 ± 1	20 ± 1
40	30 ± 2	23 ± 0	9 ± 1
60	24 ± 1	17 ± 2	3 ± 2
80	18 ± 0	11 ± 1	0 ± 0

The nodules showed three functional zones: distal zone, central zone and the proximal zone. The activity of distal zone was mainly, confined with meristematic activities adding new cells to the peripheral and core

region. Longitudinal sections revealed that the core region of the nodule was surrounded by a zone of noninfected, thin walled, approximately isodiametric cells designated as nodule parenchyma followed by nodule endodermis (Fig. 1). The cells of the nodule parenchyma were comparatively compressed accompanied with few intercellular spaces. The cells of the outer cortical region were mostly isodiametric, oval and having well defined intercellular spaces (Fig. 1). The intercellular spaces in the nodule parenchyma cells were smaller and comparatively less in number. The inner cortical layers became thinner and indistinct towards the distal region.

Fig. 1. Longitudinal section of nodule: OC - outer cortex, E - endodermis, IC - nodule parenchyma.
Fig. 2. Control nodule: BC - bacterial cells, CV - central vacuole.
Fig. 3. Plasmolysed infected cell showing degenerated symbiosomes (*arrow*).
Fig. 4. An infected symbiosome of nodule under salt treatment: CV - central vacuole.

There was reduction in the size of nodule which was attributed to the lower activity of the meristematic zone. At salinity in 60 and 80 meq dm^{-3} almost complete elimination of meristematic zone was observed.

Light micrographs of core region of control nodules showed infected cells with large number of symbiosomes. The infected cells contained large number of vacuoles and starch granules (Fig. 2). The core region of salt treated (40 meq dm^{-3}) nodules still had a large central vacuole but the number of symbiosomes was reduced (Fig. 3). Higher salinity resulted in plasmolysed cells with crimpled walls, degenerated symbiosomes, and decreased number of starch granules (Fig. 4). The cortical cells of this treatment also showed crinkled walls with reduced intercellular spaces (Fig. 5).

Electron micrograph of the control bacteroidal cell conformed the intactness of the peribacteroid membrane, a membrane envelope delimiting bacteroid (Fig. 6). Figs. 7, 8 and 9 show different degree of degradation of the symbiosome as caused by increasing levels of salt stress.

Electron dense material was present in the intercellular spaces in the cortex of nodules under salt treatment (Fig. 10). Such depositions have been previously reported by James *et al.* (1993). They have

reported the material to be a glycoprotein as it reacts with monoclonal antibodies MAC 236 (Van den Bosch *et al.* 1989) and has been implicated in the operation of the oxygen diffusion barrier (James *et al.* 1991). James *et al.* (1993) studied the structure of mature soybean nodules subjected to 50 mM NaCl. Nodules harvested one week after a 14-d salt treatment had small regions of meristematic activity at the edge of the infected zone and a number of infected cells contained vacuoles around host cell nuclei.

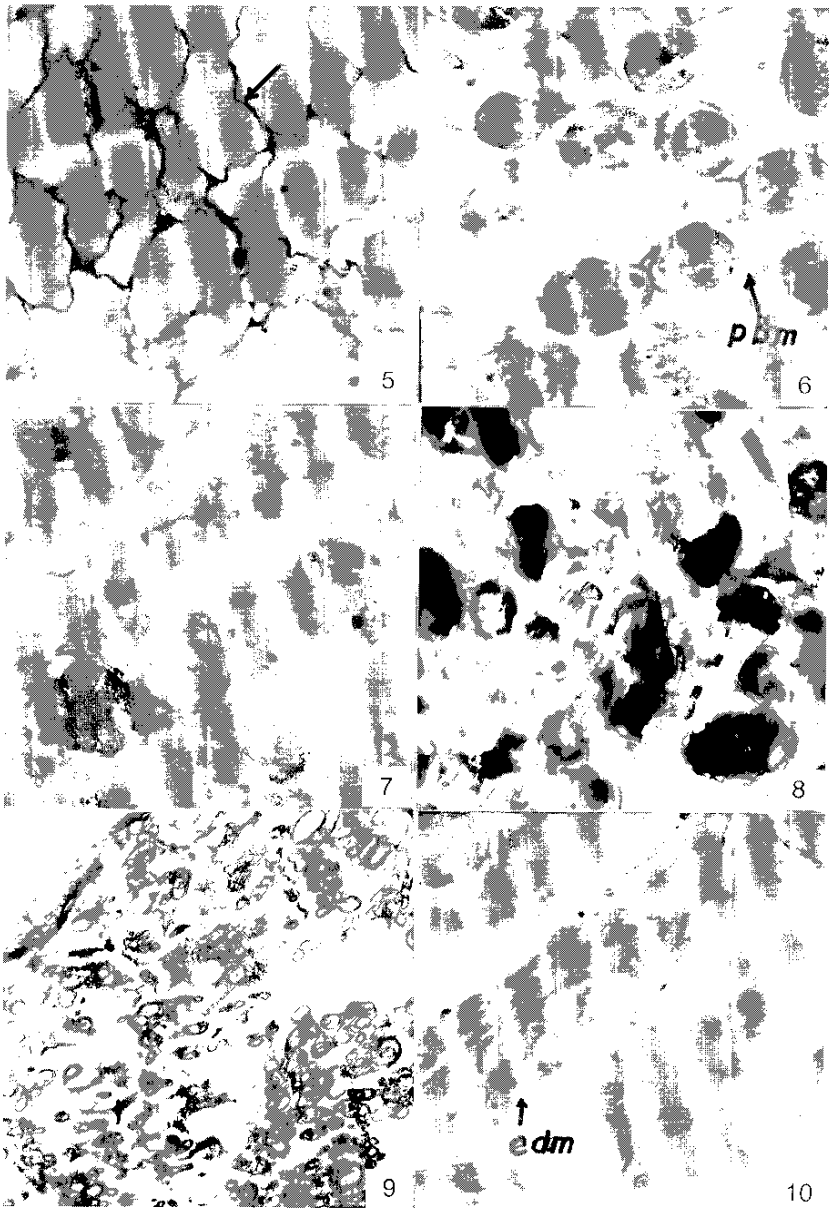

Fig. 5. Crinkled wall of cortical cells (*arrow*).
Fig. 6. Electron micrograph showing the peribacteroid membrane (pbm) of unaffected symbiosomes.
Figs. 7, 8, 9. Different degree of degeneration of the symbiosomes in the salt stressed plants.
Fig. 10. Electron dense material (edm) in the intercellular space of cortex of nodule under salt treatment.

This study reveals that the sharp decline in N_2-fixation under salinity stress is mainly due to salt-induced structural degradation of the infected cells and increased diffusion resistance for O_2 due to depositions in the intercellular spaces of the inner cortical cells.

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