

The Effects of Salinity on Nitrogen Fixation and Trehalose Metabolism in Mycorrhizal *Cajanus cajan* (L.) Millsp. Plants

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Abstract Arbuscular mycorrhizal (AM) fungi exist widely in natural ecosystems as well as in salt-affected soils and are considered suitable candidates for bio-amelioration of saline soils. Plants respond to salinity by accumulating sugars and other low-molecular-weight compatible solutes. One such compound is trehalose, which has been found to play an important role as a stress protectant. The aim of the present investigation was to study interactions between an AM fungus and salinity stress on growth, nitrogen fixation, and trehalose metabolism in *Cajanus cajan* (L.) Millsp. (pigeonpea). Two genotypes [Sel 85N (salt-tolerant) and ICP 13997 (salt-sensitive)] were subjected to saline treatments with and without mycorrhizal inoculations. Salinity reduced plant biomass (shoot and root) in both genotypes and resulted in a decline in shoot-to-root ratio (SRR); however, a smaller decline was observed in Sel 85N than in ICP 13997. AM colonization was reduced with increasing salinity levels but mycorrhizal responsiveness (MR) increased. Genotypic variability in nitrogen fixation and trehalose metabolism in response to salinity and mycorrhization was observed. An increment in nodule number was accompanied by a reduction in dry mass. Subsequently, nodular activity (leghemoglobin, acetylene-reduction activity [ARA], nitrogen content) was reduced under soil salinity, which was more profound in ICP 13997 than in Sel 85N. The symbiotic association with *Glomus mosseae* led to significant improvement in plant dry mass and nitrogen-fixing potential of nodules under salt stress. Salinity led to an increase in trehalose-6-P synthetase (TPS) and trehalose-6-P

phosphatase (TPP) activities resulting in increased trehalose content in nodules, which was accompanied by inhibition of trehalose catabolism (trehalase activity). AM plants had lower trehalase activity under saline and non-saline conditions. Thus, a symbiotic relationship between plant roots and *G. mosseae* might have resulted in salinity tolerance in a genotype-dependent manner.

Keywords *Cajanus cajan* · *Glomus mosseae* · Nitrogen fixation · Nodule · Trehalose

Introduction

Soil salinity is considered one of the most significant abiotic stress factors affecting crop production and agricultural sustainability in arid and semiarid regions of the world (Flowers 2004; Munns 2005; Ashraf and others 2008; Katerji and others 2009). It is expected that increasing salinization of arable land will have devastating global effects, resulting in 30% land loss within the next 25 years and up to 50% by the year 2050 (Wang and others 2003). Salinity stress causes low osmotic potential of soil solution (osmotic stress) and specific ion effects (mainly Na⁺ and Cl⁻) resulting in nutritional imbalances or ion cytotoxicity (Munns 2005; Munns and Tester 2008). Most crop plants are susceptible and cannot survive under conditions of higher salinity (Siddiqui and others 2009). Most legume species have been found to be either sensitive or moderately tolerant to salinity as they depend on symbiotic nitrogen fixation for their growth (Drevon and others 2001; Aydi and others 2008; Chalk and others 2010; Jebara and others 2010). In addition to its effect on the growth of the host plant, salinity affects nodule establishment (Aydi and others 2004), nodule growth (Abdelmoumen and El-Idrissi

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2009), and symbiotic activity (Bolanos and others 2006; Lopez and Lluch 2008; Al-Sherif 2009; Dulormne and others 2010; Jebara and others 2010). Salt-mediated inhibition of nitrogen-fixing activity has been attributed to a direct effect of salinity on the nitrogenase enzyme in symbiotic systems (Bolanos and others 2006; Tejera and others 2006; Al-Sherif 2009; Jebara and others 2010) or to reduction of nodule respiration and a decrease in leghemoglobin production (Lopez and Lluch 2008; Dulormne and others 2010). Although the establishment and activity of the legume-*Rhizobium* are known to be susceptible to salinity, differences in nitrogen (N₂) fixation efficiency in cultivars and genotypes within the same species such as common bean (Sassi and others 2008; Jebara and others 2010) and chickpea (Garg and Singla 2004; Tejera and others 2006) have been reported. Thus, selection and breeding of genotypes that can grow under stress conditions might be an effective tool in resolving the soil reclamation practices.

Osmotic adjustment with the accumulation of various organic compounds, collectively known as compatible solutes or osmolytes (Zhu 2002; Cortina and Culiánez-Macia 2005), has been reported in plants under salt stress (Lopez and others 2009). One such compound is trehalose (α -D-glucopyranosyl-1,1- α -D-glucopyranoside), a nonreducing disaccharide that has been found to play an important role as an abiotic stress protectant in a wide variety of organisms (Elbein and others 2003; Lopez and others 2009; Fernandez and others 2010). Symbiotic nitrogen-fixing bacteria such as *Rhizobium* have the capacity to synthesize trehalose, and its accumulation has been detected in bacteroids and in nodules of leguminous plants (Muller and others 2001). The synthesis of trehalose in root nodules has been correlated to the maintenance of efficient nitrogen fixation and whole-plant tolerance under drought and salinity stresses (Farias-Rodríguez and others 1998; Jimenez-Zacarias and others 2004; Lopez and others 2008). Jimenez-Zacarias and others (2004) have suggested that nodule trehalose may protect bacterial nitrogenase activity under drought conditions and that both trehalose and nitrogen fixation contribute to drought tolerance. Because little is known about the significance of trehalose in providing protection in legumes under salinity stress, a deeper insight is necessary for understanding the role of this disaccharide in stress tolerance.

In conjunction with using salt-tolerant plants, the role of microorganisms has become increasingly prominent in the conservation of fertility of soils (Welbaum and others 2004; Zuccarini 2007). The most efficient combinations providing better growth and resistance to salinity stress are represented by *Rhizobium* and arbuscular mycorrhizal (AM) fungi (Goss and de Varennes 2002; Chalk and others 2006; Franzini and others 2009). AM fungi exist naturally

in saline environments (Yamato and others 2008; Evelin and others 2009) and improve growth and vigor under a variety of salt-stress conditions (Giri and others 2007; Ashgari 2008; Kumar and others 2010). The AM fungi most commonly observed in saline soils are *Glomus* sp., and *G. mosseae* has been found to be the most efficient fungus in terms of plant performance and protection offered against the detrimental effects of salinity (Porrás-Soriano and others 2009). It has been well documented that AM symbiosis enhances the ability of plants to cope with salt stress (Kaya and others 2009) by improving host mineral nutrient uptake, such as P, Zn, and Fe (Ashgari 2008; Miransari and Smith 2008), and ion balance (Giri and others 2007; Wu and others 2010); accumulation of osmoregulators such as proline, glycine betaine, and others (Garg and Manchanda 2009); increase in photosynthetic rate; and water use efficiency (Colla and others 2008; Wu and others 2010), suggesting that salt-stress alleviation by AM fungi results from a combination of nutritional, biochemical, and physiological effects (Evelin and others 2009). Mycorrhizal fungi have also been shown to synthesize trehalose in the extraradical mycelium, which serves as the main storage carbohydrate and also as an abiotic stress protectant (Ocon and others 2007). Studying the accumulation of trehalose in legume nodules and mycorrhizal roots is of interest as it provides powerful insight into the response of rhizobial and AM symbioses to stress conditions.

Pigeonpea (*Cajanus cajan* [L.] Millsp.) is a short-lived, summer-growing perennial pulse or grain legume crop in semiarid tropical and subtropical areas of the world (Shanower and others 1999). In terms of global grain legume production, it is sixth after *Phaseolus*, beans, peas, chickpeas, broad beans, and lentils (FAO 1996). Pigeonpea has a total cultivated area of approximately 4.2 million hectares (ha) and a yield of three million tons worldwide, predominantly in Asia, Africa, Latin America, and Australia (Saxena and others 2000). It has the ability to fix up to 235 kg N₂/ha (Peoples and others 1995) and produces more N₂ per unit area from plant biomass than many other legumes. The effectiveness of AM fungi has been found to be highest in pigeonpea compared to cowpea and groundnut (Ahiabor and Hirata 1994) as it establishes symbiotic root associations with P-scavenging AM fungi, which increase plant uptake of nutrients and consequently improve plant growth (Ae and others 1990; Subbarao and others 1997).

Although there is some information regarding the role of trehalose in root nodules of legumes, little is known about the physiological significance of trehalose and AM fungi in alleviating the negative effects of salinity stress in root nodules of legumes. The objective of this study was to evaluate the role of *Glomus mosseae* in NaCl-induced

changes associated with nodule nitrogen fixation and trehalose metabolism in *Cajanus cajan* (L.) Millsp. (pigeonpea) genotypes differing in their salt sensitivities.

Materials and Methods

Greenhouse Evaluation Under Salinity

Greenhouse experiments were conducted from mid-June to October 2009 at the Department of Botany, Panjab University, Chandigarh, India (30.5° N, 76.5° E and elevation 305–366 m). The minimum temperature ranged from 22 to 29°C and maximum temperature ranged from 30 to 37°C. The morning relative humidity was between 55 and 92% and afternoon relative humidity was between 42 and 81%.

Plant Material and Symbiotic Inoculants (Rhizobial and Mycorrhizal)

The research material consisted of two genotypes of pigeonpea, salt-tolerant (Sel 85N) and salt-sensitive (ICP 13997). The seeds were washed with water and surface sterilized by dipping them in 15% H₂O₂ solution for 8 min, then washed several times with sterile water to remove any traces of chemical that could interfere in seed germination. Seeds were pretreated with specific rhizobial inoculum of *Sinorhizobium fredii* AR-4 and were kept for drying at room temperature. Seeds were also inoculated with fungal inoculum of *Glomus mosseae*, which was placed at 15-cm pot depth prior to sowing the seeds into the pots to facilitate fungal colonization of plant roots (approximately 300 spores/100 g of soil). Rhizobial and mycorrhizal inocula were obtained from the Division of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi, India.

Sowing and Salt–Stress Induction

The soil (mixture of sand and loam in a ratio of 1:1) was obtained from the nearby agricultural fields. It was fumigated with methyl bromide under air-tight plastic sheets and the fumigant was allowed to dissipate for 1 week (Al-Raddad 1991). Eight seeds were sown in each pot containing 7 kg of soil mixture, with pH 7.2, electrical conductivity of saturated extract (ECe) of 1.0 dSm^{−1}, total N content of 0.42% (Nelson and Sommers 1973) and P content of 20 ppm (Chapman and Pratt 1961). Seedlings

were thinned to three after 6 days of emergence. The pigeonpea plants were grown for 15 days before being exposed to sodium chloride (NaCl) irrigation to avoid salt effects on AM establishment and on pigeonpea seedlings. Two mycorrhizal treatments (with and without inoculations) and three saline treatments [1.0, 4, and 6 dSm^{−1} ECe corresponding to 0, 40 and 60 mM NaCl, respectively (Richards 1954)] were applied. Treatments were arranged in a randomized block design with six replicates. To avoid osmotic shock, NaCl was introduced gradually by successively adding NaCl solutions in each pot. Nodules and roots from control and treated plants were harvested at 60 days after treatment (DAT) for the various physiological and biochemical parameters analyzed. For dry weight measurements, the samples were dried in an oven at 70°C for 72 h until they attained constant weight.

Measurements and Analysis

Symbiotic Development

Nodules formed were visually determined through observations on the size and color of the nodules. *Rhizobium* development was estimated by counting total nodule number per root system and by measuring total nodule dry weight per root system. Arbuscular mycorrhizal (AM) colonization was estimated by using a modified Phillips and Hayman (1970) procedure. The roots were cut into small pieces of approximately 1.0 cm and dipped in KOH solution for 24 h, placed in HCl solution for 15–30 min for neutralization, and then stained with glycerol-trypan blue solution (0.05%) for 24–36 h. Each root piece was examined under a compound light microscope for AM colonization, and data regarding the presence or absence of mycorrhizal structures, arbuscules, and vesicles was recorded. The percentage occurrence of these structures was calculated as follows:

Percent root infection

$$= \frac{\text{Total number of infected roots}}{\text{Total number of roots observed}} \times 100$$

The mycorrhizal responsiveness (MR) or mycorrhizal dependency of a plant shows the extent to which a plant benefits from the presence of AM fungi compared to when it is absent and was calculated according to Hetrick and others (1992) as follows:

$$\text{Mycorrhizal Responsiveness (MR)} = \frac{\text{Dry weight of AM plants} - \text{Dry weight of non-AM plants}}{\text{Dry weight of non-AM plants}} \times 100$$

Normalized Nodule Weight (NNW)

Normalized nodule weight (NNW) was calculated by dividing the nodule dry weight (mg plant^{-1}) by the shoot dry weight (g plant^{-1}) (Olivera and others 2004).

Leghemoglobin Protein Pigment

The nodules were detached immediately after sampling and their leghemoglobin content was determined by the method of Hartree (1957), which is based upon the conversion of hematin to pyridine hemochromogen. A standard curve was prepared using graded concentrations of hemin.

Estimation of N_2 Fixation Efficiency

The acetylene-ethylene assay of nitrogenase activity was done using the method of Herdina and Silsbury (1990). The nitrogen-fixing complex (nitrogenase) of legumes is able to reduce C_2H_2 to CH_4 . The root system with intact nodules was incised from freshly separated plants and was incubated at room temperature in vials containing acetylene (C_2H_2) (10% v/v) in air and sealed with serum caps. The samples were flushed with acetylene gas by gently shaking the bottles and were incubated for 1 h. A 1-ml sample of gas from the incubation mixture was analyzed for ethylene in a Shimadzu GC-14B gas chromatograph equipped with a Porapak R column (Ligero and others 1986). Although the use of such a “closed” system for measuring acetylene reduction does create problems related to an acetylene-induced decline in nitrogenase activity (Minchin and others 1983), it is still useful for comparative purposes, especially when the assay time is short (Vessey 1994). From the standard values, the number of moles of ethylene produced in each case was calculated, the nodules were dried in an oven at 70°C for 24 h, and their dry weights were taken. The rate of enzyme activity was calculated as the number of moles of ethylene produced per mg dry weight of nodules per hour ($\text{nmol } C_2H_4 \text{ h}^{-1} \text{ mg nodule dry wt}^{-1}$).

Nitrogen Content

Nitrogen content was estimated by the colorimetric method of Lindner (1944). Fifty milligrams of dried and well-ground plant material (nodules) was taken in a 25-ml conical flask with 3 ml of digestion mixture. The flasks were heated gently over a heating plate until the solution became colorless. The digest was cooled and 0.5 ml of 30% H_2O_2 was added. The solutions were heated gently until they became clear and colorless. The solutions were cooled and diluted to 100 ml with distilled water. A 0.5-ml aliquot of the diluted digest was taken and to it 0.3 ml of 2.5 N NaOH was added to partially neutralize the excess acid. Another 0.1 ml of 10%

sodium silicate was added to the aliquot to avoid turbidity, and the final volume was attained by adding 5 ml of distilled water. After shaking thoroughly, five drops of Nessler's reagent were added. The mixture was allowed to stand for 30 min at room temperature. The optical density (OD) was recorded at 420 nm on a double-beam UV-190 spectrophotometer (Labnics Equipment) against a reagent blank. The standard curve was prepared using graded concentrations of $(NH_4)_2SO_4$.

Total Soluble Sugars (TSS)

Total soluble sugars (TSS) were determined following the method of Irigoyen and others (1992). A sample of 500 g of freshly harvested nodules was crushed in 5 ml of 95% (v/v) ethanol. The insoluble fraction of the extract was washed twice with 5 ml of 70% ethanol. All soluble fractions were centrifuged at $3,500\times g$ for 10 min. TSS were analyzed by reacting 0.1 ml of the alcoholic extract with 3 ml of freshly prepared anthrone (150 mg anthrone + 100 ml 72% [w/v] H_2SO_4) and placed in a boiling water bath for 10 min. After cooling, the absorbance at 625 nm was determined on a double-beam UV-190 spectrophotometer (Labnics Equipment).

Trehalose Content

Trehalose was separated and quantified by gas chromatography, according to Streeter and Strimbu (1998). Samples of nodules (200 mg) were ground in methanol (80% v/v) and incubated at 60°C for 10 min, followed by centrifugation at $13,000\times g$ for 10 min. The pellet was re-extracted three times more and then supernatants were collected and vacuum dried. Solids were dissolved in 125 μl of pure pyridine plus 125 μl of STOX reagent. This reagent contains hydroxylamine for conversion of anomeric forms to the oxime derivatives and also contains an internal standard, β -phenylglucose. The samples were derivatized by adding 200 μl of hexamethyldisilazane and 20 μl of trifluoroacetic acid 60 min before analysis. Trimethylsilyl (TXM)-oxime derivatives were separated on a packed column of 3% OV-17 on Chromosorb WHP using a Shimadzu GC-14B gas chromatograph.

Trehalose Metabolism

Extracts were prepared by homogenizing 0.2 g of nodules in a mortar with 33% (w/w) polyvinyl-polypyrrolidone and 2 ml of 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.3) containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM phenylmethanesulfonylfluoride (PMSF) for trehalase activity, and 2 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 2.5 mM

MgCl₂, 100 mM NaCl, and 10 mM β -mercaptoethanol for trehalose-6-phosphate synthetase (TPS) and trehalose-6-phosphate phosphatase (TPP) activities (Lopez and others 2006). The extracts were centrifuged at 30,000 $\times g$ for 20 min and the supernatant was used to determine enzyme activities. All operations were carried out at 4°C and enzyme activities were monitored between 2 and 4 h.

Trehalase Activity

Trehalase activity (EC 3.2.1.28) was determined calorimetrically according to Muller and others (1994) by measuring the glucose released. The reaction mixture contained 100 mM trehalose in 50 mM MES/KOH buffer (pH 6.3). After incubation at 37°C for 45 min, the reaction was stopped by heating at 100°C for 5 min. The glucose released was measured by the dinitrosulfosalicylic acid (DNSA) method (Miller 1959).

Trehalose-6-Phosphate Synthetase (TPS) Activity

The TPS assay (EC 2.4.1.15) was based on the method of Salminen and Streeter (1986) by measuring the release of UDP from UDP-glucose in the presence of glucose-6-phosphate. The reaction mixture (0.2 ml) contained 100 mM Tris-HCl buffer (pH 7.5), 8 mM UDP-glucose, 30 mM glucose-6P, 100 mM MgCl₂, 3 mM EDTA, and 25 mM KCl. The reaction was started by the addition of the nodule enzyme extract (0.04 ml). After 60 min at 30°C, reactions were stopped by heating at 100°C for 2 min. Samples were centrifuged at 2,000 $\times g$ for 10 min and the amount of UDP in the supernatant was measured in terms of oxidation of NADH in a linked assay with pyruvate kinase and lactic acid dehydrogenase. The assay mixture contained 50 mM Tris-HCl (pH 7.5), 5 mM phosphoenol pyruvate, 0.24 mM NADH, 10 mM MgCl₂, 3.5 U pyruvate kinase, and 5 U lactic acid dehydrogenase. The decrease in absorbance at 340 nm was measured continuously over a period of 20 min.

Trehalose-6-Phosphate Phosphatase (TPP) Activity

TPP activity (EC 3.1.3.12) was assayed by monitoring phosphate release from trehalose-6-phosphate (Padilla and others 2004). The reaction was carried out in a final volume of 0.25 ml containing 25 mM Tris-HCl (pH 7.0), 10 mM MgCl₂, and 1 μ M trehalose-6-phosphate. Samples were assayed for phosphate by the zinc acetate method (Bencini and others 1983). All enzyme activities were expressed per milligram of protein.

Statistical Analysis

Data presented are the mean values based on six biological repeats \pm standard error (SE) per treatment. All results

were subjected to analysis of variance (ANOVA) using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Duncan's multiple-range test was performed at $p < 0.05$ on each of the significant variables measured.

Results

Marked detrimental effects of increased levels of NaCl salinity on growth were observed in two genotypes of pigeonpea. Visible symptoms of salinity toxicity were clearly elicited in the form of stunted plant growth, leaf tip burn, necrosis of leaf margins, yellowing, wilting, and drying of the leaves leading to accelerated leaf senescence. Salinity (S), genotype (G), and arbuscular mycorrhizal (AM) inoculations, when considered individually, had significant effects on all the different parameters studied (Table 1). Analysis of factor interaction showed that $G \times S$ had significant effects on all the parameters except shoot-to-root ratio and normalized nodule weight. Interaction between G and AM was observed for plant biomass, AM colonization, mycorrhizal responsiveness, nodule number, nitrogen content, and trehalose metabolism. Interaction between S and AM was significant for most parameters, and interaction of all three factors with each other affected shoot dry weight, AM colonization, mycorrhizal responsiveness, nodule number, nitrogen content, and trehalose metabolism (Table 1).

Plant Biomass

Salinity stress had a detrimental effect on the growth of plants and significantly reduced plant dry matter (shoot and root) compared with controls; however, the extent of reduction was modulated according to the applied salt concentration and genotypes. Plant biomass production was significantly affected by genotype (G), salinity level (S), and arbuscular mycorrhizal (AM) inoculations as well as interactions among them. The significant $G \times S$ interaction for plant dry matter production confirmed genotypic differences in response to salinity (Table 1). The genotype ICP 13997 was much more sensitive to salt stress and accumulated less plant dry matter (shoot and root) compared to Sel 85N. Even the control plants of the two genotypes differed substantially in growth as ICP 13997 plants showed only about half the growth of Sel 85N (Table 2). There was a gradual decline in the dry weights of shoots (Table 2) under increasing salinity. The adverse effects of salinity on shoot dry matter were more apparent at the higher salinity level of 6 dS m⁻¹ compared to when the plants were subjected to the lower saline dosage of 4 dS m⁻¹. The root biomass and shoot-to-root ratio (SRR, Table 2) of both genotypes declined steadily as salinity

Table 1 Result of three-way ANOVA test for independent variables, including genotype (G), salinity treatment (S), and arbuscular mycorrhizal (AM) inoculations and interactions among them

G × S × AM	S × AM	G × AM	G × S	AM	S	G	Parameter
ns	ns	*	*	*	*	*	RDW
*	*	*	*	*	*	*	SDW
ns	ns	ns	ns	*	*	*	SRR
*	*	*	*	*	*	*	AM colonization
*	*	*	*	*	*	*	MR
*	*	*	*	*	*	*	NN
ns	*	ns	*	*	*	*	NDW
ns	ns	ns	ns	*	*	*	NNW
ns	*	ns	*	*	*	*	Lb content
ns	*	ns	*	*	*	*	ARA activity
ns	*	ns	*	*	*	*	N content
*	*	*	*	*	*	*	TSS
*	*	*	*	*	*	*	Trehalose content
*	*	*	*	*	*	*	Trehalase activity
*	*	*	*	*	*	*	TPS activity
*	*	*	*	*	*	*	TPP activity

ns no significant differences as determined using Duncan's multiple-range test

* Significant differences at 95%

Table 2 Effect of arbuscular mycorrhizal (AM) inoculations on root dry weight (RDW, g⁻¹ plant), shoot dry weight (SDW, g⁻¹ plant), shoot-to-root ratio (SRR), AM colonization (%), and mycorrhizal responsiveness (MR, %) in genotypes of pigeonpea under NaCl stress

Parameter	Control (C)	C + AM	4 dS m ⁻¹	4 dS m ⁻¹ +AM	6 dS m ⁻¹	6 dS m ⁻¹ +AM
Sel 85N						
RDW	0.865 ^b ± 0.122	1.084 ^a ± 0.135	0.795 ^b ± 0.142	0.937 ^b ± 0.132	0.758 ^b ± 0.115	0.845 ^b ± 0.126
SDW	4.775 ^c ± 0.114	5.211 ± 0.130	4.202 ^{cd} ± 0.117	5.265 ^b ± 0.120	3.317 ^{cd} ± 0.115	4.677 ^c ± 0.135
SRR	3.464 ^a ± 0.033	3.490 ^a ± 0.027	3.191 ^b ± 0.070	3.349 ^b ± 0.030	3.142 ^b ± 0.093	2.943 ^c ± 0.076
AM colonization	–	90.620 ^a ± 1.203	–	84.160 ^b ± 1.319	–	81.270 ^b ± 1.415
MR	–	11.610 ^c ± 1.283	–	24.110 ^b ± 0.943	–	35.500 ^a ± 1.192
ICP 13997						
RDW	0.309 ^b ± 0.112	0.424 ^a ± 0.113	0.232 ^b ± 0.083	0.274 ^b ± 0.144	0.196 ^b ± 0.084	0.229 ^b ± 0.024
SDW	2.447 ^b ± 0.133	3.917 ^a ± 0.186	1.205 ^{cd} ± 0.123	2.211 ^{bc} ± 0.138	0.920 ^d ± 0.114	1.827 ^{cd} ± 0.111
SRR	3.209 ^a ± 0.171	3.237 ^a ± 0.104	2.997 ^a ± 0.078	3.182 ^a ± 0.110	2.931 ^a ± 0.055	2.593 ^b ± 0.104
AM colonization	–	72.650 ^a ± 1.223	–	64.930 ^b ± 1.258	–	60.450 ^c ± 1.220
MR	–	57.510 ^c ± 1.224	–	72.920 ^b ± 1.118	–	84.220 ^a ± 1.039

Treatments are designed as uninoculated controls, saline stress (4 and 6 dS m⁻¹), arbuscular mycorrhiza (AM). Means followed by the same letter within a column do not differ ($p < 0.05$) using Duncan's multiple-range test

intensified, but roots seemed to withstand salt stress more than shoots. There was a significant influence of mycorrhizal inoculations on plant growth as AM fungi enabled the plants to grow better and accumulate higher biomass when compared with uninoculated plants. The maximum positive effects of AM symbiosis were observed under 4 dS m⁻¹, where total amelioration of negative effects of salt stress could be seen and shoot and root biomass was even

greater than untreated controls in Sel 85N. Positive effects mirrored by mycorrhizal responsiveness increased with increasing salt concentrations. The analysis of G × S × AM interactions confirmed genotypic differences in the influence of mycorrhization on shoot growth responses to salinity, whereas the positive effects of mycorrhization on root dry weight and SRR under salinity stress were similar in both genotypes (Table 1).

Arbuscular Mycorrhizal (AM) Colonization and Mycorrhizal Responsiveness (MR)

The percentage AM colonization and mycorrhizal responsiveness were significantly affected by G and S and by the interaction of both (Table 1). Irrespective of genotype and salinity levels, the greatest percentage of root colonization (Table 2) was observed under unstressed conditions which gradually decreased as salinity increased, indicating that salinity suppressed the growth of AM fungi to a certain extent. Significant differences in root colonization were observed between the two genotypes and AM colonization was much lower for ICP 13997 than for Sel 85N. The results of mycorrhizal responsiveness (MR, Table 2) calculation showed that ICP 13997 was much more dependent on AM colonization than Sel 85N and the dependency increased as salinity intensified from control to 4 dS m⁻¹ and reached a higher extent under the saline concentration of 6 dS m⁻¹.

Repercussions of Salinity on Symbiotic Effectiveness

Nodulation

Studies of symbiosis between root-nodule bacteria and pigeonpea plants showed that symbiotic efficiency was positively correlated with the number of nodules (Table 3) but not with their weight. Nodule number (NN) was significantly affected by G, S, and AM inoculations as well as interactions among them (Table 1). Similar to differences

reported in plant biomass, NN in control plants of ICP 13997 was only about one third that of Sel 85N (Table 3). Nodule dry weight (NDW) was significantly influenced by G × S and S × AM interactions (Table 1). Under salinization conditions, AM symbiosis in roots was associated with the formation of a significantly greater number of nodules in both genotypes. Sel 85N exhibited a higher increase in NN compared to ICP 13997. Although salt stress increased NN in both genotypes, it accelerated greening of nodules accompanied by reduced nodular growth, dry matter, and normalized nodule weights (NNW, Table 3), which was more apparent in ICP 13997 than in Sel 85N. The inhibitory effects of salinity on nodulation of pigeonpea plants were significantly reduced in the salt-tolerant Sel 85N compared to the salt-sensitive ICP 13997 by preinoculation with AM fungus *Glomus mosseae*. Accordingly, AM-inoculated plants had much higher NN, NDW, and NNW. The significant interactions of G, S, and AM on nodulation confirmed genotypic differences in the amelioration of salt-induced repercussions on nodulation by mycorrhizal colonization (Table 1).

Nitrogen-Fixation Efficiency

Similar to NDW, leghemoglobin (Lb) content and acetylene-reduction activity (ARA) was significantly influenced by G × S and S × AM interactions. Significant G × S interactions revealed that different responses of genotypes to salinity could be explained at least partly by differences in their inherent nitrogen-fixation efficiency under salinity

Table 3 Effect of arbuscular mycorrhizal (AM) inoculations on nodule number (NN), nodule dry weight (NDW, g⁻¹ plant), normalized nodule weight (NNW, mg nodule dry weight g⁻¹ shoot

dry weight), leghemoglobin content (Lb), nitrogen content (N), and total soluble sugars (TSS, mg g⁻¹ fresh wt) in genotypes of pigeonpea under NaCl stress

Parameter	Control (C)	C + AM	4 dSm ⁻¹	4 dSm ⁻¹ +AM	6 dSm ⁻¹	6 dSm ⁻¹ +AM
Sel 85N						
NN	62.27 ^c ± 0.719	83.04 ^d ± 1.270	91.12 ^c ± 1.332	115.08 ^a ± 1.298	94.12 ^c ± 1.145	99.68 ^b ± 1.105
NDW	0.210 ^{bc} ± 0.027	0.394 ^a ± 0.128	0.141 ^c ± 0.026	0.228 ^{bc} ± 0.061	0.110 ^c ± 0.017	0.168 ^c ± 0.036
NNW	0.099 ^{ab} ± 0.004	0.111 ^a ± 0.003	0.086 ^c ± 0.005	0.096 ^{ab} ± 0.003	0.085 ^c ± 0.002	0.091 ^{ab} ± 0.002
Lb content	123.750 ^b ± 8.759	162.940 ^a ± 10.848	87.280 ^{bcd} ± 1.609	133.490 ^{ab} ± 10.149	82.240 ^d ± 3.169	108.510 ^{bc} ± 9.878
N content	79.250 ^d ± 4.986	105.200 ^f ± 0.03	69.750 ^{bc} ± 0.05	95.520 ^e ± 0.09	62.350 ^b ± 0.07	89.200 ^e ± 0.247
TSS	6.330 ^f ± 0.234	10.210 ^e ± 0.419	11.470 ^d ± 0.488	12.890 ^c ± 0.611	13.740 ^b ± 0.603	15.630 ^a ± 0.719
ICP 13997						
NN	22.14 ^d ± 1.324	25.64 ^{bc} ± 1.311	26.51 ^{bc} ± 1.357	30.87 ^a ± 1.513	28.02 ^{ab} ± 1.298	29.20 ^a ± 1.048
NDW	0.067 ^b ± 0.007	0.097 ^a ± 0.010	0.036 ^c ± 0.004	0.071 ^b ± 0.008	0.030 ^c ± 0.009	0.047 ^{bc} ± 0.010
NNW	0.045 ^b ± 0.005	0.050 ^a ± 0.004	0.038 ^{bc} ± 0.004	0.041 ^{bc} ± 0.001	0.028 ^c ± 0.006	0.040 ^{bc} ± 0.004
Lb content	98.570 ^b ± 1.300	124.370 ^a ± 1.167	58.730 ^c ± 1.359	101.800 ^d ± 1.208	54.480 ^e ± 1.143	72.260 ^c ± 0.992
N content	70.050 ^f ± 0.754	83.050 ^g ± 0.633	48.850 ^c ± 0.778	58.250 ^e ± 0.693	38.550 ^b ± 0.962	51.250 ^d ± 0.623
TSS	5.100 ^f ± 0.231	8.770 ^e ± 0.383	9.090 ^d ± 0.398	11.320 ^c ± 0.583	12.240 ^b ± 0.557	14.840 ^a ± 0.672

Treatments are designed as uninoculated controls, saline stress (4 and 6 dS m⁻¹), arbuscular mycorrhiza (AM). Means followed by the same letter within a column do not differ ($p < 0.05$) using Duncan's multiple-range test

stress (Table 1). In our study, salinity affected the process of N_2 fixation more than nodulation. Specific sensitivity of symbiotic nitrogen fixation to salinity increased with an increment in salt concentration, and a much higher decline in Lb content (Table 3), ARA (Fig. 1), and nitrogen (N) content (Table 3) was seen at 6 dS m^{-1} than at 4 dS m^{-1} . Significant relationships between growth and nitrogen-fixation parameters were observed as the stress level increased in both genotypes. ARA showed a positive and significant correlation with both shoot dry weight ($r = 0.81^{**}$, Sel 85N; $r = 0.73^{**}$, ICP 13997) and nitrogen content ($r = 0.71^{**}$, Sel 85N; $r = 0.43^{**}$, ICP 13997). These correlation values support the close relationship between plant growth and symbiotic nitrogen fixation of the nodules. Inoculation with AM boosted nitrogen fixation under saline environments, and AM-inoculated stressed plants encountered a lower decline in Lb content, ARA, and N content than uninoculated plants. Statistical significance of $S \times AM$ interactions on nitrogen-fixation parameters confirmed the positive influence of mycorrhization as an important reason for better response of pigeonpea plants under salinity (Table 1).

Trehalose Metabolism and Total Soluble Sugars (TSS)

Trehalose metabolism and total soluble sugars (TSS) were significantly affected by G, S, and AM inoculations and interactions among them. The significant $G \times S$ interaction for trehalose metabolism again confirmed genotypic differences in response to salinity (Table 1). The osmotic challenge prompted by salinity stress promoted a significant rise in trehalose concentration (Fig. 2) and total

soluble sugars (Table 3) in a genotype- and concentration-dependent manner. The quantification of trehalose content revealed significantly higher levels of this disaccharide at 6 than at 4 dS m^{-1} in nodules. Trehalose accounted for nearly 3% of the total soluble sugars detected (Fig. 2 and Table 3). Salinity induced a remarkable reduction in trehalase activity (Fig. 3), suggesting that accumulation of trehalose was the result of concomitant inactivation of trehalase, the enzyme responsible for trehalose catabolism. The results indicated that trehalose content showed a negative and significant relationship with trehalase activity ($r = -0.71^{**}$, Sel 85N; $r = -0.63^{**}$, ICP 13997) and a positive relationship with total soluble sugars ($r = 0.76^{**}$, Sel 85N; $r = 0.71^{**}$, ICP 13997), but no relationship with growth and nitrogen-fixation efficiency was observed. Sel 85N accumulated large amounts of trehalose due to greater inhibition of trehalase activity when compared with ICP 13997. Association of pigeonpea roots with *G. mosseae* resulted in a conspicuous increase in total soluble sugars and the trehalose pool. In addition, salinity stress led to an increase in the activity of enzymes of the trehalose biosynthetic pathway, trehalose-6-phosphate synthase (TPS, Fig. 4) and trehalose-6-phosphate phosphatase (TPP, Fig. 5), which accounted for higher trehalose accumulation. The induction of TPS and TPP was correlated with the augmentation of the salt concentration of the irrigation solution in both Sel 85N and ICP 13997. AM colonization had a positive influence on the activity of TPS and TPP; thus, AM-inoculated plants showed higher trehalose synthesis at both salinity levels. Statistically significant $G \times S$, $G \times AM$, $S \times AM$, and $G \times S \times AM$ interactions for trehalose metabolism support a role for trehalose

Fig. 1 Effect of arbuscular mycorrhizal (AM) inoculations on acetylene reduction activity (ARA, $nmol\ C_2H_4\ h^{-1}\ mg^{-1}$ nodule dry wt) in genotypes of pigeonpea under NaCl stress. Treatments are designed as uninoculated controls, saline stress (4 and 6 dS m^{-1}), and arbuscular mycorrhiza (AM). Values are means based on six biological repeats \pm standard error (SE). Means followed by the same letter do not differ ($p < 0.05$) using Duncan's multiple-range test

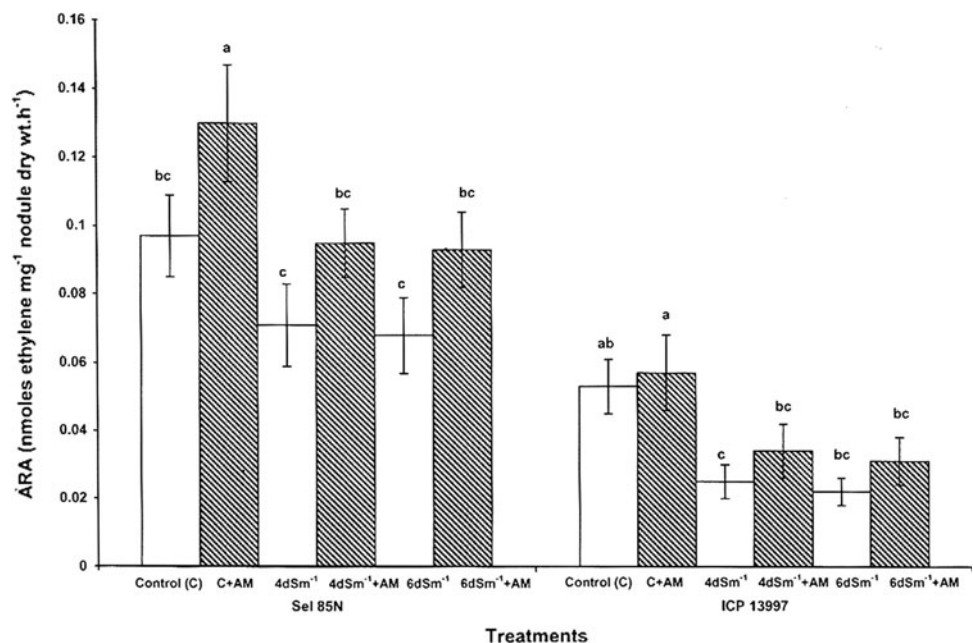


Fig. 2 Effect of arbuscular mycorrhizal (AM) inoculations on trehalose content ($\mu\text{g g}^{-1}$ fresh wt) in genotypes of pigeonpea under NaCl stress. Treatments are designed as uninoculated controls, saline stress (4 and 6 dS m^{-1}), and arbuscular mycorrhiza (AM). Values are means based on six biological repeats \pm standard error (SE). Means followed by the same letter do not differ ($p < 0.05$) using Duncan's multiple-range test

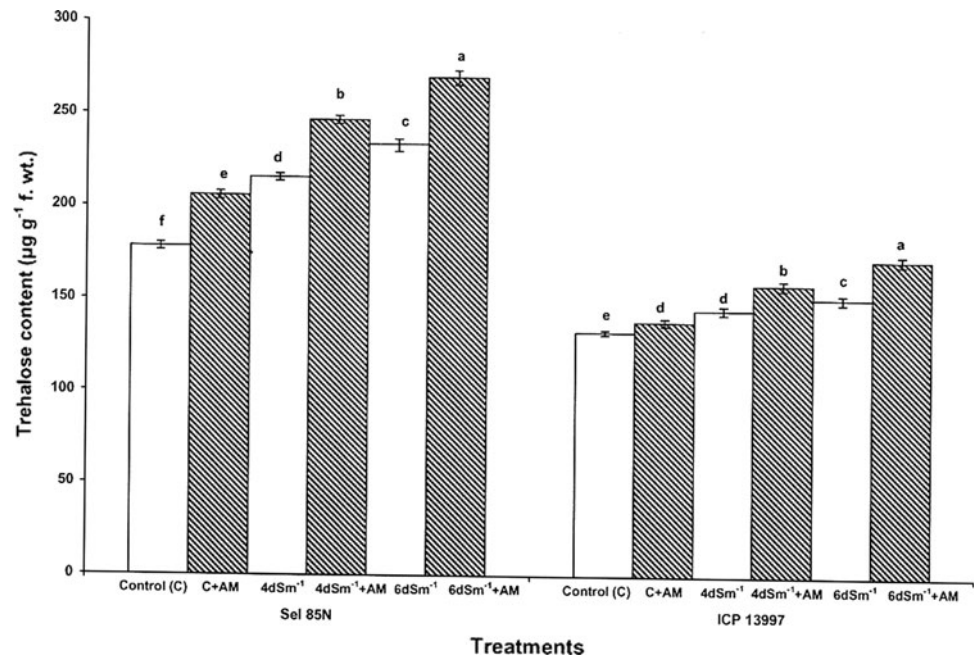
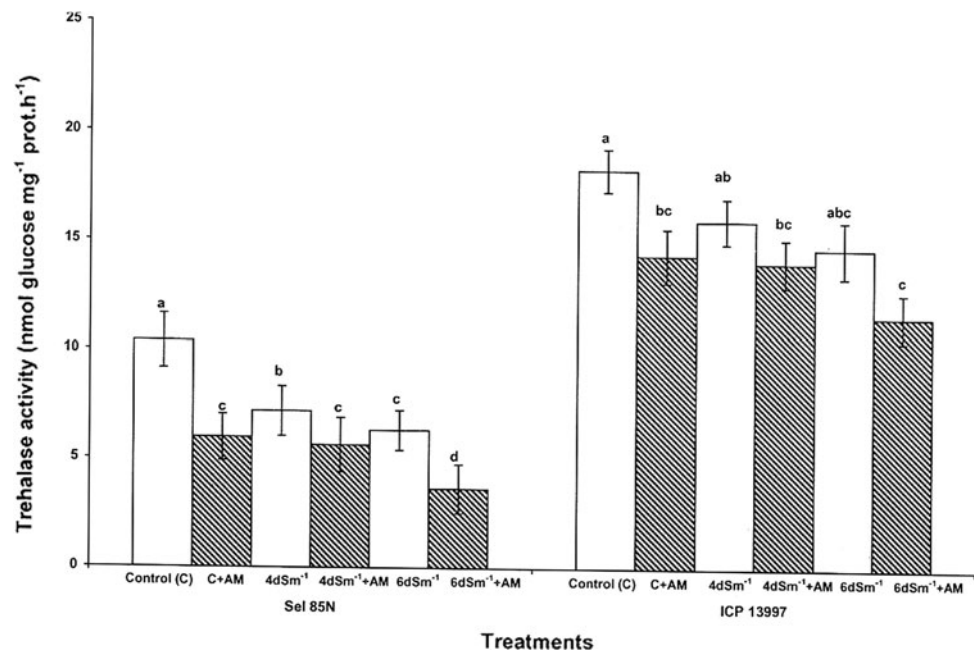


Fig. 3 Effect of arbuscular mycorrhizal (AM) inoculations on trehalase activity ($\text{nmol glucose mg}^{-1}$ protein h^{-1}) in genotypes of pigeonpea under NaCl stress. Treatments are designed as uninoculated controls, saline stress (4 and 6 dS m^{-1}), and arbuscular mycorrhiza (AM). Values are means based on six biological repeats \pm standard error (SE). Means followed by the same letter do not differ ($p < 0.05$) using Duncan's multiple-range test



in genotypic differences for mycorrhizal amelioration of salinity stress (Table 1).

Discussion

The capacity to adapt to salinity differed considerably in the two pigeonpea genotypes, and as a result they differentially responded in terms of tolerance to salt stress. This is substantiated by the higher plant biomass in the salt-tolerant genotype Sel 85N than in the salt-sensitive

genotype ICP 13997. In the present study, salinity stress diminished plant growth (shoot and root) in both genotypes; however, shoot growth was affected more extensively by salinity than root dry matter accumulation, which consequently led to a decline in the shoot-to-root ratio (SRR). This behavior is considered advantageous because it improves plant water status by increasing root requirements to explore a higher volume. Similar salt-induced growth inhibition has been observed in some important legume species (Khadri and others 2006; Lopez and Lluch 2008; Al-Sherif 2009; Dulormne and others 2010; Jebara

Fig. 4 Effect of arbuscular mycorrhizal (AM) inoculations on trehalose-6-P synthetase activity ($\text{nmol UDP glucose mg}^{-1} \text{ protein h}^{-1}$) in genotypes of pigeonpea under NaCl stress. Treatments are designed as uninoculated controls, saline stress (4 and 6 dS m^{-1}), and arbuscular mycorrhiza (AM). Values are means based on six biological repeats \pm standard error (SE). Means followed by the same letter do not differ ($p < 0.05$) using Duncan's multiple-range test

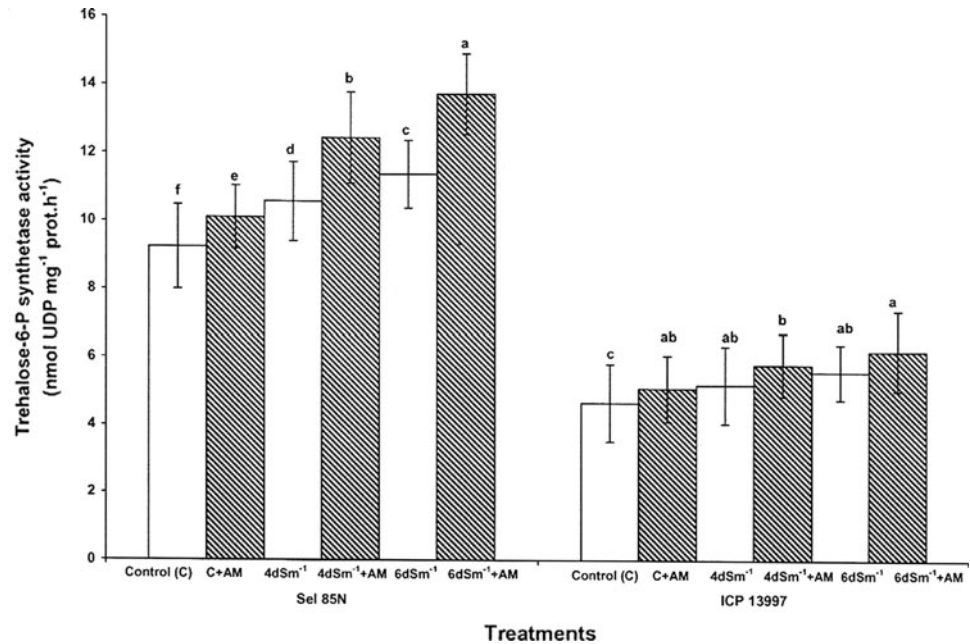
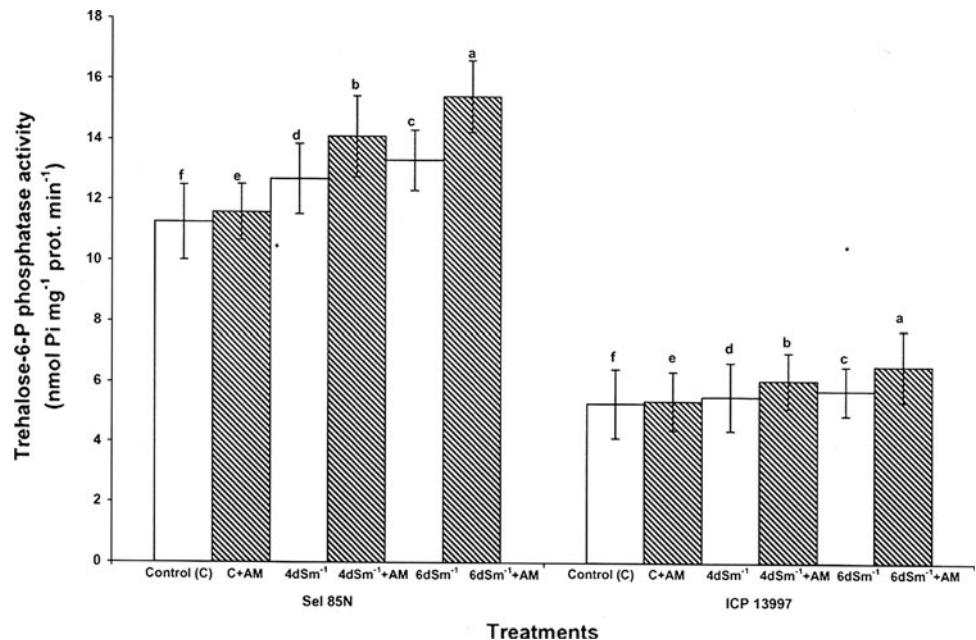


Fig. 5 Effect of arbuscular mycorrhizal (AM) inoculations on trehalose-6-P phosphatase activity ($\text{nmol Pi mg}^{-1} \text{ protein h}^{-1}$) in genotypes of pigeonpea under NaCl stress. Treatments are designed as uninoculated controls, saline stress (4 and 6 dS m^{-1}), and arbuscular mycorrhiza (AM). Values are means based on six biological repeats \pm standard error (SE). Means followed by the same letter do not differ ($p < 0.05$) using Duncan's multiple-range test



and others 2010). Our results coincide with those of Garg and Singla (2004) and of Tejera and others (2006) where the depressive effects of NaCl on plant growth and dry matter accumulation have been reported at the genotypic level in chickpea. The present study showed that mycorrhization with *Glomus mosseae* increased the fitness of host plants by enhancing shoot and root biomass and that AM-inoculated plants had higher plant biomass than did uninoculated plants at all salinity levels. The effect of AM colonization on growth improvement of salt-affected plants was highly significant, particularly in Sel 85N. Differential mycorrhizal responsiveness of genotypes could be the

result of plant- or fungus-related mechanisms. Enhancement of growth in mycorrhizal plants under saline conditions has been related partly to mycorrhizal-mediated enhancement of host plant P nutrition and other nutrients with low mobility, such as Fe, Cu, and Zn (Miransari and Smith 2008; Kaya and others 2009), and decreased uptake of Na (Al-Karaki 2006; Giri and others 2007; Garg and Manchanda 2008).

The results of the present study showed that salt stress reduced colonization of *G. mosseae*-inoculated pigeonpea plants, which was significantly higher in Sel 85N, and confirmed the previous results of Giri and others (2007),

Porras-Soriano and others (2009), Kumar and others (2010), and Wu and others (2010). Although high soil ECE in the rooting medium decreased AM colonization, mycorrhizal responsiveness (MR) increased in soil having 4 and 6 dS m⁻¹ ECE. These results show the high mycorrhizal dependence of ICP 13997 to reach optimum development under stress conditions as compared to Sel 85N. Symbiotic development between mycorrhizal fungi and pigeonpea plants seemed to be strengthened in the saline environment after the establishment of the association indicating the ecological importance of AM for plant survival and growth of plants under salinity stress.

The present study showed an increase in nodule number (NN) with increasing concentrations of salinity, with a greater increase at 6 dS m⁻¹ in Sel 85N. However, the nodules formed under salinity showed reduced growth, biomass accumulation, low nitrogen-fixation efficiency and nitrogen content than control plants. Although reports on genotypic responses are lacking, reduced nodule functioning has been reported by Anthraper and Dubios (2003) and Garg and Manchanda (2008) in some legume species. The negative effect of salt stress on plant nitrogen-fixation capacity was clearly observed in ICP 13997, although considerably higher values of these parameters were obtained in Sel 85N, even with the highest salt dosage. Shoot dry matter and acetylene-reduction activity (ARA) showed a similar inhibition by salt. This corroborates the suggestion that the negative effect of salinity on plant growth was a consequence of decline in the nitrogen-fixation process, as has been widely reported in legumes (Tejera and others 2006; Khadri and others 2007; Lopez and Lluch 2008; Chalk and others 2010). Our results showed that the application of AM fungi counteracted the harmful effects of salinity on nodulation and nitrogen fixation and thereby alleviated the salinity-induced premature nodule senescence in Sel 85N. Colonization of pigeonpea plants increased the number of nodules, indicating a positive influence of AM fungi on legume-nitrogen-fixing bacteria symbiosis under salinity stress. A lesser decline in nodule dry matter and a much higher Lb content and ARA were observed in Sel 85N inoculated with *G. mosseae* when compared to uninoculated plants under saline conditions. Evidence from previous studies indicates that the presence of AM fungi enhances nodulation and nitrogen fixation by legumes (Goss and de Varennes 2002; Patreze and Cordeiro 2004; Garg and Manchanda 2008, 2009), although reports on genotypic responses are lacking.

Our results revealed that salinity triggered an inhibition of trehalase activity and an increase of trehalose content, indicating a role for this carbohydrate in osmoprotection and osmotic adjustment against salinity stress. Trehalose content and total soluble sugars increased in stressed plants of both genotypes under increasing concentrations of NaCl, with

considerably greater sugar content in Sel 85N than in ICP 13997. The accumulation of soluble carbohydrates in plants as a response to drought and salinity stresses has been reported (Jimenez-Zacarias and others 2004; Lopez and Lluch 2008; Lopez and others 2009; Garg and Manchanda 2009). Trehalose activity is induced in root nodules of legumes by the microsymbionts (*Rhizobium* and AM fungi), and under salt stress conditions trehalose accumulates in nodules of *Medicago truncatula* when the trehalase gene (*MtTRE1*) is repressed, suggesting that the increase in the trehalose level results, at least in part, from *MtTRE1* transcriptional repression (Lopez and others 2008). Furthermore, a microarray analysis has revealed that most of the genes involved in trehalose metabolism in *A. thaliana* respond to a wide range of abiotic stresses such as cold, salt, and UV (Iordachescu and Imai 2008). Trehalose expression is a two-step pathway involving two enzymes: trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). The results from the present study showed that the activities of TPS and TPP increased under salinity, which accounted for increased trehalose accumulation depending upon the genotypes under study. Suarez and others (2008) reported that *Phaseolus vulgaris* (common bean plants) inoculated with *Rhizobium etli* overexpressing the trehalose-6-phosphate gene had more nodules with increased nitrogenase activity and higher biomass under drought stress. The increase in nodule number and nitrogenase activity in *P. vulgaris* plants overexpressing the TPS gene suggested that bacterial trehalose triggered a morphological and biochemical response leading to higher nitrogen fixation. In the present study, colonization of roots by AM fungi further stimulated the salinity-induced accumulation of trehalose by inhibiting trehalase activity in mycorrhizal plants, along with an increase in total soluble sugars. This stimulation was more significant in Sel 85N than in ICP 13997. Additionally, the trehalose biosynthetic enzymes TPS and TPP also showed more activity in stressed and unstressed mycorrhizal plants. Results showed a smaller inhibition of plant biomass and nitrogen fixation in salinized plants inoculated with *G. mosseae* compared with uninoculated plants, suggesting that an increase in trehalose content of nodules might have improved relative tolerance of Sel 85N in such conditions. The significant differences in the accumulation of trehalose upon AM colonization under NaCl stress in the two genotypes shown in this study support its role as a stress protectant.

Conclusions

In conclusion, the pigeonpea genotypes studied here displayed remarkable variability in their symbiotic performance (nitrogen fixation and AM colonization) under

stressed and nonstressed conditions. The higher salt tolerance of the Sel 85N genotype upon AM colonization, supported by higher biomass accumulation, less nitrogen-fixation inhibition, and improved trehalose synthesis, confirmed genotypic differences in amelioration of salt-induced repercussions by AM fungi. The induction of trehalose metabolism in root nodules under salt stress and its manipulation through AM symbiosis suggest its role in imparting salt tolerance in pigeonpea plants.

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