

Role of Arbuscular Mycorrhizal Symbiosis in Proline Biosynthesis and Metabolism of *Cicer arietinum* L. (Chickpea) Genotypes Under Salt Stress

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Abstract Salinity causes osmotic stress and negatively impacts plant growth and productivity. Proline is one of the most important osmoprotectants synthesized under stressed conditions. Accumulation of free proline occurs due to enhanced biosynthesis and repressed degradation, and both processes are controlled by feedback regulatory mechanisms. Arbuscular mycorrhizal (AM) fungi are considered to be bioameliorators of salinity stress due to their wide-ranging presence in contaminated soils and their role in modulation of biochemical processes. Chickpea is considered sensitive to salinity. However, reports on AM-induced osmoprotection through regulation of proline biosynthesis in chickpea genotypes are scant. The present study investigated the influence of AM symbiosis on proline metabolism in two chickpea (*Cicer arietinum* L.) genotypes (PBG-5 and CSG-9505) under salt stress and correlated the same with sodium (Na^+) ion uptake. Salinity reduced plant biomass (roots and shoots), with roots being more negatively affected than shoots. Mycorrhizal colonization with *Glomus mosseae* was much stronger in PBG-5 and was correlated with reduced Na^+ ion uptake and higher growth when compared with CSG-9505 under stressed and unstressed conditions. Mycorrhizal symbiosis with chickpea roots boosted proline biosynthesis by significantly increasing pyrroline-5-carboxylate synthetase (P-5-CS) and glutamate dehydrogenase (GDH) activities with a concomitant decline in proline dehydrogenase (ProDH) activity under salt stress. The enhancement of the activity of these enzymes was higher in PBG-5 than in CSG-9505 and could be directly correlated with the percent mycorrhizal

colonization and Na^+ uptake. The study indicated a strong role of AM symbiosis in enhancing stress tolerance in chickpea by significantly modulating proline metabolism and Na^+ uptake.

Keywords *Cicer arietinum* · Genotypes · *Glomus mosseae* · Proline · Salinity

Introduction

High salinity, most commonly mediated by sodium chloride (NaCl), is one of the major abiotic stresses globally. Increased salinization of arable land is expected to have devastating effects, resulting in 30 % land loss within the next 25 years and up to 50 % by the middle of the 21st century (Wang and others 2003). In Asia alone, 21.5 million ha of land is affected by salt, with India having 8.6 million ha of such area, which constitutes a major part of problematic soils in India (Kumar and others 2008). Salt stress affects almost all aspects of plant development, including germination, vegetative growth, and reproductive development (Chinnusamy and others 2006). Salt effects are the combined result of the complex interactions among different morphological, physiological, and biochemical processes. Morphological symptoms are indications of the injurious effects of salt stress. Salinity may directly or indirectly inhibit cell division and enlargement in the plant, reduce dry matter content (Manchanda and Garg 2008), disturb water relations (Sabra and others 2012), affect photosynthetic electron transport and inhibit PSII activity (Mateo and others 2004; López-Climent and others 2008; Noreen and Ashraf 2009), and impose ionic cytotoxicity (Tejera and others 2006; Munns and others 2006; Lee and

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others 2009; Pagariya and others 2011; Leyva and others 2011).

Biochemical studies have shown that plants under salt stress accumulate a number of metabolites, which are termed compatible solutes because they do not interfere with plant metabolism and accumulation of these solutes contributes to turgor maintenance in plants (Wani and others 2012). Proline accumulation is one of the most frequently reported modifications induced by salt stress in plants and is often considered to be involved in stress resistance mechanisms (Köşkeröglü and Levent 2010; Alamgir and others 2011). Proline is considered the only osmolyte shown to scavenge singlet oxygen and free radicals, including hydroxyl ions, and hence stabilize proteins, DNA, and membranes (Kumar and others 2010b; Wani and others 2012). The level of proline accumulation in plants under stress varies from species to species and can be 100 times greater than in control situations (Nathalie and Christian 2008). Proline is reported to reduce the enzyme denaturation caused by NaCl and act as a source of carbon, nitrogen, and energy during, and recovery from, stresses (Chen and others 2011). In plants, proline biosynthesis occurs via two pathways: from either glutamate or ornithine. Proline biosynthesis from glutamate appears to be the predominant pathway, especially under osmotic stress conditions (Hu and others 1992; Silva-Ortega and others 2008; Zhuang and others 2011). There are four steps in the process of proline metabolism catalyzed by two enzymes, P-5-CS (Δ^1 -pyrroline-5-carboxylate synthetase) and P-5-CR (Δ^1 -pyrroline-5-carboxylate reductase) (Maurizio and others 2008; Verslues and Sharma 2010; Wang and others 2011; Wani and others 2012). P-5-CS is a bifunctional enzyme (EC 2.7.2.11/1.2.41) that catalyzes the first two steps of the glutamate pathway in proline biosynthesis in plants. It is a rate-limiting enzyme in the glutamate pathway and exhibits both γ -glutamyl kinase and glutamic- γ -semialdehyde dehydrogenase activities (Silva-Ortega and others 2008; Somboonwatthanaku and others 2010; Stein and others 2011). Another possible route for generating glutamate in the mitochondria is glutamate dehydrogenase (GDH; EC 1.4.1.3), which can either directly produce glutamate from ammonium and α -ketoglutarate or deaminate glutamate to produce α -ketoglutarate (Forde and Lea 2007). Although the role of GDH is not clear, there is evidence that it can be important in supplying glutamate for proline synthesis (Skopelitis and others 2006; Verslues and Sharma 2010). Oxidative degradation of proline to glutamate is carried out in the mitochondria by sequential actions of proline dehydrogenase (ProDH) and P5C-dehydrogenase (P5C-DH) (Stein and others 2011).

Arbuscular mycorrhizal (AM) fungi exist naturally in saline environments and improve growth and vigor of plants under a variety of salt-stress conditions (Giri and

others 2007; Kumar and others 2010a). The AM fungi most commonly observed in saline soils are *Glomus* sp., and *Glomus mosseae* has been found to be the most efficient fungus in terms of plant performance and protection against the detrimental effects of salinity (Porrás-Soriano and others 2009). Proline accumulation has been found to increase when the plant is colonized by AM fungi (Sharifi and others 2007; Evelin and others 2009).

Chickpea (*Cicer arietinum* L.) was one of the earliest grain crops cultivated by man. It is the largest produced food legume in South Asia and the third largest produced food legume globally, after common bean (*Phaseolus vulgaris* L.) and field pea (*Pisum sativum* L.). Chickpea is grown mainly as a cool-season crop under both rain-fed (>90 %) and irrigated conditions. It leaves substantial amounts of residual nitrogen for subsequent crops and adds plenty of organic matter to the soil to maintain and improve soil health and fertility. Because of its deep tap root system, chickpea can withstand drought conditions by extracting water from deeper layers in the soil profile (Gaur and others 2010). To improve the adaptation of chickpeas to saline soils, it is critical to identify tolerant sources and understand the mechanisms of salinity tolerance. Although recently proline has been identified as an important osmolyte that imparts protection to plants under salinity stress, reports on the role of AM symbioses in modulating proline biosynthetic enzymes are contradictory (Porcel and others 2004). The objective of this study was to evaluate the role of *G. mosseae* in NaCl-induced changes associated with proline metabolism, Na⁺ ion uptake, and mycorrhizal dependency in *Cicer arietinum* L. (chickpea) genotypes.

Materials and Methods

Biological Material and Plant Growth Conditions

Experimental material consisted of two genotypes of chickpea, salt-tolerant (PBG-5) and salt-sensitive (CSG-9505), procured from the Department of Vegetable Crops, College of Agriculture, Punjab Agricultural University (PAU), Ludhiana, India, and from the Central Soil Salinity Research Institute (CSSRI), Karnal, India, respectively. Mycorrhizal inoculum of *G. mosseae* (UTMU 128 WM1/1) was obtained from The Energy and Resource Institute (TERI), New Delhi, India. The inoculum was bulked in an open-pot soil culture using *Zea mays* L., *Cajanus cajan* (L.) Millsp., *Sorghum bicolor* L., and *Coriandrum sativum* L. Experiments were conducted in the Department of Botany, Panjab University, Chandigarh (30.5°N, 76.5°E; elevation = 305–366 m), from November 2011 to March 2012 (minimum temperature, 11–14 °C; maximum temperature,

28–32 °C; morning relative humidity, 28 %; and afternoon relative humidity, 86 %).

Experimental Design

The soil (a mixture of sand and loam in ratio of 1:1 by volume) was obtained from the nearby agricultural fields [pH 7.2, organic carbon 0.68 % (Walkley 1947), total nitrogen content of 42 % (Nelson and Sommers 1973), and phosphorus 20 mg kg⁻¹ (Chapman and Pratt 1961)]. It was autoclaved (121 °C, 1 h twice at 48-h interval) to eliminate existing AM fungi propagules. Seeds were inoculated with fungal inocula of *G. mosseae*, placed in a pot at 1.5 cm depth prior to sowing, to facilitate fungal colonization of plant roots. Fifty grams of soil-based inoculum (containing 60–70 AM propagules per 10 g soil) along with chopped AM-colonized roots of trap plants were added to each pot. Non-AM treatments received the same weight of autoclaved inoculum (to obtain the same soil texture) together with a 10-mL aliquot of an inoculum filtrate. Seeds were surface-sterilized with 10 % hydrogen peroxide (v/v) solution for a few minutes and then rinsed by soaking in sterile distilled water. After 2 weeks of seedling establishment, NaCl was applied as a water solution of NaCl (0, 4, 6, and 8 dS m⁻¹ ECE corresponding to 0, 40, 60, and 80 mM NaCl, respectively) (Richards 1954), with and without AM inoculations. Pots were arranged in a completely randomized block design with a factorial combination of 4 × 2 with and without AM inoculations. Deionized water was used to prepare all solutions. Plants were harvested for physiological and biochemical analyses at 80 days after sowing (DAS). Samples (roots and leaves) were oven-dried at 70 °C for 72 h until they reached constant weight. Six plants per treatment were analyzed and data were calculated on per plant basis by taking the means.

Measurements and Analysis

Mycorrhizal Colonization (MC)

Mycorrhizal colonization was estimated (McGonigle and others 1990) after staining the fungal structures with Cotton Blue (Phillips and Hayman 1970). The samples were kept in staining solution for 24–36 h. The roots were cut into 50 small pieces of approximately 1 cm and observed under a compound light microscope. Root pieces that contained even a single vesicle or arbuscules along with hyphae were considered infected.

Percent root colonization

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

An index of mycorrhizal dependency (MD) was determined by expressing the dry weights of the plants concerned as a percentage of the dry weight of the control plants (Esaun and others 1987).

Sodium Content

Sodium (Na⁺) content was estimated using flame photometer according to method of Chapman and Pratt (1961). First, 10 mL of acid mixture consisting of nitric acid, sulfuric acid, and perchloric acid at a ratio of 9:4:1 was added to 2–5 g of ground samples and kept at 120 °C overnight. The samples were then maintained at 70 °C on a hot plate for 30 min; the temperature was increased to 120 °C for 30 min and then to 250 °C until only 3–4 mL of the sample was left. A final volume of 50 mL was maintained using distilled water and left overnight. The next day it was filtered using Whatman No. 1 filter paper. A blank was run without plant samples.

Proline Content

Free proline content was determined following the method of Bates and others (1973). Proline was estimated based on the formation of brick red-colored proline ninhydrin complex in acidic medium. The plant sample (0.5 g) was homogenized in 5 mL of sulfosalicylic acid (3 %) using mortar and pestle, the homogenate was filtered, and filtrate was used for the estimation of proline content. Two milliliters of extract was placed in a test tube to which 2 mL of glacial acetic acid and 2 mL of ninhydrin reagent were added and the tube was heated at 100 °C for 30 min. Six milliliters of toluene was added and then transferred to a separating funnel. The chromophore containing toluene was separated and its absorbance was read at 520 nm in the spectrophotometer against a toluene blank. The concentration of proline was estimated by referring to a standard curve made from known concentrations of proline.

Pyrroline-5-carboxylate Synthetase (P-5-CS)

Pyrroline-5-carboxylate synthetase activity was assayed as described by Garcia-Rios and others (1997). The reaction mixture (3 mL) contained 100 mM Tris-HCl buffer (pH 7.2), 25 mM MgCl₂, 75 mM sodium glutamate, 5 mM ATP, and 0.2 mL of enzyme extract. The reaction was initiated by the addition of 0.4 mM NADPH. The activity was measured as the rate of consumption of NADPH monitored by the decrease in absorbance at 340 nm.

Glutamate Dehydrogenase (GDH)

Glutamate dehydrogenase activity was assayed using the method of Kanamori and others (1972). The reaction

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

| S × AM × G | AM × G | S × G | S × AM | G | AM | S | Parameter |
|------------|--------|-------|--------|---|----|---|--------------------------|
| ns | ns | * | * | * | * | * | RDW |
| ns | ns | * | * | * | * | * | SDW |
| ns | ns | * | ns | * | * | * | RSR |
| * | * | * | * | * | * | * | AM colonization |
| * | * | * | * | * | * | * | MD |
| * | * | * | * | * | * | * | Sodium content (roots) |
| * | * | * | * | * | * | * | Sodium content (leaves) |
| * | * | * | * | * | * | * | Proline content (roots) |
| * | * | * | * | * | * | * | Proline content (leaves) |
| * | * | * | * | * | * | * | P-5-CS activity (roots) |
| * | * | * | * | * | * | * | P-5-CS activity (leaves) |
| * | * | * | * | * | * | * | GDH activity (roots) |
| * | * | * | * | * | * | * | GDH activity (leaves) |
| * | * | * | * | * | * | * | ProDH activity (roots) |
| * | * | * | * | * | * | * | ProDH activity (leaves) |

ns no significant differences

* Significant differences at 95 %

mixture contained 200 mM Tris–HCl buffer (pH 8.0), 150 mM NH₄Cl, 150 mM α-ketoglutaric acid, and 0.2 mL enzyme extract in a final volume of 3 mL. The reaction was initiated by the addition of 3 mM NADH. The GDH activity was measured by following the oxidation of NADH at 340 nm.

Proline Dehydrogenase (ProDH)

Proline dehydrogenase activity was assayed according to the method of Reno and Splittstoesser (1975). The reaction mixture contained 100 mM Na₂CO₃–NaHCO₃ (pH 10.3), 20 mM L-proline, and 0.5 mL enzyme extract in a final volume of 3 mL. The reaction was initiated by the addition of 10 mM NAD. The increase in absorbance at 340 nm was measured at 32 °C.

Statistical Analysis

Data presented are the mean values based on six biological repeats ± standard error (SE) per treatment. All results were statistically analyzed by analysis of variance (ANOVA) using SPSS 18.0 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. Pearson's correlation (r_p) was used to determine the relationship between two dependent variables for different parameters.

Results

Salinity reduced dry matter production of chickpea plants and marked detrimental effects of increased levels of NaCl salinity on growth were observed in PBG-5 and CSG-9505. However, the negative impact of salt stress was more pronounced in the latter than in the former genotype. There was severe yellowing of leaves and necrosis of leaf margins, browning of roots, and death of growing tips at the highest saline concentration in CSG-9505. 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 combined effects showed that although S × G had significant effects on all the parameters, S × AM was mainly significant with the exception of root-to-shoot ratio. The interaction between AM and G and the interaction of all three factors (S × AM × G) with each other were significant, except for plant biomass (Table 1).

Plant Dry Mass

NaCl exposure of *Cicer arietinum* L. induced a significant decline in root and shoot dry weights (Table 2) compared with controls; however, the extent of reduction was modulated according to the concentration of salt applied and genotype. The significant S × G interaction for plant dry matter production confirmed genotypic differences in response to salinity (Table 1). The salt toxicity symptoms

were more severe in CSG-9505, which accumulated less plant dry matter (roots and shoots) compared to PBG-5. Shoot biomass was depressed significantly ($p < 0.05$) by increasing the salinity level in chickpea genotypes (Table 2). The adverse effects of salinity were more apparent at the higher salinity level of 8 dS m^{-1} than when the plants were subjected to the lower saline dosage of 4 dS m^{-1} . Browning of roots was observed with increasing salinity and was accompanied by severe reductions ($p < 0.05$) in root dry weight and the root-to-shoot ratio (Table 2). In the sensitive genotype, significant reductions in the dry weight of roots were observed under all saline concentrations. A decline of 62.36 % under the salt dosage of 6 dS m^{-1} and 82.63 % under higher salt dosage (8 dS m^{-1}) was observed in CSG-9505 relative to the unstressed control at 80 DAS. The dry weights of roots declined by 36.52 % under 6 dS m^{-1} and 55.16 % under 8 dS m^{-1} in PBG-5 over controls at 80 DAS. Inoculations with *G. mosseae* improved root and shoot dry weights under saline conditions (Table 2). The growth response to AM fungal colonization was more effective in improving root growth than shoot growth, and particularly under salt stress (Table 2). Total amelioration of the negative effects of salt stress was absorbed at 4 dS m^{-1} , and root and shoot biomass was even greater than untreated controls in PBG-5.

Arbuscular Mycorrhizal Colonization (MC) and Mycorrhizal Dependency (MD)

The results of our study showed that different levels of salt stress had inhibitory effects on mycorrhizal colonization, although significant mycorrhizal colonization was observed at different saline concentrations. Irrespective of genotype and salinity level, the greatest percentage of root colonization (Table 2) was observed under unstressed conditions and it 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 as AM colonization was much lower for CSG-9505 than for PBG-5. Mycorrhizal dependency (MD) is the index that indicates the potential for AM fungi to affect the growth of the host plant. A decrease in the percent of root colonization was observed with increasing stresses, whereas MD of both genotypes increased, thereby indicating a negative correlation between the two (r_p : PBG-5 = 0.784, CSG-9505 = 0.869). The results of the MD calculation showed that CSG-9505 was more dependent on AM colonization than PBG-5, and the dependency increased as salinity intensified from control to 4 dS m^{-1} and reached a higher extent under the saline concentration of 8 dS m^{-1} . PBG-5 had a 31.46 % dependence under 4 dS m^{-1} and 33.4 %

under 6 dS m^{-1} , which further increased to 37.73 % under 8 dS m^{-1} at 80 DAS. On the other hand, MD of 34.16 % under 4 dS m^{-1} , 37.90 % under 6 dS m^{-1} , and 40.92 % under 8 dS m^{-1} were recorded in CSG-9505 at 80 DAS, indicating differential mycorrhizal responsiveness of the two genotypes.

Sodium (Na^+) Content

The concentration of Na^+ significantly increased in the roots and leaves of chickpea plants in the presence of NaCl stress (Table 2). CSG-9505 had a higher accumulation of Na^+ than PBG-5. It was seen that most of the Na^+ was held up in the roots and much less reached the leaves in both genotypes. Mycorrhization with *G. mosseae* counteracted such harmful accumulation of Na^+ in both genotypes. A gradient accretion was observed in Na^+ content when salinity was raised from 4 to 6 and 8 dS m^{-1} , with a much higher increase at 8 dS m^{-1} . At the high salinity level (8 dS m^{-1}), tissues showed the inability to control Na^+ uptake and transport where the ionic stress dominated. There was a positive correlation (r_p : PBG-5 = 0.956, CSG-9505 = 0.989) between sodium content and mycorrhizal dependency. Thus, mycorrhizal plants had significantly lower Na^+ content than nonmycorrhizal plants under stressed conditions. The mycorrhizal inoculations were effective in alleviating salt stress by preventing Na^+ absorption by roots and translocation to the shoot tissues. PBG-5 was able to recover from ionic stress and had less Na^+ content upon AM colonization than did CSG-9505. The extent of Na^+ uptake was inversely proportional to the differential percent mycorrhizal colonization in the two genotypes, which was depicted in the form of a negative correlation of sodium content with mycorrhizal colonization (r_p : PBG-5 = 0.779, CSG-9505 = 0.839).

Proline Metabolism

All parameters related to proline content as well as the enzymes involved in the proline biosynthetic pathway increased with increasing salinity. The osmotic challenge prompted by salinity stress promoted a significant rise in proline concentration. The increase in proline content had a direct correlation with increased Na^+ content in leaves of both genotypes (r_p : PBG-5 = 0.795, CSG-9505 = 0.752) and in roots (r_p : PBG-5 = 0.747, CSG-9505 = 0.729) (Fig. 1). The quantitative analysis of proline revealed significantly higher levels of this amino acid at 8 dS m^{-1} than at 6 and 4 dS m^{-1} in leaves when compared with roots. In addition, salinity stress led to a higher increase in the activity of enzymes of the proline biosynthetic pathway in leaves when compared with roots, with P-5-CS (Fig. 2) activity

Table 2 Effect of arbuscular mycorrhizal (AM) inoculations on root dry weight (RDW, g^{-1} plant), shoot dry weight (SDW, g^{-1} plant), root-to-shoot ratio (RSR), AM colonization (MC, %), mycorrhizal dependency (MD, %), and sodium content ($mg\ g^{-1}$ DW) in roots and leaves of chickpea genotypes 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 | 8 dS m^{-1} | 8 dS m^{-1} + AM |
|-----------------|----------------|-----------------|-----------------|--------------------|----------------|--------------------|----------------|--------------------|
| PBG-5 | | | | | | | | |
| RDW | 3.97c ± 0.080 | 5.63a ± 0.124 | 3.20de ± 0.230 | 4.76b ± 0.317 | 2.52e ± 0.173 | 3.79 cd ± 0.300 | 1.78f ± 0.265 | 2.87e ± 0.242 |
| SDW | 1.79bc ± 0.057 | 2.38a ± 0.051 | 1.46 cd ± 0.103 | 2.03b ± 0.188 | 1.20de ± .075 | 1.78bc ± 0.144 | 0.89e ± 0.173 | 1.41d ± 0.103 |
| RSR | 2.21b ± 0.034 | 2.35a ± 0.038 | 2.19bc ± 0.046 | 2.33a ± 0.042 | 2.10cd ± 0.034 | 2.12bcd ± 0.049 | 2.00e ± .046 | 2.04de ± 0.059 |
| MC | – | 87.33a ± 0.623 | – | 80.58ab ± 4.087 | – | 75.20bc ± 2.889 | – | 69.26c ± 2.344 |
| MD | – | 28.26c ± 0.473 | – | 31.46b ± 0.519 | – | 33.40a ± 0.539 | – | 37.73a ± 0.366 |
| Na | 3.12ef ± 0.334 | 2.48f ± 0.311 | 4.40d ± 0.161 | 3.38e ± 0.127 | 6.01b ± 0.317 | 4.38d ± 0.121 | 7.39a ± 0.311 | 5.19c ± 0.196 |
| Na | 2.14de ± 0.056 | 1.82e ± 0.046 | 2.74c ± 0.242 | 2.31d ± 0.080 | 3.51b ± 0.034 | 2.75c ± 0.051 | 4.39a ± 0.213 | 3.30b ± 0.010 |
| CSG-9505 | | | | | | | | |
| RDW | 3.800b ± 0.161 | 5.567a ± 0.277 | 2.410c ± 0.132 | 3.799b ± 0.207 | 1.43d ± 0.190 | 2.343c ± 0.242 | 0.660e ± 0.271 | 1.139de ± 0.089 |
| SDW | 1.762b ± 0.080 | 2.364a ± 0.045 | 1.234c ± 0.056 | 1.734b ± 0.152 | 0.836d ± 0.110 | 1.305c ± 0.079 | 0.469e ± 0.109 | 0.771de ± 0.207 |
| RSR | 2.156b ± 0.034 | 2.354a ± 0.061 | 1.952c ± 0.024 | 2.190b ± 0.051 | 1.710e ± 0.055 | 1.795d ± 0.050 | 1.407f ± 0.046 | 1.477f ± 0.042 |
| MC | – | 86.820a ± 1.189 | – | 77.880ab ± 5.813 | – | 70.002b ± 2.310 | – | 49.209c ± 4.046 |
| MD | – | 29.885d ± 0.377 | – | 34.161c ± 0.468 | – | 37.902b ± 0.321 | – | 40.928a ± 0.571 |
| Na | 3.210e ± 0.259 | 2.87e ± 0.386 | 5.43 cd ± 0.150 | 4.65d ± 0.225 | 7.52b ± 0.409 | 6.09c ± 0.230 | 9.49a ± 0.178 | 6.99b ± 0.051 |
| Na | 2.28e ± 0.067 | 2.13e ± 0.042 | 3.37 cd ± 0.103 | 3.05d ± 0.218 | 4.39b ± 0.235 | 3.68c ± 0.084 | 5.55a ± 0.147 | 4.39b ± 0.144 |

Treatments are designed as uninoculated controls, saline stress (4, 6, and 8 dS m^{-1}), and arbuscular mycorrhiza (AM). Each value is the mean of six replicates ± SE. Means followed by the same letter within a column do not differ ($p < 0.05$) using Duncan's multiple-range test

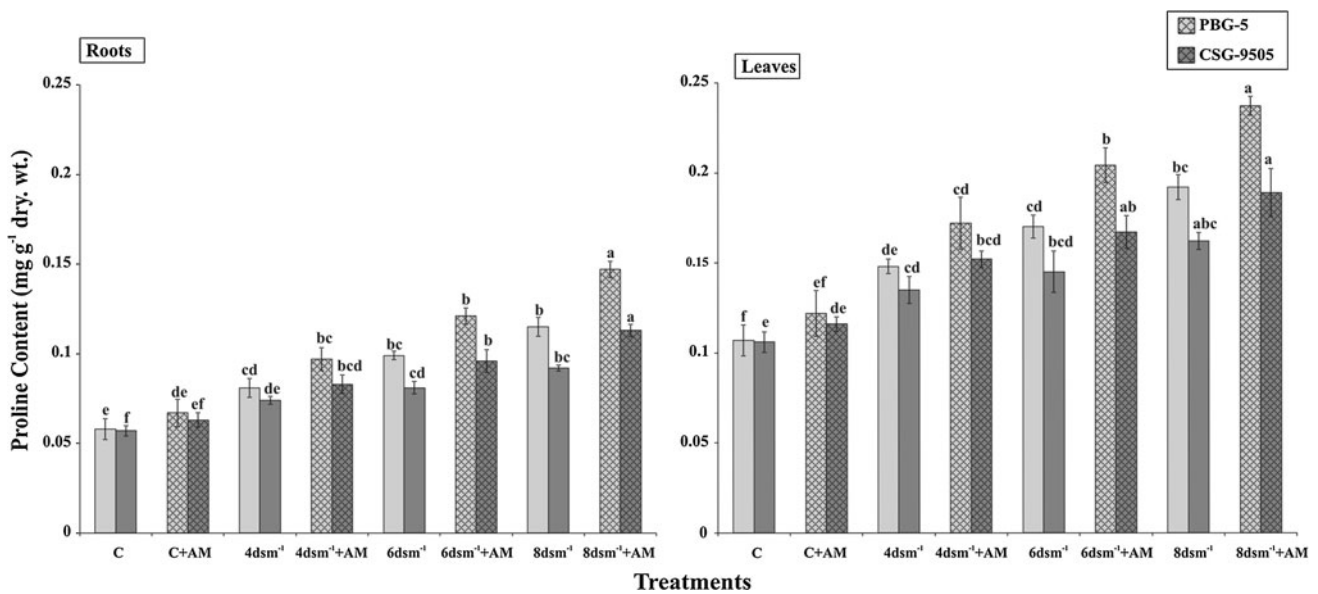


Fig. 1 Effect of arbuscular mycorrhizal (AM) inoculations on proline content (mg g^{-1} DW) in roots and leaves of chickpea genotypes under NaCl stress. Treatments are designed as uninoculated controls, saline stress (4, 6, and 8 dS m^{-1}), and arbuscular mycorrhiza (AM).

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

even higher than GDH (Fig. 3), which accounted for higher proline accumulation in leaves [P-5-CS (r_p): PBG-5 = 0.986, CSG-9505 = 0.972; GDH (r_p): PBG-5 = 0.980, CSG-9505 = 0.968] than in roots [P-5-CS (r_p): PBG-5 = 0.972, CSG-9505 = 0.950; GDH (r_p): PBG-5 = 0.970, CSG-9505 = 0.922]. The induction of P-5-CS and GDH was correlated with the augmentation of salt concentration of the irrigation solution in leaves [P-5-CS (r_p): PBG-5 = 0.854, CSG-9505 = 0.781; GDH (r_p): PBG-5 = 0.813, CSG-9505 = 0.728] and roots [P-5-CS (r_p): PBG-5 = 0.819, CSG-9505 = 0.749; GDH (r_p): PBG-5 = 0.717, CSG-9505 = 0.701]. PBG-5 accumulated large amounts of proline in leaves when compared with CSG-9505, indicating its higher potential for osmotic adjustment. On the other hand, salinity induced a remarkable reduction in proline dehydrogenase (Fig. 4), suggesting that accumulation of proline was the result of concomitant inactivation of proline dehydrogenase, thus negatively correlated with sodium content in leaves (r_p : PBG-5 = 0.759, CSG-9505 = 0.724) as well as in roots (r_p : PBG-5 = 0.616, CSG-9505 = 0.599). The results indicated that proline content showed a negative and significant relationship with proline dehydrogenase in leaves (r_p : PBG-5 = 0.873, CSG-9505 = 0.805) and roots (r_p : PBG-5 = 0.830, CSG-9505 = 0.737).

Association of chickpea roots with *G. mosseae* resulted in a conspicuous increase in the proline pool under salt stress as shown by the positive correlation of proline with plant MD [leaves (r_p): PBG-5 = 0.937, CSG-9505 = 0.917; roots (r_p): PBG-5 = 0.926, CSG-9505 = 0.854]. AM colonization had a positive influence on the activity of P-5-CS and

GDH; thus, AM-inoculated plants showed higher proline synthesis at all salinity levels, with a further decline in PDH activity. The $S \times AM \times G$ interaction for proline metabolism (Table 1) confirmed a significant and a positive role of AM in modulating the tolerance response to salinity in chickpea genotypes.

Discussion

The differential response of two chickpea genotypes was observed in terms of tolerance to salt stress. This was substantiated by the higher plant biomass in the salt-tolerant genotype PBG-5 than in the salt-sensitive genotype CSG-9505. Salinity stress led to a significant decline in plant biomass accumulation (root and shoot) in both genotypes; however, root growth was affected more extensively by salinity than shoot dry matter, which consequently led to a decline in the root-to-shoot ratio (RSR). Our results coincided with those of Garg and Singla (2004) and of Tejera and others (2006) who reported depressive effects of NaCl on plant growth and dry matter accumulation at the genotypic level in chickpea.

In the present study, the osmotic phase of salinity was accompanied by the ion-specific phase of the plant response to NaCl when Na^+ accumulated to toxic concentrations in the plant tissues (roots and leaves) in a genotype- and dose-dependent manner. The increment in values for Na^+ in roots under stress suggested that Na^+ might have accounted for poor values of growth parameters

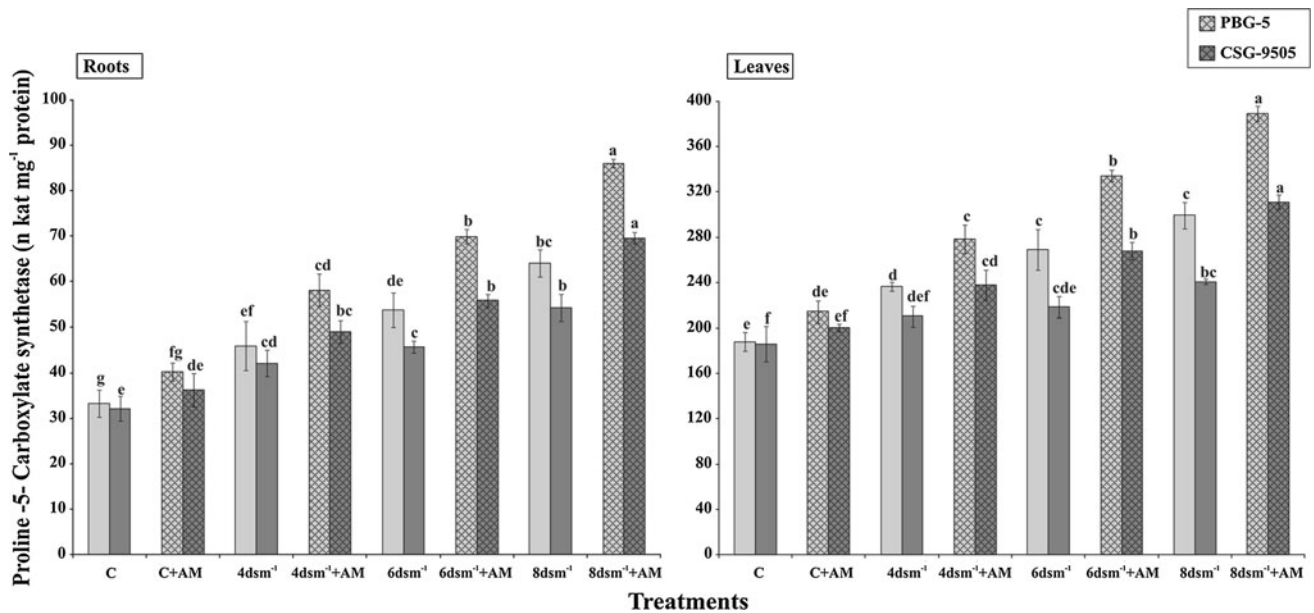


Fig. 2 Effect of arbuscular mycorrhizal (AM) inoculations on proline-5-carboxylate synthetase (nkat mg⁻¹ protein) in roots and leaves of chickpea genotypes under NaCl stress. Treatments are designed as uninoculated controls, saline stress (4, 6, and 8 dS m⁻¹),

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

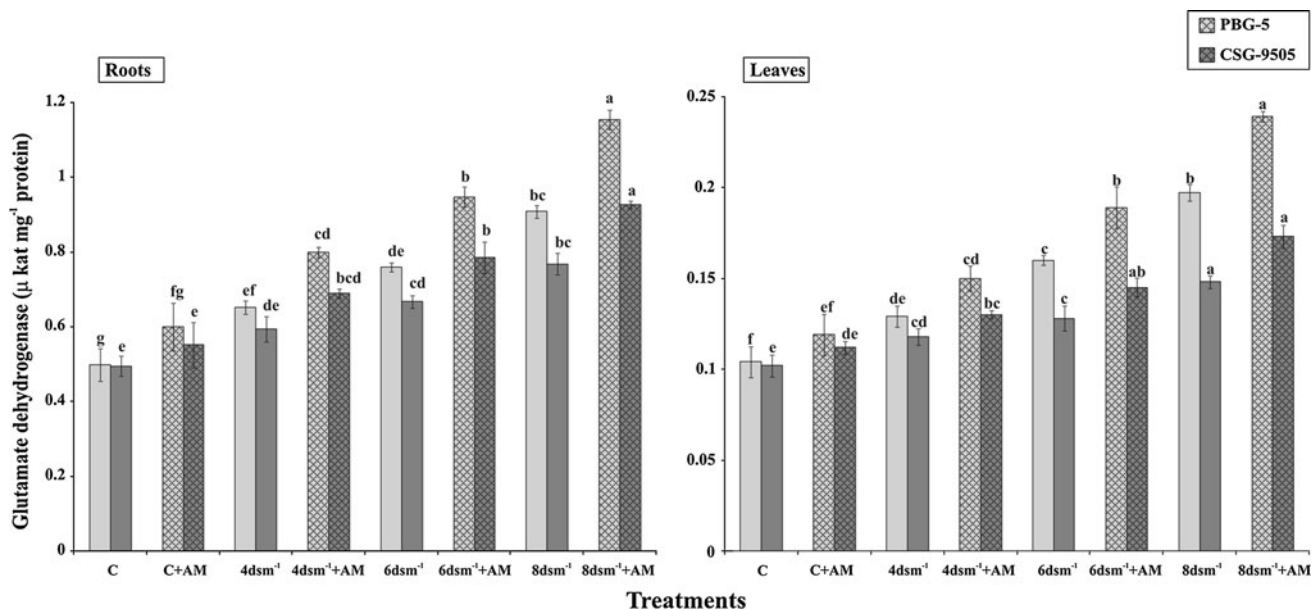


Fig. 3 Effect of arbuscular mycorrhizal (AM) inoculations on GDH (μ kat mg⁻¹ protein) in roots and leaves of chickpea genotypes under NaCl stress. Treatments are designed as uninoculated controls, saline stress (4, 6, and 8 dS m⁻¹), and arbuscular mycorrhiza (AM). Values

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

(Kchaou and others 2010; Lenis and others 2011; Tarchoune and others 2012). Roots constituted the preferential site of Na⁺ accumulation, whereas leaves showed lower Na⁺ concentrations. Higher Na⁺ concentrations were found in the sensitive genotype (CSG-9505) than in

the tolerant one (PBG-5), suggesting a positive correlation between salt tolerance and the control of Na⁺ absorption. Based on the observed data and available evidence from previous work (Tarchoune and others 2012), it can be suggested that differential tolerance to salt in chickpea

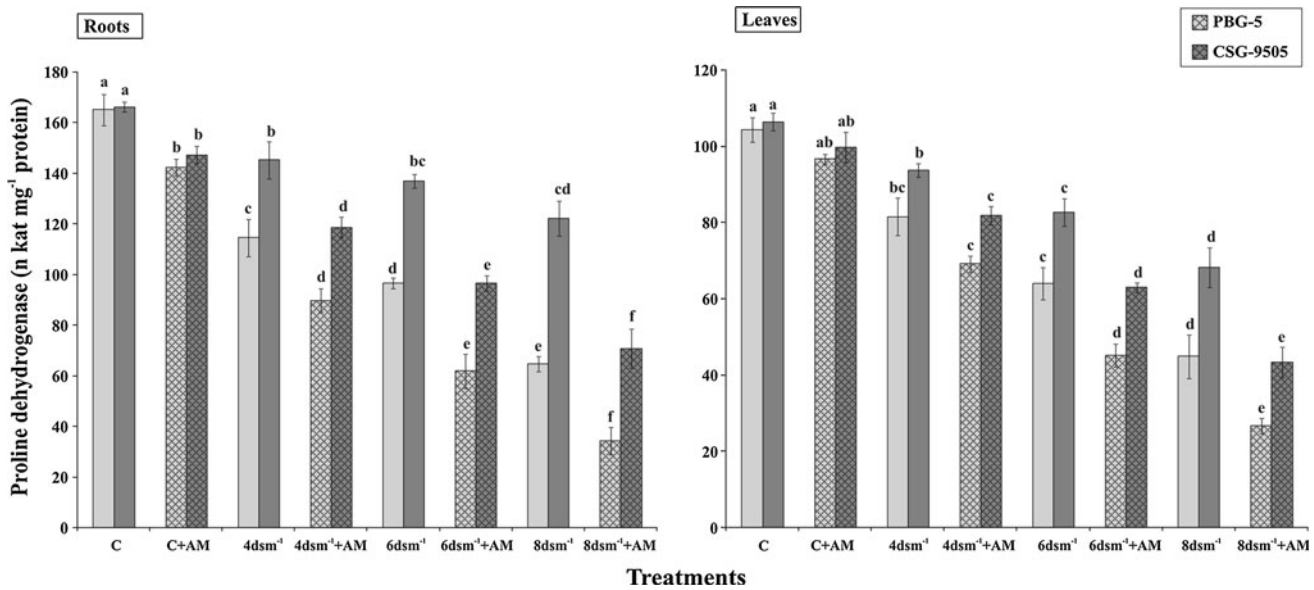


Fig. 4 Effect of arbuscular mycorrhizal (AM) inoculations on proline dehydrogenase (nkat mg⁻¹ protein) in roots and leaves of chickpea genotypes under NaCl stress. Treatments are designed as uninoculated controls, saline stress (4, 6, and 8 dS m⁻¹), and arbuscular

mycorrhiza (AM). Values are means based on six biological repeats ± standard error (SE). Means followed by the same letter do not differ (*p* < 0.05) using Duncan’s multiple-range test

genotypes was achieved by a combination of mechanisms. Roots of PBG-5 had the ability to hold Na⁺ more efficiently than CSG-9505 and in so doing prevented its transport and accumulation in leaves, the main photosynthetic organs. Higher tolerance of tissues to toxic ion build-up might be due to a more active sequestration of Na⁺ into vacuoles of PBG-5 plants which have the ability to survive and even thrive in conditions of salinity. Therefore, genotypic differences observed in the present study in the plant growth response to NaCl stress depended on the intrinsic differences in rates of toxic ion uptake, transport, and distribution within the plant (Bavei and others 2011; Lenis and others 2011; Fatehi and others 2012).

Mycorrhization of chickpea roots with *G. mosseae* increased the fitness of host plants by enhancing root and shoot biomass at all salinity levels. The effect of AM on plant dry matter was more pronounced in root biomass than aerial biomass as mycorrhizal plants develop a more efficient carbon-use root system. This behavior is considered profitable because it could improve plant water status by increasing root requirements to explore a higher volume (Miransari and Smith 2008). The effect of AM colonization on growth improvement of salt-affected plants was highly significant, particularly in PBG-5. Differential mycorrhizal responsiveness of genotypes could be the result of plant- or fungus-related mechanisms (Al-Karaki 2006). 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⁺ (Giri and others 2007; Garg and Manchanda 2008). The results of the present study showed that salt stress reduced colonization of *G. mosseae*-inoculated chickpea plants, which was significantly higher MC in PBG-5, and confirmed the previous results of Giri and others (2007), Porrás-Soriano and others (2009), and Wu and others (2010) for different plant species. Our results indicated high mycorrhizal dependence of CSG-9505 to reach optimum development under stress conditions as compared to PBG-5. Symbiotic development between mycorrhizal fungi and chickpea 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 under salinity stress.

A response to osmotic stress consists of the accumulation of compatible osmolytes in plants which are thought to protect cells against stress damage. Among these plant-compatible osmolytes, proline is considered to be of major importance, as it has been reported to accumulate in a large number of species in response to stresses (Wang and others 2011). In the present study, the proline content increased in stressed plants of both genotypes under increasing concentrations of NaCl. Salinity triggered an inhibition of proline dehydrogenase and an increase of proline content, indicating a role for this amino acid in osmoprotection and osmotic adjustment against salinity stress.

The decrease in soil water potential under salt stress might have led to an alteration of the plant water status, which might have caused stomata closure, photosynthesis

reduction, and thus growth inhibition (Apel and Hirt 2004; Garg and Manchanda 2009). The results from the present study showed that the activity of P-5-CS increased under salinity, which accounted for increased proline accumulation depending upon the genotypes under study. Our results revealed that proline biosynthesis increased by significantly increasing P-5-CS and GDH activities with a concomitant decline in ProDH activity under salt stress. The change in proline biosynthesis with higher activity of the biosynthetic enzyme in PBG-5 could be correlated with its capacity to tolerate and adapt to salinity conditions (Kumar and others 2003; Wang and others 2007).

In the present study, the association of chickpea roots with *G. mosseae* increased proline content significantly. Higher proline concentrations in AM plants of soybean and *Jatropha curcas* L. have been reported at different salinity levels (Sharifi and others 2007; Dodd and Pérez-Alfocea 2012). In our study, colonization of roots by AM fungi further stimulated the salinity-induced accumulation of proline by inhibiting proline dehydrogenase in mycorrhizal plants. Additionally, the proline biosynthetic enzymes P-5-CS and GDH also showed more activity in stressed mycorrhizal plants. Transcriptome analysis using real-time reverse transcriptase by Abo-Doma and others (2011) indicated that mycorrhiza treatment resulted in an increase in the gene expression of P-5-CS on the transcription level. Moreover, the enhancement in the activity of these enzymes in PBG-5 over CSG-9505 could be directly correlated with the percent mycorrhizal colonization of the genotypes, suggesting that an increase in proline content of roots and leaves might have improved the relative tolerance of PBG-5 in such conditions (Ruiz-Lozano and others 2012).

Conclusions

In conclusion, the chickpea genotypes studied here displayed remarkable variability in their symbiotic performance (AM colonization) under stressed and nonstressed conditions. PBG-5 was more salt tolerant and had greater stress protection through increased proline synthesis than CSG-9505. *G. mosseae* symbiosis was much stronger in PBG-5, which led to reduced Na⁺ ion uptake and higher growth when compared with CSG-9505 under stressed conditions. Mycorrhizal symbiosis with chickpea roots boosted proline biosynthesis by significantly increasing P-5-CS and GDH activities with a concomitant decline in ProDH activity under salt stress. The induction of proline metabolism under salt stress and its manipulation through AM symbiosis suggests its promising role in imparting salt tolerance in chickpea plants.

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