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Inducing salinity tolerance in chickpea (*Cicer arietinum* L.) by inoculation of 1-aminocyclopropane-1-carboxylic acid deaminase-containing *Mesorhizobium* strains

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Salinity stress severely affects growth, nodulation and yield of chickpea (*Cicer arietinum* L.). However, inoculation with *Mesorhizobium* strains containing 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase improves the plant growth by reducing the level of ethylene induced by salt stress. Fifty (50) *Mesorhizobium* isolates were obtained from nodules of chickpea plants on yeast extract mannitol agar (YEMA) medium. *Mesorhizobium* isolates were screened for ACC utilization and growth at different salt concentrations in YEMA medium. Six salt tolerant *Mesorhizobium* isolates were checked for their role in plant growth promotion under pot house conditions in chillum jar assembly. *Mesorhizobium* strains having ACC utilization ability caused an increase in the nodule number, nodule weight and shoot dry weight after plant growth for 50 and 80 days, both with and without NaCl. *Mesorhizobium* isolate MBD26 showed 294 mg/plant shoot dry weight without salt condition after 50 days of plant growth. *Mesorhizobium* isolate MBD26 increased shoot dry weight by 49.52% (without salt) and 41.53% in the presence of salt (40 mM NaCl) after 80 days of plant growth. It was observed that inoculation with *Mesorhizobium* isolates containing ACC-deaminase improved nodulation and plant growth of chickpea over ACC deaminase lacking isolates. Thus, inoculation with *Mesorhizobium* strains possessing ACC utilization ability could be a sustainable approach to improve plant growth under salinity stress.

Key words: 1-Aminocyclopropane-1-carboxylic acid (ACC) utilization, salt stress, *Mesorhizobium*, chickpea, nodulation, plant growth.

INTRODUCTION

Maintenance of sustainable agricultural crop productivity and simultaneously increasing food production to meet the demands of growing human population is a challenging task. Moreover, abiotic stresses due to the climate changes, soil environment and agricultural practices adversely affect the crop productivity. The soil environment is constantly changing, making it relatively stressful for both macro- and micro-organisms.

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Changes such as fluctuations in pH, temperature, salinity and nutrient availability greatly influence the growth, survival and metabolic activity of soil microorganisms (Zahran, 1999). Soil salinity affects about 800 Mha of arable lands worldwide (Munns and Tester, 2008) and this area is gradually expanding. Salinity affects agricultural production in arid and semiarid regions, where rainfall is limited and is not sufficient to transport salts from the plant root zone (Tester and Davenport, 2003).

Salt concentration negatively affects the growth and yield of legume plants by its harmful effect on biological nitrogen fixation, lessening supply of photosynthates to nodule of plants (Bekki et al., 1987), reduced supply of respiratory substrates supply to bacteroids (Delgado et al., 1994) and modifications in the diffusion barrier of oxygen (Serraj et al., 1994). Salt stress also enhance ethylene (C_2H_4) synthesis which in most of the cases acts as stress hormone in plant (Grichko and Glick, 2001; Arshad and Frankenberger, 2002). ACC deaminase enzyme lowers the level of C_2H_4 in plants and it protects the plants from the deleterious effects of environmental stresses (Reed and Glick, 2005; Saleem et al., 2007; Aamir et al., 2013).

Recently, plants inoculated with plant growth promoting rhizobactertia containing ACC deaminase activity have been found to thrive through the salinity menace leading to normal growth pattern (Mayak et al., 2004a; Saravanakumar and Samiyappan, 2006; Gamalero et al., 2010). Mayak et al. (2004b) reported that inoculation with rhizobacterial strains dramatically lowered the level of ethylene and growth inhibition of tomato plants was prevented when grown in the presence of high concentration of salts.

The frequency of ACC deaminase activity containing bacterial strains is relatively low and 12% of isolated *Rhizobium* spp. from various sites in Southern and Central Saskatchewan were found to possess this enzyme (Duan et al., 2009). ACC deaminase activity has been found in a wide range of bacterial isolates including *Azospirillum*, *Rhizobium*, *Agrobacterium*, *Achromobacter*, *Burkholderia*, *Ralstonia*, *Pseudomonas* and *Enterobacter* (Glick et al., 2007a; Saleem et al., 2007; Ahmad et al., 2011; Khandelwal and Sindhu, 2012).

In this study, *Mesorhizobium* isolates were obtained from legume root nodules collected from chickpea plants grown under saline areas. Selected ACC deaminase containing *Mesohizobium* isolates were evaluated for growth promotion of chickpea under salinity stress conditions.

MATERIALS AND METHODS

Isolation of *Mesorhizobium* from chickpea nodules

Healthy chickpea plants (with nodules) grown in saline soils were collected from different locations in Hisar, Bhiwani and Sirsa districts of Haryana state. Nodules were surface sterilized with $HgCl_2$ (1%), crushed with sterile glass rod and the crushed nodule suspension was streaked on yeast extract mannitol agar (YEMA) medium plates (Garg et al., 1985). The plates were incubated for three to eight days at 28±2°C. Purified cultures were maintained at 4°C in the refrigerator till further use.

Screening of *Mesorhizobium* isolates for growth at different salt concentrations

Purified *Mesorhizobium* isolates were checked for their ability to grow at different concentrations of sodium chloride (NaCl), that is, 1, 2, 3 and 4% (w/v), on YEMA medium plates containing 20 mM HEPES (N-2-hydroxyethane-sulphonic acid) (Marsudi et al., 1999). Medium plates were spotted with a loopful of bacterial isolates. The plates were incubated for three to four days at 28±2°C in a biological oxygen demand (B.O.D.) incubator. The susceptibility to NaCl was recorded as a positive or negative result.

ACC utilization by *Mesorhizobium* isolates

Minimal medium plates supplemented with 2 mM ACC were prepared (Penrose and Glick, 2003). A loopful of 48-h old growth of *Mesorhizobium* isolate was spotted on the ACC supplemented medium plates (Khandelwal and Sindhu, 2012). The minimal medium incorporated with ammonium sulphate (2 g/L) was kept as control to compare the growth of bacterial isolates to those with ACC supplemented medium plates. The growth of bacterial isolates was recorded after five days of incubation at 28±2°C. The bacterial cultures showing good growth on ACC supplemented medium plates, that is, having high efficiency of ACC utilization as nitrogen source, were scored as bacteria having ACC deaminase activity.

Effect of inoculation on plant growth of chickpea

Selected ACC utilizing and ACC non-utilizing *Mesorhizobium* isolates were checked for nodulation and plant growth using chickpea (*Cicer arietinum* L.) var. HC-1 in sterilized chillum jar assemblies (Dahiya and Khurana, 1981) containing washed river sand in the upper jar and Sloger's nitrogen-free mineral salt solution (Sloger, 1969) in the lower assembly. Surface sterilized seeds of chickpea were inoculated with 5 ml culture (containing 10^7-10^8 cells/ml of growth suspension) of selected ACC utilizing and ACC non-utilizing *Mesorhizobium* isolates individually. Uninoculated seeds were sown as control. Quarter strength Sloger's nitrogen-free mineral salt solution was used for watering whereas salinity levels of 40 mM NaCl were maintained for salt treatment. The observations for shoot biomass, nodule number, nodule weight and plant nitrogen (Lindner, 1944) were recorded at 50 and 80 days of plant growth.

RESULTS

Salt tolerance of different *Mesorhizobium* isolates

Among the fifty *Mesorhizobium* isolates tested at 1, 2, 3 and 4% NaCl concentrations, only four isolates, that is, MHD2, MHD12, MHD14 and MSD41 showed growth up to 4% NaCl concentration (Table 1 and Figure 1). Four

Table 1. Salt tolerance among various *Mesorhizobium* isolates.

Colony size (mm)	Growth of *Mesorhizobium* isolates at different NaCl concentrations			
	1%	2%	3%	4%
0-5 Group 1	MHD6, MHD9, MHD10, MBD16, MBD18, MBD19, MBD22, MBD24, MBD33, MBD40, MBD41, MBD42, MBD43, MBD47	MHD6, MHD9, MHD10, MHD15, MBD19, MBD22, MBD24, MBD25, MBD26, MBD29, MBD30, MBD32, MBD33, MSD38, MSD40, MSD41, MSD42, MBD47	MHD2, MHD6, MHD9, MHD10, MBD19, MBD22, MBD24, MBD25, MBD26, MBD29, MBD30, MBD31, MSD38, MSD40, MSD41	MHD2, MSD41
5-10 Group 2	MHD14, MHD15, MBD20, MBD25, MBD26, MBD27, MBD28, MBD29, MBD30, MBD31, MBD32, MBD34, MSD38	MHD1, MHD2, MHD14, MBD20, MBD27, MBD28, MBD31	MHD1, MHD12, MHD14, MBD20	MHD12, MHD14
10-15 Group 3	MHD1, MHD2, MHD12	MHD12	-	-
No growth Group 4	MHD3, MHD4, MHD5, MHD7, MHD8, MHD11, MHD13, MBD17, MBD21, MBD23, MSD35, MSD36, MSD37, MSD39, MSD44, MSD45, MSD46, MSD48, MSD49, MSD50	MHD3, MHD4,, MHD5, MHD7, MHD8, MHD11, MHD13, MBD16- MBD18, MBD21, MBD23, MBD34-, MSD37, MSD39, MSD43 - MSD46, MSD48 - MSD50	MHD3 - MHD5, MHD7, MHD8, MHD11, MHD13, MHD15, MBD16 - MBD18, MBD21, MBD23, MBD27, MBD28, MBD32 - MBD34, MSD35 - MSD37, MSD39, MSD42 - MSD50	MHD1, MHD3 - MHD11, MHD13, MHD15, MBD16 - MBD34, MSD35 - MSD40, MSD42 - MSD50

major groups were distinguished on the basis of different colony size on salt incorporated medium plates. In the third group, only three *Mesorhizobium* isolates MHD1, MHD2 and MHD12 showed large colony size (10-15 mm) at 1 and 2% NaCl salt concentration. At 2% NaCl concentration, twenty four *Mesorhizobium* isolates did not show growth and only MHD12 isolate showed large colony size. Seven *Mesorhizobium* isolates showed 5-10 mm colony diameter on 2% NaCl plates whereas eighteen isolates of *Mesorhizobium* showed small colony size (group 1st). At 3% salt concentration, fifteen *Mesorhizobium* isolates having small colony growth were found in 1st group, whereas, MHD1, MHD12, MHD14 and MBD20 isolates showed 5-10 mm colony size (group 2). Remaining 62% isolates did not show growth. At 4% NaCl concentration, forty six *Mesorhizobium* isolates did not show growth. Only two isolates MHD12 and MHD14 showed more growth (5-10 mm) than other two isolates MHD2 and MSD41 (Table 1). At 1% salt concentration, twenty *Mesorhizobium* isolates did not show growth (4th group).

ACC utilization by different *Mesorhizobium* isolates

All the *Mesorhizobium* isolates were screened for ACC utilization and *Mesorhizobium* isolates were divided into four major categories based upon ACC utilization (Table 2). Only two *Mesorhizobium* isolates, that is, MHD1 and MHD12 showed significant growth on ACC supplemented plates (Table 2 and Figure 2). Eight isolates, that is, MHD2, MHD4, MHD8, MHD11, MBD25, MBD26, MSD28 and MSD29 moderate growth whereas twelve isolates showed little growth on ACC plates. Twenty eight *Mesorhizobium* isolates did not grow on ACC supplemented plates. On ammonium sulphate containing plates, nine *Mesorhizobium* isolates showed significant growth whereas 33 cultures did not grow. Five *Mesorhizobium* isolates, that is, MBD27, MBD30, MBD33, MSD48 and MSD50 showed little growth.

Symbiotic effectiveness of different *Mesorhizobium* isolates

Three ACC^+ and three ACC^- *Mesorhizobium* isolates were selected for pot house experiment to check their nodulation efficiency and symbiotic effectiveness. Inoculation of *Mesorhizobium* isolate MBD26 showed significant increase in nodule number (54 nodules/plant), nodule weight (357 mg/plant) and nitrogen content (13.67 mg/plant) along with increase in shoot dry weight (294 mg/plant) as compared to uninoculated control (without salt condition) followed by *Mesorhizobium* isolates KR48 and MHD2 after 50 days of plant growth (Table 3). In salt conditions, *Mesorhizobium* isolate MBD26 formed 38

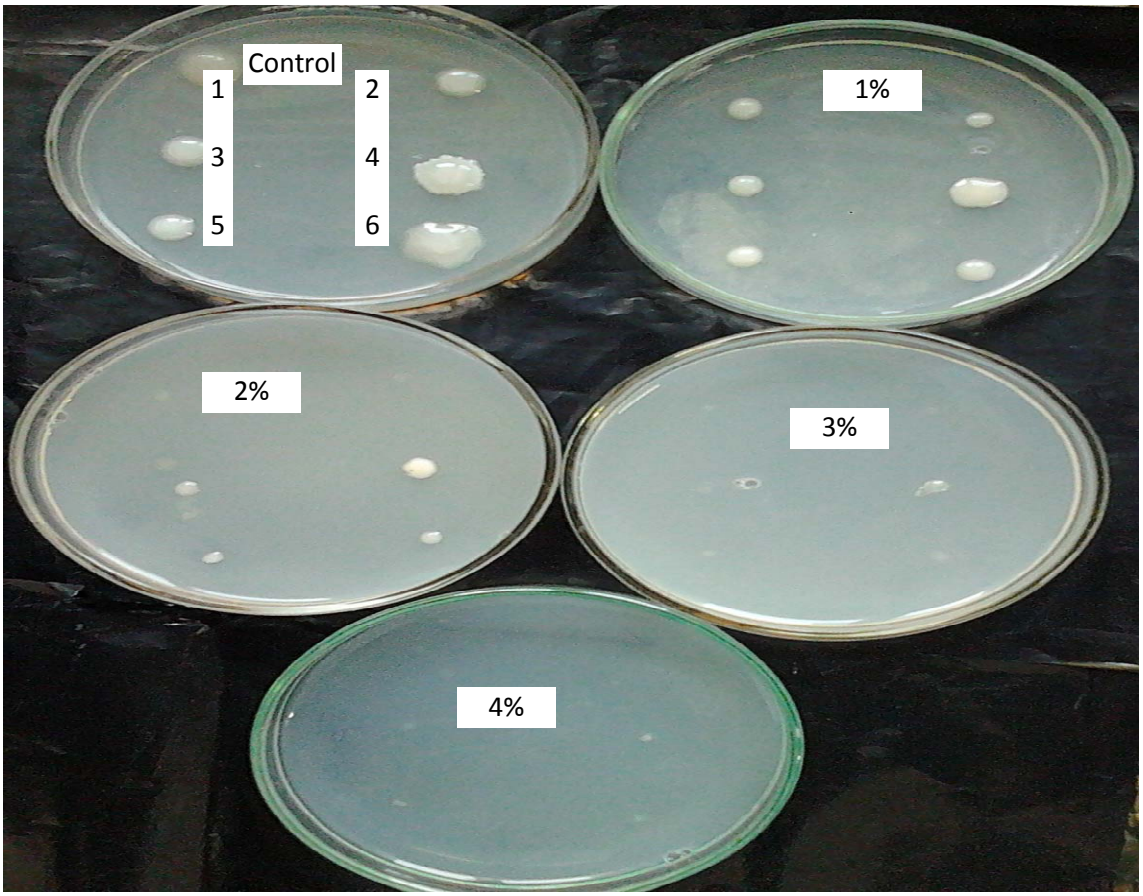

Figure 1. Growth of *Mesorhizobium* isolates on different NaCl concentrations (1-4%) incorporated in YEMA medium. The number given in the figure represents: left to right: 1st row: MBD31, MBD16; 2nd row: MBD28, MBD26; 3rd row: MHD12; MHD2

Table 2. Growth of *Mesorhizobium* isolates on minimal medium supplemented with ACC or ammonium sulphate.

No. of *Mesorhizobium* isolates	Group of ACC utilizing isolates
Growth on ACC supplemented plates	
MHD10. MHD14, MBD27. MBD30 - MBD32, MBD34, MSD40, MSD42, MSD47, MSD48, MSD50	+
MHD2, MHD4, MHD8, MHD11, MBD25, MBD26, MSD28, MSD29	++
MHD1, MHD12	+++
MHD3, MHD5 - MHD7, MHD9, MHD13, MHD15, MBD16 - MBD21, MBD22, MBD23, MBD24, MBD33, MSD35 - MSD39, MSD41, MSD43 - MSD45, MSD46, MSD49	-
Growth on $(NH_4)_2SO_4$ containing plates	
MBD27, MBD30, MBD33, MSD48, MSD50	+
MHD8, MBD32, MSD44	++
MHD1, MHD2, MHD4, MHD11,MHD12, MHD14, MBD25, MBD26, MBD28	+++
MHD3, MHD5 - MHD7, MHD9, MHD10, MHD13, MHD15, MBD16 - MBD24, MBD29, MBD31, MBD34, MSD35 - MSD43, MSD45 - MSD47, MSD49	-

Growth characteristics

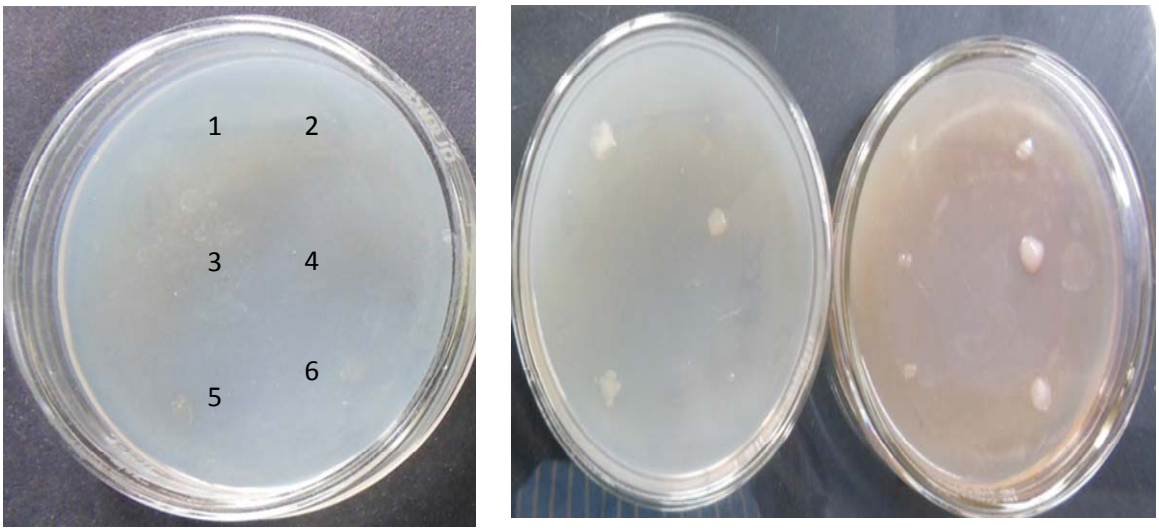

Figure 2. Growth of *Mesorhizobium* isolates on minimal medium (A), ACC supplemented medium (B) and ammonium sulphate amended medium (C). The numbers given in the figure represents: 1, MHD12; 2, MBD33; 3, MSD44; 4, MBD26; 5, MHD2; 6, MHD46

Table 3. Inoculation effect of *Mesorhizobium* isolates in chickpea for nodulation and plant growth under chillum jar conditions at 50 days of plant growth

Inoculation with *Mesorhizobium* isolates	No. of nodules /plant	Nodule weight (g/plant)	Shoot dry weight (g/plant)	Total nitrogen (mg)
Control	-	-	208	8.80
	-	-	140	6.30
MHD9	35	287	230	9.33
	23	131	146	6.57
MBD20	41	298	248	10.67
	25	142	157	7.06
MSD46	43	303	260	11.67
	28	153	162	7.29
MBD26	54	357	294	13.67
	38	192	189	8.50
MHD2	46	317	263	11.50
	29	157	173	7.78
KR48	49	337	281	13.0
	34	173	186	8.37

Values in the second line of each treatment represent the values observed when plants were grown under salt conditions.

nodules/plant having nodule weight (192 mg/plant), shoot dry weight (189 mg/plant) and nitrogen content (8.5 mg/plant) whereas *Mesorhizobium* isolate KR48 formed 34 nodules/plant and produced 186 mg/plant shoot dry weight.

At 80 days of plant growth (without salt condition), inoculation with *Mesorhizobium* isolate MBD26 resulted in nodule weight of 372 g/plant and 471 g/plant shoot dry weight was observed. Maximum plant growth stimulation and nodule formation was observed by inoculation of *Mesorhizobium* isolate MBD26 followed by KR48 and MHD2. It was observed that nodule number, nodule weight, shoot biomass and nitrogen content were increased in *Mesorhizobium* inoculated plants, as compared to uninoculated control plants (Table 4 and Figure 3). The maximum increase in shoot dry weight (49.52%) was recorded by inoculation with MBD26 without salt conditions and 41.53% increase was observed

Table 4. Inoculation effect of *Mesorhizobium* isolates in chickpea on nodulation and plant growth under chillum jar conditions at 80 days of plant growth.

Inoculation with *Mesorhizobium* isolates	No. of nodules /plant	Nodule weight (mg/plant)	Shoot dry weight (mg/plant)	Total nitrogen (mg)
Control	-	-	315	10.39
	-	-	195	7.60
MHD9	47	325	365	12.10
	28	147	215	8.38
MBD20	52	334	397	13.18
	31	153	232	9.04
MSD46	55	337	421	13.97
	33	156	243	9.47
MBD26	65	372	471	15.97
	42	178	276	10.76
MHD2	59	348	434	14.71
	35	167	257	10.02
KR48	62	353	456	15.46
	38	174	269	10.49

Values in the second line of each treatment represent the values observed when plants were grown under salt conditions.

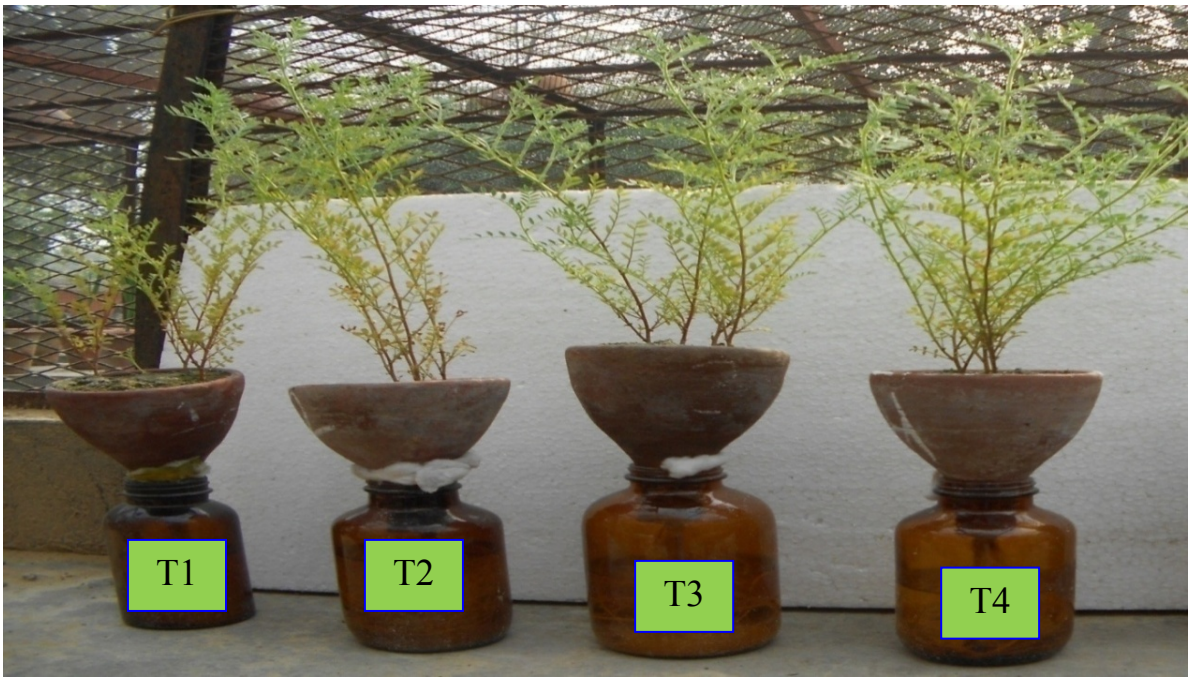

Figure 3. Inoculation effect of *Mesorhizobium* isolates on nodulation and plant growth of chickpea after 80 days under chillum jar conditions. T1: Uninoculated control; T2: *Mesorhizobium* KR48, ACC^+; T3: *Mesorhizobium* MHD2, ACC^+; T4: *Mesorhizobium* MBD26, ACC^+.

in the presence of salt (40 mM NaCl) after 80 days of plant growth. Inoculation with other ACC^+ *Mesorhizobium* isolates also showed similar enhancement for nodule number, nodule weight and nitrogen content as compared to uninoculated control plants. While ACC^- *Mesorhizobium* isolates showed comparatively less

increase in plant parameters as compared to control uninoculated plants. Maximum increase in shoot dry weight (by 24.61%) was observed by inoculation of ACC^- *Mesorhizobium* isolate MSD46 after 80 days in salt treatment. It formed 33 nodule/plant and showed 9.47 mg nitrogen content.

DISCUSSION

Biological nitrogen fixation is performed by a limited group of prokaryotic microorganisms known as diazotrophic bacteria and the nitrogenase enzyme complex of these organisms convert atmospheric inert nitrogen to plant utilizable ammonia form (Araujo et al., 2014). It is known that these microorganisms produce various plant growth-promoting substances (Sindhu et al., 2010; Malik and Sindhu, 2011). Such diazotrophic bacteria have the potential to be used as biofertilizers in different crops for a sustainable agriculture.

Fifty isolates of *Mesorhizobium* were obtained from the nodules of chickpea plants grown in salt affected fields by streaking crushed nodule suspension on the YEMA medium plates. Ogutcu et al. (2010) evaluated the symbiotic effectiveness of *Rhizobium leguminosarum* bv. *Ciceri* strains isolated from perennial wild chickpeas (*Cicer anatolicum*) in comparison with uninoculated control under NaCl salinity stress conditions. Dry weights of root and shoot, root-to-shoot ratio (RSR), number and dry weights of nodules, chlorophyll and N content of the plant, and amounts of total and fixed N decreased progressively with increasing salinity levels. In both non-saline and saline (50 and 100 mM NaCl) conditions, inoculations with *R. leguminosarum* bv. *Ciceri* strains isolated from wild chickpeas significantly increased all the symbiotic parameters when compared with the uninoculated control treatment. Garg and Sharma (2013) studied stress tolerant forms of rhizobia isolated from *Trigonella foenumgraecum*. Growth of isolates on yeast mannitol medium having variable range of pH (4-10) and different concentration of NaCl (0.05 - 5%) was determined. Among all isolates, four (RTF1, 2, 5, 10) were found salt tolerant. 5 isolates RTF1, 2, 3, 9 and 10 were pH tolerant and 6 isolates RTF1, 2, 3, 5, 7 and 8 were temperature tolerant.

Mesorhizobium isolates were divided into four major categories on ACC utilization pattern (Table 2). Several other bacterial strains that can utilize ACC as a sole source of nitrogen have been isolated from rhizosphere soil samples and subsequently used for inoculation purposes (Glick, 2003; Glick et al., 2007a, b, Govindasmay et al., 2008). Zafar et al. (2007) isolated twenty seven isolates of rhizobacteria containing ACC-deaminase from the lentil rhizosphere by using dilution plate technique. All the rhizobacterial isolates had the potential to improve the growth of lentil seedlings under axenic conditions. Khandelwal and Sindhu (2012) found that 38.9% *Pseudomonas* isolates obtained from cluster-bean rhizosphere showed good growth on ACC supplemented plates. These ACC utilizing rhizobacteria are potentially important for agricultural practice and rhizobial or *Pseudomonas* strains that are intended for use as inoculants of host legumes should first be selected/ tested for the presence of a functional ACC deaminase.

Higher level of ethylene which is applied either directly or indirectly had significant inhibitory effect on nodulation (Guinel and Sloetjes, 2000). Inoculation with ACC deaminase containing *Rhizobium* had the potential to improve plant growth by reducing the inhibitory effect of salinity. Single inoculation have shown positive response to the measured growth parameters that might be attributed to changes in endogenous ethylene level by presence of plant growth promoting bacteria containing ACC-deaminase on the roots of legumes (Shahroona et al., 2006; Nadeem et al., 2009; Ahmad et al., 2011). Compared to uninoculated plants, nodule number, nodule fresh and dry weight was considerably improved by sole inoculation of ACC^+ *Mesorhizobium* isolates (Tables 3 and 4). Shahzad et al. (2010) reported that inoculation with selected isolates increased the root length, shoot length, dry root weight, shoot dry weight, lateral root number, lateral root length and lateral root dry weight of chickpea seedlings up to 107.5, 57.4, 86.7, 83.5, 266.7, 286.6 and 121%, respectively, over uninoculated control plants. Similar enhanced nodulation and increase in plant biomass production has been reported by inoculation of legumes with different rhizobial strains (Belimov et al., 2002; Dey et al., 2004; Mayak et al., 2004a). These ACC^+ *Mesorhizobium* sp. *Ciceri* strains having the ability to improve root nodule mass and shoot biomass could be further tested for symbiotic effectiveness under pot house and field conditions. Furthermore, the use of rhizobial strains with ACC deaminase activity might be very important for developing microbial inocula for agricultural purposes.

Conflict of interests

The authors have not declared any conflict of interest.

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