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Synergistic effect of *Mesorhizobium ciceri* and 1-amino cyclopropane 1-carboxylate (ACC) deaminase producing rhizobacteria on chickpea growth and yield

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ABSTRACT

Plant growth promoting rhizobacteria IIPR-ACC-3 and IIPR-ACC-79 with ACC deaminase activity of 862.41 and 417.32 nM of a-ketobutyrate/mg protein, respectively were isolated from chickpea rhizosphere. 16S rDNA sequence analysis indicated that IIPR-ACC-3 is *Bacillus subtilis* while IIPR-ACC-79 belongs to *Pseudomonas stutzeri*. Both the bacterial strains showed other plant growth promoting traits including the production of IAA, Siderophore and can reduce negative effects of stress ethylene with ACC deaminase under rainfed as well as irrigated field conditions. Gnotobiotic root elongation assay with bacterized chickpea recorded higher root length and biomass up to 113% over stressed seedlings. Root biomass of rainfed chickpea was improved by seed bacterization with IIPR-ACC-79 and its combined inoculation with *Mesorhizobium ciceri* strain CR-13 up to 31.59 and 38.34% respectively over uninoculated control. Enhanced root nodulation at early vegetative stages of chickpea due to combined inoculation of bacterial strains helped the plant to develop 47.91% higher shoot biomass at 90 days post inoculation. ACC deaminase producing bacteria interact synergistically with *M. ciceri* strain CR-13 and enhanced plant growth and grain yield of chickpea under rainfed as well as irrigated field.

Keywords: ACC deaminase; induced systemic tolerance; moisture stress; chickpea

Chickpea (*Cicer arietinum L.*) is an important pulse crop that has been grown in India, the Middle East and parts of Africa. India produces 6 million tones of chickpea annually and contributes a major share of approximately 70% of total world production. It is one of the important crops that can be successfully grown in 12 million ha of rainfed rice lands that remain fallow during post rainy season. Though inclusion of pulses in rice based cropping system is an attractive choice for recouping impoverished soil fertility, chickpea productivity is poor in rice fallow soil due to abiotic stresses including low moisture stress and nutrient supply capacity of soil. Under stressed environment, plant hormone ethylene accumulates at root system in order to regulate plant homeostasis. 1-Amino cyclopropane 1-carboxylate (ACC), an immediate precursor of ethylene biosynthesis accumulated at root tissues and oxidized into ethylene by ACC oxidase. Accelerated accumulation of stress ethylene at root system is known to inhibit nodulation, root biomass development and cause pre-mature senescence of nodule under stressed environment (Glick *et al.* 2007). Ethylene inhibits the infection thread elongation and increases the senescence zone in early stages of root nodule development in various legumes including *Medicago sativa*, *Phaseolus vulgaris,* and *Pisum sativum* (Xie *et al.* 1996; Guinel and Geil, 2002).

Plant growth promoting rhizobacteria (PGPR) elicit physical and chemical changes through a process referred as 'induced systemic tolerance' (IST) that results in enhanced plant tolerance to drought (Yang *et al.* 2008). Certain rhizobacteria produce ACC deaminase, a pyridoxal 5'-phosphate (PLP) dependent enzyme hydrolyzed ACC into á-ketobutyrate and ammonia and utilized them as carbon and nitrogen sources, respectively (Belimov *et al.* 2005). The structural gene for ACC deaminase, *acd*S has been detected in several rhizobia including *Sinorhizobium* sp. BL3 and *Mesorhizobium loti* (Tittabutr *et al.* 2008). ACC deaminase producing *S. meliloti* strain was more competitive in nodulation than the wild-type strain and showed 35 to 40% greater efficiency in nodulating *Medicago sativa* (Ma *et al.* 2003). However, *acd*S of *Mesorhizobium loti* ICMP3153 expressed only at the bacteroid stage under the control of a NifA-RpoN dependent promoter and failed to reduce the concentration of ACC during nodule initiation and early developmental stages. Exogenous expression and high copy number of *acd*S through genetic engineering improved the nodulation efficiency, competitiveness of rhizobia and plant growth (Nascimento *et al.* 2012). Under the natural conditions, inoculation of ACC deaminase producing rhizobacteria bacteria along with *Mesorhizobium* can reduce the negative effects of ethylene and improve the plant growth. *Variovorax paradoxus* 5C-2 having ACC-deaminase activity promoted biomass of root from 20 to 25% irrespective of soil moisture levels, and total plant biomass was also improved up to 25% in pea plants grown in drying soil. Co-inoculation with *Bradyrhizobium* and PGPR isolates enhanced the nodulation, root and shoot growth in mung bean compared with inoculation with *Bradyrhizobium* alone. The present investigation aimed to isolate potential ACC deaminase producing rhizobacteria and to assess their synergistic interactions with *M. ciceri* on chickpea growth and yield.

MATERIALS AND METHODS

Isolation and characterization of ACC deaminase producing rhizobacteria

Rhizosphere soil samples were taken from healthy chickpea plants grown under moisture stressed fields during 2011 and 2012. Chickpea roots with strongly adhered rhizosphere soil were dipped in sterile water followed by shaking for 30 min. Diluted rhizosphere soil suspension was used to enrich ACC deaminase producing bacteria in DF minimal liquid medium supplemented with 3mM ACC and incubated at 100 rpm, 28±2° C for 48h (Dworkin and Foster, 1958). The enriched broth culture was serially diluted, spread on DF minimal agar medium with 3mM ACC and incubated at 28±2° C for 48h. Bacterial colonies with profuse growth were purified and stored as 25% glycerol stocks under deep refrigeration at -80° C. Cell pellets of purified isolates were suspended in sterile water and spot inoculated on DF agar plates supplemented with 3mM ACC/ $(NH_4)_2SO_4$ as nitrogen source or without nitrogen source. Bacterial colonies growing profusely on DF agar with ACC but not on N free DF agar were selected. ACC utilization potential was tested by inoculating nutrient free cell suspension of selected isolates into DF liquid medium with/ without 3mM ACC and incubated at 100 rpm, 28±2° C for 48h. Bacterial isolates with 0.2 OD_{600nm} in ACC amended growth medium were selected for further studies.

Gram's staining, production of enzymes like oxidase, catalase, amylase and gelatinase, urease and nitrate reductase were carried out using standard conventional methods (Clarke *et al*., 1952). Genomic DNA of ACC deaminase producing bacteria were isolated by following phenol-chloroform extraction method. 16S rDNA was amplified by using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') and sequenced by fluorescent dye terminator method using ABI prism TM Bigdye TM terminator cycle sequencing ready reaction kit V.3.1. DNA sequences were subjected to BLASTn analysis with the NCBI data base. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura *et al*. 2011).

Plant growth promoting traits of selected rhizobacteria

IAA production was analyzed in nutrient broth with or without tryptophan 100µg/ml Two mL of culture supernatant was mixed with 100 µL of 10 mM ortho phosphoric acid and 4 mL of reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% $HClO_4$) and incubated at 28 ºC for 25 min for color development. Absorbance of a developed pink color was read at 530 nm. Phosphate solubilizing ability of bacterial endophytes was detected by spotting them on plates with Pikovskya agar. Plates were then incubated at 28 ºC for 3 days and observed for the clearing zone around the colonies. Production of siderophore was detected by spot inoculating the isolates on Chrome Azurol S (CAS) – agar plates and incubated at 28 ºC for 3 days. Siderophore producing colonies appeared with surrounding yellow halo. ACC deaminase enzyme activity (EC 4.1.99.4) was assayed by measuring the amount of á-ketobutyrate produced when the enzyme ACC deaminase cleaves ACC (Penrose and Glick, 2003). Presence of *acd*S, structural gene for ACC deaminase was confirmed by PCR using a primer set, F-1936 5'- GHGAMGACTGCAAYWSYGGC -3' and F-1938 5'- ATCATVCCVTGCATBGAYTT -3'. PCR cycling consisted of initial denaturation at 94° C for 4 min. followed by 30 cycles of 94° C for 60 sec, 56° C for 60 sec and 72° C for 60 sec and a final extension at 72° C for 4 min (Madhaiyan et al. 2010). Plant bioassay to test the plant growth promoting trait was carried out with mungbean and chickpea seeds. Seeds were surface sterilized with 70% ethanol for 3 min and 0.1% mercuric chloride for 30 sec and washed several times with sterile water. Surface sterilized seeds were applied with 100 µl each of 3 mM ACC and/or ACC deaminase producing cell suspension (10^6 cfu/ml). Treated seeds were kept on soft agar plates and incubated at 28±2° C for 7 days and the seedling biomass, length of plumule and radical were recorded.

Synergistic effect of bacterial bioinoculants on chickpea growth and yield

Chickpea seeds of Shubhra variety were soaked in double the volume of bacterial cell suspension containing different combinations of *M. ciceri* strain 13, IIPR-ACC- 3 and IIPR-ACC- 79 for 15 min. Bacterized seeds were air dried for 2h and sown in A-14 field of new research farm (NRF), IIPR, Kanpur, India. Experiments were carried out both under irrigated and rainfed conditions. The growth and yield parameters as listed below were observed at 45, 90 DAS and at the time of harvest; 1) Plant fresh weight and dry weight, 2) Root fresh weight and dry weight, 3) Nodule fresh weight and dry weight, 4) Grain yield, 5) Number of pods/plant, and 6) Number of seeds/plant. Data from in vitro as well as field experiments were subjected to statistical analysis with SPSS to assess the significance of the treatments.

RESULTS AND DISCUSSION

Application of ACC deaminase producing rhizobacteria can significantly decrease ACC levels in plants, thereby decreasing the amount of stress ethylene and subsequent damage to the plant that might occur as a consequence of stress ethylene (Belimov et al. 2005; Grichko and Glick, 2001). Soil inoculation with ACC deaminase containing *V. paradoxus* 5C-2 promoted the growth of ethylene-overproducing Arabidopsis mutant *eto1-1* but not in ethylene-insensitive mutants (*etr1-1* and *ein2-1*) and indicated the importance of ACC deaminase on plant growth promotion under stressed environment (Chen *et al*. 2013). In the present study, ACC deaminase producing bacteria were isolated from chickpea rhizosphere through enrichment culture technique. Bacterial colonies with profuse growth on ACC containing DF minimal media were selected (Fig. 1) and tested for their potential for utilizing ACC as nitrogen source. Bacterial isolates viz., 1-13 and 1-14a with ACC deaminase activity of 862.41 and 417.32 nM of a-ketobutyrate/mg protein respectively were selected and renamed as IIPR-ACC-3 and IIPR-ACC-79, respectively. Both the bacteria isolates did not produce gelatinase and starch hydrolyzing enzymes. IIPR-ACC-3 produced urease and nitrate reductase while IIPR-ACC-79 is positive for oxidase and H_2S test. Gram staining and microscopic observation indicated that IIPR- ACC-3 is Gram variable and rod shaped bacteria while IIPR-ACC-79 is Gram negative rods (Table 1). 16S rDNA sequence based

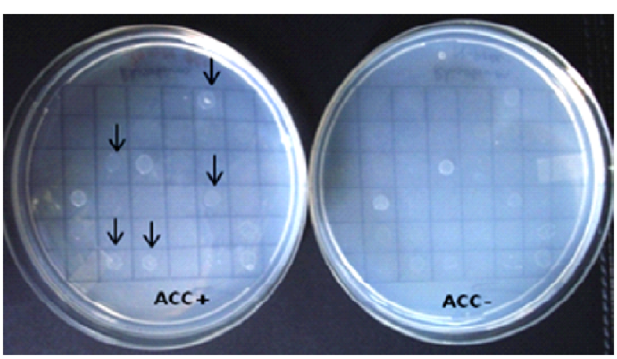

Fig. 1. Differential growth of ACC deaminase producing rhizobacteria on DF minimal medium.

Table 1: Characteristics of selected ACC deaminase producing bacteria

Characteristics	IIPR-ACC-3	IIPR-ACC-79
Hydrogen sulphide test	-	-
Urease test	+	+
Nitrate reductase test	+	+
Gelatinase test	-	-
Catalase test	+	+
Oxidase test	-	-
starch hydrolysis test	-	-
IAA production	+	+
P-solubilization	-	+
Siderophore production	+	+
Gram staining	Gram variable, Rod shaped	Gram negative, Rod shaped

phylogenetic analysis revealed that IIPR-ACC-3 is *Bacillus subtilis* while IIPR-ACC-79 belongs to *Pseudomonas stutzeri* (Fig. 2).

Presence of *acd*S, structural gene for ACC deaminase was tested through PCR amplification with degenerative primers. Expected DNA fragment size of 750bp was amplified while using DNA from IIPR-ACC-79 as template. IIPR-ACC-3 did not produce any amplified products. However, ACC utilization trait of this strain was confirmed by determining ACC deaminase activity of 862.41 nM of a-ketobutyrate/mg protein. Due to large sequence variations of *acd*S and poor availability of sequence information in public database, synthesis of universal primers is difficult for this gene. Large sequence variations of *acd*S can result in functional variations and one can expect bacteria with varying potential of ACC utilization. Both the bacterial strains are positive for the production of IAA and Siderophore. Production of IAA in the presence of a suitable precursor such as tryptophan has been reported for several plant beneficial rhizobacteria belonging to the genera *Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Pantoea, Pseudomonas*, and *Serratia*. Under stressed condition, IAA produced by the rhizobacteria loosens the plant cell walls thereby facilitating root exudation. Bacterial IAA production by rhizobacteria has been reported to increase ACC synthase expression in plants and give a competitive advantage for ACC deaminase producing bacteria over other soil microorganisms. Physiologically active IAA, produced by rhizobacteria can have pronounced effects on plant growth. It promotes root growth directly by stimulating root elongation or the formation of lateral and adventitious roots or indirectly by influencing bacterial ACC deaminase activity (Zazueta *et al*. 2013). Siderophore produced by IIPR-ACC-3 and IIPR-ACC-79 can help to suppress fungal pathogens in the rhizosphere by chelating iron. As the siderophore production is one of the mechanisms involved in the solubilization of iron-bound phosphorus by the microorganisms, bioinoculants with siderophore can also improve the P-availability in rhizosphere soil. Gnotobiotic root elongation assay was carried out with mungbean and chickpea. Seed treatment with 3mM ACC inhibited radical and plumule development due to ethylene stress (Fig. 3). Inoculation of ACC deaminase producing bacteria to treated seeds reduced the ACC levels and enhanced root length and seedling biomass up to 113% over stressed seedlings (Table 2).

ACC deaminase producing bacteria were abundant in plant rhizosphere and almost nonexistent in bulk soil samples. It has also been reported that they are much more abundant in rhizosphere of barely (*Hordeum spontaneum*) growing in stressed environment than unstressed normal field condition (Timmusk *et al*. 2011). This indicated that ACC deaminase producers more readily survive in stressed environments by mutualistic interaction with a host plant.

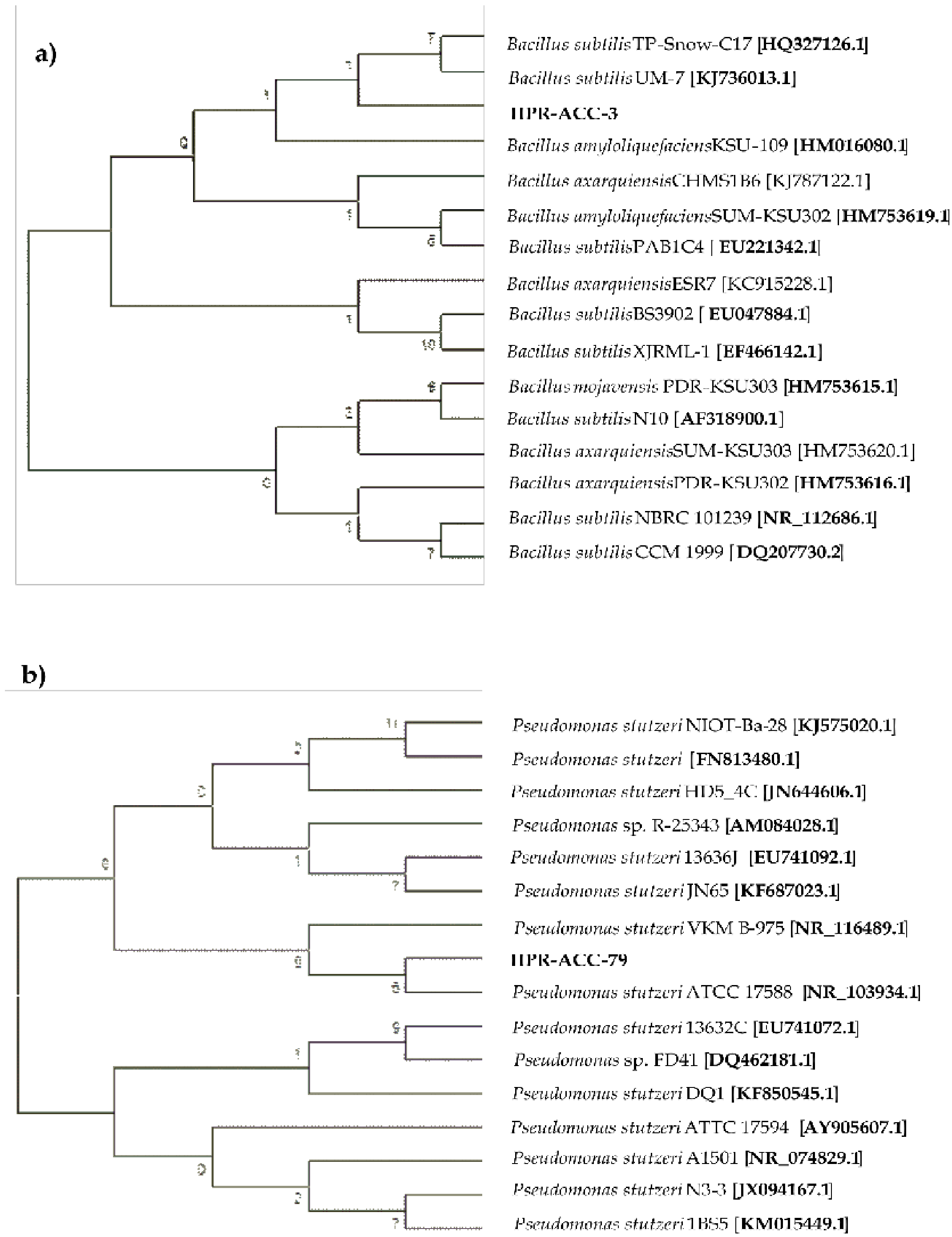

Fig. 2. Phylogenetic relationship among selected ACC deaminase producing rhizobacteria a) IIPR-ACC-3; b) IIPR-ACC-79.

Table 2: *In vitro* root elongation in chickpea by ACC deaminase producing bacteria

Treatments	Bacterial Growth (OD_{600nm})		Root length (cm)	Shoot length (cm)	Plant biomass (g/five seedlings)
	With ACC	Without ACC			
Uninoculated control	-	-	6.87±0.9	15.00±1.3	0.394±0.06
ACC (3mM)	-	-	1.17±0.4	3.10±0.7	0.195±0.03
IIPR-ACC-3	0.411	0.063	7.83±1.2	14.61±1.4	0.364±0.04
IIPR-ACC-3+ 3mM ACC	-	-	6.50±0.9	12.17±2.4	0.349±0.03
IIPR-ACC-79	0.505	0.026	8.00±0.6	13.69±1.6	0.398±0.06
IIPR-ACC-79+ 3mM ACC	-	-	7.16±0.6	12.91±1.7	0.416±0.04
CD (0.05)			1.42	2.81	0.080

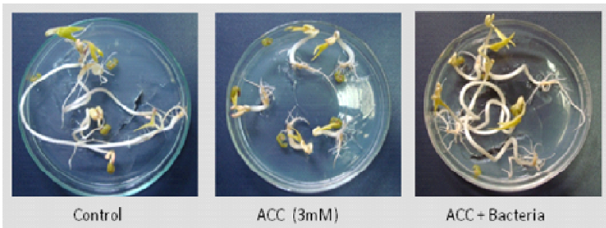

Fig. 3. Effect of inoculation of ACC deaminase producing bacterium on mungbean seedling growth. a) Uninoculated control, b) Seed treatment with 3mM ACC, c) ACC treatment with bacterial inoculation

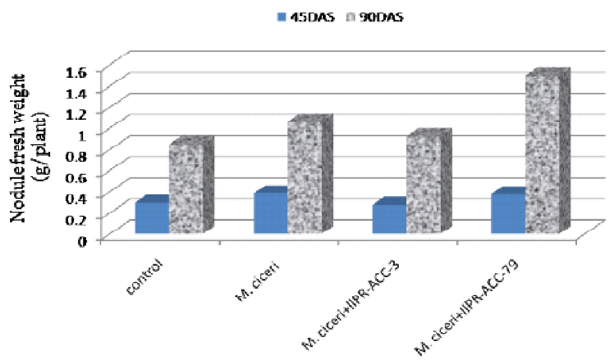

Fig. 4. Effect of ACC deaminase producing bacteria on chickpea nodulation under rainfed field condition.

These microorganisms decrease ethylene levels at root system and facilitate plant health under stress conditions. In turn, healthier plants provide their associated microorganisms with more nutrients thereby increasing the proliferation of these microorganisms. Several researchers demonstrated the ability of ACC deaminase producing bacteria to ameliorate plant stress caused by water logging, nutritional shortage, drought, high salts] and the presence of pollutants (Bal *et al*. 2013; Chookietwattana and Maneewan, 2012; Reed and Glick, 2005). *Achromobacter piechaudii* ARV8, which produces 1-aminocyclo-propane-1-carboxylate (ACC) deaminase, conferred IST to drought stress in pepper (*Capsicum annuum* L.) and tomato (*Solanum lycopersicum* L.) plants. ACC deaminase-producing *Pseudomonas* under growth chamber showed the growth promoting ability in soybean (Husen et al. 2011). However, the efficacy of ACC deaminase producing bacteria to improve plant growth under field soil conditions may vary depending on the range and variability of the environmental factors. Though several bacteria possess ACC deaminase trait as their survival strategy and one cannot expect that all ACC deaminase producing bacteria should improve the plant growth; thereby plant-microbe interaction studies under field condition are essential to identify potential bio-inoculants. In the present study, experiments were carried out to understand the synergistic effect of microbial bio-inoculants on chickpea growth and yield. Root biomass under rainfed condition was improved by seed bacterization with ACC deaminase producing bacterium IIPR-ACC-79 and its combined inoculation with *Mesorhizobium ciceri* strain CR-13 up to 31.59 and 38.34% respectively over uninoculated control. Similarly, under irrigated field condition, co-inoculation of IIPR-ACC-79 with *M. ciceri* enhanced root biomass up to 44.8% compared to uninoculated control. These results indicated that ACC deaminase producing bacteria can reduce ethylene stress under rainfed as well as irrigated conditions caused by several biotic and abiotic stresses. Though the strain IIPR-ACC-3 recorded higher ACC deaminase activity, it did not improve root biomass development during early stages of chickpea under rainfed condition.

Under rainfed condition, single inoculation of ACC deaminase producing bacteria enhanced shoot biomass up to 15% over uninoculated control. However, there is no significant enhancement on shoot biomass of plants co-inoculated with *M. ciceri* and ACC deaminase producing rhizobacteria at early stages of chickpea growth. Under co-inoculated condition, ACC deaminase producing bacteria promoted effective nodulation by efficient strain of *M. ciceri* and helped to fix more nitrogen, which is energy consuming process. At early vegetative stages, part of the photosynthates are translocated and used for higher root biomass development and nodule metabolism rather than shoot biomass development. Higher nodulation and nitrogen fixation at early stages helped the plant to develop 47.91% higher shoot biomass of at 90DAS.

ACC deaminase producing bacteria interact synergistically with *M. ciceri* strain CR-13 and recorded higher grain yield compared to uninoculated or rhizobium alone inoculated plants under rainfed as well as irrigated field. Total yield enhancement by bacterial inoculation was

Table 3: Effect of co-inoculation of ACC deaminase producing bacteria on chickpea growth

Treatment	Fresh Root Biomass at 45DAS (g/plant)		Dry Shoot Biomass at 45 DAS (g/plant)		Dry Shoot Biomass at 90 DAS (g/plant)	
	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed
Uninoculated control	1.587±0.1	1.570±0.1	0.4342±0.02	0.4442±0.03	4.172±0.4	4.30±0.4
Mesorhizobium ciceri	1.722±0.1	1.973±0.3	0.4214±0.03	0.4540±0.02	4.641±0.2	5.41±0.3
IIPR-ACC-3	1.834±0.2	1.763±0.2	0.4332±0.03	0.4998±0.03	5.683±0.3	5.32±0.3
IIPR-ACC-79	2.136±0.3	2.066±0.3	0.4934±0.02	0.5114±0.02	5.796±0.3	5.28±0.4
M. ciceri + ACC-3	2.180±0.2	1.848±0.2	0.4850±0.03	0.3610±0.02	5.361±0.3	6.29±0.2
M. ciceri + ACC-79	2.298±0.2	2.172±0.3	0.4316±0.02	0.4006±0.03	6.076±0.3	6.36±0.4
CD (0.05)	Irrigation (I): 0.170 Treatment (T): 0.294 I x T: 0.415		I: 0.0169 T: 0.0292 I x T : 0.0413		I: 0.214 T: 0.370 I x T : 0.524	

Table 4: Effect of co-inoculation of ACC deaminase bacteria and *M. ciceri* on chickpea yield

Treatments	Pods/10 plant		Seeds/10 plant		100 seed wt. (g)		Seed wt (g/10 plant)	
	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed	Irrigated	Rainfed
Uninoculated control	171±21	147±16	178±22	149±16	35.43±0.6	32.45±0.8	62.35±10.0	54.28±9.7
Mesorhizobium ciceri	173±19	152±14	184±15	180±21	33.66±0.5	31.70±0.7	65.41±8.5	62.91±6.5
IIPR-ACC-3	200±25	188±22	210±25	194±27	33.84±0.8	33.25±1.0	72.32±12.6	67.22±9.6
IIPR-ACC-79	180±27	183±16	190±21	191±15	36.16±0.6	31.72±0.7	68.87±9.4	57.62±8.4
M. ciceri + ACC-3	192±14	169±21	211±10	223±29	36.81±0.4	33.41±1.1	88.45±9.3	79.19±13.8
M. ciceri + ACC-79	199±25	173±18	238±33	228±30	33.78±0.7	33.81±0.8	81.06±12.5	79.04±13.2
CD (0.05)	Irrigation (I): 14.49		I: 14.85		I: 0.516		I: 7.52	
	Treatment (T): 25.10		T: 25.71		T: 0.893		T: 13.02	
	I x T: 35.49		I x T : 36.36		I x T : 1.263		I x T : 18.42	

correlated with 53% higher number of seeds per inoculated plant. Increase in number of pods and seeds per plants inoculated with ACC deaminase producing bacteria and *M. ciceri* strain CR-13 was directly correlated with higher yield. Further studies under multi-location trails are required to evaluate and commercialize the potential ACC deaminase producing bacterial candidates for improving chickpea production under rainfed conditions.

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