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RESEARCH ARTICLE

Potential of ACC-deaminase producing plant growth promoting rhizobacteria on water stress mitigation in lentil (*Lens culinaris* L. Medikus) under axenic conditions

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Abstract

Sixty five rhizobacteria isolated from lentil rhizosphere were evaluated for ACC-deaminase activity and other plant growth promoting traits and found belonging to genera *Bacillus* (24), *Pseudomonas* (37) and *Serratia* (4). Twenty seven of the isolates were able to utilize ACC as a sole N-source, measured in terms of growth (optical density λ_{600}). Highest ACC-utilization was observed with seven isolates and maximum being in isolate 23-B (0.463) among the *Bacillus* spp. and 6-P (0.317) amongst *Pseudomonas* spp. Efficiency of phosphorous solubilization was recorded with six isolates which ranged from 14.6 to 21.6 mg/100 ml. All the isolates produced Indole-acetic-acid ranging from 4.4-22.8 $\mu\text{g/ml}$ in presence of tryptophan. Two isolates 23-B and 6-P were evaluated for their PGP potential in lentil (LL 931) seedlings under PEG 6000 induced water stressed condition and were found to enhance root, shoot length, fresh and dry weight of seedlings over uninoculated seedlings. The most promising influence on lentil growth was observed in case of isolate 23-B which was molecularly characterized as *Bacillus cereus* on the basis of 16S rRNA gene sequencing.

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INTRODUCTION

Increased incidence of abiotic and biotic stresses has become major cause for stagnation of productivity in principal crops including legumes (Ahmed, *et al.* 2010). Among various environmental stresses, some of the important abiotic stresses include salinity, drought, flooding, heavy metal toxicity, chilling and nutrient deficiency (Saleem, *et al.* 2007). Drought or water deficit is the most important of environmental stresses that influences the production (Abdalla and El-khoshiban, 2007). Arshad, *et al.* (2008) reported that inoculation with PGPR containing ACC-deaminase was highly effective in removing the effects of water stress on growth, yield and ripening of peas. Ethylene is a gaseous plant growth hormone produced endogenously by almost all plants and plays a key role in inducing multifarious physiological changes in plants at molecular level. Enhanced endogenous production of ethylene in response to biotic and abiotic stresses adversely affects nodulation, root growth and consequently the growth of the plant as a whole (Shaharoon, *et al.* 2012). To reduce the deleterious effects of ethylene stress plant growth-promoting rhizobacteria (PGPR) that facilitate the proliferation of plants under stress conditions are a potentially viable option. These PGPR are known to affect plant growth either directly or indirectly, the indirect mechanisms include synthesis of antifungal metabolites such as lytic enzymes and hydrogen cyanide (HCN), ability to successfully compete with pathogens for nutrients and niches on the root and to induce systemic resistance (Khalid, *et al.* 2006). Glick, *et al.* (1998) have reported that some PGPR function as a sink for 1-aminocyclopropane-1-carboxylate (ACC), the immediate precursor of ethylene by hydrolysing it into α -ketobutyrate and ammonia, and in this way promote root growth by lowering endogenous ethylene levels in plant. PGPR with ACC-deaminase trait are usually reported to give very consistent results in improving plant growth and yield and thus, are good candidate

for bio-fertilizer formulation. The present study was undertaken with a view to identify ACC-deaminase positive rhizobacteria and to assess their plant growth promoting potential under water deficit conditions in lentil.

MATERIAL AND METHODS

Isolation and characterization of rhizobacteria

Rhizobacteria were isolated from ten different soil samples collected from different lentil growing fields in Punjab. The soil samples were serially diluted up to 10^{-9} dilutions and were plated on nutrient agar (for *Bacillus spp.*) and King's B (*Pseudomonas spp.*) medium followed by incubation at 30°C for 24 h. The isolates were then characterised on the basis of colony morphology, Gram staining and biochemical tests namely: catalase production, nitrate reduction, starch hydrolysis and methyl red test (Cappuccino and Sherman, 1992). These PGPRs were further evaluated for plant growth-promoting traits viz. ACC-deaminase activity, phosphorus solubilizing ability, production of indole acetic acid (IAA) equivalents and also characterized biochemically.

Aminocyclopropane-1-carboxylic acid (ACC)-deaminase activity

ACC-deaminase activity was estimated by the method of Govindasamy, *et al.* (2008). Bacterial isolates were grown overnight and cell pellet collected by centrifugation at 8000 rpm for 5 minutes, washed with sterile distilled water and resuspended in 1 ml of sterile water and spot inoculated on petri plates containing DF salt minimal medium (Dworkin and Foster, 1958) supplemented with 3mM ACC as a sole N-source DF medium alone (negative control) and DF + ammonium sulphate (positive control). The plates were incubated for 3-4 days at 28°C and observed for growth. For quantitative estimation of ACC-deaminase each selected isolate was grown individually in the above liquid media and their growth was measured at 600 nm.

Phosphate-solubilization

Phosphate solubilizing isolates were screened by spotting on Pikovskaya's medium (Pikovskaya, 1948). Inoculated plates were incubated at 30°C and the diameter of clear zone (halo) surrounding the bacterial growth as well as the diameter of colony were measured after 4 weeks and P-solubilization index was calculated.

$$\text{Phosphate Solubilization Index} = \frac{\text{Total diameter (colony + halo zone)}}{\text{Diameter of colony}}$$

The colonies forming clear halos were considered as phosphate solubilizers. Microbial solubilization of insoluble phosphates in liquid media was detected by the method of Jackson (1973). The total phosphorus was estimated with the help of standard curve using different concentrations of phosphorus.

Production of Indole-3-acetic acid (IAA)

Characterization of isolates for the production of IAA was done by method given by Gordon and Weber (1951).

Evaluation of polyethylene glycol 6000 (PEG) induced water stress mitigation potential of rhizobacteria under axenic conditions.

Out of 7 ACC positive isolates, two isolates 23-B and 6-P were selected on the basis of their relative multi-functionality traits and were evaluated for their water stress mitigation potential in lentil under axenic conditions. Seeds of lentil variety LL 931 were obtained from Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana. The experiment was conducted in completely randomized design (CRD) with three replications. Lentil LL 931 seeds were surface-sterilized with 0.1% HgCl_2 solutions for 30-60 sec (Khalid, *et al.* 2004). The treated seeds were rinsed with sterilized water thoroughly. These seeds were inoculated by dipping them in two rhizobacterial cultures (23-B and 6-P). To simulate drought stress, polyethylene glycol solutions (PEG-6000) equivalent to the following water potential -0.2, -0.4 and -0.6 MPa (Mega Pascal) were prepared. Ten seeds of each genotype were germinated in sterilized petri dishes (15 cm diameter) lined with sterilized filter paper moistened with 5 ml solution of PEG-6000 having an appropriate osmotic pressure and kept in BOD incubator at $22 \pm 2^\circ\text{C}$. Each treatment was replicated three times. A seed was considered to have germinated when the emerging radicle elongated to 1 mm. After 7 days percent germination, root and shoot length, fresh and dry weights were recorded. Shoots and roots were separated and weighed to obtain their fresh weight. These samples were dried at 70°C for 72 hrs in an oven and thereafter weighed to determine their dry weight. For Identification of potent isolate, DNA isolation and amplification of 16s rRNA using universal primers was carried out.

Molecular characterization of potential PGPR

Identification of potent isolate 23-B was done using 16s r-RNA using universal primers was carried out at macrogen. Sequencing was carried out using an automated ABI 3100 Genetic analyser at Macrogen.

Accession number and phylogenetic tree analysis

The nucleotide sequence of 16s r-RNA gene were deposited in the NCBI GenBank. The accession number of 16s r-RNA gene nucleotide sequences of the isolate were obtained from NCBI GenBank database.

RESULT AND DISCUSSION

Isolation and biochemical characterization of rhizobacteria

A total of 65 rhizobacterial isolates were isolated from lentil rhizospheric soil samples collected from 10 different locations of Punjab. All the 65 isolates were assessed for cultural, morphological and biochemical characteristics as per Bergey's manual of systematic bacteriology. The isolates were assessed for gram reaction and differences in colony morphology. Out of these, 37 were selected from Kings B medium and all of these showed yellowish green pigment, characteristic of *Pseudomonas* spp. whereas 24 isolates selected from Nutrient agar medium showed typical colony morphology which was predominantly off-white to creamish in colour, typical of genus *Bacillus* and some showed pigments typical to that of *Serratia* (4). The predominance of *Pseudomonas* and *Bacillus* spp. in legume rhizosphere has been reported by many workers (Sahu and Sindhu, 2011 and Yadav, *et al.* 2010). Most of the *Bacillus* isolates were indole and citrate negative but catalase and methyl red positive. The isolates were able to reduce nitrate and hydrolyze starch. The rest of the isolates showed rapid growth on King's B medium at 28°C with production of yellow to green pigment. All of these were gram negative rods and were tested positive for methyl red, Voges Proskauer, catalase and indole whereas negative for citrate test. They were also able to reduce nitrate. Most of them were able to hydrolyse starch also. On the basis of these tests, the isolates were tentatively assigned to genera *Bacillus* and *Pseudomonas* (Table 1).

Aminocyclopropane-1-carboxylic acid deaminase activity

Of the 65 rhizobacterial isolates tested for ability to produce ACC-deaminase, 25 were able to utilize ACC as a sole source of N however, variation in the degree of efficacy to utilize ACC was observed. Three of the *Bacillus* isolates (23-B, 4-B and 8-B) showed highest growth in terms of optical density (0.463, 0.424 and 0.406) whereas *Pseudomonas* (4-P, 6-P, 10-P & 15-P) showed medium growth (0.273, 0.317, 0.180 & 0.176 respectively) (Table 2). Several bacterial species belonging to different genera such as *Azospirillum*, *Burkholderia*, *Bacillus*, *Pseudomonas* and *Ralstonia* have been reported to exhibit variable ACC-deaminase activity which reduces the level of stress ethylene conferring resistance and stimulating growth of plants (Arshad, *et al.* 2008). Govindasamy, *et al.* (2009) in their studies reported that out of 236 bacterial isolates from the wheat rhizosphere, 40 isolates showed growth on DF minimal medium containing ACC. Seven isolates with ACC-deaminase activity were further evaluated for their PGP traits.

Phosphate-solubilization

PSB ability is widespread among many bacterial genera which include *Bacillus*, *Pseudomonas*, *Rhizobium*, *Achromobacter*, *Flavobacterium* etc (Chibuogwu and Nmesoma, 2011). Six out of 15 isolates, exhibited P-solubilization potential and 4 of these belonged to genus *Pseudomonas*. Phosphate solubilizing index ranged from 2.2 to 2.7, highest being recorded with isolate 23-B (Plate 1) followed by 6-P. Under liquid culture conditions P-solubilized ranged from 14.6-21.6 mg/100ml. The bacterial isolate 6-P was found to be the potent P-solubilizer as it showed maximum P-solubilization on 6th day of incubation (21.6 mg/100ml) followed by 8-B (20.2 mg/100ml) and 23-B (17.9 mg/100ml) (Table 3). Mahalakshmi and Reetha, (2009) reported that 83.3% of *Pseudomonas* isolates were P-solubilizers. Gupta *et al.* (2002) described *Pseudomonas* species as a potent phosphate solubilizer. This is in corroboration with the results of the present study.

IAA production

Diverse soil microorganisms are capable of producing auxins, such as indole-3-acetic acid which is an important hormone for plant growth and development, especially associated with root initiation and elongation (Yasmin, *et al.* 2009). As shown in Table 3, all the seven ACC positive isolates also exhibited the production of IAA. Amongst the *Bacillus* isolates, equivalents of IAA produced ranged from 18-22.6ug/ml whereas, *Pseudomonas* isolates produced IAA ranging from 10-21.6 ug/ml in presence of tryptophan, highest being produced by *Bacillus* isolate 23-B (22.2 ug/ml). In the present study isolates of *Bacillus* genus were found to be strong IAA producers as compared to genus *Pseudomonas* and higher IAA production was observed in presence of tryptophan (Plate 2). These results are in accordance to Etesami, *et al.* (2009), they reported that higher IAA production was observed in presence of L-TRP and found significant difference in the potential production of IAA amongst the isolates.

PEG-6000 induced water stress mitigation by rhizobacteria

Rhizobacterial isolate 23-B and isolate 6-P showed relatively good levels of ACC-deaminase production,

P-solubilization, IAA synthesis hence these were selected for germination assay under axenic conditions and for checking water stress tolerance.

Germination assay

Results of germination experiment revealed that inoculation with rhizobacterial isolates 23-B and 6-P exhibited maximum germination (93.3%) at 0.2 MPa and that was equivalent to percentage germination found in case of absolute control. At 0.4 MPa, 23-B exhibited 83.3% germination which reduced to 40% at 0.6 MPa, which was still higher than control (28.3%). Thus significant increase in percentage germination was observed in seeds inoculated with isolates 23-B and 6-P as compared to control at higher stress levels (Fig 1). Application of PGPR isolates have been reported to improve the percentage of seed germination under saline conditions (Mishra, *et al.* 2010).

Effect on seedling growth

In general the seedling growth was reduced under water stress, however as evident from data presented in Table 4 inoculation with ACC-deaminase positive rhizobacterial isolates was effective even under water deficit conditions. Isolate 23-B increased root length significantly at all three osmotic levels viz 2.1, 2.0 & 1.7 cms which was higher than control seedlings, whereas in case of 6-P maximum increase in root elongation was observed at 0.2 MPa (2.8 cm) there after it was comparable to control (Table 4). Similar trend was observed with respect to root fresh weight. Shoot traits were also observed to be affected by water stress. A similar response was recorded in terms of shoot traits, isolate 23-B showed maximum shoot length and at all three osmotic potentials (2.4, 1.6, 1.4 cms), which was relatively higher than the shoot growth in control treatments.

Although the isolate 6-P treated seedlings exhibited comparatively lower growth as compared to 23-B, however the shoot length recorded were higher than the control. Increase in shoot fresh weight in response to inoculation with rhizobacteria containing ACC-deaminase was found to be 81% as compared to uninoculated control. The most promising increase was once again observed in case of 23-B (16.3mg/seedling) followed by 6-P (13.9mg/seedling), more prominently this response was recorded even upto 0.6MPa and was significantly higher than the uninoculated seedlings. This may be due to variable potential of the ACC-deaminase enzyme of the two rhizobacteria to hydrolyse ACC as is evident from the growth studies and also their ability to produce IAA and solubilise phosphorus may be an added contributing factor. It is clear from the data recorded that 23-B and 6-P exhibited profound effect on lentil growth under water stress (Plate 3). These results are in agreement with observations of many researchers who reported better root and shoot growth in plants inoculated with ACC-deaminase positive bacteria (Shaharoon, *et al.* 2012, Abdalla and El-khoshiban, 2007). Ahmad, *et al.* (2011) reported that salinity stress significantly affected some physiological parameters, nodulation, growth and yield in mungbean but co-inoculation with PGPR containing ACC-deaminase and rhizobia reduced the inhibitory effect of salinity.

Identification of potent isolate 23-B using 16s r-RNA sequence homology with Genbank database.

The 16S rRNA sequences obtained after sequencing was deposited in the NCBI GenBank nucleotide sequence database under the accession numbers KP406594 for isolate 23-B. The nucleotide sequence of alkaline protease gene was processed by NCBI Blast function (available at www.ncbi.nlm.nih.gov/BLAST/) for 'multiple alignments' with GenBank database. Phylogenetic analysis based on 16S rRNA gene sequences of isolate 23-B available from NCBI showed 100% similarity with *Bacillus cereus*.

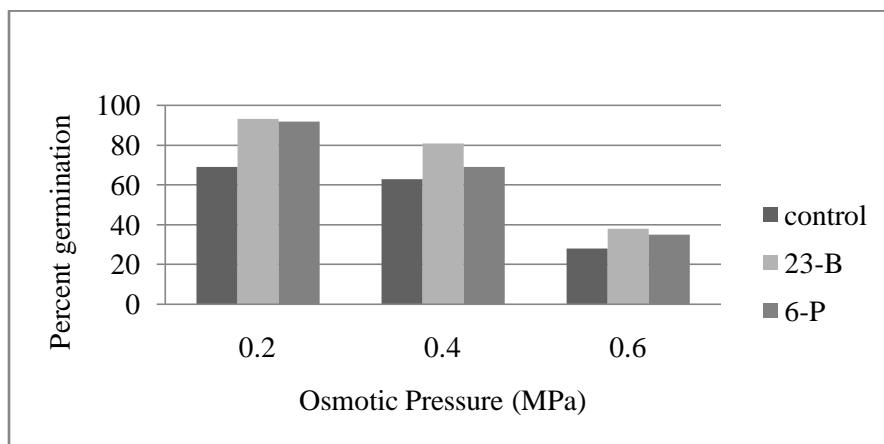


Fig 1: Effect of ACC-deaminase positive rhizobacterial isolates on germination of lentil LL 931 seeds under water stress

Table 1: Cultural and biochemical characteristics of rhizobacterial isolates

Characteristic of organism	test	<i>Pseudomonas</i> spp.	<i>Bacillus</i> spp.
Gram's reaction		-ve	+ve
Shape		Rods	Rods
Pigment		+	-
Pigment colour		Yellow green	White
Starch hydrolysis		+	+
Catalase production		+	+
Methyl red test		+	+
Citrate		-	-
Nitrate production		+	+

Table 2: Relative efficacy of ACC-utilization by rhizobacterial isolates

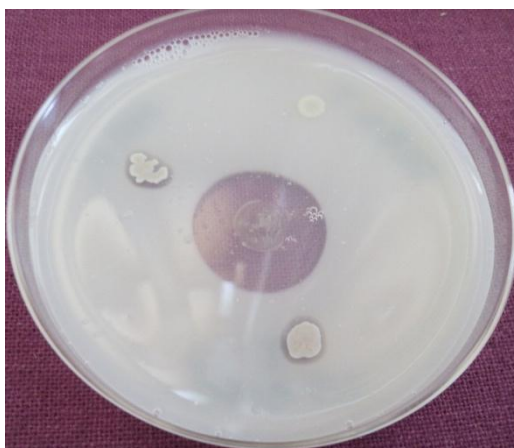
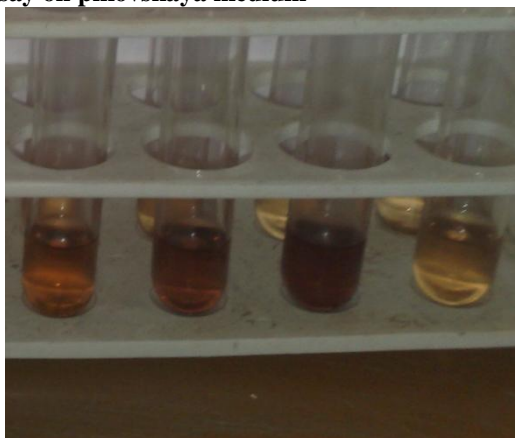
Rhizobacterial Isolates	ACC (+)	OD ₆₀₀		Ammonium sulphate
		ACC (-)		
4-B	0.424	0.020		0.621
6-B	-	-		-
8-B	0.406	0.035		0.644
10-B	-	-		-
13-B	-	-		-
17-B	-	-		-
18-B	-	-		-
23-B	0.463	0.015		0.730
4-P	0.273	0.013		0.670
6-P	0.317	0.053		0.693
10-P	0.180	0.022		0.250
11-P	-	-		-
12-P	-	-		-
14-P	-	-		-
15-P	0.176	0.060		0.347

Table 3: Relative plant growth promoting functionality traits of ACC-deaminase positive rhizobacteria

Rhizobacterial Isolates	P-solubilization index	PGP traits			
		IAA equivalents (µg/ml)		P-solubilization(mg/100ml)	
		L-TRP (-)	L-TRP (+)	6th Day	9 th Day
4-B	-	1.4	19.4	-	-
8-B	2.2	8.8	18.2	20.2	17.2
23-B	2.7	4.7	22.8	14.6	17.9
4-P	-	4.2	10	-	-
6-P	2.4	5.6	21.6	21.6	18.2
10-P	-	0.5	4.4	-	-
15-P	-	3.4	8.2	-	-

Table 4: Effect of ACC-deaminase positive rhizobacterial isolates on root and shoot traits of lentil LL 931 under water stress

Rhizo-bacterial isolates	PGP traits											
	Root Length (cm)			Shoot Length (cm)			Root Fresh Weight (mg/seedling)			Shoot Fresh Weight (mg/seedling)		
	Doses of PEG (MPa)											
	0.2	0.4	0.6	0.2	0.4	0.6	0.2	0.4	0.6	0.2	0.4	0.6
Control	1.6	1.2	1.0	1.4	1.2	1.0	12.3	7.8	7.6	9.0	7.5	8.0
23-B	2.1	2.0	1.7	2.4	1.6	1.4	12.8	11.2	7.7	16.3	12.7	13.1
6-P	2.8	1.2	1.2	1.8	1.3	1.1	14.7	10.7	9.7	13.9	10.7	10.3
Absolute control	4.1	4.1	4.1	3.7	3.7	3.7	22.7	22.7	22.7	19.3	19.3	19.3
CD P = 0.05	0.73	0.54	0.64	1.24	0.97	1.2	5.8	4.38	4.4	5.3	6.3	5.97

**Plate 1: P-Solubilization plate assay on pikovskaya medium****Plate 2: Indole acetic acid production by rhizobacteria in presence of tryptophan**

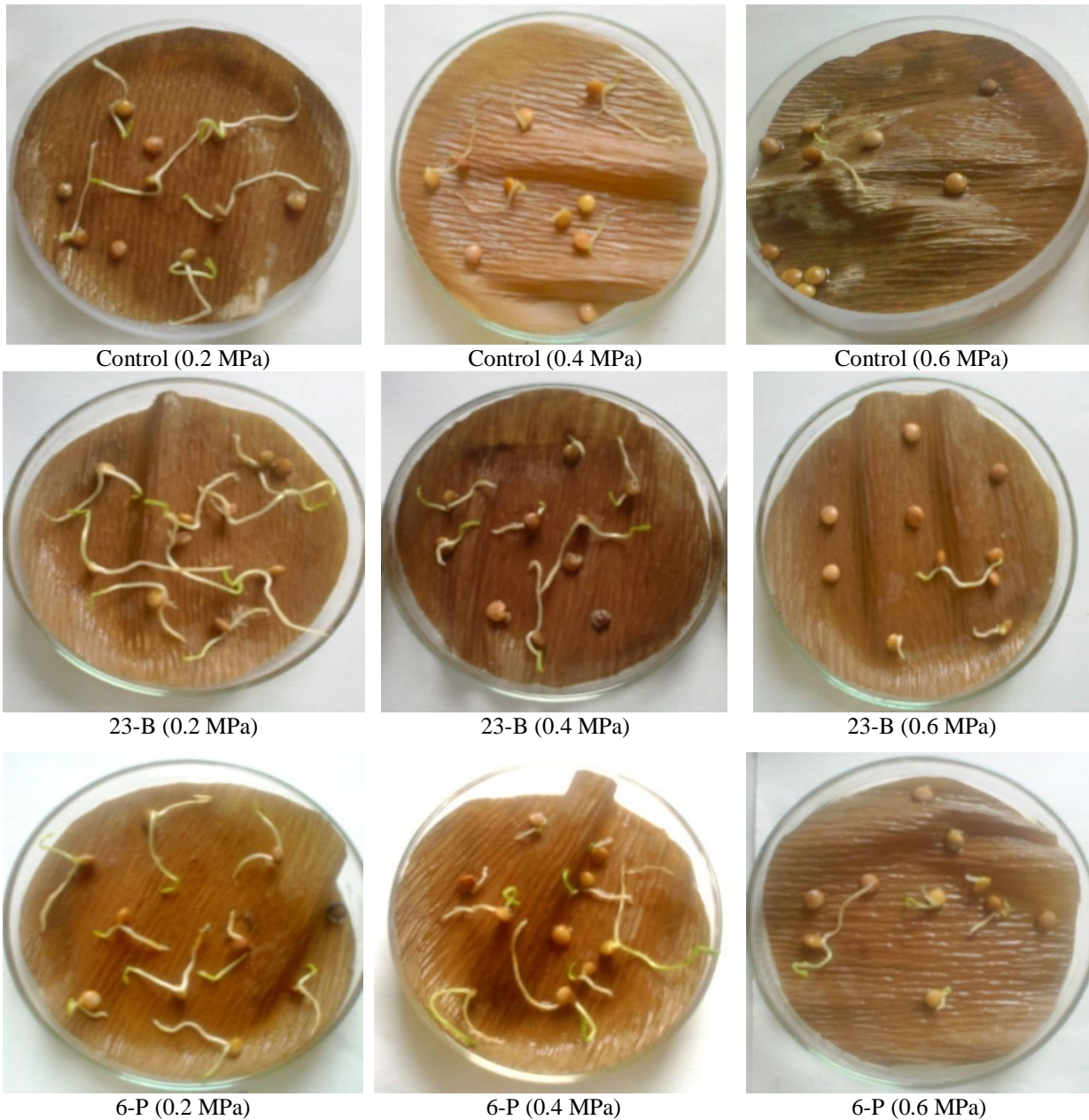


Plate 3: Effect of ACC-deaminase positive rhizobacterial isolates on germination and growth of lentil (LL-931) under water stress

CONCLUSION

The results of present study indicate that inoculation with rhizobacteria containing ACC-deaminase significantly promoted growth of lentil seedlings under water stressed conditions probably through lowering of ethylene in plant at early stage of development as a result of ACC-deaminase activity. The efficiency of application of such inoculants under field conditions would further elucidate their potential in stress amelioration. Screening efficient PGPR's containing ACC-deaminase activity compatible with the environment and host could be very useful approach to enhance the nodulation, growth and yield of legumes under stress conditions.

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