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Preliminary Screening for ACC-deaminase Production by Plant Growth Promoting Rhizobacteria

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Ethylene is well thought-out stress hormone because its synthesis is induced by a variety of stresses. Nodulation and subsequent nitrogen fixation by lentil plants are inhibited by accelerated ethylene concentration in the root zone. Some plant-growth-promoting rhizobacteria (PGPR) promote plant growth by lowering the endogenous ethylene synthesis in the roots through their 1-aminocylopropane-1-carboxylate (ACC)-deaminase activity. A total of 9 rhizobacterial isolates obtained from lentil rhizospheric soils were characterized and found belonging to genera *Bacillus* (6), *Pseudomonas* (3), which were able to utilize ACC as the sole N-source, their ACC-metabolizing rate measured as optical density (OD_{496}) and (OD_{600}) ranged from 0.54-1.39 and 0.165-1.120 respectively. The isolates also showed compatibility with *Rhizobium leguminosarum* under axenic conditions and also promoted root/shoot growth in lentil seedlings. Our results reveal that inoculation with PGPR containing ACC-deaminase and *Rhizobium* could be utilized as expeditious biofertilizers to increase the growth as well as nodulation in legume plants.

Key words: ACC-deaminase, lentil, PGPR, *Rhizobium,* legumes, symbiosis, biofertilizer.

Lentil (*Lens culinaris* Medikus), is a winter crop belonging to the family Leguminoseae. Its seed is rich in proteins, minerals and vitamins for human nutrition and the straw is a valuable animal feed. India is the largest producer and consumer of legumes in the world, the total production being 14.66106 t in. (Singh *et al*. 2008 [1]). However low average yields are largely due to cultivation in neglected marginal lands, poor crop management, inadequate fertilizer inputs.

It is well known that ethylene - a plant hormone plays a regulatory role in almost all the plant development aspects. Ethylene applied directly as a gas or indirectly as 1-Aminocyclopropane-1-carboxylase (ACC), an ethylene precursor in higher plants has been reported to inhibit nodulation. Endogenously synthesized ethylene negatively affects the nodulation, while inhibitors of ethylene synthesis promote nodulation (Ligero *et al*. 1999 [2]). Yuhashi *et al*. 2000[3] stated that the reduced levels of ACC resulted in lower levels of ethylene concentration and consequently promoted the root growth. Plants inoculated with ACC-deaminase producing bacteria have been reported to have better root systems and better ability to resist inhibitory effects of ethylene stress on plant growth imposed by phytopathogens. Thus through these traits some rhizobacteria can also promote plant growth indirectly by enhancing symbiotic nitrogen fixation, nodulation or nodule occupancy. Keeping this in view, the present work was undertaken to isolate and evaluate PGPR from lentil rhizosphere and to evaluate their effect on germination and plant growth parameters of lentil under axenic conditions.

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MATERIALS AND METHODS

The rhizobacterial isolates (nine) were collected from different lentil growing fields, during the year 2009-10 by standard practice of serial dilution and pour plating, using Nutrient agar (NA) for *Bacillus* and King's B (King *et al*. 1954) [4] for *Pseudomonas*.

Production of ACC-deaminase

Qualitative method

The qualitative estimation was done by the method prescribed by Govindasamy *et al.* 2008[5]. Bacterial isolates were grown in LB medium for 24 hours at 150 rpm at 28°C. Cell pellet collected by centrifugation at 8000 rpm for 5 minutes was washed with sterile distilled water and resuspended in 1 ml of sterile water and spot inoculated on Petri plates containing DF salt minimal medium supplemented with 3 mM ACC. Growth of isolates on ACC supplemented plates was compared to positive ($(NH_4)_2SO_4$ as N-source) and negative controls (DF minimal medium without ACC) after 3-4 days incubation at 28°C. Isolates grown well on ACC plates were selected.

Quantitative estimation of ACC-deaminase was done by following two methods:

(a) Each selected isolate was grown individually in liquid DF minimal medium with and without ACC and their growth was measured at 600 nm using a spectrophotometer.

(b) ACC metabolism assay was proceeded according to the method (modified) described by Jacobson *et al.*1994[6]. Isolates were inoculated in Luria broth. Cultures were incubated for 48 hours at room temperature along with shaking. In 96-well micro-titer plate, 122 µl DF (Dworkin and Foster 1958)[7] medium containing $(NH_4)_2 SO_4$ was added in lane 5 and 6 while 6 µl of thawed ACC was filled along with DF medium without any nitrogen source in the lane 1 and 2. Two other lanes 3 and 4 were filled with DF medium without $(NH_4)_2 SO_4$ or substrate ACC. For inoculation of each well 22 µl bacterial culture was used. In uninoculated control wells, 22 µl of distilled water was used in place of bacteria. Optical density (OD_{496}) was measured after 0, 24, 48, 72, and 96 hours at 496 nm. Value of ACC, without ACC/ammonium sulphate and $(NH_4)_2SO_4$ well was compared along with distilled water wells to determine the ability of bacteria to metabolize ACC.

Effect of rhizobacteria on germination and growth of lentil under axenic conditions

Roll towel method

The experiment was conducted to assess the influence of selected efficient isolates of PGPR on seed germination and tested for their plant growth promotion ability by the standard roll towel method (ISTA 1985)[8] in growth chamber.

RESULTS AND DISCUSSION

The isolates were evaluated in detail for their cultural, morphological and biochemical characteristics as given in Bergey's manual of systematic bacteriology. On the basis of these tests, the isolates were tentatively placed into two genera, *Bacillus*[6] and *Pseudomonas*[3].

Production of ACC-deaminase

In the present study, all of the rhizobacterial isolates were able to produce ACC-deaminase in the presence of the substrate ACC. The ability of rhizobacterial isolates to utilize ACC as a source of N was assessed on the basis of bacterial growth on plates containing substrate ACC. All of the isolates were found to show growth but considerable variation was observed with respect to growth, when compared with negative control. Further, the isolates were tested in liquid DF medium first at 600 nm and then by the method of Jacobson *et al.,* (1994) at 496 nm. All the test isolates utilized ACC as N source (i.e. positive for ACC-deaminase enzyme activity) but with different degrees of efficacy and these were grouped into 3 groups on the basis of growth measured as optical density. Rhizobacterial isolates (B-15, B-23 and P-1) showed highest growth ($OD_{600} > 0.50$). Similarly, 5 isolates (B-1, B-2, B-37, B-40 and P-16) showed medium growth ($OD_{600} = 0.50$-0.20), while only 1 isolate (P-23) exhibited least growth ($OD_{600} < 0.20$) by first method. These results were corroborated by the method of Jacobson *et al.,* (1994), B-15, B-23 and P-1 showed highest growth ($OD_{496} > 0.75$) in ACC supplemented medium, 4 isolates (B-1, B-37, B-40 and P-16) showed medium growth ($OD_{496} =$ 0.50-0.75), while 2 isolates (B-2 and P-23) exhibited least growth ($OD_{496} < 0.50$). Similar observations

have been reported by different workers. Govindasamy *et al.,* (2009)[9] observed that from the initial 236 bacterial isolates screened from the wheat rhizosphere, 40 isolates showed growth on DF minimal medium containing ACC. Their OD_{600} values were also higher in liquid DF medium with ACC, when compared to DF medium without any N source. These results are in corroboration with Zafar-Ul-Hye *et al.,* (2007) [10], who grouped lentil rhizobacterial strains into three groups, as strains with higher (OD> 0.7), medium (OD= 0.5-0.69) and lower (OD< 0.5), depending on their growth measured as optical density at 550 nm for ACC substrate as compared to ammonium sulphate and found a direct correlation between *in vitro* bacterial ACC-deaminase activity and root growth.

Table 1. Relative efficacy of ACC-utilization by rhizobacterial isolates

Rhizobacterial isolates	OD_{600}	
	ACC	Ammonium sulphate
B-1	0.214	1.120
B-2	0.230	0.300
B-15	0.788	0.803
B-23	0.644	0.668
B-37	0.201	0.287
B-40	0.271	0.664
P-1	0.534	0.804
P-16	0.240	0.380
P-23	0.165	0.250

Effect of rhizobacteria on germination and growth of lentil under axenic conditions

Germination tests were carried out to determine the effect of inoculation with rhizobacteria showing plant growth promotional traits on seed germination. Results of germination experiment revealed that inoculation with rhizobacteria did not significantly affect the percent germination, however, increase in root by 10% and

Table 2. Grouping of isolates based on their ACC-metabolism assay

Rhizobacterial isolates	OD_{496}		
	Group H O.D. >0.75	Group M O.D.=0.75-0.50	Group L O.D. <0.50
B-1		√	
B-2			√
B-15	√		
B-23	√		
B-37		√	
B-40		√	
P-1	√		
P-16		√	
P-23			√

Table 3. Potential of rhizobacterial isolates to promote germination and growth of lentil seedlings under axenic conditions

Rhizobacterial isolates	PGP traits						
	% Germination	Root Length (cm)	Shoot Length (cm)	Root Fresh Weight(mg)	Shoot Fresh Weight(mg)	Root Dry Weight(mg)	Shoot Dry Weight(mg)
Control	95	3	4.7	105.5	221.5	16.4	26.8
B-40	95	3.3	4.9	146.1	284.4	25.4	36.4
P-1	100	5	4.9	178.7	262.8	26.9	38.7
CD at 5%	NS	NS	NS	NS	NS	3.4	NS

Values represent mean of three replicates with 10 seeds per replication.

66.6% as compared to uninoculated control was recorded. Pseudomonades *isolate* P-1, increased root length up to 66.6% over uninoculated control. Increase in root fresh weights was also recorded by P-1 (178.7 mg/seedling) and B-40 (146.1 mg/ seedling) as compared to uninoculated control (105.5 mg/seedling). The maximum increase (64%) in root dry weight of lentil seedlings was observed in response to inoculation with rhizobacterial isolate P-1, followed by isolate B-40 (54.8%). A marginal increase in shoot length of lentil seedlings was recorded with both the inoculants as compared to uninoculated control, however, 18.6 and 28.4% increase in shoot fresh weight was recorded over uninoculated control. Significant increase in shoot dry weight was observed in case of inoculation with the rhizobacterium P-1 (44.4%) and with isolates B-40 (35.8%). All the parameters recorded were on par with both the rhizobacterial isolates (Table 3). These results are in agreement with the findings of many researchers who reported better plant growth on inoculation with bacteria containing ACC-deaminase (Shahroona *et al*. 2006 [11]; Zahir *et al*. 2008 [12]). It is highly credible that rhizobacteria promoted root growth by lowering ethylene levels in plants and/or in the vicinity of roots. These changes in root architecture of the inoculated plants could be attributed to bacterial ACC-deaminase activity. A co-relation has been observed between root and shoot growth with ACC-deaminase production rates, in the present study, as described earlier by Mayak *et al*. 2004 [13].

These are the preliminary studies for the selection of effective PGPR strains for consequent use as bioagents. These findings may imply that rhizobacteria with ACC-deaminase activity could prove to be effective inoculants for improving growth of lentil plants. Thus ACC-deaminase activity may be a useful criterion for the selection of effective plant growth promoting rhizobacteria.

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