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Screening and characterisation of ACC deaminase producing rhizobacteria from root nodules of clusterbean (*Cyamopsis tetragonoloba*)

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ABSTRACT

Clusterbean plants are suffered with poor nodulation and nitrogen fixation due to ethylene concentration in the root zone. Plant growth-promoting rhizobacteria (PGPR) can help overcome these detrimental effects. Recently, rhizobacteria containing ACC (1-aminocyclopropane-1-carboxylate) deaminases have been found to enhance nodulation and plant growth in various legumes by lowering the levels of ethylene in the plants. The enzyme has been identified in limited number of bacteria and plays a significant role in sustaining plant growth and development under biotic and abiotic stress conditions by reducing stress induced ethylene production in plants. In the present study eighteen rhizobial isolates obtained from the root nodules of clusterbean using trap plant method were screened for drought tolerance using different concentration (0-50%) of polyethylene glycol 6000 (PEG 6000). Twelve of these isolates were tolerant to 30% PEG 6000. All the drought tolerant isolates were screened for ACC utilization using ACC as the sole nitrogen source on Dworkin and Foster's minimal agar medium. Rhizobial isolates GB-15c and GB-22b showed good growth on ACC supplemented plates and these could be used as biofertilizer after evaluating them under field conditions.

Key words: Rhizobia, phytohormone, ACC, legume

INTRODUCTION

Agricultural crops are exposed to many stresses that are induced by both biotic and abiotic factors. These stresses reduce yields of legumes and represent barriers to the introduction of legumes into areas that are not suitable for legume cultivation. The occurrence and activity of soil microorganisms are affected by a variety of environmental as well as plant-related factors. Abiotic stress factors include high and low temperature, salinity, drought and heavy metals (Hu, 2005). The PGPR containing ACC deaminase are present in various types of soils and offer promise as a bacterial inoculum for improvement of plant growth, particularly under unfavourable environmental conditions such as flooding, heavy metals, phytopathogens, drought and high salt. Ethylene is an important phytohormone, but over-produced ethylene under stressful conditions can result in the inhibition of nodulation formation, plant growth or death, especially for seedlings. Plant growth promoting rhizobacteria containing ACC deaminase can hydrolyze ACC, the immediate precursor of ethylene, to α-ketobutarate and ammonia and in this way promote plant growth. Inoculation of crops with ACC deaminase-containing PGPR may assist plant growth by alleviating deleterious effects of salt stress ethylene (Seshadri *et al.,* 2007; Castango *et al.,* 2008). Rhizobabacteria that form symbiosis with legumes having active ACC deaminase, contained a relatively low level of ethylene activity compared with the amount of enzyme activity generally found in free-living soil bacteria (Glick, 2005). As a consequence of reduction in ethylene synthesis, nodulation by rhizobia on legumes was enhanced in alfalfa and *Lotus japonicas* (Nukui *et al.,* 2000), peas (Ma *et al.,* 2004), chickpea (Mann *et al.,* 2002) and *Medicago truncatula* (Prayitno and Mathesius, 2010). Earlier, coinoculation studies of PGPR with *Rhizobium/Bradyrhizobium* spp. have been observed in improving nodulation, root and shoot weight, plant vigor and grain yield of various legumes. Therefore, the postulate of this study is that screening of ACC deaminase-containing rhizobacteria having symbiotic interactions with *Rhizobium/Bradyrhizobium* may result in growth-promoting effects leading to improved legume yield as well as productivity. Clusterbean (*Cyamopsis tetragonoloba*) is a drought tolerant crop and it is grown during the summer season in the northern arid zone of India. Clusterbean is a rich source of high quality galactomannan gum which is in great demand in the world market because of it multi-purpose use in textiles, foods, cosmetics, mining, explosives and oil industries. However, nodulation status of this crop is poor

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and only 5 to 10 nodules are formed per plant by native strains (Rao *et al.,* 1984). Despite multipurpose use of clusterbean, no systematic work has been done to improve the nodulation, nitrogen-fixing ability and crop productivity using bioinoculants. Thus, it is desired that efficient *Rhizobium/Bradyrhizobium* cultures should be isolated and introduced in clusterbean growing areas to improve its nodulation, seed quality and crop productivity.

MATERIALS AND METHODS

Collection of Soil samples: Soil samples were collected from the different fields located at three districts of Haryana state namely, Hisar, Bhiwani and Mahendergarh. Further isolation and characterization of rhizobial isolates from clusterbean root nodules was carried out in screen house at the Department of Microbiology, CCS Haryana Agricultural University, Haryana, India

Isolation of native rhizobia nodulating cluster bean using trap plant method: Five seeds of clusterbean were grown in pots and at later stage three healthy plants were left and rest were removed by thinning process. Each pot contains 2 kg soil collected from South-Western Haryana to trap the rhizobia nodulating cluster bean. After 45 days of growth when proper nodule formation took place the healthy pink nodules were removed separately from each host plant and were surface sterilized by using 0.1% $HgCl_2$ and 70% ethanol. After that the nodules were washed (5-6 times) with sterilized distilled water and crushed. A loopful of nodule sap was streaked on YEMA plates containing Congo red dye (Vincent, 1970). The plates were incubated at 30ºC and growth was observed daily for 3-7 days. The rhizobial isolates were picked up from the plates and were restreaked for purification. Single rhizobial pure isolates were picked up from the plates and maintained on YEMA slants. The slants were stored at 4ºC in a refrigerator for further studies.

Characterization of rhizobial isolates by Gram's staining and Peptone water tests: All the eighteen clusterbean rhizobial isolates obtained from nodules were characterized for Gram staining and peptone water test to check the authenticity of rhizobia. The isolates were inoculated individually in different peptone water containing tubes and incubated at 30ºC for 3-4 days.

Screening of rhizobial isolates for drought tolerance: The effect of drought on rhizobia-growth was studied using polyethylene glycol (PEG) 6000. One hundred microliters of YEM overnight culture was transferred to 10ml of the same YM broth supplemented with 10, 20, 30 and 40% PEG, after incubation at 30^{o}C with shaking at 120 rpm for five days the bacterial growth was measured spectrophotometrically The growth was measured spectrophotometrically at OD 420 nm (Abdel-salam *et al.*, 2010).

Screening of rhizobial isolates for utilization of ACC: The medium plates were prepared with minimal medium (Dworkin and Foster, 1958) supplemented with ammonium sulphate ($2gl^{-1}$) or 3 mM ACC. A loopful of log phase culture of rhizobia was spotted on the medium plates. The growth of bacterial isolates on ACC supplemented medium plates was recorded after 2-5 days of incubation at 30^{o}C. The bacterial cultures showing good growth on ACC supplemented medium plates were scored as ACC positive.

RESULTS AND DISCUSSION

Isolation and screening for drought tolerance

Eighteen rhizobial isolates were obtained from different soil samples collected from arid and semi-arid regions of Haryana. It was observed that all the isolates were found to be Gram negative with small rods. Moreover, the isolates obtained from same soil samples showed identical cell shape and size. Thus, on the basis of Gram staining and plant infectivity test, all rhizobial isolates were selected for drought tolerance. In the present study growth of rhizobial isolates were measured after their exposure to 10 to 40% PEG 6000, for five days. It was observed that as increasing the PEG concentration in the broth, the survival rate of rhizobial isolates decreases. Tweleve rhizobial isolates viz., GB-10a, GB-11c, GB-12a, GB-15c, GB-18a, GB-19a, GB-21a, GB-22b, GB-23c, GB-34a, GH-7c and GM-5b could grow at 30% PEG concentration (Table 1). These results are

orthodoxy with the results obtained by Abdel-Salam *et al.* (2010). The growth and persistence of *Rhizobia* and *Bradyrhizobia* in soils are negatively impacted by drought conditions (Cytryn *et al.*, 2007). Uma *et al.* (2013) studied 30 isolates using YEM broth supplemented with PEG. All the 30 isolates grew well in YEM broth without PEG. As the concentration of PEG increased, the growth was found to decrease. The isolates SBJ-2, SBJ-10, SBJ-14 and SBJ-23 were found to grow at 30% PEG 6000.

Table 1: Drought tolerance by rhizobial isolates

Rhizobial Isolate No.	PEG concentration (%)				
	0	10	20	30	40
GB-10a	0.823	0.396	0.196	0.079	-
GB-11c	0.930	0.442	0.230	0.067	-
GB-12a	0.612	0.408	0.129	0.044	-
GB-15c	0.944	0.628	0.290	0.131	0.008
GB-18a	0.680	0.373	0.100	0.049	-
GB-19a	0.946	0.317	0.205	0.047	-
GB-21a	0.654	0.476	0.261	0.033	-
GB-22b	0.798	0.397	0.245	0.064	-
GB-23c	0.678	0.284	0.086	0.055	-
GB-25a	0.745	0.225	0.122	0.008	-
GB-34a	0.929	0.311	0.269	0.030	-
GH-6a	0.562	0.347	0.104	0.008	-
GH-7c	0.416	0.600	0.409	0.108	0.041
GM-2a	0.760	0.394	0.078	-	-
GM-5b	0.682	0.281	0.131	0.077	-
GM-8b	0.692	0.217	0.065	-	-
GM-13b	0.853	0.423	0.112	-	-
GM-16c	0.645	0.268	0.093	-	-

(- =nogrowth)

Screening for ACC deaminase activity

Plants are constantly exposed to abiotic stress, especially drought, which is one of the most serious problems associated with plant growth and development ultimately affecting agricultural demands. Introduction of drought tolerant rhizobacteria containing ACC deaminase enzyme in the soil having drought conditions can reduce stress in the legume plants by lowering production of ethylene. Drought tolerant microorganisms could survive in these habitats and bound to seed coat or root of developing seedlings and cause deamination of ACC the immediate precursor of ethylene in plants by ACC deaminase leading to lowering of plant ethylene level and thereby facilitating the growth and development of plants (Glick *et al.*, 1998). All the twelve drought tolerant rhizobial isolates were screened for ACC deaminase based on the enrichment method, where ACC was used as the sole nitrogen source. Among twelve isolates, two rhizobial isolates (GB-15c and GB-22b) grew well on minimal medium with either ACC or ammonium sulfate serving as the sole nitrogen source which was compared with minimal medium without nitrogen source. On the basis of rhizobial growth on two medium plates, rhizobial isolates were divided into 4 categories (Table 2). Seven rhizobial isolates i.e., GB-10a, GB-11c, GB-12a, GB-18a, GB-21a, GB-23c and GB-34a could not grow on ACC medium plates and they may require some amino acid for growth on Dworkin and Foster medium. In 2nd category, rhizobial isolates GB-19a and GH-7c showed slight growth on both the plates. Two rhizobial isolates GB-15c and Gb-22b showed similar growth on both the medium plates. Another rhizobial isolates GM-5b showed significant growth on ammonium sulphate plate and less growth on ACC supplemented plates (Table 2).

Table 2: Screening of rhizobial isolates for ACC deaminase activity

Category	Rhizobial isolates	Ammonium sulphate 2 gl^{-1}	ACC (3 mM)
1	GB-10a, GB-11c, GB-12a, GB-18a, GB-21a, GB-23c, GB-34a	+++	-
2	GB-19a, GH-7c	++	+
3	GM-5b	+++	++
4	GB-15c, GB-22b	+++	+++

(Growth of rhizobial isolates were tested on minimal medium supplemented with ammonium sulphate 2gl^{-1} or 3mM ACC. On the basis of colony diameter after 3-5 days of incubation at 30^{o}C, the growth of rhizobial isolates scored as -= no growth, += poor growth, ++= moderate growth and +++= good growth)

In this study, 58.3% of rhizobial isolates showed the ability to utilize ACC. Ma *et al.* (2003b) also observed that only 38.7% rhizobial strains (five positive out of 13 strains tested) possess ACC deaminase enzyme. Similarly, twenty bacterial strains out of 236 rhizobacteria were found to contain ACC deaminase activity based on their ability to grow on ACC containing plates using ACC as a sole nitrogen source (Govindasamy *et al.,* 2009). Our results suggest that, the selection and use of ACC deaminase-producing drought tolerant rhizobia, with multiple PGP activities for the facilitation of plant growth in drought environments, may be a highly important area for future research. Hence,

further evaluation of these drought tolerant bacterial strains is needed to uncover their efficiency as plant growth promoting bacteria in soil plant systems.

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