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Elongation of Shoot and Root in Wheat by ACC Deaminase of *Rhizobium* Spp. Indigenous to Soils of Iran

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ABSTRACT

This study was conducted to investigate the role of 1-aminocyclopropane-1-carboxylate (ACC) deaminase in *Rhizobium* strains indigenous to Iranian soils and their ability to reduce stress ethylene and promote the elongation of the roots of wheat seedlings. One hundred different rhizobial strains isolated from some Iranian soils were cultured on Rhizobial Minimal Medium (RMM) with two different sources of nitrogen (ACC & ammonium chloride). RMM without any nitrogen was taken as the control. From the rhizobial strains tested, sixty five were positive for ACC deaminase and were then divided into three groups of strong, mild and weak based on ACC deaminase production. According to this classification, all of the strong group members and most of the mild ones belonged to *Sinorhizobium meliloti*. In addition, some strains were tested for their ability to promote the root elongation of wheat seedling under gnotobiotic conditions. ACC deaminase containing rhizobia could increase the length of root and also shoot of wheat seedlings significantly, in comparison with the negative control. Those strains belonged to strong group were more successful in elongation of wheat seedling roots.

Key Words: Rhizobia; ACC deaminase; Ethylene; Root elongation; Wheat

INTRODUCTION

An important phytohormone, ethylene, has ubiquitous regulatory functions in almost every stage of plant life. The dramatic effects of ethylene on the growth and development of etiolated seedlings were the basis for the discovery by Neljubov of the physiological action of ethylene. The so-called "triple response" involves a reduction in elongation, swelling of the hypocotyl and a change in the direction of growth (Abeles *et al.,* 1992). For many plants a burst of ethylene is required to break seed dormancy (Esashi, 1991) but, following germination, a sustained high level of ethylene may inhibit root elongation (Jackson, 1991). Roots respond markedly to the ethylene content of their environment (Jackson, 1982). Typically, root growth is stimulated at low concentrations of ethylene and inhibited at higher concentrations. Another interesting response of roots of a number of species to ethylene is the massive production of root hairs (Jackson, 1982). Ethylene may also modify shoot growth. The reduced growth and improved trunk diameter of un-staked shade trees is probably due to ethylene produced in response to flexing stress (Neel & Harris, 1971). It is well documented that plants respond to a variety of different environmental stresses by synthesizing stress ethylene. In stressful conditions, ACC which is the immediate precursor of ethylene in higher plants accumulates and then produces stress ethylene (Abeles *et al.,* 1992). In fact a significant portion of the damage to plants from environmental stress may occur as a direct result of the response of the plant to the increased level of stress ethylene (Van Loon, 1984).

The enzyme of ACC deaminase has been found in various plant growth-promoting rhizobacteria (PGPR) as well as some yeast and fungi (Shah *et al.,* 1998; Jia *et al.,* 2000). This enzyme enables these microorganisms to utilize ACC as a sole nitrogen source by metabolizing it to ammonia and α-ketobutyrate. It was previously proposed that rhizobacteria attached to the surface of plant roots or seeds can take up some of the ACC exuded by the plant and degrade it through the action of ACC deaminase (Glick *et al.,* 1998). To maintain the equilibrium between the internal and external ACC levels, plants exude more ACC and as a result, the biosynthesis of ethylene inside the plant decreases (Penrose & Glick, 2001). PGPR that contain this enzyme, when bound to the seed coat of a developing seedling, may act as a mechanism for ensuring that the ethylene level does not become elevated to the point where root growth is impaired. By facilitating the formation of longer roots, these bacteria may enhance the survival of some seedlings, especially during the first few days after seeds are sown (Glick, 2005).

Plants treated with ACC deaminase-containing bacteria have longer roots (Hall *et al.,* 1996; Shah *et al.,* 1998) and can better resist the inhibitory effects of stress ethylene on plant growth imposed by heavy metals (Burd *et*

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al., 2000), pathogens (Wang *et al.,* 2000) and flooding (Grichko & Glick, 2001). The existence of ACC deaminase has also been found in some strains of rhizobia (Ma *et al.,* 2003). Rhizobia and Bradyrhizobia are well known as the microbial symbiotic partners of legumes, forming N_2 fixing nodules. However these bacteria also share many characteristics with other PGPR and they can colonize the roots of many non-legume plants (Antoun *et al.,* 1998).

Ma *et al.* (2003) reported the presence of ACC deaminase in *Rhizobium* for the first time and this study is the first one conducted in Iran and documenting the presence of this enzyme in rhizobial strains indigenous to Iranian soils. According to the problems farmers face in Iran such as drought, pathogens, flooding occasionally, salinity, etc. and also by considering this fact that native strains can proliferate and act more efficiently in soils of a region. The current study was designed to determine the rhizobial strains in Iran soils having the ability to produce ACC deaminase and survive in the rhizosphere of wheat for promoting the elongation of seedling roots and shoots.

MATERIALS AND METHODS

One hundred strains of rhizobia including *Rhizobium leguminosarum* bv. *Viciea*, *R. leguminosarum* bv. *Phaseoli, Sinorhizobium meliloti, Mesorhizobium ciceri* and *Bradyrhizobium* sp. were selected from the collection of Soil Science Department of Tehran University. They had been isolated from soil samples collected from different regions of Iran, and stored in -80°C.

Growth of rhizobia. All rhizobial strains were streaked on YMA (Yeast extract Mannitol Agar) plates and incubated in 28°C for 3 days to obtain individual colonies. Test tubes containing 8 mL TY broth medium (0.5% Tryptone, 0.3% Yeast extract, 0.044% $CaCl_2.2H_2O$, w/v) were inoculated with an individual colony of each strain and incubated at 120 *g* in a shaker for 2-3 days at 28°C, until they reached stationary phase.

Preparation of ACC solution and RMM. ACC solution (0.3 M), prepared, filter-sterilized through a 0.2 µm membrane and the filtrate collected, aliquoted (150 µL) and frozen at -20°C. Just prior to inoculation, one aliquot was added to the surface of solid RMM in Petri dishes and was spread. RMM was prepared as described by Broughton *et al.* (1986).

ACC deaminase activity. To measure ACC deaminase activity, rhizobial cells (grown in TY medium) were collected using a centrifuge for 10 min at 11,000 × g, washed twice with 0.85% NaCl solution and suspended in 5 mL modified M9 minimal medium (Ma *et al.,* 2003) (5.8 $g.L^{-1}$ Na_2HPO_4, 3 $g.L^{-1}$ KH_2PO_4, 0.5 $g.L^{-1}$ NaCl, 0.25 mM $CaCl_2$, 1 mM $MgSO_4$, 0.15% glucose and 0.3 $\mu g.mL^{-1}$ biotin) supplemented with 4 mM ACC as the sole source of nitrogen and then incubated at 28°C with shaking (120 g) for another 36-40 h, for induction.

The prepared bacterial cells (as described above) were re-suspended in 50 µL 0.85% NaCl solution and used to inoculate RMM plates.

A 4×4 grid was drawn at the back of RMM plates to indicate the right place of inoculation. Three series of RMM plates were prepared: The first and second series were spread with a 150 µL aliquot of 0.3 M NH_4Cl and a 150 µL aliquot of 0.3 M ACC solution, respectively as the sole source of nitrogen, just prior to incubation and were allowed to dry fully. The third series didn't receive any nitrogen source. These three series were inoculated with a 2 µL aliquot of the bacterial suspension (which made a small circle with the diameter of 2 mm on the solid RMM). Each plate was inoculated with eight different strains in two replications in 16 grids, incubated at 28°C and grown colonies diameter measured after 10 days incubation. Following the indication of ACC deaminase containing rhizobia, all positive strains were categorized into three groups of strong, mild and weak. Three strains from strong group, two from mild one, one strain from weak group and two strains not able to produce ACC (negative control) were selected for root elongation assay.

Root elongation assay. The bacterial cell pellet of selected strains as described above was suspended in 1 mL sterile 0.03 M $MgSO_4$ and cell number adjusted evenly by using optical density (600 nm).

Agar slant test tubes were prepared for the gnotobiotic assay by adding nitrogen free Hoagland solution into 1% agar test tubes, sealed fully, wrapped in aluminum foil and autoclaved at 121°C for 15 min.

Wheat seeds (*Triticum aestivum* cv.pishtaz) were soaked in 70% ethanol for 1 min in glass Petri dishes followed by 1% sodium hypochlorite for 10 min. The seeds were thoroughly rinsed with sterile distilled water at least five times and then inoculated with bacterial suspension (sterilized 0.03 M $MgSO_4$ used as negative control) and incubated at room temperature for 24-48 h for germination. Following the incubation period, germinated seeds were placed in each test tube with sterilized forceps under fully sterile conditions, in four replications. The test tubes were placed upright in a rack and incubated in a growth chamber of which max and min of temperature maintained at 28°C and 20°C, respectively with a cycle beginning with 14 h of dark followed by 10 h of light (10000 lux). The longest primary roots and also the length of shoots were measured after 14^{th} day of growth.

Statistical analysis. RMM and test tube experiments were set up in a completely randomized design (CRD) with factorial arrangement. Data were analyzed by means of analysis of variance (ANOVA) and treatment means were compared by LSD test.

RESULTS AND DISCUSSION

The results showed that rhizobial strains indigenous to Iranian soils had active ACC deaminase. About 65% strains could utilize ACC as the sole source of nitrogen (Table I), however this ability was not just the same in all positive strains.

Table I. Grouping of Rhizobial strains based on using ACC from growth media

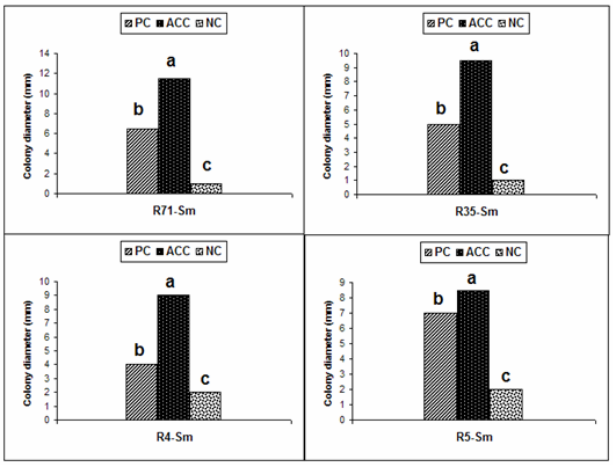
Strains	Strong	Mild	Weak	ACC-d$^-$	Strains	Strong	Mild	Weak	ACC-d$^-$
R71-*Sm*	+	−	−	−	R284-*Rlp*	−	−	+	−
R35-*Sm*	+	−	−	−	R281-*Rlp*	−	−	+	−
R4-*Sm*	+	−	−	−	R261-*Rlp*	−	−	+	−
R5-*Sm*	+	−	−	−	R24-*Sm*	−	−	+	−
R122-*Sm*	+	−	−	−	R240-*Bsp*	−	−	+	−
R17-*Sm*	−	+	−	−	R239-*Bsp*	−	−	+	−
R121-*Sm*	−	+	−	−	R165-*Sm*	−	−	+	−
R81-*Sm*	−	+	−	−	R149-*Sm*	−	−	+	−
R167-*Sm*	−	+	−	−	R144-*Sm*	−	−	+	−
R159-*Sm*	−	+	−	−	R13-*Sm*	−	−	+	−
R133-*Sm*	−	+	−	−	R128-*Sm*	−	−	+	−
R123-*Sm*	−	+	−	−	R127-*Sm*	−	−	+	−
R397-*Mc*	−	+	−	−	R117-*Sm*	−	−	+	−
R277-*Rlp*	−	+	−	−	R10-*Sm*	−	−	+	−
R21-*Sm*	−	+	−	−	R126-*Sm*	−	−	+	−
R16-*Sm*	−	+	−	−	R160-*Sm*	−	−	−	+
R14-*Sm*	−	+	−	−	R384-*Mc*	−	−	−	+
R94-*Sm*	−	+	−	−	R267-*Rlp*	−	−	−	+
R119-*Sm*	−	+	−	−	R315-*Rlv*	−	−	−	+
R6-*Sm*	−	+	−	−	R47-*Sm*	−	−	−	+
R23-*Sm*	−	+	−	−	R279-*Rlp*	−	−	−	+
R137-*Sm*	−	+	−	−	R497-*Rlv*	−	−	−	+
R64-*Sm*	−	+	−	−	R377-*Mc*	−	−	−	+
R226-*Bsp*	−	−	+	−	R139-*Sm*	−	−	−	+
R163-*Sm*	−	−	+	−	R324-*Rlv*	−	−	−	+
R15-*Sm*	−	−	+	−	R20-*Sm*	−	−	−	+
R155-*Sm*	−	−	+	−	R116-*Sm*	−	−	−	+
R22-*Sm*	−	−	+	−	R136-*Sm*	−	−	−	+
R11-*Sm*	−	−	+	−	R272-*Rlp*	−	−	−	+
R69-*Sm*	−	−	+	−	R263-*Rlp*	−	−	−	+
R494-*Rlv*	−	−	+	−	R275-*Rlp*	−	−	−	+
R325-*Rlv*	−	−	+	−	R254-*Rlp*	−	−	−	+
R297-*Rlp*	−	−	+	−	R68-*Sm*	−	−	−	+
R26-*Sm*	−	−	+	−	R148-*Sm*	−	−	−	+
R18-*Sm*	−	−	+	−	R161-*Sm*	−	−	−	+
R158-*Sm*	−	−	+	−	R270-*Rlp*	−	−	−	+
R141-*Sm*	−	−	+	−	R157-*Sm*	−	−	−	+
R140-*Sm*	−	−	+	−	R19-*Sm*	−	−	−	+
R129-*Sm*	−	−	+	−	R162-*Sm*	−	−	−	+
R7-*Sm*	−	−	+	−	R350-*Rlv*	−	−	−	+
R493-*Rlv*	−	−	+	−	R345-*Rlv*	−	−	−	+
R492-*Rlv*	−	−	+	−	R336-*Rlv*	−	−	−	+
R491-*Rlv*	−	−	+	−	R302-*Rlp*	−	−	−	+
R406-*Mc*	−	−	+	−	R135-*Sm*	−	−	−	+
R380-*Mc*	−	−	+	−	R9-*Sm*	−	−	−	+
R348-*Rlv*	−	−	+	−	R138-*Sm*	−	−	−	+
R341-*Rlv*	−	−	+	−	R8-*Sm*	−	−	−	+
R321-*Rlv*	−	−	+	−	R490-*Rlv*	−	−	−	+
R310-*Rlp*	−	−	+	−	R496-*Rlv*	−	−	−	+
R289-*Rlp*	−	−	+	−	R414-*Mc*	−	−	−	+

Rlv: R.leguminosarum bv. *Viciea, Rlp: R.leguminosarum* bv.*phaseoli, Bsp: Bradyrhizobium spp. Mc: Mesorhizobium ciceri, Sm*: *Sinorhizobium meliloti.*
ACC-d$^-$: containing no ACC deaminase enzyme
+, present
−, absent

Table II. The abundance of ACC deaminase containing strains in each group

Strains	Number of strains	Strong	mild	weak	Total
R. leguminosarum bv.*phaseoli*	15	0	1	6	7
R. .leguminosarum bv. *viciea*	15	0	0	8	8
S. meliloti	61	5	15	24	44
M. ciceri	6	0	1	2	3
Bradyrhizobium spp.	3	0	0	3	3
Total	100	5	17	43	65

Fig. 1. Comparison of colony diameters of strong strains on RMM containing ACC, NH_4Cl (PC) and negative control (NC) after 10 days. Bars with the same letter are not significantly different according to Fisher's protected (*P*=0.05) least significant difference (LSD) test. Values represent the mean of two replicates



In this study, the colony diameter of any strain grown on RMM plates with ACC after 10 days was related to the ability of those strains in utilizing ACC as the sole source of nitrogen, which implies the existence of the enzyme ACC deaminase. Therefore all positive strains were categorized into three groups of weak (colony diameter less than 2.5 mm), mild (colony diameter between 2.5 & 5.5 mm) and strong (colony diameter more than 5.5 mm) strains. Five, seventeen and forty-three strains belonged to strong, mild and weak classes, respectively. It implies that although 65 out of 100 of tested strains could utilize ACC as the sole source of nitrogen, just five strains could make a colony diameter more than 5.5 mm after 10 days; more interestingly, all of these strong strains were belonged to *S.inorhizobium meliloti*. Furthermore, most of strains in species, other than *S. meliloti*, were belonged to weak class and mild strains were also very rare in those species (Table II). Considering strong strains, it was observed that the colony diameter on RMM+ACC was even greater than which on RMM+NH_4Cl, even though NH_4Cl is a favorable source of nitrogen for most rhizobia (Fig. 1). The results revealed that all strong strains had a better growth on

Fig. 2. Illustration of a strong strain grown on three plates with different sources of nitrogen. Up left: RMM+NH4Cl, Up right: N-free RMM, and down: RMM+ACC

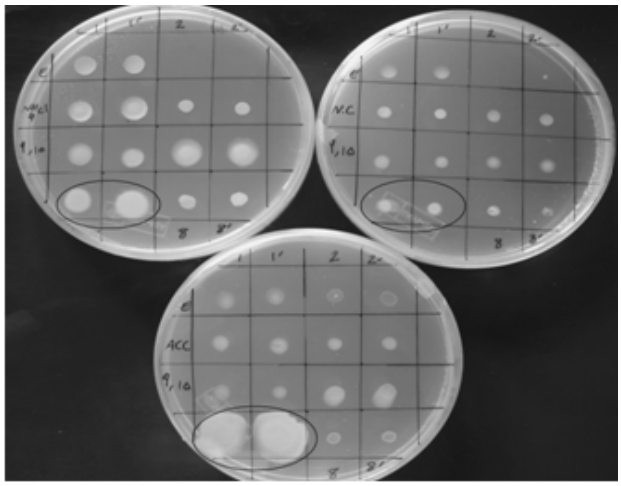
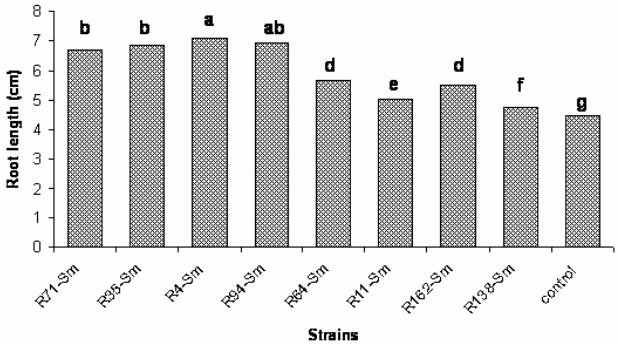


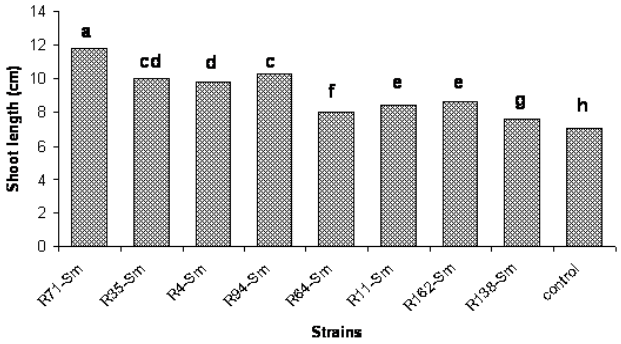
Fig. 3. Comparison of mean length of the longest primary root in different treatments. Bars with the same letter are not significantly different according to Fisher's protected (*P*=0.05) least significant difference (LSD) test. Values represent the mean of four replicates



RMM+ACC compared to RMM+ NH_4Cl. It implies that these strains have a high ACC deaminase activity, which enabled them to grow in a medium with ACC as the sole source of nitrogen. It is also shown that even when apparently nitrogen-free agar is used and there is no additional source of nitrogen in the medium, it is almost impossible to obtain plates with absolutely no bacterial growth but it is possible to get plates with very, very low growth. This phenomenon might be the result of cell lysis, which provides enough nitrogen sources to grow and develop a colony. The difference among colony diameters of one strain grown on three different plates has been illustrated clearly in Fig. 2.

The main role of ACC deaminase enzyme of inoculated plants is to prevent the harmful effect of stress

Fig. 4. Comparison of mean shoot length of different treatments. Bars with the same letter are not significantly different according to Fisher's protected (*P*=0.05) least significant difference (LSD) test. Values represent the mean of four replicates



ethylene and thereby increase the length of plant roots.

But reviewing other research (Grichko & Glick, 2001; Nie *et al.,* 2002; Ma *et al.,* 2004) convinced us to measure the length of shoots as well. Various species and genera of rhizobia are different in consumption pattern of ACC and plant root-bacteria interaction (Belimov *et al.,* 2001). Therefore, the isolates used in the next step for inoculation of wheat seeds and for root elongation test were selected from *S. melliloti.*

All of the rhizobial strains show significant difference in their ability to increase root length (Fig. 3). Strain of R4-Sm, which was considered strong from the standpoint of ACC deaminase production in the first part of this experiment, has caused most root elongation. Control treatment (with no bacteria), showed the least root elongation. R94-Sm strain increased root elongation even though it is considered mild as far as enzyme production is concerned. R11-Sm strain, being in the weak group, did not show much ability for root elongation. However, it is still in a higher level compared to the control treatment.

Comparison of mean shoot length for different treatments of rhizobial strains are showed that shoot length in control treatment is the least and statistically differs from other treatments (Fig. 4). No significant difference was seen among treatments with the isolates of R35-Sm, R4-Sm and also R11-Sm and R162-Sm. The highest increase in shoot length was observed with R71-Sm. Treatments of R4-Sm, R35-Sm and R94-Sm were listed in the lower level with no significant difference.

According to the results obtained by Etesami *et al.* (2005) from the same collection of rhizobial strains, the IAA production test for selected strains showed that none of these strains were classified in very high and high producers of IAA, which is more than 200 µg L^{-1} and 150-200 µg L^{-1} IAA production, respectively. R35-Sm and R138-Sm were classified as moderate (100-150 µg L^{-1}); R71-Sm, R4-Sm, R94-Sm and R11-Sm as low (50-100 µg L^{-1}); and R64-Sm as very low (less than 50 µg L^{-1}) IAA producers. Therefore, we can relate both root and shoot elongation to ACC deaminase enzyme, because those strains which were strong in ACC deaminase activity (i.e., R71-Sm & R4-Sm), were among low producers of IAA. On the other hand, we see that the strain which was considered as none-producer of ACC deaminase (i.e., R138-Sm), was among moderate IAA producers. So, the results for root and shoot elongation were in agree with ACC deaminase activity in comparison with IAA production.

Results obtained from this study revealed that some strains of rhizobia indigenous to Iranian soils had the ability to produce ACC deaminase enzyme. Previous studies (Glick *et al.,* 1998; Burd *et al.,* 2000; Wang *et al.,* 2000; Grichko & Glick, 2001) have shown that all of ACC deaminase containing bacteria may lower the stress ethylene in plant. Ma *et al.* (2004) showed that Alfalfa seedlings inoculated with ACC deaminase-containing *S. meliloti* strains had significantly more nodules, higher shoot length and shoot dry weight than non-inoculated seedlings. Nie *et al.* (2002) found that in the presence of arsenate, transgenic canola plants (that express the *Enterobacter cloacae* UW4 ACC deaminase gene) grew to a significantly greater extent than non-transformed canola plants. Grichko and Glick (2001) stated that although ACC deaminase producing bacteria can also improve the growth of shoot, their major role seems to be on the root development. Our results also support these findings.

According to Prof. Glick (personal communication) ACC deaminase detected in Rhizobia is 10 to 30 times weaker than those recognized in free living bacteria. To sum up, our results show that rhizobial strains indigenous to Iranian soils also have the ability to produce ACC deaminase. In this regard, some strains of *S. meliloti* seem to be more effective and stronger.

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