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Short Communication

Isolation and screening of rhizobacteria containing ACC- deaminase for growth promotion of sunflower seedlings under axenic conditions

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Abstract

The use of rhizobacteria containing 1-Aminocycloprpane-1- carboxylic acid (ACC) deaminase may have beneficial effect on plant growth by regulating endogenously synthesized ethylene. Forty isolates of rhizobacteria containing ACC-deaminase were isolated by using dilution plate technique and screened for growth promotion of sunflower seedlings under axenic conditions. Selected isolates revealed the differential ability to utilize ACC as sole N source. On the basis of determined ACC qualitative assay, 12 isolates were noted for highest, 15 medium and 13 lower ACC-deaminase activity. The results showed that 31 isolates (out of 40) increased the root length, shoot length, root fresh weight, shoot fresh weight, root dry weight and shoot dry weight up to 58, 63, 71, 53, 67 and 55%, respectively, over uninoculated control, while 9 isolates had negative effect on root and shoot growth as compared to control. The isolates showing positive effects were taken as plant growth promoting rhizobacteria (PGPR) for further experimentation. The results indicated that screening effective isolates of rhizobacteria containing ACC-deaminase for growth promotion of sunflower under axenic conditions could be a valuable approach for selecting efficient isolates before testing their potential under natural conditions.

Key words: Inoculation, rhizobacteria, ACC-deaminase, ethylene, sunflower

The activities of the rhizosphere microbial communities extensively influence several aspects of plant physiology and growth and therefore are important for terrestrial ecosystems and agriculture (Dazzo and Ganter, 2009). Rhizobacteria are very important catalysts involved in various interactions which take place between the plant, soil and other microbes in the root zone influenced by compounds exuded by the roots and by microorganisms feeding on them (Antoun and Prevost, 2005). Beneficial rhizobacteria which aid in the growth promotion of plants via synthesis of phytohormones, regulating plant ethylene synthesis, disease suppression improving nutrient uptake and solubilising inorganic phosphate are termed as plant growth promoting rhizobacteria (PGPR) (Morrone *et al.,* 2009; Mehta *et al.*,2010). A bacterium qualifies as PGPR when it is able to produce a positive effect on the plant upon inoculation, hence demonstrating good competitive skills over the existing rhizosphere communities. Generally, about 2 to 5% of rhizosphere bacteria are PGPR (Antoun and Prevost, 2005).

Ethylene is a potent plant hormone and its presence in extremely low concentration could have tremendous effect on plant growth and development (Khalid *et al.,* 2006 a, b). It plays an important role in root initiation, elongation, senescence, abscission and ripening as well as in stress signalling and the rate of ethylene production increases during germination and seedling growth (Arshad and Frankenberger, 2002; Geisler-Lee *et al.,* 2010).

The synthesis of ethylene in plants is directly related to the concentration of ACC in plant tissue (Arshad and Frankenberger, 2002). Some PGPR stimulate plant growth through the activity of ACC-deaminase (Glick *et al.,* 1998). The ACC (an immediate precursor of ethylene) is hydrolyzed into ammonia and α-ketobutyrate by bacterial enzyme ACC-deaminase (Glick *et al.,* 1998; Kamala-Kannan *et al.,* 2010). The uptake and subsequent hydrolysis of ACC by the PGPR, decreases the amount of ACC in the plant, thus plant may develop a better root system by regulating ethylene levels. The presence of ACC-deaminase has been studied in various plant growth promoting bacteria like *Enterobacter cloacae* (Penrose *et al.*, 2001) and *Pseudomonas*, *Variovorax*, *Alcaligenes, Bacillus* (Belimov *et al.,* 2001).

As ethylene is mainly a stress hormone, which at low concentration, facilitates the formation of longer roots by the action of ACC-deaminase therefore, such rhizobacteria may enhance the survival of plant seedlings under various abiotic and biotic stresses (Kausar and Shahzad, 2006; Nadeem *et al., 2006;* Nadeem *et al.,* 2010; Wang *et al.,*

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2000). Thus, the ability to promote root elongation by PGPR is a direct result of the presence of ACC-deaminase and to a greater extent experimental evidence indicates that ACC-deaminase is one of the key mechanisms by which rhizobacteria enhance the growth of plants, especially root elongation (Shahzad *et al.,* 2010). Since the bacterial enzyme ACC-deaminase lowers the level of ethylene in roots, inoculation with PGPR containing ACC-deaminase could be effective for improving growth of sunflower plants.

In the present study, efforts were made to isolate and screen effective rhizobacteria containing ACC-deaminase for their potential to promote growth of sunflower seedlings under axenic conditions.

Isolation of rhizobacteria containing ACC-deaminase

Several rhizobacterial isolates were isolated from the rhizosphere of sunflower. For this purpose, sunflower plants along with roots were collected from University of Agriculture, Faisalabad (UAF), Chiniot and Ayub Agricultural Research Institute, Faisalabad (ARRI). The plants were uprooted and taken to the laboratory in polythene bags. Non-rhizosphere soil from respective plants was removed by gentle agitation of the roots and the soil strictly adhering to the roots was separated to be used as rhizosphere. The rhizobacteria were isolated by using DF-minimal salt medium (MSM) containing ACC as sole N source (Glick *et al.,* 1994).

Dilution plate technique (Wollum II, 1982) was employed for isolation under aseptic conditions. Sterilization of MSM was carried out at 121 ^{o}C for 20 min. The substrate ACC was sterilized by filtering through 0.2 µm membrane filter. Forty fast growing rhizobacterial colonies were recovered. Further streaking on fresh agar plates (2-3 times) was done to purify the collected rhizobacterial isolates coded as QS1, QS2, QS3, QS4, QS5 ……..QS40, and stored at -20^{o}C for further use.

ACC-metabolism assay

For qualitative analysis, an *in vitro* ACC-metabolism assay was carried out to confirm the rhizobacterial isolates for their ability to utilize ACC as sole N source. ACC utilization rates of rhizobacterial isolates were examined on two different N sources [ACC and $(NH_4)_2SO_4$] along with a mineral source $MgSO_4$ as a control. The growth rate of the selected isolates for ACC substrate in parallel to $(NH_4)_2SO_4$ was observed by measuring the optical density at 550 nm by the Microlog System Release 4.2 (Biolog Inc., Hayward, CA). On the basis of their growth on ACC, rhizobacterial isolates were grouped into three categories, as isolates with higher (OD_{550} > 0.80), medium (OD_{550}: 0.80-0.50) and lower (OD_{550} < 0.5) ACC-deaminase activity. ACC-metabolism assay was conducted as described by Jacobson *et al.* (1994).

Five millilitre of ½ tryptic soy broth (TSB) was inoculated with rhizobacterial isolates. The cultures were incubated for 48 hours at 25 ^{o}C under shaking conditions and diluted 10 times by 0.1 M $MgSO_4$. DF-minimal salt medium was used as a principle medium to examine the ACC metabolism assay. In 96-well micro-titer plate, 122 µL DF salt were added in all wells. Lane 3, 6, 9 and 12 were filled with 15 µL 0.1 M $MgSO_4$ and 15 µL 0.1 M $(NH_4)_2SO_4$ was added in lane 2, 5, 8 and 11 whereas lane 1, 4, 7 and 10 were filled with 15 µL of thawed ACC. Twenty two microliter 0.1 M $MgSO_4$ was used in place of bacteria in uninoculated control wells. Optical density (OD_{550}) was measured after 0, 24, 48, 72 and 96 h at 550 nm. Value of ACC and $(NH_4)_2SO_4$ was compared with $MgSO_4$ wells to determine the ability of bacteria to metabolize ACC.

Screening rhizobacteria containing ACC deaminase for growth promoting activity

Rhizobacterial isolates exhibiting ACC-deaminase activity were tested for growth promotion of sunflower seedlings under axenic conditions. Sterilized plastic jars were used for the experiment. For this purpose, each jar was filled with 350 g sand. Twenty millilitre of distilled water was added to each jar before autoclaving. All jars after wrapping in papers were autoclaved thrice at 121 ^{o}C for 30 min. Broth culture was prepared by using MSM having ACC as a source of N. Selected rhizobacterial isolates were used to inoculate the respective test tubes containing 60 mL sterilized broth culture. These test tubes were cultured in a shaking incubator at 30 ^{o}C for 48 h at 100 rpm. Optical density was measured at 550 nm by spectrophotometer and uniform cell density containing OD_{550} = 0.5 (10^8-10^9 CFU mL^{-1}) was achieved.

Seeds of sunflower (*Helianthus annuus* L.) cv. Hysun-33 were surface-disinfected by momentarily dipping them in 95% ethanol followed by 3-5 minutes dipping in 0.2% $HgCl_2$ solutions and 6-7 times repeated rinses with distilled water. Surface-sterilized sunflower seeds were inoculated by dipping them in the respective rhizobacterial broth culture for 15 min. Five inoculated seeds were sown in each autoclaved plastic jars containing autoclaved sand. Uninoculated control was treated with sterilized broth. Jars were incubated at 25±2 ^{o}C in the growth room using completely randomized design (CRD) with three replications for each treatment. Nutrients were supplied by adding 20 mL of sterilized Hoagland solution (½ strength) (Hoagland and Arnon, 1950). While after germination, two

plants per jar were maintained. Light and dark period was adjusted to 10 and 14 h, respectively. After 15 days, the data regarding root and shoot growth were recorded. Standard error of means was calculated (Steel *et al.,* 1997).

ACC-metabolism assay of rhizobacterial isolates was determined and then tested for their intrinsic ability to promote sunflower seedlings under axenic conditions. The results are elucidated below:

ACC-metabolism assay

Principle capacity of rhizobacterial isolates to make use of ACC as a source of N was determined. ACC-utilization assay disclosed that all 40 rhizosphere bacterial isolates metabolized ACC with variable range of effectiveness. On the basis of growth, measured in terms of OD_{550}, 40 isolates were divided into three groups (Table 1) i.e. 12 isolates possessed high (OD_{550} > 0.80), 15 medium (OD_{550}: 0.50-0.80) and 13 Low (OD_{550} < 0.5) ACC-deaminase activity. These isolates were further tested for growth promotion of sunflower seedlings under axenic conditions.

Table 1: Sunflower rhizobacterial isolates showing differential growth on the media containing ACC as sole N source

Group	Isolates
High (OD_{550}>0.80)	QS2,QS5,QS10,QS13,QS14,QS17,QS20, QS24,QS26,QS27,QS31,QS40
Medium (OD_{550} = 0.80-0.50)	QS6,QS7,QS9,QS12,QS15,QS16,QS19,Q S22,QS25,QS29,QS34,QS35,QS37,QS38, QS39
Low (OD_{550}<0.50)	QS1,QS3,QS4,QS8,QS11,QS18,QS21,QS 23,QS28,QS30,QS32,QS33, QS36

Screening rhizobacteria containing ACC-deaminase under axenic conditions

Rhizobacteria exhibiting ACC-deaminase activity, upon inoculation, increased root length over uninoculated control (Table 2). Isolate QS10 showed maximum increase in root length up to 58% over control. Next effective isolates were QS5, QS17, QS26, QS31 and QS40 which promoted root growth up to 57% as compared to uninoculated control. Rest of the isolates gave increase in root length up to 47%, except 8 isolates which showed negative effect on root length up to 7.36% as compared to control. QS24 was the least effective isolate with 15% increase over control on root growth.

Inoculation with ACC-deaminase containing rhizobacteria extensively increased root fresh weight (71.4%) in response to inoculation with QS10 (Table 2). Other prominent root associated bacterial isolates QS2, QS5, QS13, QS14, QS17, QS20, QS24, QS26, QS27, QS31 and QS40 also showed effective increase in root fresh weight ranging from 21 to 69.8% over uninoculated control (Table 2). Remaining isolates also increased the root fresh weight up to 19.32%. But seven isolates showed decrease in root fresh weight compared with control.

The most promising increase (67%) in root dry weight was obtained in response to inoculation with rhizobacterial isolate QS10 followed by QS27 that showed increase in root dry weight of 63.52% as compared with uninoculated control (Table 2). Rest of the rhizobacterial isolates also gave effective increase in root dry weight up to 6.15% compared with control.

The most promising increase (63%) in shoot length was recorded due to inoculation with QS10 as compared to control (Table 3). Next effective isolates were QS5, QS13, QS14, QS24, QS26, QS27 and QS40 which induced shoot length ranging between 41-59% over uninoculated control. Remaining isolates also exhibited their prominence over control but least effective isolate was QS3 while isolates QS6, QS7, QS8, QS18, QS21, QS22, QS29, QS35 and QS36 showed negative effect on shoot length that was up to 17.19% less than control.

Data regarding shoot fresh weight revealed that maximum (53%) shoot fresh weight was recorded due to inoculation with isolate QS40 followed by QS10 isolate (Table 3). Next effective isolates were QS2, QS5, QS13, QS14, QS17, QS20, QS24, QS26, QS27 and QS31 which also promoted shoot fresh weight up to 48.82% in comparison to uninoculated control. Remaining isolates also increased shoot fresh weight up to 22% but QS15 showed only 3.15% increase over uninoculated control. Nine isolates showed decrease in shoot fresh weight as compared to control.

Maximum increase (55%) in shoot dry weight was recorded in case of inoculation with Q20 isolate as compared to control (Table 3). Other efficient isolates were QS2, QS5, QS10, QS13, QS14, QS17, QS24, QS26, QS27, QS31 and QS40 which showed increase in shoot dry weight ranging from 42 to 53% as compared to control. Rest of the isolates also exhibited variable range of effectiveness to increase shoot dry weight in comparison to uninoculated control. While 8 isolates showed negative effect on shoot dry weight up to 4.21% as compared to control.

Plant hormone ethylene is essential in the regulation of several developmental processes and stress responses in plants. Stimulatory effect of ethylene involvement in various physiological processes of the plants has been widely reported (Arshad *et al.,* 2007; Love *et al.,* 2009).

Table 2: Effect of inoculation with rhizobacteria on root growth of sunflower seedlings under axenic conditions

(Mean of three replications ± SE†)

PGPR isolates	Root length (cm)	Root fresh weight plant^{-1} (g)	Root dry weight plant^{-1} (g)
Control	12.50 ± 1.07	1.19 ± 0.014	0.244 ± 0.010
QS1	14.42 ± 1.10	1.37 ± 0.010	0.253 ± 0.013
QS2	18.00 ± 1.13	1.44 ± 0.014	0.301 ± 0.011
QS3	15.50 ± 1.20	1.37 ± 0.015	0.254 ± 0.014
QS4	15.33 ± 1.01	1.31 ± 0.021	0.255 ± 0.010
QS5	19.42 ± 1.12	1.99 ± 0.013	0.365 ± 0.012
QS6	12.00 ± 1.11	1.17 ± 0.015	0.239 ± 0.014
QS7	12.17 ± 1.04	1.17 ± 0.010	0.229 ± 0.009
QS8	12.33 ± 1.13	1.23 ± 0.011	0.252 ± 0.011
QS9	13.58 ± 1.15	1.28 ± 0.014	0.281 ± 0.013
QS10	19.75 ± 1.14	2.04 ± 0.012	0.408 ± 0.014
QS11	15.25 ± 1.10	1.38 ± 0.010	0.249 ± 0.012
QS12	15.58 ± 1.13	1.38 ± 0.013	0.260 ± 0.011
QS13	18.42 ± 1.11	1.79 ± 0.016	0.342 ± 0.010
QS14	17.58 ± 1.10	1.53 ± 0.011	0.363 ± 0.012
QS15	12.58 ± 1.15	1.39 ± 0.010	0.279 ± 0.011
QS16	17.08 ± 1.09	1.35 ± 0.012	0.250 ± 0.013
QS17	19.58 ± 1.12	2.01 ± 0.014	0.299 ± 0.015
QS18	11.58 ± 1.02	1.18 ± 0.013	0.261 ± 0.012
QS19	14.92 ± 1.05	1.42 ± 0.015	0.254 ± 0.013
QS20	17.50 ± 1.07	1.64 ± 0.011	0.346 ± 0.010
QS21	12.03 ± 1.11	1.29 ± 0.009	0.236 ± 0.012
QS22	12.25 ± 1.13	1.12 ± 0.010	0.233 ± 0.011
QS23	16.42 ± 1.16	1.38 ± 0.012	0.238 ± 0.014
QS24	14.33 ± 1.12	1.62 ± 0.011	0.267 ± 0.016
QS25	15.25 ± 1.14	1.31 ± 0.013	0.265 ± 0.012
QS26	19.08 ± 1.17	2.02 ± 0.014	0.310 ± 0.013
QS27	17.33 ± 1.10	1.76 ± 0.010	0.399 ± 0.009
QS28	14.08 ± 1.03	1.34 ± 0.008	0.248 ± 0.005
QS29	12.25 ± 1.10	1.18 ± 0.006	0.242 ± 0.009
QS30	14.75 ± 1.13	1.25 ±0.012	0.264 ± 0.010
QS31	19.08 ± 1.11	1.97 ±0.013	0.327 ± 0.012
QS32	16.58 ± 1.14	1.29 ± 0.011	0.257 ± 0.011
QS33	14.50 ± 1.06	1.40 ± 0.014	0.257 ± 0.007
QS34	14.92 ± 1.10	1.25 ± 0.012	0.275 ± 0.011
QS35	12.08 ± 1.12	1.18 ± 0.011	0.240 ± 0.010
QS36	13.17 ± 1.14	1.16 ± 0.013	0.243 ± 0.008
QS37	15.25 ± 1.13	1.37 ± 0.015	0.261 ± 0.012
QS38	15.00 ± 1.15	1.35 ± 0.010	0.247 ± 0.010
QS39	14.42 ± 1.08	1.28 ± 0.011	0.260 ± 0.011
QS40	19.37 ± 1.20	1.92 ± 0.014	0.366 ± 0.014

†SE: Standar error of means

Keeping in mind the significance of ethylene regulation at extremely low concentration, its production can be controlled by altering endogenous levels of ethylene through ACC-deaminase enzyme possessed by a number of plant growth promoting rhizobacteria, thus preventing the ethylene from becoming inhibitory for plant growth as enzyme converts ACC into α-ketobuyrate and ammonia, instead of ethylene (Arshad *et al.,* 2007; McDonnell *et al.,* 2009; Hao *et al.,* 2010). Lowering of endogenous ethylene levels in plants facilitates better root system. This study demonstrates the effectiveness of rhizobacteria containing ACC-deaminase to increase sunflower growth under axenic conditions.

Table 3: Effect of inoculation with rhizobacteria on shoot growth of sunflower seedlings under axenic conditions

(Mean of three replications ± SE†)

PGPR isolates	Shoot length (cm)	Shoot fresh weight plant^{-1} (g)	Shoot dry weight plant^{-1} (g)
Control	13.50 ± 1.43	1.27 ± 0.11	0.285 ± 0.013
QS1	14.92 ± 1.64	1.34 ± 0.10	0.294 ± 0.016
QS2	17.67 ± 1.12	1.85 ± 0.13	0.347 ± 0.011
QS3	13.75 ± 1.22	1.48 ± 0.17	0.310 ± 0.019
QS4	17.00 ± 1.20	1.38 ± 0.12	0.313 ± 0.021
QS5	21.42 ± 1.76	1.91 ± 0.14	0.436 ± 0.022
QS6	11.08 ± 1.02	1.23 ± 0.11	0.280 ± 0.019
QS7	13.17 ± 1.12	1.25 ± 0.09	0.278 ± 0.014
QS8	12.58 ± 1.10	1.25 ± 0.13	0.273 ± 0.016
QS9	17.57 ± 1.47	1.43 ± 0.16	0.312 ± 0.017
QS10	22.00 ± 2.04	1.93 ± 0.17	0.431 ± 0.021
QS11	15.08 ± 1.34	1.55 ± 0.15	0.361 ± 0.018
QS12	16.42 ± 1.17	1.32 ± 0.11	0.295 ± 0.013
QS13	21.50 ± 1.67	1.92 ± 0.16	0.404 ± 0.014
QS14	19.00 ± 1.41	1.68 ± 0.13	0.405 ± 0.020
QS15	14.25 ± 1.08	1.31 ± 0.08	0.298 ± 0.012
QS16	15.50 ± 1.21	1.35 ± 0.12	0.288 ± 0.011
QS17	17.92 ± 1.32	1.58 ± 0.13	0.331 ± 0.015
QS18	11.50 ± 1.11	1.21 ± 0.10	0.283 ± 0.011
QS19	14.83 ± 1.14	1.48 ± 0.15	0.294 ± 0.014
QS20	17.33 ± 1.23	1.87 ± 0.18	0.441 ± 0.023
QS21	12.50 ± 1.12	1.18 ± 0.13	0.272 ± 0.013
QS22	12.58 ± 1. 02	1.24 ± 0.11	0.263 ± 0.012
QS23	15.75 ± 1.13	1.45 ± 0.10	0.316 ± 0.015
QS24	21.42 ± 2.11	1.64 ± 0.14	0.298 ± 0.014
QS25	17.25 ± 1.09	1.41 ± 0.16	0.297 ± 0.010
QS26	19.33 ± 1.48	1.66 ± 0.19	0.363 ± 0.012
QS27	20.67 ± 1.62	1.89 ± 0.15	0.404 ± 0.018
QS28	14.92 ± 1.22	1.44 ± 0.11	0.312 ± 0.011
QS29	12.67 ± 1. 13	1.22 ± 0.13	0.295 ± 0.013
QS30	14.25 ± 1.19	1.34 ± 0.12	0.364 ± 0.014
QS31	17.58 ± 1.89	1.81 ± 0.15	0.419 ± 0.011
QS32	15.75 ± 1.39	1.40 ± 0.09	0.328 ± 0.010
QS33	15.83 ± 1.31	1.54 ± 0.12	0.293 ± 0.015
QS34	16.42 ± 1.29	1.46 ± 0.10	0.341 ± 0.017
QS35	13.25 ± 1.06	1.24 ± 0.11	0.274 ± 0.013
QS36	13.17 ± 1.12	1.25 ± 0.13	0.280 ± 0.015
QS37	15.92 ± 1.51	1.40 ± 0.11	0.328 ± 0.014
QS38	17.00 ± 1.69	1.43 ± 0.13	0.299 ± 0.019
QS39	17.08 ± 1.86	1.48 ± 0.08	0.316 ± 0.012
QS40	20.00 ± 1.56	1.94 ± 0.16	0.416 ± 0.018

†SE: Standard error of means

A jar study was conducted for screening rhizobacterial isolates having ACC-deaminase for their growth promoting activity under axenic conditions. Results showed that majority of rhizobacterial isolates were able to improve the root and shoot growth of sunflower seedlings. Considerable increase in root length (58%), shoot length (63%), root fresh weight (71%), shoot fresh weight (53%), root dry weight (67%) and shoot dry weight (55%) as compared to uninoculated control was attributable to the intrinsic ability of these rhizobacteria to express ACC deaminase activity and subsequent reduction in the inhibitory ethylene level in roots which also influence shoot growth positively. These results are in conformity with the findings of Asghar *et al.* (2004) and McDonnell *et al.* (2009) as they reported positive

influence of rhizobacterial strains containing ACC-deaminase activity on plant growth. Similar kind of results have also been reported by some other researchers (Bohm *et al*., 2007; Patel *et al*., 2008).

On the other hand, some rhizobacterial isolates did not show competence for growth promotion of sunflower seedlings. It clearly elucidates that the presence of rhizobacteria in the root zone may exert neutral, harmful or beneficial effect on plant growth. Deleterious rhizobacteria are presumed to adversely affect plant growth and development through the production of metabolites like phytotoxins but also through competition for nutrients. These results are in the line of findings of (Zeller *et al.*, 2007; Dooley and Beckstead, 2010) which guarantees the existence of rhizobacteria able to induce a strong negative effect on growth and vegetative development.

Conclusion

These are initial exploratory studies for the selection of effective PGPR isolates. From the present study, it can be concluded that rhizobacterial isolates showed variation in improving plant growth promoting traits of sunflower seedlings. However, it was shown that the PGPR strains having high ACC-deaminase activity were superior for their effectiveness to improve the growth of sunflower seedlings. Thus ACC-deaminase activity may be a useful criteria for the selection of effective plant growth promoting rhizobacteria. Further research should be directed towards the potential use of these efficient PGPR isolates as a bioenhancer for plant growth improvement under natural conditions.

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