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SCREENING RHIZOBACTERIA CONTAINING ACC-DEAMINASE FOR GROWTH PROMOTION OF WHEAT UNDER WATER STRESS

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Abstract

Due to climate changes globally, rainfall has become more erratic causing water shortages in the rainfed regions, resulting in reasonable yield reduction in cereal crops including wheat. Plant growth is inhibited due to higher levels of ethylene production in the rhizosphere under water stress conditions. Rhizobacteria containing ACC-deaminase can facilitate plant growth to overcome these harmful effects. Isolation of rhizobacteria containing ACC-deaminase from the rhizosphere of wheat grown in different districts of Chakwal, Attock and Rawalpindi, were screened for growth promotion of wheat seedlings under axenic conditions. The results of the laboratory experiments conducted on wheat revealed that some rhizobacterial isolates increased root and shoot growth as compared to the uninoculated control. The selected strains were again tested in soil to assess their effectiveness for improving growth of wheat seedlings under water stress soil conditions. The results revealed that inoculation with selected isolates increased the root length (upto 54.6%), shoot length (up to 80.2%), dry root weight (up to 54.2%) and dry shoot weight (up to 95.4%) of wheat seedlings grown at different water levels (i.e., 60, 45, 30 and 15% water holding capacity, WHC) over uninoculated control.Isolated strains belonging to the genus *Serratia* and *Aerococcus* were found to be most effective strains in improving the growth of wheat seedlings in water stress conditions.The results clearly indicate that rhizobacteria isolated from rainfed regions were effective in promoting growth of wheat seedlings under axenic conditions.

Introduction

Water availability is the major limitation to crop yields in the rain fed regions all over the world (Khan *et al*., 2012). As the growing season progresses and surface soil water is depleted, much of the water and nutrient supply function of the roots is determined by a relatively small proportion of roots that are at the leading edge of root system development. The rate of progress of the root system down the soil profile is therefore an important determinant of yield. Ethylene is a plant stress hormone released in response to stress conditions such as water shortage (Saleem *et al*., 2007, Kang *et al*., 2012). During stress conditions, ethylene concentrations increase resulting in plant growth inhibition, especially root growth. The inhibition of root growth impairs the ability of plants to capture water and nutrients resulting in reduced yield (Kulkarni&Phalke, 2009).

ACC deaminase is an enzyme that catalyzes ACC into a-ketobutyrate and ammonia (Glick *et al*., 1998). This enzyme is found in some microorganisms and thus enables them to grow on minimal salts media containing ACC as the sole nitrogen source. Ethylene is synthesized from ACC by using the enzyme ACC oxidase and derived from S-adenosylmethionine by using enzyme ACC synthase (Shaharoona*et al*., 2006).

The presence of rhizobacteria having ACC deaminase enzyme on the roots reduces the accelerated endogenous ethylene synthesis and thus promotes plant growth and yield. So inoculation of seeds with competitive rhizobacteria having ACC deaminase could be one of the most effective approaches for growth promotion of wheat seedlings under axenic as well as field conditions. As the bacterial enzyme ACC deaminase lowers the level of ethylene in roots, so inoculation with rhizobacteria having ACC deaminase could be a successful toolfor promotion of growth in wheat. Keeping in view the above discussion, a study was conducted to isolate and screen the rhizobacteria under axenic conditions from different rainfed areas of Pakistan.The use of PGPR containing ACC-deaminase may prove useful in developing strategies to facilitate plant growth under drought conditions.

Materials and Methods

Laboratory experiments were conducted at the Department of Environmental Sciences, PirMehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan for the isolation of rhizobacteria possessing ACC-deaminase activity for improving wheat growth under water stress (drought) conditions.

Sampling site: The sampling sites covered three districts (Rawalpindi, Chakwal and Attock) of northern Punjab province of Pakistan. Wheat plants (45-60 days old) were collected from 12 different sites of rainfed area during wheat growth (November-April). The rainfall ranged from 39   310 mm per month and temperature ranged from 5   32ºC.

Isolation of rhizobacteria containing ACC-deaminase: Bacterial isolates were isolated from rhizosphere soil of wheat plants (45-60 days old). For this purpose, the plants were uprooted from 12 different sites of rainfed area and were brought to the laboratory in polythene bags. The non-rhizospheric soil was removed by gentle agitation while the soil strictly adhering to the roots (rhizosphere soil) removed by shaking roots in sterilized water.Dilution plate technique (Wollum II, 1982) was used for isolation of bacteria under aseptic conditions. A series of dilutions were prepared from the suspension of rhizospheric soil and then 200 ml of the suspension from each dilution were plated onto agar mineral salt mediumcontaining ACC at the rate of 50 mg L^{-1}as the source of nitrogen. A mineral salt medium of pH 7.2 was used for the isolation of bacteria. The medium was composed (g L^{-1}) of glucose (1.5), KH_2PO_4 (0.5), $MgSO_4 \cdot 7H_2O$ (0.1) and $Fe_2SO_4 \cdot 7H_2O$ (trace amount).

These petri plates were incubated at 30 ˚C for 48 h. Primarily 260 rhizobacterial isolates were selected. These isolates were again tested on the medium spiked with ACC and about 100 isolates exhibiting prolific growth were selected for evaluating their growth promoting activities under axenic conditions.

Effect of rhizobacteria on growth of wheat: A laboratory experiment was conducted in petri plates for screening bacterial isolates with their effective growth promoting activities. Seeds of wheat (var. GA-2009) were disinfected by dipping them in 95% ethanol solution for 20 sec, following a dip in 0.2% (w/v) $HgCl_2$ solution for 30 sec as described by Khalid *et al*., (2004). The treated seeds were rinsed with sterilized water for several times. These seeds were then inoculated with the inocula (OD 0.6 ± 0.02 at 600 nm) of the selected bacteria. The inoculated seeds (6 seeds per plate) were sandwiched between two sterilized filter paper sheets (Whatman filter paper No. 42). Five milliliters of sterilized water were also applied to wet paper sheets. The plates were incubated at 25 ±1 ºC for six days under axenic conditions. Experiment was conducted in a completely randomized design with four replications for each treatment. Data regarding root growth was recorded.

Based on root growth of wheat (petri plate experiment), the most effective 10 isolates of rhizobacteria with ACC deaminase activity were further tested for their potential to enhance wheat growth by conducting jar and growth pouch experiments under axenic conditions. Jars (385 cm^3) filled with 150 g sterilized sand were used. Seeds were sown in pre-sterilized petri plates for germination. Slightly germinated seeds were separated and inoculated with the inocula of respective rhizobacterial isolates (OD 0.6 ± 0.02at 600 nm). Inoculation was done by dipping pre-germinated seeds in the broth culture. Sterilized medium was used for the control treatment. Five milliliter half strength Hoagland solution (Hoagland &Arnon, 1950) was applied in each jar to provide nutrition. The jars were arranged in a completely randomized design with six replications. Jars were placed in a growth chamber at 25 ±1 ºC. Data regarding root length shoot length and dry root weight and dry shoot weight were recorded after 2 weeks.

Another experiment was performed in jars containing soil at different water levels (i.e. 60% of water holding capacity WHC, 45% WHC, 30% WHC and 15% WHC). Water level in each treatment was maintained by adding water daily on weight loss basis. The increase in plant weight was neglected while maintaining water levels. Pre-germinated seeds of uniform size transplanted to jars containing 150g soil. Five rhizobacterial strains (CS5, CC7, CK3, RT7 and RR2) isolated from rainfed area (barani region) were tested in this experiment. Inoculation was done directly by injecting the inocula to soil around the germinated seedlings after one day of transplanting. Sterilized medium was used for the control treatment. The experiment was consisted of four replications for each inoculated and uninoculated treatment. Data regarding root length, shoot length, dry root weight and dry shoot weight were recorded after three weeks.

Bacterial identification: The 16S rRNA gene analysis was performed to identify the most effectivebacterial strains (CC7 and CK3) as described by Mahmood *et al*. (2012).Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). Sequences data of the strains CC7 and CK3 were submitted to BankltNCBIwithaccessionnumbers KC425221 and KC425222, respectively. Advance blast search results showed that the strains CC7 and CK3 had more than 99% resemblance with the previously described spp. *Serratiaodorifera* and *Aerococcusviridians*, respectively.

Statistical analysis: The data were analyzed in a Microsoft® Excel 2007 spreadsheet for calculating means and standard deviations

Results and Discussion

The isolated rhizobacteria were first tested for their ability to utilize ACC as source of N and later on the rhizobacteria capable of utilizing ACC were screened for improving growth of wheat seedlings under axenic conditions. Out of 260 isolates, 102 bacterial isolates showed prolific growth on ACC medium (Table 1). The ability of rhizobacterial isolates to utilize ACC as a source of N was assessed on the basis of bacterial growth. This implies that ACC-deaminase enzyme in different rhizobacteria might have potential to hydrolyze ACC and, thus could have different effect on growth of inoculated plants. Out of the 102 isolates, 56% were isolated from the Chakwal district; whereas 35.3 % and 8.8 % were isolated from Attock and Rawalpindi districts, respectively. Several bacterial species belong to different genera such as *Azospirillum, Agrobacterium, Achromobacter, Burkholderia, Enterobacter, Pseudomona*s and *Ralstonia*have been reported to possess variable ACC-deaminase activity (Nadeem*et al.,* 2007; Arshad *et al.,* 2008).

The Petri plate experiment was conducted under axenic conditions and the results obtained revealed that the isolates had a positive effect on the root growth of wheat seedlings (Table 1). Inoculation with rhizobacteria containing ACC-deaminase increased the root length of the wheat seedlings ranging from 21 to 23% over uninoculated control.The maximum (25.5 cm) root length of wheat seedlings was observed in response to inoculation with an individual rhizobacteria isolated from Chakwal district.Similarly,increase in root dry weight in response to inoculation with rhizobacteria containing ACC-deaminase was found up to 285%as compared to uninoculated control. The most promising increase was observed in case of inoculation with the rhizobacteria isolated from Rawalpindi district. It was also observed that indigenous bacterial isolates showed increasein number of lateral roots that were 25 to 50 % higher in comparison to uninoculated control. It is highly likely that rhizobacteria promoted root growth by lowering ethylene levels in plant and/or in the vicinity of roots because of their ACC-deaminase activity. Many researchers have reported betterroot growth in plants inoculated with bacteria containing ACC-deaminase (Glick *et al.,* 1995; Mayak*et al.,* 2004; Shaharoona*et al.,* 2006).

Table 1. Isolation of bacteria from different sites of rainfed area capable of growing on ACC medium and improving root growth of wheat (var. GA-2009) under axenic conditions.

Sampling sites	Bacteria isolated	Petri plate experiment under axenic condition[a]							
		Root length (cm)		No. of lateral roots		Fresh root weight (g $plant^{-1}$)		Dry root weight (g $plant^{-1}$)	
		Range	Mean ± SE[b]	Range	Mean ± SE	Range	Mean ± SE	Range	Mean ± SE
Control			8.440		4.000		0.089		0.014
Chakwal	57	5.16-25.5	10.261±0.43	2.0-6.0	3.793±0.11	0.023 - 0.439	0.169±0.002	0.003 - 0.058	0.018±0.011
Attock	36	4.9-20.75	10.437±0.49	3.0-6.0	3.833±0.12	0.047 - 0.416	0.171±0.003	0.003 - 0.112	0.020±0.014
Rawalpindi	9	6.4-12.21	10.415±0.68	2.0 -5.0	3.455±0.26	0.004 - 0.46	0.150±0.004	0.004-0.46	0.059±0.021

[a]The experiment was conducted in petri plates under controlled conditions and the data are average of four replications
[b]Standard error.

Table 2. Effect of selected rhizobacterial isolates on root growth of wheat (var. GA-2009) at different water levels under axenic conditions.

Rhizobacterial Isolates	Root length (cm)				Dry root weight (g $plant^{-1}$)			
	60%[a]	45%	30%	15%	60%	45%	30%	15%
Control	7.63±1.82[b]	8±2.63	8.8±0.84	12.4±2.17	0.024±0.001	0.018±0.001	0.014±0.002	0.021±0.001
CS5	5.7±2.16	8.6±2.41	10.1±1.67	10.43±0.12	0.006±0.001	0.037±0.001	0.024±0.003	0.031±0.001
CK3	9.73±1.45	11.8±1.76	12.3±1.24	10.83±0.73	0.028±0.001	0.028±0.002	0.028±0.001	0.03±0.001
CC7	11.8±1.48	12.5±1.30	11.5±2.52	12.13±0.57	0.037±0.00	0.028±0.001	0.031±0.001	0.037±0.002
RT7	5.35±0.97	11.15±1.12	12±0.65	12.7±2.37	0.01±0.003	0.015±0.001	0.018±0.001	0.023±0.001
RR2	8.05±0.00	11.05±2.45	9.7±0.94	17.5±1.80	0.019±0.001	0.015±0.002	0.015±0.001	0.015±0.001

[a]Water holding capacity (WHC)
[b]Standard error

Table 3. Effect of selected rhizobacterial isolates on shoot growth of wheat (var. GA-2009) at different water levels under axenic conditions.

Rhizobacterial Isolates	Shoot length (cm)				Dry shoot weight (g $plant^{-1}$)			
	60% [a]	45%	30%	15%	60%	45%	30%	15%
Control	5.05±1.73 [b]	10.3±0.38	11.3±0.27	8.15±0.57	0.022±0.001	0.02±0.002	0.02±0.001	0.016±0.001
CS5	1.85±0.55	11.37±2.07	12.9±0.18	10.67±0.41	0.006±0.001	0.035±0.001	0.03±0.001	0.032±0.001
CK3	9.1±2.31	12.57±0.32	12.7±1.48	10.4±0.75	0.025±0.001	0.043±0.001	0.041±0.001	0.03±0.003
CC7	2.85±2.09	13.05±0.92	12.6±0.51	10.3±0.32	0.035±0.001	0.039±0.001	0.06±0.001	0.032±0.001
RT7	6.6±1.71	13.8±0.32	13.2±0.37	11.37±1.27	0.006±0.002	0.025±0.003	0.029±0.001	0.03±0.001
RR2	3.85±0.97	7.95±0.20	11.5±2.00	12.7±0.78	0.012±0.001	0.016±0.001	0.016±0.002	0.02±0.001

[a]Water holding capacity (WHC)
[b]Standard error

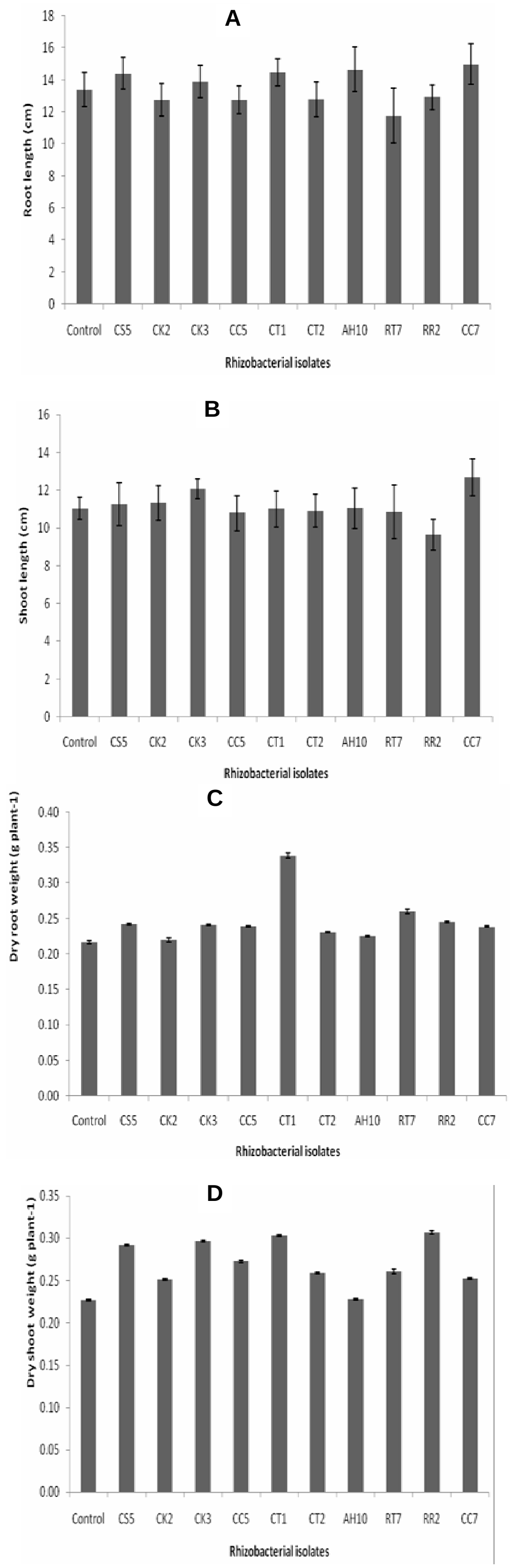

Fig. 1. Effect of inoculation on a) root length, b) shoot length, c) dry root weight and d) dry shoot weight of wheat seedlings in jar trial under controlled conditions.

Results of jar trial revealed that inoculation with rhizobacteria containing ACC-deaminase increased root length up to 10% over uninoculated control (Fig. 1a). Isolates CC7, AH10, CT1 and CS5were found to be the most effective. The remaining isolates also showed effectiveness in promoting root growth.Increase in shoot length in response to PGPR inoculation was found more than 20% over uninoculated control by isolate CC7 (Fig. 1b). The most promising increase was observed in case of inoculation with isolates CC7 and CK3. The results obtained for dry root weight showed that inoculation with ACC-deaminase containing rhizobacteria increased dry root weight extensively.Maximum increase was observed in case of CT1 and RT7 isolates that increased root weight up to 50% over uninoculated control (Fig. 1c). The dry shoot weight in response to inoculation with PGPR containing ACC-deaminasewas more than uninoculated control (Fig. 1d). Most promising increase (30%) was observed in case of inoculation with isolates RR2, CT1 and CK3 as compared to uninoculated control.

Based on the potential of different rhizobacterial isolates to promote growth (primarily root growth), five more efficient PGPR isolates were further studied for their effectiveness to improve wheat growth under different water levels. The wheat seedlings were grown at 4 different levels of water (WHC) and the results regarding the effect of PGPR isolates (CS5, CK3, CC7, RT7 and RR2) on the root length of wheat seedlings are shown in Table 2. At 60% WHC, CC7 was the most effective isolate in increasing root length of wheat up to 60% over uninoculated control.Next to it, CK3 and RR2 isolate significantly increased root length upto 30 % compared to the control. Similarly, CC7 also increased root length by 60% at 45% WHC compared to uninoculated control. Maximum increase in root length was caused by strain CC7 and it was followed by isolate CK3, RT7 and RR2 (50% increase over control). At 30% WHC, all isolates increased root length ranging from 10 to 40% over uninoculated control. The highest root length (12.3 cm) was recorded for the isolate CK3 (up to 40 %over uninoculated control) and differed significantly from all other isolates. Under high water stress (15% WHC), isolate RR2 showed the highest increase in root length (40% over the control). The isolates RT7 and CC7 had almost similar effect on root length and up to 2.41% increase was observed as compared to control.The rhizobacterial isolate RR2 significantly increased the number oflateral roots (up to 33%) at 60% WHC compared to control. All other isolates produced number of lateral roots similar to that observed in case of control. At 45 and 30% WHC, maximum increase of 33% in number of lateral roots was observed in response to inoculation with PGPR isolate CC7 as compared to control. However, increase in number of lateral roots was not observed at 15% WHC. In case of dry root weight, CC7 significantly increased dry root weight at all water levels compared to control. The increase was 76% over the control under high water stress (15% WHC). Significant increase in dry root weight was also recorded for isolate CK3 at all water levels except 15 % WHC.

Among newly isolated PGPR, CK3 was most effective in increasing shoot length at 60% WHC, which was 80% greater than control (Table 3). The next effective isolate was RT7 increasing the root length by 30.7% over the control; however, increase was not observed in CS5, CC7 and RR2. Significant increase in shoot length was recorded for RT7 that increased shoot length by 34 and 16.8% at 45 and 30% WHC respectively. At 45% WHC all other isolates had similar results increasing the shoot length up to 31%. However at 30% WHC, isolate RT7 was followed (descending order) by CS5, CK3 and CC7. They all differed significantly from the control. At 15% WHC, all strains showed significant increase in shoot length over the uninoculated control. The most promising increase was noted for the isolate RR2 followed by RT7, increasing the shoot length by 55.8 and 39.5% respectively. At 60% WHC, maximum increase (59%) in dry shoot weight was recorded in case of isolate CC7 followed by CK3 increasing the dry shoot weight by 13.6%, whereas the other isolates did not show positive increase in dry shoot weight. All isolates revealed significant increase (up to 115%) over the control at 45 and 30% WHC. Isolates CS5 and CC7 were most effective in increasing dry shoot weight by 97.5% at high water stress (15% WHC). Isolate CC7 was highly effective to enhance total biomass at all water levels and significantly increased it by 56.6, 76.3, 167 and 277% at 60, 45, 30 and 15% WHC, compared to uninoculated control. Isolate CS5 was the most effective isolate to increase total biomass (up to 89.4% over control) at 45% WHC.

The rhizobacterial isolates were capable of promoting dry root weight and root length of wheat seedlings. Rhizobacterial strains belonging to *Aerococcus* and *Serratia* spp. were very effective in improving plant growth and this change in the root architecture is credited to the bacterial ACC deaminase activity. Similarly the isolates had the potential to promote shoot growth such as shoot length and dry shoot weight.Ghosh*et al*.,(2003) reported that rhizobacteria containing ACCdeaminase promoted plant growth, while root length was significantly increased under axenic conditions. Similar kinds of findings have been documented by other researchers (Dodd *et al.,* 2004; Sergeeva*et al.,* 2006). These findings may imply that rhizobacteria with ACCdeaminase activity could prove to be effective inoculants for improving growth of wheat plants. The isolates CC7 and CK3 (isolated from water stress area of Chakwal district) were very effective in improving root growth and architecture at different water levels applied to soil. It is highly probable that these isolates promoted root growth of wheat by lowering ethylene levels in roots (Glick *et al*., 1998; Arshad *et al*., 2008). Suchrhizobacteria might also have promoted shoot growthindirectly through increased root growth that provides nutrients to the plant. The inoculated wheat seedlings were tolerant to water stress as compared to uninoculated seedlings at different water levels.

Ethylene is a stress hormone and is produced at higher concentration under any kind of stress including water stress. It is very likely that the rhizobacterial isolates promoted root and shoot growth by lowering the endogenous inhibitory levels of ethylene in roots because of its high ACC metabolizing ability (Kang *et al*., 2010).

Such promising isolates could possibly be used under field conditions in rainfed environment where agriculture is exclusively reliant on rainfall (Hamayun*et al*., 2010).

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