Pak. J. Bot., 38(5): 1491-1499, 2006.

1-AMINOCYLOPROPANE-1-CARBOXYLATE (ACC)-DEAMINASE RHIZOBACTERIA EXTENUATES ACC-INDUCED CLASSICAL TRIPLE RESPONSE IN ETIOLATED PEA SEEDLINGS

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Abstract

Ethylene is well thought-out stress hormone because its synthesis is induced by a variety of stresses. 1-Aminocyclopropane-1-carboxylate (ACC) is the immediate precursor of ethylene in higher plants. Some rhizobacteria can hydrolyze ACC into ammonia and α-ketobutyrate because of their ACC-deaminase activity. This study was conducted to investigate the influence of ACC-deaminase rhizobacteria on the ACC-induced classical "triple" response in etiolated pea seedlings. Etiolated pea seedlings were exposed to different concentrations of ACC (0, 2, through 10 mmol L^{-1}), in 100 ml glass beakers placed in airtight mason jars wrapped in green foil and incubated under dark for seven days. In another study pea seedlings were inoculated with five strains of rhizobacteria which vary in their ACC-deaminase activity. These inoculated pea seedlings were exposed to 10 mmol L^{-1} ACC and incubated in the darkness at 25 ± 3 ºC. Results revealed that exogenous application of ACC had a concentration-dependent effect in creating classical "triple" response in etiolated pea seedlings. Inoculation with rhizobacteria decreased the ACC-imposed classical "triple" response in etiolated pea seedlings, as significant increases in seedling length (up to 4.6-folds) and root elongation (up to 3.9-folds) were recorded over uninoculated ACC-stressed control. Stem diameter was significantly decreased (up to 31%) than uninoculated ACC-stressed control in response to inoculation. A significant ($P < 0.05$) positive correlation ($R^2 = 0.91$) was recorded between ACC-deaminase activity and seedling length which implies that ACC-deaminase activity of rhizobacteria was responsible in decreasing the classical "triple" response in etiolated pea seedlings. Our study concludes that the inoculation with rhizobacteria containing ACC-deaminase could be used to decrease ACC which produces due to a variety of biotic and abiotic stresses in plants.

Keywords: ethylene, classical triple response, ACC deaminase, Rhizobacteria, etiolation

Introduction

The gaseous plant hormone ethylene participates in the regulation of many developmental processes throughout the life cycle of plants (Abeles *et al*., 1992; Reid, 1995). Besides its physiological roles in different developmental stages, ethylene is also regarded as a stress hormone because its synthesis is induced by a variety of stress signals, such as mechanical wounding, chemicals and metals, drought, extreme temperatures and pathogen infection (Kende, 1993; Morgan & Drew, 1997; Johnson & Ecker, 1998). 1-Aminocyclopropane-1-carboxylate (ACC) is an immediate precursor of ethylene in higher plants. Production of ethylene in plants is highly dependent on endogenous levels of ACC (Lürssen *et al.*, 1979; McKeon *et al*., 1982). Therefore, in the

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early stages of plant response to stress, ACC accumulates concomitantly with a rapid burst in ethylene production (Morgan & Drew, 1997).

It has been discovered that certain microorganisms contain an enzyme ACC-deaminase that hydrolyses ACC into ammonia and α-ketobutyrate (Glick *et al.,* 1994a,b; 1998; Mayak *et al.,*1999; Shaharoona *et al.,* 2006a) instead of its conversion into ethylene. The uptake and cleavage of ACC by ACC-deaminase containing rhizobacteria decrease the amount of ACC, as well as ethylene, in the roots, thereby acting as a sink for ACC. Decreased levels of ACC result in lower levels of endogenous ethylene, which eliminate the potentially inhibitory effects of stress-induced higher ethylene concentrations (Glick *et al.,* 1998). Recently, several reports have indicated that under gnotobiotic conditions, inoculation with rhizobacteria containing ACC-deaminase increased growth of the inoculated plants primarily through regulation of ethylene synthesis in the inoculated roots (Jacobson *et al.,* 1994; Glick *et al.,* 1995; Li *et al.,* 2000; Penrose *et al.,* 2001; Ghosh *et al.,* 2003; Shaharoona *et al.,* 2006a, b). Also, the plants that are treated with PGPR containing ACC-deaminase are dramatically more resistant to the deleterious effects of stress ethylene that is synthesized as a consequence of stressful conditions such as heavy metals (Grichko *et al.,* 2000; Burd *et al.,* 1998) flooding (Grichko & Glick, 2001a, b) drought and high salt (Mayak *et al.*, 2004a,b, Arshad *et al.,* 2008).

Etiolated pea seedlings are very sensitive to ethylene. The most widely renowned example of the effect of ethylene on plant growth is the classical "triple" response in etiolated dicot seedlings in the presence of ethylene. This effect consists of three distinct morphological changes in the shape of seedlings, inhibition of stem elongation, increase in stem diameter and horizontal growth (Akhtar *et al*., 2005; Khalid *et al*., 2006). This "triple" response reaction of etiolated seedlings has been a reliable bioassay for ethylene action (Guzman & Ecker, 1990).

In a previous study (Shaharoona *et al.,* 2007), the effect of inoculation with ACC-utilizing and ethylene-producing rhizobacteria had been compared through highly ethylene specific classical "triple" response bioassay. In this study, the effect of inoculation with rhizobacteria having different ACC-deaminase activities on extenuating the classical "triple" response in etiolated pea seedlings was investigated.

Materials and Methods

A series of experiments were conducted under axenic conditions to study the response of etiolated pea seedlings to inoculation with rhizobacteria containing ACC-deaminase. For this, rhizobacteria were isolated from the pea rhizosphere by dilution plate technique using DF salt minimal medium (Dworkin & Foster, 1958) containing ACC as sole nitrogen source (enrichment technique). The collected rhizobacterial isolates were purified by further streaking on fresh plates and stored in 20% glycerol at -20 °C.

Rhizobacterial isolates were characterized for ACC-deaminase activity, auxin (indol acetic acid, IAA) production and root colonization. ACC deaminase activity was assayed according to a modification of the method of Honma & Shimomura (1978) which measures the amount of α-ketobutyrate produced upon the hydrolysis of ACC. The number of µmol of α-ketobutyrate produced by this reaction was determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate ranging between 0.1 and 1µmol. A stock solution of 100 mmol L^{-1} α-ketobutyrate was

prepared in 0.1 mol L^{-1} Tris-HCl (pH 8.5) and stored at 4˚C. Just prior to use, the stock solution was diluted with the same buffer to make 10 mmol L^{-1} solution from which a standard concentrations curve was generated. In a series of known α-ketobutyrate concentrations, 2 mL of the 2, 4-dinitrophenyl-hydrazine reagent (0.2% 2, 4-dinitrophenyl-hydrazine in 2 mol L^{-1} HCl) was added, the contents were vortexed and incubated at 30 ºC for 30 min, during which time the α-ketobutyrate was derivitized as a phenylhydrazine. The color of phenylhydrazine was developed by the addition of 2 mL, 2 mol L^{-1} NaOH, the absorbance of the mixture was measured after mixing by using spectrophotometer at 540 nm.

For determining ACC deaminase activity, the bacterial cell pellets were suspended in 5 mL of 0.1 mol L^{-1} Tris-HCl, pH 7.6, and transferred to microcentrifuge tube. The contents of the tubes were centrifuged at 16000 rpm for 5 min and supernatant was removed. The pellets were suspended in 2 mL 0.1 mol L^{-1} Tris HCl, pH 8.5. Thirty µL of toluene were added to the cell suspension and vortexed for 30 s. Two hundered µL of the toluenized cells were placed in a fresh microcentrifuge tube, 20 µL of 0.5 mol L^{-1} ACC were added to the suspension, vortexed, and then incubated at 30 ^{o}C for 15 min. Following the addition of 1 mL of 0.56 mol L^{-1} HCl, the mixture was vortexed and centrifuged for 5 min at 16000 rpm at room temperature. Two mL of the supernatant was vortexed together with 1 mL of 0.56 mol L^{-1} HCl. Thereupon, 2 mL of the 2, 4-dinitrophenylhydrazine reagent (0.2% 2, 4-dinitrophenylhydrazine in 2 mol L^{-1} HCl) was added to the glass tube, and the contents were vortexed and then incubated at 30^{o}C for 30 min. Following the addition and mixing of 2 mL of 2 mol L^{-1} NaOH, the absorbance of the mixture was measured by using spectrophotometer at 540 nm.

In vitro auxin production by selected isolates was determined as indole acetic acid (IAA) equivalents in the presence and absence of L-tryptophan (an auxin precursor) by using the protocol described by Khalid *et al.* (2004). The ability of different bacterial strains to colonize wheat roots under axenic conditions was assessed using the method described by Simons *et al.* (1996).

In order to study the relationship between [ACC] and classical triple response, pea seeds (cv. 2000) were surface sterilized by dipping in 95% ethanol solution for 5 min, 0.2% $HgCl_2$ solution for 3 min and washed thoroughly with sterilized water (Khalid *et al.*, 2004). Two seeds sandwiched in the folds of sterilized filter papers were sown in 100 ml beaker and then placed in air tight mason jar wrapped in green foil. Seeds were exposed to 0, 2, 4, 6, 8, and 10 mmol L^{-1} ACC. All the treatments were replicated four times. The jars were incubated in complete darkness throughout the experiment at 24 ± 3°C. After seven days, classical "triple" response was observed by recording seedling length and stem diameter.

In another experiment, surface disinfected pea seeds were dipped for 5 min in the respective rhizobacterial (ACC4, ACC5, ACC6, ACC10 and ACC14) inoculum, each containing 10^{7}-10^{8} cfu ml^{-1}. In case of uninoculated control, seeds were dipped in sterilized inoculum. Two seeds were sown in the folds of sterilized filter paper in 100 ml beaker containing 10 mmol ACC and placed in air tight mason jars wrapped in green foil to provide "safe" green light. Each treatment was replicated four times. Jars were incubated in complete darkness at 24 ± 3°C. After seven days, seedling length, stem diameter and root elongation were recorded.

Results

Rhizobacteria containing ACC-deaminase were characterized for ACC-deaminase activity, IAA production both in the presence and absence of L-tryptophan (L-TRP) and root colonization. Results divulge that different strains of rhizobacteria varied in their ACC-deaminase activity (Table 1). ACC5 was found to be the most efficient in hydrolyzing ACC (as it produced 174 nmol α-ketobutyrate g^{-1} biomass h^{-1}) followed by ACC4. All the rhizobacterial isolates were capable of producing IAA both in the presence and absence of L-TRP. Maximum IAA production in the absence of L-TRP was recorded in case of ACC14, whereas in the presence of L-TRP, ACC6 was the maximum IAA producer (Table 1). Rhizobacterial isolates also varied in their ability to colonize pea roots and ACC5 was found to be the most efficient colonizer of pea roots (8.0×10^7 cfu g^{-1} root) followed by ACC6 (4.0×10^7 cfu g^{-1} root).

The effect of different concentrations of ACC in creating classical "triple" response in etiolated pea seedlings is clearly visible Fig. 1. The exogenous application of ACC had concentration-dependent effect on pea seedlings as there was a linear reduction in the seedling length (R^2 = -0.93**) with the increase in exogenously applied [ACC]. Diameter of etiolated pea seedlings increased with increasing [ACC] and had significant positive correlation (R^2 = 0.92**) with [ACC].

Table 1. Characterization of rhizobacteria containing ACC-deaminase (average of four replicates).

Isolate	ACC-deaminase activity (nmol α-ketobutyrate g^{-1} biomass h^{-1})	IAA production (mg L^{-1})		Root colonization (cfu g^{-1} root)
		Without L-tryptophan	With L-tryptophan	
ACC4	114 ± 1.3	4.4 ± 0.5	9.6 ± 0.7	1.2×10^7
ACC5	174 ± 3.2	3.8 ± 1.0	7.5 ± 0.9	8.0×10^7
ACC6	13.6 ± 1.6	3.6 ± 0.8	37.4 ± 0.6	4.0×10^7
ACC10	16.1 ± 1.0	4.5 ± 0.2	5.6 ± 1.3	2.6×10^6
ACC14	25.7 ± 2.5	10.8 ± 1.3	20.8 ± 0.8	2.0×10^7

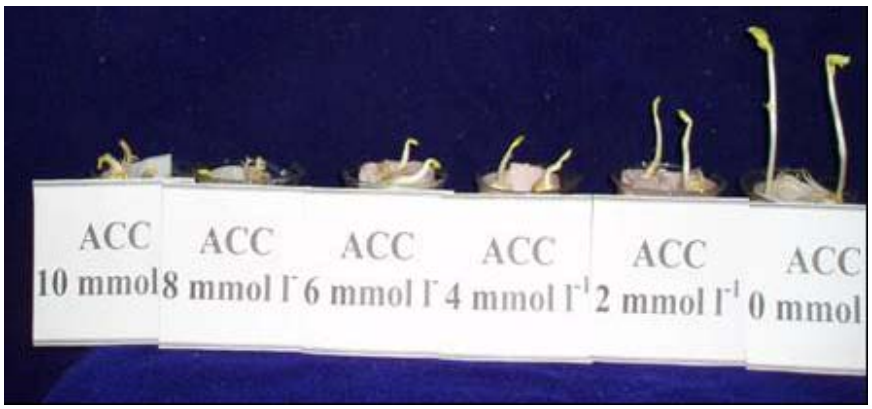

Fig. 1. Effect of different levels of exogenously applied ACC on the classical triple response of etiolated pea seedlings under axenic conditions.

Table 2. Effect of inoculation with rhizobacteria containing ACC-deaminase on seedling length, stem diameter and root length of etiolated pea seedlings in the presence of 10 mmol L^{-1} ACC (average of four replicates).

Rhizobacteria	Seedling length (cm)	Stem diameter (mm)	Root elongation (cm)
Uninoculated (without ACC)	6.4 ± 0.4	0.95 ± 0.02	3.9 ± 0.4
Uninoculated + ACC*	1.75 ± 0.2	2.81 ± 0.01	1.50 ± 0.3
ACC4 + ACC	9.75 ± 0.9	1.93 ± 0.01	4.75 ± 0.2
ACC5 + ACC	6.60 ± 0.3	2.00 ± 0.03	7.35 ± 0.5
ACC6 + ACC	4.45 ± 0.5	2.05 ± 0.05	2.95 ± 0.1
ACC10 + ACC	1.95 ± 0.3	2.03 ± 0.01	4.00 ± 0.2
ACC14 + ACC	2.50 ± 0.6	1.94 ± 0.02	4.50 ± 0.3

* 10 mmol L^{-1}

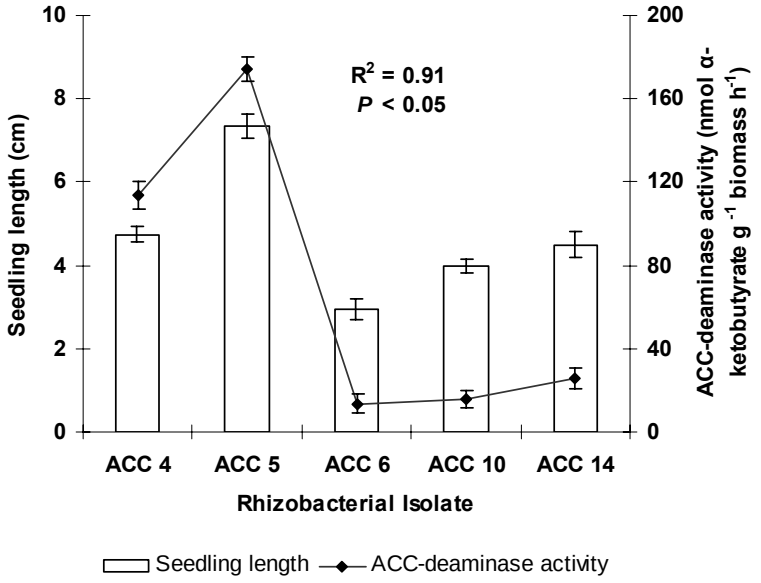

Fig. 2. Correlation between ACC-deaminase activity and length of etiolated pea seedlings under axenic conditions.

Data regarding the effect of inoculation with rhizobacteria containing ACC-deaminase on seedling length, stem diameter and root elongation is summarized in Table 2. Results indicate that exposure of etiolated pea seedlings to ACC caused substantial reduction (72%) in seedling length as compared to without ACC uninoculated control, although, inoculation with most of the rhizobacterial isolates caused significant increase in seedling length than uninoculated ACC-stressed control. Maximum increase in seedling length was recorded in response to inoculation with ACC5 that was 4.6-folds higher than that recoded for ACC-stressed uninoculated control and it was 52% higher than uninoculated control without ACC. Next effective isolate was ACC4 that caused 2.8-folds increase in seedling length than uninoculated ACC-stressed control. Minimum increase in seedling length was recoded in case of inoculation with ACC10 that was 11%

higher than ACC-stressed uninoculated control. Stem diameter was significantly increased in response to exogenous application of ACC. Again, inoculation with rhizobacteria caused significant decrease in stem diameter as compared with ACC-stressed uninoculated control. Among all the isolates, ACC4 and ACC10 were found the most effective they caused 31 and 30% decreases in stem diameter, respectively, than ACC-stressed uninoculated control. Root elongation of etiolated pea seedlings was also negatively affected upon addition of ACC in the growth medium that was 62% lesser than uninoculated without ACC control (Table 2). Inoculation with rhizobacteria significantly increased root elongation than uninoculated ACC-stressed uninoculated control. ACC4 was found the most effective in increasing root elongation of etiolated pea seedlings as it caused 3.9-folds increase in root elongation than ACC-stressed uninoculated control.

In order to explore the role of rhizobacteria containing ACC-deaminase in extenuating triple response, the correlation was calculated between ACC-deaminase activity and seedling length of ACC-stressed etiolated pea seedlings. It is obvious from the results shown in Fig. 2 that there was a significant correlation ($R^2 = 0.91$*) between ACC-deaminase activity and seedling length. The effect of inoculation with rhizobacteria containing ACC-deaminase in extenuating the ACC-induced classical "triple" response is clearly visible in Fig. 3.

Discussion

Rhizobacteria containing ACC-deaminase have been reported to decrease the inhibitory effects of ethylene both under normal and stress conditions due to their ACC-deaminase activity. This study was conducted to investigate the role of rhizobacteria having different ACC-deaminase activities in decreasing ACC upon inoculation in etiolated pea seedlings through highly ethylene specific classical "triple" response bioassay. It is evident from the results that increase in the concentration of ACC increased the intensity of classical triple response (Fig. 1) in etiolated pea seedlings. The linear correlation between seedling length ($R^2 = -0.93$) and stem diameter ($R^2 = 0.92$) implies that ethylene production increases with increase in exogenous ACC application. Many researchers have reported that exogenously applied ACC dramatically stimulate ethylene production in plant tissues (Lürssen *et al*., 1979; McKeon *et al*., 1982; Khalid *et al*., 2006). Production of classical "triple" response due to exogenously applied ACC in etiolated tomato and *Arabidopsis* seedlings has also been reported (Barry *et al.*, 2001; Ton *et al.*, 2001; Shaharoona *et al.,* 2007).

Inoculation with most of the rhizobacterial isolates decreased the intensity of ACC-induced classical "triple" response in etiolated pea seedlings with variable efficacy. Isolate ACC5 was more efficient in increasing seedling length and root elongation whereas ACC4 and ACC14 were more effective in decreasing stem diameter which might be due to variation in other traits of rhizobacteria like IAA production (which has direct effect on ACC production) and root colonization, in addition to ACC-deaminase activity.

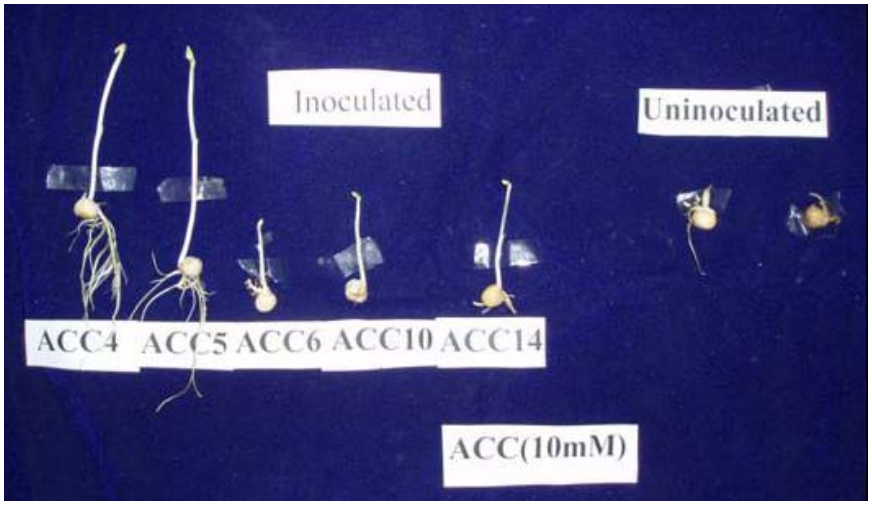

Fig. 3. Effect of inoculation with rhizobacteria containing ACC-deaminase on the classical triple response of etiolated pea seedlings in the presence of 10 mmol L^{-1}.

A significant correlation ($R^2 = 0.91$*) between ACC-deaminase activity and increase in seedling length of ACC-stressed etiolated pea seedlings was recorded. It is highly likely that ACC-deaminase activity of rhizobacteria was responsible in diluting the ACC-imposed effect on seedling length of etiolated pea seedlings. Also, inoculation with rhizobacteria containing ACC-deaminase increased the seedling length and root elongation as compared with uninoculated control without ACC which implies that endogenous ACC levels might also be decreased in response to inoculation. Results are in conformity with the findings that seed and/or root inoculation with certain rhizobacteria decrease endogenous ethylene levels and promote root growth through ACC-deaminase activity under gnotobiotic conditions (Wang *et al*., 2000; Belimov *et al*., 2002; Ghosh *et al*., 2003; Shaharoona *et al*., 2006a). In a previous study it has been reported that inoculation with ACC-utilizing rhizobacteria decreased the intensity of classical "triple" response whereas inoculation with ethylene-producing rhizobacteria further enhanced the intensity of classical "triple" response (Shaharoona *et al.,* 2007). The finding of this study that rhizobacteria with more ACC-deaminase activity have more ability to decrease the intensity of ACC-induced classical "triple" response (Fig. 3) further confirms the premises that ACC-deaminase activity of rhizobacteria is responsible for decreasing endogenous as well as exogenous ACC in inoculated plants.

This study also suggests the inoculation with rhizobacteria containing ACC-deaminase could be used to decrease ACC, which produces due to a variety of biotic and abiotic stresses in plants.

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(Received for publication 18 September, 2006)

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Published by the Pakistan Botanical Society.
Printed at Karachi University Press, Karachi-75270, Pakistan. Phone: 9243186, 4969237.
Copies available from the Chief Editor, Pakistan Journal of Botany, Department of Botany, University of Karachi, Karachi-75270, Pakistan. Phone: 4387867.
Price: Each issue Rs. 100/ US$20; Vol. Rs. 600/US$120 or in exchange.