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Differential Response of Etiolated Pea Seedlings to Inoculation with Rhizobacteria Capable of Utilizing 1-Aminocyclopropane-1-Carboxylate or L-Methionine

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The majority of soil microorganisms can derive ethylene from L-methionine (L-MET), while some rhizobacteria can hydrolyze 1-aminocyclopropane-1-carboxylate (ACC) due to their ACC-deaminase activity. In this study, three strains having either ACC-deaminase activity (*Pseudomonas putida* biotype A, A_7), or the ability to produce ethylene from L-MET (*Acinetobacter calcoaceticus*, M_9) or both (*Pseudomonas fluorescens*, AM_3) were used for inoculation. The highly ethylene specific bioassay of a classical "triple" response in pea seedlings was used to investigate the effect of the inoculation with the rhizobacteria in the presence of 10 mM ACC or L-MET. The exogenous application of ACC had a concentration-dependent effect on the etiolated pea seedlings in creating the classical "triple" response. The inoculation with *P. putida* diluted the effect of ACC, which was most likely due to its ACC-deaminase activity. Similarly, the application of Co^{2+} reduced the ACC-imposed effect on etiolated pea seedlings. In contrast, the inoculation of *A. calcoaceticus* or *P. fluorescens* in the presence of L-MET caused a stronger classical "triple" response in etiolated pea seedlings; most likely by producing ethylene from L-MET. This is the first study, to our knowledge, reporting on the comparative effect of rhizobacteria capable of utilizing ACC vs L-MET on etiolated pea seedlings.

Keywords: Ethylene, 1-aminocyclopropane-1-carboxylate, L-methionine, pea seedlings, rhizobacteria, ACC-deaminase

The gaseous plant hormone ethylene (C_2H_4) participates in the regulation of many developmental processes and the responses to environmental stresses throughout the life cycle of plants (Abeles *et al.*, 1992; Reid, 1995). Ethylene is produced by plants as well as by microorganisms; however, they produce ethylene via different biosynthetic pathways (Arshad and Frankenberger, 2002). In higher plants, L-methionine (MET) is converted into S-adenosylmethionine (SAM) which is converted to 1-aminocyclopropane-1-carboxylate (ACC) in the presence of ACC-synthase. ACC-oxidase then oxidizes ACC into ethylene (MET → SAM → ACC → C_2H_4). The production of ethylene in plants is highly dependent on the endogenous levels of ACC (Lürssen *et al.*, 1979; McKeon *et al.*, 1982). Paradoxically, ethylene is synthesized in microorganisms by a pathway that does not include ACC (Arshad and Frankenberger, 1998, 2002; Jia *et al.*, 1999).

Numerous inhibitors of ACC-synthase and ACC-oxidase that affect ethylene synthesis in plants have been recognized. Cobalt (Co^{2+}) acts as a chemical inhibitor of ACC-oxidase and prevents the conversion of ACC into ethylene when applied in the range of 10-100 μM (McKeon *et al.*, 1995). Several authors have recently reported that some plant growth promoting rhizobacteria (PGPR) containing the enzyme ACC-deaminase lower endogenous levels of ethylene by hydrolyzing ACC into α-ketobutyrate and ammonia, which affects plant growth (Glick *et al.*, 1998; Li *et al.*, 2000; Penrose and Glick, 2001; Ghosh *et al.*, 2003; Shaharoona *et al.*, 2006a, 2006b). The majority of soil microorganisms produce ethylene from L-MET via the 2-keto-4-methlythiobutyric acid (KMBA) pathway (Primrose, 1977; Ince and Knowles, 1986; Nazli *et al.*, 2003).

Etiolated pea seedlings are very sensitive to ethylene. The most widely documented example of the effect of ethylene on plant growth is the classical "triple" response phenotype exhibited by dicot seedlings grown in darkness in the presence of ethylene. This effect, which was first described by Neljubow (1901), consists of three distinct morphological changes in the shape of the seedlings, including the inhibition of stem elongation, radial swelling of the stem and a change in the direction of growth (Guzman and Ecker, 1990; Akhtar *et al.*, 2005; Khalid *et al.*, 2006). This "triple" response reaction of etiolated seedlings has been a reliable marker/bioassay for ethylene action and has been used to quantify the dose-response characteristics of ethylene (Chen and Bleecker, 1995). The "triple" response has also been used to identify components in the ethylene signal transduction pathway in mutant screens (Bleecker *et. al.*, 1988; Guzman and Ecker, 1990).

In this study, a classical "triple" response bioassay was employed to investigate the effect of microbially induced changes in the ethylene found in etiolated pea seedlings in

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response to inoculation with ethylene-lowering (ACC-deaminase-containing) and ethylene-producing rhizobacteria. The effect of inoculation with rhizobacteria containing ACC-deaminase (a biological inhibitor of ethylene) vs. Co^{2+} (chemical inhibitor) on pea seedlings was also investigated.

Materials and Methods

Isolation of rhizobacteria

Rhizobacteria were isolated from the rhizosphere soil of wheat and maize by the dilution plate technique using DF salt minimal medium (Dworkin and Foster, 1958) containing ACC or L-MET as the sole source of N (enrichment technique). The selected strains were further tested for their ability to grow on alternate substrates.

Characterization of rhizobacterial isolates

The ACC-deaminase activity was determined by monitoring the amount of ammonia generated due to the hydrolysis of ACC by the rhizobacterial isolates containing ACC-deaminase. In Erlenmeyer flasks, 500 ml DF salt minimal medium containing 2 g/L ammonium sulfate as a nitrogen source was inoculated with each strain and incubated at 28±1°C in an orbital shaking incubator at 100 rpm for 48 h. The broth was then centrifuged at 8,300×g for 10 min and the cell pellets were collected in phosphate buffer (0.5 M, pH 7) and recentrifuged. Cell pellets were transferred to DF salt minimal medium containing 10 mM ACC substrate and incubated in an orbital shaking incubator for 1 h at 28± 1°C. After one hour, the broth was centrifuged at 8,300×g for 10 min and the ammonia in the supernatant solution was determined by the protocol described by Nagatsu and Yagi (1966).

The production of ethylene from L-MET by the selected isolates was examined using 125 ml Erlenmeyer flasks capped with mininert valves (Pierce, USA). No air was flushed from the head space; however, the flasks were autoclaved at 121°C for 20 min and the mininert valves were sterilized prior to use using 95% ethanol. Five milliliters of filter-sterilized (0.2 μm pore size membrane filter, Whatmann) L-MET (100 mM) solution were added to the flasks containing 49 ml of salt minimal medium and inoculated with 1 ml of inoculum to achieve a final concentration of 10 mM. The valves were air-locked and the flasks were completely capped during incubation. Ethylene production was determined after incubation for 48 h by gas chromatography using a gas-tight glass hypodermic syringe to withdraw 1 ml gas samples from the headspace above the culture medium. The gas chromatograph (UNICAM-4600) was equipped with a flame-ionization detector (FID) and a 2 m Porpak N (particle size, 0.14-0.18 mm) column. The column was operated isothermally at 70°C. The following conditions were used for operating the GC: sample size, 1.0 ml; carrier gas (N_2), 13 ml/min; H_2 flow, 30 ml/min; air flow, 300 ml/min; detector temp, 200°C; and injector temp, 120°C. The peak area and retention times for ethylene were compared to the reference standards made by diluting 99.5% ethylene obtained from Matheson (Secaucus, USA). The minimum detection limit of the GC was 50 nmol.

Three rhizobacterial strains capable of utilizing either ACC, L-MET, or both, were identified using the Biolog® identification system (Microlog™ System Release 4.2, USA). The Biolog® identification system has been reported to be at par with the 16S rRNA system (Flores-Vargas and O'Hara, 2006). The root colonization ability was studied under axenic conditions as described by Simons *et al.* (1996).

Selection of rhizobacteria

Three rhizobacterial isolates were selected for use as the inocula. *Pseudomonas putida* biotype A (A_7) carried ACC-deaminase but it was unable to grow on L-MET and produce ethylene from L-MET. *Pseudomonas fluorescens* (AM_3) was able to grow on both ACC and L-MET and, therefore, had the ability to hydrolyze ACC as well as to produce ethylene from L-MET. *Acinetobacter calcoaceticus* (M_9) was not found to exhibit ACC-deaminase activity; however, it showed prolific growth on L-MET and produced ethylene from L-MET.

Response of etiolated pea seedlings to exogenous application of ACC

The 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 they were thoroughly washed with sterilized water in order to study the relationship between ACC and the classical triple response (Khalid *et al.*, 2004). Two seeds sandwiched between two sterilized filter papers were sown in a 100 ml beaker and placed in an air tight Mason jar wrapped in green foil to provide "safe" green light. The seeds were exposed to 0, 2, 4, 6, 8, and 10 mM ACC. All of the treatments were replicated six times. Incubation was conducted in complete darkness throughout the experiment at 24±3°C. The classical "triple" response was observed after seven days by recording the seedling length and stem diameter.

Response of etiolated pea seedlings to inoculation with selected rhizobacteria in the presence of ACC or L-MET

In the second experiment, surface disinfected pea seeds were dipped for 5 min in the respective bacterial (A_7, M_9 and AM_3) inoculum, each containing 10^7-10^8 cfu/ml. The seeds used as the uninoculated control were dipped in sterilized inoculum. Two seeds in the folds of sterilized filter paper were sown in a 100 ml beaker containing either 10 mM ACC or L-MET, and were placed in air tight Mason jars wrapped in green foil to provide "safe" green light (devoid of photosynthetic radiation). Each treatment was replicated six times. The jars were incubated in complete darkness at 24±3°C. The seedling length and stem diameter were recorded after seven days.

Comparative effect of Co^{2+} and inoculation with strain A_7 and AM_3 on etiolated pea seedlings

In the third experiment, the effect of inoculation with rhizobacterial strains containing ACC-deaminase was compared with that of the Co^{2+} treatment, which is a chemical inhibitor of ethylene. Pea seeds were inoculated with rhizobacteria (A_7, AM_3) containing ACC-deaminase or exposed to 100 μM Co^{2+} ($CoCl_2$) in the growth medium with or without 10 mM ACC. The experiment was performed with six replications. The incubation conditions were the same as those described

Table 1. Characteristics of the selected strains of rhizobacteria

Strain	Source	Ability of rhizobacteria to grow on medium containing		ACC-deaminase activity (nmol NH_3 per g biomass per h)	Ethylene production (nmol per 72 h per 50 ml culture)	Root colonization (cfu/g root)
		ACC	L-MET			
Pseudomonas putida biotype A (A_7)	Maize rhizosphere	+	-	278±17	ND	8.3×10^4
Pseudomonas fluorescens (AM_3)	Wheat rhizosphere	+	+	329±12	523±10.6	1.1×10^5
Acinetobacter calcoaceticus (M_9)	Maize rhizosphere	-	+	ND*	609±12.5	3.1×10^2

* Not detected

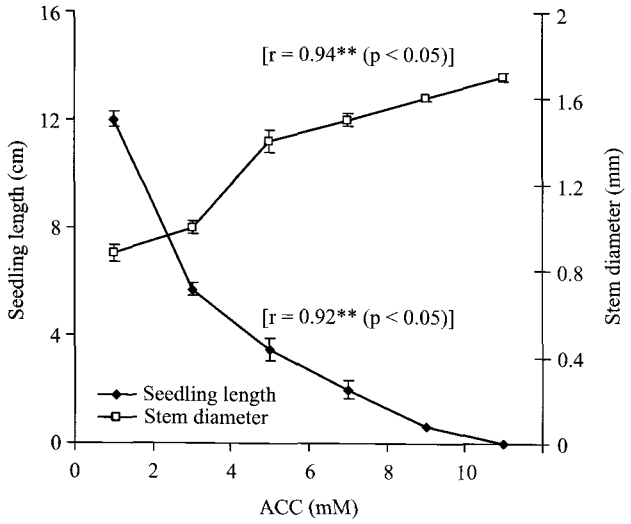

Fig. 1. Effect of different levels of exogenously applied ACC on the seedling length and stem diameter of etiolated pea seedlings.

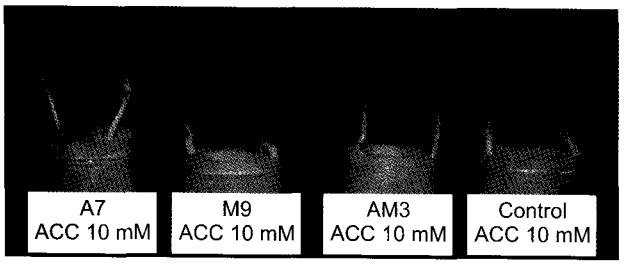

Fig. 2. Effect of inoculation with the selected strains of rhizobacteria on the classical "triple" response of etiolated pea seedlings in the presence of 10 mM ACC. The rhizobacteria are capable of utilizing ACC (A7), L-MET (M9) or both ACC and L-MET (AM3).

above. The seedling length and stem diameter were recorded after seven days.

Statistical analysis

The correlation between ACC and the seedling length or swelling of hypocotyls (stem diameter) was determined using the MSTATC (Michigan State University, USA) software. The significance of correlation was determined by applying a student's t-test. The data regarding the effects of inoculation with ACC or L-MET utilizing rhizobacteria exposed to either 10 mM ACC or L-MET on the seedling length and stem diameter of etiolated pea seedlings were analyzed by applying two-factorial completely randomized designs, and the means were compared by a least significant difference (LSD) test. The effects of inoculation with rhizobacteria, containing ACC-deaminase or Co^{2+} exposed to two levels of ACC (0 and 10 mM), were also analyzed by applying two-factorial completely randomized designs, and the means were compared by a least significant difference (LSD) test. All the tests were performed at $p<0.05$, using the MSTATC (Michigan State University, USA) software.

Results

The selected strains of rhizobacteria identified as *Pseudomonas putida* biotype A (A_7), *Pseudomonas fluorescens* (AM_3) and *Acinetobacter calcoaceticus* (M_9) were characterized for their *in vitro* ACC-deaminase activity, ethylene production, and root colonization under gnotobiotic conditions (Table 1). The ACC-deaminase activity of *P. putida* and *P. fluorescens* varied from 278 to 329 nmol NH_3 per g biomass/h. *Acinetobacter calcoaceticus* did not grow on the medium containing ACC as the sole source of nitrogen. This strain was unable to hydrolyze ACC and consequently no NH_3 was produced. The maximum ethylene production (609 nmol per 72 h per 50 ml culture) from L-MET was recorded in the case of *A. calcoaceticus* inoculation and it was followed by *P. fluorescens*. No ethylene production was detected in the pure culture of *P. putida*, even after 72 h. *Pseudomonas fluorescens* was found to be the most active strain to colonize (1.1×10^5 cfu/g root biomass) pea roots when compared to the other two strains, and *A. calcoaceticus* was found to be the least efficient in colonizing pea roots (3.1×10^2 cfu/g root biomass).

The data regarding the seedling length and stem diameter of etiolated pea seedlings revealed that the exogenous application of ACC had a concentration-dependent effect on the pea seedlings and created the classical "triple" response (Fig. 1). A linear reduction in the seedling length was observed with an increase in exogenously applied ACC and had a highly significant negative correlation (r=-0.92**). The diameter of etiolated pea seedlings increased with increasing ACC and showed a significantly positive correlation (r=0.94**) with ACC.

The exogenous application of 10 mM ACC created a

Table 2. Classical "triple" response in etiolated pea seedlings inoculated with rhizobacteria capable of utilizing ACC or L-MET

Selected rhizobacteria	Seedling length (cm)		Stem diameter (mm)	
	ACC*	MET*	ACC	MET
Uninoculated control	2.0 d**	09.2 a	1.48 b	0.88 c
Pseudomonas putida biotype A (A_7)	3.8 c	10.3 a	0.99 c	0.89 c
Pseudomonas fluorescens (AM_3)	2.1 d	06.8 b	0.97 c	0.86 c
Acinetobacter calcoaceticus (M_9)	1.6 d	06.5 b	1.79 a	1.08 b

* Each substrate was applied @ 10 mM concentration.
** Means that strains sharing the same letter(s) in a column do not differ significantly according to least significant difference test ($p<0.05$).

Table 3. Response of etiolated pea seedlings to inoculation with rhizobacteria containing ACC-deaminase or exposed to cobalt (Co^{2+}) in the presence or absence of ACC

Treatment*	Seedling length (cm)		Stem diameter (mm)		Root elongation (cm)	
	Without ACC	With ACC (10 mM)	Without ACC	With ACC (10 mM)	Without ACC	With ACC (10 mM)
Uninoculated	6.7 b**	1.0 c	0.91 b	1.46 a	4.5 b	1.7 c
$CoCl_2$	12.1 a	11.7 a	0.89 b	0.96 c	8.8 a	8.9 a
Pseudomonas putida biotype A (A_7)	11.6 a	10.3 a	0.88 b	0.95 c	12.5 a	9.2 a
Pseudomonas fluorescens (AM_3)	10.5 a	1.9 c	0.93 b	1.14 b	12.5 a	1.1 c

* Cobalt was used as a chemical inhibitor of ethylene production while *Pseudomonas* spp. were used as biological inhibitor.
** Means that strains sharing the same letter(s) in a column do not differ significantly according to least significant difference test ($p<0.05$).

classical "triple" response in etiolated pea seedlings; however, the inoculation with ACC utilizing rhizobacterium *P. putida* (A_7) diluted the effect of ACC, increased the seedling length and decreased the stem diameter of etiolated pea seedlings in comparison to the uninoculated control in the presence of ACC alone (Fig. 2). Inoculation with the L-MET utilizing rhizobacterium *A. calcoaceticus* (M_9) plus ACC resulted in a stronger "triple" response in etiolated pea seedlings compared with the uninoculated control. Inoculation with *P. fluorescens* (AM_3) in the presence of 10 mM ACC partially eliminated the "triple" response, and the seedling length was higher than in the uninoculated control but less than that of the seeds inoculated with *P. putida*.

The pea seedlings showed different responses when inoculated with ACC or L-MET utilizing rhizobacteria in the presence of either exogenous ACC or L-MET (Table 2). The exogenous application of ACC produced a classical "triple" response in etiolated pea seedlings, while no such response was observed in case of an exogenous application of L-MET. The inoculation of etiolated pea seedlings with *P. putida* significantly increased the seedling length (90%) and decreased the stem diameter (33%) in the presence of ACC as compared with the uninoculated control. Inoculation with *P. fluorescens* had a non-significant effect on seedling length but significantly decreased the stem diameter in comparison to the uninoculated control. Inoculation with *A. calcoaceticus* caused a 20% decrease in the seedling length and a 17% increase in the stem diameter in comparison with the uninoculated control.

In the case of the exogenous application of L-MET, a "triple" response was observed only in the case of inoculation with *A. calcoaceticus,* which caused significant decrease in the seedling length (29%) and an increase in the stem diameter (21%) in comparison to the uninoculated control (Table 2). Inoculation with *P. fluorescens* also caused a significant decrease in the seedling length but had a non-significant effect on the seedling diameter.

The data regarding the seedling length and stem diameter of the etiolated pea seedlings inoculated with rhizobacteria containing ACC-deaminase (*P. putida* and *P. fluorescens*) or exposed to Co^{2+} in the presence or absence of exogenous ACC are summarized in Table 3. The exposure of etiolated pea seedlings to Co^{2+} or inoculation with rhizobacteria containing ACC-deaminase in the absence of ACC caused a significant increase in the seedling length and root elongation in comparison to the control; however, the effect was not significant in the case of stem diameter. The exogenous application of ACC produced a classical "triple" response; however, the addition of Co^{2+} reduced the ACC-imposed effect on the etiolated pea seedlings and showed a dramatic effect in increasing the seedling length and root elongation (10.7 and 4.2 fold, respectively) over the uninoculated control. The exposure of etiolated pea seedlings to Co^{2+} also significantly decreased the stem diameter (34% less than control). Similarly, the inoculation of etiolated pea seedlings with rhizobacteria containing ACC-deaminase *P. putida* reduced the ACC-imposed effect and caused a significant increase in the seedling length (9 fold) and root elongation (2 fold), and a decrease in stem diameter (35%). The inoculation of etiolated pea seedlings with the rhizobacteria that had the ability to hydrolyze ACC as well as to produce ethylene from L-MET (*P. fluorescens*) had no significant effect on the shoot length and root elongation when compared to the control. However, the stem diameter in this strain significantly decreased (22%).

Discussion

This study compared the effects of the inoculation of etiolated pea seedlings with three types of rhizobacteria that were either capable of utilizing ACC, MET or both ACC and MET. The exogenous application of ACC had a concentration-dependent effect on the etiolated pea seedlings and caused a classical "triple" response, which was most likely due to an increase in the synthesis of ethylene. Many researchers have reported that exogenously applied ACC dramatically stimulated ethylene production in plant tissues (Lürssen *et al.*, 1979; McKeon *et al.*, 1982; Khalid *et al.*, 2006). The production of a classical "triple" response due to the exogenous application of ACC to etiolated tomato and *Arabidopsis* seedlings has also been reported (Barry *et al.*, 2001; Ton *et al.*, 2001).

The inoculation with *P. putida* biotype A (A_7) containing ACC-deaminase diluted the effect of ACC on etiolated pea seedlings and significantly increased the seedling length and decreased the stem diameter in the presence of 10 mM ACC in comparison to the seedlings inoculated with *A. calcoaceticus* (M_9), *P. fluorescens* (AM_3) or the uninoculated control. The intensity of the "triple" response most likely decreased in response to the lowering of endogenous levels of ethylene in etiolated pea seedlings because of the hydrolyses of ACC by the ACC-deaminase activity of *P. putida*. Penrose and Glick (2001) reported that the ACC from seeds or plant roots along with other small molecules exudes in the rhizosphere and thus the endogenous production of ethylene reduces in response to decreased ACC levels within the plant. Furthermore, the ethylene production in the rhizosphere also decreases due to bacterial ACC deaminase activity. These results are in agreement with the findings that seed and/or root inoculation with certain rhizobacteria decreases endogenous ethylene levels and promotes 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). Interestingly, the inoculation of etiolated pea seedlings with ethylene producing rhizobacterium (*A. calcoaceticus*, M_9) in the presence of ACC further increased the intensity of the "triple" response. Since this strain (M_9) is unable to use ACC, it is likely that the endogenous L-MET of pea seedlings exuded in the rhizosphere might have been converted to ethylene by the MET utilizing rhizobacteria.

Exogenously applied L-MET could not produce a "triple" response in etiolated pea seedlings under axenic conditions. This may imply that the exogenous application of L-MET could not increase endogenous ethylene synthesis by the MET ACC C_2H_4 pathway, which was most likely due to poor or no uptake by the pea seedlings. This premise is supported by others who reported that the exogenous application of L-MET alone could not produce a classical "triple" response in etiolated pea seedlings (Arshad and Frankenberger, 1988; Akhtar *et al.*, 2005). In the presence of L-MET, a classical "triple" response was observed only in the case of the inoculation with the L-MET utilizing rhizobacteria *A. calcoaceticus* (M_9) and *P. fluorescens* (AM_3). Since these two strains are able to derive ethylene from L-MET, a "triple" response was observed in the inoculated pea seedlings. The effect of microbially produced ethylene on etiolated pea seedlings in the presence of L-MET has also been previously reported (Arshad and Frankenberger, 1988; Akhtar *et al.*, 2005; Khalid *et al.*, 2006).

This study also revealed that the inoculation of pea seedlings with rhizobacteria containing ACC-deaminase eliminated the classical "triple" response just like the chemical inhibitor Co^{2+}, which inhibits the activity of ACC-oxidase and prevents the conversion of ACC into ethylene. The similarity in behavior of both biological and chemical ethylene inhibitors strongly supports the premise that rhizobacteria containing ACC-deaminase decrease endogenous levels of ethylene, and thereby decrease the ACC concentration in plants.

This study suggests that plant growth could be modified by either changing the ethylene synthesis endogenously and/or in the close vicinity of the root through the careful selection of the rhizobacteria to be used as inocula.

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