

Soybean Response to 1-Aminocyclopropane-1-Carboxylate Deaminase-Producing *Pseudomonas* under Field Soil Conditions

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Abstract: Problem statement: 1-Aminocyclopropane-1-Carboxylate (ACC) deaminase-producing bacteria have been known to promote plant growth by decreasing ethylene inhibition of various plant processes. However, their efficacy under field soil conditions may vary depending on the range and variability of the environmental factors. This study examined the ability of eight promising isolates of ACC deaminase-producing *Pseudomonas* to enhance soybean growth under acidic and low fertility status of field soil conditions. **Approach:** The bacteria were formulated into peat-based carrier and used to inoculate soybean seeds. Cell viability in the carrier was evaluated periodically. The number of bacterial population at the time of seed inoculation was above 10^7 cell g^{-1} . Treated and untreated seeds were grown in plots (5×4 m²) and set in a randomized complete block design with 3 replicates. Observations were made at 30 d after planting for shoot height and weight, number of nodules and at harvesting for number of pods and yield. **Results:** Three out of eight isolates significantly increased soybean growth exhibited by higher number of nodules and pod filling and higher seed dry weight than those of untreated control. Those five remaining bacteria, on the contrary, inhibited soybean growth indicating that other unknown external factors influenced or covered the beneficial trait of ACC deaminase. **Conclusion:** Bacteria having ACC deaminase activities could be truly plant growth promoting bacteria providing that their beneficial effects are consistent at a wide range of environmental conditions.

Key words: ACC deaminase, environmental factors, growth promotion, *Pseudomonas*, soybean

INTRODUCTION

A number of 1-Aminocyclopropane-1-Carboxylate (ACC) deaminase-producing bacteria have been known to promote plant growth by ameliorating plant growth inhibition caused by ethylene production (Glick *et al.*, 1995; Penrose *et al.*, 2001; Mayak *et al.*, 2004; Ma *et al.* 2003). These bacteria hydrolyze root ACC (ethylene precursor) into ammonia and α -ketobutyrate as their sources of carbon and nitrogen (Honma and Shimomura 1978; Jacobson *et al.*, 1994; Glick *et al.*, 1998); thereby reducing ethylene synthesis. Various findings in the last few years also demonstrate their ability to ameliorate plant stress caused by various biotic and abiotic conditions, such as caused by high concentration of indole-3-acetic acid (Mayak *et al.*, 1997), water logging (Grichko and Glick 2001), nutritional shortage (Belimov *et al.*, 2002), drought (Mayak *et al.*, 2004),

high salts (Saravanakumar and Samiyappan, 2007) and the presence of pollutants (Reed and Glick, 2005; Belimov *et al.*, 2001) and plant pathogens (Wang *et al.*, 2000; Dey *et al.*, 2004; Shaharoon *et al.*, 2006). Our previous experiments using local isolates of ACC deaminase-producing *Pseudomonas* under growth chamber showed the ability of these isolates in enhancing soybean growth (Husen *et al.*, 2009). However, the extent by which plant responds to these bacteria under field soil conditions may vary since the environmental factors are variable during plant growth.

Reports by Shaharoon *et al.* (2006; 2007) confirmed that the effectiveness of ACC deaminase-producing bacteria on plant growth was influenced by nutrient status of the media which could be related to ethylene production (Abeles *et al.*, 1992). On the other hand, Glick *et al.* (2007) concluded that the efficacy of these bacteria on plant growth may not be clearly

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observed at stress-free conditions of the plants. Moreover, unusual findings by Belimov *et al.* (2007) found that a single strain (*P. brassicacearum* Am3) producing ACC deaminase may have growth-promoting, neutral, or pathogenic effects on plant growth according to the environmental conditions. These facts imply the importance of field trials to acquire a conclusive evidence whether the bacteria are truly plant growth-promoting bacteria or their beneficial effects depend on a certain environmental condition.

The present study aimed to evaluate the effects of eight promising isolates of ACC deaminase-producing *Pseudomonas* on soybean growth and yield under field soil conditions. Peat-based inoculants of the isolates were formulated to ensure cell viability before and after seed inoculation.

MATERIALS AND METHODS

Bacterial Isolates: Eight promising isolates of ACC deaminase-producing *Pseudomonas* were used in the study. The isolates, designated as Crb5, Crb12, Crb17, Crb24, Crb46, Crb47, Crb49 and Crb56 were selected based on previous research results in the laboratory and growth room experiments (Husen *et al.*, 2009). The origin of the bacteria is from the rhizosphere of soybean grown in Plumbon agricultural area in Cirebon, West Java, Indonesia. Besides producing 1-Aminocyclopropane-1-Carboxylate (ACC) deaminase (E.C.4.1.99.4) and Indole-3-Acetic Acid (IAA), the isolates were also not detected to inhibit rhizobial strains based on co-culture tests using *Bradyrhizobium japonicum* Bjj11 and *Sinorhizobium fredii* Rif5.

Preparation of peat-based Inoculants: Peat-based formulation of the isolates for seed treatments was performed in the Laboratory of Soil Biology and Health, Indonesian Soil Research Institute (ISRI) and the Laboratory of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University (IPB). Each isolate from stock culture of King's B (KB) medium was first grown in Dworkin-Foster (DF) minimal salts medium (Dworkin and Foster, 1958) supplemented with either ACC or ammonium sulfate to induce and maintain the activity of ACC deaminase and then subsequently transferred to the economically modified KB medium. The KB medium contained 20 g protease peptone, 10 mL glycerol, 1.5 g K₂HPO₄, 1.5 g MgSO₄.7H₂O, 15 g agar (for solid media) and 1000 mL distilled water. The modified KB medium was KB medium, except the glycerol and protease peptone were replaced with 15 mL molasses, 20 mL skimmed milk and 10 mL freshly coconut water. The composition of DF minimal salt

medium were 4 g KH₂PO₄, 6 g Na₂HPO₄, 0.2 g MgSO₄.7H₂O, 1 mg FeSO₄.7H₂O, 10 µg H₃BO₃, 10 µg MnSO₄, 70 µg ZnSO₄, 50 µg CuSO₄, 10 µg MoO₃, 2 g glucose, 2 g gluconic acid, 2 g citric acid, 1000 mL distilled water + either 0.3033 g L⁻¹ ACC or 2 g L⁻¹ ammonium sulfate and solidified with 15 g agar. Except the heat-labile ACC that was filtered-sterilized using 0.2 µm membrane filter, all media constituents were mixed and sterilized by autoclaving at 121°C for 15 min.

Inoculant production and peat-carrier material were prepared according to the procedures described by Somasegaran and Hoben (1994). A loop full of each isolate from DF minimal salt medium was transferred to 100 mL KB medium and incubated in a shaker at 125 rpm for 48 h at room temperature. After 48 h incubation, a 50 mL of bacterial suspension containing 1×10⁸ cells mL⁻¹ was transferred to 1000 mL modified KB medium and incubated at constant shaking as described above. The growth of bacteria in the medium was periodically measured spectrophotometrically at 600 nm for 48 h. Prior to peat inoculation, bacterial suspension was adjusted to give an absorbance of 0.55 at 600 nm which was equal to 10⁹ cells mL⁻¹ (Cattelan *et al.*, 1999). Peat material as the inoculant carrier was homogenized using 200 mesh sieve and enriched with calcium carbonate (4% of peat material) to increase the pH. The mixtures were packed 40 g each in autoclavable plastic bags and sterilized twice (2 consecutive days) at 121°C for 30 min. A 10 mL of bacterial suspension was injected to each of 40g sterile peat under aseptic conditions and the products were stored at room temperature.

Observations for cell viability, pH and water content of the carrier were made at week-1, week-4 and week-12 after peat inoculation. Recovery of bacteria from the carrier was conducted according to the procedure of Zuberer (1994) and the data were present on the dry weight basis.

Soil Properties, experimental design and seed treatment:

The experiment was conducted in farmer upland agricultural area in Lengkong Village, Karangpawitan Sub district, Garut District, West Java Province, Indonesia. The area is located at 07°12'35.3" SE and 107°57'09.4" E. A composite soil sample of Ultisol surface layer (0-20 cm depth) was collected and analyzed for soil texture, pH, C-organic and selected nutrients according to the standard procedures of the Soil Chemistry Laboratory of Indonesian Soil Research Institute (ISRI) in Bogor. Based on soil sample analyses (Table 1), the soil is acidic with fine texture (clay loam), low organic content and low fertility status.

Table1: Texture and some chemical characteristic of soils used for the experiment in Lengkong Village, Karangpawitan, Garut, West Java, Indonesia

	Texture	pH	C	N	(Extracted HCl 25%)			
					P ₂ O ₅	K ₂ O	CEC	BS
Unit -	-	----- g kg ⁻¹ -----	----- mg kg ⁻¹ -----		cmol ₍₊₎ kg ⁻¹	%		
Value	Clay loam	4.9	11	0.9	260	70	22.07	57
Status	Fine	Acidic	Low	Very low	Medium	Very low	Medium	Medium

CEC = cation exchange capacity; BS = base saturation

The experiment used plot size of 5×4 m², which were arranged in a randomized complete block design with 3 replications for each treatment. Eight isolates and one untreated control were used as treatments. Planting distance and fertilizer application followed the existing farmer practices.

Seeds of soybean (*Glycine max* L. Merr.) cv. Wilis as test-plant were obtained from the Indonesian Center for Biotechnology and Genetics Resources Research and Development, Bogor. Seed treatment was conducted by mixing moist seeds (previously soaked with tap water for several min) with freshly peat-based inoculants. Treated and untreated seeds were sowed at 40×20 cm planting distance. Observations were made at 30 d after planting for shoot height, shoot weight and number of nodules and at harvesting for number of pods, pod filling and seed yield.

Statistical analysis: Plant growth and yield data were analyzed independently by Analysis Of Variance (ANOVA) and treatment means were separated by the Duncan Multiple Range Test (DMRT) using the SAS systems for Windows v6.12.

RESULTS AND DISCUSSION

Characteristics and viability peat-based Inoculant:

Enrichment of peat material with calcium carbonate (CaCO₃) increased pH value (from 6.0-7.1) and micro nutrient contents (data not shown). Besides increasing pH materials, calcium carbonate made the materials stickier; as such it increased the binding ability of the inoculant to seed surface as described by Somasegaran and Hoben (1994).

Water content of the inoculants decreased from 40% (value at the time of peat inoculation) to about 34.1-36.6% after 12 weeks of storage periods. Similar to water content, cell viability in the carrier also decreased across inoculant formulas and storage periods (Fig. 1). Higher decrease was exhibited by inoculants Crb17, Crb46, Crb47 and Crb56. The decrease of inoculants Crb17 and Crb56 have been started since the first week of storage periods. The results suggest that higher cell density at the time of peat inoculation is required to anticipate cell viability reduction; otherwise they cannot be stored for a long time period. The results also confirm that viable cell density of freshly peat-

based inoculants, used to treat the soybean seeds is above 10⁷ cell g⁻¹.

Soybean Response to Inoculation: Response of soybean seeds treated with *Pseudomonas* inoculants varied across treatments and parameters measured (Table 2). At vegetative stage (30 day after sowing), shoot height of treated plants was not significantly different from uninoculated control. The significant effects were exhibited by the number of nodules of plants treated with inoculants Crb5, Crb46, Crb47 and Crb49. Interestingly, plants treated with Crb12, Crb17, Crb24, Crb47 and Crb56 showed significant lower shoot dry weight than that of untreated control. These results were consistent with data at harvesting that showed lower number of pods and yields of plants treated by these isolates which indicate that these five inoculants have injurious effects on soybean growth. On the other hand, plants treated with Crb5, Crb46 and Crb49 significantly increased yields suggesting that these three isolates are truly plant growth promoting bacteria. In short, the overall results showed that interactions between soybean and ACC deaminase-producing *Pseudomonas* in this study felled into two categories, i.e., beneficial and deleterious depending on the isolates.

As previously described, the beneficial effects of *Pseudomonas* Crb5, Crb46 and Crb49 could be due to the action of their ACC deaminase activities in reducing ethylene synthesis and ameliorating growth inhibition. This result is consistent with previous experiments under growth chamber conditions, which showed growth enhancement of plants treated with these isolates (Husen *et al.*, 2009). On the other hand, the injurious effects of those five isolates (Crb12, Crb17, Crb24, Crb47 and Crb56) as exhibited by growth inhibition and yield reduction of soybean upon inoculation are beyond the expectation of the study. Whether the result is the coincidence or an accidental circumstance, the evidence that a bacterium producing ACC deaminase shows both growth-promoting as well as deleterious properties is possible as reported by Belimov *et al.* (2007). Further investigation on environmental factors that influence the beneficial effects of bacterial ACC deaminase activity on plant growth is required.

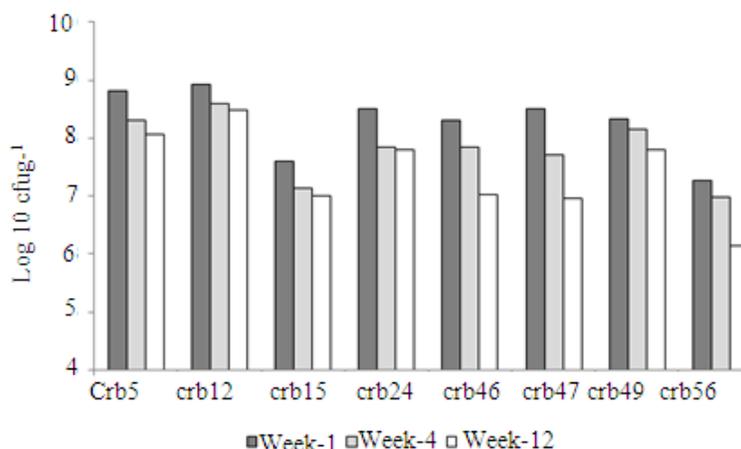


Fig.1: Cell viability in peat-based inoculants of *Pseudomonas* producing ACC deaminase (isolates Crb5 to Crb56) at three periods of storage

Table 2: Soybean response to ACC deaminase-producing *Pseudomonas* under field soil conditions

Isolates	Shoot height (cm)	Shoot dry weight (g)	No. of nodules	No. of pods	No. of Pod filling	Seed dry weight (g)
Crb5	16.8ab	15.0a	21.2b	44.2a	19.7a	3.29a
Crb12	14.5ab	12.3d	15.0d	33.4c	11.9e	1.84fg
Crb17	17.2a	13.8b	20.4bc	43.2a	15.8c	2.57d
Crb24	14.8ab	12.5d	16.6d	33.5c	12.0e	2.03fg
Crb46	16.1ab	14.9ab	21.4b	36.6bc	17.8b	3.04b
Crb47	14.4b	13.7bc	25.8a	38.5b	13.5d	2.28e
Crb49	14.7ab	14.5ab	21.4b	39.5b	19.1a	3.41a
Crb56	15.1ab	12.6cd	16.8d	33.9c	12.8de	2.07f
Untreated	15.0ab	15.6a	17.8cd	43.0a	15.8c	2.77c

Means in a column followed by the same letter are not significantly different at 5% level by DMRT

CONCLUSION

Bacteria having ACC deaminase activities could be truly plant growth promoting bacteria providing that their beneficial effects on plant growth are consistent at a wide range of the environmental conditions. Three out of eight ACC deaminase-producing *Pseudomonas* used in the study increased soybean growth and yield under field soil conditions, whereas those five remaining bacteria showed deleterious effects. The possibility that some environmental factors influence or cover the beneficial effects of bacterial ACC deaminase activity cannot be excluded from consideration.

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