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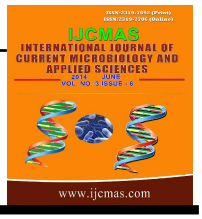

Original Research Article

Role of Antibiosis and production of Indole-3-Acetic acid by bacilli strains in suppression of root pathogens and growth promotion of Alfalfa seedlings

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Keywords

Biological control, PGPR, Antibiosis, IAA, *Chalara paradoxa*, *Fusarium solani*, *Rhizoctonia solani*, *Bacillus amyloliquefacaciens*, alfalfa, date palm, Potato

A B S T R A C T

Many economical crops grown in Qassim region, Saudi Arabia, are subjected to root diseases caused mainly by *Chalara paradoxa*, *Fusarium solani* and *Rhizoctonia solani*. Screening and evaluating of native strains of plant growth promoting rhizobacteria (PGPR) for control of alfalfa, date palm and potato root pathogens was conducted in this study. 170 rhizobacterial strains were isolated from alfalfa, date palm, potato and wheat roots from five different locations in Qassim region. The isolated rhizobacteria were grouped into 35 distinct strains according to their colony morphological characteristics. Tests were conducted on the antibiosis activities against root pathogens, growth promotion to alfalfa seedlings and production of indole-3-acetic acid. The most effective antagonists to *C. paradoxa*, *F. solani* and *R. solani* were *Bacillus amyloliquefacaciens subsp. plantarum* ME 3, *B. amyloliquefacaciens* subsp. *plantarum* ME 106, *B. subtilis* ME 105, *Pseudomonas fluorescence* ME1, *Bacillus spp.* ME 209 and *Paenibacillus polymyxa* ME6. *C. paradoxa* and *R. solani* were more sensitive to antibiosis than *F. solani.* Eleven isolates produced detectable levels of IAA in the growth media. The most active isolates in production of IAA were *B. subtilis* ME 105, *B. amyloliquefacaciens* subsp. *plantarum* ME 3, *P. polymyxa*, and *B. amyloliquefaciens* subsp. *plantarum* ME8 (188- 107 µg/ ml medium). It could be concluded that *B. amyloliquefaciens* subsp. *plantarum* are the most dominant PGPR associated with plant roots in Qassim region. Moreover, screening for promising biocontrol rhizobacteria should consider testing antibiosis, seedling growth promotion as well as secretion of IAA.

Introduction

Date Palm (*Phoenix dactylifera* L.), alfalfa (*Medicago sativa* L.) and potato (*Solanum tuberosum* L.) are the most common crops cultivated in Qassim region where 37,822, 14,785 and 2,500 hectares are planted with these crops respectively (Anonymous 2010). Root rot caused by *Fusarium spp. and Chalara paradoxa* are among the most important diseases of date palm Al-Qassim region (El-Meleigi 2008, El-Meleigi *et al.* 1993).

Potato crop in Saudi Arabia, is also affected by *Rhizoctonia solani*, *Macrophomina* spp,

Pythium spp., *Phoma* spp. and *Fusarium* spp. (Al-Kherb *et al*. 1996, El-Meleigi *et al*. 1984, El Meleigi 2008). Alfalfa, is the main forage crop in the Qassim region and commonly infected by crown and root rot diseases caused by *Fusarium* solani and *Rhizoctonia solani* (El Meleigi 2008).

Farmers in Saudi Arabia and elsewhere became more and more dependent on agrochemicals as a relatively reliable method of crop protection helping with economic stability of their operations. However, increasing use of chemical inputs causes several negative effects, i.e., development of pathogen resistance to the applied agents and their non target environmental impacts (De Weger *et al*. 1995) Gerhardson 2002). Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Gerhardson 2002, Postma. *et al.* 2003, Welbaum, *et al.* 2004).

Plant growth-promoting rhizobacteria (PGPR) colonizing the root surfaces and the closely adhering soil interface, the rhizosphere that are associated with many, if not all, plant species and are commonly present in many environments (Kloepper *et al*. 1992 & 1999). Corn, sorghum and wheat grains treated with certain PGPR isolates were protected against root pathogens and significant increases in corn grain yields were reported (El Meleigi 1989). In recent research, a *Bacillus subtilis* strain was used to protect rice against *R. solani* the causal agent of rice sheath blight (Kumar *et al*. 2013). *Paenibacillus polymyxa (Bacillus polymyxa*) was isolated from wheat roots and used successfully in Qassim fields for control of wheat root rot caused by *Chocliobolus sativus* and *Fusarium graminearum* (El-Meleigi *et al*. 2007) and cucurbits gummy stem blight (black rot) caused by *Didymella bryoniae* (El-Meleigi and Al-Rehiani, 2004).

Screening of a large number of PGPR to select the most effective isolates is a complicated process that is usually begins by detecting those isolates with strong antibiosis activities against the targeted plant pathogen. However, the mechanisms by which PGPR promote plant growth are not yet fully understood, many different traits of this bacterium are responsible for growth promotion activities (Cattelan *et al*. 1999, Bakker *et al*. 2003& 2007). Rhizobacteria promote plant growth by several mechanisms. These include: production or changing the concentration of indoleacetic acid (IAA), giberillic acid, nitrogen fixation, suppression of the growth of deleterious organisms by production of siderophore,b-13-glucanase, chitinases, antibiosis and cyanide, and phosphate solubilization and other nutrients (Cattelan *et al*. 1999).

Our main goal is to screen and select effective native PGPR strains for control of the main root pathogens of alfalfa, date palm and potato in the Qassim region. Success of our efforts will be positively reflected on human health, environment and economics of the farming community and Saudi society.

Materials and Methods

Collection of soil and plant samples

Ten random samples were collected in winter of 2011 from each of 5 fields of alfalfa, potato, wheat and date palm which were separated by a minimum of 5 km. Root samples and attached soil were collected from apparently healthy plants, placed in plastic bags and transferred to the laboratory under cooled conditions. Samples were refrigerated at 5 ºC until processed within 48 h.

Isolation of root pathogenic fungi

Roots of diseased plants showing root rot symptoms were collected from each field, washed thoroughly with running tap water for one hour, surface sterilized with 10% Clorox (NaOCl 5.25%) amended with drops of ethanol, platted dry on sterile filter paper, dissected and plated on Potato Dextrose Agar (PDA), and Corn Meal Agar (CMA) media. Plated samples were incubated at 28 ºC for 5-7 days. Pure cultures of the fungi associated with root rots were obtained by hyphal tip technique and maintained on acidified PDA (pH 6.0) slants and stored at 5ºC in the refrigerator.

Isolation of plant growth promoting rhizobacteria: (PGPR)

Roots of alfalfa, potato, wheat or date palms and adhering soil were blended for 2 min. in a sterile solution of 0.1 M MgSO4 (1g/10 ml). A series of dilutions were made and 0.1 ml aliquots of the appropriate dilutions spread onto PDA, Pseudomonas agar F (Diffco) and Nutrient Agar (NA) media (Diffco). The plates were incubated at 25-28C for 48 to 72 h. Single colonies of distinct species were then transferred into PDA and stored at 4ºC (El-Meleigi 1989.

Antibiosis tests

Rhizobacteria isolates were tested for antibiosis activity against the isolated root pathogens. Each PGPR isolate was streaked across the center of five PDA plates and incubated at 28ºC for 48 h. Five-millimeter-diameter disks obtained from the edge of an actively growing colony of each tested fungus were placed at 3 cm distance from the bacterial growth. The inoculated plates were incubated at 25ºC in the dark. The inhibitions of mycelia growth of the pathogens were measured daily until growth of the control treatment was reached by using the formula:

$$I = \frac{100\ (C-T)}{C}$$

Where, I = inhibition of mycelial growth of pathogen (%), C = radial growth of pathogen in the control plate (mm), and T = radial growth of pathogen in plates challenged with PGPR (mm).

Effect of rhizobacteria on alfalfa seed germination and growth:

For testing the effect of the PGPR isolates on germination and growth of alfalfa, seeds treated with PGPR were germinated in wet paper towel (20 seeds/replicate) and five replicates per treatment (Fig 1). Plant vigor was determined visually by comparison to check treatment (non treated seeds) on a scale of 0-100, where the check plants were considered 100% and by recording the fresh weight of ten seedlings per replica, two weeks after sowing.

Assay for IAA production:

Bacterial isolates were grown on nonfat milk broth medium (NFM) containing tryptophan (1.0 mg/L). The cultures were grown for 72 h at 26 °C in a shaking incubator. The bacterial cells were separated by centrifugation at 10,000 rpm for 15 min. The pH of the supernatant was adjusted at 2.8 with HCL and then extracted 3 times with equal volumes of ethyl acetate (Tien *et al*., 1979). The extract was evaporated to dryness and the residue was re-suspended in 2 ml of ethanol. The samples were analyzed on HPLC using a UV detector and Tec sphere 5-ODS C-18 column. A methanol: acetic acid: water (30:1:70) mixture was used as mobile phase with flow rate 1.5ml/min. For identification, 20 µl samples, filtered through 0.45 µl filter, were injected into the HPLC column. The growth

hormone was identified on the basis of retention time of the standard IAA by using a refractive index detector (RI). The concentration was calculated on the basis of peak height and peak area in comparison with standard. The standard treatment was non inoculated broth of NFM media .

Identification of effective PGPR isolates through DNA sequencing

Identity of PGPR isolates showing significant growth promotion, antibiosis or detectable amounts of IAA were confirmed by CABI Europe UK, Bakeham Lane, Egham, Surrey TW20 9TY. Bacteria samples were processed using partial 16S rDNA sequencing homology technique analysis. All procedures were validated and processing undertaken in accordance with CABI's in-house methods as documented in TPs 61-68.

Results and Discussion

Diversity of PGPR isolates

One hundred and seventy six rhizobacteria strains were isolated from roots of alfalfa, date palm, potato and wheat plants collected from Qassim fields. These isolates were grouped according to culture and colony characteristics into 35 distinct strains. One representative strain from each group was used in further tests.

The designated code number and source of each isolate are presented in Table 1. Fifteen of the PGPR strains were obtained from wheat, 9 strains from alfalfa, 7 strains from potato, and 4 strains were isolated from date palm roots (Table 1). Most of the effective and promising PGPR strains as shown by our results below were obtained from wheat roots (Table 1).

Effect of rhizobacteria on alfalfa seed germination and fresh weight

Statistical analysis of the fresh weight date (Table 1), showed significant differences among PGPR strains in their effect on fresh weight of alfalfa in the laboratory. Two PGPR strains *P. polymyxa* ME6 (#7), obtained from wheat roots and *B.amyloliquefacaciens subsp. plantarum* ME 106 (#13), obtained from alfalfa roots increased alfalfa seedlings growth significantly over check treatment (Fig. 1&2). Meanwhile, 14 PGPR isolates had no significant effect of growth of alfalfa seedlings and 19 PGPR isolates significantly suppressed alfalfa seedlings growth in the laboratory (Table 1). The most suppressive rhizobacteria were isolates # 5, 23 and 25 that were isolated from roots of date palm and wheat, respectively **(Table 1, Fig. 1&2).**

Antibiosis of 35 PGPR strains to three root pathogens

Rhizoctonia solani and, *F. solani* were isolated from diseased alfalfa and potato plants respectively while *C. paradoxa* was isolated from the roots of diseased date palm trees. All the 35selected rhizobacteria isolates were screened in the laboratory on PDA medium for their antifungal activities against *R. solani* from alfalfa *F. solani* from potato and *C. paradoxa* from date palm. Six rhizobacteria (17.1%) showed significant antibiosis activities against one or more of the tested pathogens (Table 2).

Antibiosis activities varied according to rhizobacteria isolate and species of challenged fungal root pathogen. *Chalara paradoxa* was the most suppressed by rhizobacteria isolates followed by *F. solani* and *R. solani*, respectively (Table 2, Fig. 3&4) *Bacillus amyloliquefacaciens subsp. plantarum* isolates ME 3 and ME 106

demonstrated considerably higher antibiosis on all of the fungal pathogens than did the other rhizobacteria

Production of IAA

Eleven PGPR isolates showed detectable levels of IAA in the growing media (Table 3). The production of IAA by different bacteria varied significantly among tested PGPR isolate. The highest levels of IAA production were associated with *B. subtilis* ME 105, *B. amyloliquefacaciens* subsp. *plantarum* ME 3, *P. polymyxa*, and *B. amyloliquefaciens* subsp. *plantarum* ME8 (188, 151.9, 108.1 and 107 µg/ ml, respectively) (Table 3). The production of IAA by the other seven rhizobacteria isolates was 56.2-26.2 µg/ ml (Table 3).

Statistical analysis of the correlation of alfalfa growth promotion, antibiosis activities and production of IAA for the six tested bacteria showed no correlations between the three traits R= 0.01-0.293 (Table 4). Rhizobacteria are an important functional group of beneficial bacteria used for plant growth promotion and control of soil borne pathogens (Hoflich *et al*., 1994; Rajkumar *et al*., 2004). Screening studies in our lab resulted in the selection of six efficient strains out of approximately 35 strains isolated from the rhizosphere of alfalfa, date palm, potato and wheat. Most of the isolated PGPR strains were found in wheat roots while the least number of isolates were isolated from date palm . This may be related to the nature of root growth of each plant. Wheat roots are extensive adventitious roots that grow in top soil where microbial activity is at its optimal growing conditions, while the alfalfa, potato and date palm roots grow more deeply in the soil where microbial activities and diversity are gradually declining.

The objective of this investigation was to select the most efficient antagonists against soil borne infection of *C. paradoxa* , *R. solani* and *F. solani* isolates from diseased roots of date palm, alfalfa and potato grown in fields of the Qassim region of Saudi Arabia. The 35 strains were subjected to different screening methods to select a suitable strain for the control of *C. paradoxa R. solani* and *F. solani*. First, the traditional *in vitro* dual culture assay on culture media was taken as a measure of antagonistic potential of the bacterial strains. Of the 35 isolates, *B. amyloliquefacaciens subsp. plantarum* ME 3, *B. amyloliquefacaciens* subsp. *plantarum* ME 106, *B. subtilis* ME 105, *P. fluresens* ME1, *Bacillus spp.* ME 209 and *P. polymyxa* produced detectable inhibition against two or all of the tested root pathogen.

B. amyloliquefacaciens subsp. plantarum ME 3, *B. amyloliquefacaciens* subsp. *plantarum* ME 106 and *B. subtilis* ME 105 suppressed the mycelial growth of *C. paradoxa, F. solani* and *R, solani* at an average of 84.7, 76.5 and 51.6, respectively. While the other three PGPR isolates *B. subtilis* ME 105, *Florescent pseudomonas* ME1, *Bacillus spp.* ME 209 and *P. polymyxa* were not effective antagonists to *R. solani* and suppressed mycelial growth of *C. paradoxa* and *F. solani* at an average of 13.7-28.3% . The results show that *C. paradoxa* was the more sensitive to the antibiosis activities of the six PGPR isolates compared to *F. solani* and *R. solani*.

The second test performed in this study was conducted to select the most effective growth promoters of the 35 PGPR isolates was the alfalfa seed germination and growth test. *B. amyloliquefacaciens subsp. plantarum* ME 3, and *B. amyloliquefacaciens* subsp. *plantarum* ME 106 were the only isolates that increased seedlings growth over the non-treated seeds (5.71%) of the tested isolates).

Table.1 Plant growth promoting rhizobacteria isolated from alfalfa, date palm and wheat roots in the Qassim region of Saudi Arabia and their effect on fresh weight of two week old California 1 alfalfa seedlings, in vitro.

PGPR Code	Source	Fresh Weight (g/10 seedlings)*	Identification**	PGPR Code	Source	Fresh Weight (g/10 seedlings)*	Identification**
1	Wheat	0.554 e		19	Wheat	0.412 c	
2	Date Palm	0.408 c		20	Wheat	0.502 cde	
3	Date Palm	0.420 c		21	Potato	0.424 c	
4	Wheat	0.410 c	*B. subtilis* ME 105	22	Wheat	0.522 cde	
5	Date Palm	0.278 b		23	Wheat	0.156 a	
6	Alfalfa	0.552 e		25	wheat	0.138 a	
7	Wheat	0.710 f	*Paenibacillus polymyxa* ME6	26	wheat	0.548 e	
8	Alfalfa	0.518 cde	ME 8	27	Wheat	0.534 e	
9	Alfalfa	0.548 e		28	Wheat	0.512 cde	
10	Wheat	0.528 de	*Bacillus* spp. ME7	29	Potato	0.422 c	
11	Alfalfa	0.546 e		30	Potato	0.524 cde	
12	Alfalfa	0.540 e	*Bacillus amyloliquefacaciens subsp. plantarum* ME 3	31	Potato	0.280 b	
13	Alfalfa	0.706 f	*Bacillus amyloliquefacaciens subsp. plantarum* ME 106	32	Potato	0.280 b	*Pseudomonas fluresens*
14	Date Palm	0.430 cd		33	Wheat	0.282 b	
15	Alfalfa	0.416 c		34	Potato	0.422 c	
16	Alfalfa	0.408 c	*Bacillus* spp. ME209	35	Potato	0.384 b	*Bacillus amyloliquefaca ciens subsp.*
17	Alfalfa	0.548 e		None		0.568 e	
18	Wheat	0.414 c					

Table.2 Antibiosis activity of six plant growth promoting rhizobacteria (PGPR) isolates against *Rhizoctonia solani*, *Fusarium solani* and *Chalara paradoxa* on potato dextrose agar medium incubated at 24-27 °C.

Rhizobacteria Isolate	*Rhizoctonia solani*	*Fusarium solani*	*Chalara paradoxa*	Mean
	% Inhibition of radial growth			
B. amyloliquefacaciens subsp. plantarum ME 3	79	83	92	84.7
B. amyloliquefacaciens subsp. *plantarum* ME 106	57	80	92	76.3
B. subtilis ME 105	40	65	50	51.6
Pseudomonas fluresens ME1	0	33	52	28.3
Bacillus spp. ME 209	0	18	28	15.3
Paenibacillus polymyxa ME6	0	21	20	13.7

Table.3 Production of IAA by plant growth promoting r isolates in a descending order.

Rhizobacteria isolates	IAA µg/ ml medium
B. subtilis ME 105	188.c
B. amyloliquefacaciens subsp. plantarum ME 3	151.9bc
P. polymyxa	108.1b
B. amyloliquefaciens subsp. plantarum ME8	107.0b
ME 116	56.2a
ME189	56.1a
P. fluresens ME1	50.2a
ME190	34.2a
B. amyloliquefacaciens subsp. *plantarum* ME 106	30.4a
ME186	28.0a
Bacillus spp. ME 209	26.2a

Averages followed by same letter with in a column are not significantly different from each other at P ≤ 5% (Duncan's multiple range test)

Table.4 Simple correlation coefficients of alfalfa seedlings fresh weight, antibiosis activity and production of Indole-3-acetic acid (IAA) by PGPR isolates

Traits	Antibiosis (% inhibition)	IAA (µg/ml)
Alfalfa fresh weight	0.293	0.010
Antibiosis (% inhibition)	-	0.135

(Critical value at 0.05 level of probability = 0.81)

Figure.1 Growth of alfalfa seeds treated with PGPR strain ME 5(right) compared with seeds treated with *Bacillus amyloliquefacaciens subsp. plantarum* ME 106 (left).

Figure.2 Germination of untreated Alfalfa seeds (20 seeds) (C), and those treated with PGPR strain ME 5 (5) or *Bacillus amyloliquefacaciens* subsp. *plantarum* ME 106 (13).

Figure.3 Antibiosis of *Bacillus amyloliquefacaciens subsp. plantarum* ME 3, *Bacillus amyloliquefacaciens* subsp. *plantarum* ME 106, *Bacillus. subtilis* ME 105, *Pseudomonas fluresens* ME1 to *Chalara paradoxa* (C), *Fusarium solani* (F), and or *Rhizoctonia solani* (R)

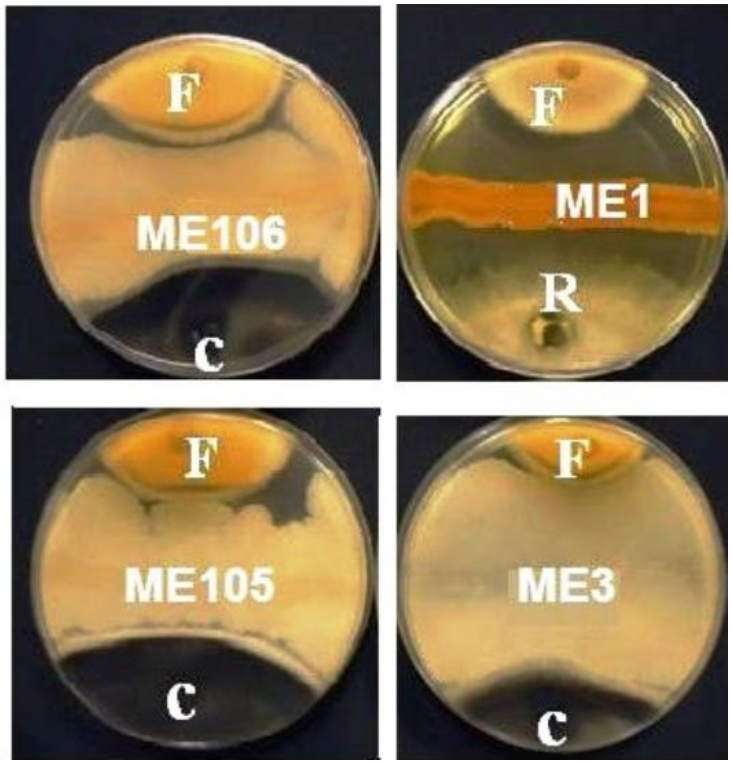
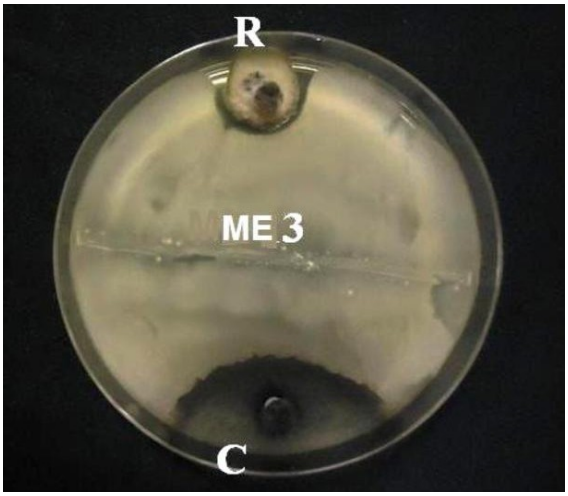


Figure.4 Rhizobacteria *Bacillus. amyloliquefacaciens subsp. plantarum* ME 3 over grow and limit radial growth of *Chalara paradoxa* (C) and *Rhizoctonia solani* (R).



The other isolates were either neutral 40% (14 isolates) or suppressive to alfalfa germination 54.3% (19 isolates). However, the results of this test may be different if it is conducted in the presence of the pathogens. Therefore, it is suggested that these two isolates *B. amyloliquefacaciens subsp. plantarum* ME 3, and *B. amyloliquefacaciens* subsp. *plantarum* ME 106 are growth promoters to alfalfa as well as good antagonists to its root pathogens *R. solani* and *F. solani*.

This suggests the presence of fungistatic metabolites and antibiotics as well as growth promotion materials are secreted by certain PGPR isolates. Previous research indicated that PGPR isolates produced β 1, 3-glucanase, salicylic acid, and HCN when inhibiting the mycelial growth of *R. solani* (Nagarajkumar *et al*., 2004).

Only 11 isolates out of 35 tested, produced detectable amounts of IAA ranged from 26.2 to 188 µg/ ml (Table 3). Up to 80% of rhizobacteria can synthesize IAA (Loper and Scroth 1986). However, secretion of high levels of IAA by rhizobacteria was not always related to growth plant promotion activities, high levels of IAA production was associated with some free living rhizobacteria (Barazani and Friedman 1999). This results suggest similar findings, high production of IAA by PGPR isolates was not constantly associated with plant growth promotion activity in the lab and the greenhouse (results under publication). Therefore, the amount of IAA production by PGPR isolates does not necessarily relate to growth promotion of tested plants. It is known that IAA is the major root growing hormone and its synthesis depend on the plant and its environment more than the rhizosphere living rhizobacteria. In our study *B. amyloliquefacaciens* subsp. *plantarum* ME 106 and *P. polymyxa* ME 6 were the best strong growth promoters to alfalfa and offered the best protection against the soilborne *R. solani* and *F. solani*, meanwhile B. *amyloliquefacaciens* subsp. *plantarum* ME 106 produced relatively low level of IAA (30.4 µg/ml) while *P. polymyxa* ME6 produced high level of IAA (108.1 µg/ml) (Table 3).

As the host plant plays an important role in supporting the introduced antagonists in field conditions, a screening method involving the host plant, pathogen, and the antagonist is expected to give a more realistic picture than the dual culture plate assay (Rajkumar *et al*., 2004). Therefore, we are conducting greenhouse experiments on PGPR isolates with best results obtained from the three screening methods described above.

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