PRODUCTION AND OPTIMIZATION OF INDOLE ACETIC ACID BY *PSEUDOMONAS* SPP. ISOLATED FROM RICE PHYLLOSPHERE

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ABSTRACT

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Experiments were conducted in the laboratory of Crop Protection Department at Universiti Putra Malaysia (UPM), Malaysia in 2013 to determine indole acetic acid (IAA) production ability and to optimize the production condition of phylloplan bacteria. All the experiments were laid out in RCB design with 6 replications. A total of 14 bacterial isolates were isolated following appropriate methodologies from rice plant samples collected from different rice fields of Peninsular Malaysia. Among them, 5 isolates namely, UMB20, UMB22, KMB28, BMB42 and BMB59 were found capable to produce IAA and they were identified as *Pseudomonas* spp. according to Bergy's Manual of Determinative Bacteriology. These bacterial isolates were monitored for inhibition ability against *Rhizoctonia solani* causing sheath blight disease. Irrespective of bacterial isolates Tryptic Soya Broth medium showed the highest amount of IAA production ranged 29.66 to 51.29 µg/mL. The rate of L-Tryptophan 3mg/mL was the best concentration to produce higher amount in IAA by all the isolates. The higher range of IAA production (55.9-72.89 µg/mL) by all the isolates was observed with pH 9. The optimum temperature was 35°C to produce highest amount of IAA. Subsequently, the effect of culture filtrate on plant growth was tested by the pot assay in glass house. Rice seeds were treated with culture filtrate and allowed to grow them for 12 days. Analysis of seedling growth parameters showed that seedling length, percentage of seed germination, seedling vigour index, fresh weight of seedlings and number of adventitious roots were significantly increased compared to control but statistically similar to standard IAA. Dry weight of seedlings didn't vary significantly compared to standard IAA and control. In conclusion, *Pseudomonas* spp. isolated from plant sample can produce IAA and they can be used as biofertilizer inoculants to promote plant growth.

Key words: Growth factor, Growth promotion, Indole Acetic acid, *Pseudomonas* spp.

INTRODUCTION

Several bacterial species under the genera *Bacillus*, *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Bradyrhizobium*, and *Rhizobium* have demonstrated plant growth promoting (PGP) activities in many crops (Wahyudi *et al*. 2011). Among them *Pseudomonas* is widely recognized and often considered predominant group that possesses almost all the plant growth promoting traits (Ownley *et al*. 2003). The direct beneficial effects of fluorescent pseudomonads have been attributed to their ability to produce various compounds including phytohormones like indole-3-acetic acid (IAA), gibberellins and cytokinins (Podile and Kishore, 2006). The indirect mechanisms involve inhibition of different phytopathogens (Bhasan and Holguin 1998) in crops. IAA is a product of L-Tryptophan metabolism in different microorganisms including bacteria and plays an important role in plant growth and development process. Bacterial auxins have the potential to change any of these processes by altering the plant auxin pool. It depends on the amount of IAA produced and the sensitivity of plant tissue to changing levels of IAA. The roots are the most sensitive organs and respond to the changing levels of IAA by elongation of primary roots, formation of adventitious and lateral roots, or cessation of growth (Davies 1995). Indole 3 acetic acid does not function as a hormone in bacterial cells but their ability to produce the same may have evolved as it is important in plant-bacteria relationship (Paten and Glick 2002; Spaepen *et al*. 2007). Bacteria produce IAA by using L-Tryptophan as a precursor (Sarwar and

Frankenberger 1994) and *in vitro* IAA production by bacteria depends on the culture condition.

Extensive studies have been performed on IAA as a plant hormone synthesized by microorganisms including *Pseudomonas* associated with rhizosphere. Other than rhizobacteria, some phyllosphere bacterial strains of *Pseudomonas* spp., *Pantoea* spp. and *Agrobacterium* spp. have been reported to increase plant growth and nutrient uptake of grasses, cereals, and legumes (Boddy *et al*. 1995). *Pseudomonas* bacteria originating from the phyllosphere can be possessed diverse beneficial traits and their potency need to be explored. With this view, the study was undertaken to isolate IAA producing bacteria from rice plant (phylloplane) and to increase IAA production by varying different parameters such as L-Tryptophan concentrations, pH, and temperature, so that we can get the intended conditions at which the IAA production is maximized and to see the effect of isolates on seed germination and seedling growth.

MATERIALS AND METHODS

Isolation of *Pseudomonas* bacterial isolates

Experiments were conducted in the laboratory of Plant Protection Department at Universiti Putra Malaysia, Malaysia in 2013. A total of 100 rice plant samples were collected from irrigated paddy fields in different rice growing areas of Peninsular Malaysia. Bacterial isolates were isolated from the samples following dilution technique on Kings B Agar medium. Bacterial colonies were selected based on morphological characteristics. Single colonies were maintained on nutrient agar (NA) slants as pure culture and preserved in refrigerator (at 4°C) for further use.

Screening of bacterial isolates for Indole acetic acid (IAA) production

All the isolates were screened for IAA production following the methods described by Oh *et al*. (2011) with slight modifications. In this case, nutrient broth (NB) was used instead of DF minimal medium. Loop full bacterial cultures were inoculated in nutrient broth (NB) amended with L-Tryp at the rate of 1g/L and incubated for 5 days at 30C with an orbital shaker at 130 rpm. After incubation the cultures were centrifuged at 8000 rpm for 10 minutes. Two mL of supernatant was transferred into the tube and mixed vigorously with 4 mL of Salkowski's reagent (2 mL of 0.5M $FeCl_3$ in 98 mL of 35% $HClO_4$) and allowed to stand at room temperature for 25 minutes. Development of pink to red colour in the tubes indicated IAA production. Results obtained from this test scored as either positive or negative. Bacterial isolates showing negative response to IAA production were discarded from this experiment.

Cultural and biochemical characterization of bacterial isolates

All the *Pseudomonas* isolates were characterized for pigmentation, fluorescence under UV light, mobility, shape, colour, odour, gram reaction, indole production, Methyl Red (MR), Voges Proskaeur (VP), catalase, nitrate reduction, oxidase and gelatin liquefaction starch hydrolysis according to Bergy's Manual of Determinative Bacteriology (Cappuccino and Sherman 1992).

Evaluation of bacteria for antagonism to *Rhizoctonia solani*

Selected bacterial isolates were evaluated for inhibition potency against *R. solani* following dual culture technique. Four millimeter diameter plug with 3 day-old *Rhizoctonia solani* was placed in the centre of potato dextrose agar (PDA) plate. Bacterial isolates (48-hour old) grown on NA was inoculated to the plate at 4 places in equal distance. Each spot was 3 cm apart from the centre of the plate. The plates were incubated for 72 hours at room temperature (28±2 C). Petri dishes having no bacterial cultures served as control.

Selection of medium for IAA production by bacterial isolates

Four types of media e.g. Nutrient broth (NB), Tryptic Soya Broth (TSB), Luria Bertany Broth (LBB) and modified Succinate Broth (SB) were used in this test. Media were amended with L-Tryp at the rate of 1g/L. Bacteria were individually inoculated to the media in the conical flask containing 100 mL of each media. After incubation for 5 days at 150 rpm on rotary shaker at 30 C, test of IAA production was done following the same procedure described earlier. Colour produced in the different medium was visually examined and the intensity of colour was measured by UV-visible spectrophotometer (Cary 50 Bio). Amount of IAA was calculated using the equation came from standard curve using each medium separately. For quantitative determination of IAA production by bacterial isolates, the absorbance was measured at 530 nm and the quantity of IAA produced was estimated against the IAA (Sigma Chemicals) standard from standard curve. Uninoculated broth served as control. Medium producing higher range of IAA was selected for further study.

Preparation of standard graph of IAA

Different concentrations were prepared as aqueous solution of IAA (standard) ranging from 5 µg/mL to 100 µg/mL. To each 1 mL solution, 2 mL of Salkowaski reagent was added and readings were taken after 25 minutes at 530 nm by UV-Visible spectrophotometer. Standard graph is prepared by plotting concentration of IAA in micrograms/mL Vs Optical Density (OD).

Effect of concentration of L-Tryptophan on the production of IAA

Selected TSB medium was inoculated with the isolates amended with 1, 3 and 5 mg/mL L-Tryp. The amount of IAA was measured (following the same methodology described earlier) 7 days after incubation. After optimization of the standard concentration of L-Tryp in the medium was used for other factors affecting production of IAA *in vitro*.

Effect of temperature

Optimization of temperature was carried out by incubating the medium amended with 3 mg/mL L-Tryp containing bacterial isolates at 20, 25, 30, 35 C on orbital shaker at 130 rpm for 7 days.

Effect of pH of the media

To study the effect of pH on the production of IAA by the different isolates, 3mg/mL of L-Tryp was added to TSB medium and adjusted to 5, 7, 9 and 11 pH levels. Medium was inoculated with one loop of 48 hours old bacterial culture and incubated at 30 C for 5 days at 130 rpm on orbital shaker. After 5 days of incubation the amount of IAA was assessed.

Confirmation of IAA by Thin Layer Chromatography (TLC)

Confirmation of IAA production was done following the methodology of Ahmad *et al.* (2005).

Extraction of crude IAA

Single bacterial colonies of 3 isolates of *Pseudomonas* spp. were inoculated in 200 mL of NB amended with 1 and 5 mg/mL of L-Tryp and incubated at optimum temperature for 1 week on a shaker incubator. Bacterial cells were separated from the supernatant by centrifugation at 8000 rpm for 10 minutes. The supernatant was filtered with syringe filter (0.22µm) and acidified to pH 2.5 to 3.0 with 1N HCl. Extraction was done twice with ethyl acetate at double volume of the supernatant. Extracted ethyl acetate fraction was evaporated to dryness in a rotatory evaporator at 40 C. The extract was dissolved in 3 mL of methanol and kept at -20 C.

Thin layer chromatography

Ethyl acetate fractions (10-20 mL) were placed on TLC plates (Silica gel G f254, thickness 0.25 mm) and developed in a mixture of eluents ethyl acetate: chloroform: formic acid in the ratio of 55:35:10. Spots with retention factor (Rf) values identical to authentic IAA were identified under UV light (254 nm) by spraying the plates with Salkaosky reagent. Retention factor value was calculated using the following formula (Sudha *et al*. 2012):

$$RF = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

The effect of crude extract of IAA on the growth of rice seedlings

Culture filtrate of bacterial isolate UMB20 was used for this test. After 5 days of incubation culture filtrate of isolate UMB20 was obtained following the previous method described elsewhere in the article. Surface sterilized 25 seeds of rice cultivar MR219 were soaked in the supernatant for 1-hour blot dried. Seeds were placed in wet blotters and incubated in a growth chamber for 12 days. Seeds soaked in sterile water were used as controls. The vigour index was calculated using the following formula: vigour index = percent germination × seedling length (shoot length + root length) (Abdul-Baki and Anderson 1973).

All the quantitative data were subjected to analysis of variance (ANOVA) with SAS software (version 9.2) and significant differences among the treatments were determined according to least significant differences ($P \leq 0.05$).

RESULTS AND DISCUSSION

Isolation and screening of *Pseudomonas* bacterial isolates for IAA production

A total of 14 bacterial isolates were isolated from the samples. Among them, 5 isolate namely UMB20, UMB22, KMB28, BMB42 and BMB59 produced pink to red colour responding to IAA production which was visible in the supernatant after adding the Salkawosky reagent. The rests of the isolates did not produce pink or red colour indicating they were negative to this test (Fig:1). In the environment, most of the *Pseudomonas* bacteria have intrinsic ability to produce IAA. The results are in agreement with the results of Ritika *et al*. (2012). They reported that different isolates of *Pseudomonas* sp. produced auxin like substances in the stationary phase of growth.

A total of 5 isolates were positive to IAA production and tentatively identified as *Pseudomonas* bacteria on the basis of cultural and biochemical characteristics as described in the Bergy's Manual of Determinative Bacteriology (Table 1).

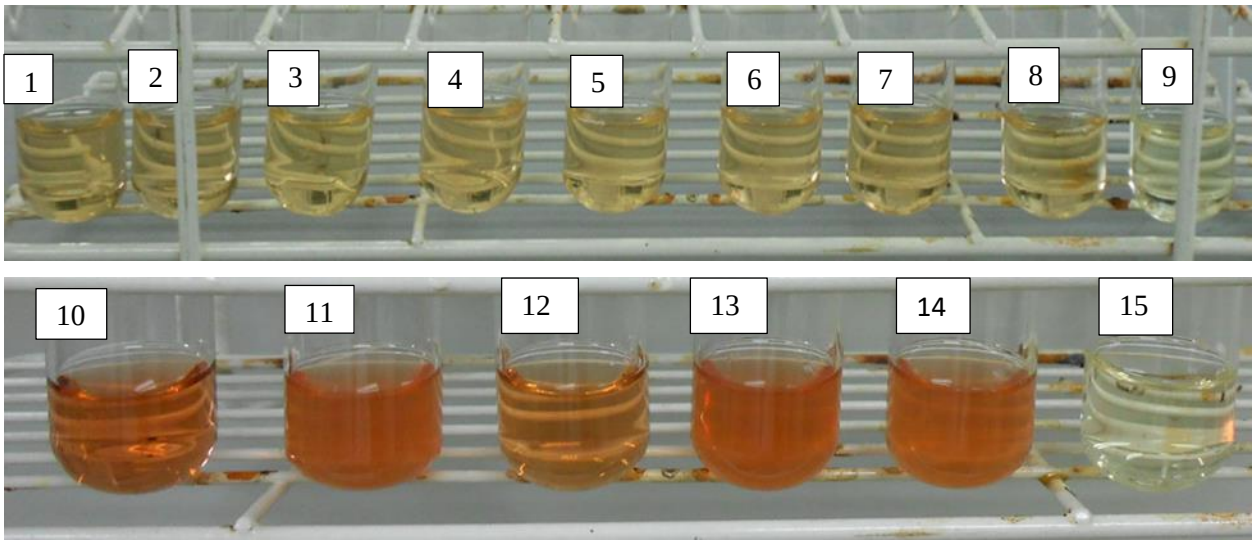

Figure 1: Reaction of bacterial isolates to IAA production; 1= KMB25, 2= KMB26, 3= KMB29, 4= TMB32, 5= TMB33, 6= PMB35, 7= PMB38, 10= UMB20, 11=UMB22, 12= KMB28, 13= BMB42, 14=BMB59, 9 and 15=control

Table 1: Cultural and biochemical characteristics of bacterial isolates

Characteristics of test organisms	Reaction of test bacteria					
	UMB20	UMB22	KMB28	BMB39	BMB42	BMB59
Pigmentation	+	+	+	+	+	+
Fluorescence under NUV light	+	+	+	+	+	+
Gram reaction	-	-	-	-	-	-
Motility	+	+	+	+	+	+
Biochemical tests						
Indole	-	-	-	-	-	-
MR	-	-	-	-	-	-
VP	-	-	-	-	-	-
Oxidase test	-	-	-	-	-	-
SH	-	-	-	-	-	-
NR	+	+	+	+	+	+
UP	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
GL	-	-	-	-	-	-

Note: MR= Methyl Red; VP= Voges Prokaeur; GL= Gelatin liquefaction; NR= Nitrate reduction; SH= Starch hydrolysis; UP= Urease Production; + means positive response; - means negative response

Evaluation of antagonistic activity of bacterial isolates against *R. solani*

Inhibition capacity of test isolates (UMB20, UMB22, KMB28, BMB42 and BMB59) against the fungus ranged 45.11 to 50.00 % (Table 2). Among the isolates, UMB20 was found to reduce the fungal growth by 50% which was followed by BMB42 where the magnitude of growth inhibition was 48.22 %. Percent growth inhibitions ranged 45.11-46.44% obtained by other bacteria were statistically similar. All the isolates produced clear inhibition zone around the fungal colony measuring 8.0-10 mm. The biggest zone (10.00 mm) was produced by isolate BMB42 which was followed by UMB20 (9.5 mm). KMB28 (9.0 mm), UMB 22 (8.5 mm) and the lowest zone (8.0 mm) was produced by the isolate BMB59. The assay can be used as standard test for the selection of biocontrol agent and in many reports it has been shown cumulative effect of all mechanisms undergoing for biocontrol due to production of different secondary metabolites (Compant *et al*. 2005). These compounds may be dissolved the cell wall of the pathogen mycelium and blocked the normal growth. *Pseudomonas fluorescens* strain Pf 003 was found to be effective against sheath blight pathogen (Reddy *et al.* 2010).

Table 2: Effect of bacterial isolates on mycelial growth of *Rhizoctonia solani* after 3 days of incubation

Bacterial isolates	Inhibition zone (mm)	Growth inhibition (%)
UMB20	9.5ab	50.00a
UMB22	8.5bc	45.11b
KMB28	9.0abc	45.77b
BMB42	10.00a	48.22ab
BMB59	8.0c	45.11b
Control	0.00	0.00

Means followed by a common letter are not significantly different according to least significant difference test (P≤0.05)

Selection of medium

Irrespective of bacterial isolates TSB medium showed the highest amount of IAA production ranged 29.66 to 51.29 µg/mL (figure 2). Succinate medium had the little effect on the production of IAA by the isolates. Based on the higher production rate of IAA, TSB medium was selected for further study. Furthermore, comparing with other media, TSB gave the typical colour for indication of IAA production. This might be happened due to the composition of the medium which enhanced IAA production.

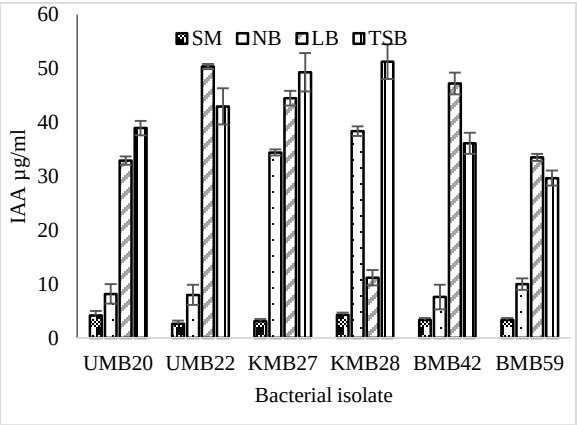

Figure 2: Indole acetic acid produced by bacteria in four media amended with L-Tryptophan at the rate of 1mg/mL (SM= Succinate medium; NB= Nutrient broth; LB= Luria Bertany broth and TSB= Tryptic soya broth; Bar = mean ± standard deviation).

Effect of L-Tryptophan concentration

The medium was supplemented with different concentration of L-Tryp ranging from 0 to 5 mg/mL to see their effect on IAA production. The quantitative analysis by spectrophotometer revealed the great variation in the production of IAA by the bacterial isolates with different concentrations of L-Tryp (Table 3). After 7 days of incubation, IAA production was recorded 7.5-38.21µg/mL without tryptophan. Increasing trend in producing IAA by increasing the concentration of L-Tryp was observed until 3mg/mL. At this concentration of L-Tryp range of the hormone was 75.04-83.33 µg/mL. The rate of L-Tryp 3mg/mL was the best concentration to produce higher amount in IAA by all the isolates. The production of IAA was decreased at the higher concentration of L-Tryp (5g/mL). Ghosh and Bashu (2002) reported that *Rhizobium* sp. isolated from root nodules of *Dalbergia lanceolaria* produced higher amount of IAA at 2.5 mg/mL while another *Rhizobium* sp. isolated from root nodules of *Ryostonia regia* reported as maximum IAA producer at 3 mg/mL of L-Tryp. Khalid *et al.* (2004) showed the variable amount of auxins produced by the rhizobacteria *in vitro*, and amendment of the culture media with L-Tryp further stimulated auxin biosynthesis. In the present studies, L-Tryp concentration used (0-5 mg/mL) in the medium resulted in an increase in IAA production where 3 mg/mL L-Tryp concentration gave maximum IAA production whereas at higher concentrations L-Tryp (5 mg/mL) decreases the production. This might be happened due to production of IAA degrading enzymes. These types of enzymes such as IAA oxidase and peroxidase were reported earlier in *Rhizobium* sp. from *Cajanus cajan* (Datta and Basu 2000). Ahmad *et al.* (2005) reported that rhizosphere *Azotobacter* spp. and *Pseudomonas* spp. produced a high level of IAA when theses bacteria were cultured in a nutrient broth amended with 2 and 5 mg/mL of L-Tryp. Karnwal (2009) tested fluorescent *Pseudomonas* isolates for their ability to produce indole acetic acid in pure culture in the absence and presence of L-Tryp and found that for both strains, indole production enhanced with increases in L-Tryp concentration.

Effect of temperature

The effect of temperature (20-35°C) on production of IAA was shown in the figure 2. All bacterial isolates produced maximum amount of IAA with the levels of 84.52-89.82 µg/mL and lower amount of IAA with the levels (25.58-65.72 µg/mL) while incubated at 35 and 20 C, respectively. At 20 C temperature isolate UMB22 produced the lowest level (25.58) of IAA and the second lowest was recorded in UMB20 (32.77). Almost the same amount was produced by other isolates. At higher temperature the production of IAA by all isolates was found higher. Aldesuquy *et al.* (1998) found that temperature in the range of 25-30 C was suitable for growth and IAA production of *Streptomyces* sp.

Table 3: Effect of L-Tryptophan concentration in the medium on IAA production by bacterial isolates after 7 days of incubation

Bacterial isolate	Production of IAA (µg/mL) at different concentration of L-Tryptophan			
	0 mg/mL	1mg/mL	3mg/mL	5mg/mL
UMB20	38.21a	46.15c	75.45ab	60.61cd
UMB22	22.52d	45.00c	83.33a	75.27a
KMB28	7.50e	49.08b	68.07b	57.68d
BMB42	28.57b	48.89b	78.80b	66.47b
BMB59	26.00c	56.10a	75.04ab	64.28ab
LSD	1.62	2.22	8.90	5.29

Means followed by a common letter are not significantly different according to least significant difference test (P≤0.05)

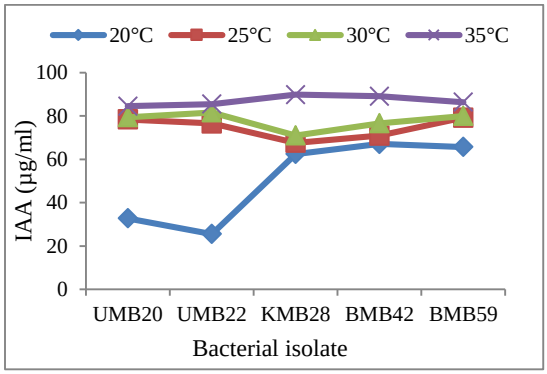

Figure 3: Effect of temperature on IAA production by bacterial isolates

Effect of pH

To determine the optimum level of pH for IAA production, the test isolates were inoculated in TSB amended with 3mg/mL L-Tryp having pH 7, 9 and 11 (Fig 4). The higher range of IAA production (55.9-72.89 µg/mL) by all the isolates was observed with pH 9 which may create favourable condition in the medium to produce the hormone. The production of IAA by all the isolates decreased at pH level 11. The optimum condition of the medium for higher production IAA by the isolates having pH with 7-9. From these it is clear that too much basic condition is not suitable for production of IAA by bacteria. It may affect the function of enzyme systems as well as the solubility of many substances that are important for bacterial growth. Our findings of IAA production with the pH level in bacterial isolates are in agreement with other researchers. Yurekli *et al.* (2003) reported that the synthesis of high levels of IAA was determined in cultures with pH level 7.5. In another study, *Streptomyces* sp. produced maximum IAA in the medium having pH 7 (Khamma *et al.* 2010). Maximum amount of IAA was produced by some bacteria when the pH of medium was 7-9 (Mohite 2013). According to Madhuri (2011), different *Rhizobium* strains produced maximum IAA at pH level 7.0.

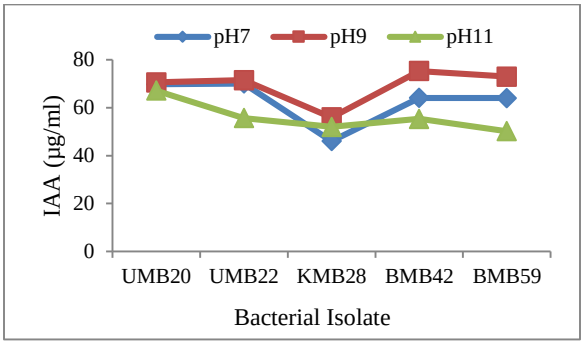

Figure 4: Effect of pH in growing medium on IAA production by bacterial isolates

Thin layer chromatography

Upon spraying with the developing agent, the obtained Rf value of culture spots was (0.87) which was almost equal to the standard value of IAA (data were not presented here). Thin-layer chromatography and comparison with Rf value of the standard revealed that the compound obtained from our bacterial culture to be IAA. Ahmad *et al.* (2005) also found the same Rf value from the extracted IAA by TLC with authentic IAA. The findings are also in agreement with the reports by Xie *et al.* (1996).

Effect of culture filtrate on seedling growth of rice

Analysis of the seedlings after 12 days of growth evident that culture filtrates of *Pseudomonas* could promote plant growth based on seedling height, seed germination, seedling vigour index, fresh weight and number of adventitious roots in comparison with the control (Table 4). The highest percentage (97.00 %) of seed germination obtained by bacterial culture filtrate

treatment which is statistically similar to standard IAA treatment. Other parameter like seedling length, seedling vigour index, fresh weight of seedlings and number of adventitious roots were significantly increased compared to control but statistically similar to standard IAA. Dry weight of seedlings didn't vary significantly compared to standard IAA and control. It has been demonstrated in previous work that culture filtrates of *S. olivaceoviridis* containing IAA stimulated growth and yield of wheat plants (Aldesuquy *et al*.1998). El-Tarabily (2008) reported that *Streptomyces* spp. from a tomato rhizosphere had the ability to produce IAA and improved tomato growth by increasing root dry weight.

Some genera of bacteria like *Azospirilum, Rhizobium, Bacillus,* and *Pseudomonas* have been reported to produce auxin (Patten and Glick 1996) and shown to stimulate the growth in different plants. Inoculation of canola seeds with *Pseudomonas putida* GR12-2, which produces low levels of IAA, resulted in 2 to 3 folds increase in the length of seedling roots (Caron *et al*. 1995).

Table 4: Effect of culture filtrate from *Pseudomonas fluorescens* UMB20 on seedling growth parameters under *in vitro* condition

Treatments	Seedling length (cm)	% Germ	SVI	Wet weight (g)	Dry weight (g)	No. of Ad. root
Culture filtrate (40µg/mL)	15.50b	97.00a	1503.78b	0.82ab	0.19a	5.05ab
Standard IAA (40µg/mL)	17.90a	97.95a	1753.78a	0.87a	0.19a	5.42a
Sterile distilled water	13.58c	90.91b	1236.43c	0.76b	0.18a	3.92b

Note: Germ=Germination SVI= Seedling vigour index; Ad. root= Adventitious root

CONCLUSION

The findings of the present investigation highlighted that IAA producing bacteria from rice phylloplane could be easily isolated and may be exploited after strain improvement for local use. However, further studies using IAA mutant strains of these isolates are needed to explore the exact contribution of IAA production in the promotion of plant growth as well as the contribution of other PGP traits.

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