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Indole Acetic Acid Production by the Indigenous Isolates of *Azotobacter* and Fluorescent *Pseudomonas* in the Presence and Absence of Tryptophan

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Abstract: A total of 21 bacterial isolates (*Azotobacter* sp., 10 and fluorescent *Pseudomonas* sp., 11) were isolated from different rhizospheric soils in the vicinity of Aligarh city and characterized as per standard methods. These isolates were further tested for the production of indole acetic acid (IAA) in a medium with 0, 1, 2 and 5 mg/ml of tryptophan. A low amount (2.68-10.80 mg/ml) of IAA production was recorded by *Azotobacter* strains without tryptophan addition. Seven *Azotobacter* isolates showed high level (7.3 to 32.8 mg/ml) production of IAA at 5 mg/ml of tryptophan while at 1 and 2 mg/ml the production was in the range of 1.47 to 11.88 and 5.99 to 24.8 mg/ml, respectively. Production of IAA in fluorescent *Pseudomonas* isolates increased with an increase in tryptophan concentration from 1 to 5 mg/ml in the majority of isolates. In the presence of 5mg/ ml of tryptophan, 5 isolates of *Pseudomonas* produced high levels (41.0 to 53.2 mg/ml) of IAA while 6 other isolates produced IAA in the range of 23.4 to 36.2 mg/ml. Production of IAA was further confirmed by extraction of crude IAA from 3 isolates of *Azotobacter* (Azs_1, Azs_6 and Azs_9) and three isolates of *Pseudomonas* (Ps_1, Ps_4 and Ps_7) and subsequent TLC analysis. A specific spot from the extracted IAA preparation was found corresponding with the standard spot of IAA with same R_f value. *Pseudomonas* isolates (Ps_1, Ps_4 and Ps_7) showed inhibitory effects on the growth of root elongation of *Sesbania aculeata and Vigna radiata* at all concentrations of tryptophan compared to the control. However, the isolates of *Azotobacter* (Azs_1, Azs_6 and Azs_9) demonstrated stimulatory effects on both plants. Increasing the concentration of tryptophan from 1 mg/ml to 5 mg/ml resulted in decreased growth in both *S. aculeata* and *V. radiata.* On a comparative basis isolate Azs_9 was most promising in promoting plant growth. On the other hand, high concentration of exogenous tryptophan could exhibit toxic effects on plant growth.

Key Words: Indole acetic acid, Tryptophan, *Azotobacter*, *Pseudomonas*

Introduction

Plant growth promoting rhizobacteria (PGPR) are considered to promote plant growth directly or indirectly. PGPR can exhibit a variety of characteristics responsible for influencing plant growth. The common traits include production of plant growth regulators (auxin, gibberellin, ethylene etc.), siderophores, HCN and antibiotics (1). Indole acetic acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L-tryptophan metabolism by several microorganisms including PGPR (2,3).

Microorganisms inhabiting rhizospheres of various plants are likely to synthesize and release auxin as secondary metabolites because of the rich supplies of substrates exuded from the roots compared with non-rhizospheric soils (4,5). Plant morphogenic effects may also be a result of different ratios of plant hormones produced by roots as well as by rhizosphere bacteria (6). Diverse soil microorganisms including bacteria (6), fungi (7) and algae (8) are capable of producing physiologically active quantities of auxins, which may exert pronounced effects on plant growth and establishment.

Azotobacter paspali secreted IAA into culture media and significantly increased the dry weight of leaves and roots of several plant species following root treatment (9). It was found that inoculation of wheat seedlings with *Azospirillum brazilance* increased the number and length of lateral roots (10). Inoculation of canola seeds with *Pseudomonas putida* GR12-2, which produces low levels of IAA, resulted in 2 - or - 3 fold increases in the length of seedling roots (11,12). It is presumed that PGPR producing plant growth regulators play a critical role in plant growth promotion. Effects of plant growth regulators including IAA on the plant will be concentration dependent. To assess this hypothesis, local isolates of *Azotobacter* and *Pseudomonas* sp. were screened for their intrinsic ability to produce IAA in the presence of varying amounts of L-tryptophan and their effect on root elongation of germinating seeds of test plants.

Materials and Methods

Isolation and biochemical characterization of indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas*

Rhizospheric soils of different crops (wheat, berseem, mustard, cauliflower) in the vicinity of Aligarh city, UP, India were collected in October to December, 2002, for the isolation of *Azotobacter* and *Pseudomonas* spp. *Azotobacter* isolates were isolated from the soil on nitrogen free Jensen's medium (sucrose 20 g, di-potassium hydrogen phosphate 1 g, magnesium sulfate 0.5 g, sodium chloride 0.5 g, ferrous sulfate 0.1 g, sodium molybdate 0.005 g, agar 20 g, for 1 liter, pH 6.9). Each isolate showing characteristic growth, pigmentation and biochemical reactions as described in Bergy's Manual of Determinative Bacteriology for *Azotobacter chroococcum* and related species was purified and given an isolate number. Similarly, fluorescent *Pseudomonas* strains were isolated on nutrient agar medium or King's medium as per the standard method (13). Microbiological media were purchased from Hi-Media lab. Pvt. Mumbai, India.

Biochemical characterization of the test isolates

All the 10 isolates of *Azotobacter* sp. and 11 isolates of *Pseudomonas* sp. were biochemically characterized for Gram reaction, carbohydrate fermentation, H_2S production, NO_3^- reduction, IMViC tests, oxidase test, starch hydrolysis, and gelatin liquefaction as per the standard methods (14).

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

All the test strains of A*zotobacter* and *Pseudomonas* spp. were screened for IAA production (15). Briefly, test bacterial culture was inoculated in the respective medium (Jensen's/nutrient broth) with tryptophan (1, 2, and 5 mg/ml) or without tryptophan incubated at 28 ± 2 ^{0}C for 15 days for *Azotobacter* and 1 week for *Pseudomonas* spp. Cultures were centrifuged at 3000 rpm for 30 min. Two milliliters of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of Solawaski's reagent (50 ml, 35% perchloric acid; 1 ml 0.5 $FeCl_3$). Development of a pink colour indicates IAA production. O.D. was read at 530 nm using Spectronic 20D$^+$. The level of IAA produced was estimated by a standard IAA graph.

Extraction of crude IAA

Single bacterial colonies of 3 isolates of *Pseudomonas* spp. (Ps_1, Ps_4 and Ps_7) and 3 strains of *Azotobacter* (Azs_1, Azs_6, Azs_9) were inoculated in 200 ml of nutrient broth amended with 1 and 5 mg/ml of tryptophan and incubated at 28 ± 2^{o}C for 1 week on a shaker incubator. Bacterial cells were separated from the supernatant by centrifugation at 10,000 rpm for 30 min. The supernatant was acidified to pH 2.5 to 3.0 with 1 N HCl and extracted twice with ethyl acetate at double the volume of the supernatant. Extracted ethyl acetate fraction was evaporated to dryness in a rotatory evaporator at 40 ^{o}C. The extract was dissolved in 300 ml of methanol and kept at –20 ^{o}C.

Thin layer chromatography

Ethyl acetate fractions (10-20 ml) were plated on TLC plates (Silica gel G f_{254}, thickness 0.25 mm) and developed either in ethyl acetate:chloroform:formic acid (55:35:10) or benzene:n-butanol:acetic acid (70:25:5). Spots with R_f values identical to authentic IAA were identified under UV light (254 nm) by spraying the plates with Ehmann's reagent (16).

Effect of rhizobacteria at different concentrations of tryptophan on root elongation

Different concentrations of tryptophan (0, 1, 2 and 5 mg/ml) were incorporated in the respective media for *Azotobacter* and *Pseudomonas*. One-fifth of each plate was streaked with test bacteria and plates were incubated for 48 -72 h at 28 ± 2 ^{0}C. Surface sterilized seeds of *S. aculeata* and *V. radiata* were placed 1 cm away from the bacterial growth. Ten seeds were placed in each plate and 10 replicate plates of each treatment were made. Plates were further incubated for seed germination for 96 h at 28 ± 2^{0}C. The roots of the germinated seeds were then measured.

Results and Discussions

A total of 21 isolates of *Azotobacter* and fluorescent *Pseudomonas* sp. were isolated from rhizospheric soil and tentatively identified on the basis of biochemical tests and sugar fermentation behavior as described in Bergy's

Manual of Determinative Bacteriology (Table 1). These bacterial isolates were screened for their ability to produce plant growth regulator, IAA. Varying levels of IAA production were recorded with different concentrations of tryptophan, i.e. 0, 1, 2 and 5 mg/ml (Tables 2 and 3). The range of IAA production in *Azotobacter* isolates without tryptophan was 2.68-10.80 mg/ml. A significant increase in the production of IAA was recorded in the presence of 1, 2 and 5 mg/ml of tryptophan, i.e. 1.47-11.88 mg/ml, 5.99-24.8 mg/ml and 7.3-32.8 mg/ml respectively (Table 2). Our findings of IAA production in *Azotobacter* isolates are in agreement with those of other researchers (17 - 19).

Similarly 11 *Pseudomonas* isolates were able to produce IAA without tryptophan in the range 5.34 to 22.4 mg/ml. A further increase in IAA production was observed in the presence of tryptophan (1, 2 and 5mg/ml) as depicted in Table 3. These isolates varied greatly in their intrinsic ability to produce IAA. Production

Table 1. Cultural and biochemical characteristics of *Azotobacter* and *Pseudomonas* isolates.

Characteristics of test organisms	Test bacteria	
	Azotobacter isolates 10*	*Pseudomonas isolates* 11*
Growth on N_2 free medium	+	_
Pigmentation	+/_	+
Fluorescence under UV light	_	+
Gram reaction	G-ve	G-ve
Biochemical tests		
Indole, MR and VP	+ (100)	-(100)
H_2S production	+ (40)	- (100)
Citrate, catalase and NO_3^- test	+ (100)	+ (100)
Oxidase test	+ (100)	+ (100)
Starch utilization	+ (80)	+ (36.37)
Sugar fermentation		
Lactose	+ (50)	- (100)
Dextrose	+ (90)	+ (72.73)
Sucrose	+ (30)	+ (27.27)
Mannitol	+ (40)	- (100)

% of positive or negative test is given in parentheses.
* Total number of isolates

Table 2. IAA production by *Azotobacter* isolates after 15 days of incubation.

Treatments of tryptophan (mg/ml)	IAA production (µg/ml) by test isolates									
	AZS_1	AZS_2	AZS_3	AZS_4	AZS_5	AZS_6	AZS_7	AZS_8	AZS_9	AZS_{10}
0	7.40	7.98	2.68	7.00	4.43	10.80	3.73	3.40	4.40	4.60
1	11.53	8.04	9.38	11.88	5.88	14.36	1.47	9.62	7.25	10.72
2	19.8	16.80	15.80	14.9	13.90	24.80	5.99	18.77	23.8	16.8
5	27.6	26.80	25.80	23.8	21.80	32.80	7.30	27.80	28.9	26.9

Table 3. IAA production by *Pseudomonas* isolates after 7 days of incubation.

Treatments of tryptophan (mg/ml)	IAA production (µg/ml) by test isolates										
	Ps_1	Ps_2	Ps_3	Ps_4	Ps_5	Ps_6	Ps_7	Ps_8	Ps_9	Ps_{10}	Ps_{11}
0	9.64	5.34	8.24	8.60	8.00	6.59	22.4	9.28	8.2	5.64	6.46
1	14.7	11.6	10.4	24.1	24.1	16.0	24.8	28.3	20.2	16.2	19.9
2	27.7	20.8	24.8	32.8	34.9	21.3	36.9	37.5	30.2	23.6	24.5
5	53.2	23.4	36.2	43.0	41.6	32.9	52.8	46.4	41.0	28.2	32.3

of high levels of IAA by fluorescent *Pseudomonas* is a general characteristic; our test isolates showed a similar high level of IAA production to those recorded by other researchers (12, 20, 21).

On the basis of IAA production level culture filtrates of fluorescent *Pseudomonas* (Ps_1, Ps_4 and Ps_7) and *Azotobacter* (Azs_1, Azs_6 and Azs_9) were used to extract IAA for characterization by TLC. The spots of ethyl acetate extracts of the respective culture and standard IAA were tested in solvent systems (A) ethyl acetate:chloroform:formic acid (11:7:2) and (B) benzene:n-propanol:acetic acid (14:5:1). Chromatograms of culture spots and standard IAA, sprayed with Ehmann's reagent, showed almost the same R_f values. Our TLC findings are in agreement with reports by other scientists (22). In addition to IAA, other compounds were also detected on TLC plates, which remain to be identified.

The effect of *Pseudomonas* and *Azotobacter* isolates on root elongation was evaluated at the different concentrations of tryptophan, i.e. 0, 1, 2, and 5 mg/ml. Without tryptophan, the root elongation of germinating seeds of *S. aculeata* and *V. radiata* was highest with *Azotobacter* isolate Azs_9, followed by Azs_1 and Azs_6, compared to the control, whereas the root length decreased in *Pseudomonas* isolates at 1 and 2 mg/ml tryptophan concentrations in *S. aculeata.* Similar trends were also found with *V. radiata* (Tables 4 and 5). In the case of *V. radiata* at 2 mg/ml of tryptophan only Azs_9 showed significant root elongation. At a 5mg/ml tryptophan concentration in both *S. aculeata* and *V. radiata* the root elongation decreased in the presence of all isolates, which indicated that tryptophan at a 5 mg/ml concentration is toxic in the presence of test bacteria. However at a higher concentration of tryptophan, the production of IAA is higher which might exert an adverse effect on plant growth.

The findings of the present investigation highlighted that IAA producing bacteria from local soil 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.

There are numerous soil microflora involved in the synthesis of auxins in pure culture and soil (23). Some microorganisms produce auxins in the presence of a suitable precursor such as L-tryptophan. The effects of auxins on plant seedlings are concentration dependent, i.e. low concentration may stimulate growth while high concentrations may be inhibitory (24). Different plant seedlings respond differently to variable auxin concentrations (25) and type of microorganisms.

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Table 4. *In vitro* effect of *Azotobacter* isolates on root elongation at different concentrations of tryptophan.

Tryptophan concentration (mg/ml)	Root elongation (mm) *Sesbania aculeata*			
	Control	AZS_1	AZS_6	AZS_9
0	38.88 ± 1.4	42.50 ± 0.32*	42.80 ± 1.12*	49.88 ± 0.50*
1	45.53 ± 0.34	49.00 ± 0.10*	47.20 ± 1.01*	55.25 ± 0.50*
2	40.24 ± 0.21	46.10 ± 0.10*	45.20 ± 0.44*	50.09 ± 0.02*
5	37.88 ± 0.87	27.53 ± 0.40	29.18 ± 0.10	32.95 ± 0.10
	Root length (mm) *Vigna radiata*			
0	27.65 ± 0.25	31.38 ± 0.31*	32.38 ± 0.84*	36.35 ± 0.40*
1	35.65 ± 0.40	36.90 ± 0.60*	38.00 ± 0.55*	41.25 ± 0.58*
2	32.63 ± 0.15	31.37 ± 0.47	32.00 ± 0.10	38.23 ± 0.25*
5	25.50 ± 0.20	25.50 ± 0.42	19.15 ± 0.10	21.70 ± 0.40

* Statistically significant difference from control (C.D.<0.05)

Table 5. *In vitro* effect of *Pseudomoas* isolates on the of root elongation at different concentration of tryptophan.

Tryptophan concentration (mg/ml)	Root elongation (mm) *Sesbania aculeata*			
	Control	Ps_1	Ps_4	Ps_7
0	38.88 ± 1.4	10.42 ± 0.69	12.45 ± 0.31	10.45 ± 0.39
1	45.53 ± 0.34	15.32 ± 0.80	11.75 ± 0.50	12.65 ± 0.80
2	40.24 ± 0.21	12.59 ± 0.31	11.48 ± 0.15	11.07 ± 0.060
5	37.88 ± 0.87	7.80 ± 0.21	12.38 ± 0.76	10.65 ± 0.36
	Root length (mm) *Vigna radiata*			
0	27.65 ± 0.25	9.38 ± 0.25	5.20 ± 0.26	5.42 ± 0.86
1	35.65 ± 0.40	12.53 ± 0.10	7.00 ± 0.00	8.48 ± 0.10
2	32.63 ± 0.15	8.63 ± 0.15	6.50 ± 0.10	7.00 ± 0.10
5	25.50 ± 0.20	5.55 ± 0.10	6.65 ± 0.10	3.95 ± 0.20

The data are not statistically significant from the control (C.D.<0.05)

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