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Isolation, Characterization, and Plasmid pUPI126-Mediated Indole-3-Acetic Acid Production in *Acinetobacter* Strains from Rhizosphere of Wheat

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Abstract

Thirty-seven strains of *Acinetobacter* isolated and characterized from rhizosphere of wheat were screened for indole-3-acetic acid (IAA) production. Only eight *Acinetobacter* strains showed IAA production. The genus *Acinetobacter* was confirmed by chromosomal DNA transformation assay. Biotyping of eight strains was carried out and they were found to be genospecies of *A. junii*, *A. baumannii*, *A. genospecies 3*, and *A. haemolyticus*. Five of eight strains produced IAA at the early stationary phase: *A. haemolyticus* (A19), *A. baumannii* (A18, A16, A13), and *Acinetobacter* genospecies 3 (A15). *A. junii* A6 showed maximum IAA production at log phase and *A. genospecies 3* and *A. baumannii* (A28, A30) at late stationary phase. IAA was extracted by ethyl acetate and purified by preparative thin-layer chromatography. Purified IAA was confirmed by ^{1}H-nuclear magnetic resonance and infrared spectrum analysis. Pot experiments showed a significant increase in plant growth inoculated with eight *Acinetobacter* genospecies as compared to control plants. IAA production was found to be encoded by plasmid pUPI126. All eight strains of *Acinetobacter* contain a plasmid pUPI126 with a molecular weight of 40 kb. Plasmid pUPI126 was transformed into *Escherichia coli* HB101 at a frequency of 5×10^{-5}, and *E. coli* HB101 (pUPI126) transformants also showed IAA activity. PUPI126 also encoded resistance to selenium, tellurium, and lead. This is the first report of plasmid-encoded IAA production in the genus *Acinetobacter*.

Index Entries: *Acinetobacter* spp.; wheat rhizosphere; tryptophan; indole-3-acetic acid production; plasmid pUPI126; transformation; infrared analysis; ^{1}H-nuclear magnetic resonance; plant growth promotion.

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Introduction

Acinetobacter species are ubiquitous in nature *(1,2)*. *Acinetobacter* is commonly found in soil, water, food, as well as on healthy human skin *(3–5)*. *Acinetobacter* is one of the known opportunistic human pathogens *(5)*. It also possesses a number of naturally occurring plasmids exhibiting resistance to antibiotics and heavy metals *(6,7)*. There are few reports on the presence of *Acinetobacter* in soil, and detailed studies regarding its occurrence, distribution, growth pattern, physiology, and interactions with other soil microorganisms are not available. There is only one report on the presence of *Acinetobacter* in wheat rhizosphere *(8)*, but detailed information about the role of *Acinetobacter* in rhizosphere is not known.

Soil is a rich environment for the growth of microorganisms, and specifically, rhizosphere is a highly specialized environment in soil for the growth of microorganisms. Since rhizosphere contains a large number of microorganisms, one would expect plasmid transfer and the dynamics of plasmid transfer from *Acinetobacter* to other microorganisms and vice versa in the rhizosphere environment. The rhizosphere of each and every plant is very specific with respect to the root exudates since it is the main source of nutrients for rhizosphere microorganisms *(9)*.

Until now there has been no report on the involvement of plasmids in the production of indole-3-acetic acid (IAA) from the genus *Acinetobacter*. IAA is one of the major plant growth–promoting hormones produced by plants as well as some bacteria and fungi *(10)*. Many species of bacteria produce IAA, especially when growth media are supplemented with tryptophan, a precursor of IAA. A number of microorganisms such as *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, *Pseudomonas savastanoi*, *Pseudomonas* spp. *(11)*, *Rhizobium* spp. *(14)*, *Bradyrhizobium* spp., and *Azospirillum* spp. *(13)* present in the rhizosphere of plants are known to produce IAA *(14)*. The biosynthesis of plant growth–promoting substances such as auxins from phosphate-solubilizing rhizobacteria from rhizosphere of wheat and ray has been reported *(11)*. The aim of the present study was to isolate and characterize *Acinetobacter* from rhizosphere of wheat and to find out the role of *Acinetobacter* in plant growth promotion in general and the involvement of plasmids in the production of plant growth–promoting substances such as IAA.

Materials and Methods

Sample Collection

Acinetobacters were isolated from the rhizosphere of wheat. The variety of wheat plant was HD 2189 ICAR, from New Delhi, India. The rhizosphere soil was collected from December to March 1998, the growing season at the different stages of the life cycle of wheat plant: control soil (0 d), elongation (30 d), flowering stage (45 d), fruiting stage (60 d), and ripened fruiting stage (75 d) from three areas—rhizosphere soil (RS),

rhizoplane (RP), and nonrhizosphere soil (NRS). The samples were collected from an agricultural field of Mahatma Phule Agriculture College, Shivajinagar, Pune, Maharashtra, India. Samples were brought to the laboratory and processed within half an hour.

Culture Media

Five different culture media were used for isolation of *Acinetobacter* and other Gram-negative bacteria from wheat rhizosphere. The media used were violet red bile agar (VRBA) medium *(8)* and cystine lactose electrolyte-deficient (CLED) medium (HiMedia, Mumbai, India) for all Gram-negative bacteria, Acinetobacter minimal medium (AMM) *(2)* and Holtons selective medium *(15)* for growth of *Acinetobacter*, and standard plate count agar (SPCA) for estimation of total count of culturable bacteria present in all three samples (RP, RS, and NRS).

Isolation of Acinetobacter *from Rhizosphere*

The dilution plate method *(9)* was used for isolation of *Acinetobacter* and all other Gram-negative bacteria from wheat rhizosphere and RP. All types of colonies were isolated from four selective media and total bacterial counts were taken from SPCA. Colonies from VRBA medium, AMM, CLED medium, and Holtons medium were characterized by their morphologic features and tentatively identified up to genus level by Gram character and morphology, motility, oxidase test, catalase test, and capsule staining.

Chromosomal DNA Transformation Assay

Chromosomal DNA transformation assay was carried out using naturally competent auxotrophic mutant *A. calcoaceticus* BD413 trp E27. Transformation was done by a modified method of Juni *(2)*. In brief, the temperature used for lysis was 65°C for 90 min using crude DNA *(2)* as well as purified DNA *(16)* isolated from *Acinetobacter*. The growth of transformants of *Acinetobacter* on AMM without tryptophan was considered a positive result of DNA transformation.

Identification of Acinetobacter *Strains to Species Level*

Acinetobacter strains were classified to species level by the Bouvet and Grimont *(17,18)* classification system. API 20NE was also employed for biotyping of *Acinetobacter* strains isolated from wheat rhizosphere *(19)*.

Detection of IAA Production in Acinetobacter *Strains*

IAA production was detected by two methods as described next.

Nitrocellulose Paper Assay

All 37 *Acinetobacter* strains were tested by nitrocellulose paper assay *(20)* for the production of IAA. The *Acinetobacter* strains were spot inocu-

lated on Luria-Bertani (LB) medium supplemented with 5 m*M* tryptophan (LBT). The spot-inoculated agar surface was overlaid with a nitrocellulose membrane filter and incubated at 28°C for 48 h. The membrane filter was aseptically removed from the plate after 48 h and transferred to Whatman filter paper no. 2, and 500 µL of Salkovaski reagent (2% 0.5 *M* $FeCl_3$ in 35% perchloric acid or 2.025 g of $FeCl_3$ in 300 mL of conc. H_2SO_4 and 500 mL of distilled water) was added on the nitrocellulose paper and kept for 1 to 2 min at room temperature. IAA production was indicated by a red ring around the colony.

Salkowaski Method

For the Salkowaski method *(21)*, 37 *Acinetobacter* strains were grown at 28°C in LB broth supplemented with 1 mg/mL of tryptophan. After 48 h of incubation, cells were harvested by centrifugation at 8000 *g* for 15 min at room temperature, and 1 mL of sample (supernatant) and 4 mL of Salkowaski reagent *(21)* were mixed and allowed to react in the dark at room temperature for 30 min. One milliliter of uninoculated LBT and 4 mL of Salkowaski reagent was treated as blank. Optical density (OD) was checked at 540 nm. Red color formation was considered positive evidence for IAA production *(20)*.

Time Course of IAA Producing Acinetobacters

IAA production by *Acinetobacter* strains at different growth phases was also studied. *Acinetobacter* strains were inoculated in LBT medium and incubated at 28°C at 90 *g*. Production of IAA was checked every 2 h up to 108 h by the Salkovaski method.

Extraction and Purification of IAA by Preparative Thin-Layer Chromatography

IAA produced by *Acinetobacter* genospecies was purified by the method described by Koga et al. *(22)*. In brief, all strains were grown in LBT medium until they showed maximum IAA production. Culture broth (150 mL) was centrifuged at 5000 *g* (Remi, RMI2C) for 20 min at room temperature. The pH of the supernatant was adjusted to 7.0 (neutral extract) and the supernatant was extracted with 1:1 (v/v) ethyl acetate. The aqueous phase was carefully separated and the pH was adjusted to 2.8 with HCl (acid extract). This acid extract was again extracted with 1:1 (v/v) ethyl acetate. Organic phases from both extractions were mixed together and evaporated in rota-vapor at 60°C (Buchi, Switzerland) to get powdered IAA. At each phase of extraction, the Salkovaski test was done for the organic as well as aqueous phases. The preparative thin-layer chromatography (TLC) was run for the extracted samples with the standard IAA (Sigma, St. Louis, MO) using methanol:chloroform (10:90) as a solvent system. TLC was carried out on a polygram G/UV 254 20 × 20 × 60 mm precoated aluminum sheet (Merck, Darmstadt, Germany). The TLC spots

were observed under ultraviolet light (245 nm) and compared with standard IAA sample (Sigma). IAA spots were scratched with a fine spatula, and a sample with silica gel was collected in a clean glass bottle. The sample was dissolved in 2 mL of chloroform or ethyl acetate and filtered through cotton to remove the silica. TLC of the sample was done to check the purity of the sample.

Identification of IAA by Infrared Spectrum and Melting Point

Infrared (IR) spectrum of extracted IAA sample was taken using a Perkin-Elmer 1600 FTIR Spectrophotometer, and the spectra were recorded in nujol mull using KBr cells and expressed in wave number (cm^{-1}). The melting point of extracted IAA was tested on a Thomos Hoover melting point apparatus in degrees Celsius.

Analysis of IAA by ^{1}H-Nuclear Magnetic Resonance

The purified sample of IAA was analyzed by Mercury ^{1}H-nuclear magnetic resonance (NMR) (300 MHz; Vavion), and the peaks were identified for IAA. The ^{1}H-NMR of standard IAA was also checked. The two ^{1}H-NMR were compared. The purified IAA was dissolved in 25 µL of dimethyl sulfoxide (DMSO).

Effect of pH on IAA Production

To study the effect of pH on IAA production, buffered LBT broth was prepared in the standard buffers such as acetate, phosphate, and Tris-HCl *(23)*. pH in the range of 4.0–9.0 was checked. The maximum IAA production phase at different pHs was determined by the Salkovaski test.

Effect of IAA Production by Acinetobacter *on Growth of Wheat Plant*

The effect of IAA production on the growth of wheat plant was tested by pot experiments. All eight *Acinetobacter* strains were grown separately in each of four media (LB, LBT, AMM, and AMMT) containing 1 mg/mL of tryptophan. LB and AMM were used as control media and LBT and AMMT were used for IAA production. Eight *Acinetobacter* genospecies were inoculated in all four media and incubated at 90 *g* at 28°C up to 48 h. The wheat seeds were surface sterilized by 2% $HgCl_2$ *(24)* and washed with sterile distilled water for six to seven times to remove the $HgCl_2$ completely. After washing, seeds were added to the aforementioned culture and kept for 2 h at 28°C and 90 *g*. After 2 h, wheat seeds were aseptically collected and inoculated in sterile soil in pots. Wheat seeds mixed with uninoculated media as well as with distilled water were treated as control. The pots were kept in sunlight and raised under close supervision. The growth of plants was observed every day for 21 d. After 21 d the plants were carefully uprooted and root and shoot length were measured. The same experiment was done simultaneously using large pots up to 4 mo for the overall life cycle of the wheat plant.

Statistical Analysis

Root length and shoot length were considered the main parameters in determining the effect of IAA on wheat plant. Statistical analysis was done with the help of mean, SD, and analysis of variance (ANOVA) *(25)*.

Isolation of Plasmid

Eight strains were checked for the presence of plasmid(s). Plasmids were isolated by three different methods described by Kado and Liu *(26)*, Sambrook et al. *(27)*, and Birnboim and Doly *(28)*. The presence of plasmid(s) was tested by 0.7% agarose gel electrophoresis in TAE buffer at 52 V for 6–8 h. Ethidium bromide–stained gels were observed under a gel documentation system (Alpha Imager™ 2200 Documentation and Analysis System; Alpha Innotech) and photographed. Molecular weight was determined by comparing with a 1-kb DNA ladder.

Plasmid Curing

Plasmid curing was done for all eight strains of *Acinetobacter* genospecies using ethidium bromide (1024 µg/mL) as well as heat (52°C) as described by Deshpande and Chopade *(29)*.

Transformation

Escherichia coli HB101 (rif^r) was used as a recipient for transformation. Plasmid DNA isolated from *A. haemolyticus* (A19) was used for transformation of DNA because this strain showed good IAA production and also exhibits other interesting characteristics such as resistance to selenium, lead, and tellurium (which were used as genetic markers for plasmid transformation); chitinase production; and antimicrobial activity against plant as well as human pathogenic fungi and bacteria *(30)*. Transformation was carried out by preparing *E. coli* HB101 (rif^r)–competent cells by the $CaCl_2$ method *(27)*, and competent *E. coli* cells were mixed with plasmid pUPI126 DNA (10 µL). The transformants were selected and checked for IAA production using the Salkovaski test and parent-recipient *E. coli* HB101 (rif^r) was used as control.

Results

Isolation, Identification, and Confirmation of Acinetobacter Genospecies

Colonies showing mucoid character were selected and tested for Gram character and morphology, motility, presence of capsule, as well as oxidase and catalase production. Gram-negative, coccobacilli, nonmotile, oxidase negative, catalase positive, and capsulated strains were considered as tentative *Acinetobacter* spp. and confirmed by chromosomal DNA transformation assay. Thirty-seven *Acinetobacter* strains isolated from five stages of

wheat plant were confirmed as genuine *Acinetobacter* strains. *Acinetobacter* strains were identified to species level on the basis of biochemical tests. These *Acinetobacter* strains were not biotypeable by the API 20NE system. Eight IAA-producing strains were identified as *A. baumannii* A18, A16, A13, and A30, *A. haemolyticus* A19, *A. junii* (A6), and *Acinetobacter* genospecies 3 A15, A28 (Table 1).

Production of IAA by Acinetobacter *Strains*

All 37 *Acinetobacter* strains were tested for IAA production by nitrocellulose paper assay. Of the 37 *Acinetobacter* strains only 8 showed a red ring around the growth of the bacterial colony within 1 min on addition of Salkovaski reagent. IAA production for these eight *Acinetobacter* strains was also detected by the Salkovaski method, and the development of a red color indicated the presence of IAA. One milliliter of LB and 4 mL of Salkovaski reagent was used as negative control.

Time Course of IAA-Producing Acinetobacter *Strains*

Five strains of *Acinetobacter*—*A. haemolyticus* (A19), *A. baumannii* (A18, A16, A13), and *A. genospecies 3* (A15)—showed maximum IAA production in the early stationary phase (48 h). *A. junii* (A6) showed maximum IAA production in the log phase (24 h) and *A. genospecies 3* (A28) and *A. baumannii* (A30) in the late stationary phase (60 and 72 h). Figure 1A,B represents the growth phase with maximum IAA production by each *Acinetobacter* spp.

Extraction and Purification of IAA by TLC

Extraction of IAA was done by ethyl acetate. The Salkovaski test was done at each step of the extraction and showed that only the organic phase contained IAA. The dry powder obtained after evaporation of ethyl acetate in a Bouchi evaporator showed the presence of IAA, which was further fractionated by preparative TLC. The band pattern of purified IAA was comparable with that of standard IAA (Fig. 2). When these bands from TLC were scratched and again tested by TLC, a single band was shown as that of standard IAA with an R_f value of 0.5. The amount of auxins found in the culture filtrate was 4 mg/L.

Identification of IAA by IR Spectrum and by Melting Point

The IR spectrum of pure IAA showed an OH frequency at 3384.9 cm^{-1} and a C=O frequency at 1698.4 cm^{-1} (Fig. 3). The IR spectrum of standard IAA also showed the same results. The melting point of purified IAA was found to be 168°C, also the same as that of standard IAA.

Analysis of IAA by ^{1}H-NMR

^{1}H-NMR of the purified eight IAA samples was found to be same as that of ^{1}H-NMR of standard IAA (Fig. 4). The first peak from the right in Fig. 4 is of acid, of value 9.0 δ bs (–OH), and the lateral peaks are of protons

Table 1
Identification of IAA-Producing *Acinetobacter* genospecies Isolated from Rhizosphere of Wheat[a]

	A. genospecies 3 (A15, A28)	*A. baumannii* (A13, A16, A18, A30)	*A. haemolyticus* (A19)	*A. junii* (A6)
Grams nature	–	–	–	–
Morphology	Coccobacilli	Coccobacilli	Coccobacilli	Coccobacilli
Motility	-	–	–	–
Oxidase	–	–	–	–
Catalase	+	+	+	+
Capsule staining	+	+	+	+
Growth at				
44°C	–	+	+	+
41°C	+	+	+	+
37°C	+	+	+	+
28°C	+	+	+	+
Gelatin hydrolysis	–	–	+	+
Hemolysis	+	–	+	–
Citrate	+	+	+	+
Acid from glucose	+	+	+	+
Utilization of				
Glutarate	+	+	+	+
L-phenyalanine	+	+	+	–
Phenylacetae	–	–	+	–
Malonate	+	+	+	+
L-Histidine	+	+	+	+
D-Malate	+	+	+	+
L-Aspartate	+	+	+	+
L-Leucine	+	+	–	+
L-Tyrosine	+	+	–	+
B-Alanine	+	+	+	+
Ethanol	+	+	+	+
trans-Aconitate	+	+	+	–
L-Arginine	+	+	+	+
DL-4-Amino butarare	+	+	+	–
DL-Lactate	+	+	+	+

[a]–, negative; +, positive.

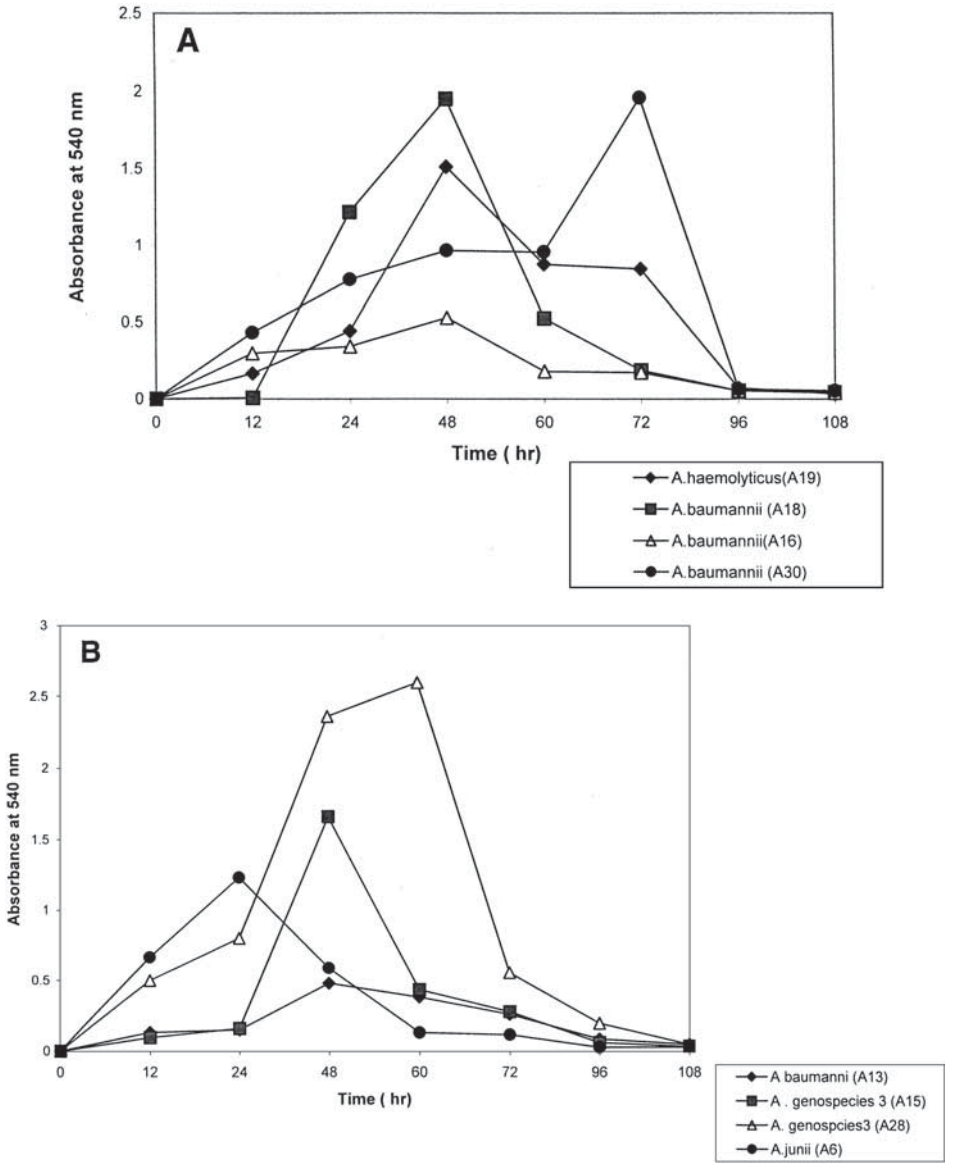

Fig. 1. **(A)** IAA production by four *Acinetobacter* genospecies—*A. haemolyticus* (A19) and *A. baumannii* (A18, A16, and A30)—isolated from rhizosphere of wheat; **(B)** IAA production of four *Acinetobacter* genospecies—*A. baumannii* (A13), *A. genospecies 3* (A15 and A28), and *A. junii* (A6)—isolated from rhizosphere of wheat.

having values 7.8 δ d 1H (C_8H), 7.5 δ d 1H (C_5H), 7.31 δ d 1H (C_2H), and 7.07 δ m 2H (C_6 & C_7H). The large peak in the middle is of DMSO having a value of 3.04 δ S (d^6) and moisture. The next peak is of carbon and hydrogen, having value of 2.59 δ S 2H (–CH_2–), and the last peak is of internal standard, tetra-methyl-silnate, of value 0.08.

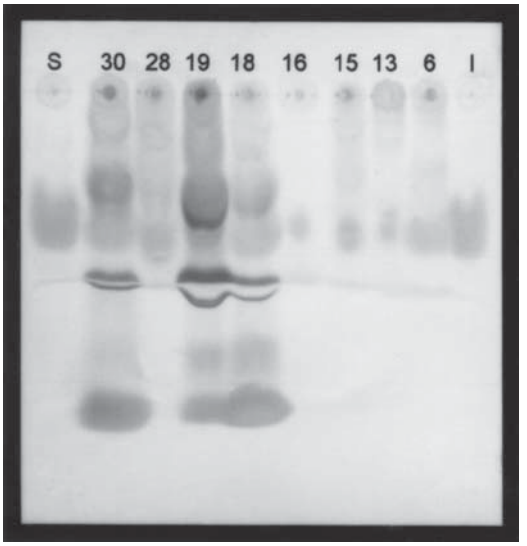

Fig. 2. TLC of purified IAA produced from *Acinetobacter* genospecies. S, purified IAA; 30, *A. baumannii* (A30); 28, *A. genospecies 3* (A28); 19, *A. haemolyticus* (A19); 18, *A. baumannii* (A18); 16, *A. baumannii* (A16); 15, *Acinetobacter* genospecies 3 (A15); 13, *A. baumannii* (A13); 6, *A. junii* (A6); I, standard IAA.

Table 2
Effect of pH on IAA Production by *Acinetobacter* genospecies

	A. genospecies 3		*A. baumannii*				*A. junii*	*A. haemolyticus*
pH[a]	A15	A28	A16	A18	A30	A13	A6	A19
6.0	0.02	0.08	0.07	0.81	1.23	0.06	1.46	1.22
7.0	0.13	0.14	0.32	1.10	1.90	1.45	1.43	1.95
8.0	0.11	0.23	0.21	0.92	1.12	1.46	1.35	1.01
9.0	0.10	0.35	0.29	0.58	0.88	1.52	1.28	0.55

[a]At pH 4.0 and 5.0 there was no growth of *Acinetobacter* genospecies; hence, there was no IAA production. OD at 540 nm.

Effect of pH on IAA Production

It was observed that at acidic pH (pH 4.0 and 5.0) *Acinetobacter* genospecies did not grow. Growth was observed from pH 6.0 to 9.0 and pH 7.0 was the optimum for IAA production by *A. baumannii* A16 and A18, *Acinetobacter* genospecies 3 (A15), *A. haemolyticus* (A19), and *A. junii* (A6). Two strains, *A. baumannii* (A13) and *A. genospecies 3* (A28), showed optimum IAA production at pH 9.0 (Table 2).

Effect of IAA Produced by Acinetobacter *on Growth of Wheat Plant*

Statistical analysis showed a significant difference in root and shoot length of test wheat plants (21 d) compared with control plants (Table 3).

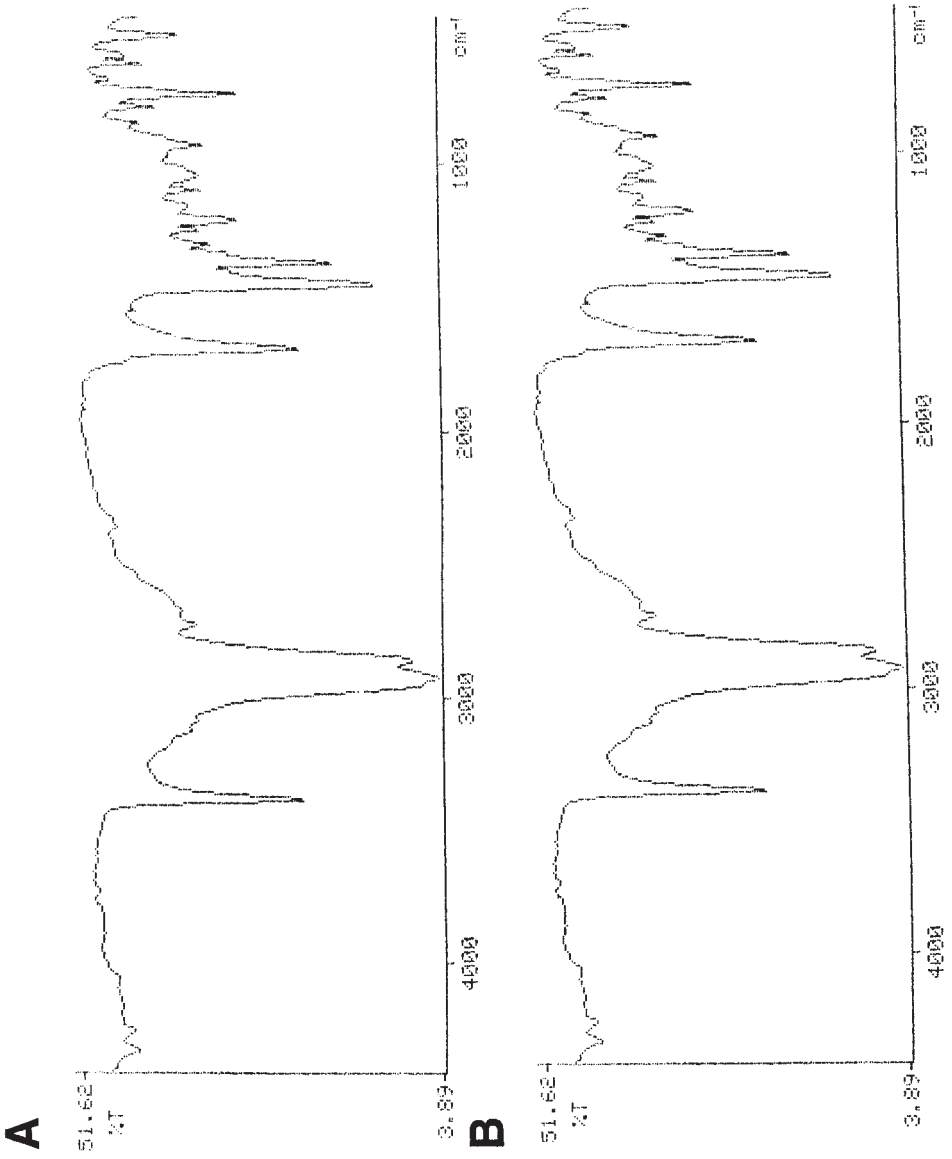

Fig. 3. **(A)** IR spectrum of purified IAA *Acinetobacter* genospecies. **(B)** IR spectrum of standard IAA from Sigma. %T, percentage of transmission; cm^{-1}, wavelength in centimeters.

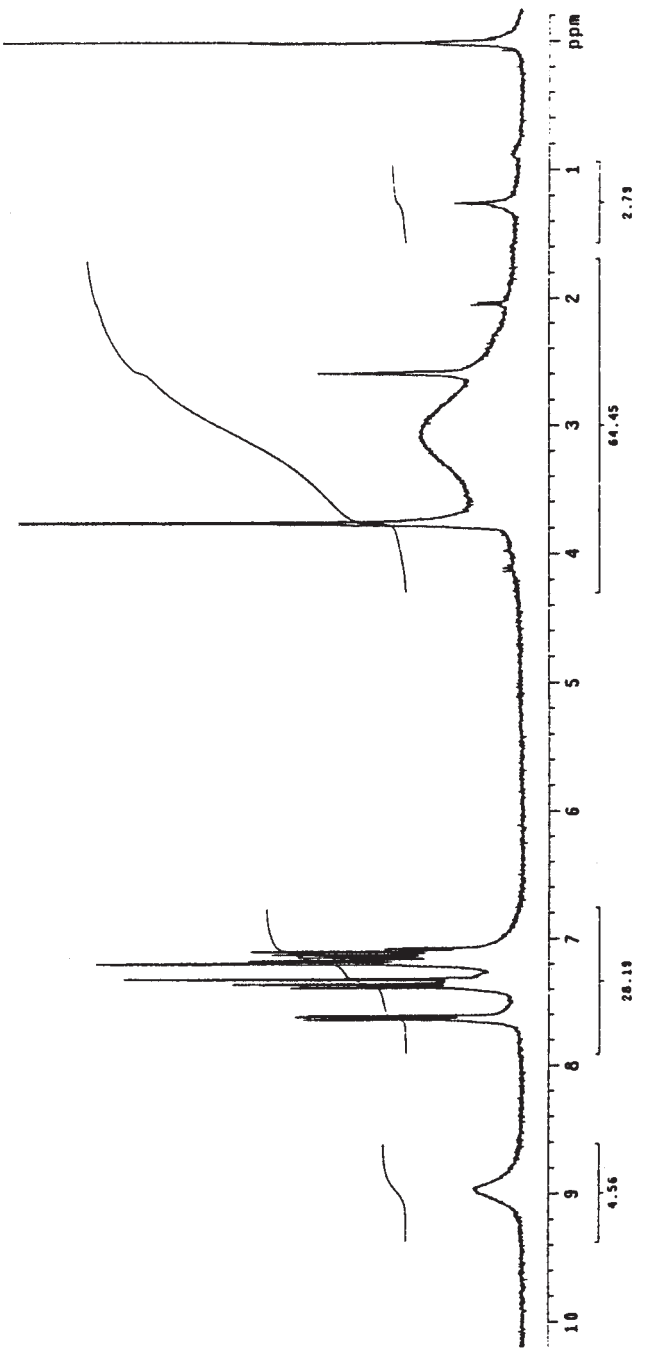

Fig. 4. ^{1}H-NMR analysis of purified IAA from *Acinetobacter* genospecies.

Table 3
Effect of IAA Producing *Acinetobacter* genospecies
on Root Length and Shoot Length of 21-d Wheat Plant by ANOVA[a]

	Root length (cm)				Shoot length (cm)			
Source	df	SS	MS	*F*	df	SS	MS	*F*
Medium[b]	4	2193.7	548.4	33.2	4	3407.8	851.9	42.5
Bacteria[c]	8	3900.4	487.5	29.5	8	1770.0	221.2	11.0
Interaction[d]	24	3814.7	158.9	9.6	24	2669.7	111.2	5.5
Error	1069	17,622.6	16.4		648	154,726.4	20.0	
Total	1106	27,531.4			684	162,573.9		

[a]df, degrees of freedom; SS, sum of squares; MS, mean squares; *F*, *F*-test.
[b]AMM, LB, AMMT, and LBT.
[c]*Acinetobacter* spp., *Pseudomonas* spp., *Moraxella* spp., and *Serratia* spp.
[d]Interaction takes place between bacteria and bacteria and between media and bacteria.

Table 4
Effect of IAA Produced by *Acinetobacter* genospecies
on Root and Shoot Length of Wheat Plant at Ripened Fruiting Stage (75 d)[a]

	Root length (cm)		Shoot length (cm)	
Genospecies	Mean	SD	Mean	SD
A. baumannii				
A18	36.7	5.02	40.1	0.1
A13	32.3	2.5	36.9	5.7
A30	32.4	4.8	35.1	4.8
A16	37.2	2.3	33.5	5.5
A. haemolyticus				
A19	35.3	3.5	41.7	2.8
A. junii				
A6	35.6	2.5	40.1	0.1
Acinetobacter genospecies 3				
A15	37.2	4.3	40.3	0.3
A28	41.4	2.1	42.6	2.08

[a]Values are based on three sets of experiments.

A similar difference was observed when the same experiment was done using large pots (Table 4). It was observed that there was a difference in the color of the leaves of control and inoculated plants. Leaf color of plants inoculated with *Acinetobacter* strains was dark green, compared with pale green of control. Interestingly, the width of the shoots of inoculated plants was found to be almost double compared with that of control. It was also observed that the development of the inflorescence stage (flowering stage) and fruiting stage was 10 d earlier in inoculated plants compared with that of control (Fig. 5).

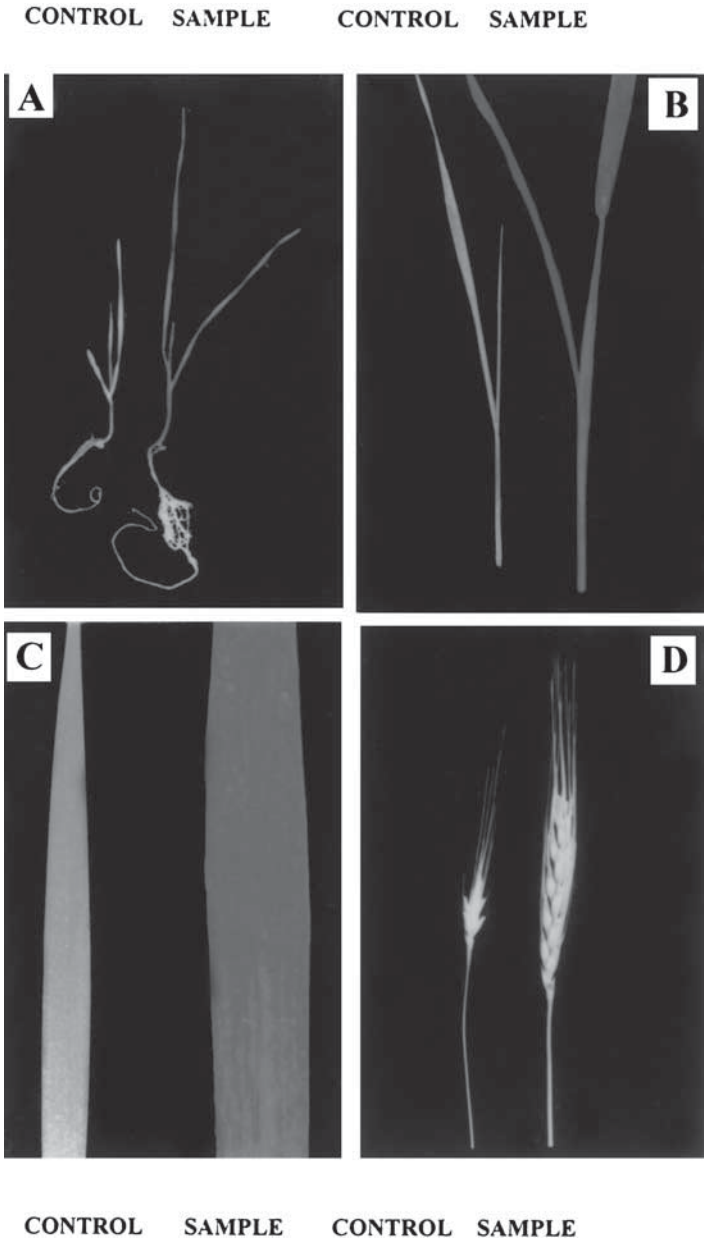

Fig. 5. Effect of IAA produced by *Acinetobacter* genospecies on growth of wheat plant: **(A)** Root and shoot length of 21-d wheat plant; **(B)** shoot width of 60-d wheat plant; **(C)** leaf width of 60-d wheat plant; **(D)** fruiting size and number of grains of 75-d wheat plant.

Isolation of Plasmid

All eight *Acinetobacter* strains contained one megaplasmid of 2.6×10^3 kDa mol wt. All three methods of isolating plasmid DNA showed the presence of only one plasmid in all eight strains. This plasmid was designated pUPI126 (Fig. 6).

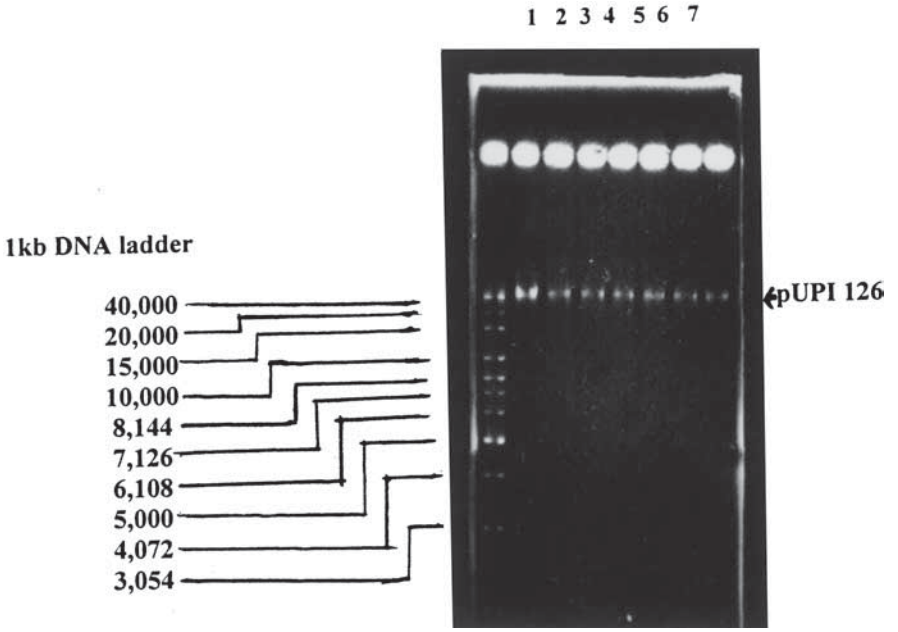

Fig. 6. Plasmid pUPI126 in IAA-producing *Acinetobacter* genospecies. Lane 1, *A. hemolyticus* (A19); lane 2, *A. baumannii* (A13); lane 3, *A. baumannii* (A16); lane 4, *A. genospecies 3* (A15); lane 5, *A. baumannii* (A18); lane 6, *A. juni* (A6); lane 7, *A. genospecies 3* (A28).

Plasmid Curing

Plasmid pUPI126 was not cured by ethidium bromide even at a concentration of 1024 μg/mL and 52°C.

Transformation of Plasmid pUPI126

Plasmid pUPI126, which showed resistance to selenium, tellurium, and lead, was transformed to *E. coli* HB101 rif^r at a frequency of 5×10^{-5}. Along with IAA, selenium, tellurium, and lead were cotransferred almost at the same frequency. Transformants showed IAA production as checked by the Salkovaski test. The color developed was pink, as compared with red developed by the original host *A. haemolyticus* strain (A19). The negative control *E. coli* HB101 (rif^r) did not show any color formation with the Salkovaski reagent. Plasmid pUPI126 was isolated from transformants and observed by 0.7% agarose gel electrophoresis.

Discussion

Acinetobacter is commonly found in soil *(1,5)*. Therefore, it was logical to believe that it may be present in rhizosphere. However, there is no report on the presence of *Acinetobacter* in the rhizosphere of plants including wheat plant, which is an economically important plant. There is only

a short report on the presence of *Acinetobacter* in wheat rhizosphere *(8)*. *Acinetobacter* is a relatively recently studied group of microorganisms because previously this bacteria was known by 40 different names, and hence, there has been a lot of confusion about the systematics of *Acinetobacter* strains *(31,32)*. With the development of chromosomal DNA transformation assay by Juni *(2,33)* a genuine method of confirmation of *Acinetobacter* was developed. This assay is specific only for *Acinetobacter* genospecies, and based on this assay, the authenticity of genus *Acinetobacter* was established *(2,33)*. At present, the systematics of *Acinetobacter* is well defined and consists of 21 genospecies *(17,18,34)*.

Our work on the effect of IAA production by *Acinetobacter* strains on the overall life cycle of wheat plant was done systematically because of the routine use of the chromosomal DNA transformation assay for confirming genus *Acinetobacter (2,5)*. Interestingly, all 37 isolates of *Acinetobacter* were confirmed by this assay. It is important to note that of 21 genospecies, only 5 *Acinetobacter* genospecies were detected from the rhizosphere of wheat. Our findings have revealed that *Acinetobacter* is present in the rhizosphere of wheat in significant numbers *(30)*. This finding gave us the most valuable clue that *Acinetobacter* may have some role in the wheat rhizosphere. Note, however, that *Acinetobacter* is not a plant pathogen. To find out its role in plant growth promotion, we screened *Acinetobacter* strains for IAA production. Until now there has been no report on the production of IAA in the genus *Acinetobacter*. The biology of *Acinetobacter* is very similar to that of *Pseudomonas*, which is also found in the wheat rhizosphere. Moreover, *Pseudomonas* spp. from wheat rhizosphere also produce IAA *(8,14)*. Like *Pseudomonas savastanoi*, *Acinetobacter* genospecies also produced IAA by using tryptophan as a precursor. Determination of IAA-producing capacity of a microorganism is useful in its identification and provides a valuable marker when examining the physiological role or ecological significance of IAA in the establishment and persistence of an organism in the rhizosphere *(20)*. Compared with other IAA-producing bacteria, the production of IAA with respect to the growth phase of *Acinetobacter* was similar in that it produced IAA in the stationary phase, but *A. junii* (A6) produced IAA in the log phase. Interestingly, IAA production by *Acinetobacter* was qualitatively strong because it produced a dark red color within 1 min when it reacted with Salkovaski reagent on nitrocellulose paper.

TLC of extracts clearly showed the presence of IAA in all eight *Acinetobacter* genospecies (Fig. 2). The ^{1}H-NMR, IR, and melting point of extracted samples matched with the standard IAA. We found that pH also affected IAA production and neutral pH *(7)* was the best for production of IAA for six *Acinetobacter* genospecies: *A. genospecies 3*, *A. baumannii*, *A. junii*, and *A. haemolyticus* (A15, A16, A18, A13, A6, and A19). Of eight *Acinetobacter* genospecies, in two of them, *A. baumannii* and *A. genospecies 3* (A13, A28), IAA was produced maximally at alkaline pH compared with acidic pH. This fact has ecological significance because the pH of clay soil used for

cultivation of wheat in Maharashtra, India, is alkaline (pH 8.0–10.0). The effect of IAA on the length and width of plant roots and shoots, fruiting capacity, and health of the plants compared with control plant clearly indicates that IAA is produced by *Acinetobacter* and is directly involved in the promotion of plant growth. The *Acinetobacter* genospecies grown in AMMT or LBT promoted maximum growth of wheat plants in pots since the growth media were supplemented with tryptophan, the precursor for IAA production, whereas the *Acinetobacter* strains grown in AMM and LB promoted less growth of plants because there was a lack of tryptophan. This observation indicates that promotion of plant growth was definitely owing to the IAA produced by *Acinetobacter* genospecies. Similarly, the effect of an inoculation with IAA producing three *Pseudomonas* and one *Acinetobacter* on root growth resulting in increased shoot growth of maize plant has been demonstrated *(35)*.

Plasmid isolation and transformation of plasmid pUPI126 to *E. coli* HB101 (Rif^r) provided evidence that production of IAA and resistance to selenium, tellurium, and lead genes are encoded on the plasmid pUPI126 in *A. haemolyticus* (A19) and that the *E. coli* HB101 transformants also produced IAA in the stationary phase. Our findings are very similar to previously published results of *P. savastanoi* in which IAA-producing genes are also encoded on plasmid pIAA1 *(14,36)*.

To the best of our knowledge, ours is the first report of the IAA production in the genus *Acinetobacter*. It is observed that plasmid pUPI126 is not cured by either ethidium bromide or heat, which suggests that this plasmid is very stable in its original host, *Acinetobacter*. Plasmid transfer and behavior are well established in *Acinetobacter (29,37,38)*. It would be worth investigating the behavior of this plasmid pUPI126 in rhizosphere microorganisms.

Besides IAA encoded by plasmid pUPI126, there may be genes that may specify the production of other hormones such as cytokine, as has been reported in plasmid pP4TH in *Erwinia harbicola pv. Phypsophiloe (39)*, and this warrants further investigation. The present work has indeed established the role of *Acinetobacter* in wheat rhizosphere. It is expected that this work will provide stimulus to study *Acinetobacter* plant interactions in a variety of economically important plants. We have successfully shown the effect of IAA on the growth of wheat plant. Large-size pot experiments have confirmed this observation. Besides enhancement of the growth of wheat plant, the flowering (inflorescence) and fruiting stages of the life cycle were reached about 10 d earlier and persisted longer then the control. The overall health of the wheat plant was very much improved compared with that of the control. This indicates the potential of *Acinetobacter* as a novel bioinoculant for wheat. Further studies on cloning of IAA genes and its regulation, the pathway of IAA biosynthesis, and field studies on the effect of IAA produced by different *Acinetobacter* genospecies on the growth and yield of wheat plant and the development of bioinoculant are under progress.

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