Research Article

Plant Growth Promoting Substances (Phytohormones) Produced by Rhizobacterial Strains Isolated from the Rhizosphere of Medicinal Plants

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are a group of microorganisms which can enhance growth parameters of host plants and can be used as biofertilizers. Indole acetic acid (IAA) production is a major property of rhizosphere bacteria that stimulate and facilitate plant growth. The present work deals with isolation, characterization and identification of indole acetic acid producing bacteria from the rhizospheric soil. A total of 219 bacterial strains isolated from the rhizosphere of different medicinal plants collected from different locations of Telangana state (India). Among the 186 indole acetic acid producing isolates, 3 bacterial strains were selected as efficient IAA producers and investigated to establish the plant growth substances produced by the bacteria. Partial purification of IAA was done and identify plant growth substances produced by the bacteria in liquid culture. Purity was confirmed with thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC). In this study, we showed the presence of a substances associated with auxin, cytokinin activity, and indolic compounds were extracted from the supernatant. Great variation was observed in the IAA production capacity among selected PGPR isolates. *Pantoea agglomerans* (Cf 7) produced 25.0 µg/ml of IAA, *Pseudomonas putida* (Te 1) 46.6 µg/ml of IAA while, in the case of *Pseudomonas* sp. (Av 30) 60.0 µg/ml of IAA was detected in the presence of tryptophan supplements. All the selected strains of PGPR were able to produce plant growth promoting phytohormones affirming the natural ability of PGPR in synthesizing IAA. In conclusion the study suggests the IAA producing bacteria used as an efficient biofertilizer inoculants to promote plant growth.

Keywords: PGPR, Plant growth promoting substances, IAA, Rhizobacteria, Phytohormones.

INTRODUCTION

Rhizosphere is a rich niche of microbes and should be explored for obtaining potential plant growth promoting rhizobacteria (PGPR). Bacteria that colonize the rhizosphere and plant roots, and enhance plant growth by any mechanism are referred to as PGPR, which can be useful in developing bio-inoculants for enhancement of growth and yield of crop plants. Some microorganisms found in the soil are able to produce substances which regulate plant growth. Plant growth regulators (phytohormones) are organic substances, which at low concentrations (less than 1mM), promote, inhibit, or modify the growth and development of plants. Commonly six major groups of phytohormones are recognized: gibberellins, cytokinins, abscisic acid, ethylene, brassino steroids and auxins[1,2].

Indole acetic acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L-tryptophan metabolism produced by several microorganisms including Plant Growth-Promoting Rhizobacteria (PGPR)[3]. Indole acetic acid helps in the production of longer roots with increased number of root hairs and root laterals which are involved in nutrient uptake[4]. IAA stimulates cell elongation by modifying certain conditions like, increase in osmotic contents of the cell, increase in permeability of water into cell, decrease in wall pressure, an increase in cell wall synthesis and inducing specific RXA and protein synthesis. It promotes embial activity, inhibit or delay abscission of leaves, induce flowering and fruiting[5]. Many root colonizing bacteria including *Pseudomonas* sp., *Azatobacter* sp., *Azospirillum* sp., *Bacillus* sp., *Enterobacter* sp. etc. are known to produce plant growth promoting substances.

In previous studies we have isolated screened for multiple PGP activities like ammonia, IAA, HCN production, Phosphate solubilization etc. and evaluate their plant growth properties[6]. Main objectives of this study are isolation, characterization and identification of rhizobacteria which are producing phytohormones from the rhizospheric soil of medicinal plants.

MATERIALS AND METHODS

Isolation of IAA producing bacteria from rhizospheric soil

Sampling was done from different rhizospheres of medicinal plants *viz.*, *Coleus forskohlii, Withania somnifera, Ocimum sanctum, Andrographis paniculata, Mentha spicata, Aloe vera, Lycopersicum esculentum, Tagetes erecta, Artemisia vulgaris, Acorus calamus* and *Mimosa pudica* grown in Botanical garden, Dept. of Botany, Osmania University, CIMAP (Central Institute of Medicinal and Aromatic Plants) centre, ANGRAU, (Rajendra nagar), Hyderabad, and SILPA, Agro farms Pvt. Ltd. Zaheerabad, Medak (Dt), Telangana state, India.

Rhizobacteria were isolated from rhizosphere samples by employing soil dilution plate method[7]. One gram soil was mixed in 10 ml sterile distilled water and serial dilutions were made to get final dilutions of 10^{-6}-10^{-8}. One ml of

each dilution were aseptically transferred to sterile petri plates and melted cool medium (45°C) (Nutrient and King's B agar medium) was added. The plates were gently moved to mix the diluted soil solution uniformly with the medium. Plates were incubated at room temperature (28±2°C) for 24-48h. Colonies were picked from these plates and maintained as pure cultures in respective media with periodic transfer to fresh media. These strains were maintained at -80°C with 50% glycerol. Total 219 isolates were obtained and well isolated colonies were observed for morphological characterization. The isolates were further checked for IAA production.

Identification of isolates

The isolates based on micromorphological observation and biochemical characterization were identified. The tests involved, were Gram staining, amylase and gelatinase, catalase, citrate utilization, indole test, Vogus Proskaur test, methyl red test, H_2S production[7] etc.

Characterization of IAA production

Production of indoles by rhizobacteria was assayed as described by Patten and Glick[8] method. Bacterial isolates were propagated in Luria Bertani (LB) broth (25 ml) amended with tryptophan (50µ/ml). They were incubated for 24 h at 28°C on rotary shaker. Bacterial cells were harvested by centrifugation at 10,000rpm for 15min. 2ml of supernatant was taken in a test tube, 2 to 3 drops of orthophosphoric acid and 4 ml of salkowsky reagent (1mL of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) was added and incubated for 30min in dark at room temperature. Development of pink colour indicated the presence of indoles. Absorbance was read at 530 nm using spectrophotometer[9].

Extraction and purification of IAA

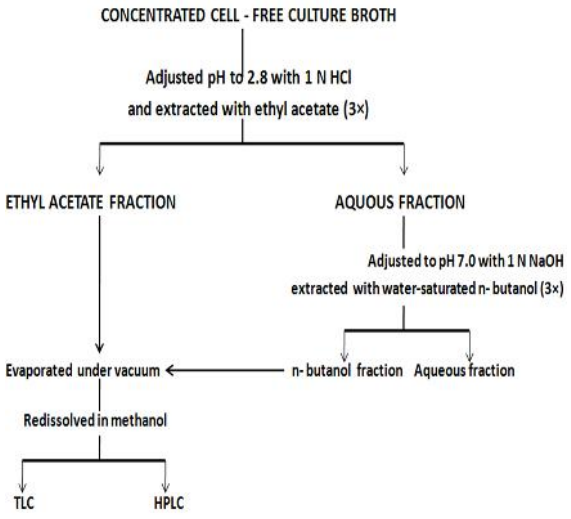

Figure 1: Procedures used in extracting and partitioning bacterial cultures

The bacteria were grown in 500ml flasks containing 200ml of King's B broth amended with 100µg of L-tryptophan/ml at 28°C for 48h. The bacterial population was determined by standard plate count techniques. Bacterial cultures (200ml) were centrifuged at 7,700rpm for 30min. The supernatant was reduced to 50 ml by evaporation under vacuum and extracted with ethyl acetate for the separation of indole compounds and gibberellins and extracted with n-butanol for the separation of cytokinins in bacterial supernatant. Preparation of ethyl acetate and n-butanol fractions procedure outlined in Fig.1.

These extracts were filtered through 0.22 µm membrane filters, and chromatographed by thin-layer chromatography (TLC) and high pressure liquid chromatography (HPLC).

HPLC analysis

HPLC chromatograms were produced by injecting 5 to 10µl of the filtered extracts onto a 10-µm reverse phase column (Shimadzu's Prominence HPLC system Japan C18, 2 mm by 15 cm) liquid chromatograph equipped with a differential PDA detector absorbing at 254 nm. Two solvent systems were used to separate indole compounds and cytokinins. Solvent A was water: acetonitrile: acetic acid (85:15:1 [vol/vol]), flow rate was 1.5 ml/min, and the operating pressure was 1,400 lb/in^2 (95 atm). Solvent B was 30% methanol in water, flow rate was 1.5 ml/min, and the operating pressure was 1,600lb/in^2 (108 atm). Co-chromatography with authentic compounds by TLC, specific color reactions with chromogenic reagents, HPLC, and bioassays were used to establish identity.

Thin layer chromatography (TLC)

TLC chromatograms were run on 0.50-mm-thick preparative silica gel plates. Solvent systems were chloroform: ethyl acetate: formic acid (50:40:10 [vol/vol]) to separate indole compounds and gibberellins in ethyl acetate fractions, and n-butanol: acetic acid: water (12:3:5 [vol/vol]) to separate cytokinins in n-butanol fractions. IAA and other indole compounds were detected on TLC plates by spraying with Ehrlich reagent. Gibberellins were detected by spraying the chromatograms with ethanolic sulfuric acid (90:10 [vol/vol]) and heating to induce fluorescence of the compounds in ultraviolet light.

RESULTS AND DISCUSSION

Isolation of IAA producing isolates

IAA, a member of the group of phytoharmones, is generally considered to be the most important native auxin. Medicinal plants support a great diversity of microflora in their rhizosphere including PGPR. The rhizosphere of medicinally important plants was investigated to explore the diversity of plant growth promoting rhizobacteria from different regions of Telangana state. The rhizosphere soils supported a total of 219 PGPR isolates with diversified characteristics suggesting the importance and richness of the niche as a source of plant microbe interactions. All the 219 isolates screened for IAA production. 186 isolates showed 84.9% of IAA production. From these only 3 strains (Cf 7, Te 1,

Av 30) were selected for further studies and they were potential IAA producers. Studies from the earlier work showed that IAA producing organisms are Gram negative[10,4].

Few gram positive strains belong to *Bacillus* strain also produce IAA[11].

In the present study selected three IAA positive strains were Gram negative.

Identification of bacterial isolates

The isolates were identified based on morphological observation and biochemical characterization (Table 1). Bergey's manual of determinative of bacteriology[12] was used as a reference to identify the isolates. The isolates were identified as *Pantoea* sp. (Cf 7) and *Pseudomonas* sp. (Te 1, Av 30), and the results are presented in Table 1.

Table 1: Morphological and Biochemical characterization of IAA producing strains

S. No	characteristics	Pantoea sp. (Cf 7)	Pseudomonas sp. (Te 1)	Pseudomonas sp. (Av 30)
1	Shape	Short rods	Short rods	Short rods
2	Capsules	-	-	-
3	Gram stain	Gm -ve	Gm -ve	Gm -ve
4	Spore stain	-	-	-
5	Buds or sheaths	-	-	-
6	Motility	Motile	Motile	Motile
7	Nutrition	Autotroph	Autotroph	Autotroph
8	Colonies	Golden	Glistening	Golden
9	Growth	Abundant	Abundant	Abundant
10	Form	Entire	Circular	Rhizoid
11	Margins	Flat	Entire	Serrate
12	Elevation	Autotroph	Raised	Convex
13	Density	Golden	Translucent	Translucent
14	Surface growth	None	None	Pellicle
15	Clouding	Heavy	Heavy	Heavy
16	Sediment	Scanty	Granular	Granular
17	Citrate utilization	+	+	-
18	Lysine utilization	+	+	+
19	Ornithine utilization	+	-	+
20	Urease	-	-	-
21	Phenylalanine deamination	-	-	-
22	Nitrate	-	-	-
23	H_2S production	-	-	-
24	Glucose	-	+	+
25	Adonitol	-	-	+
26	Lactose	+	+	+
27	Arabinose	+	+	+
28	Sorbitol	+	+	+
29	Indole	+	+	+
30	Methyl red	+	+	+
31	Voges Prokaur	-	-	-
32	Catalase	+	+	+
33	Gelatinase	-	+	+

Table 2: Indole compounds and their retention times produced by Cf 7, Te 1 and Av 30

Solvent A (Cf 7)		**Solvent B (Cf 7)**	
TTP	5.298		
Tryptamine	6.909	IAA	6.721
Indole lactic acide	7.801	Indole pyruvic acid	8.911
Indole 3- acetaldehyde	11.376	Butyric acid	17.047
Indole 3- butyric acid	44.591		
Solvent A (Te 1)		**Solvent B (Te 1)**	
Indole 3- acetaldehyde	11.948		
Indole acetic acid	12.813	Indole pyruvic acid	10.427
Indole propionic acid	22.180	Indole acetonitrile	14.247
Indole butyric acid	42.254	Indole butyric acid	17.267
Solvent A (Av 30)		**Solvent B (Av 30)**	
		IAA	5.674
IAA	12.717	Indole pyruvic acid	8.149
Indole propionic acid	20.305	Indole 3-acetaldehyde	10.538
		Indole acetonitrile	14.518
		Indole butyric acid	15.665

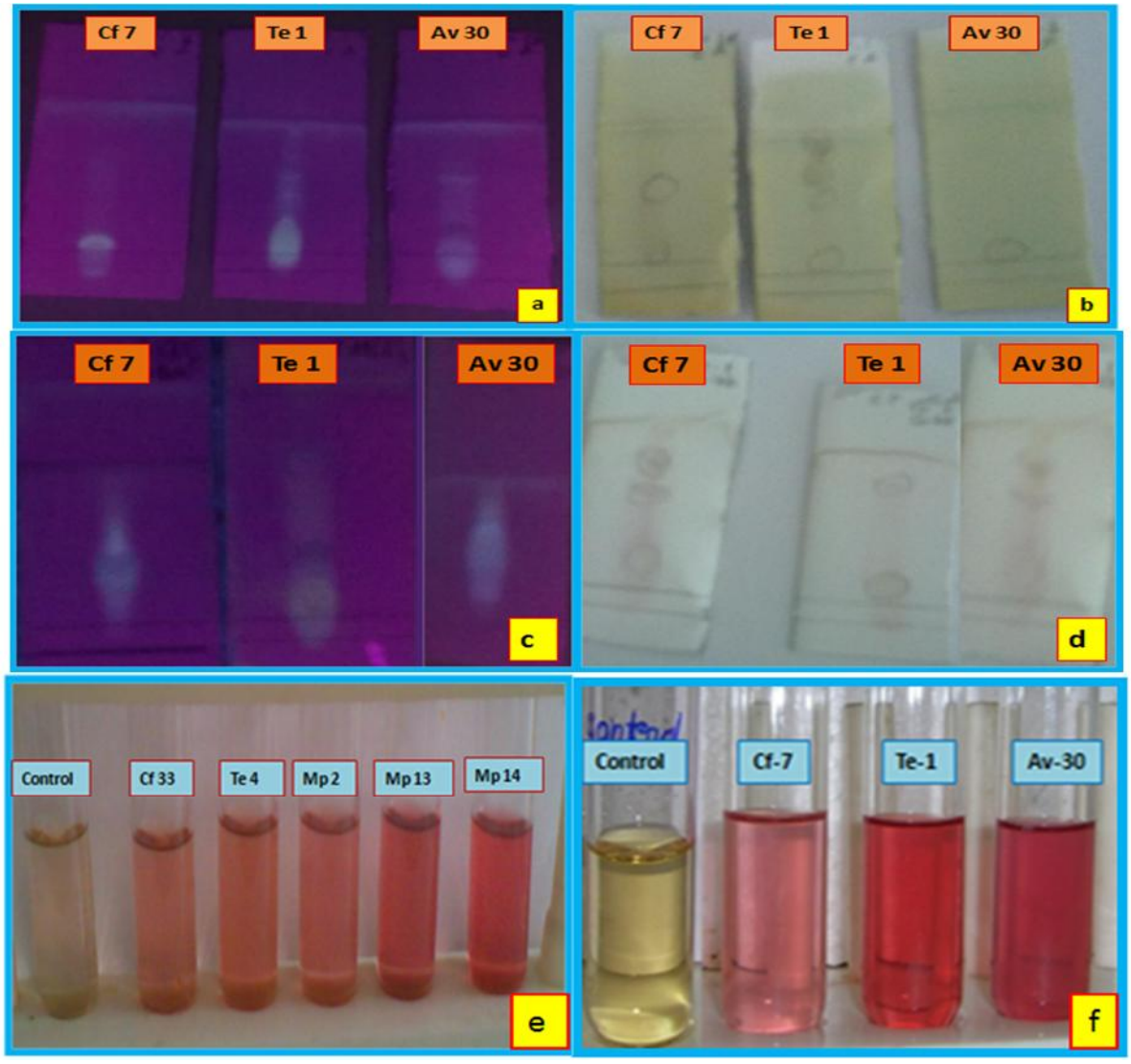

Figure 2: (a) Indole compounds and Gibberellins under UV light (b) Indole compounds after treatment with Ehrlich reagent (c) Cytokinins under UV light (d) Cytokinins under normal light (e) Varied levels of IAA produced by different rhizobacterial isolates (f) IAA Produced by selected rhizobacteria

Characterization of IAA production potential

It has been reported that IAA production by bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability. Moreover, isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil[13]. Great variation was observed in the IAA production capacity among selected PGPR isolates (Fig.2e). All the selected strains of PGPR were able to produce plant growth promoting phytohormones, IAA (indole-3-acetic acid), affirming the natural ability of rhizobacteria in synthesizing IAA. The amount of IAA produced varied among the bacteria, Nine isolates produced high amounts of IAA ranging from 100-170 µg/ml. 23 strains produced 50-100 µg/ml. 13 produced 10-50 µg/ml, 135 strains produced a minimum quantity in the range of 0.5-10 µg/ml 22 strains failed to produce IAA. *Pantoea agglomerans* (Cf 7) produced 25.0 µg/ml of IAA, *Pseudomonas putida* (Te 1) 46.6 µg/ml of IAA while, in the case of *Pseudomonas* sp. (Av 30) 60.0µg/ml of IAA was detected in the presence of tryptophan supplements (Fig.2f). However when strains were grown in the presence of 10 µg of tryptophan per ml for 48-72h, the tested PGPR isolates responded by producing higher levels of IAA.

Detection and Identification of indoles by HPLC and TLC

Numerous bacteria also have the ability to synthesize plant growth regulators such as indole- 3- acetic acid, indole 3- butyric acid, indole-3-acetaldehyde, indole lactic acid and other indole related compounds[14]. The production of IAA from tryptophan amended bacterial cultures was confirmed by TLC and HPLC bioassay. Production of IAA and other indole compounds in bacterial cultures was confirmed by HPLC analysis. Cultures were extracted with ethyl acetate and n-butanol according to Tein[12] and filtered through 0.22 µM membrane filters. Indole compounds were identified based on their retention times in reference peaks from literature. They were quantified at 254 nm and by using PDA detector. Retention times for peaks were compared to those of authentic standards in literature, which was added to the medium and extracted by the same procedures used with bacterial cultures[12]. Quantification was done by comparison of peak heights. A mixture of indoles compounds were identified by using two solvent systems described in materials and methods. Indole compounds and their retention times produced by Cf 7, Te 1 and Av 30 in HPLC analysis reports are shown below.

Indole compounds were determined by using thin layer chromatography (TLC). Ethyl acetate extractions of Cf 7, Te 1 and Av 30 were spotted on TLC plate and run chloroform:ethyl acetate:formic acid (50:40:10) as a solvent system. Blue colour spots were visualized under UV light (Fig. 2a,b,c,d), and are identified by spraying Ehrlich reagent. Ethyl acetate extracts of *Pantoea agglomerans* (Cf 7), *Pseudomonas putida* (Te 1), and *Pseudomonas* sp. (Av 30) cultures showed a clear blue spot corresponding to some indole compounds when chromatograms treated with ehrlich ragent. Indole compounds from crude extract into individual components on TLC when visualized under UV irradiation, indicated presence of more than one spot in case of most of the isolates.

There is firm evidence that indole 3-acetic acid (IAA), gibberellins[15-19] and cytokinins[20-22] are produced by plants which are essential to their growth and development, and are also produced by various bacteria which live in association with plants. There is also evidence that the growth hormones produced by the bacteria can in some instances increase growth rates and improve yields of the host plants[15,23]. In the present study, to validate the auxin production of the strains assessed in this study, HPLC analysis was performed to determine IAA production. In all cases, it was possible to measure IAA levels. Although different values among bacterial species and within the same genus. *Pseudomonas* sp., and *Pantoea* sp., produced high levels of IAA. Production of IAA was further confirmed by 3 isolates (Cf 7, Te 1, Av 30) and subsequent TLC analysis. A specific spot from the extracted IAA preparation was found. Ethyl acetate extracts were prepared from bacterial cultures of *Pantoea* sp, *Pseudomonas* sp. grown under stationary conditions in Kings'B medium for 3 days. Gibberilic acids do not absorb ultraviolet light 254 nm, only TLC and bioassays were used for their detection. TLC showed unidentified substances which fluoresced green under ultraviolet light. A mixture of gibberellins may be present, but these could not be separated with the solvents used.

The n-butanol fraction of bacterial culture was used for detection of cytokinins. Most cytokinins especially Zeatin and Zeatin riboside are retained in this fraction[24,25]. We cannot identify this cytokinin on HPLC chromatograms because of the presence of interfering substances. Further purification is needed before analyzing the n-butanol fraction by HPLC. Biosynthesis of indol 3-acetic acid, zeatin, and gibberellic acid showed differences among strains and are summarized in Table 2. In the present study three rhizosphere bacterial isolates which are producing auxins, cytokinins were detected by using HPLC analysis. Ethyl acetate extractions of *Pantoea agglomerans* (Cf 7) producing tryptamine at the retention time of 6.909, indole lactic acid at 7.801, indole 3 acetaldehyde at 11.376, indole butyric acid is producing at the the retention time of 44.591 by using solvent A (water: acetonitrile: acetic aid in the ratio of 85:15:1) and indole acetic acid at 6.721, indole pyruvic acid at 8.911 and butyric acid at 17.047 are produce by using solvent B (30% methanol). All the indole compouds are showing prominent peaks in the HPLC analysis reports (Table.2).

Pseudomonas putida (Te 1) also producing indole compounds *i.e.,* indole 3-acetadehyde at the retention time of 11.948, indole lactic acid at 12.813, indole propionic acid 22.180, indole butyric acid at the retention time of 42.254 by using solvent A

(water:acetonitrile:acetic acid in the ratio of 85:15:1) and indole pyruvic acid (IAA) at the retention time of 10.427, indole acetonitrile at 14.247, indole butyric acid at 17.267 by using sovent B (30% methanol). All the indole compouds are showing prominent peaks in the HPLC analysis reports (Table.2).

Pseudomonas sp. (AV 30) producing indole acetic acid (IAA) at the retention time of 12.717 and Indole propionic acid at 20.305 by using solvent A (water:acetonitrile:acetic acid in the ratio of 85:15:1) and indole acetic acid (IAA) produced at the retention time of 8.149, indole 3-acetaldehyde (10.538), indole acetonitrile (14.518), indole butyric acid (15.665) by using sovent B (30% methanol). All the indole compounds are showing prominent peaks in the HPLC analysis reports. Tien[12] and Vikram Patil[26] reported similar type of results for plant growth substances produced by *Azospirillum* sp. The results can be related with the reports of Dey[27] who isolated the PGPR's that produced IAA like substances from the rhizosphere of *Arachis hypogaea.*

Effect of L-Tryptophan concentration on IAA

L-Tryptophan is generally considered as an IAA precursor; because of its addition to IAA producing bacterial culture enhances IAA biosynthesis[28]. All 5 isolates preferred Tryptophan for IAA production. The present study clearly revealed that all isolates tested in this study had the ability to produce IAA and consequently, considered as IAA producing rhizobacteria. Most studies have shown that IAA biosynthesis is greatly influenced by L-TRP precursor. L-TRP is believed to be the primary precursor for formation of IAA in several microorganisms[29]. Addition of L-TRP (an auxin precursor) to the media increased the auxin production by several fold.

The use of the technique for the detection of IAA using the salkowski reagent is an important option for qualitative and semi-qualitative determination that assure the presence of the hormone in the supernatant of bacterial cultures or liquid formulations of biological inoculants. The amount of IAA produced by the bacteria was within the detection limits of salkowski reagent[30]. The reagent gives reaction with IAA and does not interact with L-tryptophan and Na-acetyl-L-tryptophan and used by and large[31]. Among the isolates 186 strains Cf 7, Te 1 and Av 30 were found to be the best producers of IAA (Fig.2f). Hence for further characterization these isolates were selected.

The concentration of IAA produced varied between bacterial strains. Other studies have shown the ability of *Pseudomonas* and *Pantoea* species produce IAA ranging from 20-60 µg/ml in the presence of tryptophan and 7.7-21.4 µg/ml without tryptophan. Asghar[32] had shown that the high concentration of IAA (111.50 mg L-1) was produced by plant growth-promoting rhizobacteria S88 when L-TRP was added in the medium. In natural condition, plant roots excrete organic compounds including L-TRP which can then be utilized by the rhizobacteria for IAA biosynthesis[34]. Asghar[32] showed that hundred bacterial isolates from rapeseed plant were able to produced IAA in the absence of precursor L-TRP and the highest concentration of IAA produced by one of the isolates was 11.40 µg/ml.

CONCLUSION

From this study, it is clear that rhizospheric soil can provide a rich source of IAA producing bacteria and has the ability to produce a significant amount of IAA in a tryptophan-supplemented medium. Overall 186 isolates were identified as IAA producing strains among which three efficient IAA producing bacteria were characterized and media components, physical parameters were optimized for IAA production. It is concluded that presence of such growth promoting rhizoflora accountable for the beneficial effects on crop growth and yield. In the present study *Pantoea* sp.(Cf 7), *Pseudomonas* sp. (Te 1, Av 30) could be stated as the potential of IAA producing strains and optimization study for IAA production will flourish the growth and ultimately IAA production in the field prevent environmental pollution by avoiding excessive applications of industrially produced fertilizers to cultivated fields.

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