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Studies on Indole acetic acid production and phosphate solubilization from forest soil bacteria

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ABSTRACT

Indole acetic acid (IAA) is a naturally occurring auxin with broad physiological process including cell enlargement and cell division, tissue differentiation and responses to light and gravity. Phosphate solubilizing bacteria (PSB) solubilize insoluble phosphate and make it available to the plants. In this study, sample was collected from two different areas, Thirupathi and Kerala. Totally 30 bacterial colonies were selected and used for further screening process. The isolated strain ware screened for IAA production by using Solawaski's reagent in nutrient broth. The color change shows the production of IAA production. Out of 30 isolates, three (TS2, TS13 and KS3) strains showed pink color which is positive for IAA production. TS2, TS13 and KS3 are extracted using separating funnel and the extract was dissolved in methanol and kept in cool temperature. The crude compound was separated by using TLC and the Rf value was noted. Estimation of IAA shows TS2 that strain able to produce maximum IAA ranges between 11.31-26.5µg/ml in the absence and presence of tryptophan. *In vitro* studies, showed a maximum growth in the plants containing TS2, TS13 and KS3 isolates than that of normal plants. Among 30 isolates, 12 strains showed good phosphate solubilization activity in the Pikovskaya medium.

KEYWORDS

Indole acetic acid (IAA);
Phosphate solubilization;
Solawaski's reagent;
Pikovskaya medium.

INTRODUCTION

Indole acetic acid (IAA)

Auxins were discovered early in the twentieth century as plant-regulating substances. Indole-3-acetic acid (IAA) is a naturally occurring auxin with broad physiological processes including cell enlargement and division, tissue differentiation, and responses to light and gravity. Although many plant genes that are transcriptionally regulated by IAA have been characterized in recent years, our understanding of the auxin signal transduction pathway(s) in plants is still incomplete. IAA biosynthesis in plants can occur via different pathways, which are classified according to their intermediates: Indole-3-Acetamide (IAM), indole-3-Pyruvate (IPyA), tryptamine, and Indole-3-Acetonitrile. To further un-

derstanding of plant growth and development in nature, microbial released auxins, which can have a pronounced effect on plants, should be considered. The two most common routes for IAA biosynthesis in bacteria are the IAM and the IPyA pathways[10].

IAA is a common product of L-tryptophan metabolism by several microorganisms including PGPR (plant-growth promoting rhizobacteria)[1]. Microorganisms inhabiting rhizospheres of various plants are likely to synthesize and release auxins as secondary metabolites because of the rich supplies of substrates exuded from the roots compared with nonrhizospheric soils.

The list of species synthesizing IAA is steadily growing, and presently includes gram negative, gram positive, plant pathogenic, symbiotic and nitrogen fixing bacteria. Many of these also can produce and excrete in their cultures more than one hormone type: *Rhizobium sp.* synthesizes gibberellins (GA) and auxin, *Azotobacter sp.* synthesizes GA, auxins and cytokinins , *Acetobacter sp.* and *Herbaspirillum sp.* synthesizes isolates IAA and GA and pink-pigmented facultative methylotrophic bacteria (PPFMs) and plant-associated bacteria that are ubiquitously distributed on plant surfaces[3].

Enhanced plant growth may result directly from microbial production of plant-growth regulators, including indole-3-acetic acid[2]. The IAA produced by microbes colonizing the seed or root surfaces is proposed to act in conjunction with endogenous IAA in plant to stimulate cell proliferation and or elongation and enhance the host's uptake of minerals and nutrients from the soil[12].

Among plant growth regulators, indole-3-acetic acid (IAA) is the most common natural auxin found in plants and its positive effect on root growth and morphology is believed to increase more nutrients from the soil[14].

IAA produced by bacteria improves plant growth by increasing the number of root hairs and lateral roots[9]. Microbial biosynthesis of IAA in soil is enhanced by tryptophan from root exudates or decaying cells. Phosphate solubilizing bacteria are potential to increase available phosphate for plant, especially in soils with amounts of precipitated phosphate.

Phosphate solubilization

Phosphorous is an essential nutrient of plants, but is often not available due to its fixation in soil. Phosphate solubilizing bacteria (PSB) solubilize insoluble phosphate and make it available to the plants. Indian soils on an average contain 0.05% Phosphorous that constitutes 0.2% of plant dry weight. Even applied Phosphorous combines with metal ions PSB are required for its release. PSB secrete organic acids and enzymes that act on insoluble phosphate and convert it into soluble form thus, providing Phosphorous to plants. PSB also produce amino acids, vitamins and growth promoting, which promote plants growth. Increased growth and yield of oats, coffee, tea, banana, soya bean, maize, rice, sorghum, barley, cabbage, and tomato to using of PSB[13].

MATERIALS AND METHODS

Collection of sample

The soil samples were collected from two different areas at Thirupathi (Andhra Pradesh) and Athirampalli plantation soil (Kerala). The samples were transported in a sterile polythene bag with pre sterilized spatula. Then the collected samples were brought to the lab in ice box and processed within one day.

Isolation of microorganism

The samples were serially diluted up to 10^5 dilution using 9 ml sterile distilled water blanks. 0.1 ml of aliquot from 10^2 to 10^5 dilutions was transferred to nutrient agar plates and spreaded using sterile L rod. Plating was done in duplicate and all the plates are incubated at 28°C for 24-48 hours. After enumeration, morphologically different bacterial colonies were selected, purified and sub cultured on nutrient agar slants.

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

All the isolated strains were screened for IAA production. Briefly, test bacterial culture was inoculated in the nutrient broth medium with tryptophan (2 mg/ml) and without tryptophan incubated at 28 ± 2°C for 5 to 7 days. After incubation, 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 0.5M $FeCl_3$). Development of a pink color indicates IAA production[1].

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Extraction of crude IAA

The screened strains which showed positive for the production of IAA were inoculated in 200 ml of nutrient broth with tryptophan (2 mg/ml) and incubated at 28 ± 2°C for 1 week on a shaking incubator. After incubation the bacterial cells were separated by centrifugation at 10,000 rpm for 30 minutes. The resulting supernatant was acidified to pH 2.5 to 3.0 using 1N HCl and extracted twice with two volume of ethyl acetate to the supernatant. Extracted ethyl acetate fraction was evaporated to dryness. The extract was dissolved in methanol and kept in cool temperature[1].

Partial purification of crude extract by thin layer chromatography (TLC)

The crude extract was purified by Thin Layer Chromatography (TLC) using Silica gel coated plates. To find out the best solvent system for good separation of crude compound, solvents systems such as Ethyl acetate: chloroform: formic acid (55:35:10)[4] and benzene: n-butanol: acetic acid (70:25:5) were used[1].

The crude extract was dissolved in 200 µl of methanol. With the help of capillary tube, the sample was spotted. The solvent was allowed to run till it reaches about half a centimeter below the top of the plate. After running, the plate was kept in room temperature for the complete drying of the plate. Spots with IAA were identified by spraying the plates with Ehmann's reagent. Rf value of the spot on the TLC plate was determined.

Rf value = Movement of solute / Movement of solvent

Estimation of IAA

A modified colorimetric method was used for the estimation of IAA. The selected isolates were grown in nutrient broth without tryphtophan and with tryphtophan (2, 4, 6 mg/ml), and incubated at 28°C for 48 hours and then centrifuged at 1000 rpm for 10 minutes, after centrifugation 1 ml of culture supernatant was placed in a test tube and mixed with 2 ml of Salkowski's reagent. After 20 to 25 min, the color absorbance was read using spectrophotometer at 535 nm. Pure IAA was used for preparing the standard 0, 5, 10, 15, 20, 25, 30, 35, 40 µg/ml[15].

Plant growth promoting activity of IAA producing strain

Seeds of *Amaranthus sp* were surface sterilized with acid: alcohol (H_2SO_4: ethanol, 7:3, v/v) for 5 min and washed with sterilized water[6]. The surface sterilized seeds were inoculated with broth cultures of IAA producing strains. The seeds were placed on sterile Petri plate lined with blotting paper and incubated at 28 ± 2°C for 3 days[11]. The germinated seeds were transferred in series of six sterile polythene bags. In which, the first three bags contain sterile soil, minimal potassium and nitrogen sources and seeds which are inoculated with IAA producing strains TS2, TS13 and KS3 respectively., The fourth bag contains sterile soil, uninoculated seeds and minimal nutrition medium which contains basal doses nitrogen (15.24 mg/100 g of soil) and potassium (5 mg /100 g of soil) it is maintained as positive control. The fifth bag contains sterile soil and uninoculated seeds (negative control 1), sixth bag contain unsterile soil and uninoculated seeds (negative control 2).

Solubilization of phosphate

Phosphate solubilization test were conducted qualitatively by plating the bacteria in agar containing precipitated tricalcium phosphate, the medium was a modification of Pikovskaya medium. Bacterial culture were spotted in the plates and incubated at room temperature for overnight[7].

Identification of IAA producing strains

The IAA producing strains are characterized based on their phenotypic characteristics such as micromorphology (gram staining, capsule staining, endospore staining and motility), cultural characteristics (on basal media, differential media and selective media), and biochemical characteristics (catalase, oxidase, IMViC). Based on the results of studied phenotypic characteristics all the selected IAA producing bacterial strains are identified with the help of Bergey's Manual of Determinative Bacteriology.

RESULTS AND DISCUSSION

Isolation of bacteria from forest soil

After incubation, morphologically different colonies were observed on Nutrient agar plates. In Thirupathi soil sample, 20 bacterial colonies were isolated, named as TS1 to TS20 and the bacterial populations were

TABLE 1 : Estimation of IAA

S. no	Strain	IAA production (µg/ml) with Tryptophan concentration (mg/ml)			
		0	2	4	6
1	TS2	11.31	20.0	22.57	26.50
2	TS13	8.42	17.14	17.71	20.85
3	KS3	10.52	17.42	19.42	23.79

estimated as 6.45×10^8 CFU/gram of sample. In Kerala soil sample, 10 bacterial colonies were isolated, named as KS1 to KS10 and the bacterial populations were estimated as 8.20×10^7 CFU/gram of sample.

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

After one week the culture supernatant was centrifuged and collected, and then Solawaski's Reagent was added and kept at room temperature for 30 minutes. Out of 30 isolates, pink color was observed in three strains, TS2, TS13 and KS3. It confirms the IAA production.

Extraction of crude IAA

After one week of incubation, supernatant was extracted with two volumes of ethyl acetate. After extraction, the extract was mixed with methanol and used for TLC.

Partial purification of crude extract by Thin Layer Chromatography (TLC)

Among the two solvent systems tested, benzene: n-butanol: acetic acid (70:25:5) showed bluish violet color spot and its Rf value was noted. Rf values of the strains TS2, TS13 and KS3 were calculated as 0.58, 0.68, and 0.54 respectively.

Culture filtrates of *Pseudomonas* and *Azotobacter* were used to extract IAA for characterization by TLC[1]. The extracted compound was collected and dissolved in methanol and kept in cool temperature and used for TLC. Bluish violet color spot in the TLC plate shows the presence of IAA in the compound[4].

Estimation of IAA

Among the three strains tested, TS2 were able to produce maximum IAA both in the absence and presence of tryptophan ranges between 11.31 to 26.5 µg/ml. The results were given in the TABLE 1. Yasmin et al.[15] reported IAA production by *Pseudomonas* and *Acinotobater* isolated from wheat and rye rhizospheres ranging from 0.01 to 3.98 mg/ml. Hundred bacterial isolates produced IAA in the absence of precursor L-TRP and the highest concentration of IAA produced by one of the isolate was 11.40 µg/ml.

TABLE 2 : Plant growth promoting activity

S. no	Strain	Root weight	Length (cm)
1	TS2	0.137	7.8
2	TS13	0.121	7.4
3	KS3	0.123	7.6
	Controls		
4	Positive control	0.119	7.3
5	Negative control(1)	0.83	4.8
6	Negative control (2)	0.96	6.3

Plant growth promoting activity of IAA producing strain

After 25 days, inoculated plant root was observed. *In vitro* study reveals that a plant growth promoting activity of the soil contains IAA producing isolates showed good growth rate than that of the positive control and negative control. Dry weight and length was measured and it was shown in the TABLE 2.

Solubilization of phosphate

After incubation, a clear zone was observed in the modified Pikovskaya medium. Out of 30 strains, 12 strains TS2, TS3, TS6, TS7, TS8, TS9, TS10, TS16, KS4, KS5, KS9 and KS10 showed good activity for the Phosphate solubilization. Microorganisms capable of producing a clear zone due to solubilization of organic acids in the surrounding medium are selected as potential phosphate solubilizers and are routinely screened in the laboratory by a plate assay method using either Pikovskaya agar. Several reports on bacteria and fungi isolated from soil have evaluated their Mineral Phosphate Solubilizing (MPS) activity with various phosphate sources such as calcium phosphate tribasic [$Ca_3(PO_4)$] iron phosphate ($FePO_4$)[8] and aluminium phosphate ($AlPO_4$). An increase in Phosphate availability to plants through the inoculation of PSBs has also been reported previously in pot experiments and under field conditions[5].

Identification of IAA producing strains

Based on the morphological, cultural and biochemi-

cal characteristics, The potential strain was compared with Bergey's Manual of Determinative Bacteriology and identified as, *Bacillus sp.* (TS2), *Klebishella sp.* (TS13) and *Pseudomonas sp.* (KS3). In the present study, it was found that the isolates TS2, TS13 and KS3 are responsible for IAA production and phosphate solubilization.

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