International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.7, No.1, pp 099-107, 2014-2015

Isolation of Plant Hormone (Indole-3-Acetic Acid) Producing Rhizobacteria and Study on their Effects on Tomato (*Lycopersicum esculentum*) Seedling

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Abstract: Twenty one rhizobacteria were isolated from rhizospheric soils of Uttarakhand Tarai region. The isolated soil bacteria were screened for the production of indole acetic acid, phosphate solubilisation and siderophore production. Out of 21 isolates, 3 isolates (P3, P9, and P19) were screened for indole-3-acetic acid (IAA) production that are Gram negative, catalase positive and starch hydrolysis positive. Isolate P19 was the best IAA producer strain (50.25µg/ml) while isolate P3 was lowest IAA producer (15.25µg/ml) comparatively. All three isolates showed siderophore production while phosphate solubilisation was shown by only P19 isolate. All the three isolate showed nitrogen fixing activity in G-NFMM media. Effect of IAA producing PGPR alone and in different combinations was studied on seed germination of tomato plant. Treatment with PGPR isolates in combination of P3+P9+P19 on tomato seedling showed maximum shoot length (8.6cm), root length (3.0cm), shoot fresh weight (0.104g), root fresh weight (0.005g), fresh plant weight (0.109g) and dry plant weight (0.018g) as compared to control and other PGPR combinations. The consortia of three isolated bacterial strains showed significant effects on seed germination rate. Production of indole acetic acid (IAA) and siderophore was also observed by isolated strains. Thus, the use of combination of PGPR isolates is advocated for excellent growth performance of plants.
Keywords: Plant hormone, Siderophore, Phosphate solubilisation, Indole acetic acid, PGPR.

Introduction

Tomato (*Lycopersicum esculentum*), according to the FAO, is the second most cultivated vegetable in the world, after the potato, with an annual production of nearly 108 t in 3.7×10^6 ha worldwide. China, USA and Turkey are being the leading producers[1, 2]. In addition to its economic importance, tomato consumption has recently been demonstrated to be beneficial to human health, because of its content of phytochemicals such as lycopene, β-carotene, flavonoids, vitamin C and many essential nutrients[2,3]. This composition explains the high antioxidant capacity in both fresh and processed tomatoes associating the fruit with lower rates of certain types of cancer and cardiovascular disease[2, 4, 5]. In the last century, chemical fertilizers were introduced and this made farmers to be happy of getting increased yield in agriculture in the beginning. But slowly chemical fertilizer started displaying their ill-effects such as leaching, polluting water basins, destroying microorganisms and friendly insects, making the crop more susceptible to the attack of diseases, reducing the soil fertility and thus causing irreparable damage to the overall system. One of the other most important effective factors in increasing plant yield is seed inoculation or priming with plant growth promoting rhizobacteria (PGPR)[6]. Also, plant growth promoting rhizobacteria (PGPR) are a group of bacteria that actively colonize plant roots and increase plant growth and yield[7, 8, 9]. The mechanisms by which PGPRs promote plant growth are not fully understood. But, several mechanisms have been suggested by which PGPR can promote plant growth and this include auxins[10], enhancing stress resistance, symbiotic N_2 fixation[11], solubilisation of inorganic phosphate and mineralization of organic phosphate or other nutrients[12,13] increasing the supply or availability of primary nutrients to the host plant and antagonism against phytopathogenic microorganisms by production of

siderophores, synthesis of antibiotics, enzymes or fungicidal compounds and competition with detrimental microorganisms[6,8,14,15,16]. Microorganisms inhabiting rhizosphere of plants utilize the rich source of substrates from the roots and are expected to synthesize and release auxins as secondary metabolites[17, 18, 19, 20]. Kloepper and Beauchamp[21] have been shown that cereal yield increased up to 30% with *Azotobacter* inoculation and up to 43% with *Bacillus* inoculation. Strains of *Pseudomonas putida* and *Pseudomonas fluorescens* could increase root and shoot elongation in canola[22]. Bashan and Cakmake[23, 24] reported that inoculation of plants with *Azospirillum* could result in significant changes in various growth parameters, such as increase in total plant biomass, nutrient uptake, plant height, leaf size, leaf area index and root length of cereals[6, 23]. IAA 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[25, 26]. The soil microorganisms used in biofertilizers are phosphate solubilising microbes, mycorrhizae, *Azospirilum sp*, *Azotobacter sp*, *Rhizobium sp*, *Sesbania*, Blue green algae, *Nitrosomonas sp*, *Nitrobacter sp* and *Azolla sp*. Thus, the aim of this study is IAA production of isolated PGPR and to determine the effect of IAA on seed germination and plant growth promotion of tomato plant (*Lycopersicum esculentum*).

Materials and Methods

Isolation, Purification and Identification of Isolates

Rhizospheric soil of 2 months old paddy field in different areas of Kashipur region (Tarai Region of Uttarakhand) in India was used for isolation of PGPR organisms. After removal of plant from soil, root portion was cut and packed in sterile labelled plastic bags. The rhizosphere soil was collected in a separate bag. The bags were transported to laboratory under cold conditions for immediate processing. Adhering soil was carefully brushed off, and the plant roots were vigorously shaken and washed off with sterile saline solution so as to remove microorganisms closely associated with roots. Soil suspension was prepared by suspending approximately 1 gm of soil in sterile distilled water and vortexed. The suspension was serially diluted up to 10^8 and 100 µl of inoculum was plated in triplicate on nutrient agar and yeast extract mannitol agar medium. Plates were incubated at 28˚C for 3 days. Well-isolated colonies were selected based on morpho-phenotypic characteristics [27, 28], purified and maintained on nutrient agar and Jensen's agar media. IAA-producing isolates were selected by growing them in IAA production medium. The isolate producing maximum amount of IAA was further selected for the optimization of IAA production.

Evaluation of Plant Growth Promoting Activities of Soil Bacteria

The isolated soil bacteria were screened for the production of indole acetic acid, phosphate solubilisation and siderophore production. Siderophore production was tested qualitatively using chrome azural (CAS) agar as described by Alexander and Zuberer[29]. The bacterial isolate was streaked on the CAS agar plates and incubated at 28±2 ˚C for 3-4 days.

Phosphate solubilisation test was carried out by plating the bacteria on tricalcium phosphate agar medium[30]. The presence of clearing zones around the bacterial colonies following incubation at 28±2˚C for 24 hrs indicated positive test for phosphate solubilisation.

Bacterial indole acetic acid production was examined by growing isolates in nutrient broth supplemented with tryptophan[31]. The growth cultures were centrifuged at 6,000 rpm for 30 mins. 1 ml of the supernatant was mixed with 2 drops of orthophosphoric acid and 2 ml of salkowski reagent (50 ml, 35% perchloric acid and 1 ml 0.5 M $FeCl_3$) in the ratio 1:2. The mixture was then incubated in dark for 30mins for the development of pink color (IAA production) and absorbance was measured at 535 nm using spectrophotometer.

Screening of Nitrogen-Fixing Activity

The visual detection of nitrogen-fixing activities of the selected IAA producing strains was observed using glucose nitrogen free mineral medium (G-NFMM). Single colony grown on nitrogen free medium was taken and inoculated into G-NFMM containing BTB solution (bromothymol blue solution). It was observed after one week incubation for the appearance of blue green color that confirms nitrogen fixing activity.

Collection and Preparation of Soil Sample

The soil sample was collected from Dhyan Nagar village, Ramnagar village and Mahuadabra located at Jaspur, Mahuakheraganj and Kunda village at Kashipur, Uttarakhand. The soil was collected from the top 15cm depth with a trowel and evenly distributed into sterile planting pots. The planting pots were labelled appropriately and watered carefully awaiting the application of seeds.

Preparation of Inoculum for Field Inoculation

The test organisms from the stock culture were resuscitated by sub-culturing into enrichment media and incubated for 24hrs at 28 ± 2^0C. After incubation, nutrient broth was prepared by aseptically decanting the components into 800 ml of deionised water in eight different 250 ml conical flask. It was sterilized by autoclaving and allowed to cool. The three test organisms P3, P9, P19 and their different mixtures: P3+P9, P3+P19, P9+P19, P3+P9+P19 were inoculated into each of the conical flask respectively with one un-inoculated control. It was incubated for 24hrs at 28 ± 2^oC.

Germination Assay

Preparation of Seeds

To study the effect of IAA producing PGPR on seed germination, the present experiment was performed under laboratory condition using tomato (*L.esculentum* Mill.) Var. T-3, purchased from G. B. Pant University of Agriculture & Technology, Pantnagar. Healthy seeds were selected and surface sterilized with 2% sodium hypochlorite for 3 minutes and then they were washed by sterile distilled water for 5 times before transferring into Petri dish having double layer of filter paper.

Seed Treatment and Germination

The sterilized seeds were treated with 50 ml of each mixture at room temperature for 12 hours and then transferred onto the two sheets of sterilized filter paper inside the petridishes. Eight seeds were put into each dish. The dishes were arranged in a simple randomized design and in three replicates. The treatments of PGPR were applied as follows (1) Sterilize broth treated (control), (2) Isolate 3, (3) Isolate 9, (4) Isolate 19, (5) Consortia of isolate 3+9, (6) Consortia of isolate 3+19, (7) Consortia of isolate 9+19, (8) Consortia of isolate 3+9+19. After treatment, the dishes were sealed with paraffin tape, and placed in the dark in an incubator at 28 ± 3^oC. The numbers of seeds germinated were counted every day. At the end of the 7^{th} day, the potential of seed germination was assessed in terms of percent seed germination.

Pot Trial Experiment for Tomato Seedling

Tomato (*L. esculentum* Mill.) was used as plant model in this experiment. Tomato seeds were soaked in 2% sodium hypochlorite for 3 minutes and the seeds were washed with sterile water about 5 times. Sterilized seeds were incubated with 50 ml of each mixture at room temperature for 24 hours. After 24 hours incubation, the soaked 5 seeds (in the depth of 2 cm) were sowed in a plastic cup filling with equal amount of soil and four replicates were set for each treatment. To supply the IAA requirement for plant, 100 ml of each treatment was added to four cups in equal amount after five day planting. At 30^{th} day, the tomato plants were harvested separately according to the treatment. The effects of different treatments were observed and plant indexes were recorded such as plant height (cm), root length (cm), shoot length (cm), fresh weight of root and shoot (gm), total dry mass of the plant. The planting pots were 1m apart from each other. The treatment consisted of

C. Garden soil (without biofertilizer) (Control)

1. Garden soil + Biofertilizer (P3)
2. Garden soil + Biofertilizer (P9)
3. Garden soil + Biofertilizer (P19),
4. Garden soil + Biofertilizer (P3+P9).
5. Garden soil + Biofertilizer (P3+P19),
6. Garden soil + Biofertilizer (P9+P19), and
7. Garden soil + Biofertilizer (P3+P9+P19)

Results

Morphological and Biochemical Characterization of Rhizobacteria

Total 21 rhizobacteria were isolated from paddy rhizospheric soils of tarai region and were identified by morphological and biochemical characteristics. Among them, 3 isolates (P 3, P9, P19) were recognized as *Pseudomonas sp*. (Table 1).

Table 1. Biochemical characterization of selected IAA producing PGPR isolates

Biochemical Test	P3isolate	P9 isolate	P19 isolate
Gram Reaction	-	-	-
Catalase	+	+	+
Starch Hydrolysis	-	-	-
Gelatin Hydrolysis	+	+	+
Casein Hydrolysis	-	-	-
Methyl Red test	+	-	-
Voges-Proskauer	+	-	-

where '+' indicates positive, '-' indicates negative.

Production of IAA, Siderophore and Solubilization of Phosphorus

The plant growth promoting properties of the test bacterial isolates are shown in Table 2. Selected all three isolates of *Pseudomonas sp*. tested for their IAA production showed a significant amount of IAA production in L-tryptophan supplemented medium. It has been reported that IAA production by PGPR can vary among different species and it is also influenced by culture condition, growth stage and substrate ability[32, 33]. Maximum IAA production was obtained in isolate 19.

As shown in Table 2, isolates P3, P9 and P19 induced the IAA production and siderophore production. Orange halos around the colonies indicated siderophore production. (Fig. 2).

Isolate P3, P9 were found negative for phosphate solubilisation while isolate P19 was positive. It has been reported that IAA production by PGPR can vary among different species and it is also influenced by culture condition, growth stage and substrate ability[32, 33]. PGPR have been shown to solubilize precipitated phosphates and enhance phosphate availability to *Lycopersicum esculentum* that represent a possible mechanism of plant growth promotion under field condition[33, 34]. In comparison to non-rhizospheric soil, higher concentration of phosphate solubilising bacteria is commonly found in the rhizosphere[33]. Suresh[35] indicated that most of the isolates tested in their study possessed plant growth promoting traits and that these isolates can be used as potential biofertilizers and also as biocontrol agents.

Table 2: Plant Growth Promoting properties of the test bacterial isolates

Phytohormones	P 3 isolate	P 9 isolate	P19 isolate
Indole Acetic Acid	15.25 µg/ml	28.3 µg/ml	50.25 µg/ml
Siderophore production	+	+	+
Phosphate solubilisation	-	-	+

Note: '+' indicates positive, '-' indicates negative

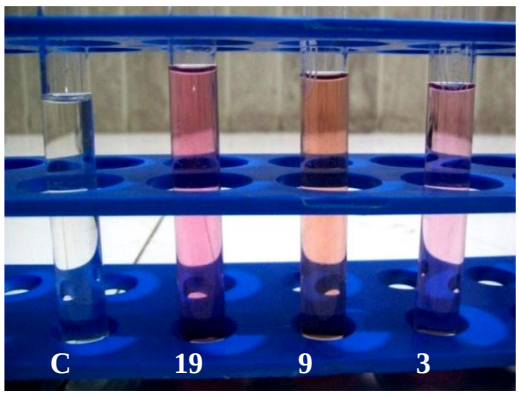

Fig.1. IAA production by test bacterial isolates

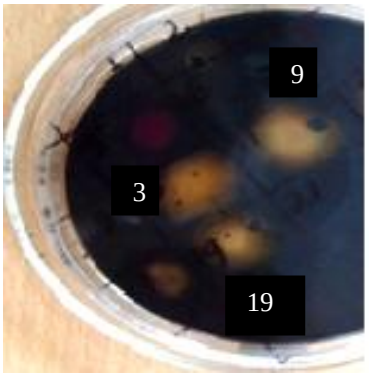

Fig 2.Siderophore production by isolates

Screening of Nitrogen Fixing Activity of the isolated bacteria

The selected three IAA producing isolates were also screened for nitrogen fixing activity on G-NFM medium with BTB as indicator to study the release of ammonium in the culture as shown in Fig. 3. At glucose 0.5%, all three isolates were observed to fix nitrogen by changing the dark green color to blue green color distinctly.

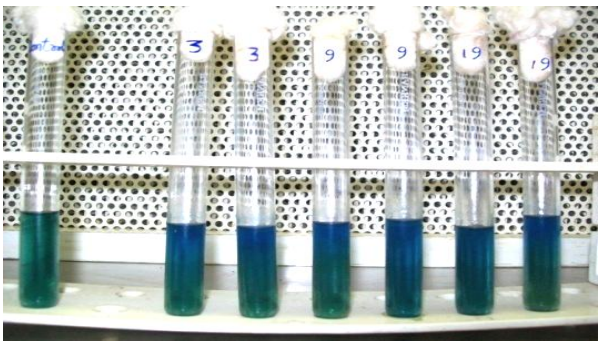

Fig 3. Screening of nitrogen fixing activity

Germination rate of tomato seeds by the IAA producer strains

The germination was influenced by different treatments. Result showed that the maximum number of seedling emergence was in 6th and 7th treatment, which contains isolate P9+P19 and consortia of all three isolated strains (P3+P9+P19) respectively, in comparative to other treatments.

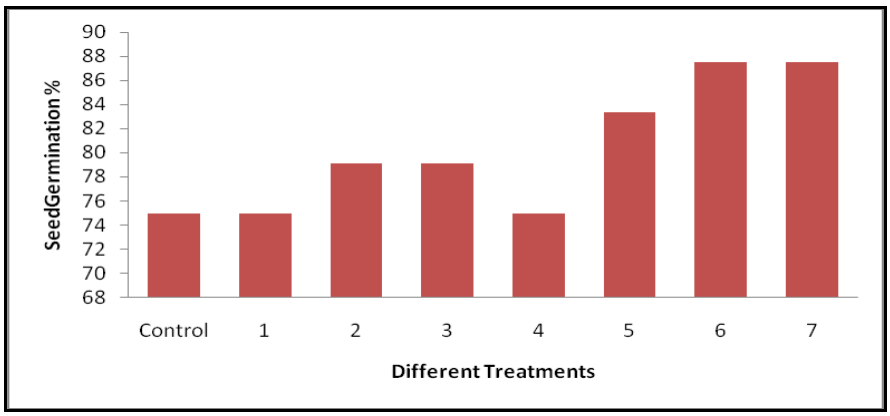

Fig 4. Effect of different treatments on seed germination (%).

Effect of the IAA producer strains on tomato seedling

PGPR isolates treated and non-treated seedlings showed significant difference in the germination rate. Results of various growth parameters of treated and non-treated seeds of tomato are listed in table 3. Significant

increase in the seedling growth of the plants when PGPB (Plant growth promoting bacteria) treated plants were compared with non-treated control is shown in figure 5. There was increase by 63% in overall fresh biomass of tomato seedling. Also notable increase in the range of 12% of plant dry mass after 30 days was observed. Moreover, tomato seedling showed increase by 8% and 32% in root length, shoot length, respectively. The overall fresh shoot biomass and root biomass after 30 days of sowing were 62% and 1%. Comparing overall growth parameters namely shoot length, root length, fresh and dry biomass of plant showed significant difference in the growth of PGPB treated and non-treated seedlings and this was true for the test plants under study. All the pot experiments replicated four times.

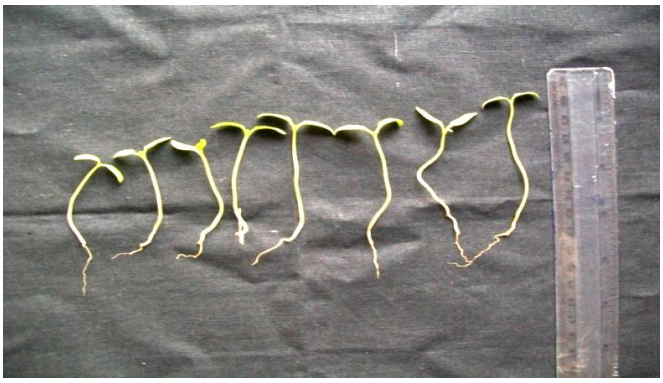

Fig 5. Effect of different treatments on tomato seedling growth

Table 3. Effect of plant growth promoting rhizobacterial strains on different growth parameters in tomato pot trials.

S No	Treatments	Shoot length cm)	Root length (cm)	Shoot fresh weight (g)	Root fresh weight (g)	Fresh weight of plants (g)	Dry weight of plants (g)
C	Control	5.4	2.2	0.042	0.004	0.046	0.006
1	(P3)	6.0	1.4	0.046	0.003	0.049	0.006
2	(P9)	6.1	1.8	0.053	0.004	0.057	0.007
3	(P19)	6.3	1.9	0.065	0.004	0.069	0.007
4	(P3+P9)	6.7	2.4	0.067	0.004	0.071	0.007
5	(P3+P19)	6.8	2.0	0.063	0.004	0.067	0.009
6	(P9+P19)	7.0	2.5	0.077	0.004	0.081	0.009
7	(P3+P9+P19)	8.6	3.0	0.104	0.005	0.109	0.018

Discussion

In this study, out of 21 isolates, 3 isolates were tested for their ability to produce IAA. The isolate P19 produces maximum amount of IAA. In selected three isolates, P19 produces maximum IAA (50.25 µg/ml) at 1 mg/ml of tryptophan concentration. The IAA producing strains also had the nitrogen fixing activity with 0.5% glucose in BTB containing G-NFM medium. This study revealed that the tomato plants that were grown with combination of the three microbial inoculants (at 7th treatment) had greater value in all the growth parameters monitored such as shoot length, root length and the root and shoot fresh weight of the plant and dry weight of plants than the plants that was treated with one microbial inoculant (Treatments 1,2,3) and also the control which was not treated with any biofertilizer had the lowest value. These results were similar with the findings of Dobbelaere[36] who assessed the inoculation effect of PGPR *Azospirillum brasilense* on growth of spring wheat. They observed that inoculated plants resulted in better germination, early development and flowering. Dobbelaere[36]and Cakmakı[37] have been reported that PGPR can increase yield and leaf area index, shoot and root weight and delay leaf senescence. Ordookhani[2] reported that in all their treatments, shoot and fruit potassium increased when PGPR and Arbuscular Mycorrhiza Fungi (AMF) were used together. He also found that the application of Pseudomonas + Azotobacter + Azosprillum + AMF treatment had the most effect on lycopene, antioxidant activity and potassium contents on tomato. Significant increases in growth and yield of agronomical important crops in response to inoculation with PGPR have been reported by Biswas[38] and Asghar[39]. Fluorescent Pseudomonads are nonpathogenic rhizobacteria[40] and several isolates of *P. fluorescens, Pseudomonas putida, P. aeruginosa,* and *P. aureofaciens* suppressed the soilborne pathogens through different

proposed mechanisms including rhizosphere colonization, antibiosis, and iron chelation by siderophore production[40].

Trials with Plant growth-promoting rhizobacteria indicated that yield and dry matter accumulation increase in wheat[41,42], maize[6, 43, 44], sugarcane[45], rice[46], and barley[47, 48]. Mishra[33] reported that most of isolates used in their study resulted in a significant increase of shoot length, root length and dry matter production of shoot and root of *Cicer arietinum* seedlings. Application of PGPR isolates significantly improves the percentage of seed germination under saline conditions[33]. The results of the study by Sharifi[44] showed that seed priming with Plant Growth Promoting Rhizobacteria affected grain yield, plant height, number of kernel per year, number of grains per ear row significantly. Maximum of these characteristics were obtained by the plots which seeds were inoculated with *Pseudomonas* bacteria.

From this study, it has been shown that the combined use of the three bacterial inoculants had the highest value of the growth parameters monitored as comparative to control. Biofertilizer has been widely used with excellent result for the growth of different kinds of plant in several countries. Most of the isolates significantly showed increase in plant length, root length and internode length root of *Lycopersicum esculentum*. Our results suggested that PGPR are able to enhance the production of IAA, solubilization of phosphorus, and siderophore production, thereby improving growth of *Lycopersicum esculentum* plant. The use of PGPR as inoculants biofertilizer could be an efficient approach to replace chemical fertilizers and pesticides for sustainable *Lycopersicum esculentum* cultivation in Tarai region of Uttarakhand in India.

Acknowledgement

Author is thankful to Department of Biotechnology, Graphic Era University, Dehradun, (U.K.) and R&D Unit of Sri Ram Solvent Ext. Pvt. Ltd. Jaspur- 244712, Kashipur Road, Uttarakhand, India for providing necessary facilities.

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