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Indole-3Acetic Acid Production by Plant Growth Promoting Rhizobacteria of Saline Soil, Odisha and its Role in Root Elongation in Rice Plant

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Abstract **Original Research Article**

In the present study five PGPR were isolated from Casuarina rhizosphere, coastal Odisha, India and were identified as *B. acidiceler*, *Sphingomonas paucimobilis*, *Kocuria kristinae*, *B. subtilis* and *B. megaterium* by 16SrDNA sequencing. The production of IAA by five PGPR ranges from 1.34 µg/ml to 2.509 µg/ml in absence of tryptophan whereas 3.103 µg/ml to 7.929 µg/ml in presence of tryptophan by using Salkowski's reagent. The optimum activity of five PGPR was recorded at 2 mg/ml of tryptophan concentration. The optimum temperature and pH for IAA production by five PGPR were recorded at 30°C and 7.0. Partial purification of tryptophan was also done by TLC with R_f value 0.9. It is also found that the five PGPR have potential in the elongation of root length of rice plant grown in saline soil.

Key words: IAA, PGPR, rhizosphere, Salkowski's reagent, TLC and tryptophan.

1. INTRODUCTION

Plant growth and development involve the combination of environmental factors and its indigenous effects like, various growth regulators such as auxin, cytokinin, gibberellins, abscisic acid, ethylene, brassinosteroids and jasmonic acid, which are collectively called as plant hormones. Indole-3-acetic acid is one of the most physiologically active naturally occurring principal auxin in plants (Simon and Petrassek, 2011), which functions in cell enlargement, cell division, tissue differentiation, response to light and gravity (Teal *et al.*, 2006). Microbial production of IAA may directly enhance plant growth (Chung *et al*., 2005) by modifying certain conditions like, increasing osmotic contents of the cell, enhancing water permeability into cell, decreasing turgor pressure, stimulating cell wall and protein synthesis. It promotes embial activity (***embial*** *is a collective name for a group of closely related lipids that contain substitutions on the 2H-1-benzopyran-6-ol nucleus and a long hydrocarbon chain of isoprenoid units. They are antioxidants by virtue of the phenolic hydrogen. Tocopherols react with the most reactive form of oxygen and protect unsaturated fatty acids from oxidation*), inhibit or delay abscission of leaves, induce flowering and fruiting. (Zhao, 2010) L-tryptophan serves as a primary precursor of IAA for PGPR as well as for plant (Lynch, 1985; Ahmad, 2005 and Patil *et al.*, 2011). However it has also proved that IAA biosynthesis can occur via tryptophan independent pathway (Venis and Napier, 1991 and Normanly, 1997). More than one pathway for the production of IAA can be present in bacterium (Pattern and Glick, 1996).

Bacteria that colonize the rhizosphere and enhance plant growth by any mechanism are referred to as Plant growth promoting rhizobacteria (PGPR) (Mohite, 2013), which enhance the plant growth directly or indirectly (Kloepper, 1980). In the present scenario of world, soil salinity is accounted as the most important environmental limiting factor for crop production (Jain *et al*., 1989). Therefore, in this study rhizobacteria were isolated from the Casuarina rhizosphere of coastal saline soil of Odisha and screened for indigenous IAA production ability and their effect on the root elongation of rice plant under saline stress. Temperature, pH and L-tryptophan concentration were optimized for the maximum production of IAA and the crude IAA extract was also purified by TLC. The effects of isolated rhizobacteria on the root elongation of *Oryza sativa* var. Padmini were also studied.

2. MATERIALS METHOD

2.1 Isolation of rhizobacteria

Rhizobacteria were isolated from the saline soil collected from the rhizosphere zone of Casuarina plants of three coastal districts of Odisha, India, which were away from any human interference and inhabited with Casuarina plants. The sample collection regions were

Balipada (19.8111°N, 85.8201°E) and *Penthakata* (19.7905°N, 85.8517°E) of *Puri*, *Balaramgadi* (21.4701°N, 87.0228°E) and *Chandipur* (21.4345°N, 87.0126°E) of *Baleswar* and Haripur (19.1501°N, 84.7654°E) and *Mantridi* (19.3156°N, 84.7811°E) of *Ganjam*.

Stock solution was prepared by suspending 1 g of soil sample in 9ml of sterile distilled water and incubated on rotary shaker at 120 rpm for 10 min. The samples were serially diluted (Mohite, 2013) and 0.1 ml of diluted sample from each dilution was spreaded on sterile nutrient agar plates (0.5% peptone, 0.3% beef extract and 1.8% agar) in triplicate.

2.2 Molecular identification of rhizobacteria

The 16S rDNA of five rhizobacteria was amplified by using universal 27F forward primer (5′-AGGCCTAACACATGCAAGTC-3′) and 1492R reverse primer (5′-GGGCGGWGTGTACAAGGGC-3′) (Das *et al*., 2014). The amplified PCR product was purified by HiPura ™ PCR product purification kit (HIMEDIA) and nucleotide sequences were determined using Big dye terminator v 3.1 cycle sequencing kit using an automated 3500 genetic analyzer system (Applied Biosystems, Hitachi, USA) and submitted to genbank, NCBI.

2.3 Quantitative estimation of in vitro IAA production by rhizobacteria

Single colony from each of five isolated rhizobacteria was inoculated into nutrient broth medium and incubated at 28°C for 24h in triplicate. 1ml of each overnight grown culture was inoculated into 10ml of nutrient broth having 2mg/ml L-tryptophan and incubated in rotary shaker at 100rpm and 28°C in triplicate. After 7 days of incubation each culture was centrifuged at 10,000rpm for 30 mins. 1 ml of culture supernatant was mixed properly with 2ml of freshly prepared Salkowski's reagent (50 ml 35% $HClO_4$ + 1 ml $FeCl_3$). Absorbance was measured at 530nm by UV-Visible spectrophotometer for the pink colored complex after 30 mins of incubation in dark (Bent *et al*., 2001). Standard graph of IAA was plotted by taking known concentrations of pure IAA (0, 5, 10, 15, 20, 25, 30, 35 and 40µg/ml) (Yasmin *et al*., 2007) to determine the concentration of IAA.

2.4 Extraction and Partial purification of crude IAA by thin layer chromatography (TLC)

The five isolated rhizobacteria were inoculated in 100ml nutrient broth medium supplemented with 2mg/ml and incubated for 1 week at 28°C in shaking incubator in triplicate. After incubation the bacterial cells were separated by centrifugation at 10,000rpm for 30 mins. The supernatant collected in triplicates was acidified to pH 2.5-3.0 using 1N HCl and extracted twice with two volume of ethyl acetate. Extracted ethyl acetate fraction was allowed to dry through evaporation (Ahmed *et al*., 2005). The crude extract was dissolved in 300ml methanol and kept in refrigerator. The crude extract of IAA was purified by TLC. By the help of capillary tube the sample was spotted on TLC plate along with standard IAA (10mg/100ml). The solvent system used for TLC was propanol and water (8:2). The spots with R_f value were identified by spraying Salkowski's reagent to the airdried TLC plate (Kuang-Ren *et al*., 2003).

2.5 Optimization of incubation period for IAA production

1ml of each overnight grown culture was inoculated into 100ml of nutrient broth having 2mg/ml L-tryptophan and incubated in rotary shaker at 100rpm and 28°C in triplicate. At every 24 h of intervals, 5ml of each culture was analyzed for IAA production (Bent *et al*., 2001) in triplicate.

2.6 Optimization of tryptophan concentration for IAA production

For the production of maximum quantity of IAA the five isolated rhizobacteria were grown in nutrient broth medium having tryptophan concentration (0, 2, 4 and 6 mg/ml) in triplicate (Senthil *et al*., 2010) keeping other parameters constant.

2.7 Effect of hydrogen ion concentration and temperature for IAA production

The five isolated rhizobacteria were inoculated in broth medium having pH in a range of 5-9 while other parameters remained same. The pH was maintained with the help of phosphate buffer (Mohite, 2013). The culture media was incubated in temperature ranging from 25°C to 40°C. All the experiments are done in triplicate to optimize the pH of the medium and the incubation temperature for the growth of the five isolated rhizobacteria.

2.8 Effect of rhizobacteria on root elongation of rice plant

Surface sterilized seeds of *Oryza sativa* var. Padmini were inoculated with five rhizobacteria separately and allowed to germinate on Petri plates with un-inoculated control in triplicates. The germinated seeds were sowed in pots having autoclaved saline soil and un-inoculated germinated seeds also sowed in same way as control (Saber *et al*., 2012). The root length of the rice plants were measured after 120 days of sowing.

2.9 STATISTICAL ANALYSIS

The experimental data were statistically processed using DMRT. All results are expressed as mean ± SEM at α=0.05 (Mihalache *et al*., 2017).

3. RESULTS AND DISCUSSION

The five isolated rhizobacteria were identified by 16S rDNA sequencing as *B. acidiceler*, *Sphingomonas paucimobilis*, *Kocuria kristinae*, *B. subtilis* and *B. megaterium*. The accession numbers from Genbank, NCBI submission were given in table 1.

Table-1: Accession No. of identified strains by Gen bank, NCBI

Rhizobacteria	Identification	Accession No.
P1	*Bacillus acidiceler*	MK214384
P2	*Sphingomonas paucimobilis*	MK091525
P6	*Kocuria kristinae*	MK201658
B1	*Bacillus subtilis*	MK091526
B2	*Bacillus megaterium*	MK105917

P1= B. acidiceler, P2= Spingomonas paucimobilis, P6= Kocuria kristinae, B1= B. subtilis and B2= B. megaterium.

The wild cultures of above five rhizobacteria were analyzed for IAA production quantitatively by Spectrophotometr for the developed pink colour by Salkowski's reagent after 30 mins of incubation in the dark (**figure 1A**) and the results were represented in table 2. After 48h of incubation, maximum quantity of IAA production ranging from 5.11-7.93µg/ml was observed by the five rhizobacteria in the NB medium supplemented with 2mg/ml tryptophan (**figure 2A**). The highest amount of IAA was produced by *B. megaterium* in compare to other four rhizobacteria (**table 2**). Mohite, (2013) reported the production of IAA by *B. megaterium, Lactobacillus casei, B. subtilis, B.cereus* and *Lactobacillus acidophilus* from rhizospheric soil. Verma *et al*., (2015) reported IAA production by *Kocuria kristinae* from the sample collected from Nothern hill zones of India. In this investigation, for the first time the IAA production potentialities of *B. acidiceler* were found.

Table-2: Incubation period optimization for IAA production

Incubation period (h)	Concentration of IAA (µg/ml)*				
	B. acidiceler	***Spingomonas paucimobilis***	***Kocuria kristinae***	***B. subtilis***	***B.megaterium***
24	4.61 ± 0.012^{b}	4.74 ± 0.01^{b}	3.98 ± 0.003^{b}	3.92 ± 0.013^{b}	5.82 ± 0.046^{b}
48	5.46 ± 0.018^{a}	5.19 ± 0.007^{a}	5.11 ± 0.009^{a}	5.55 ± 0.009^{a}	7.93 ± 0.007^{a}
72	4.47 ± 0.013^{c}	4.63 ± 0.009^{c}	3.93 ± 0.013^{b}	3.91 ± 0.012^{b}	4.44 ± 0.001^{c}
96	3.84 ± 0.012^{d}	3.94 ± 0.015^{d}	2.99 ± 0.044^{c}	3.23 ± 0.022^{c}	3.11 ± 0.112^{d}

***=**Mean of triplicates ± standard error, α=0.05,
Small alphabets in letter indicate significant difference.
In a column, same alphabets don't have any significant difference.

Fig-1A: Estimation of IAA by Salkowski's reagent

C= control and B2= *B. megaterium*

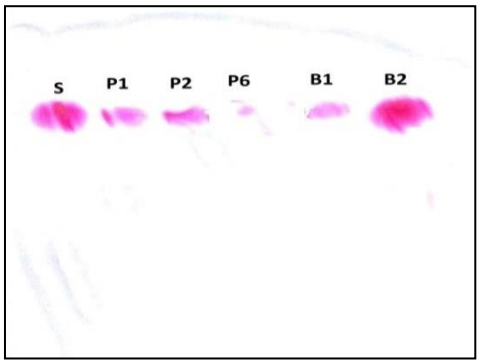

Fig-1B: Purification of IAA by TLC

C= control, S= Standard, P1= B. acidiceler, P2= Spingomonas paucimobilis, P6= Kocuria kristinae, B1= B. subtilis and B2= B. megaterium.

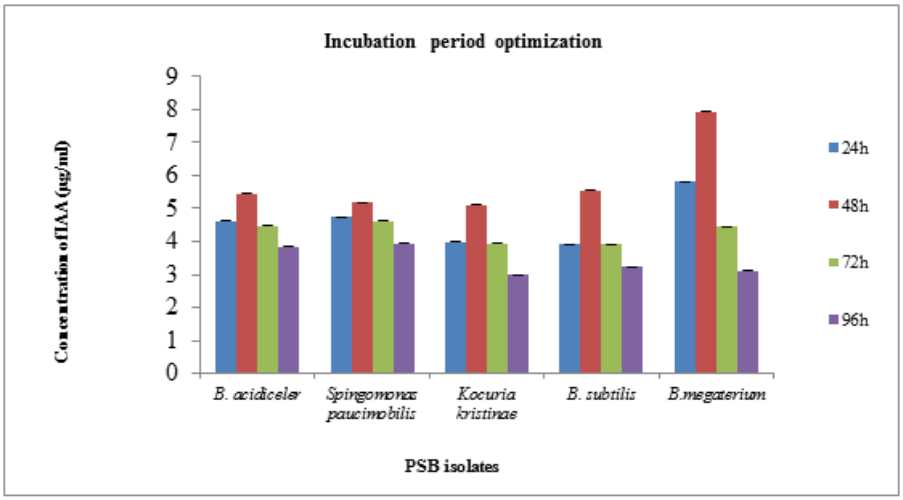

Fig-2A: optimization of incubation period for IAA production by five rhizobacteria

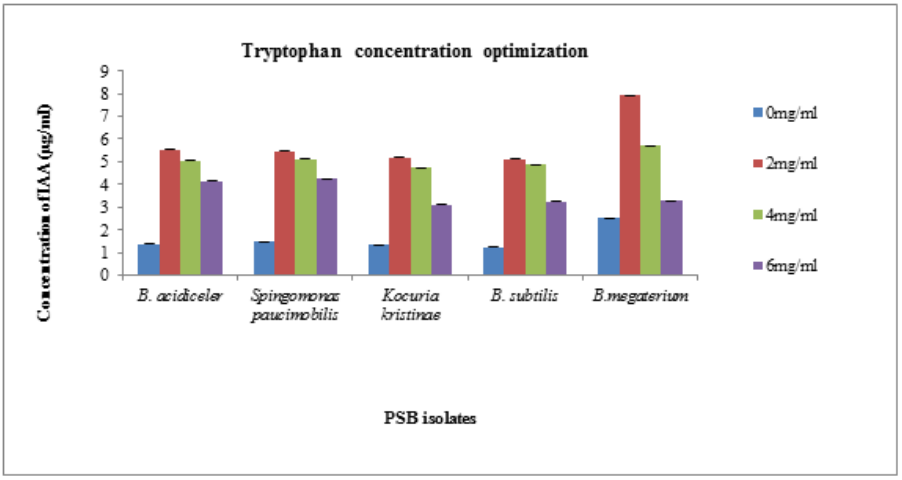

Fig-2B: optimization of tryptophan concentration for IAA production by five rhizobacteria

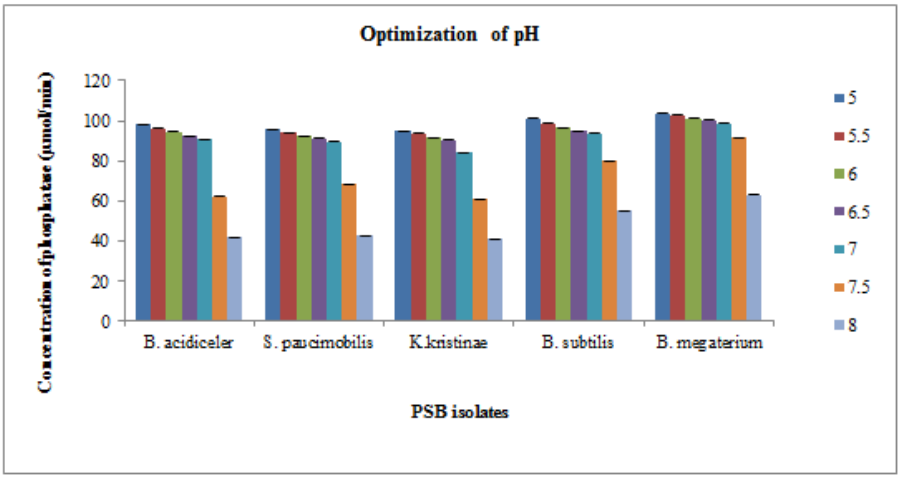

Fig-2C: optimization of pH for IAA production by five rhizobacteria

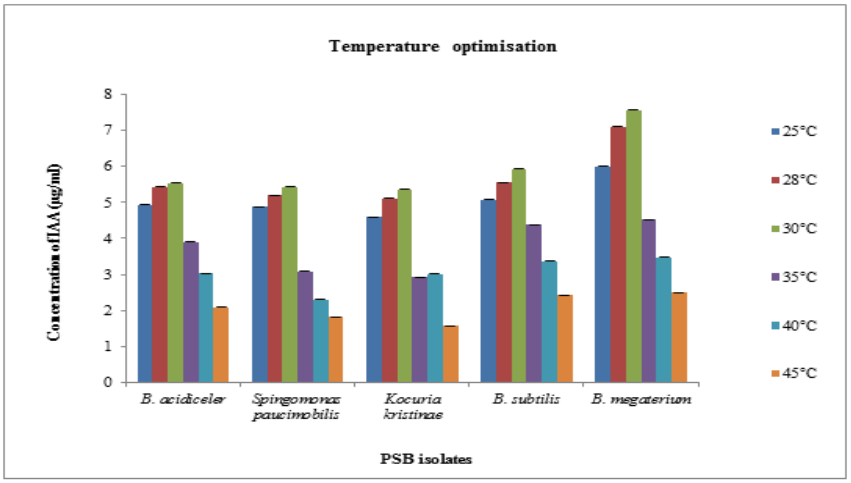

Fig-2D: optimization of temperature for IAA production by rhizobacteria

After 48h of incubation, the amount of IAA production was decreased gradually in production medium (**figure 2A**), which was in agreement with Hunter, (1989) and Harikrishnan *et al*., (2014) who observed the reduction in IAA production after its maximum value. It might be due to the release of IAA degrading enzymes such as IAA oxidases, peroxidases by the bacteria or by transformation to IAA derivatives Chin *et al*., (2012).

In this study, it was also found that the quantity of IAA production by five rhizobacteria was less (ranging from 1.34 to 2.509) in absence of tryptophan as compare to presence of tryptophan in the culture medium (**figure 2B**), which proves that for the five PSB isolates tryptophan is the precursor for IAA production. The similar report was also given by Pant and Agrawal, (2014) for *Bacillus* sp. Mohite, (2013) reported optimum growth at 0.1% tryptophan for *B. megaterium*, *Lactobacillus casei* and *B. subtilis* at 0.05% *B. cereus* and at 1.5% *Lactobacillus acidophilus*.

On the basis of IAA production level, culture filtrates of five rhizobacteria were used to extract IAA for characterization by TLC. TLC chromatogram of of ethyl acetate bacterial extract showed pink colour spot at the R_f corresponding to the standard authentic IAA (figure 1B). The R_f value was found to be 0.85, where the distance travelled by solute and solvent was 4.1cm and 4.8cm respectively, which confirmed the IAA producing potential of the above five rhizobacteria. Mohite, (2013) supported the above report where R_f corresponding to the standard IAA was 0.57. Abd-Alla *et al*., (2013) also confirmed IAA by TLC. Pant and Agrawal, (2014) also confirmed IAA by TLC where R_f was 0.9 corresponding against the standard IAA.

For all the above five rhizobacteria, incubation period was optimized at 48h (figure 2A) and tryptophan concentration in the NB medium at 2mg/ml (figure 2B). The pH and temperature were optimized at 7.0 and 30^{o}C respectively (figure 2C and 2D).

Mohite, (2013) reported optimum growth at pH 8.0 for the isolates *B. megaterium*, *Lactobacillus casei* and *B. subtilis* while pH 7.0 for *Lactobacillus acidophilus* and pH 9.0 for *B. cereus*. The effect of incubation temperature on the above five rhizobacteria was optimized at 30^{o}C (figure 2D). Khamna *et al*., (2010); Abd-Alla *et al*., (2013) and Harikrishnan *et al*., (2014) have reported optimum temperature is 30°C for production of IAA by *Streptomyces* sp.

The above five IAA producing rhizobaceria were applied to rice seeds and grown in saline soil in green house experiment along with un-inoculated control. After 120 days of sowing, the root length of five rhizobacteria treated rice plants was found to be longer than the control plant (**table 3**), which was due to the IAA production by the rhizobacteria.

Table-3: Effect of five rhizobacteria on root elongation of rice plant

Treatment	Root length (cm)*
Control	11.43±0.067^d
Bacillus acidiceler	18.87±0.088^a
Spingomonas paucimobilis	16.50±0.0^b
Kocuria kristinae	15.90±0.058^c
Bacillus subtilis	18.87±0.033^a
Bacillus megaterium	18.93±0.033^a

*****=Mean of triplicates ± standard error, α=0.05,
Small alphabets in letter indicate significant difference.
In a column, same alphabets don't have any significant difference.

This was supported by Egamberdieva and Kucharova, (2009), who reported the production of IAA, gibberellins and many unknown determinants by PGPR enhanced root length, root surface area and number of root tips. Chen *et al*., (2007) also reported the accumulation of osmolytes and phytohormone signaling help the plants to overcome osmotic shock due to salinity stress. The maximum root length was found in rice plants inoculated with *B. megaterium* 18.933cm. The findings of Lopez-Bucio *et al*., (2007) showed dramatic changes in root-system architecture that include increased growth of lateral roots and increased root-hair elongation in *Arabidopsis thaliana* inoculated with *B. megaterium*. Saleemi *et al*., (2017) reported increased root length 27.8cm in wheat plant coinoculated with PGPR and PSB.

4. CONCLUSION

The research of the present investigation concluded that the five isolated rhizobacteria have great potential to enhance soil fertility and plant growth promotion through the production of IAA. As they have efficient role in elongation of root length in rice plant, they can be called as PGPR. However, this assessment of plant growth needs further study in field conditions.

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Abbreviation

µg/ml- microgram/ml
h- hour
IAA- Indole acetic acid
mg/ml- microgram/ml
min- minute
NB- nutrient broth
^{o}C- degree Celsius
PGPR- Plant Growth Promoting Rhizobacteria
R_f- Retention factor
rpm- revolution per minute
TLC- Thin layer chromatography
Var- variety

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