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Research Article

ISOLATION AND SCREENING OF PHYTOHORMONES PRODUCING PGPR FROM COTTON PLANT RHIZOSPHERIC SOIL

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ABSTRACT

Plants are subjected to various abiotic stresses, such as drought, extreme temperature, salinity, and heavy metals. Plants employ several tolerance mechanisms and pathways to avert the effects of stresses that are triggered whenever alterations in metabolism are encountered. Phytohormones are among the most important growth regulators; they are known for having a prominent impact on plant metabolism. PGPR, root - associated rhizobacteria that produce phytohormones may prove to be important for plant growth. In our study, cotton (*Gossypium hisrsutum*) samples were collected from four different sampling sites rhizospheric soil (rhizosphere, rhizoplane, endorhizosphere) from agriculture farm, cotton research centre, Surat, Gujarat. Out of 81 rhizobacteria from wild and transgenic cotton rhizospheric soil, we isolated 16 IAA, 11 gibberellins and 8 ACC deaminase producing PGPR. *Sphingomonas pseudosanguinis strain G1-2, Bacillus circulans strain NBRC 13626, Stenotrophomonas rhizophila strain e-p10*with ACC deaminase is balancing ethylene level in stress condition. Other hormones were used in every stages of plant growth. *Pseudomonas species*, produced highest IAA (0.059 mg/ml) and gibberellic acid (0.53mg/ml). They help in increasing the shoot and root length. So, rhizobacteria play a vital role in the stimulation of plant growth and defense response mechanisms.

Keywords: PGPR, Rhizosphere, Phytohormones.

1. INTRODUCTION

Cotton is the most important plant fibre to make textile products. Cotton, kharif crop is cultivated by many countries around the globe. India has a potential market for cotton textile industries and also has a successful export business in various countries having similar weather such as Pakistan, Bangladesh, China etc.[1].

The live microorganisms which grow in, on, or around plant tissues, utilized for improving plant growth and crop productivity are generally referred to as biofertilizers or microbial inoculants, known as plant growth promoting rhizobacteria. They promote plant growth, increase soil fertility and control plant diseases. Microorganisms can directly influence plant growth by synthesizing growth-stimulating hormones [2-4] and metabolizing growth-inhibitory hormones [5, 6]. Researchers have reported the beneficial use of auxins, cytokinins, gibberellins, ethylene and absicisic acids (ABA) in plants, which helped in boosting their growth and increasing the plant yield [7-10].

2. MATERIAL AND METHODS

2.1. Sample collection

Samples for PGPR study were collected from Agriculture Farm, Cotton Research Centre, Surat, Gujarat. Soil consisting mainly of two nonrhizospheric zone (Bulk soil) along with three different sites from rhizospheric zone (rhizosphere, rhizoplane and endorhizosphere) of *Gossypium hisrsutum* (cotton) was selected for study.

2.2. Isolation of PGPR

Bacterial species were isolated from the nonrhizospheric soil and rhizospheric soil samples using various appropriate selective media such as Pikovskaya agar, Nitrogen free media, Ashby's Mannitol agar, King's media, Bacillus media, Yeast Extract Mannitol Agar. Plates were incubated at room temperature until visible growth was observed. All the isolates were coded according to selective media/sample plant/sample site/number. Gram staining and motility test were performed for differentiating test and primary screening.

2.3. *In Vitro* screening of phytohormone producing PGPR

Promotion of plant growth by one of the direct mechanisms by PGPR is by the production of plant growth regulators or phytohormones. The rhizobacteria were screened for three different Phytohormones production.

2.3.1. Indole -3- acetic acid

Fifty (50) ml of nutrient broth containing 0.1% DL-tryptophan was inoculated with 500 µl of 24 hours old bacterial cultures. Broth was incubated in incubator shaker at 30±0.1 °C at 180 rpm for 48 hours in dark. Bacterial cultures were then centrifuged at 10000 rpm for 10 minutes at 4 °C. Quantitative estimation of IAA in the supernatants was done using colorimetric assay [11]. For the assay 1 ml of supernatant was mixed with 4 ml Salkowski reagent and absorbance of the resultant pink color developed was checked after 30 minutes at 535 nm in an UV/Visible spectrophotometer. Appearance of pink color in the test tubes indicated the production of IAA [12].

2.3.2. Gibberellic acid

Gibberellic acid production by bacteria was determined by the method of Borrow [13]. For the assay 1 ml broth of bacteria was added separately in 100 ml sterilized nutrient broth and incubated at 37°C for seven days. After incubation, the bacterial culture was centrifuged at 9000 rpm for 10 minutes in order to remove the bacterial cells. 15 ml of the culture was pipetted out separately into the test tubes and 2 ml of zinc acetate solution was added to it. After 2 minutes, 2 ml of potassium ferrocyanide solution was added and centrifuged at 9000 rpm for 10 minutes.5 ml of supernatant was added to 5 ml of 30% hydrochloric acid and the mixture was incubated at 25 °C for 75 minutes. Blank was prepared with 5% hydrochloric acid and was considered as control. Absorbance was measured at 254 nm using a UV-VIS spectrophotometer.

2.3.3. Ethylene production (ACC deaminase production)

ACC deaminase activity was determined by the method of Glick [14]. For this, 1 µl of each luria broth pure bacterial culture was inoculated into agar plates containing NFb or NFb-ACC modified by addition of 1-aminocyclopropane-1-carboxylate (5.0 g/l) as unique nitrogen source. Plates were incubated at 28 °C and observed daily for colony formation. Colonies were re-inoculated and incubated in the same experimental conditions. Newly formed colonies in NFb with addition of ACC were considered positive for ACC deaminase activity which changes theclour of the media from green to blue.

2.4. Molecular Characterizations and phylogenetic study

Microbial Identification was done with the use of 16S rDNA according to multitraits PGPR on the basis of quantitative analysis. DNA was isolated from the bacterial culture. Its quality was evaluated on 1.0% Agarose Gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rDNA gene was amplified by PCR. A single discrete PCR amplicon band was observed when resolved on Agarose. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with 8F &1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The 16S rDNA sequence was used to carry out BLAST with the database of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software programs and their phylogenetic relationships were determined.

3. RESULTS AND DISCUSSION

The rhizopheric soils of crop plants have more flora and fauna due to availability of more macronutrients and micronutrient [15]. Soil microbes are found to dominate the niche by assisting the continuous delivery of nutrients from plant roots which helps in plant growth promotion under a number of mechanisms [14].

81 bacterial species were isolated from the three different rhizospheric sites: Rhizosphere, Rhizoplane and Endorhizosphere of wild and transgenic cotton from Cotton Research Centre, Surat:

List of rhizobacterial species grown on different selective media is shown in Table 1.

Diversity of rhizobacteria has been observed to be more in the rhizospheric zone (46 isolates) of transgenic cotton plant than that which was observed in the wild cotton plant in similar rhizospheric soil conditions (35 isolates). The number of isolates present in rhizospheric soil may vary due to the root exudation or due to chemotactic action of bacteria. Several scientistshave carried out similar research and reported that majority of PGPR are present in rhizospheric soil because of the availability of

nutrients which include sugars, amino acids, organic acids, and also other small molecules from plant root exudates. These root exudates are responsible for the enhanced microbial diversity [16-19].

Table 1: Total number of isolates on various selective media

Sample site	Selective Media	Transgenic Cotton Plant	Wild Cotton Plant
Rhizosphere	Pikovskaya's medium	3	1
	Nitrogen free medium	2	2
	King's medium	2	1
	Ashby's mannitol agar medium	2	1
	Yeast extract mannitol agar	2	3
	Bacillus medium	2	2
	Total number of isolates	**13**	**10**
Rhizosplane	Pikovskaya's medium	3	2
	Nitrogen free medium	1	3
	King's medium	5	3
	Ashby's mannitol agar medium	2	1
	Yeast extract mannitol agar	3	3
	Bacillus medium	1	1
	Total number of isolates	**15**	**13**
Endorhizosphere	Pikovskaya's medium	2	2
	Nitrogen free medium	3	2
	King's medium	4	1
	Ashby's mannitol agar medium	2	1
	Yeast extract mannitol agar	2	5
	Bacillus medium	5	1
	Total number of isolates	**18**	**12**
		46	**35**
	Total isolates (Transgenic + Wild)	**=**	**81**

Table 2: Phytohormone production by PGPR

	Phytohormone production		
	IAA	Gibberellic acid	Ehtylene
Positive PGPR (Out of 81)	16	11	08
Range of production capability	0.02-0.053 mg/ml	0.023-0.59 mg/ml	Qualitative assay
Highest production	0.053 mg/ml	0.59 mg/ml	

In characterization of PGPR, we found majority of rhizobacteria to be Gram negative, motile short rods. Several scientists have carried out similar studies and reported that majority of PGPR are Gram negative motile bacteria [20, 21].

3.1. Phytohormone production

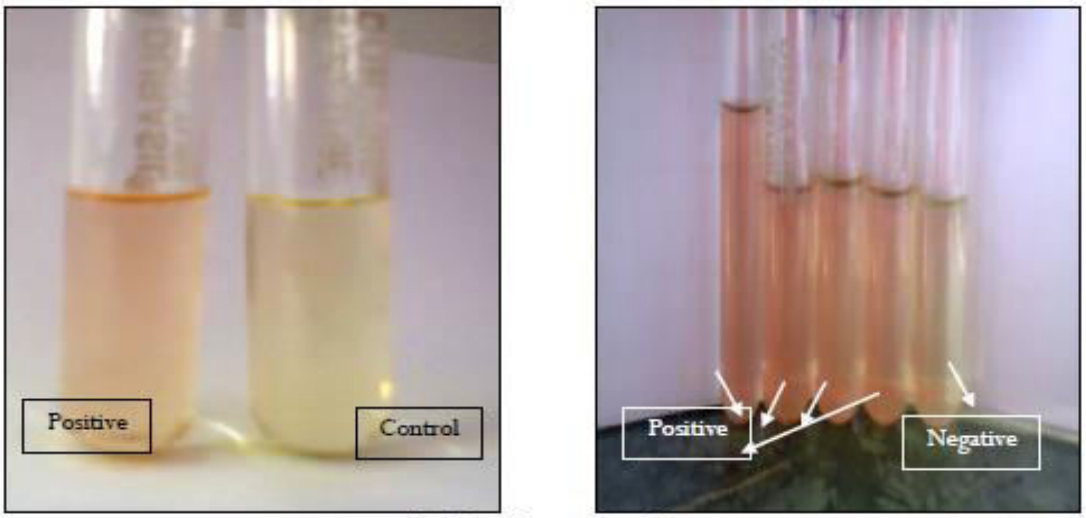
Phytohormones play a key role in regulating plant growth and development. They also function as molecular signals in response to environmental stress/factors that otherwise limit plant growth and/or become lethal when uncontrolled [22]. PGPR excrete hormones for root uptake or manipulate hormone balance in the plants in order to boost growth and stress response.

Many PGPR are capable of producing auxins [6, 23] which are capable of exerting particularly strong effects on root growth [24] and architecture. IAA producing bacteria *Azotobacter chroococcum* improved seed germination, seedling development, increased plant height, boll number and boll weight when applied as a seed treatment [25]. Boll is the round, fluffy clumps form of cotton which grows on a cotton plant. Some strains of PGPR can produce relatively large amounts of gibberellins, leading to enhanced plant shoot growth [24].

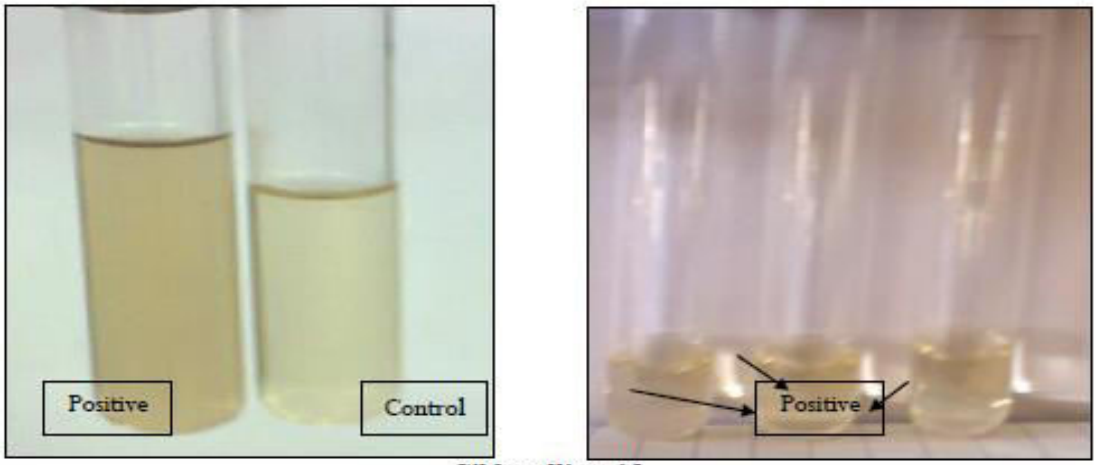
Rhizobacterial isolates produced the highest IAA (0.053 mg/ml), being lower than bacterial isolates from the rhizosphere of potato, which was 5.816 mg/l [26]. Higher percentage (26.67%) of IAA producing rhizo-

bacteria wwa obtained from transgenic cotton rhizoplane.

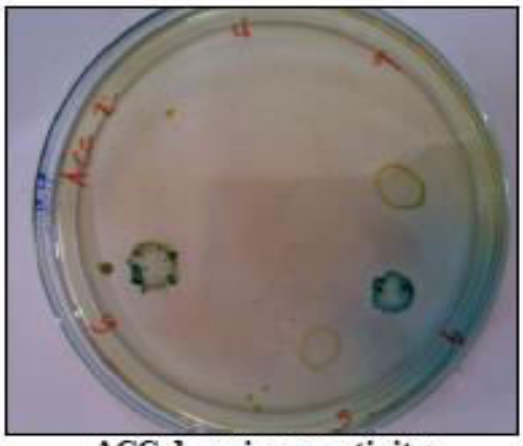
Indole-3-Acetic Acid is a key hormone for many aspects of plant growth that is able to regulate many physiological processes [27, 28]. Indole-3-acetic acid (IAA) can stimulate growth such as cell lengthening, cell division and differentiation [29]. The process of bacterial synthesis in generating IAA is stimulated by the presence of L-tryptophan. In soil microorganisms, IAA biosynthesis can be triggered by the presence of L-tryptophan derived from root exudates.



Indole – 3 - acetic acid



Gibberellic acid



ACC deaminase activity

Fig. 1: Phytohormone production

Rhizobacteria can produce optimal gibberellin when depending on several factors such as isolate or strain species and isolate culture conditions [30]. The culture conditions of the isolates were influenced by pH growth medium, temperature, incubation time, and incubation conditions–moving or still, and dark or bright. Gibberellins are plant growth-promoting hormones, which play a prominent role in seed germination [31], response to abiotic stress [32], increased stem elongation [33], better flowering [34], and other physiological effects that occur in its interaction with other phytohormones [35].

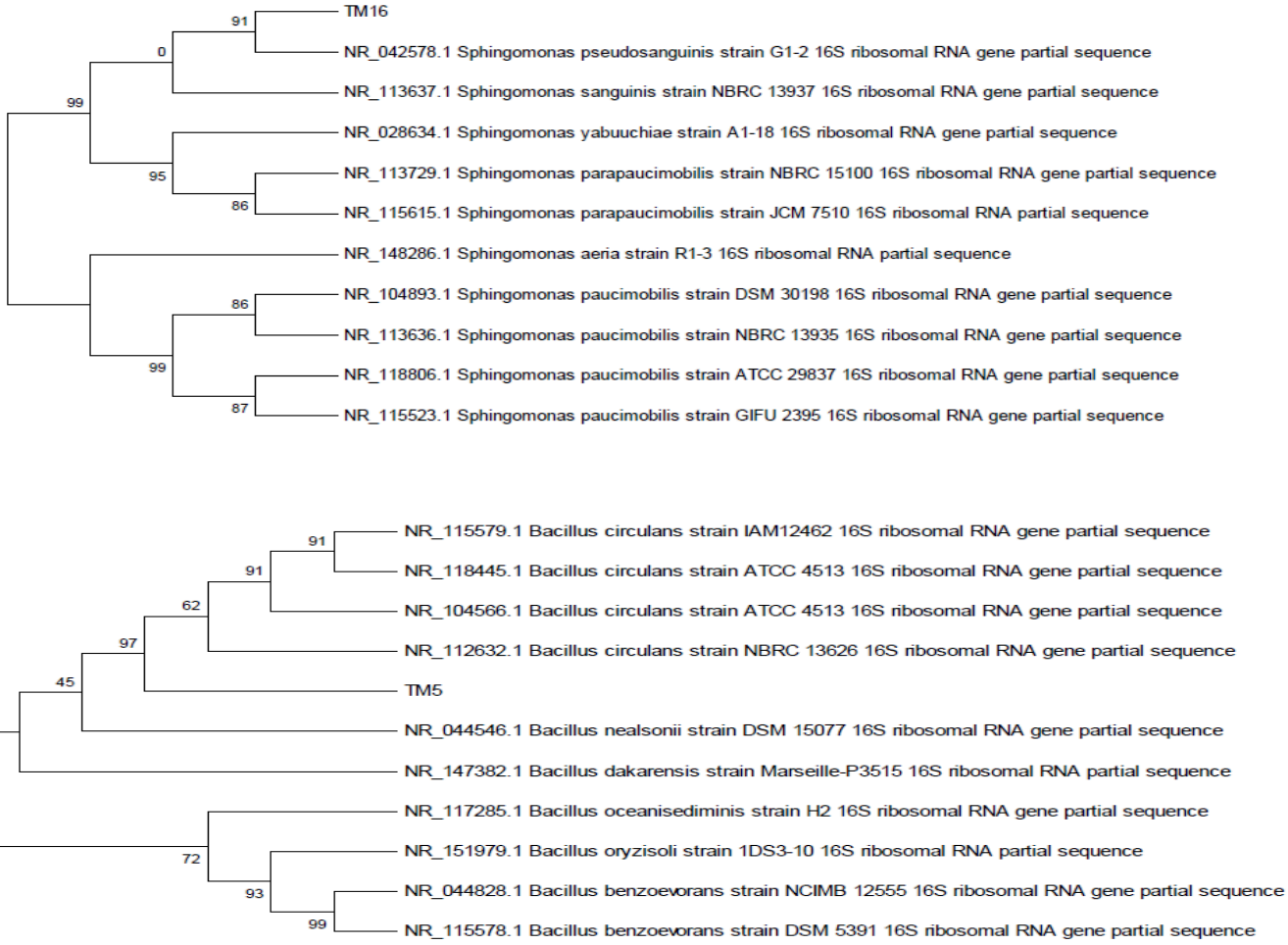
One of the most considered traits of PGPR is production of ACC deaminase to control ethylene level in plants. This trait was studied in detail of *Pseudomonas putida* by Glick [14].

In 2010, Martines- Viveros [36] concluded from study, that decreasing the ethylene level by PGPR namely *Achromobacter, Bacillus, Enterbacter, Pseudomonas* increase the plant resistance during flooding day in Chili, South America. We found eight positive ACC deaminase PGPR in our study. This capability of PGPR is most important to re-established healthy root study in environmental cold stress condition.

3.2. Molecular analysis of PGPR

The highest phytohormone producing PGPR identified by16S rDNA. *Sphingomonas pseudosanguinis strain G1-2, Bacillus circulans strain NBRC 13626, Stenotrophomonas rhizophila strain e-p10* with ACC deaminase is balancing ethylene level. *Pseudomonas species*, produced highest IAA (0.059 mg/ml) and gibberellic acid (0.53mg/ml) were used in every stages of plant growth for shoot and root production.

Characteristic of PGPR, naturally occurring soil bacteria are capable to colonize the root surface, survive, multiply and compete with other microbiota, and also needed to express their plant growth promotion/ protection activities and promote plant growth. They can be exploited commercially as biofertilizers, phytostimulator and biopesticites for good cotton production.



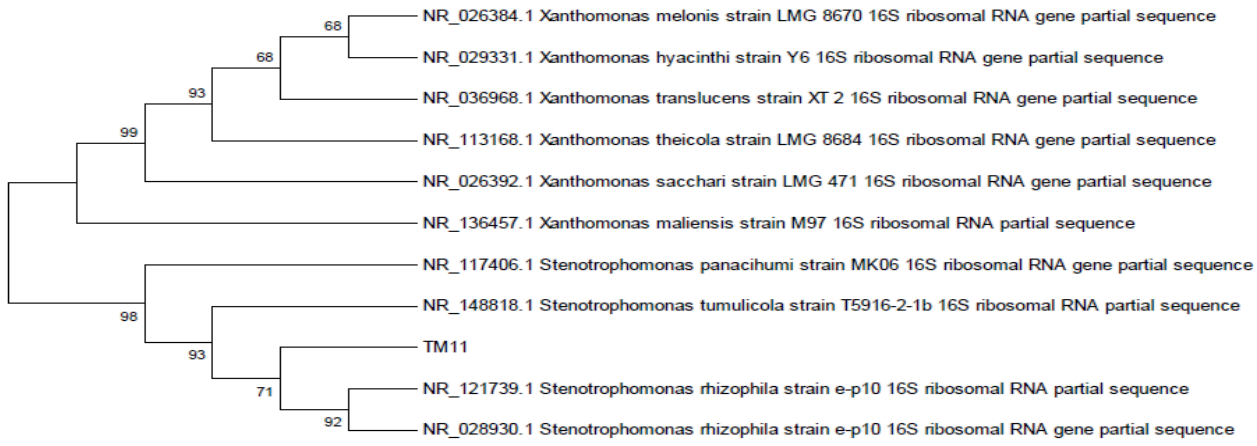


Fig. 2: Phylogenetic analysis of PGPR

4. CONCLUSION

We concluded that, the phytohormone like Indole-3-acetic acid and Gibberellic acid are produced by most of therhizobacteria under study which aids the plant growth promotion and satisfying hormonal need. On the other hand, stress hormone (ethylene) was found less in kharif crop than rabi crop season where environmental stress is less. PGPR may have practical biological application in plant growth characteristics which can potentially replace the use of chemical fertilizers. The use and application of such bioformulation in the fields can result in reduction of application of harmful chemicals; protect the environment and biological resources.

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