

Auxin Hormon Production and Plant Growth Promotion by Phosphate Solubilizing Bacteria of Groundnut Rhizosphere

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ABSTRACT: Groundnut (*Arachis hypogaea*) rhizosphere was screened for Phosphate solubilization. Total fifty phosphate solubilizing bacteria were isolate. 32 bacterial isolates with zone diameter more than 5 mm were further selected for screening of their characteristic of auxin harmon production. Quantitative estimation for auxin was done by colorimetric method with 2 days interval for up to 10 days of incubation period. Thirteen isolates were found to show the production of auxin by salkowaski reagent. Among these thirteen organisms four were remarkable for their high level of auxin production. All auxin producers showed different optimum periods for auxin production. Strain GST 3 was the best producer of auxin with amount 8.02 µg/ml, at eighth day of incubation whereas strain GSD 2 was least in amount of auxin production. Strain GST 3, was also efficient for phosphate solubilization. Four efficient auxin harmon producers were checked for plant growth promotion by seed germination assay and pot experiment study. Bacterial isolate with high potential for phosphate solubilization and auxin production were found to show the enhanced seed germination and plant growth promotion for groundnut seeds, as compared with uninoculated control. All auxin producers were characterized phenotypically. Four efficient auxin producers: GSP 4, GSB 3, GST 3, and GSV 3 were further identified by 16s rRNA sequence analysis as *Burkholderia kururiensis*, *Burkholderia cenocepacia*, *Enterobacter cloacae* and *Bacillus subtilis*. The study suggested that, auxin producing bacteria as efficient biofertilizer inoculants to promote plant growth. Therefore, there is a need for these isolates for further study due to their potential to be developed as plant growth promoting rhizobacterial inoculants in the field application.

KEYWORDS: Phosphate solubilizing bacteria, Auxin, Seed germination, Plant growth promotion.

I. INTRODUCTION

Continuously rising world population have given a danger alarm to the food scarcity for the future population. This leads to the practice of raising the world production by employing many technologies and strategies which are not in favour of the sustainability of soil health (Fox *et al.*, 2007; Kumar *et al.*, 2010). However, increase in the agriculture production must not be at the expense of our ecological heritage. Solution for this problem has opened the avenues for exploitation of various biofriendly strategies that leads to raise the production in sustainable manner. Hence, the use of biofriendly biological methods such as use of various biological techniques, organic manures and bio-fertilizers etc. have been recommended to increase the productivity from last few years.

Rhizosphere is the zone of rich microbial community compare with nonrhizosphere (Zaidi *et al.*, 2009). Bacteria colonizing, the plant roots are in many cases beneficial for plant growth, development, and productivity. Bacteria that can increase plant growth through many different direct and indirect mechanisms are known as plant growth promoting bacteria (Glick, 1995; Gupta *et al.*, 2000). Major beneficial activities of soil bacteria include solubilization of minerals, fixation of nitrogen, production of growth-promoting hormones and competitive suppression of pathogens (Gaskinsol *et al.*, 1985).

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Auxin is a general name for a group of hormones that are involved with growth responses (i.e., elongate cells, stimulate cell division in callus). The term "auxin" is derived from the Greek word means "to increase or grow" (Dr. Stephen G. *et al.*, 2009). This was the first group of plant hormones discovered. The auxin are a group of indole ring compounds which have the ability to improve plant growth by stimulating cell elongation, root initiation, seed germination and seedling growth (El-Tarabily, 2008). Auxin in plants also mediates the tropism, response to gravity and light (M. Kelen *et al.*, 2004). Microorganisms inhabiting rhizosphere of various plants are found to synthesize and release auxin as secondary metabolites because of the rich supplies of substrates exuded from the roots compared with non rhizospheric soils (Kampert *et al.*, 1975; Strzelczyk and Pokojaska-Burdziej, 1984). Indole acetic acid (IAA) is a common natural auxin and is a product of L-tryptophan metabolism in microorganisms including PGPR (Frankenberger and Brunner, 1983). Various species of PGPR are found to produce auxin harmon, includes bacteria like *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Bacillus* and *Rhizobium* (Dubeikovsky *et al.*, 1993; Taghavi *et al.*, 2009). Although, there are several types of auxin, IAA is the main auxin found in plant tissues and produced by microorganisms. Therefore, IAA was referred to as auxin in the scientific literature unless specifically referring to a type of auxin. The property of synthesizing indole acetic acid is considered as an effective tool for screening beneficial microorganism as there have been reports suggesting that IAA producing bacteria having profound effect on plant growth (Kuklinsky *et al.*, 1999; Ali B and Hashin S, 2007). The improved plant growth by PGPR is due to its ability to produce phytohormones such as indole acetic acid (IAA) (Pattern and Glick, 2002).

Therefore, the present experiment is designed to access an efficient multitrait PGPR, groundnut (*Arachis hypogaea*) rhizospheric isolates of PSBs were screened for their intrinsic ability to produce auxin harmon and their effect on plant growth promotion was also studied.

II. MATERIALS AND METHODS

Isolation and screening for phosphate solubilizing bacteria (PSB)

For isolation, screening, purification and preservation of phosphate solubilizing bacteria, soil samples were collected from rhizosphere of cultivated groundnut cropping fields of different locations of the district 'Sabarkantha' Gujarat, one of the sampling location, shown in the figure 1. (a) and soil sampling from rhizosphere in figure 1. (b).



Rhizosphere diversity was explored for phosphate solubilizing bacteria by plating serial dilutions of rhizosphere soil samples on Pikovaskya's agar and Nutrient agar media. Bacterial isolate bearing different morphology was screened separately for their phosphate solubilization attribute, by both qualitatively and quantitatively using culture media : Pikovaskya's agar bromophenol blue dye (Nautiyal *et al.*, 2002) and Pikovaskya's broth (Olsen *et al.*, 1954). All of the

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bacterial isolates with phosphates solubilization trait were purified by subculturing, preserved in 40% glycerol and by stabbing in soft agar and further screened for IAA production.

Bio assay for Auxin Production

1. Screening for Auxin Production

PSBs were screened for their trait of auxin harmon production by using the method of Zakry *et al.*, (2010). 24 hours growth culture of test isolates were inoculated in the nutrient broth with tryptophan (0.1 g/l), incubated at $28 \pm 2^\circ\text{C}$ for 3 days. Uninoculated growth medium was used as a control. 3 ml of bacterial cultures were centrifuged at 6000 rpm for 30 minutes. To the 2ml of supernatant, 2 drops of Orthophosphoric acid and 4ml of Solawaski's reagent (Solawaski's reagent – 50ml of 35% Perchloric acid and 1ml of 0.5 (M) FeCl_3) was added and was kept for 25 minutes in dark at room temperature. Production of auxin was confirmed by observation of color change from yellow to pink or red.

Salkowski's reagent works as it react with IAA and does not interact with L-tryptophan therefore this reagent is an important for qualitative and semi qualitative determination for the presence of the auxin hormone.

2. Colorimetric assay

The bacterial isolates positive for auxin production were assayed for amount of auxin production using the method developed by (Gorden and Paleg, 1957) with some modification. The whole assay was performed twice and readings were taken in triplicates. 2 ml of bacterial culture was collected from incubated broth for up to 10th day with 2 days interval and centrifuged at 6000 rpm for 30 minutes. 2 ml of the supernatant was mixed with 2 to 3 drops of orthophosphoric acid and 4 ml of Salkowski's reagent and kept in the dark for 25 minutes at room temperature. Absorbance of color change was measured by using UV-VIS spectrophotometer at 530 nm. Concentration of auxin produced by isolates was measured by preparing standard graph using indole acetic acid (IAA) (Sigma-Aldrich) as standard.

Seed Germination Assay

Healthy seeds of groundnut variety G-20 were selected and washed with sterile distilled water. Selected seeds were surface sterilized by soaking in 70% ethanol for 5 min and 2% sodium hypochlorite for 3 minutes and then they were washed by sterile distilled water for 5 times. Sterilized seeds were incubated with 50 ml of each treatment at room temperature for 24 hour, along with uninoculated media as control. After 24 hours incubation, the soaked seeds were placed in sterilized petri dish containing wet cotton and were incubated at room temperature up to 8 days (Khin Mya Lwin *et al.*, 2012).

Pot Study

Experimental setup

Pot experiment was carried out by using plastic pot of 20 cm diameter and filled with mixture of sterilized sand and soil in a ratio of 1:2 wt/wt. Healthy groundnut seeds of variety G-20 were collected from, "Agriculture Research Centre" of "Dantivad Agriculture University" located at Talod. Surface sterilized seeds were mixed with each selected inoculum singly, using vermicompost as carrier (vermicompost) and were sown in each pot. Three seeds were sown per pot. Three replicate were used for each treatment. Three plants of each treatment (1 plant/replicate/treatment) were uprooted at 20th day of growth to determine the selected physical parameters (shoot length & dry plants weight).

Phenotypic characterization of selected Rhizobacteria

13 auxin hormone producers were assigned with codes as: GSS 25, GSP 4, GSH 8, GSB 3, GST 3, GSB 13, GSO 1, GSH 1, GSB 2, GSD 2, GSV 4 and GSV3, characterized phenotypically by using morphological appearance and some biochemical reactions.

Biochemical characterization was done by performing some suitable biochemical test reactions includes: Gram staining, catalase, oxidase, citrate utilization, triple sugar iron (TSI), gelatin agar test (Aneja, K.R., 2003), and nitrate reduction, bile esculine agar test and motility indole ornithine (MIO) (Jason woodland, 2004).

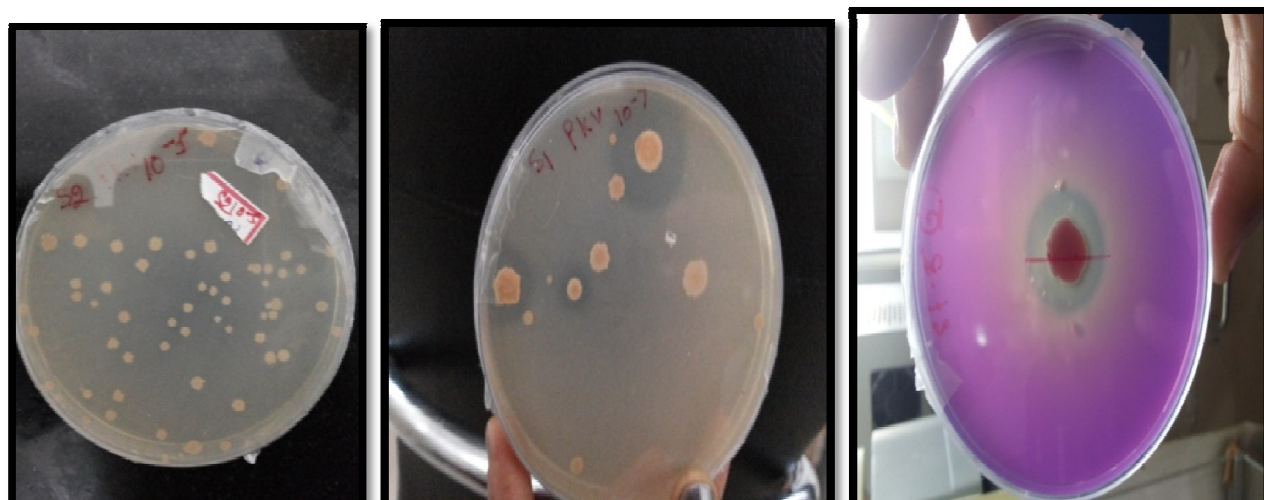
Genotypic characterization of potent strains

Four potent auxin producers GSP 4, GST 3, GSB 3 and GSV 3 were selected for their genotypic identification by sequencing their 16S rRNA and deposited in the Gene bank repository of Gujarat state biotechnology mission (GSBTM) Gandhinagar.

III. RESULT

Isolation and selection of phosphate-solubilizing bacteria

Isolation and screening for phosphate solubilizing bacteria were done basis of their formation of halo zone of phosphate solubilization, as shown in fig.: 2. (a) & (b) isolation of phosphate solubilizing rhizobacteria, 2. (c) selection of phosphate solubilizers.



(a)

(b)

(c)

Figure 2: (a) & (b) Isolation of PSB's Rhizobacteria, (c) Selection of phosphate solubilizers

A total of 50 isolates of phosphate solubilizing bacteria were obtained based on the formation of halo zone of phosphate solubilization on Pikovaskaya agar containing bromophenol blue (Nautiyal *et al.*, 2002).

Bacterial strains, with zone diameter equal and more than 5 mm were selected for further study. 32 isolates were found with zone diameter more than 5 mm. and were further quantified for their amount of phosphate solubilization by colorimetric assay.

Auxin harmon production

Thirteen bacterial isolates were found positive for auxin production, as per their appearance of color change to red on assay for auxin production, shown in figure 3 (a) and (b). Varying levels of auxin production were recorded. These isolates varied greatly in their intrinsic ability to produce auxin. Our findings of auxin production by PSBS isolates are in agreement with those of other researchers (Khin Mya Lwin *et al.*, 2012)

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(a) (b)
Figure 3: (a) and (b) Auxin harmon production assav by PSBs. positive reaction is shown by a color change to red.

Quantitative assay for auxin production

The standard calibration curve of IAA was set up by using the different concentrations of IAA at 530 nm with UV spectrophotometer. Different amount of auxin production, for thirteen selected isolates for up to ten days, with two days intervals are recorded in table 1.

Different pattern of auxin production by selected isolates, also presented by bar diagram in figure: 4.

From the recorded value of auxin production as per table: 1, it was found that the optimum time of incubation for auxin productions by all isolates were different. Among all isolates, strain GST 3 was the best auxin producer with amount 8.02 µg/ml, at its eighth day of incubation followed by GSB 3, producing 3.98 µg/ml, at its sixth day of incubation whereas strain GSD 2 is found to produce only small amount of IAA.

Table 1: IAA Production by PSPs

IAA concentration in days (µg/ml)	Day 2	Day 4	Day 6	Day 8	Day 10
Isolates					
GSS 25	0.33	0.98	2.47	1.76	0.89
GSP 4	3.76	0.91	0.68	0.58	0.31
GSH 8	0.63	0.8	0.36	0.21	0.13
GSB 3	0.34	3.55	3.98	2.47	1.76
GSB 5	0.36	1.69	0.92	0.42	0.27
GST 3	1.52	6.89	7.18	8.02	5.36
GSB 13	0.49	0.75	0.57	0.37	0.12
GSO 1	2.85	1.18	0.88	0.41	0.26
GSH 1	0.24	0.73	0.9	0.60	0.35
GSB 2	0.19	0.94	0.73	0.42	0.28
GSD 2	0.36	0.72	0.318	0.29	0.14
GSV 4	1.094	1.87	1.99	2.35	1.47
GSV 3	3.75	0.91	0.68	0.36	0.14

Strain GST 3 was the most efficient producer of auxin, as it was showing to produce a good amount of auxin during the whole periods incubation, in comparison to all others selected isolates. From this result, four strains: GSP 4, GST 3, GSB 3 and GSV 3 were found as the best auxin producers and were selected for further study.

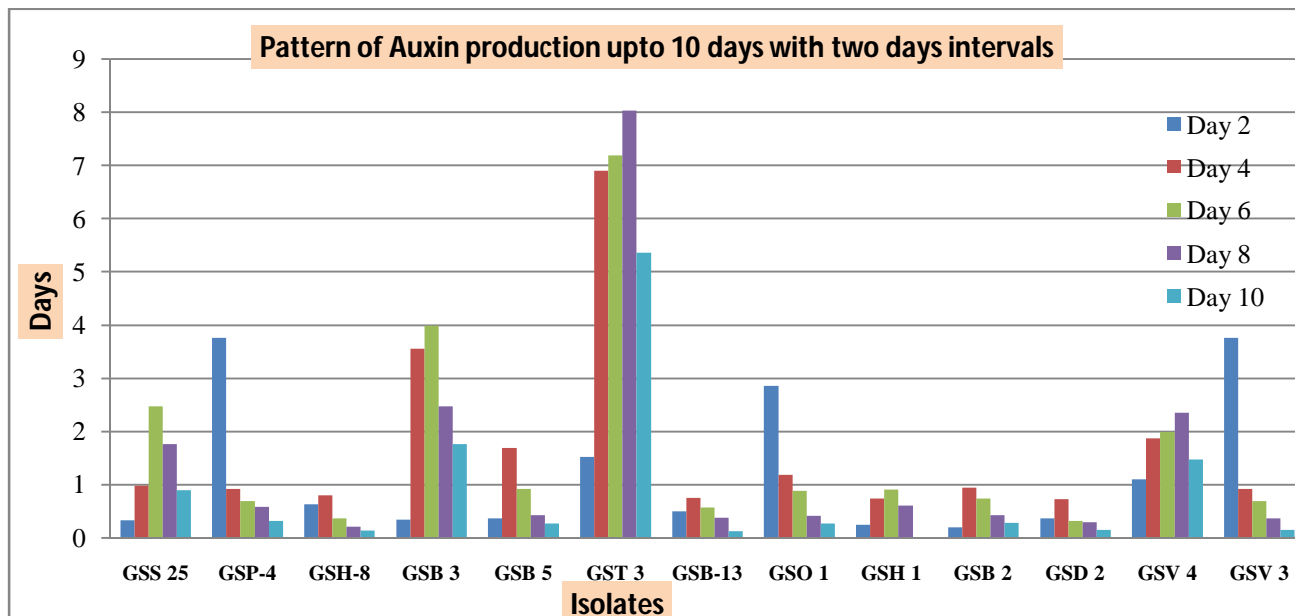


Figure 4: Bar diagram showing pattern of production of auxin after two day interval for up to ten days of incubation period.

Seed germination assay for groundnut seed by the selected strains

In vitro studies for the effects of selected potential isolates (GSP 4, GST 3, GSB 3 and GSV 3) on seed germination of groundnut seeds, after incubation periods of 10 days were found to show the enhanced seed germination for groundnut seeds in contrast to uninoculated control as shown in Fig.5a-e.

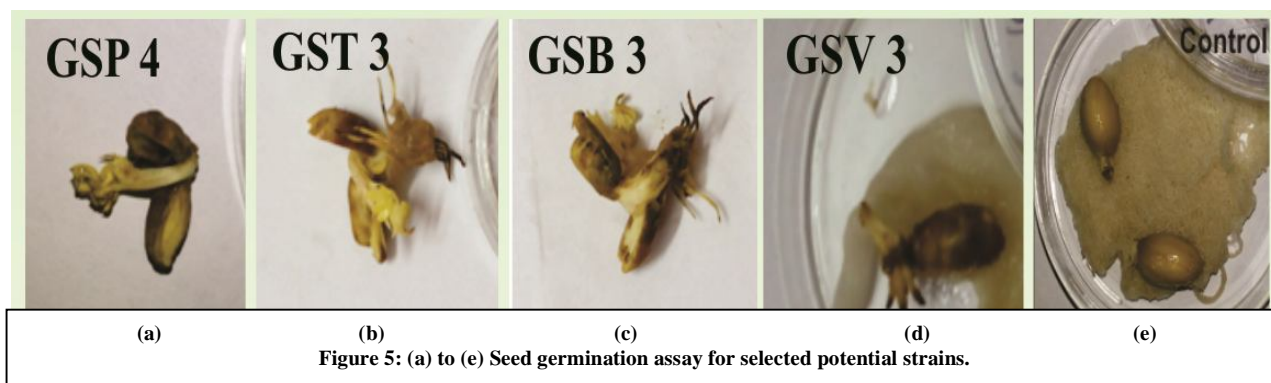


Figure 5: (a) to (e) Seed germination assay for selected potential strains.

Strain GST 3, highest producer of IAA was found to give the highest result for enhanced seed germination assay followed by GSP 4, GSB 3, whereas the strains GSV 3 shows the minimum effect out of all, 4 selected strains but more than control.

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Pot experiment result

Pot experiment was done to find out the effect of selected potential auxin harmon producing PSBs (GSP 4, GST 3, GSB 3 and GSV 3) on the growth of groundnut plant. Selected strains were also found to be potent for their property of phosphate solubilization. The effect on the shoot height and weight of groundnut plants, on treatment with the each selected isolates singly was recorded after 20 days of their growth in pots as shown below in table: 2 and presented by bar diagram in figure : 6.

Table 2: Effect of application of selected treatment of rhizobacterial isolates on shoot height (cm/plant) and dry weight (g/plant) of groundnut.

Inoculum	Shoot length (cm/ plant)	Dry weight (g/ plant)
Control	16.6	11.23
GSP-4	28.6	29.2
GSB 3	27.3	34.8
GST 3	28.4	36.7
GSV 3	26.2	32.4

Plant height

It was revealed from the results that there was a significant increase in plant height, when treated with selected isolates over uninoculated control. Maximum increase in plant height was recorded with the strain GSP 4 (28.8 cm) followed by GST 3 (28.4 cm), GSB 3 (27.3 cm) and then GSV 3 (26.2 cm) over uninoculated control (16.6 cm), showing that highest the production of auxin, more the effect on plant height.

Plant dry weight

The dry weights of plants were also showing a significant increase with selected strains compared to control. The highest plant dry weight was recorded for inoculation with isolate GST 3 (36.70 g), followed by GSV 3 (32.4 g), than by GSB 3 (34.8 g), the least increase was shown by GSP 4 (29.2 g), and most least by uninoculated control (11.23 g) respectively. Therefore it was revealed from the results that plant dry weight was also increased significantly when treated with auxin producers PSBs strains.

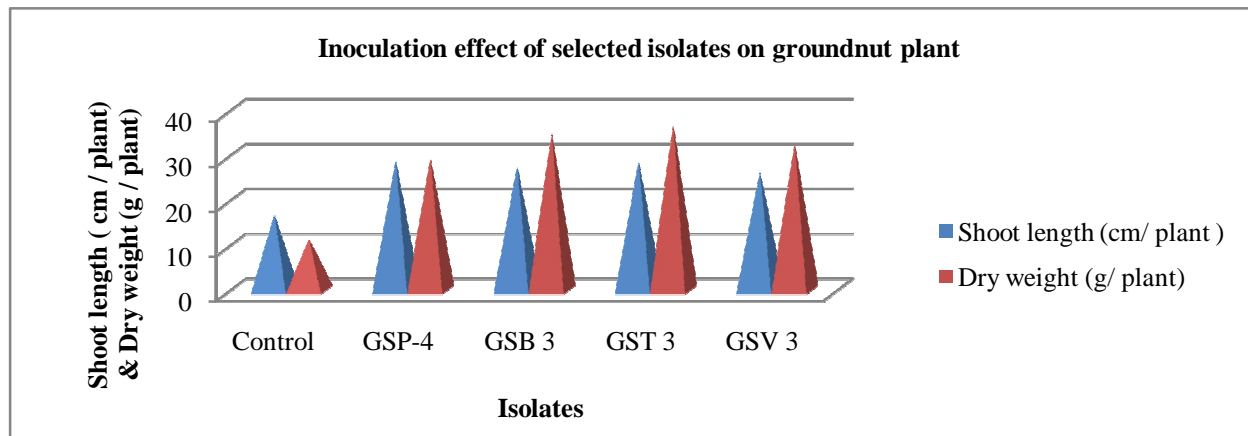


Figure 6: Bar diagram showing pattern of plant growth promotion by the selected efficient strains.

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Biochemical characteristic of selected Auxin producers PSBs

13 selected auxin hormone producers were characterized for their biochemical properties, listed in table: 3. All the isolates were found positive for catalase production and all have the property of nitrate production. Some of the gram negative isolates were showing positive reaction for oxidase, citrate production, along with no reaction for triple sugar iron test, revealing their similarity to *Pseudomonas*. None of the isolates were positive for motility indole agar test. Maximum was negative for triple sugar iron test. Three gram positive bacilli are found to give positive reaction for bile esculine agar test, while all other ten isolates are negative. Most of isolates were found positive for gelatin hydrolysis.

Table 3: Some Biochemical test result for selected auxin producing PSBs

Code	Morphology	Gram stainin g	Oxidase	Catalase	Citrate	Triple sugar iron	Motility indole agar	Nitrate reduction	BILE esculine	Gelatin agar
GSS 25	Flat , rhizoidal, white, rough	GPB*	Negative	Positive	Negative	K/K/G	M/T/O	Positive	Negative	Negative
GSP 4	Small,irregular , low convex, shiny, greyish color	GNB**	Positive	Positive	Positive	K/K/G	M/T/O	Positive	Negative	Positive
GSH 8	Small, round, low convex, translucent	GNB	Positive	Positive	Negative	K/K/G	M/T/O	Positive	Negative	Positive
GSB 3	Small, irregular, entire margin, golden color	GNB	Positive	Positive	Negative	K/K/G	M/T/O	Positive	Negative	Positive
GSB 5	Small, round, low convex, yellowish brown color	GPB	Negative	Positive	Negative	K/K/G	M/T/O	Positive	Negative	Positive
GST 3	Small, low convex, translucent, shiny	GNB	Negative	Positive	Negative	K/K/G	M/T/O	Positive	Negative	Negative
GSB 13	Small, round, centrally up umbonation, dull color	GPB	Negative	Positive	Negative	K/A/G	M/T/O	Positive	Positive	Positive
GSO 1	Big, mucoid, convex, translucent	GPB	Positive	Positive	Negative	K/K/G-	M/T/O	Positive	Negative	Negative
GSH 1	Irregular, ovalshape, translucent	GNB	Negative	Positive	Negative	K/K/G-	M/T/O	Positive	Negative	Negative
GSB 2	Small, round, low convex, opaque, cream color	GPB	Positive	Positive	Positive	K/A/G	M/T/O	Positive	Negative	Positive
GSD 2	Big,round,convex,opaque,cream color	GNB	Positive	Positive	Negative	K/K/G-	M/T/O	Positive	Negative	Positive

GSV 4	Small, round, low convex, rough, opaque, dark color	GPB	Negative	Positive	Negative	K/A/G ⁻	M/T/O ⁻	Positive	Positive	Positive
GSV 3	Small, round, low convex, rough, opaque	GPB	Negative	Positive	Negative	K/A/G ⁻	M/T/O ⁻	Positive	Positive	Positive

Sing indication: * GNB – Gram negative bacilli, **GPB – gram positive bacilli.

In microscopic examination, most of the isolates were found to be gram positive bacilli, as shown in figure 7 (a) & (b) while only five isolates are found to be gram negative as shown in below mentioned figure 7 (c).



a. Gram Positive Bacilli

b. Gram Positive Bacilli

c. Gram Negative Bacilli

Figure 7: Microscopic Examination of Isolates

Genotypic Identification

Selected efficient strains GSP 4, GSB 3, GST 3 and GSV 3 were deposited in gene bank at biogene - GSBTM with biogene accession number BAB 4868, BAB 4878, BAB 4883 and BAB 4896 respectively. 16S rRNA gene sequences of the selected strains obtained by sequencing were identified using BLAST at the National Centre for Biotechnology Information web site (www.ncbi.nlm.nih.gov) and submitted to GenBank EMBL with accession number: BAB 4868 - *Burkholderia kururienensis* (KT462732), BAB 4878 - *Burkholderia enocepacia* (KT462738), BAB 4883 - *Enterobacter cloacae* (KT462763), and BAB 4896 - *Bacillus subtilis* (KT462749). Similarity among the sequences studied was 99%.

IV. CONCLUSION

In this study, 50 different phosphate solubilizing rhizobacteria were isolated from groundnut rhizosphere. Among thirteen, selected phosphate solubilizers produced different amounts of auxin and the highest amount of auxin (8.02 µg/ml) was produced by GST 3 at 8th day of incubation.

Most efficient auxin producer GST 3 was found to belong to family Enterobacteriaceae identified as *Enterobacter cloacae* with accession number KT462763 in NCBI.

All auxin producer strains shows different periods incubation time for their optimum amount of auxin hormone production. The selected strains of best auxin producers (GST 3) and phosphate solubilizers have shown a significant influence for the enhancement of the plant growth as per our finding from seed germination and pot experiment results. In pot experiment the treatment with desired selected strains show a significance enhancement of the shoot height as well as the plant dry weight than untreated controls. Productions of auxin and phosphate solubilization by the PSBs were examined as possible contributing factors for plant growth promotion. Therefore the property of production of auxin along with phosphate solubilization is considered as an effective tool for screening of plant growth promoting microorganism.

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In conclusion the study suggests the auxin producing PSBs as efficient biofertilizer inoculants to promote plant growth. Thereby, resulting a green tool for enhanced agricultural productivity to replenish the threatened alarm to the World's food scarcity, in a sustainable ecofriendly way.

Help our farmer for a balanced fertilizer application which will be synchronized with crop demand and also will reduce the cost of production.

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