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Isolation of Plant Growth Promoting Bacteria from Fermented *Panchagavya* and their Effect on *Vigna Radiata*

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Abstract

Panchagavya is a mixture of five cow products viz. cow dung, urine, milk, curd and ghee containing many beneficial microorganisms mentioned in ancient Indian scripts. Two bacterial cultures (PG-7 and PG-34) isolated from fermented panchagavya were studied for gibberellic acid (GA)production and phosphorus (P) solubilization, respectively. Initially, PG-7 produced 0.81 mg/ml GA and was increased to 5.70 mg/ml after media optimization. PG-34 was able to solubilize considerable amount of tricalcium phosphate (213.36 µg/ml). The effect of pure cultures (PG-7, PG-34) and mixed culture (PG-7 plusPG-34) on *Vigna radiata* was studied in the pots. The application of pure as well as mixed culture showed the significant positive effect on the growth of *Vigna radiata*. The mixed culture increased about 2.07, 2.16, 1.50, 7.50, 2.50, 2.72 times fresh weight, dry weight, shoot length, root hair numbers, leaf numbers and leafarea,respectively on 20thday.The bacterial isolates (PG-7, PG-34) offermented panchagavya have a potential to be used as biofertilizer.

Keywords: Bacteria, Biofertilizer,Fermented panchagavya, *Vigna radiata*.

INTRODUCTION:

Panchagavya (PG) means mixture of five cow products viz. dung, urine, milk,curd and ghee. Microbial fermentation of the panchagavya resulted in a rich repertoire of compounds and microbes that are beneficial for plant growth. NMR metabolomics of panchagavya, showed several primary metabolites such as plant growth regulators consisting of indole acetate; gibberellic acids; vitamins and co-factors; nucleobases; that make up many of the biochemical pathways (Ukale, etal. , 2016). Microbes obtained from panchagavya have been said to possess the property of being able to produce phytohormones (Amalraj, et al., 2013).

Biofertilizers are living microorganisms that increase the growth as well as development of plant by amassing the accessibility of mineral nutrients, solubilizing phosphorus, and phytohormonesproduction (Mushtaq, et al., 2021). Individual or mixed cultures microorganism inoculant, have been proved to significantly increase crop production at a very low cost when compared to chemical fertilizers. Plant growth promoting bacteria (PGPB) alleviates the effect of biotic and abiotic stress factors directly or indirectly. PGPB have some properties such as phosphate solubilization, ACC deaminase production and phytohormone production. PGPB are colonized at/near the plant root, their wave of efficacy hits the doorstep of major functional aspects of the entire plant (Mondal, 2020).

The production of such growth regulators by microorganisms delivers several benefits to the host plant with the facilitation of root system expansion, which increases the absorption of water and nutrients and improves plant survival (Verma, et al., 2017). Phytohormone comprise cell wall and cuticle thickening, structural changes of roots, stems, and leaves.Stem elongation is stimulated by gibberellic acid (GA). Gibberellins are plant hormones affecting germination, stem elongation, flowering, abolition of dormancy, leaf

and fruit senescence, and enzymatic stimulation (Singh, et al., 2020).

Soil is a foremost source of nutrients required by plants for growth. The three chief nutrients are nitrogen (N), phosphorus (P) and potassium (K). Phosphorous is the essential elements that are necessary for plant development and growth; it makes up about 0.2% of a plant's dry weight. Phosphorousis second only to nitrogen among mineral nutrients, most commonly limiting the crops growth (Azziz, et al., 2012).On average, the phosporous content of soil is about 0.05% (w/w); however, only 0.1% of this phosphorus is available for plant use. Phosphate solubilizing microorganisms (PSMs) increases the availability of insoluble soil phosphorus for plant use. PSMs secrete organic acids and phosphatases which convert insoluble phosphates into soluble phosphate which can be utilized by plants (Hii, et al., 2020).

In order to scrutinize plant, soil and microbial interactions, pot experiments are normally used as an imperative tool for exploring plant responses to treatment manipulations. The pot study is a preliminary experiment that provides us evidence of the impact our test bacteria on the experimental crop. In the present study, *Vigna radiata* (mung bean) crop was used for studying the effect of bacteria isolated from fermented panchagavya on their growth.

MATERIAL AND METHODS

Bacterial isolates

We have reported the bacteria showing plant growth promoting activities isolated from fermented panchagavya (Gohil, et al., 2020). Out of these, two bacterial isolates, viz. PG-7and PG-34 were selected for further study. PG-7 was studied for GA production and PG-34 for phosphorus solubilization and their effect on the growth of *Vigna radiata* was tested.

Maintenance and preservation of isolates

The pure cultures of bacterial isolates were grown on Nutrient agar medium. They were preserved by monthly transfer onto Nutrient agar slants and were kept in the refrigerator at 4°C.

Inoculum preparation

For inoculum preparation, 20.0ml of sterile Nutrient broth was inoculated with freshly grown cultures and incubated at 30°C,120 rpm on an orbital shaker for 24 h. Bacterial suspension having a cell density ~10^8cells/ml was used to analyze the optimized parameter. The prepared culture suspension was used as 1% (v/v) inoculum.

Furthermore, the cells were separated by centrifugation at 5000 rpm for 15 minutes and the supernatant was removed. Cell pellet was resuspended in sterile normal saline to obtain a cell density ~10^8cells/ml and it was used for pot treatment.

Plant growth promoting traits of PG-7and PG-34

Gibberellic acid production by PG-7

To study the effect of various carbon sources on the production of gibberellic acid, 100 ml Nutrient broth having pH 7.0 was supplemented with different carbon sources viz. dextrose, lactose and fructose and the concentration of these sugars was kept 2% (w/v). The flask was inoculated with inoculum of PG-7 and incubated at 30±2℃, at 150 rpm on an orbital shaker.

To investigate the effect of nitrogen source, peptone was replaced by $NaNO_3$, urea, and glycine at 0.5% (w/v) in 100 ml Nutrient broth. It was inoculated with PG-7and incubated at 30± 2℃, 150 rpm on an orbital shaker.

For optimum GA production, 100 mlnutrient brothin 250 ml flask was supplemented with 1% (w/v) dextrose and 0.2 % (w/v) $NaNO_3$. The medium was inoculated with 1% (v/v) PG-7and incubated at 30± 2 ℃, 150 rpm for 72 h on shaker.

Gibberellic acid estimation (Graham and Thomas, 1961) : 2.0 ml broth was taken

aseptically in an Eppendorf tube, and centrifuged at 5000 rpm for 10 minutes. 1.0 mlsupernatant and 1.0 ml ethyl acetate was mixed and shook vigorously. Ethyl acetate layer was allowed to separate and carefully transferred in a 50 mlglass beaker, the ethyl acetate extraction was repeated thrice. The ethyl acetate extract was allowed to evaporate at room temperature. The residue was dissolved in 2.0 mlmethanol and collected in screw cap tubes. To this 1.0ml of DNPH (2,4-Dinitrophenylhydrazine) was added and incubated at 100°C for 5 minutes. After cooling, 5.0 ml alcoholic potassium hydroxide (10%, w/v) and 15.0 ml sterile distilled water was added. Red wine color developed was measured at 430nm. Standard calibration curve of GA (0.8 mg/ml) was prepared in methanol.

Phosphate solubilization by PG-34

Solubilization of different phosphorus sources like Aluminum phosphate (AP), Zinc phosphate (ZP) and Tricalcium phosphate (TCP) was studied using Pikovskaya's broth. The phosphate solubilized by PG-34 was estimated by Stannous chloride method (King, 1932).In three 250 ml flaskscontain100ml sterile Pikovskaya's broth (pH 7.0±0.2),1.0 gm% different phosphate sources (AP, ZP and TCP) were added. Each flask was inoculated with 1% (v/v) inoculum and incubated at 25°C, 150 rpm on a shaker. Phosphate solubilization was tested after five and ten days.

2.0ml sample was centrifuged at 10,000 rpm for 15-20 minutes to remove cells. From this, 0.1 ml supernatant was taken and 0.9 ml double distilled water and 1.0 ml of chloromolybdic acid was added. Contents in test tubes were diluted to 4.0 ml by adding double distilled water. To this, 25.0 µL chlorostannous acid reagent was mixed till blue color was developed. Final volume was made up to 5.0 ml with double distilled water as quickly as possible. Absorbance was taken at 600nm. Total amount of phosphate solubilized was calculated against the standard curve of K_2HPO_4 (100 µg/ml).

Soil sample

The 12.0 kg soil sample was collected in sterile plastic bag from a depth of 4.0 inches from the central library garden area of Gujarat University and transported to the laboratory for pot experiment.

Seeds

*Vigna radiata*seeds were collected from the local market Ahmedabad for pot experiments. Seeds were disinfected by washing them with tap water to remove all the dirt and dust followed by washing twice with distilled water to remove any remaining tap water. The seeds were treated with 0.1% $HgCl_2$ solution for 3 minutes. Seeds were cleaned with 70% ethanol 3 times followed by rinsing them with sterile distilled water 5 times.

Pot study

The pot study was conducted in plastic pots with a height of 14.0 cm and a diameter 6.0 cm between the month of February and March 2020 in Ahmedabad, when the average temperature was 35±2°C.

For the pot preparation, garden soil was autoclaved twice for 20 minutes at 121°C. Each pot was filled with approximately 300 grams sterile soil having a neutral pH. *Vigna radiata*(V) seeds were surface sterilized and watered slightly after transplanting at a depth 0.5cm.

Pots were prepared and named as control, PG-7, PG-34 and mixed culture, respectively. A mixed culture was prepared by mixing the bacterial suspensions of all the two isolates (PG-7and PG-34). To check the culture effect on *Vigna radiata*growth, 5.0 ml bacterial suspension were given on 3rd and 10th day after sowing (DAS). Control plants were treated with sterile distilled water.

The plants from each pot were harvested on the 10th and 20th day to studythe morphological characteristics (fresh weight, dry weight, shoot length, root length, root number, root hair number, leaf number and leaf area).

RESULTS AND DISCUSSION

Gibberellic acid production by PG-7

Gibberellic acid is a plant growth regulator produced by bacteria and fungi that helps in the plants growth. The exploration of various plant growth promoting substances produced by the bacteria makes them an effective biofertilizer. PG-7 was able to produce 0.8mg/ml gibberellic acid after 48 h in Nutrient broth (Figure 1 A).PG-7 bacteria isolated from fermented panchagavya having good capacity to produce gibberellic acid within 48 h, which is as good as fungal

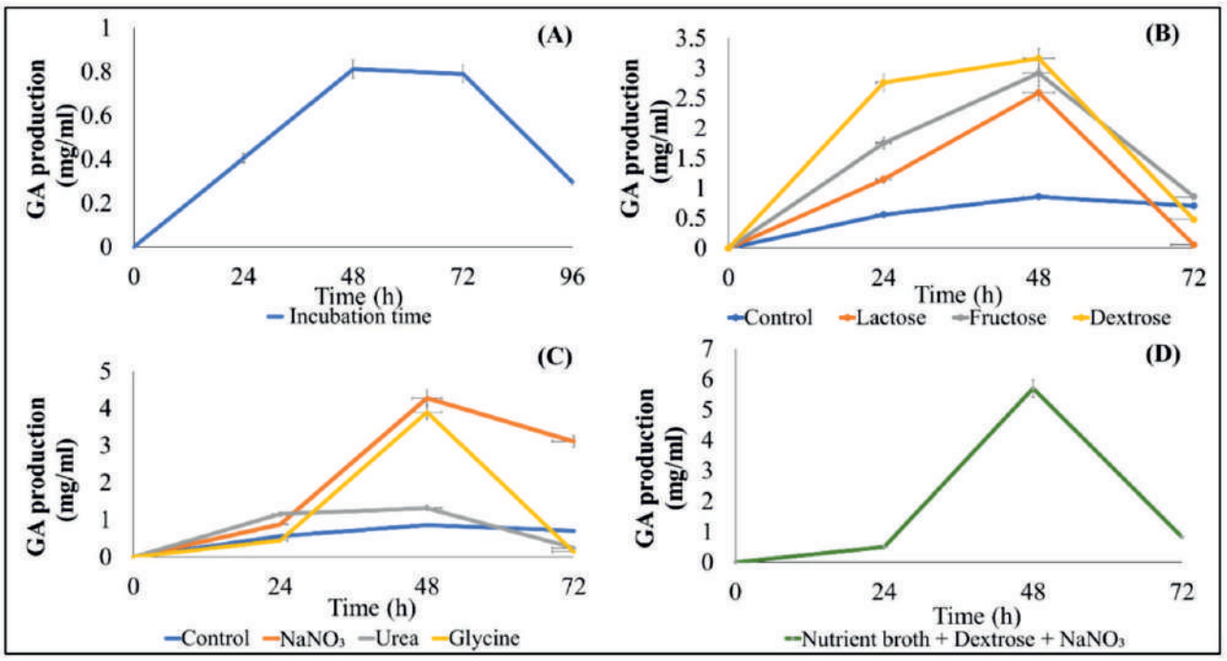

Figure 1 Optimization of gibberellic acid production by PG-7 (A) GA production with time (B) Effect of carbon sources on GA production (C) Effect of nitrogensources on GA production (D) Gibberellic acid production using optimized medium.

production.The gibberellic acid production is primarily reported by various fungi and were used for commercial production.

In comparison to bacteria, fungi require more time to grow and produce GA. *Pseudomonas stutzerii* solated from rhizospheric soil produced 0.03 mg/ml GA, after 72 h (Patel & Saraf 2017). Rhizospheric isolate *Pseudomonas monteilii* produce0.09 mg/ml G Aafter 48h (Pandya & Desai 2014). Panchgavya isolate PG-7 produced significant GA in compare to above isolates. *Fusarium moniliforme* isolated from soil generated 0.4 mg/ml GA at 144 h (Sleem, 2013). PG-7 has produced about double gibberellic acid than the above fungi within 48 h.

Gibberellic acid production was optimized by adding carbon and nitrogensources in the nutrient medium. Medium optimization for GA production can decrease the production costs on large scale.The effect of different sugars on GA production was studied and it boosted the production up to four times. The GA production was increased to 3.1 mg/mlupon addition of dextrose (2% w/v), whereas fructose and lactose showed 2.9 mg/ml and 2.5 mg/ml GA productionafter 48 h, respectively(Figure 1 B). *G. fujikuroi* produced 0.3 mg/ml GA after 168 h in a basal medium supplemented with glucose (Lale & Gadre 2010). Ammonium mineral salt broth supplemented with 0.5% (w/v) Na-succinate as a carbon source showed 0.12 mg/ml GA production by *M. Oryzae* (Siddikee, et al., 2010).About 0.08 mg/ml Gibberellic acid production was obtained with 1% glucose by rhizospheric bacterial isolate K8 (Desai, 2017).

The nitrogen sources $NaNO_3$, Urea, and Glycine

were added to the nutrient broth as a substitute of peptone and 4.2, 1.3, and 3.8 mg/ml GA was produced, respectively after 48 h. The addition of $NaNO_3$ and glycine resulted to about 5 times increase in GA production, whereas urea gave double production. The medium containing $NaNO_3$ as the nitrogen source produced the highest (4.2 mg/ml) GA at 30°C after 48 h (Figure 1 C).

The nitrogen source plays an important role in GA production, 0.29 mg/ml GA with NH_4NO_3 and 0.15 mg/ml GA was produced with glycine (Shukla et al., 2005). Similar results were obtained by Karakoc and Aksoez (2004) and addition of $NaNO_3$increased the GA production (0.14 mg/ml). Desai (2017) investigated the effect of nitrogen supplementation on GA production by *Azotobacter* spp. and found that nutrient broth supplemented with 0.5% ammonium chloride produced 0.07 mg/ml GA.

Optimized medium supplemented with dextrose and $NaNO_3$ showed 5.6 mg/ml GA production after 48 h in shake flask conditions (Figure 1 D). The optimized medium showed 7 times increase in GA production.

Phosphate solubilization by PG-34

Phosphate solubilizing bacteria improve the phosphates availability by solubilizing them and making them available to the plant. PG-34 was capable of solubilizing 216 µg/mlinsoluble inorganic phosphorus after 10 daysin Pikovskay's broth (Figure 2 A). *Bacillus cereus* able to solubilize 159µg/ml phosphate after 10 days (Salwan, et al. 2021). PG-34 was able to solubilize phosphate sources along with pH drop from 7.0 to 4.5 after 10 days. The pH of medium shifted from neutral to acidic due to organic acid production.PS Mswas able to solubilize different inorganic phosphatessources in liquid media and it was also associated with pH reduction (Lacava, et al., 2021).

PG-34 was found to solubilizemore TCP (213.36 µg/ml) than ZP (7.7µg/ml) and AP (2.04 µg/ml) after 10 days (Figure 2 B). PG-34 solubilized maximum ZP (76µg/ml) after 5days, thereafter no significant increase of available phosphorus was observed. PG-34 showed the maximum solubilization of TCP, moderate solubilization of ZP and least solubilization of AP (Figure 2 C).

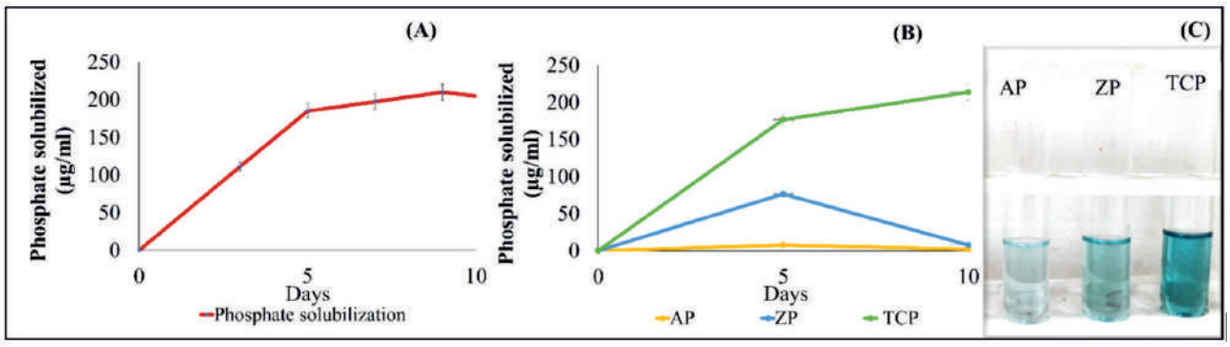

Figure 2 Phosphate solubilization by PG-34 (A) Phosphate solubilization with time (B) Solubilization of different phosphorus sources C) Detection of solubilized aluminium phosphate (AP), zinc phosphate (ZP) and tricalcium phosphate (TCP) on 10th day.

Similarly, Sagervanshi , *et al*., (2012) studied the solubilization of phosphorus sources (AP, ZP, TCP) and reported TCP as the best phosphate source. Phosphate solubilization was 4.73 mg/ml with single super phosphate and 2.89 mg/ml by TCP by *Piriformospora indica* (Seshagiri & Tallapragada 2016). Phosphate solubilization capability varies with different microorganisms. PG-34 may be useful in resolving calcareous soils by increasing the accessible plant phosphate levels.PSBs are present in fermented panchagavya and have higher capacity for phosphate solubilization makes them useful for liquid biofertilizer.

Pot study

In the primary test, isolates were tested for their beneficial role in producing phytohormone and releasing phosphorus in the broth medium. Their biological contribution to improve the mung bean plant was investigated.The effect of pure culture PG-7, PG-34 and mixed culture was tested and they showed significant increase in plant growth compared to control. Variation in the efficiency was apparent and mixed culture performed better than the pure cultures.

Plant growth promotion activity of PG-7 and PG-34 on *Vigna radiata*

The impact of PG-7 (gibberellic acid-producing) and PG-34 (phosphate-solubilizing bacteria) on *Vigna radiata*in pots was studied. Pure cultures (PG-7 and PG-34) and mixed culture effect on the growth of mung plant over control is presented inTable 1. On the 20th day, an improvement in root length, shoot length root hairs and leaf numbers were observed. Leaves were wider and lengthyin treated plants compared to untreated plants. Plant inoculated with PG-7showed significant increase in the fresh weight, dry weight shoot length, root length, root hair number, leaf number and leaf area by 1.97, 2.0, 1.45, 1.03, 6.0, 2.0and 2.7 times, respectively compared to the untreated plant. Similarly, plant inoculated with PG-34 showed significant enhancement in the fresh weight, dry weight, shoot length, root length,root hair number, leaf number and leaf area by 1.71, 1.66, 1.3, 1.08, 4.5, 2.5 and 2.4 times, respectively over control. The plant inoculated with mixed culture showed considerable growth in the fresh weight, dry weight, shoot length, root length, root hair number, leaf number and leaf area by 2.07, 2.16, 1.50, 1.41, 7.50, 2.50 and 2.72 times compared to thecontrol after 20th day.

The difference in growth promotion effects between fresh and dry weights between control and treated might be attributed to PGPB capacity to provide more water holding capacity to plant. Gibberellic acidstimulates seed germination, shoot growth, vegetative to flowering, determines sex expression and grain development along with an interaction of different environmental factors (Gupta & Chakrabarty 2013). PSM increases the bioavailability of soil insoluble phosphorus for plant use. PSMs solubilize and mineralize insoluble organic phosphorus. Therefore, soil or crop inoculation with PSM is a promising strategy for improving plant phosphorus absorption and thus reducing the use of chemical fertilizers that have a negative impact on the environment (Alori et al., 2012). Application of PGPB improved vegetative growth characteristics, notably in leaf area, when compared to control plants(Abdelaal et al., 2021).Our results are in general agreement with numerous studies reported for beneficial effect of the PGPB on plant growth promotion.

Vegetative parameters	Treatments							
	Control		PG-7		PG-34		Mixed culture	
Day	10	20	10	20	10	20	10	20
Fresh weight (g)	0.21	0.38	0.35	0.75	0.38	0.65	0.26	0.79
Dry weight (g)	0.03	0.06	0.08	0.12	0.06	0.10	0.05	0.13
Shoot length (cm)	9.5	10.0	13.0	14.5	12.0	13.0	13.0	15.0
Root length (cm)	1.9	6.0	5.0	6.2	1.0	6.5	2.4	8.5
Root number (No.s)	1	1	1	1	1	1	1	1
Root hair number (No.s)	0	2	1	12	2	9	2	15
Leaf number (No.s)	2	2	2	4	2	5	2	5
Leaf area (cm^2)	2.40	2.50	5.25	6.75	5.60	6.00	5.25	6.80

Table 1 Effect of PG-7, PG-34 and mixed culture on growth of Vigna radiata.

Sangeetha & Thevanathan(2010) stated that panchagavya application encouraged the lateral rootsdevelopment, leaves, leaflets and lamina growth compared to control in experimental plants (*Vigna radiata, Vigna mungo, Arachis hypogea*and *Cicer arietinum*). Phosphate solubilizing bacterial inoculums to assess their ability to promote tomato (*Lycopersicon esculentum*) seedling and plant growth in in-vitro and greenhouse experiment respectively, and reported that isolates significantly enhanced all parameters (shoot length, root length, shoot and root dry weight) compared to control (Pathak et al., 2017). Positive effects of biofertilizers on improving crop growth might be due to increased synthesis of plant growth promoting substances.

CONCLUSION

The present study demonstrates that fermented panchagavya contains potential bacteria with plant growth promoting abilities like gibberellic acid productionand phosphate solubilization. Application of isolatesPG-7 and PG-34, in pure and mixed culture significantly increased the plant growth compared to control. It showed considerable improvements in the fresh weight, shoot length, root length, root hair number, leaf number and leaf areaof *Vigna radiata.* Since most soils in the world are deficient in plant-available nutrients, the use of bioinoculants would increase the nutrients availability and increase the soil fertility. This will reduce the use of chemical fertilizers and encourage the sustainable agriculture. The bacterial isolates of fermented panchagavya are potential candidates to be used as bioinoculants and biofertilizers.

CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

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