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Study of Bacterial Strain Isolated from Pigeon Pea Rhizosphere and their Role as Biofertilizers

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Abstract: The transition toward sustainable farming necessitates a reduction in chemical fertilizer dependency through the use of biologically active alternatives. Rigorous and rigid contraction can increase the quantity of chemical fertilizers that will deplete the soil because of decreasing the penetration capacity of water into the soil. This paper outlines a comprehensive framework for the isolation of potent nitrogen-fixing bacteria, specifically *Pseudomonas, Azotobacter, and Rhizobium*, from the rhizosphere soil of the pigeon pea plant at MIET, Meerut, Uttar Pradesh Morphological and biochemical tests were performed after the successful isolation of the bacterial strains in which all the bacterial strains were found gram-negative bacteria under morphological test. Catalase, MR-VP, Carbohydrate fermentation, urea and indole tests were also done for biochemical characterization. These isolated strains are proposed to be formulated into a multi-strain biofertilizer consortium designed for application on ornamental flowering plants. In the present study, we have conducted test trials in pots for observing the effect of isolated bacterial strains on flowering plants, and all the three bacterial strains were cultured in the Urea broth. The broth was applied as Seed Treatment, soil application by enriching compost at the time of sowing and later by drenching in the flowering plants. As a result, the proposed approach aims to enhance soil fertility, balanced pH, EC, increased OC and N, early blooming of flowers, improve plant growth metrics, and promote sustainable agricultural practices. This study provides a structured methodology and evaluation plan to validate the efficacy of rhizosphere bacterial consortiums in horticultural applications.

Keywords: *Sustainable, Penetration, Biofertilizers, Ornamental, Horticultural.*

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I. INTRODUCTION

Soil is a rich habitat for various organisms. One of the most important habitants of soil is the microbial community. The greatest microbial diversity is found in soil out of any other environment. Pigeon pea regularly is known as tur, dal, or red gram and has second position among the pulse crop in India, which is an essential food legume crop in semi-arid regions worldwide (Gupta et al.2020). Today, pigeon pea is one of the most important food legumes for many of the resource-deprived farmers worldwide and previously neglected crop is considered as one of the core commercial crops in present times (Arora et al. 2018). Pigeon pea crop can grow under poor soil conditions and tolerate water scarcity. It is a nutritious, high-protein legume crop used as a source of dietary protein. India alone accounts for about 80% of total production of pigeon pea. Nowadays, modern agriculture relies on the excessive use of chemical fertilizers and pesticides to increase crop production, which caused severe adverse effects on soil health and environment (Aktar W.et al. 2009). However, use of Plant growth promoting rhizobacteria (PGPR) as biofertilizer in agriculture to enhance the growth of plants via circulating the nutrients in the soil is an eco-friendly strategy to minimize the need of synthetic fertilizers as much as possible (Verma P., Saharan B.S. et al. 2015,2011). Plant growth promoting rhizobacteria (PGPR) is the group of beneficial bacteria that enhance plant growth and biocontrol by a wide variety of mechanisms (Kloepper J.W. et al. 1978). Various kinds of researchers have been reported that PGPRs are the most important agents in the mass production of crops. PGPRs, also known as bio-stimulants, are effectively used to increase the plant growth, yield and food security (Sun et al. 2024).

PGPR when applied to the crop and seed it enhanced the yield and growth of plant (Teja et al. 2023) and freed the living rhizobacteria that strongly remain attached to rhizosphere plant roots. Due to release of metabolite, PGPR generates plant growth promoting effect in the plant then enhanced the growth. PGPR represents the microbial group colonizing the roots, and has capacity to directly enhance the plant growth and protect from damage or disease and pathogen attack.

PGPR has been isolated and screened from rhizosphere soil of pigeon pea to enhance growth, seed emergence, crop yield and production (Farah A. et al. 2006). PGPR has been commercialized as microbial bioinoculants or biofertilizers to

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increase crop production (Adesemoye A.O. et al. 2009). PGPR offers an attractive strategy for replacement and reduction of heavy application of chemical pesticides and fertilizers (Banerjee M.R. et al. 2006). Hence, the present work was aimed to isolate, screened and characterize the PGPR from rhizosphere soils of pigeon pea which can be utilized in the future for increasing growth and yield of crops.

II. METHODOLOGY

➢ *Collection of Soil Sample*

The soil sample was collected from the rhizosphere soil of pigeon pea from MIET, Meerut, Uttar Pradesh. Soil sample was collected up to the depth of 5cm to 10cm from the rhizosphere of pigeon pea. The soil sample was transferred into a small sterilized polythene bags and brought to laboratory for further studies.

➢ *Isolation of Rhizosphere Bacteria*

Serial dilution was used for the isolation of bacteria from pigeon pea rhizosphere soil. One gram of rhizosphere soil sample was suspended in 9 ml of sterile distilled water blank and kept in a rotary shaker at 160 rpm for 30–45 minutes for constant mixing of soil. Then 1ml of sample was introduced into the first test tube with the help of sterile micropipette and labelled 10^{-1} and mixed thoroughly again. 1 ml was taken and transferred to the second test tube using a 1 ml micropipette which gives 10^{-2} dilution. This step was followed repeatedly to obtain concentrations 10^{-3}, respectively. 0.1 ml sample was taken from 10^{-1}, 10^{-2} and 10^{-3} dilutions with the help of micropipette and inoculated on each nutrient agar plate by the spread plate technique. The plates were then incubated at 37ºC for 24-48 h and the colonies growth can be shown in Fig.1.A respectively. Then colonies were calculated as CFU per plate and differential colonies were marked for the pure culture.

➢ *Pure Culture Isolation*

Based on colonies appearance, 4 different colonies from two incubated plates (10^{-1} and 10^{-2}) were taken loopful using sterile loop and streaked on the nutrient agar plate which can be shown in Fig.1.B respectively.

➢ *Biochemical Characterization*

- *Catalase Test:*

It is used to ascertain the ability of bacteria to produce catalase that reduces hydrogen peroxide to water and oxygen. Growth of bacterial colony is picked with sterile loop and suspended in a drop of 3% H_2O_2 on a glass slide, then observe for bubble formation. The effervescence on the glass slide confirms the presence of catalase production by the bacteria and no effervescence shows negative results for catalase production.

- *Methyl Red Test:*

This test is used to identify bacterial ability to produce stable acid end products by means of a mixed-acid fermentation of glucose. MR-VP broth was prepared, sterilized and inoculated with bacteria and incubated for 24-48 hours at 37ºC. After incubation, 6-7 drops of methyl red solution were added. Occurrence of bright red colour indicates positive result, red-orange colour indicates weak positive result and yellow-orange colour indicates negative result.

- *Voges-Proskauer Test:*

VP test is a biochemical test that detects the ability of bacteria to metabolize the pyruvate into a neutral intermediate product called 'acetylmethylcarbinol' or 'acetoin'. If present, acetylmethylcarbinol is converted to diacetyl in the presence of α-naphthol, 40% KOH and atmospheric oxygen. MR-VP broth was prepared, sterilized, inoculated with bacteria and incubated for 48 hours at 37ºC. After incubation, 6-7 drops of Barritt's reagent A and 3-4 drops of Barritt's reagent B was added to the tubes and the tubes were shaken at intervals to show aeration. Occurrence of red-pink colour at the surface of the medium by continuously shaking of tubes, indicates positive result and no colour change indicates negative result.

- *Carbohydrate Fermentation Test:*

It is used to access the ability of bacteria to ferment a specific carbohydrate and to differentiate bacteria based on their carbohydrate fermentation pattern and identify them. Lactose broth was prepared, sterilized, inoculated with bacteria and incubated for 48 hours at 37ºC. After incubation, 6-7 drops of phenol red indicator were added to test tube. Occurrence of yellow colour indicates positive result and occurrence of red colour indicates negative result or becomes more alkaline dark red/purple.

- *Urea Test:*

This biochemical test is designed to measure urease activity, which determines the ability of soil microorganisms to hydrolyse urea into ammonia (NH_3) and carbon dioxide (CO_2). Urea broth was prepared, sterilized, inoculated with bacteria and incubated for 24 hours at 37ºC. After completion of 24 hours, 6-7 drops of phenol red indicator were added to test tube. Bright pink/magenta colour indicates positive result and no colour change indicates negative result.

- *Indole Test:*

It is a biochemical test conducted on bacterial strains to detect their ability to produce indole from tryptophan. Tryptophan broth was prepared, sterilized, inoculated with bacteria and incubated for 24 hours at 37ºC. After completion of incubated period, 3-4 drops of Kovac's reagent were added down the side of the test tube. The formation of pink-red ring at the top of the broth indicates positive result and no ring formation or yellow ring formation indicates negative result.

➢ *Preparation of Microbial Consortium*

All the three isolated bacterial strains (*Pseudomonas, Azotobacter, and Rhizobium*) were formulated into biofertilizer by culturing them with Jaggery, K_2HPO_4(dipotassium hydrogen phosphate) and $MgSO_4$(magnesium sulphate). Jaggery acts as carbon source and boosts microbial growth, $MgSO_4$ provides Mg^{2+} and sulphur which is essential for enzyme activation, cell growth and protein synthesis meanwhile K_2HPO_4 acts as a phosphate source and buffering agent. Separate cultures were prepared

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for three bacterial strains and then one mixed culture was prepared for observing best result.

- *Quantity:*

✓ Jaggery- 10gm (for each)
✓ K_2HPO_4- 0.5gm (for each)
✓ $MgSO_4$- 0.2gm (for each)
✓ Distilled water- 1L (for each)

➢ *Procedure (for 1L):*

10gm jaggery was mixed in 1L distilled water until fully dissolved and filtered (cloth/filter paper) to remove impurities then, 0.5gm K_2HPO_4 and 0.2gm $MgSO_4$ was added and stir well until everything dissolved. The formulated solution was sterilized by autoclaving at 121ºC for 15-20 minutes. After autoclaving, bacterial strain was inoculated with the help of sterilized loop and incubated at 30ºC for 48 hours.

➢ *Inoculation in Flowering Plants*

The formulated biofertilizer was inoculated in three different flowering plants i.e. Petunia, Gazania and Marigold in pots at MIET college, Meerut, Uttar Pradesh, for observing the impact of microbial consortium on flowering plants. 200ml of formulated biofertilizer was applied during sowing of seeds and rest of was applied with the interval of 5 days upto 5 times with specific amount of 200ml. Total of 4 pots were taken for each flowering plant in which 1 pot was taken as control and rest of three pots were taken to show impact of formulated biofertilizer.

➢ *Analysis of Soil Fertility Parameters*

After the period of 30 days the soil samples were taken from each pot and sent to the environment department of AES laboratories Pvt Ltd., Noida, Uttar Pradesh, for analyzing the soil fertility parameters i.e. pH, EC (electric conductivity), OC (organic carbon) and N (nitrogen).

III. RESULT AND OBSERVATION

The dilution plates and the streak plates are presented in Fig.1.A and Fig.1.B respectively.

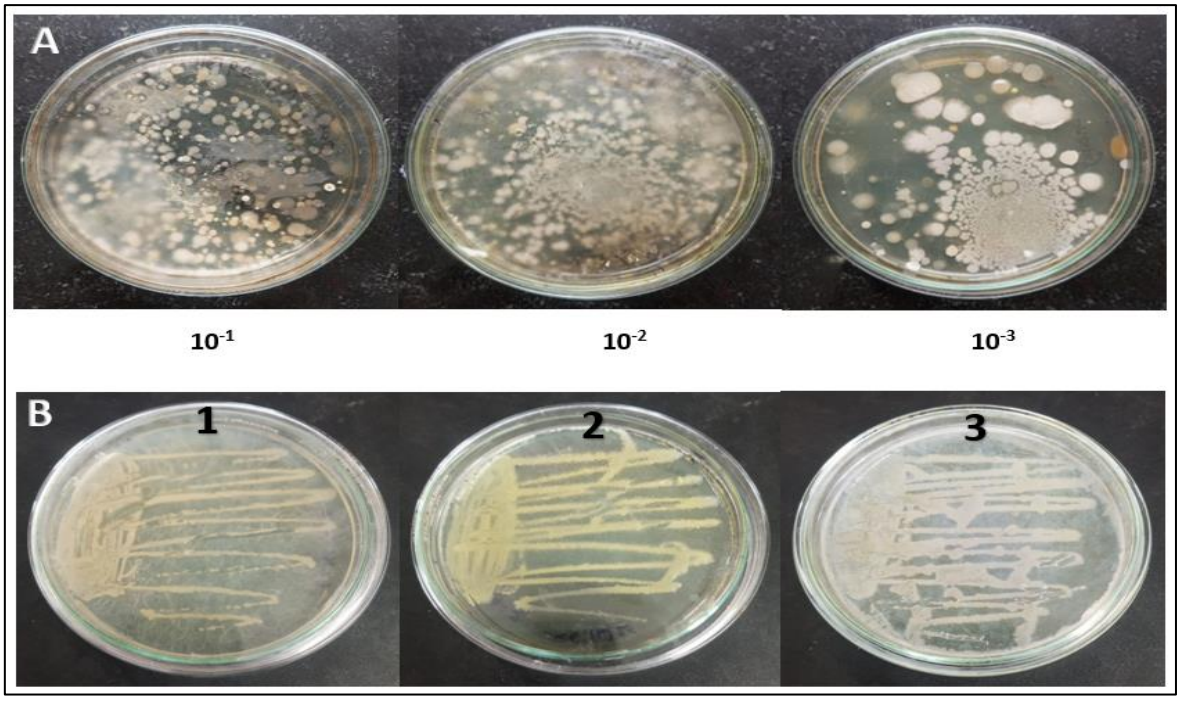

Fig 1 (A) CFU of Soil Samples (B) Streak Plates of Isolates

➢ *Catalase Test:*

All three bacterial isolates gave positive result which shows that they are producing catalase enzyme which breaks down harmful hydrogen peroxide (H_2O_2) into harmless substances.

➢ *Methyl Red Test:*

After the incubation of 48 hours at 37ºC, methyl red indicator was added to each tube. All the bacterial isolates show bright red colour which means it performs mixed acid fermentation and indicate lower pH below 4.4, hence isolates were methyl red positive.

➢ *Voges-Proskauer Test:*

In VP test the bacteria follow the butylene glycol pathway which produces acetoin and 2,3-butanediol. No bacterial isolate shows colour change that indicates the absence of acetoin, hence isolates were VP negative.

➢ *Carbohydrate Fermentation Test:*

After the incubation of 48 hours at 37ºC, phenol red indicator was added to the tubes. All the bacterial isolate shows red colour which indicates that no bacterial isolate ferment a specific sugar, hence the isolates were CFT negative.

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➢ *Urea Test:*

Urea test checks whether the bacteria produce the enzyme urease or not. After the incubation, phenol red indicator was added to the tubes. All the bacterial isolate shows pink colour which indicates that the isolates were urease positive.

➢ *Indole Test:*

After the incubation for 24 hours at 37ºC, Kovac's reagent was added to the tubes. All the bacterial isolates show red ring formation at top of the broth which indicates that isolates were indole positive.

The summary of the biochemical tests carried out for the bacterial isolates are present in Table 1 and Fig.2.

Table 1 Summary of Biochemical Tests Performed and Identified Bacteria

Isolates	Catalase test	Methyl red test	VP test	Carbohydrate fermentation test	Urea test	Indole test
1.Azotobacter	Positive	Positive	Negative	Negative	Positive	Positive
2.Pseudomonas	Positive	Positive	Negative	Negative	Positive	Positive
3.Rhizobium	Positive	Positive	Negative	Negative	Positive	Positive

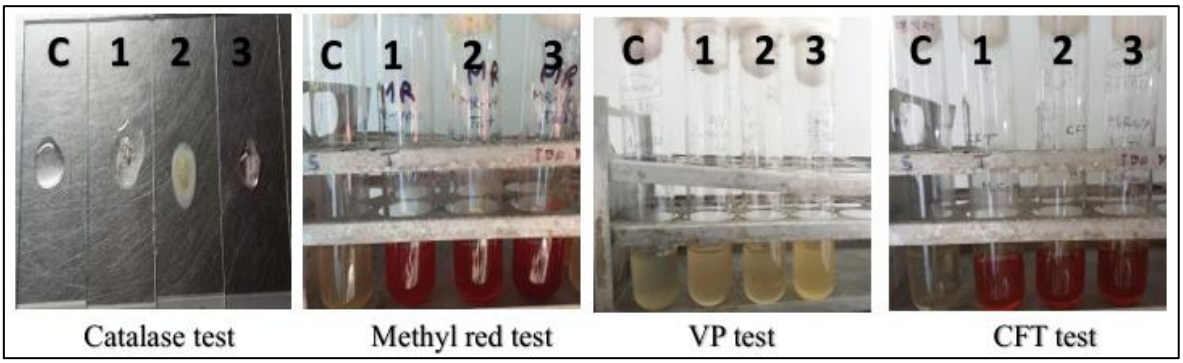

Fig 2 Representative Pictures of the Biochemical Tests (C)Control (1) Isolate 1 (2) Isolate 2 (3) Isolate3

➢ *Soil Analysis:*

Different soil parameters like pH, EC, OC and N were tested for three flowering plants i.e. Petunia, Gazania and Marigold. Calculative data of these parameters can be shown in Table 2, 3, and 4. Impact of the bacterial strains *(Pseudomonas, Azotobacter, and Rhizobium)* can be shown in Fig.3, Fig.4, and Fig.5.

Table 2 Soil Analysis of Petunia Plant

Petunia inoculated with *Azotobacter*				
Parameters	**pH**	**EC (dS/m)**	**OC (%)**	**N (kg/ha)**
POT-1 (control)	7.4	1.5 (slightly Saline)	0.42 (low)	180 (low)
POT-2	6.9	0.59 (normal)	0.80 (high)	360 (medium)
POT-3	7.1	0.55 (normal)	0.75 (high)	310 (medium)
POT-4	7.1	0.42 (normal)	0.72 (medium)	280 (medium)

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Fig 3 Impact of Bacterial Strain (*Azotobacter*) on Petunia Plant

Table 3 Soil Analysis of Marigold Plant

Marigold inoculated with *Pseudomonas*				
Parameters	**pH**	**EC (dS/m)**	**OC (%)**	**N (kg/ha)**
POT-1 (control)	7.4	1.4 (Slightly Saline)	0.45 (low)	200 (low)
POT-2	7.0	0.66 (normal)	0.77 (high)	290 (medium)
POT-3	7.0	0.68 (normal)	0.78 (high)	390 (medium)
POT-4	7.1	0.75 (normal)	0.75 (high)	375 (medium)

Fig 4 Impact of Bacterial Strain (*Pseudomonas*) on Marigold Plant

Table 4 Soil Analysis of Gazania Plant

Gazania inoculated with *Rhizobium*				
Parameters	**pH**	**EC (dS/m)**	**OC (%)**	**N (kg/ha)**
POT-1 (control)	7.4	1.5 (Slightly Saline)	0.49 (low)	190 (low)
POT-2	7.1	0.57 (normal)	0.77 (high)	380 (medium)
POT-3	7.1	0.46 (normal)	0.71 (medium)	435 (medium)
POT-4	6.9	0.51 (normal)	0.76 (high)	470 (medium)

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Fig 5 Impact of Bacterial Strain (*Rhizobium*) on Gazania Plant

Table 5 Soil Analysis of Gazania Plant

Gazania inoculated with *Rhizobium*+ *Pseudomonas*+ *Azotobacter*				
Parameters	**pH**	**EC (dS/m)**	**OC (%)**	**N (kg/ha)**
POT-1 (control)	7.4	1.6 (Slightly Saline)	0.49 (low)	190 (low)
POT-2	7.0	0.58 (normal)	0.77 (high)	480 (medium)
POT-3	7.0	0.62 (normal)	0.80 (high)	550 (high)
POT-4	7.0	0.57 (normal)	0.76 (high)	580 (high)

Fig 6 Impact of Bacterial Strains (*Rhizobium*+ *Pseudomonas*+ *Azotobacter*) on Gazania Plant

IV. CONCLUSION

Pseudomonas, Azotobacter and *Rhizobium* were successfully isolated from the rhizosphere soil of pigeon pea plant that exhibited significant traits that could be applied as formulated biofertilizer. Biochemical characterization showed that the isolates reveal metabolic capabilities, identification traits, specific mechanisms for enhancing plant growth, and producing extracellular enzymes such as catalase.

Formulated biofertilizer was applied to the flowering plants specifically Petunia, Marigold and Gazania. As an impact of formulated biofertilizer, the pH was neutralized from alkali, electric conductivity turns normal to saline, organic carbon and nitrogen value increases as compared to the control. Changes in physical nature of flowering plants

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was also noticed i.e. early blooming of flowers, better vegetative growth and greener leaves (due to increased nitrogen).

In summary, the isolated bacterial strains from rhizosphere of pigeon pea soil confer multi plant growth promoting traits- further demonstrating their promise as a sustainable biofertilizer. The application of rhizosphere bacteria isolated from pigeon pea plant as defined through this research enhance soil fertility, balanced pH, EC, increased OC and N, early blooming of flowers, improve plant growth metrics, promote sustainable agricultural practices and reduces the need for reliance of chemical fertilizer to achieve eco-friendly practices.

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