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Isolation and Biochemical Characterization of *Pseudomonas* spp. for Sustainable Abiotic Stress Management

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ABSTRACT

Five *Pseudomonas* isolates were isolated from the rhizosphere of the Chickpea plant (Cultivar RKGK-13-414). Native strains were identified as plant growth-promoting Rhizobacteria, frequently reported earlier. This study shows that these five novel *Pseudomonas* sp. isolates can be effective new plant growth-promoting *Rhizobacteria,* for that purpose it was necessary to characterize the strains. The market of biofertilizers is expected to reach 3.8$ billion by 2025 from 2$ billion in 2019. The use of biopesticides is increasing slowly at a rate of 8% annually based on the different types of microbial pesticides. Therefore, the present research has been undertaken to discuss PGPR- as bio-controlling agent and promote the widespread adoption of biofertilizers, to promote sustainable agriculture.

Figure : 00 References : 10 Tables : 03
KEY WORDS : Biocontrol, Biofertilizers, PGPR, Phosphate solubilization, Phytohormones, Siderophores

Introduction

The demand for food grain is increasing with the increase in the world population. At present, this requirement is fulfilled majorly by using chemical fertilizers, pesticides, and insecticides. These should be replaced by an eco-friendly substitute for sustainability in agriculture.

Plant growth-promoting *Rhizobacteria* (PGPR)[6] is an indispensable part of the rhizospheric biota and is the most beneficial bacteria that colonize plant roots.[4] Plant growth-promoting *Rhizobacteria* (PGPR) are chemical-free, eco-friendly alternatives to hazardous chemical fertilizers and alternatives to conventional crop protection in agriculture. PGPR increases the growth, yield, and stress tolerance of crop plants. They also improve plant growth through enhanced nutrient uptake from the soil and a wide variety of mechanisms such as siderophore production, phosphate solubilization, biological nitrogen fixation, ammonia production, IAA production, phytohormone production, antifungal activity, and systemic resistance induction. Their emergence as a potent alternative has come in response to the overuse of agrochemical products such as fertilizers and pesticides, which lead to the contamination of soil, fruits, and vegetables[1]. This threat to agriculture and human health has prompted researchers to seek viable alternatives to reduce the use of chemical products. According to studies conducted on this issue, PGPR can be included as biofertilizers, as the most effective organic alternative. The presence of *Pseudomonas spp*. in inoculants as bioformulation in microbial fertilizers plays an effective role in stimulating the yield and growth

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TABLE-1 : Standard Plate Count/ Total Plate Count

S. No	Dilution	Replica	Colony Observed	Av/Mean Value
1.	10^{-1}	A	TNTC	TNTC
		B	TNTC	
2.	10^{-2}	A	90	94
		B	98	
3.	10^{-3}	A	10	11.5
		B	13	
4.	10^{-4}	A	0	0
		B	0	
5.	10^{-5}	A	0	0
		B	0	

traits of crops (increasing seed germination, seedling vigour, enhanced shoot/root ratio and overall plant growth). Some strains of *Pseudomonas spp*. produce chelating agents called siderophores with a high affinity for iron absorption. There is also a proven antifungal activity of pseudomonads VOCs against *Botrytis cinerea*[9] and *Penicillium italicum*[10]. Moreover, *Pseudomonas aeruginosa* strain FG106 was able to produce extracellular proteases and lipases which may contribute to its antagonistic activity against *Alternaria alternata, Botrytis cinerea, and Rhizoctonia solani*[5]. Henceforth, inoculation of bacterial isolates associated with Chickpea (*Cicer arietinum*) crops that have the capability to alleviate drought stress could be used for environmentally sustainable agriculture. The combination of processes that PGPR contributes to make them alternative to replace synthetic chemicals. The above qualities make PGPR potentially usable biofertilizers in agriculture.

Material and Methods

Experiments were carried out to precisely evaluate the *Pseudomonas* strain for its Morphological and Biochemical Analysis.

Isolation of *Pseudomonas* sp. The bacterial isolates were isolated from the different rhizosphere of chickpea (*Cicer arietinum*), Cultivar RKGK-13-414 from experimental fields of Agricultural University, Kota, Rajasthan (India) *23*45 26*33 N* and 75* 27 and 77*26 E** Location in 2019. For each sample, 500 g of soil was collected from the rhizosphere of healthy chickpea plants. Samples were kept in the laboratory at 4°C before analysis. The roots were carefully shaken for bacterial isolation. Around 1 g of rhizospheric soil was added to 9 ml of sterile water, and the mixture was agitated for 2 min, then the surface of root segments was subsequently disinfected by using 2.5% sodium hypochlorite solution for 3 min, rinsed three times with sterile distilled water and 1g was blended in 9 ml of sterile physiologic water. Serial dilutions were separately prepared and 0.1 ml of each dilution was poured onto King B medium to isolate and quantify fluorescent *Pseudomonas* spp. Two replicates were made for each extracted sample, and the plates were incubated for 48 hrs at 28°C. Fluorescent colonies on King B medium under UV light were seen. These colonies were subculture and stored at 4°C for further use. The results obtained were mainly based on the method,[2] which showed that the rhizosphere of chickpea was rich in bacteria. The fluorescent *Pseudomonas* were found in abundance in the

TABLE-2 : The appearance of *Pseudomonas* on different Selective media

S. No	Isolates	Morphology	MacConkey Agar	Starch Agar	*Pseudomonas* Agar	Cetrimide Agar
1.	*Pseudomonas* strain	Size	2-3 mm	2 mm	-	-
2.	-	Shape	Round	Viscous	Round	-
3.	-	Colour	Florescence	Colourless (lactose non fermenter)	Florescence	Florescence Blue green
4.	-	Elevation	Raised	Flat	Raised	Raised
5.	-	Margin	Raised	Entire smooth	Lobate	Entire smooth
6.	-	Texture	Shows florescence under UV Light	Turbid and Florescence	Slimy, moist	Slimy moist

rhizoplane comparatively to the rhizosphere. In our study from the 5 fluorescent *Pseudomonas* isolates, 3 isolates (PSUEDO 1A, PSUEDO 2A and PSUEDO 4A) were isolated from the rhizoplane, 1 isolate (PSUEDO 3A) from the end rhizosphere and 1 isolate (PSUEDO 5A) from the rhizosphere.

Results

Serial Dilution and Standard Plate Count/ Total Plate Count

10 g of soil sample was taken from the rhizosphere of the chickpea (*Cicer arietinum*) plant (Cultivar RKGK-13-414) Agricultural University, Kota, Rajasthan in December'2019. Serial dilution was performed aseptically, after which plating was performed for each dilution on EMB agar (for *Pseudomonas*) after 24 hrs of incubation @ 37°C. The standard plate counts were recorded and given in Table 1.

At 10^{-1} dilution majority of colonies were lactose non-fermenter (colourless) and at 10^{-2} dilution colonies were lactose fermenter (Pink colour).

Control: No growth seen (perfectly OK)

Typical *Pseudomonas* colonies growing on MacConkey Agar plates were inoculated in Asparagine Broth, which is a selective media for the growth of *Pseudomonas sp.* on the basis of fluorescence due to the pigment formation by *Pseudomonas sp.* The colonies from MCA were also selected on the basis of fluorescence after giving a short exposure to UV light. Asparagine Broth after inoculation was incubated at 37ºC for 24 hours. Asparagine Broth tubes were taken out from the incubator and examined visually for the presence of turbidity and fluorescence. It was observed that both the tubes which were inoculated a day before from two MCA replicates having similar fluorescence colonies were turbid as well as florescent colour developed. Both the tubes along with the negative control tubes were again examined under UV light. Under UV light fluorescence became more prominent and later on Cetrimide Agar and *Pseudomonas* Agar plates were used for streaking the inoculum from Asparagine Broth (AB tubes). Both the Agar plates were freshly prepared on the same day. After streaking both the plates were incubated at 37ºC for 24 hrs. The instruction manual Laboratory microbiology by Cappuccino was followed for gram staining. A pure culture of gram-negative rods (Pink color) was observed under a 100X lens using oil emersion, which is one of the confirmatory microscopic examinations of *Pseudomonas.*

Morphological characterization of *Pseudomonas*

Pseudomonas was cultured on different medium for morphological characterization. On Cetrimide Agar the colonies were entirely smooth, raised, slimy moist, and florescent blue-green in colour.

On *Pseudomonas* Agar, the colonies were raised, slimy moist, round in shape with lobate margins, and florescent blue and green in colour. Colonies on Starch Agar were viscous, flat, entirely smooth, turbid and

TABLE-3 : Biochemical characterization of *Pseudomonas* isolates

S. No	Name of tests	Reaction	Indication	Remarks
1.	Gram staining	-ve	Rod shaped, Pinkish in colour	Gram staining showed gram –ve bacteria
2.	Motility	+ve	Growth area extending away from the inoculation line	Gram negative bacteria showed motile extending growth area formation
3.	Simmon's Citrate test	+ve	Colour changes from green to Blue	Citrate metabolizing gram -ve bacteria
4.	Methyl Red test	+ve	Colour turns from Yellow to red ring formation	Isolated bacteria have ability to utilize Glucose
5.	Catalase test	+ve	There were no oxygen bubbles	Isolated bacteria formed bubbles resulting from production of O_2 gas
6.	Oxidase Test	+ve	The colour of oxidase disc change to purple	Isolated bacteria changed the colour of oxidase disc to purple
7.	TSI	+ve	Colour changed from red to yellow.	Bacteria produced H_2S at the . butt confirming Lactose fermentation
8.	Urease test	-ve	Colour did not change from yellow to pink	Bacteria does not have ability to utilize urea
9.	VP test	-ve	Colour does not change from yellow to red ring on the surface.	Bacterial is not able to produce acetyl methyl Carbinol from glucose fermentation
10.	Indole test	-ve	No formation of pink colour ring on the surface.	Does not have ability to decompose Tryptophane to Indole

colourless. On MacConkey Agar the colonies appeared to be round, raised, 2mm in size, and showed fluorescence under UV light.

Biochemical characterization of *Pseudomonas* isolates.

Bacterial colonies having enzymatic potential can be characterized using major biochemical tests. Selected isolates were tested for major biochemical characteristics that include oxidase test, gelatin hydrolysis, catalase test, MR-VP test, indole utilization test, triple sugar iron test, urease test etc.

The organisms show a positive starch hydrolysis test, oxidase test, catalase test, triple sugar iron test, simmon's citrate test and methyl red test. The motility test showed diffuse, hazy growths which shows a positive test for *Pseudomonas.*

Urease test, gelatine hydrolysis test, indole production test, and Voges-Proskauer test (VP) were reported negative (-ve).

Simmons's Citrate Agar plates developed blue colour on the slope portion after incubation of *Pseudomonas* culture. Hence *Pseudomonas* shows (+ve) positive for Simmon's Citrate Agar Test. The starch has been hydrolysed; a clear zone of hydrolysis

surrounds the growth of the organisms which indicates positive result.

Triple Sugar Iron Test for *Pseudomonas*

The Triple sugar iron agar slants turned into pinkish colour whereas there was no change in the butt (bottom) this indicates there was no gas formation. Although an isolated organism (*Pseudomonas*) was able to ferment one of the sugars which resulted in colour change of the slope portion of the slant.

Under ultraviolet light (360 nm) these five isolates (PSU1A, PSU2A, PSU3A, PSU4A and PSU5A) showed fluorescence production and yellowish-green pigment in KB medium. These isolates showed positive tests for oxidase and motility. They were gram-negative. The isolates PSU2A and PSU5A were positive for catalase activity, whereas PSU5 and PSU4 were negative PSU3 and PSU1 showed positive responses for nitrate reduction, while PSU5 and PSU4 were negative. Phosphate solubilization test (+ve) for *Pseudomonas*.

Anti-biotic sensitivity test for *Pseudomonas*

A sensitivity test was done for +ve *Pseudomonas*. In the sensitivity test, Ofloxacin, revealed the highest significant antibacterial activity with showing an inhibitory zone. Antibacterial activity study suggested that the rhizospheric soil of chickpea, had a broad spectrum of intermediate antimicrobial activity against isolated bacteria with prominent inhibition zone. The above tests provided detailed information about the isolation, characterization, and antibiotic sensitivity assay against *Pseudomonas*. The present investigation would be a good source of information for the molecular detection of this bacteria and design of a suitable control technique. This study confirms us the competency of some antibiotic and soil bacteria as natural antimicrobials and suggests the possibility of employing them as biocontrol agents for the management of infectious diseases caused by *Pseudomonas*.

Discussion

Although possessing various PGP traits, there are fewer publications regarding their PGP effects,[7] so there was an instant need to uncover this mistry for sustainable agriculture. Hence, we decided to isolate *Pseudomonas* from cultivar RKGK-13-414 from the different rhizosphere of Chickpea (*Cicer arietinum*) from Agricultural University, Umaidganj, Kota, Rajasthan. These studies suggested that the rhizospheric soil of chickpea was abundant in PGPR

Previously, substantial work was done on fluorescent *Pseudomonas*-mediated drought stress tolerance in mung beans (*Vigna radiata*). Research based on physiological and molecular levels have established *Pseudomonas aeruginosa* GGRJ21 as a very prominent osmotic stress-tolerant strain. It has capabilities to alleviate drought stress tolerance in host plants.[8] So, native strain of *Pseudomonas* can be proved to drought and heat stress, usually consists of a wide range of PGP traits like nitrogen fixation, ACCD aminase activity, antibiotic production and production of plant-beneficial compounds such as plant hormones, HCN, siderophores, EPS, IAA, and ammonia etc. These plant hormones play a significant role in the alleviation of drought and heat stress in various cereal crops. Growth stimulation mechanisms, including the ammonia production, phosphate solubilization, production of phytohormones, and colonization of plant roots, are the most efficacious mechanisms that explain PGPR effects.[3] This research will help future researchers to reveal the capability of new strains in the alleviation of drought stress with their collaborative approaches. The overall impact of microbe-mediated elicitation responses in plants, whether at the physical, biochemical, or molecular level, may be helpful in alleviating drought stress tolerance and, in a cumulative manner, constitutes the basis of eco-friendly stress management strategy. The idea to isolate PGPR from a chickpea plant opens new ways to mitigate drought stress. Past researchers observed that the fertilizer treatments containing the biocontrol agent resulted in a striking increase in the relative density of the genus *Pseudomonas* spp., with one specific dominant OTU in particular, OTU7, showing the strongest response.

Further researchers may use this new species for biofertilizers on other cereal crops, aiming at preventing damage to ecosystem. We should elucidate the pathways of the mechanisms involved in this approach such as siderophores and phosphate solubilization. In the future, plant growth-promoting *Pseudomonas* strains might be an efficient alternative to chemical fertilizers and pesticides for increasing agricultural production in environment friendly and several promising ways.

Conclusion

Future research should be targeted on the potential of these five PGPRs, as well as their co-inoculants, to enhance plant growth and overall development of the crop and for practical applications of these inoculants' bioformulation as biofertilizers in agricultural fields. A huge biological potential of *Pseudomonas sp.* is suggested by various scientific research, and they can be used as an alternative to chemical fertilizers and pesticides. This study will also be useful to other researchers to take forward the

references for the scientific evaluation of PGPR. Our experimental design allowed us to disentangle the importance of different components of bioorganic fertilizer in alleviating drought tolerance. We could gain insight into how *Pseudomonas* spp. are promoted in soils leading to a combined action together with the biocontrol agent to achieve disease suppression as well as abiotic stress management. These insights provide new mechanistic underpinnings to how specific management measures lead to suppressing abiotic stress, opening up new opportunities for more effective applications. For instance, future biocontrol strategies might involve co-inoculating synergistically interacting bacterial species to specifically promote drought stress tolerance. Potential strains not only screened for their ability to minimize abiotic stress but also for their ability to stimulate potentially synergistic resident populations.

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