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Antifungal Activity of Plant Growth-promoting Rhizobacteria Isolates Against *Fusarium* and *Pythium* spp. in Saffron Plant

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ABSTRACT: Sixteen plant growth-promoting rhizobacteria (PGPR) strains were isolated from the rhizoplane and rhizosphere of saffron (*Crocus sativus* L.) plant from five different sites of four different seasons of pampore area of Kashmir valley. These strains were analyzed for production of indole acetic acid (IAA), phosphorous solublization capability, production of hydrogen cyanide (HCN) and inhibition of *Fusarium* and *Pythium* spp. Thirteen strains produced indole acetic acid (IAA) ranging from 13.4 to 52.5 µg. ml^{-} and all possessed phosphorus solublization capability ranging from 70.9 to 292.0. Eleven strains produced HCN ranging from low to maximum range. Out of these sixteen strains twelve showed percentage inhibition against the growth of mycelium of *Fusarium* ranging from 48.66 to 65.42 and thirteen showed percentage inhibition against mycelium growth of *Pythium* species ranging from 48.32 to 71.22.

Keywords: Plant Growth-Promoting Rhizobacteria, *Fusarium* spp. *Pythium* spp., Antifungal activity, Saffron.

INTRODUCTION

Bacteria that colonize the rhizosphere and plant roots, and enhance plant growth by any mechanisms are referred to as plant growth promoting rhizobacteria (PGPR). For sustainable agriculture production, these interactions play a pivotal role in transformation, mobilization, solubilization, *etc*from a limited nutrient pool in the soil and subsequent uptake of essential plant nutrients by the plants to realize full genetic potential of the crops (Kloepper, 2003; Fatima *et al*., 2009; Pattnaik *et al*., 2021; Bhat *et al.*, 2023; Sanjeevani *et al.*, 2023). In the biogeochemical cycles of both inorganic and organic nutrients in the soil and in the maintenance of soil health and quality, soil microorganisms are very important (Jeffries *et al*., 2003). The beneficial effects of PGPR have been observed in many crops including horticultural, oilseed crops etc (Dey *et al.*, 2004; Hamid *et al*., 2021) However in saffron, reports are scantly especially as biocontrol aspects. Biological control of plant diseases is gaining attention due to increased pollution concerns because of pesticides use for crop protection and development of pathogen resistance (Wisniiewski and Wilson 1992). The use of environmental friendly microorganisms has proved useful in plant-growth promotion and disease control in modern agriculture (Weller, 1988; Nazneen *et al*., 2022; Tanvi *et al*., 2024). Kravchenko *et al.* (2002) observed that PGPR inoculation is a promising agricultural approach that plays a vital role in crop protection, growth promotion or biological disease control. Despite extensive evidence of the growth-promoting and biocontrol potential of PGPR in many crops, their role in saffron particularly in disease management remains poorly studied, highlighting a significant knowledge gap for sustainable saffron cultivation.

MATERIALS AND METHODS

Jammu and Kashmir State is located between 32.17º and 37.6º north latitudes and 73.26º and 80.30º east longitudes. The Kashmir division which is a "Bowl shaped" valley surrounded by north-west Himalayan folds on all sides, has a continental climate, characterized by marked seasonality. On the basis of temperature and precipitation in the valley, the climate of Kashmir is divided into the following four seasons:
(i) March to May (Spring season)
(ii) June to August (Summer season)
(iii) September to November (Autumn season)
(iv) December to February (Winter season)
The agro climatic conditions of Kashmir are ideal for the cultivation of saffron plant, mainly in Pampore (district Pulwama) which is a hub for its production. The selected plant for the present study was "Saffron

plant" and the area of study during the tenure were the saffron fields of Pampore. Soil samples were collected in four different seasons of Kashmir encompassing the four major stages in the life cycle of saffron plant.The four being:

Winter (Mid Dec-Mid-Jan)
Spring (Mid Mar-Mid Apr)
Summer (Mid Jun- Mid Jul)
Autumn (Mid Sep-Mid-Oct)

Isolation and characterization of PGPR from saffron rhizosphere: In the present study soil from the rhizosphere of the saffron plant along with the roots (corms) were collected at a depth of 10 to 25 cm from the surface with the help of sterilized suitable equipment and packed in sterilized zip polythene bags. Replicates of the soil samples were taken at five different random sites of same field during each sampling in four different seasons and these samples were kept in 4°C of the refrigerator till analysis.

Soil pH and Electrical conductivity (EC)

The soil pH was determined by following Jackson (1967) with a slight modification of the method. It was obtained by taking 10 gm of soil sample in 90ml distilled water at 1: 9 soil water suspension with the help of combined electrode (glass and calomel) in digital pH meter. Electrical conductivity of the soil sample was determined in the same ratio 1: 9 soil water suspension as used for measuring pH with the help of a Conductivity Meter following modified Jackson (1967) method. It is expressed in deci Siemens per meter (dS m^{-1}) at 25°C.

Serial dilution/Microbial count: The samples of each season rhizosphere soil were mixed thoroughly to make a composite soil. 10g of soil sample was suspended in 90 ml of sterile distilled water considered as a stock solution and then transferred 1ml of the soil suspension into 9 ml sterile distilled water with the help of a sterile pipette to yield 10^{-1} dilution. Similarly, a series up to 10^{-7} dilutions were prepared under aseptic conditions. Bacteria were isolated by employing serial dilution plate technique using nutrient agar (Peptone 5g/l; Beef extract 3g/l; NaCl 5g/l; Agar 15g/l) a general purpose medium. Soil suspension 0.2 ml was introduced into sterilized nutrient agar media in Petri dishes and was spread thoroughly on the media. Incubation at 37°C was done for 24 to 48 hours. For each dilution the plates were taken in triplicates. After incubation was complete morphological characterisation on the basis of colour, shape, size, elevation etc. and also number of colonies were counted. Colonies exhibiting good variable growth were selected for further streaking on fresh plates. Further purification and multiplication of isolates was done by streaking on fresh plates. The CFU. ml^{-1} was calculated using relation.

$$CFU.ml^{-1} = \frac{\text{No. of colonies (average of triplicate)}}{\text{Volume plated (ml)}} \times \text{Dilution Factor}$$

Streak plate method: The streak plate method was standardized for obtaining discrete colonies and pure cultures. The inoculating loop or transfer needle was flame sterilized till red hot, cooled and dipped in a diluted suspension of organisms or touched with a single bacterial colony. This was then streaked on the surface of an already solidified agar plate to make a series of parallel, non overlapping streaks. After streaking the loop was again flame sterilized. The petriplates were sealed with a parafilm and incubated at 28-30°C for 24-48 hours in an inverted position.

***In- vitro* screening of isolates for their plant growth promoting (PGP) activities**

(a) Phosphate Solubilising activity of bacteria: All isolated bacterial strains were first screened for phosphate solubilization on the selective Pikovskaya's agar medium (PAM). Bacterial culture were inoculated on centre of plate containing Pikovskaya's agar media by inoculation loop under aseptic condition and incubated at 30±2ºC for 5 days, a clear zones developed around the colonies showing phosphate solubilization activity of the bacteria (Parikh and Jha 2012).

Pikovskaya's agar media composed as fractions of grams per litre

Yeast extract	_	0.50 g
Ferrous sulphate	_	0.00001 g
Dextrose	_	10.0 g
Calcium phosphate	_	5.0 g
Ammonium sulphate	_	0.50 g
Potassium chloride	_	0.20 g
Magnesium sulphate	_	0.10 g
Manganese sulphate	_	0.0001 g
Agar	_	15.0 g
Distilled water	_	1000 ml

Quantitative estimation of Phosphate: - Quantitative estimation of phosphate solubilization of those isolated bacteria which had already shown PSB activity on PA media, then these strains were introduced inPikovskaya's broth containing ($MgSO_4.H_2O$ (0.25 g/l), KCl (0.2 g /l), $(NH_4)_2SO_4$(0.1g/l), $Ca_3(PO_4)_2$ (5g/l), glucose (10 g/l, yeast extract 0.50g/l, Ferrous sulphate 0.00001g/l, Manganese sulphate (0.0001g/l) and this test was carried out using Erlenmeyer flasks (250 ml) containing 100 ml medium which was inoculated with 500 µl of the bacterial strain. Un-inoculated medium served as control and it was also subjected to similar conditions. The flasks were kept on a shaker incubator at 120 rpm at 28°C- 30°C. The cultures were harvested by centrifugation at 10,000 rpm for 10 min in a Remic centrifuge. The phosphate in supernatant was estimated by vandate-molybdate reagent. The 1ml culture supernatant was mixed with 10ml vandate-molybdate reagent. The absorbance of the resultant yellow color was read at 470 nm in a Systronic spectrophotometer. The total soluble phosphate was calculated from the standard curve of stock solution made from KH_2PO_4 containing ascending concentration which were logrithimic. The values of soluble phosphate thus liberated were expressed as µg.ml^{-1} (Nautiyal, 1999).

(b) Production of IAA (indole-acetic acid):-50 ml of Nutrient broth (NB) containing 0.1% DL-tryptophan was inoculated with 500 µl of 24 h old bacterial cultures and incubated at 28±2 °C for 3 days. The bacterial cultures were centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4ml of the Salkowski reagent (50 ml of 35% percholoric acid, 1ml

0.5M $FeCl_3$ solution). Development of pink colour indicated IAA production. Optical density was taken at 530nm with the help of Systronic spectrophotometer. Concentration of IAA produced by cultures was measured with the standard curve obtained from stock solution of IAA 5µg/ml and diluted to ascending logrithimic concentrations. The values of IAA were expressed as $\mu g.ml^{-1}$ (Kumar *et al*., 2012; Kaur and Sharma 2013).

(c) Production of HCN:-Isolates were screened for the production of hydrogen cyanide. Nutrient broth was amended with glycine (4.4 g/l) and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with parafilm and incubated at 30°C for 4 days. Development of orange to red colour in the picric acid saturated filter paper discs indicated HCN production (Kumar *et al*., 2012).

(d) In vitro antifungal activity of PGPR: Bacterial isolates were assayed for antagonist activity against fungi by using dual culture technique. Isolated bacteria were spot inoculated on nutrient agar plates and also introduced with the 4mm disc of fungi at the centre. The plates were incubated for 5–6 days at 28°C. Bacteria were evaluated for showing antagonist activity against fungi and further to be used as potential in the biological control of the fungi. The percentage inhibition of fungal colony growth was calculated by the following relation

Percentage inhibition (I) = C-T/C × 100

Where "C" = mycelial growth of pathogenic fungal colony in control fungal culture that is measured in "mm" from the centre of colony up to the margin of the colony taken 5 to 6 times and then average of these is taken as the value of "C".

"T" = mycelial growth of pathogenic fungal colony in dual plate culture measured in "mm" from the centre of the colony up to the margin towards the bacterial colony that is inhibitory to the fungal growth but value of "T" is taken as the average of three replicates of the same test organism.

RESULTS

The soils of Pampore where the saffron is grown in the Kashmir valley of Jammu and Kashmir state, India are karewa soils. These are basically loamy sandy soils with light yellow texture. The four seasons worked here for PGPR analysis in consonance with the major events in the saffron plant life cycle show pH variation in the soils. These range from 6.83, 7.83, 6.9 and 6.82 from winter, spring, summer and winter seasons respectively (Table 1). The soils shows variation in electric conductivity ranging from minimum in summer 0.13 and maximum in winter 0.32 $ds.m^{-1}$ (Table 1).

The mean CFU ml^{-1} presenting the bacterial load of the rhizosphere of saffron shows maximum presence between mid June to mid July which are the temperate summer months in Kashmir. This is the dormancy time of the saffron plant with mother corm staying underground. The maximum $CFU.ml^{-1}$ count was 1.6×10^6 in summer whereas on lower side it was 7.5×10^5 in spring when extreme cold ebbs out (Table 2).

Of these colonies only 16 such bacteria were selected with effort which were active as far as plant growth promoting rhizobacterial characteristics were concerned. All these sixteen isolates were different for each season represented by different species. Assessment based on phosphate solubilising capacity (PSB), the 16 bacterial isolates showed variations in their activity (Table 3). The bacterial isolates were also tested for their plant growth promotion trait *viz.* production of IAA in which 13 of the 16isolatesshowed variations in their IAA producing capacity and maximum production by isolate DZ1 and minimum by PZ2 as 52.18 and 12.32 respectively (Table 3). It was seen that in some of these PGPR isolated, phosphate solubilisation was as high as 292 $\mu g.ml^{-1}$ in isolate DZ1 and low 70.92 $\mu g.ml^{-1}$ in MZ2 isolate. In the present study test performed showed that eleven isolates of the 16 show HCN positive test, seven show small, while four shows medium production of HCN (Table 3).

Antifungal activity of bacterial isolates against species of *Fusarium* and a species of *Pythium* using agar streak method

A test was performed for these isolates to check their antagonism against main pathogenic fungi of saffron corm showed that most of these isolates were inhibitory for a species of *Fusarium* and a species of *Pythium* (Table 4). Out of sixteen isolates twelve shows inhition against *Fusarium* while thirteen shows inhibition against *Pythium* spp. Some of the isolates induced more than 70 percent inhibition in *Pythium* spp. growth and in any case majority of the forms showed between 50 to 60 percent inhibition (Table 4).

Table 1: Some physical properties of soil collected from saffron fields in and around Pampore area in Kashmir valley.

Sr. No.	Season	Mean pH	Average electric conductivity ($ds.m^{-1}$)
1.	Winter (Mid Dec-Mid-Jan)	6.83±0.24	0.32±0.02
2.	Spring (Mid Mar-Mid Apr)	7.83±0.36	0.18±0.02
3.	Summer (Mid Jun- Mid Jul)	6.9±0.24	0.13±0.1
4.	Autumn (Mid Sep-Mid-Oct)	6.82±0.42	0.24±0.2

Table 2: Rhizobacteria enumeration as shown by the saffron plant rhizosphere soil.

Sr. No.	Soil samples	Dilution Factor (D)	Amount of Sample (µl) (V)	Average No. of Colonies (N)	Mean CFU.ml^{-1}
1.	Mid Dec-Mid Jan	10^{-3}	0.2	276	8.0×10^5
		10^{-4}	0.2	164	
		10^{-5}	0.2	42	
2.	Mid Mar-Mid Apr	10^{-3}	0.2	265	7.5×10^5
		10^{-4}	0.2	148	
		10^{-5}	0.2	36	
3.	Mid Jun-Mid July	10^{-3}	0.2	540	1.6×10^6
		10^{-4}	0.2	330	
		10^{-5}	0.2	95	
4.	(Mid Sep-Mid-Oct)	10^{-3}	0.2	461	1.2×10^6

Table 3: The quantitative phosphate and IAA levels and qualitative HCN production of PGPR shown by the 16 isolates during four seasons of saffron life cycle.

Sr. No.	Isolates	Phosphate solubilisation µg.ml^{-1}	Production of IAA µg.ml^{-1}	HCN production
1.	DZ1	292.0	52.18	–*
2.	DZ2	261.23	32.5	++
3.	DZ3	75.14	–	+
4.	DZ4	259.12	19.68	–
5.	MZ1	192.41	26.87	++
6.	MZ2	70.92	–	++
7.	MZ3	71.62	–	+
8.	MZ4	83.62	13.43	–
9.	JZ1	115.16	16.87	–
10.	JZ2	117.00	26. 37	+
11.	JZ3	143.96	19.32	++
12.	JZ4	236.65	32.5	+
13.	PZ1	148.32	18.36	+
14.	PZ2	132.25	12.32	–
15.	PZ3	98.65	18.14	+
16.	PZ4	107.16	19.14	+
17.	Control	000.00	00	00

(+*) and (–*) represents presence or absence of activity and respectively +++ represents maximum activity, ++ medium and + represents minimum activity.

Table 4: Percentage inhibition of common pathogenic fungi- *Pythium* spp. and *Fusarium* sp. of saffron underground corm as shown by various of PGPR isolates.

Sr. No.	Bacterial Isolates	Percentage of inhibition *Fusarium* sp.	Percentage inhibition of *Pythium* sp.
1.	Control	–	–
2.	DZ1	58.52	52.64
3.	DZ2	64.34	61.2
4.	DZ3	52.91	64.23
5.	DZ4	–	–
6.	MZ1	66.2	71.22
7.	MZ2	65.42	63.33
8.	MZ3	–	55.52
9.	MZ4	58.05	48.32
10.	JZ1	60.32	–
11.	JZ2	–	52.41
12.	JZ3	48.66	54.22
13.	JZ4	52.94	67.33
14.	PZ1	–	58.32
15.	PZ2	52.46	–
16.	PZ3	49.86	58.36
17.	PZ4	58.32	56.84

DISCUSSION

The soils of Pampore where the saffron is grown in the Kashmir valley of Jammu and Kashmir state, India are karewa soils. These are basically loamy sandy soils with light yellow texture.The pH of the soil is an important soil physical trait for root level microorganisms to grow. A meager variation can mean variations in the microbes at the root level of plants having different root systems. Interestingly the winter season constitutes the main active vegetative phase of the plant when roots which come out from the base of the corm grow underground and temperatures in open fields are extreme low. The pH variation does not seem to affect the electric conductivity which shows a continuous recession from winter to summer through spring. Overall the pH seems neutral. Neutrality of the saffron soils has also been reported by Ambardar and Vakhlu (2013). The developmental seasonal periodicity was reported earlier by Koul and Farooq (1984).

The summer bulk soil bacterial load almost matches with that reported by Ambardar and Vakhlu (2013) for the flowering season starting October to November. However, they have reported 40 fold higher load in rhizosphere soil than the bulk soil. At every stage of analysis for the bacterial presence it was always more than hundred colonies presented by lower dilutions on plating and the colonies receded on further dilutions.

It is now established that PGPR bacteria have an inherent and main characteristic of solubilising phosphate and then making the free phosphorous available for the plant (Bhattacharya and Jha 2012; Selvakumar *et al*., 2009; Goldstein, 1994; Oves *et al*., 2009; Mohamed *et al.,* 2021).

The IAA production and its enhancement in the vegetative growth of PGPR bacteria was of significance here. Such report in case of saffron rhizosphere PGPR is not new, others also (Parray *et al.,* 2013) in saffron and in various other plant root systems PGPR are shown to bring about phosphorous mobilisation and also solubilisation of insoluble forms of phosphate by the secretion of organic acids around the root ecosystem (Zaidi *et al.,* 2009; Vikram and Hamzehzarghani 2008).

Certain PGPR also produce HCN as cyanogenic activity. This they do in response to antagonise the growth of pathogens otherwise pathogenic for host plant root and also for their own sustenance. This is also considered an additional trait effecting indirect increase in the crop production (Wani *et al.,* 2008; Ahemad and Khan, 2011; Pathak *et al*., 2019; Pattnaik *et al*., 2021: Bhat *et al.,* 2023; Lone *et al*., 2023). Some of the isolates induced more than 70 percent inhibition in *Pythium* spp. growth and in any case majority of the forms showed between 50 to 60 percent inhibition. Such antagonism of PGPR against soil fungal pathogenic species is well documented for PGPR bacteria (Gulati *et al*., 2008; Gupta *et al.,* 2011; Gopalakrishnan, 2012; Bhat *et al.,* 2023; Noor *et al*., 2023; Nisar *et al.,* 2023).

The present study thus through various analytical traits and isolations can be utilised as the identified species for PGPR activity in saffron to improve plant growth and development and thereby improve yield and quality of the commercial produce. The identifications of these sixteen isolates of these forms and should constitute a part of any further study on these as done by Ambardar and Vakhlu (2013) for the PGPR of saffron plant at its flowering time.

SUMMARY

The study investigated sixteen plant growth–promoting rhizobacteria (PGPR) strains isolated from the rhizoplane and rhizosphere of saffron (*Crocus sativus* L.) collected across different sites and seasons in the Pampore area of the Kashmir Valley. Most isolates showed strong plant growth–promoting traits, including indole acetic acid (IAA) production, phosphorus solubilization, and hydrogen cyanide (HCN) production. Several strains also exhibited significant antagonistic activity against major soil-borne pathogens, *Fusarium* and *Pythium*, indicating their dual role in enhancing plant growth and suppressing diseases.

FUTURE SCOPE

The promising PGPR strains identified in this study can be further developed as biofertilizers and biocontrol agents for sustainable saffron cultivation. Future research should focus on molecular characterization, field-level validation, formulation development, and evaluation of their long-term effects on saffron yield and quality. Integration of these native PGPR strains into saffron agroecosystems could reduce dependence on chemical fertilizers and fungicides, promote eco-friendly agriculture, and support the revival and productivity of saffron cultivation in the Kashmir Valley.

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