INVESTIGATION FOR BENEFICIAL CHARACTERISTICS OF PLANT GROWTH PROMOTING RHIZOBACTERIA FROM OKRA PLANTS COLLECTED FROM DIFFERENT REGIONS

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Abstract. Plant growth-promoting rhizobacteria (PGPR) are known to enhance soil fertility, crop yield, and plant growth through various direct and indirect mechanisms. This study aimed to investigate the beneficial characteristics of PGPR isolated from okra plants collected from district Mansehra, Khyber Pakhtunkhwa, Pakistan. Twenty-three morphologically distinct strains were isolated and subjected to a series of biochemical tests, including gram staining, phosphate and zinc solubilization, hydrogen cyanide (HCN) production, indole-3-acetic acid (IAA) production, exopolysaccharide (EPS) production, ammonia production, hydrolytic enzyme production, and nitrogen fixation. The results revealed that 15 strains were capable of solubilizing phosphate, and 13 strains could solubilize zinc. None of the tested strains produced HCN, while 2 strains were able to producers IAA. EPS production was observed in 11 strains, and ammonia production was noted in 20 strains. Hydrolytic enzyme assays showed that 13 strains produced amylase, 16 produced cellulase, 16 produced pectinase, and 20 produced proteases. Notably, all isolated strains demonstrated the ability to fix nitrogen. These findings highlight the potential of these PGPR strains as biofertilizers to improve soil fertility and promote plant growth, particularly in okra cultivation.
Keywords: *PGPR, Okra, soil fertility, crop yield, HCN*

Introduction

Worldwide, in many countries, including Pakistan, *Abelmoschus esculentus* (Okra) is considered as one of the important vegetables. It is cultivated in summer and depending upon regional climactic conditions its peak cultivation occur between late spring and early autumn. It needs nutrient-rich and well drained soils with 25–35°C as optimum growing temperature (Khan et al., 2013). It is widely cultivated in Pakistan and every year its cultivation area increased (Hussain et al., 2016; Javed et al., 2009). In Pakistan, the annual production of okra is 109.3 thousand tons from cultivation area of 14.5 thousand hectares contributing 2% to the total global production (Kassi et al., 2018). Whereas the area under okra cultivation in Khyber Pakhtunkhwa (KPK) is 1957 ha with production of 15630 tons (Ramay, 2014). Mansehra District located in Eastern-western mountainous zone of Khyber Pakhtunkhwa Province of Pakistan. Okra is cultivated in this area from mid-March to mid-May. It is an agriculturally significant area known for its diverse agro-climatic zones,

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ranging from lowland valleys to mid-altitude regions. The fertile soil of this area and extensive vegetable farming practices creates a favorable environment for rhizosphere microbial diversity (Naheed et al., 2013). Okra has good nutritional value with high demand of consumers, comparing with this the production per hectare is very low, and this is because of abiotic stress such as soil salinity, osmotic stress, ionic imbalance and biotic stress like various soil-borne pathogens like bacteria, fungi and nematodes (Dudley et al., 2008; Afzal et al., 2013; Sultana et al., 2005).

The excessive growth in world population demands high food production and continuous change in the climate make it difficult to meet the demand. For increasing the fertility of agricultural soil different inorganic fertilizers, manure and pesticides are being used which are associated with harmful effects on environment for example absorption of hazardous chemical like cadmium in the soil, eradication of useful insects and different human health problems (Azeem et al., 2025; Naveed et al., 2024; Zameer et al., 2023; Khatri and Tyagi, 2015). Continuous and intensive use of these inorganic fertilizer result in acidity of soil and imbalance in nutrients which reduce crop yield (Shafique et al., 2015).

To overcome these problems, substitutive methods are being implemented by using biotechnological applications. The most effective and ecofriendly substitute of inorganic fertilizers are plant growth-promoting (PGP) microbes. In past few years, these PGP are used by researchers as biofertilizers for increasing growth in agricultural soil (Vurukonda et al., 2018). Rhizosphere is the soil area present around plant roots and contain number of microbes which influence the growth of plants by providing nutrition (van de Mortel et al., 2012; Bonfante and Anca, 2009). Bacteria that colonize plant roots and increase their growth are known as plant growth promoting rhizobacteria (PGPR) (Kloepper, 1978). They have ability to promote plant growth, provide resistance against root diseases and environmental stresses (Papenfus et al., 2015). These PGPR influence plant growth by several mechanisms that are (1) plant growth promoting chemicals like gibberellins, auxins, nitric oxide and cytokinins, (2) abscisic acid jasmonic acid as stress-associated plant growth regulators, (3) production of antimicrobial agents, (4) nitrogen fixation, (5) siderophores production for iron mobilization, (6) phosphate solubilization and (7) ACC (1-amino-cyclopropane-1-carboxylic acid) production (Mayak et al., 2004; Cassán et al., 2014; Costa et al., 2013).

In addition to these mechanisms PGPR need some other characteristics for example having broad spectrum of action, ability to promote plant growth, high competence in rhizosphere, ecofriendly, compatible with rhizobacters and resistant against stress factors like temperature, radiations (UV) and oxidizing agents (Vejan et al., 2016). Biochemical characterization of potent PGPR help us to use them in future technologies like formulation of PGPR biofertilizers is revolutionize by their nano encapsulation (Javed et al., 2024; Duhan et al., 2017). The combined effect of symbiotic and non-symbiotic microbes in the improvement of plant growth have been studied by different researchers. A number of species of rhizobacteria from genera *Serratia, Streptomyces, Rhodococcus, Pseudomonas, Mesorhizobium, Klebsiella, Flavobacterium, Enterobacter, Burkholderia, Bradyrhizobium, Bacillus, Azotobacter, Azospirillum, Arthrobacter, Alcaligenes,* etc are recognized for promoting plant growth, enhancing crop production, emerging of seed and agricultural development (Tariq et al., 2014; Ahmad et al., 2008; Rafique et al., 2018). The use of these microbes with diverse characteristics as PGPR not only increase crop yield but also decreases the use of hazardous chemical fertilizer (Ullah and Bano, 2015). The present study focusses on the isolation and characterization of PGPR from okra to confer their plant growth promoting and biocontrol activities.

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Material and methods

Sample collection

For investigating plant growth promoting rhizobacteria, the experimentation was performed in Microbiology lab of Abbottabad University of Science and Technology. The study area was Mansehra district, situated in Eastern-western mountainous zone of Khyber Pakhtunkhwa province of Pakistan. The sampling area was selected based on soil type, cultivation practices and altitude. Okra plants were collected in the peak cultivation season from 5 distant agricultural areas. These areas were Baffa (34.4323°N, 73.2622°E), Garhi Habibullah (34.3190°N, 73.4703°E), Dhodial (34.3752°N, 73.2103°E), Labarkot (34.4550°N, 73.2717°E) and Potha (34.3000°N, 73.2000°E). The soil adhered to the roots of the plants was removed and stored in labeled sterile polythene bags at 4°C for further process.

Isolation of rhizobacteria

Rhizobacteria were isolated from the soil by using serial dilution technique on Luria Bertani agar media. The cultured plates were incubated at 30°C for 24 h. Different colonies were appeared and single colonies were selected and streaked on LB agar. The bacterial colonies which were purified are preserved at 80°C in Eppendorf tube containing LB broth and also in glycerol (20%) (Dastager et al., 2011; Singh et al., 2023).

Morphological characterization

The isolated rhizobacterial colonies were morphologically characterized on basis of their size, shape, margin, color, opacity and elevation. Furthermore, Gram staining of each strain was performed according to protocols described in Bergey’s Manual of Determinative Bacteriology (Holt et al., 1994).

Screening of rhizobacteria for plant growth promoting properties

Phosphate solubilizing test

Qualitative analysis

Qualitative estimation of phosphate solubilizing rhizobacteria was performed by using Pikovaskaya’s agar medium. The chemical composition of the media (g/l) was, 0.2 g potassium chloride, 10 g Glucose, 0.2 g Sodium chloride, 5 g Calcium phosphate, 0.1 g magnesium sulfate heptahydrate, 0.5 gYeast extract, 0.5 g Ammonium sulfate, trace amount of FeSO4.7H2O and MnSO4 and 15 g Agar. The bacterial isolates were spot inoculated on the medium and were incubated for 7 days at 30°C. The positive isolates were indicated by the appearance of clear halo zone around colonies (Pikovskaya, 1948; Verma and Pal, 2020).

Quantitative analysis

The quantitative estimation of phosphate solubilizing rhizobacteria was performed by using Pikovaskaya’s broth medium. The 250 ml acid washed flasks were poured with 100 ml of Pikovaskaya’s broth and sterilized. The isolates were inoculated in sterilized flasks and kept on rotary shaker for 7 days at room temperature. From each flask 15 ml of culture was taken in sterilized tube and centrifuged at 3000 rpm for 15 min. After

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centrifugation 0.5 ml supernatant was diluted up to 6 ml (2.5 ml distilled water, 3 ml color reagent). The medium in tubes were mixed by using vortex and kept for 30 min (Murphy and Riley, 1962; Mahdi et al., 2020). The absorbance was measured by spectrophotometer at 600 nm and solubilized phosphate was compared with phosphate standard curve using tricalcium phosphate.

Indole production test

Salkowski approach defined by Glickmann and Dessaux (1995) is a colorimetric technique used to check rather bacteria can produce indole acetic acid (IAA) or not. For this purpose, bacterial isolates were inoculated in LB broth containing tryptophan (100 μg/ml) as IAA precursor and kept in shaking incubator at 250 rpm for 72 h to 96 h at 30°C. The culture was centrifuged for 10 min at 10,000 rpm, 2 ml of supernatant was taken and mixed with 4 ml of Salkowski reagent and 2 drops of orthophosphoric acid. Positive results for indole production were indicated by the appearance of pink color in tubes. Optical density of samples was measured at 530 nm in spectrophotometer and the obtained values were compared with IAA standard graph (Mahdi et al., 2020).

Ammonium production test

The qualitive assay for production of ammonia by rhizobacterial isolates was performed by using peptone water. Tubes containing 10 ml of peptone water were inoculated with fresh culture and incubated at 30°C for 2 days. Nessler's reagent was mixed in the fermenting media, change in color from brown to yellow shows positive results. For quantitative analysis, absorbance of the media was measured at 630 nm, obtained values were converted into microgram per milliliter unit and standard curved graph was generated from the results (Kumar et al., 2012).

Nitrogen production test

To check the nitrogen fixing ability of isolated strains, nitrogen free semi-solid malate media was prepared. This media contains 0.5% D1 malic acid, 1% sucrose, trace amount of KH2PO4·H2O, K2HPO4· H2O, CaCL2·H2O, NaCl, Na2MO4·2H2O and FeCL3 and 1.5% agar. Isolates were inoculated on media and incubated at 30°C for 7 days. The growth appearance on the media directs the facilitation of nitrogen fixation by isolates (Arfarita et al., 2018; Jaiswal et al., 2023).

Zinc solubilizing test

The Zn solubilizing activity of isolated strains were checked by using Bunt & Rovira media which contain 0.1% of Zinc oxide (insoluble zinc compound). Strains were spot inoculated on media and incubated for 7 days at 28°C. Clear zone around positive colonies indicates positive results (Bunt and Rovira, 1955).

Screening of rhizobacteria for biocontrol abilities

Test for amylase production

Qualitative analysis

Amylolytic activity of the isolates were screened by using 1% starch supplemented LB agar plate technique. Isolates were spot inoculated on media and incubated at 37°C

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for 24 h. The positive results were indicated by the appearance of the clear zone around the colonies treated with 1%-gram iodine (Khattab, 2022).

Quantitative analysis

The amylolytic screened PGPR were cultivated in 1% starch broth media and incubated for 48 h in shaking incubator (150 rpm) at 37°C. Post incubation, culture was centrifuged (8000 rpm) for 15 min and supernatant was collected. This enzyme solution (1 ml) was mixed with 1% substrate solution in 1:1 and kept for 10 min at 50°C. To check the released reducing sugar, Enzyme-substrate solution was mixed with dinitro salicylic acid reagent (DNS) and in boiling water bath incubated for 5 min. At 540 nm, the optical density of the mixture was observed and obtained released sugar values were compared with standard glucose curve graph (Toy et al., 2022; Ajithkumar et al., 2015).

Test for cellulase production

Qualitative analysis

Cellulase production by rhizobacteria was tested using cellulose agar media. A spot was inoculated on media and incubated at 30°C for 48 h. Congo red dye was used as in indicator. Clear colorless zone were appeared around the cellulolytic strain when flooded with indicator reveal cellulose hydrolysis (Khianngam et al., 2014).

Quantitative analysis

Cellulolytic bacterial strains were inoculated in broth media having 0.05% CMC, 0.1% K_2HPO_4, 0.1% $NaNO_3$, 0.05% $MgSO_4$, 0.1% KCl, 0.05% yeast extract and were incubated for 24 h in shaking incubator (150 rpm) at 37°C. Cell free supernatant was obtained by centrifugation of production media for 10 min at 10,000 rpm and used in cellulase assay as crude enzyme. Miller method was used to determine cellulase activity. A reaction mixture containing enzyme solution (0.2 ml) was mixed with 0.5% CMC (1.8 ml) and 50 mM sodium phosphate buffer (pH 7) followed by incubation at 37°C for 30 min in a shaking water bath. The dinitrosalicylic acid reagent (3 ml) was added to stop the reaction. The reaction mixture was kept at room temperature for 5 min. Change in color was observed by checking absorbance at 575 nm. Standard graph of glucose was used to calculate enzyme activity in terms of 1 μmol of glucose released in 1 min (Maravi and Kumar, 2020).

Test for pectinase production

Qualitative analysis

Pectinase production by rhizobacteria was observed by spot inoculation of strains on 2.5% pectin mixed LB agar media incubated at 30°C for 24 h. After the incubation period plates were flooded with 2%-gram iodine (indicator). Clear colorless zone were appeared around the positive strains (Tabssum and Ali, 2018).

Quantitative analysis

For quantitative analysis, submerged fermentation was used, in which 1% freshly prepared inoculum was added 100 ml media and incubated in shaking incubator at 150 rpm, 37°C for 24 h. Supernatant was collected by centrifugation of media at

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8000 rpm for 15 min. This enzyme solution (1 ml) was mixed with 1% substrate solution in 1:1 and kept for 10 min at 50^{o}C. To check the released reducing sugar, Enzyme-substrate solution was mixed with dinitro salicylic acid reagent (DNS) and in boiling water bath incubated for 5 min. At 540 nm, the optical density of the mixture was observed and obtained released sugar values were compared with standard galacturonic acid curve graph (Ejaz et al., 2018).

Test for protease production

Qualitative analysis

Protease production by rhizobacteria was tested using skimmed milk agar medium. This medium contains 10% skimmed milk, 0.1% peptone, 0.5% NaCl and 2% agar. A spot was inoculated on media and incubated at 30°C for 48 h. Clear halo zone resulted in the hydrolysis of milk proteins were appeared around the proteolytic isolates (Chang et al., 2009).

Quantitative analysis

Protease activity of the strains were determined by the method used by Subba Rao et al. (2009). For this purpose, 10 g/l of casein solution was prepared in 50 mmol/l glycine/NaOH buffer at 11 pH. The crude enzyme solution (1 ml) was mixed with 1 ml casein solution and incubated for 20 min at 70°C. To stop the reaction, 4 ml of trichloroacetic acid was add and filtered the solution using Whatman filter paper. The optical density of the filtrate was measured at 280 nm and by using tyrosine standard curve protease activity was calculated.

Test for HCN (hydrogen cyanide) production

HCN production was assayed by streaking strains on media containing glycine (4.4 g/l). Filter paper soaked with picric acid solution (0.5%) and sodium carbonate (2%) were kept in the upper lid of petri plates and sealed with parafilm to avoid gas emission. The plates were then incubated for 92 h at 30°C. After the incubation period change in color (from deep yellow to reddish-brown) of filter paper indicates HCN production (Castric, 1975).

Test for exopolysaccharide production

This test was performed to check either rhizobacteria can synthesis exopolysaccharide or not. EPS media was used to identify the exopolysaccharide producing bacteria. The isolates were streaked on agar plates and incubated for 7 days. Positive strains produces mucous or slimy growth around the colonies (Tallgren et al., 1999).

Statistical analysis

For each strain all the experiments were performed in triplicate to confirm the results. Analysis was conducted using SPSS (Version 20.0) and Microsoft Office Excel 2019.

Results

Isolation and morphological identification of rhizobacteria

Bacterial strains were isolated from the rhizosphere of okra plants using the serial dilution method on LB media. Post-incubation, several bacterial colonies emerged on

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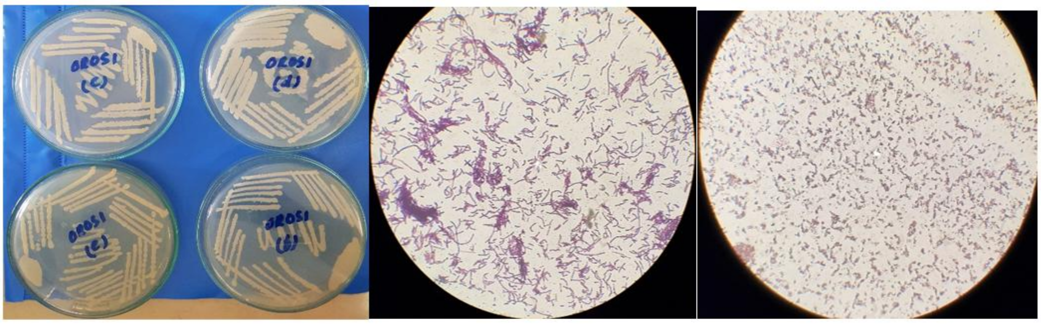
the plates. Based on morphological traits such as shape, color, colony margins, opacity, and elevation, 23 bacterial isolates were selected for further studies (*Fig. 1a*). Gram staining was performed on these isolates, and they were examined under a light microscope. This examination revealed that 18 isolates were Gram-negative, appearing pink in color, with most being cocci and a few rod-shaped. Additionally, 5 isolates were Gram-positive, displaying a purple color and rod shape (*Table 1*).

Table 1. *Morphological identification of rhizobacteria*

Code	Source	Size	Morphological characterization of colonies					Gram staining
			Shape	Color	Margin	Elevation	Opacity	
OROS1a	Soil	Large	Rod	Yellow	Mucoid	Raised	Opaque	-
OROS1b	Soil	Medium	Cocci	White	Entire	Raised	Translucent	-
OROS1c	Soil	Medium	Cocci	White	Entire	Raised	Translucent	-
OROS1d	Soil	Medium	Cocci	White	Entire	Raised	Translucent	-
OROS1e	Soil	Medium	Cocci	White	Entire	Flat	Translucent	-
OROS1f	Soil	Large	Rod	White	Entire	Raised	Translucent	-
OROS1g	Soil	Medium	Cocci	Yellow	Entire	Raised	Translucent	+
OROS1h	Soil	Medium	Cocci	White	Curled	Raised	Opaque	-
OROS1i	Soil	Large	Rod	Brown	Entire	Raised	Translucent	-
OROS1j	Soil	Medium	Cocci	Yellow	Entire	Raised	Translucent	-
OROS1k	Soil	Large	Rod	White	Entire	Raised	Transparent	+
OROS1l	Soil	Large	Rod	White	Entire	Raised	Translucent	+
OROS1m	Soil	Medium	Cocci	White	Entire	Raised	Translucent	+
OROS1n	Soil	Medium	Cocci	Yellow	Entire	Raised	Translucent	-
OROS1o	Soil	Medium	Cocci	White	Entire	Raised	Transparent	-
OROS1p	Soil	Medium	Cocci	White	Mucoid	Flat	Transparent	-
OROS1q	Soil	Medium	Cocci	Brown	Entire	Raised	Translucent	-
OROS1r	Soil	Large	Rod	White	Entire	Raised	Opaque	-
OROS1s	Soil	Medium	Cocci	White	Entire	Raised	Translucent	-
OROS1t	Soil	Medium	Cocci	White	Curled	Raised	Translucent	-
OROS1u	Soil	Large	Rod	White	Entire	Flat	Opaque	-
OROS1v	Soil	Medium	Cocci	White	Entire	Raised	Transparent	-
OROS1w	Soil	Medium	Rod	White	Entire	Raised	Transparent	+

+ = Gram-positive, - = Gram-negative



(a) **(b)** **(c)**

Figure 1. *(a) Pure bacterial culture, (b) gram staining (positive) and (c) gram staining (negative)*

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Screening of rhizobacteria for plant growth promoting properties

The 23 isolated rhizobacteria were screened for various plant growth-promoting properties, including phosphate solubilization. Qualitative analysis on Pikovaskaya's agar plates revealed that 15 out of the 23 isolates could solubilize phosphate. Among these, strain OROS1c exhibited the highest solubilizing indices (SI) of 4.3 cm^2 and solubilizing efficiency (SE) at 333%, while strain OROS1q showed the lowest SI of 1.3 cm^2 and SE 33% (*Fig. 2a*). Strains that did not form clear zones were considered non-phosphate solubilizing and were excluded from further quantitative assays. The phosphate-solubilizing strains were subjected to quantitative analysis in Pikovaskaya's broth. After a seven-day incubation period, absorbance measurements validate earlier qualitative results, strain OROS1c with highest solubilizing capacity at 43.08 µg/ml and strain OROS1q with lowest at 0.94 µg/ml (*Table 2*).

Indole production test

Indole production is one of the remarkable traits of PGPR. To estimate the indole production ability of bacteria, LB broth media supplemented with 1% tryptophan was employed. Four days post incubation of quantitative assayed that only two isolates among 23 can produce IAA. *Table 2* depict that the highest amount of IAA production was obtained by OROS1r with concentration of 1.3718 µg/ml.

Ammonium production test

The peptone water falcon tubes inoculated with the bacterial strains were assessed for ammonium production using Nessler's reagent. The colorimetric test results were determined by the color change of the medium, ranging from yellow to brown. The assay conducted on 23 strains revealed that 20 isolates were capable of producing ammonia, while 3 strains were non-producers. As shown in *Table 2,* strain OROS1c produced the highest amount of ammonia, with a concentration of 0.81 µg/ml, whereas strain OROS1t produced the lowest amount, at 0.06 µg/ml.

Nitrogen production test

Another plant growth-promoting property assessed in the rhizobacteria was their ability to fix nitrogen using Burk's modified nitrogen-free media plates. All pure culture strains were streaked onto the semi-solid media plates. The visible growth of the isolates indicated that all the strains possessed nitrogen-fixing capabilities.

Zinc solubilizing test

The qualitative assay of zinc solubilization of PGPR was examined by zone of inhibition appeared around colonies inoculated on LB supplemented with 0.1% ZnO. The results reveal that except 10 strains all 13 isolates can covert insoluble ZnO into soluble Zn. Isolate OROS1k exhibit highest SI (3.27cm^2) and SE (270%) whereas the strain OROS1w exhibit the lowest SI (0.8cm^2) and SE (20%). Zinc solubilizing strains were further processed for quantitative assay by incubating strains within broth LB media blended with ZnO. Post inoculation results of absorbance measurements mentioned in *Table 2* shows that OROS1k not only exhibits highest SI and SE values but also shows maximum zinc concentration of 36.33 µg/ml and OROS1w produce minute amount of soluble zinc with concentration of 0.52 µg/ml.

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Table 2. *Screening of rhizobacteria for plant growth promoting properties*

Code	Phosphate solubilization				Production of			Zinc solubilization			
	Zone	SI (cm^2)	SE %	P (µg/ml)	IAA (µg/ml)	Ammonia (µg/ml)	Nitrogen	Zone	SI (cm^2)	SE %	Zn (µg/ml)
OROS1a	+++	3.5	250	35.45	0	0.7	+++	+	1.4	40	1.22
OROS1b	++	2.6	160	17.83	0	0.5	+++	+	1.4	40	1.27
OROS1c	+++	4.3	333	43.08	0	0.81	+++	+	1.3	33	1.03
OROS1d	++	2.09	109	13.27	0	0.6	+++	+	1.3	33	1.07
OROS1e	++	1.6	60	3.42	0	0.13	+++	++	1.5	50	1.35
OROS1f	++	3	200	18.32	0	0.07	+++	-	0	0	-
OROS1g	++	2.4	144	12.22	0	0.66	+++	-	0	0	-
OROS1h	+	1.7	70	5.8	0	0.34	+++	-	0	0	-
OROS1i	++	2.25	125	9.89	0	0.21	+++	++	1.75	75	1.45
OROS1j	++	2	100	13.21	0.8081	0.11	+++	-	0	0	-
OROS1k	++	1.4	40	1.032	0	0.31	+++	+++	3.27	270	36.33
OROS1l	-	0	0	-	0	-	+++	-	0	0	-
OROS1m	-	0	0	-	0	0.54	+++	-	0	0	-
OROS1n	++	2.86	186	12.5	0	0.39	+++	+++	2	100	
OROS1o	-	0	0	-	0	0.44	+++	-	0	0	-
OROS1p	++	2.28	128	11.36	0	-	+++	-	0	0	-
OROS1q	+	1.3	33	0.94	0	0.57	+++	-	0	0	-
OROS1r	+	1.6	60	3.16	1.3718	0.18	+++	+++	3.33	233	15.6
OROS1s	-	0	0	-	0	0.23	+++	-	0	0	-
OROS1t	-	0	0	-	0	0.06	+++	++	1.6	60	2.97
OROS1u	-	0	0	-	0	0.7	+++	++	2.4	140	11.99
OROS1v	-	0	0	-	0	-	+++	++	3.5	250	34.39
OROS1w	-	0	0	-	0	0.43	+++	+	1.2	20	0.52

+++ = high producer, ++ = average producer, + = weak producer, - = non-producer, green highlight = lowest value, yellow highlight = highest value

Screening of rhizobacteria for biocontrol abilities

There are several enzymes and compounds enable bacteria to interact with their environment, compete with other microorganisms, and potentially establish symbiotic relationships with plants, which are all aspects of biocontrol abilities. To find out the biocontrol abilities of several tests were performed which shows some potential positive results.

Test for amylase production

To check the amylolytic activity of the bacteria, the 23 isolates were subjected for qualitative screening by spot inoculation on 1% starch supplemented LB media plates. A total of 13 isolates were tested amylase positive indicated by the formation of starch lysis zone around colonies when treated with 1%-gram iodine (*Fig. 2i*). Quantitative screening of amylolytic bacteria was performed in 1% starch mixed LB broth. After 24 h of incubation, absorbance of centrifuged DNS treated supernatant at 540 nm was measured. *Table 3* illustrate that values obtained from enzyme assay proves that OROS1g possessed highest enzyme activity while OROS1p possessed lowest enzyme activity among other isolates, concentration 8.33 IU/ml and 0.045 IU/ml, respectively.

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Test for cellulase production

Cellulase production of 23 PGPR strains was checked on 1% cellulose agar media. Out of 23 strains, 16 isolates were cellulase producers and form clear halo zone when flooded with Congo red dye (*Fig. 2h*). Cellulolytic activity of 16 isolates was measured at 540 nm in spectrophotometer. Among 16 cellulolytic isolates, OROS1u was the most potent cellulase producer with highest SE and cellulolytic activity, 550% and 5.954 IU/ml, respectively (*Table 3*).

Table 3. *Screening of rhizobacteria for biocontrol abilities*

Code	Hydrolytic enzyme production												Production of	
	Amylase			Cellulase			Pectinase			Protease				
	SI (cm^2)	SE %	Activity (IU/ml)	SI (cm^2)	SE %	Activity (IU/ml)	SI (cm^2)	SE %	Activity (IU/ml)	SI (cm^2)	SE %	Activity (IU/ml)	HCN	EPS
OROS1a	2.8	180	2.211	1.86	86	1.076	2.89	189	2.32	1.73	73	1.067	-	+++
OROS1b	3.37	237	7.76	2.50	150	1.758	3.33	233	2.655	2.46	146	3.138	-	++
OROS1c	-	-	-	2	100	1.14	-	-	-	2	100	0.007	-	-
OROS1d	2.09	109	1.3	2.67	167	1.67	2.67	167	2.716	1.65	65	1.56	-	+++
OROS1e	4	300	6.94	2.33	133	2.537	3	200	2.735	3.5	250	2.053	-	+++
OROS1f	-	-	-	-	-	-	-	-	-	2.6	160	1.042	-	-
OROS1g	4.14	314	8.33	2.33	133	2.549	3.25	225	2.932	1.25	25	1.035	-	+++
OROS1h	2.50	150	1.156	-	-	-	2.86	186	1.132	1.5	50	0.056	-	+++
OROS1i	-	-	-	-	-	-	-	-	-	3	200	1.056	-	-
OROS1j	2.67	167	1.933	3.25	225	3.831	2.67	167	1.511	3	200	2.042	-	+++
OROS1k	-	-	-	1.5	50	1.0045	1.15	15	0.456	2.6	160	1.034	-	+++
OROS1l	-	-	-	-	-	-	2.25	125	1.875	1.66	67	1.036	-	-
OROS1m	-	-	-	1.5	50	1.0056	3.2	220	3.037	1.42	43	1.009	-	-
OROS1n	-	-	-	-	-	-	-	-	-	2.87	187	1.056	-	-
OROS1o	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OROS1p	1.2	20	0.045	4	300	3.778	2.2	121	1.232	1.5	50	1.006	-	+++
OROS1q	2.6	167	6.55	1.5	50	1.023	2.6	163	1.87	-	-	-	-	-
OROS1r	2.3	129	3.78	1.5	50	1.074	1.8	80	0.97	2.45	145	2.027	-	++
OROS1s	1.5	46	0.0975	2.5	150	2.043	1.16	17	0.013	2	100	1.035	-	-
OROS1t	-	-	-	-	-	-	-	-	-	2	100	0.014	-	-
OROS1u	2.6	158	0.0486	6.5	550	5.954	2	100	1.037	2	100	0.038	-	-
OROS1v	1.6	64	0.089	1.5	50	1.046	2.5	150	1.0958	2.9	190	1.038	-	+++
OROS1w	-	-	-	1.3	33	0.021	-	-	-	-	-	-	-	-

+++ = high producer, ++ = average producer, + = weak producer, - = non-producer, green highlight = lowest value, yellow highlight = highest value

Test for pectinase production

The 23 strains were assayed for the production of pectinase on 1% pectin substrate containing fermenting media. The isolates treated with 2%-gram iodine after 24 h which shows that 16 strains are pectinolytic bacteria. To check the quantity of pectinase, produce by 16 isolates enzyme activity assay was performed, by using procedure described above. The obtained colorimetric results reveal that OROS1m was the highest pectinase producer (3.037 IU/ml) while the lowest pectinase producer was OROS1s (0.013 IU/ml) as mentioned in *Table 3*.

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Test for protease production

Skimmed milk agar media was used for the identification of proteolytic bacteria. Among 23 strains, except 3 all other were identified as proteolytic bacteria. Quantitative assay results vary from the results obtained from the qualitive screening as shown in *Table 3*. Strain OROS1e shows highest SI (3.5 cm^2) while OROS1b shows highest enzyme activity (3.138 IU/ml). Similarly, the lowest SI was shown by OROS1g, 1.25 cm^2 whereas lowest enzyme was observed by OROS1c, 0.0078 IU/ml.

Test for HCN (hydrogen cyanide) production

The test preformed for the detection of hydrogen cyanide producing strains yielded negative results. No color change was observed in 23 tested strains.

Test for exopolysaccharide production

Exopolysaccharide production help PGPR to tolerate stressful condition. To check the EPS production ability of 23 PGPR, the test was performed, which shows that 11 isolates produces mucous layer around the colony while 12 isolates did not.

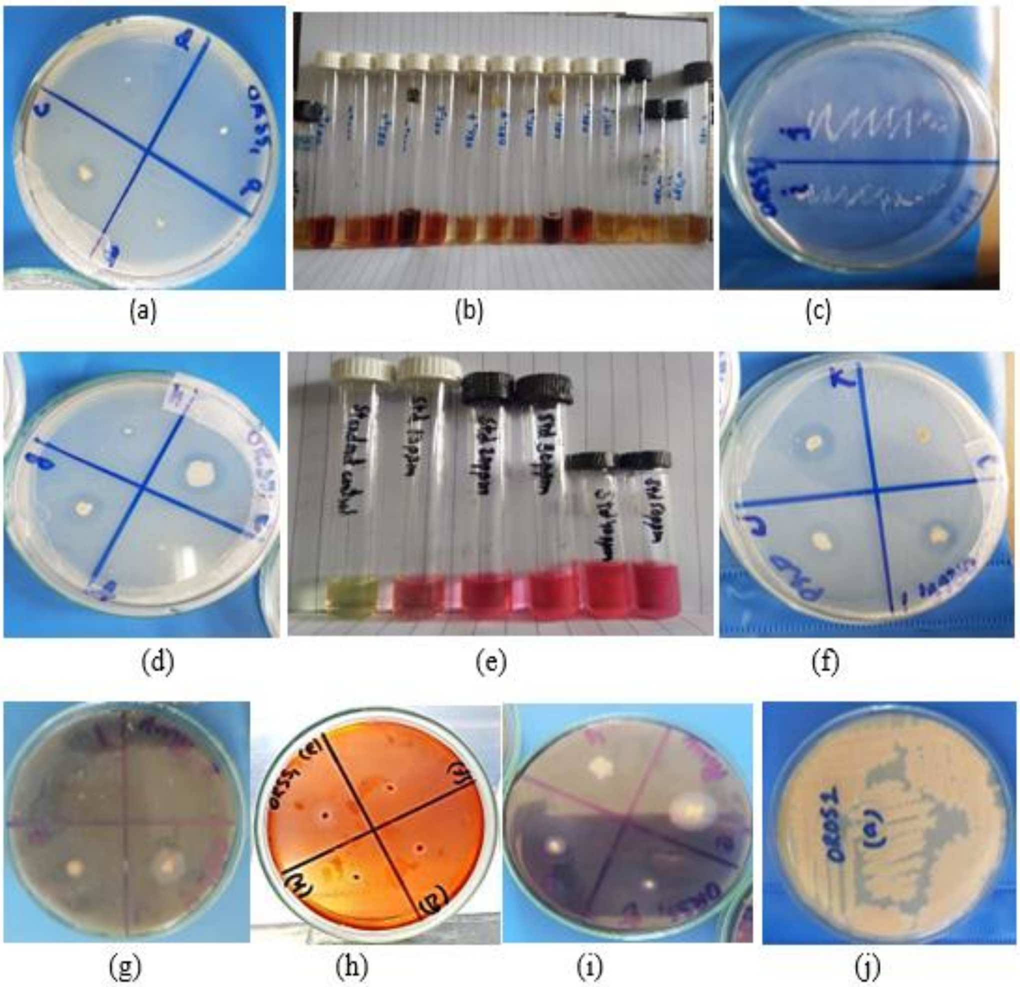

Figure 2. *(a) Phosphate solubilization (b) Ammonia production (c) Nitrogen fixation (d) Zinc solubilization (e) IAA production (f) Protease production (g) Pectinase production (h) Cellulase production (i) Amylase production (j) EPS production*

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Discussion

For maintenance of agricultural soil fertility and to increase crop productivity different novel plant growth promoting rhizobacteria are used as biofertilizers. These PGPR colonizes the roots or surrounding area and provide essential nutrients (phosphate, nitrogen), protection against diseases and resistance against abiotic stress (UV, temperature) (Tsegaye et al., 2017). The aim of this study was to investigate plant growth promoting bacteria from the rhizosphere of okra plant. For this purpose, the rhizospheric soil sample was collected from Mansehra District, KP. Serial dilution on agar media was performed and 23 isolated strains were selected based on their morphology. These strains were screened for different biochemical, biocontrol and plant growth prompting traits. Gram staining of 23 isolates shows that only 5 strains were gram positive showing purple color whereas 18 strains appear in pink color resulting as gram negative. In microscopic inspection it was examined that most of the bacteria were cocci and few were rod in shape. Articles on the screening and beneficial effects of PGPR from other crops like maize, sugar cane and rice have been reported in real-time but there are few reports and data on okra plant that discuss their importance.

Mostly the rhizosphere yields numerous amounts of potent PGPR strains when isolated, as reported in many publications (Jaiswal et al., 2023; Deka et al., 2015). Nitrogen plays crucial role in the development of plant like in the production of enzyme, proteins, formation of nucleic acid and chlorophyll. Plants cannot fix atmospheric nitrogen (N_2), although it is present in abundant amount. The symbiotic or free-living microorganism synthesize nitrogenase which utilize this inert form of nitrogen and convert it into ammonium or nitrate used by plants (Santi et al., 2013). To assess nitrogen fixation efficiency, PGPR were cultured on Burk’s media. All the bacterial strains exhibit excellent growth and proliferation. These results are mirror to the findings of Bashir et al. (2023), who conducted nitrogen fixation test using similar media. Phosphorous is second most important macronutrient and in soil as compared to fungi bacteria are predominantly present as phosphate solubilizers help to solubilize organic and inorganic phosphate and provide it to plant roots for uptake. This trait of PGPR enable plants to stand abiotic stress like salinity, nutrient deficiency and drought (Zhou et al., 2016). To evaluate phosphate-solubilizing potential, qualitative and quantitative assays were conducted using Pikovaskaya’s media. The results demonstrate, 15 strains can solubilize phosphate and strain OROS1c was the promising PSB 43.08 μg/ml. The results were compatible with the finding of Sarker et al. (2014) but significantly lower than the value reported by Mahdi et al. (2020). Plant growth and development is regulated also by IAA, which is principle phytohormone. IAA play crucial role in auxin production, which in turn increase nutrient and water absorption by elongating roots (Höflich et al., 1994). In our findings, only two strains were IAA producer, OROS1r and OROS1rj with concentration of 1.3718 μg/ml and 0.8081 μg/ml, respectively. These obtained values were too low as compared with the research work (Kumar et al., 2012; Mahdi et al., 2020). This may be due to different bacterial species, soil sample, media components and conditions.

Soil is enriched by organic nitrogen or complex forms of nitrogen, which are converted by PGPR into ammonium and released back into the soil. This ammonia is utilized by plants which help in their development by increasing growth of root and shoot and increase antifungal activity (Grover, 2012). In current study, except 3 strains all other 20 PGPR strains were capable of producing ammonia. The highest amount of ammonia was recorded as 0.81 μg/ml which is supported by results recorded by Mahdi

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et al. (2020), 0.7 µg/ml. Zinc is an important micronutrient which play pivotal role in physiological and metabolic processes of plant. Zinc solubilization by PGPR increases agricultural productivity by maintaining soil nutrients and promoting plant health and growth (Pastore et al., 2020; Mpanga et al., 2020). Our results aligned with previous findings by Prathap et al. (2022) and Ali et al. (2023) on the role of PGPR in zinc solubilization and plant nutrition. The observed prevalence of nitrogen-fixing and zinc-solubilizing bacteria in the Mansehra District can be attributed to the region's historically rich but now eroding soils. This highlights the necessity for PGPR-based interventions to sustain soil fertility while reducing chemical inputs.

PGPR not only promotes plants growth but also help them to develop defense against pathogen like fungi and nematodes. The hydrolytic enzymes like amylase, cellulase pectinase and proteases produced by PGPR, degrade the cell wall of fungi and boost plant defense system by promoting release of signaling molecules (Grover, 2012; De la Lastra et al., 2021; Riseh et al., 2024). In present study amylolytic activity of 23 PGPR strains was screened using 1% starch LB media, OROS1g strains showed highest activity, at 8.33 IU/ml which is higher than units reported by Kizhakedathil and Chandrasekaran (2018), 1.57 U/ml. However, this result contradicts the higher activity of 101.503 U/ml reported by Kazemi et al. (2015). For cellulolytic activity 16 strains showed positive results, with a maximum activity of 3.037 IU/ml, which conflict with the results of Irfan et al. (2012). The pectinolytic and proteolytic activity 23 strains exhibit that 16 strains were pectinase producers and 20 were protease producers. These results consistent with the findings of Merín et al. (2014) and Haniya et al. (2017), respectively. HCN production by PGPR plays significant role in biocontrol of plant pathogens by inhibiting cytochrome c oxidase, interfering cellular respiration (Ghadamgahi et al., 2022). In our investigation, none of strains were able to produce HCN, similar to the findings of Bashir et al. (2023). Exopolysaccharides production by PGPR help plants to enhance growth and tolerate stressful conditions like drought, heat, salinity and antibiotic stress (Mukhtar et al., 2020; Morcillo and Manzanera, 2021). Considering the importance of EPS, the test was performed which reveals that only 11 strains were able to produce EPS. Similarly, CHOMPA et al. (2024) reported 5 strains which can tolerate salinity and among them UPMRB9, produced the highest amount of exopolysaccharides. The finding of current study can be practically implemented for sustainable okra cultivation. The identified isolated strains, predominantly OROS1c and OROS1k, shows promising biofertilizer traits, which can enhance soil fertility and plant growth by providing nutrients under local conditions. Scaling these finding to field trials is essential for developing commercially viable biofertilizer formulations.

Conclusion

Increase in world population demands high food production for which crops are cultivated on large area. To protect these crop different chemical fertilizer and pesticide are used which adversely affect human health and environment. To avoid these problems now PGPR are used as biofertilizers. They are the normal flora of soil, ecofriendly and help in plant growth by producing different chemicals and phytohormones. This study successfully identified plant growth-promoting rhizobacteria from the rhizosphere of okra in Mansehra District. The isolated strains possess best traits of PGPR like phosphate and zinc solubilization, indole production, nitrogen fixation, EPS production and synthesis of hydrolytic enzymes. These PGPR

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can be used in the formulation of commercially viable biofertilizers to promote the plant growth and other biotechnological applications.

Conflict of interests. The authors declare no conflict of interests.

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