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In vitro Qualitative and Quantitative Assessment of PGPR Potential Attributes against *Rhizoctonia bataticola* in Chickpea

Bethala Kumeera[1], Swapnil Matikhaye[2], P.W. Ramteke[3] and Suchit A. John[4*]
[1]Ph.D. Scholar, Department of Biological Sciences, NAI, SHUATS, Prayagraj, (Uttar Pradesh), India.
[2]Ph.D. Scholar, Department of Biological Sciences, NAI, SHUATS, Prayagraj, (Uttar Pradesh), India.
[3]Emeritus Professor, Department of Biological Sciences, NAI, SHUATS, Prayagraj, (Uttar Pradesh), India.
[4]H.O.D., Department of Biological Sciences, NAI, SHUATS, Prayagraj, (Uttar Pradesh), India.

(Corresponding author: Suchit A. John)*
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ABSTRACT: The present study confirmed that out of thirty one isolates collected from chickpea rhizoshpere, four isolates PR7, PR10, PR30, PR31 found to be potent and identified PR7, PR10 as *E. cloacae* gram negative and PR30, PR31 as *B. subtills* gram positive in clear microscopic view at 100x magnification. These strains were identified on basis of 16s rRNA sequencing and were resulted to be antagonistic against *R. bataicola* with 65.75% of higher zone inhibition observed in PR30 strain. Protease ranged between 27.75 to 19.00 µg/ml of production, siderophore with 1.29 in PR30 to 0.98 µg/ml which helps in iron uptake, and low production in PR7. Chitin assay was 192.25 to 147.75 U/ml of production reported high in PR7, Cellulase was 482.0 U/ml observed in PR30 assisted in production of lytic enzymes that is responsible for breakdown of fungal cell wall. Whereas HCN was only able to change color in PR10 only. All these mentioned four isolates in this study, performed PGPR traits such as assessment of qualitative and quantitative screening were pronounced to be potential, thus considered as an attractive solution for the challenges facing towards the reported pathogen *R. bataticola* in chickpea under *in vitro* conditions that exhibited antagonistic activity.

Keywords: PGPR, *R. bataticola, E. cloacae, B. subtills,* Chickpea, Qualitative and Quantitative traits.

INTRODUCTION

In chickpea, among numerous factors related to biotic and non-biotic, susceptibility to diseases is the most important cause for the low productivity. Among several diseases, dry root rot caused by *Rhizoctonia bataticola* is one of the major constraints registered so far which declines 10-60% of production every year (Sundravandana *et al*., 2012). *R. bataticola* is mainly soil and seed borne fungal pathogen highly affects matured chickpea plants at podding and flowering stages in comparison to seedlings. During dry soil, temperature exceeding 30°C, the intensity of dry root rot increases with increasing the frequency of water deficit (Mamta *et al.,* 2015).

The *Rhizoctonia* genus split a numerous features in their anamorphic states which are similar and considered to be a fungal filamentous taxon in heterogeneous group has wide host range (Garcia *et al.,* 2006). Formerly, the dry root rot has not gained much significant importance, but in modern years, it has become a most important threat to chickpea production predominantly due to altered weather conditions on the account of severe and prolonged drought (Sharma *et al.,* 2013).

Bacteria that colonize roots and encourage plant growth are denoted as plant growth promoting rhizobacteria (PGPR). They have significant impacts on growth and development as they improve the availability of micro-nutrients to their host plant by assembly of growth promoting chemicals. They are also well identified for their role in improving growth patterns of roots. A huge diversity of organic compounds ooze out from the roots as exudates that act as a signal for attracting soil microbes as they are rich source of carbon supply within the soil (Drogue *et al*., 2013). The uptake of mineral nutrition and plant growth regulators are augmented by PGPR directly and/or stimulate immunity of chickpea against fungal pathogens indirectly. Use of bacterial inoculation in particular, plant growth promoting rhizobacteria (PGPR) is effective and eco- friendly to improve drought tolerance (Rolli *et al.,* 2015; Balloi *et al.,* 2010).

Generally, these fungal pathogens which are considered as soil borne are managed by chemicals, but this process leads to other health and environmental issues. Approximately 2.5 million tons of pesticides are utilized per annum universally also accumulates into the environment (Rao *et al.,* 2015). Hence alternative management strategies are most pleasing. PGPR use many different mechanisms to protect against various abiotic stresses. Several organisms have been successfully used as biocontrol agents such as *Pseudomonas* spp., *Bacillus* spp., *Enterobacter* spp. for

the management of dry root rot in chickpea (Christy *et al.,* 2012). These are interrelated multifaceted processes which include fixation of atmospheric nitrogen (N), phosphorus (P) solubilization, acquisition of nutrients, modulation of plant hormone (IAA) are having direct involvement in growth promotion. Whereas, hydrocyanic (HCN) production, volatile compounds, antibiotics, systemic resistance, siderophore production, antagonistic action against biotic pathogens for disease control and other mechanisms indirectly support the growth of chickpea plants (Abd El Daim *et al.,* 2014).

These PGPR usage in agriculture is increasing constantly because of its growth promoting activities and for the promotion of nutrient uptake by these rhizobacteria (Ali *et al.,* 2020). The suppression of disease causing organisms because of a broad-spectrum antifungal activity reported by several supporting literatures (Kudoyarova *et al*., 2019) and the promotion of positive effects on phytohormones production for the growth and development of the plant (Park *et al*., 2017). Inorder to suppress fungal diseases, biocontrol method of approaching in indirect mechanism which is suitable to act as antagonistic role (O' Brien, 2017). Many organic and inorganic compounds such as phosphorus, iron, potassium, nitrogen, and zinc are well known phenomenon for plant growth promotion available directly through PGPR (Olanrewaju *et al.,* 2017).

Therefore, the objective of the present study was to isolate the rhizospheric bacteria from chickpea (*C. arietinum*) and screen them for PGPR traits like production of IAA (Indole acetic acid, protease, siderophore, HCN (Hydrocyanic acid), cellulase, chitinase, phosphate solubilization and capability to provide tolerance against *Rhizoctonia bataticola* by dual culture assay under *in vitro* conditions.

METHODOLOGY

Screening of bacteria from the rhizosphere soil: Thirty one isolates were screened for PGPR traits from chickpea rhizosphere. For the Bacterial isolation the serial dilution technique has done and King's B media was used for the isolated bacterial bio control agents. Four isolates were found to be potential for biocontrol activity against *Rhizoctonia bataticola*.

Indole-3-acetic acid (IAA) estimation: The IAA production was estimated by method of (Gorden and Paleg 1957). The bacterial isolates was raised in Luria Bertani broth for 72 hours at 37°C under shake conditions. Then supernatant collected by the centrifugation of cultures at 15,000 rpm and Salkowski's reagent (2 mL) was mixed, and placed in dark for 30 minutes for the development of pink color. The color intensity was measured OD at 535 nm.

HCN production: The test cultures streaks on prepoured plates of King's medium B. The Whatman's No.1 filter paper strips soaked in 0.5% picric acid in 0.2% sodium carbonate and placed in between the petriplates (Baker and Schippers 1987). The petri plates was sealed with parafilm and then incubated at 37°C for 1-4 days. Uninoculated control also maintained for comparison. The plates was observed for color change in filter paper from yellow to orange brown to dark brown.

Chitinase enzyme assay: For chitinase assay, the bacterial isolates grown in 100 mL fresh medium (3% w/v chitin; 0.1% KH_2PO_4; 0.05% $MgSO_4.7H_2O$; 50 mM sodium phosphate buffer, pH 6.0) for three days at 30°C. After incubation, the supernatant was collected by centrifuging the mixture at 12,000 rpm for 20 minutes. For qualitative assay method, along with above said media, yeast (0.5g/L), colloidal chitin (30g/L) and agar was added for plates. Bacteria inoculated and incubated for 3-4 days at 28 °C. The plates observed a clear hallow zone that indicated growth of bacteria in petriplates (Berger and Reynolds 1958).

Gram stain technique: The cells that were stained with crystal violet and iodine was treated with a decolorizing agent such as 95% ethanol or a mixture of acetone and alcohol. Gram-positive bacteria retain purple iodine-dye complexes after the treatment with the decolorizing agent. To visualize decolorized Gram-negative bacteria, a red counter stain such as safranin has been used after decolorization treatment (Danish physician Hans Christian Gram 1984).

Siderophore production: The quantitative estimation of siderophore production by isolates was performed by liquid chrome azurol-S (CAS) assay method (Schwyn and Neilands 1987). The cell-free extract of supernatant (0.1 mL) mixed with 0.5 mL CAS assay solution along with 10 µl of shuttle solution (0.2 M 5-sulfosalicylic acid). The mixture kept at room temperature for 10 minutes and absorbance noted at 630 nm using UV-VIS spectrophotometer. A blank reference was maintained using all above components, except cell-free extract of supernatant. The siderophore units was calculated as:

$$\text{Percent siderophore unit} = \frac{Ar - As}{As \times 100}$$

Where Ar was the absorbance of reference at 630 nm, and As was absorbance of test solution at 630 nm.

Phosphate solubilization: Diameters of clear zones around the colonies after 5 days of incubation at 28°C was measured on Pikovskaya agar inoculated plates. whereas for quantitative assay, Pikovskaya broth was used to inoculate and incubated at 37°C, then centrifuged at 1000rpm for 30min, collected supernatant was observed reading in spectrophotometer at 410nm (Pikovskaya 1948).

Cellulase Production: Isolates were grown on LB medium supplemented with 1% carboxy methylcellulose (CMC), Congo red dye 1% and destaining with 1 M NaCl (Slama *et al*., 2019). Cellulase activity was checked by the presence of clear zones surrounding individual isolated colonies. For quantitative assay, KH_2PO_4 (0.5g), $MgSO_4$ (0.25g), gelatin (2g) added in 1L of distilled water, CMC (0.1g),

bacteria inoculated and incubated at 30°C for 3-4 days. Centrifuged at 15000 rpm for 10min, 4°C. To this supernatant solution citrate buffer was added and incubated 50°C for 30min. Dns reagent (3ml) added and OD was observed at 540nm.

Protease Production: Protease activity was checked by measuring clear zone after inoculation of bacterial strains on skimmed milk agar media containing (g/L): Yeast extract, casein peptone, agar and supplemented after autoclaving with 250 mL of sterile skimmed milk (Mefteh *et al.,* 2017). For quantitative assay, 50mM potassium phosphate buffer, pH 8.0 at 37°C, 1% (w/v) casein solution, 10% trichloro acetic acid reagent, 500mM sodium carbonate solution (Na_2CO_3) in nutrient broth 13g autoclaved and bacteria inoculated then kept under shaking incubator for 3-4 days at 35°C with 800rpm and observed OD at 280nm.

Dual culture assay: Dual inoculated plates with fungus alone as control containing PDA medium, were incubated at 28 °C with a 12 h photoperiod. The inhibition zone between the 2 cultures was measured 3 days after inoculation for *R. bataticola* (Krishna Kishore *et al.,* 2005).

Inhibition percentage = RC-RT /RC 100

RESULTS

Qualitative and quantitative screening of bacterial isolates for assessment of PGPR traits:

Thirty one bacterial isolates of chickpea rhizosphere were screened against *R. bataticola* for antagonistic activity and plant growth promotion traits. Out of these, four isolates named PR7, PR30, PR10 and PR31 were found to be potential in this study.

16S rRNA identification: 16S rDNA PCR amplification were performed as stated by **(**Ovreas *et al*., 1997) using the universal primers. PCR product was purified with a PCR purification kit (Qiagen, U.S.A) and nucleotide sequence data under accession number KP966499 for the strain PR31 identified as *Bacillus subtilis.* PR30 strain identified as *Bacillus subtilus* under accession number KP966505. PR7 strain identified as *Enterobacter cloacae* under accession number KP226581. PR10 strain identified as *Enterobacter cloacae* under accession number KP226575 and was deposited in Gen-Bank sequence database. The online program BLAST was used to find the related sequences with known taxonomic information in the databank at NCBI.

Protease activity: All the four isolates PR7, PR30, PR10, PR31 were capable for clear hallow zone formation in protease qualitative activity in petri-plates recorded high zone diameter with 29.00 mm, and low zone formation in PR7 with 12.0mm diameter shown in Table 1. During quantification assay, production of protease ranged from 19 µg/ml to 27.75 µg/ml, where highest recorded in PR30 and least in PR7 over control (22.25 µg/ml) shown in Table 2.

Siderophore production: All these reported isolates were also capable of inductive formation of siderophore activity for production of an orange hallow surface around the colony on the CAS agar plates, however highly produced zone diameter 29.0 mm in PR30, least siderophore producing isolate was PR7 with 2.0 mm zone diameter resulted in Table 1. Whereas, in quantification test mentioned isolates shown in Table 2, produced 1.29 µg/ml as highest siderophore production and 0.98 µg/ml was considered as least production over control (0.58 µg/ml).

Chitinase activity: Chitinase was also produced periodically clear zone around the colony by these isolates highly with 13.3mm of zone diameter in PR31, and least isolate producer of chitin was PR14 with 5.3 mm of diameter measurement shown in Table 1. However, the same bacterial isolates recorded highly at the range of 192.25 U/ml and low production of chitin was 147.75 U/ml at quantitative assay over control (120.20 U/ml) shown in Table 2.

Phosphate solubilization: A clear yellow zone of phosphate solubilization was highly produced in PR10 with 20.6mm, and least in PR31 with 14.3mm in diameter shown in Table 1. However, in quantitative estimation results in Table 2, showed that these isolates ranged between 1.68 µg/ml to 1.30 µg/ml with PR10 being maximum producer of phosphate over control with (1.05 µg/ml) and least recorded in PR31 strain.

Indole-3-acetic acid (IAA) production: Only two isolates were able to produce IAA indicating the production of pink color in bacterial isolates of PR30 and PR10 shown in Fig. 4, while in quantitative assay with high range of 0.67 µg/ml of IAA production was in PR30 strain and least recorded as 0.27 µg/ml of production in PR7, as same isolate along with PR31 was found to be negative with no color formation of IAA as phytoharmone shown in Table 1 and 2.

Table 1: Screening of PGPR traits for qualitative tests.

Pgpr strains	Organisms	Protease (µg/ml)	Siderophore (µg/ml)	Chitinase (U/ml)	Phosphate (µg/ml)	IAA (µg/ml)	Cellulase (U/ml)
	Control	**22.25**	**0.58**	**120.20**	**1.05**	**0.05**	**360.0**
PR7	***E. cloacae***	19.00	0.98	192.25	1.64	0.27	446.0
PR30	***B. subtilis***	27.75	1.29	158.75	1.62	0.67	480.0
PR10	***E. cloacae***	27.00	1.19	147.75	1.68	0.45	350.0
PR31	***B. subtilis***	26.75	1.02	171.50	1.30	0.34	458.0

Table 2: Screening of PGPR quantitative traits

Strains	Organisms	Protease		Siderophore		Chitinase		Phosphate		Cellulase		IAA	HCN	Dual culture Control (90mm)	Gram staining
		Q.A	Z.D (mm)	Q.A	Z.D (mm)	Q.A	Z.D (mm)	Q.A	Z.D (mm)	Q.A	Z.D (mm)	Q.A	Q.A	Inhibition (%)	
PR7	***E. cloacae***	+	12.0	+	2	+	15.2	+	20.3	+	8.6	-	-	55.74	-
PR30	***B. subtilis***	+	29.0	+	29	+	7.6	+	16.6	+	18.2	+	-	65.76	+
PR10	***E. cloacae***	+	24.3	+	27	+	5.3	+	20.6	-		+	+	60.4	-
PR31	***B. subtilis***	+	23.3	+	27	+	13.3	+	14.3	+	15.3	-	-	59.04	+

Cellulase production: Cellulase production was indicated with a clear hallow zone formation of congo red plate in three out of four mentioned isolates, highly produced in PR30 strain with 18.2mm of zone diameter and least in PR7 with 8.6mm of diameter in Table 1. These cellulase produced at quantification asaay with high range of 480.0 U/ml of cellulase production and least in PR10 at 350.0 U/ml production in Table 2 yet less than control (360.0U/ml), as this strain (PR10) was pronounced negative during qualitative assay in petri plates with no zone formation.

HCN determination: HCN production was recorded in only PR10 isolate shown in Fig. 2**,** while other isolates were unable to change color from yellow to orange brown as there was absence of hydrogen cyanide producing capability in remaining PGPR isolates.

Gram staining: Gram staining of PR7 and PR10 isolates were found to be negative with pink color of microscope view, the remaining mentioned two isolates namely PR30 and PR31 were found to be gram positive with rod shaped bacteria, violet color of microscopic view at 100x magnification clearly shown in Fig. 1.

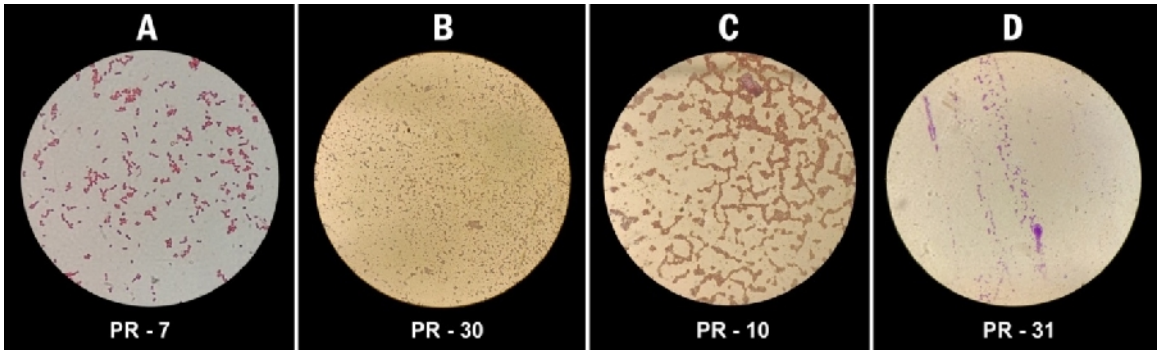

Fig. 1. Microscopic view of PGPR isolates at 100x magnification.

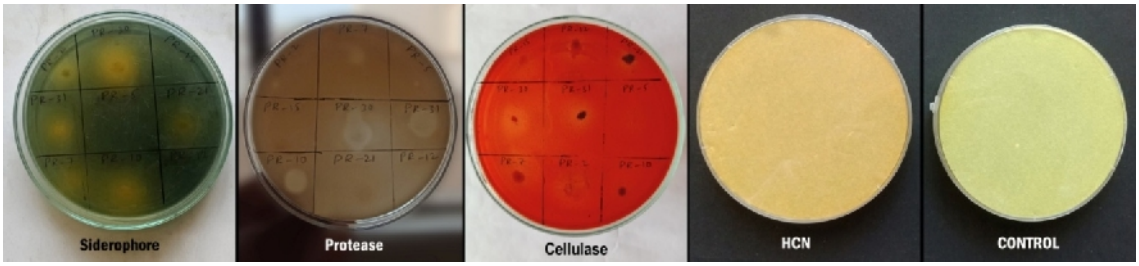

Fig. 2. Qualitative test indicated zone formation for the production of Siderophore, Protease and color change in HCN (Hydrogen cyanide) test along with control in petri plates.

Antagonistic activities against *R. bataticola* as pathogenic fungi: In this present study, PR30 identified as *B. subtilis* reported high inhibition of 65.76% with antagonistic activity, followed by PR10 identified as *E. cloacae* with high inhibition percentage of 60.4%, neatly by isolate PR31 identified as *B. subtilis* with 59.04% of inhibition and least in comparison to four isolates was PR7 identified as *E. cloacae* with 55.74 inhibition percentage resulted against *R. bataticola* during dual culture assay, where control was maintained at 90mm in petriplate clearly shown in Fig. 3. Inhibition zone in mm was recorded and inhibition percentage was calculated accordingly, the results thus obtained were presented in Table 1.

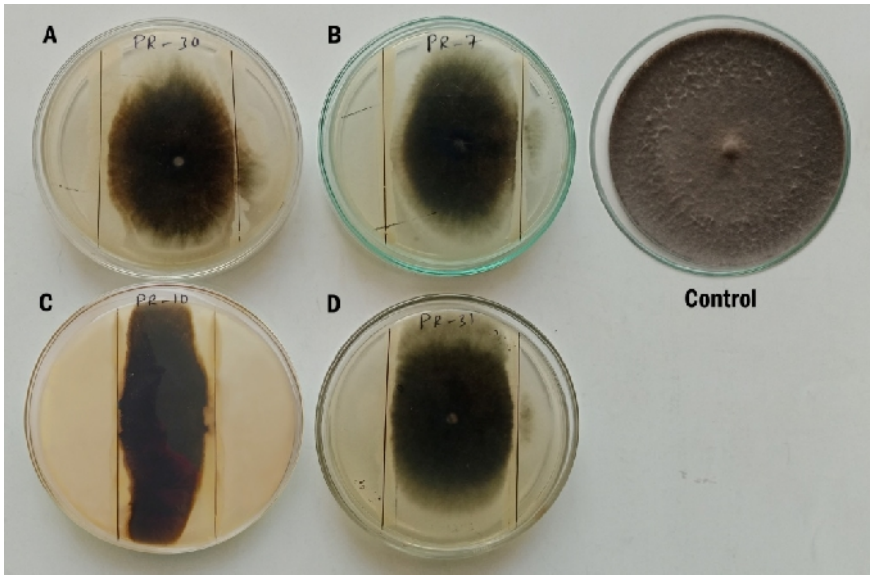

Fig 3. Dual culture assay against *Rhizoctonia bataticola* with control.

DISCUSSION

PGPR traits works combinely, as it was suggested in the ‘‘additive hypothesis,’’ that multiple mechanisms, such as phosphate solubilization, IAA, siderophore antifungal activity and biosynthesis etc. are accountable for the yield and plant growth promotion (Bashan and Holguin, 1997). Phosphorous is widely available second most nutrients in both organic and inorganic forms of soil. The abundant amount of P present is of no use to plants since they absorb only in the ionic form of monobasic (H_2PO^{-4}) and dibasic (HPO_2^{-4}) (Bhattacharyya and Jha 2012). The conversion and release of inorganic form of phosphorous to organic form, were considered as phosphate solubilizing bacteria (PSB), the results of these isolates proved that it acts as potential agent. The present study were in agreement with several literatures reporting phosphate solubilization by *Bacillus* spp. and *Enterobacter* spp. as the most significant PSB and also as an another important traits of PGPR (Panhwar *et al.,* 2014). It can produce both organic acids that can improve the chemical availability of P and growth promoting substances by enhancing various physiological functions (Maliha *et al.,* 2004). P plays a vital role in early root development, yield quality and resistance to plant diseases in chickpea (Islam *et al*., 2013a).

IAA as phytohormone production by *Enterobacter spp.* followed by *B. subtilis* shown in Fig. 4, which helps in plant growth promotion, development as well as defense responses is a major tool employed by PGPR is significant (Reetha *et al*., 2014). This diversity of function is reflected by the extraordinary complexity of IAA transport, biosynthetic and signaling pathways (Santner *et al*., 2009). Indole acetic acid production stimulated by tryptophan and reduced by anthranilate, a precursor for tryptophan. By this mechanism, IAA biosynthesis is fine tuned because tryptophan inhibits anthranilate formation by a negative feedback regulation on the anthranilate synthase, resulting in an indirect induction of IAA production (Spaepen *et al.,* 2007). Since, IAA is engaged in multiple processes like vascular bundle formation, cell division and differentiation, these three processes are also indispensable for nodule formation and also assists in seedling growth and flower development (Glick, 2012).

The PGPR isolates reported in this study, promotes plant growth by assisting nitrogen fixation, providing indole acetic acid production (IAA), phosphate solubilization and iron nutrition through siderophore (Hamid *et al.,* 2021). They produced in both Gram-positive and Gram-negative strains were able to chelate iron from surrounding environment altered to green color from blue as shown in Fig. 2, due to removal of iron from the CAS blue media (Padda *et al.,* 2017). Siderophore production by these rhizobacteria plays an important role in Fe nutrition from soil to form complexes and thereby improving the growth in chickpea. Iron (Fe^{3+}) in siderophore complex on bacterial membrane is reduced to (Fe^{2+}) which is further released into the cell through gating mechanism linking the inner and outer membranes. During this reduction process, the siderophore may be recycled or destroyed. Therefore, performs as solubilizing agents from minerals or organic compounds under iron limitations (Rajkumar *et al*., 2010). Siderophore produced by these mentioned PGPRs is an indirect mechanism that majorly takes part in preventing negative effects caused by reported pathogen by exhibiting antagonistic effects and by preventing the rise of other fungal pathogens in plant rhizosphere (Abdul Malik *et al.,* 2020). These are low molecular in weight usually less than 1 kDa, and

capable to sequester ferric forms of ions in the rhizosphere zone in a counter track by making iron unapproachable to the phytopathogens, thus preventing their growth (Sayyed *et al*., 2019).

These PGPR were capable of releasing extracellular enzymes like chitinase and β-1,3- glucanase, which contributes in cell wall lysis (Singh *et al*., 2013; Liu *et al.,* 2019). Chitinase producing *Enterobacter spp.* followed by other strains in this study, revealed antagonistic activities by eliminating fungal phytopathogens, as it is primarily composed of β-1,4-N-acetyl-glucosamine, chitinase and β-1,3-glucanase are reported as potent antifungals that suppress pathogen growth (Riaz *et al.,* 2021). They were capable of hydrolyzing the β-1, 4-linkages, an abundant N-acetyl-β-D-glucosamine polysaccharide which is an integral structural component of fungal cell walls. This helps in degrading cell walls of *R. bataticola* by production of such enzymes (Ramyasmruthi *et al.,* 2012). Detection of β–1,3–glucanase and cellulase activity in Fig. 2, was confirmed after the translucent zones with U/ml high production were observed in positivity of results in these isolates (Patil *et al*., 2015).

HCN production by PGPR indirectly increases Phosphate availability by metal chelation and sequestration of these geo chemical entities (Rijavec *et al.,* 2016). This may also contribute the capability to synthesize cell wall degrading enzymes for suppression of pathogen by releasing different kind of enzymes, metabolites shown in Fig. 4 specially protease, siderophore, chitinase in chickpea (Kumar *et al.*, 2019). Interactions between these PGPR with reported isolates may be synergistic or antagonistic and the beneficial effects of such interactions could be exploited for economic gain. It also secretes cellulose, protease, glucanase for degrading cell walls of fungus (Abd El-Daim *et al*., 2019). HCN also increases phosphate availability for rhizobacteria and Synthesis of lytic enzymes that breaks the glycosidic linkages in the cell wall of phytopathogens via limiting their vicinity and growth. HCN have been shown by genera *Enterobacter spp.* appears to act synergically with other methods of biocontrol employed by these resulted PGPR (Nandi *et al*., 2017).

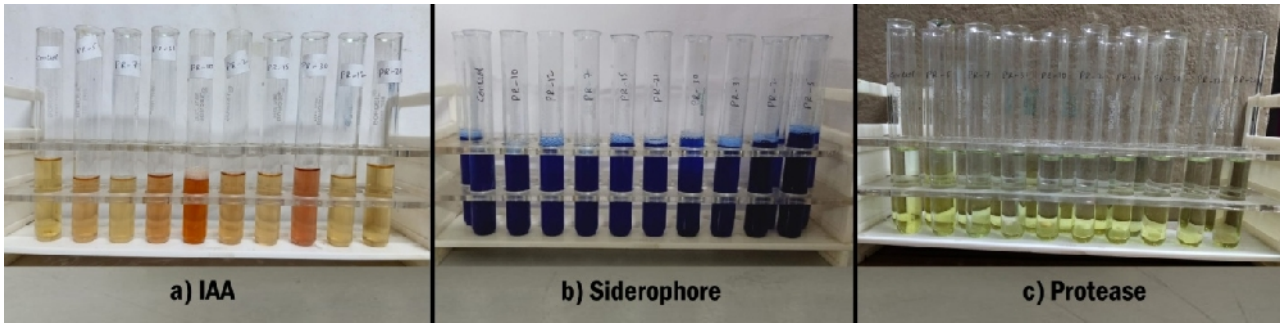

Fig. 4. Quantification of bacterial isolates for production of a) IAA (Indole acetic acid), b) Siderophore and c) Protease activity.

The major indirect mechanism of plant growth promotion in rhizobacteria is through acting as biocontrol agents. The four bacterial isolates in this study from chickpea rhizosphere was reported to have antifungal activities against major soil borne fungal pathogen (Srivastava *et al*., 2016). Amongst these isolated strains, PR30 identified as *Bacillus subtilis* were used as powerful biocontrol agents exhibited clear antagonistic activity when initially checked against dry root rot caused by *R. bataticola* in comparing to other strains. In addition, due to its broad host range, its ability to form endospores and produce different biologically active compounds with a wide spectrum of activity displayed in Fig. 3 during *in vitro* conditions (Saraf *et al.,* 2008). The inhibition of reported fungal pathogen growth by plant growth promoting rhizobacteria occurs synergistically through producing antibiotics, enzymatic lysis, volatile organic compound (VOC) production and other mediated various principal mechanisms (Nishad *et al*., 2020).

CONCLUSION

Plant growth promoting rhizobacteria (PGPR) has a prominent role but dominated by harsh chemicals, pesticides to manage dry root rot caused by *Rhizoctonia bataticola*, which negatively impacts on environment and crop productivity. Due to vast usage of these agrochemicals, a serious threat facing in global and health issues annually. Inorder to overcome this criteria, PGPR is an alternative solution for managing soil borne fungal pathogen, thus being beneficial for crop growth, yield in chickpea as well as significantly helpful for marginal farmers in present agricultural scenario organically and even to reduce environment and health concerns. In this present study, out of thirty one root colonizing chickpea (*C. arietinum*) rhizosphere bacterial isolates, four isolates reported highly antagonistic activity by suppressing *R. bataticola* growth mainly by showing its bio-control activity under *in vitro* conditions. PGPR attributes including of both indirect and direct traits, all these four mentioned

isolates pronounced as actively potential in this current study.

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Conflict of Interest. Nil.

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