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In vitro Screening of Zn solubilizing and Potassium releasing isolates for Plant Growth Promoting (PGP) characters

Y. Nagaraju*, S. Triveni, A. Vijaya Gopal, G. Thirumal, B. Prasanna Kumar and P. Jhansi
Department of Agricultural Microbiology, Professor Jayashankar Telangana State Agricultural University (PJTSAU), Rajendranagar, Hyderabad-30.
*Corresponding author: Emai: nagarajulvrth62@gmail.com +91 9482306802

ABSTRACT
Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that can be found in the rhizosphere, in association with roots which can enhance the growth of plant directly or indirectly. A large number of bacteria including species of Pseudomonas, Derixia, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, Rhizobium, Methanobacterium, Phyllobacterium and Serratia have reported to enhance plant growth. In the present study, fifteen bacterial and seven fungal isolates of Zn solubilizing and potassium releasing isolates were isolated and they were screened for Plant Growth Promoting (PGP) characters. All the isolates are positive for ammonia production, ten isolates were positive for in vitro P solubilization, eight isolates were positive for siderophore production, seven isolates have shown inhibition of Rhizoctonia solani and Sclerotium rolfsii and five isolates were positive for HCN production. These isolates have multiple roles in exerting the growth of plant. Among all isolates ZnSB-2 and ZnSF-4 were promising in exhibiting almost all PGP characters. As PGPR are environmental friendly and offer sustainable approach to increase production of crops and health. Therefore, these isolates can be utilized for biofertilizer formulation under local agroclimatic conditions.
Key words: *Siderophore, HCN, Biofertilizer, Zn solubilizers, PGP, Ammonia production*

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INTRODUCTION

The rhizosphere zone has been defined as the volume of soil directly influenced by the presence of living plant roots or soil compartment influenced by the root (Hiltner, 1904). Rhizosphere supports large and active microbial population capable of exerting beneficial, neutral and detrimental effects on the plants. Rhizobacteria (root colonizing bacteria) that exert the beneficial effects on the growth of the host plant via direct or indirect mechanisms are termed as plant growth promoting rhizobacteria (PGPR) (Juanda, 2005). The plant-microbe interactions in the rhizosphere are responsible for increasing plant health and soil fertility (Khan, 2006).

In modern cultivation process indiscriminate use of fertilizers, particularly the nitrogenous and phosphorus, has led to substantial pollution of soil, air and water. Excessive use of these chemicals exerts deleterious effects on soil microorganism, affects the fertility status of soil and also pollutes environment (Youssef and Eissa, 2014). The application of these fertilizers on a long term basis often leads to reduction in pH and exchangeable bases thus making them unavailable to crops and the productivity of crop declines. To obviate this problem and obtain higher plant yields, farmers have become increasingly dependent on chemical sources of nitrogen and phosphorus. Besides being costly, the production of chemical fertilizers depletes nonrenewable resources, the oil and natural gas used to produce these fertilizers, and poses human and environmental hazards (Joshi *et al.,* 2006). Conversely, Plant Growth Promoting Rhizobacteria (PGPR) are capable of promoting plant growth either directly or indirectly. These mechanisms can be active simultaneously or independently at different stages of plant growth (Ahmad *et al.,* 2008). For instance, these bacteria can act as biofertilizers, phytostimulators, or stress controllers (Lugtenberg and Kamilova, 2009).

The use of PGPR inoculants for the promotion of plant growth and biological control of plant diseases has gained focus as an alternative to the use of chemical fertilizers and pesticides. This collection of traits is called rhizosphere competence (Bevivino *et al.,* 1998; Raaijmakers *et al.,* 2009). Some PGP bacteria like

Bacillus and *Paenibacillus spp*. are ubiquitous in soil and have been isolated from the rhizosphere of many different plants (Costa *et al.,* 2014; Govindasamy *et al.,* 2011). There are many studies reporting the beneficial effects of these genera on plants and their biocontrol of soil borne diseases (Bevivino *et al.,* 2000; Chen *et al.,* 2013; Haggag and Timmusk, 2008; Kumar *et al.,* 2012; Lorentz *et al.,* 2006; Shaharoona *et al.,* 2007). These microorganisms can promote plant growth directly through nitrogen fixation, phosphate solubilization, and the production of phytohormones and ACC deaminase; and indirectly by the production of antagonistic compounds like hydrolytic enzymes, siderophores and a range of antibiotics (Costa *et al.,* 2014; Govindasamy *et al.,* 2011; Parke and Gurian-Sherman, 2001; Suárez-Moreno *et al.,* 2012). Several soil bacteria particularly belonging to genera *Bacillus* and *Pseudomonas*, possess the ability to change insoluble forms into soluble form by secreting organic acids as formic acid, acidic, propionic, lactic, glycolic, fumaric and succinic acid (Vazquez *et al.,* 2000). In the present study we have tried to explore the multiple PGP roles of Zn solubilizing and K releasing bacterial and fungal isolates

MATERIALS AND METHODS

Collection and processing of rhizosphere soil

Rhizosphere soils of different crops like Green gram, Soybean, Maize, Rice, Cotton and Sorghum were collected from a depth of 0-15 cm. Soil samples were collected from college farm and student farm at College of Agriculture, Rajendranagar, Hyderabad. Soils were shade dried for 24 hr and removed coarse particles and unwanted plant debris.

Isolation of zinc solubilizing rhizospheric microorganisms

Isolation of bacteria was carried out on Nutrient Agar (NA) and Fungi is isolated by using Potato Dextrose Agar (PDA) under axenic conditions. All the bacterial and fungal isolates were screened for zinc solubilization by using TRIS minimal agar medium amended with 0.1 % of either insoluble zinc oxide (ZnO) or zinc phosphate $Zn_3(PO_4)_2$. The actively growing cultures (5 µl) was spot inoculated onto the medium, incubated at 28 °C. Detected zinc solubilization efficiency by different rhizobacterial isolates based upon the ability of solubilization zone formation (Fasim *et al.,* 2002). The diameter of colony and clear zone around the colony was measured for calculating the solubilization efficiency in percent and area in mm^2.

Isolation of Potassium Releasing Bacteria (KSB)

The potassium releasing bacteria was isolated from the rhizosphere soil by serial dilution plate method using modified Aleksandrov's medium containing 0.2 % insoluble mica powder or potassium alumino silicate as insoluble potassium source. The serial dilutions of the soil samples were made up to 10^{-5} and 0.1 ml of diluted soil suspension was plated on Aleksandrov medium plates (Prajapati and Modi, 2012). The plates were incubated at 28 ± 2 °C in biological oxygen demand (BOD) incubator for 3 - 4 days. Detection of potassium solubilization by different rhizobacterial isolates were based upon the ability of solubilization zone formation. The rhizobacterial isolates were maintained by transfer on Aleksandrov agar medium slants. These bacterial cultures were stored at 4 ℃ in refrigerator for further use.

Screening of isolates for Plant Growth Promoting Properties (PGPP)

Pure isolates were isolated by streak plate method, isolates were maintained on respective media plates and screened for following Plant growth promoting properties.

Phosphate Solubilization

Sterilized Pikovskaya's agar medium was poured as a thin layer on to the sterilized petri plates and incubated for 24 hr. After incubation the Pikovskaya's plates (Pikovskaya, 1948) were spot inoculated with isolates and incubated at 28 ± 1°C for 4 - 5 days. Formation of a clear zone around the colonies were considered as positive result for phosphate solubilization.

PSE (Phosphate Solubilization Efficiency) = Z / C x 100

Where,

Z- Clearance zone including bacterial growth

C- Colony diameter

IAA Production

Indole acetic acid production was tested according to Gorden and Weber (1951). The active culture of each test isolate was raised in 5 ml respective broth tubes and incubated at determined temperature and time. After incubation these cultures were centrifuged at 10000 rpm for 10 min. Two drops of O - phosphoric acid was added to 2 ml of supernatant and incubated for 30 min to develop the color. Development of pink color considered as positive for IAA production.

Antifungal activity for biocontrol ability

Pure isolates of common disease causing soil phytopathogens viz., *Rhizoctonia solani, Sclerotia rolfsii* and *Fusarium oxysporum p.var odium* were obtained from the Dept. of Plant Pathology, College of Agriculture, Rajendranagar. Antagonistic activity was verified by following dual culture technique (Skidmore and

Dickinson, 1976). First, the bacterial isolates were streaked on respective media plates and incubated at respective temperature and time. Loopful of each bacterial isolate was streaked on the potato dextrose agar plate at one end, which was pre-inoculated with 5 days old and place 5 mm mycelial disc of test fungal pathogen at the other end. Control plate was maintained by placing only pathogen mycelial disc in the center without bacteria. The assay plates were incubated at 28 ± 1 °C for 5 days and observations were made on inhibition of mycelial growth of the test pathogens. For each bacterial isolate three replications were maintained with suitable controls. The per cent growth inhibition over control was calculated by using the formula:

$$\mathbf{P.I=}\frac{\text{Growth of pathogen in control (mm)} - \text{Growth of pathogen in treatment (mm)}}{\text{Growth of pathogen in control (mm)}} \times 100$$

Where,
P.I is Percent Inhibition
Note: In this the percent inhibition in control is taken as zero percent.

Ammonia Production

The isolates were tested for ammonia production by inoculating the isolates into 10 ml of presterilized peptone water in test tubes. The tubes were incubated for 48 - 72 hr at 36 ± 2 °C. After that Nessler's reagent (0.5 ml) was added in each tube. Change in color of the medium from brown to yellow color was taken as positive test for ammonia production.

HCN Production

The HCN production was tested by the method of Castric and Castric (1983). First respective media plates were prepared separately and incubated for 24 hr. After that, 1ml of culture /fungal disc of each test isolate was inoculated on respective media plates separately. A disc of whattman filter paper No.1 of the diameter equal to the petri plate size, impregnated with alkaline picric acid solution (0.5 % picric acid (w/v) in 1 % sodium carbonate) was placed in the upper lid of the inoculated petri plates under aseptic condition. The control plate did not receive the inoculum. The plates were incubated at 28 ± 2 °C for 48 - 72 hr. Change in color from yellow to light brown, moderate or strong reddish brown was taken as indication of HCN production.

Siderophore Production

Siderophore production was estimated qualitatively. Chrome Azurol S (CAS) Agar medium (Schwyn and Neilands, 1987). For the detection of siderophores, each isolate was grown in synthetic medium, containing 0.5 µM of iron and incubated for 24 hr on a rotary shaker at room temperature. Chrome Azurol S (CAS) assay was used to detect the siderophores. The CAS plates were used to check the culture supernatant for the presence of siderophores. Culture supernatant was added to the wells made on the CAS agar plates and incubated at room temperature for 24 h. Formation of yellow to orange colored zone around the well indicates siderophore production.

RESULTS AND DISCUSSION

Phosphate solubilization ability of Zn solubilizing and potassium releasing isolates

Among fifteen bacterial and seven fungal isolates eight bacterial and two fungal isolates were able to solubilize phosphate on Pikovskaya's media containing Tri calcium phosphate in the range of 6 mm to 22 mm. Among eight bacterial isolates ZnSB-8 recorded the highest solubilization zone (17 mm) (Fig:1 and 2) followed by ZnSB-2 (13 mm). On par results were observed among the isolates ZnSB-1 and ZnSB-3. Among fungal isolates ZnSF-4 (22 mm) followed by KSF-2 (15 mm) had recorded more efficiency. Fungal isolates have more efficiency than bacterial isolates. Singh and Ghosh (2012) obtained similar results in evaluation of phosphate solubilizing potential on Pikovskaya's media. Out of 30 isolates, five isolates showed positive result on Pikovskaya's medium producing clear zones. Ranjan *et al*. (2016) found, day wise kinetics of phosphate solubilization by *Burkholderia tropica*, *Burkholderia unamae* and *Burkholderia cepacia* against $Ca_3(PO_4)_2$ and suggested P4 isolate (580.56 ± 13.38 g ml^{-1}) as maximum mineral phosphate solubilizer followed by P9 (517.12 ± 17.15 g ml^{-1}) and P10 (485.18 ± 14.23 g ml^{-1}) at 28 °C.

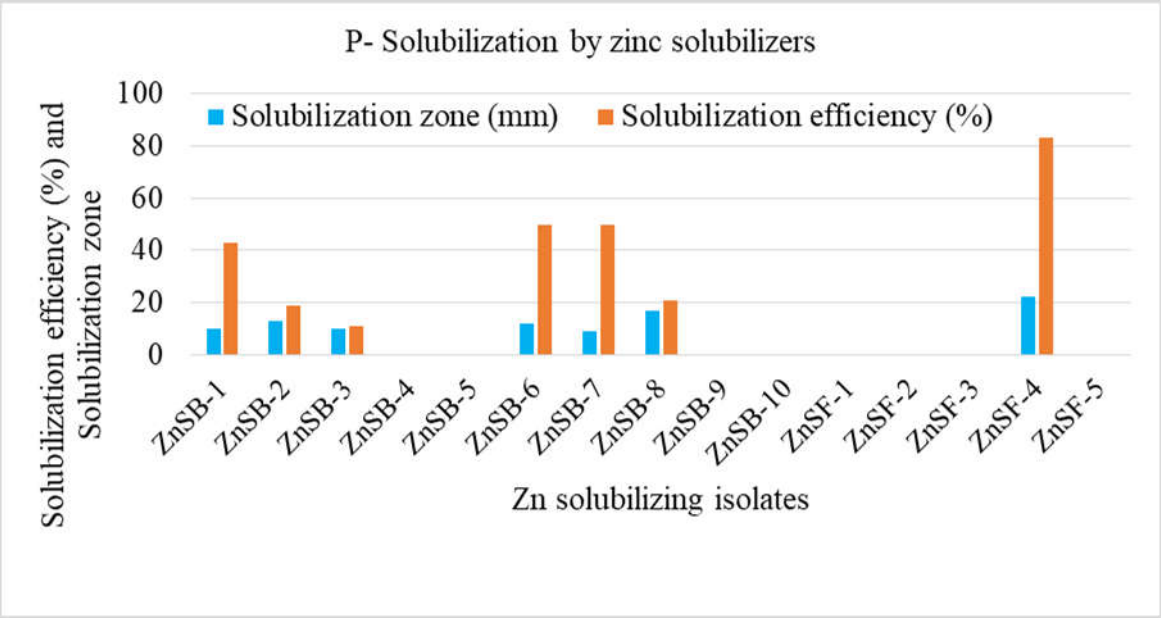
IAA production

Among fifteen bacterial and seven fungal isolates IAA production varied with supplementation of L-tryptophan and without supplementation of L-tryptophan. Out of fifteen bacterial isolates ZnSB-1, ZnSB-5, ZnSB-7 and KSB-3 showed IAA production (Table: 1). The isolates showed higher auxin production in the presence of precursor (L-tryptophan) as compared to without supplementation of L-TRP in the media. In the present study IAA production and phosphate solubilization by zinc solubilizing and potassium releasing isolates were in agreement with the earlier reports available on PGPR strains which were isolated from wheat rhizosphere showed IAA productionSimilar results were found by Reetha *et al*. (2014); Mohite *et al*. (2013); and Sadaf *et al.* (2009) with different isolates..

Antifungal activity for biocontrol ability

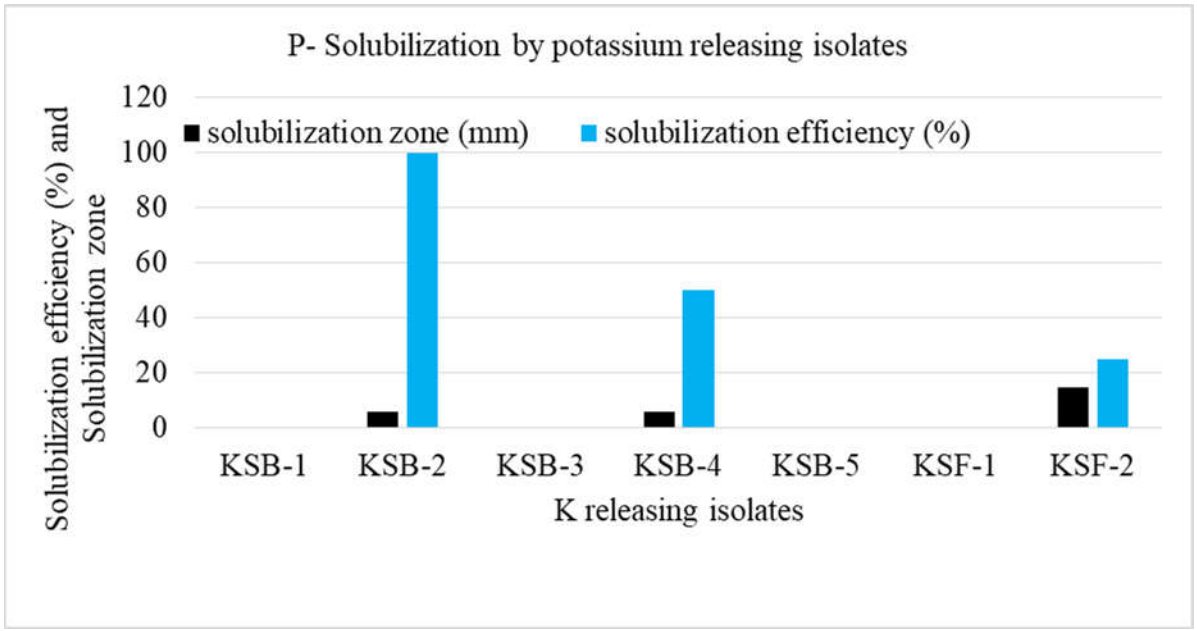
Antifungal activity of fifteen bacterial and seven fungal isolates were screened against *Rhizoctonia solani, Sclerotium rolfsii* and *Fusarium oxysporum* under *in vitro* conditions using PDA media (Table: 2).

Fig:1 Screening of zinc solubilizers for phosphate solubilization efficiency



ZnSB- Zinc Solubilizing Bacteria ZnSF- Zinc Solubilizing Fungi

Fig:2 Screening of Potassium releasing isolates for Phosphate solubilization efficiency



KSB- Potassium Releasing Bacteria KSF- Potassium Releasing Fungi

Antifungal assay with *Rhizoctonia solani*

Among fifteen bacterial and seven fungal isolates, ZnSB-2, ZnSB-10 and KSB-2 has showed weak inhibition of *Rhizoctonia solani* after 1 week of incubation at 37±2 ^{0}C. The inhibition zones obtained are as follows, 0.5 mm inhibition found with ZnSB-2, 0.4 mm inhibition was found with ZnSB-10 and 0.3 mm inhibition was found with KSB-2. Similar results were reported by Mansoureh and Halimi (2012); Nicolas (2011); and Fatima *et al.* (2009).

Table:1 Production of IAA and ammonia by Zinc solubilizing and Potassium releasing isolates

+Positive result; - Negative result

S. No	Isolates	IAA production	Ammonia production
1	ZnSB-1	+	+
2	ZnSB-2	-	+
3	ZnSB-3	-	+
4	ZnSB-4	-	-
5	ZnSB-5	+	-
6	ZnSB-6	+	+
7	ZnSB-7	-	+
8	ZnSB-8	-	-
9	ZnSB-9	-	+
10	ZnSB-10	+	+
11	ZnSF-1	-	-
12	ZnSF-2	+	+
13	ZnSF-3	-	+
14	ZnSF-4	+	+
15	ZnSF-5	-	+
16	KSB-1	-	-
17	KSB-2	-	-
18	KSB-3	+	-
19	KSB-4	-	-
20	KSB-5	-	+
21	KSF-1	-	+
22	KSF-2	-	+

Antifungal assay with *Sclerotium rolfsii*

Among fifteen bacterial and seven fungal isolates, ZnSB-2, ZnSB-10, ZnSF-2 and KSB-2 isolates were found to have antifungal activity against *Sclerotium rolfsii* after 1 week of incubation at 37±2 ^{0}C. Inhibition zone ranged from 0.4 to 0.2 mm. Highest inhibition zone was found with ZnSB-2 and ZnSF-2 (0.4 mm), followed by ZnSB-10 (0.3 mm) and KSB-2 (0.2 mm). Similar results was obtained by Raghavan *et al.* (2015). Only five PGPR showed >70 % suppression of *P. myriotylum* out of 100 PGPR isolates. Ines *et al*. (2013) reported that all the strains of *Pseudomonas spp* significantly inhibited *Alternaria alternata*, particularly in 25 % TSA medium.

Antifungal assay with *Fusarium oxysporum*

Among fifteen bacterial and seven fungal isolates, none of the isolate showed antifungal activity against *Fusarium oxysporum*.

Ammonia production

Among the zinc solubilizing and potassium releasing isolates some isolates were positive for ammonia production and some were negative in peptone water tubes (Dye, 1962; Olivera *et al.,* 2011). The positive isolates include ZnSB-1, ZnSB-2, ZnSB-3, ZnSB-6, ZnSB-7, ZnSB-9, ZnSB-10, ZnSF-2, ZnSF-3, ZnSF-4, ZnSF-5, KSB-1, KSB-5, KSF-1 and KSF-2 produced ammonia and no ammonia production was observed in ZnSB-4, ZnSB-5, ZnSB-8, ZnSF-1 KSB-2, KSB-3 and KSB-4 (Table: 1). Saravanan *et al.* (2016) showed that among 17 isolates, 5 (29.4 %), 9 (33.3 %) and 11 (40.7 %) showed positive results for ammonia. Geetha *et al.* (2014) showed enhanced growth of green gram due to the production of ammonia, IAA, HCN, phosphate solubilization, and also having antifungal activity against phytopathogenic fungi.

HCN production

HCN production is attributed as one of the mechanisms of bio control activity of the PGPR, the ability of the fifteen bacterial and seven fungal isolates to produce HCN was determined by the picric acid assay. Out of 22 isolates five isolates produced HCN (Table: 2) of which ZnSB-2, ZnSB-10 and ZnSF-2 produced HCN moderately (++) ZnSB-8 and KSB-2 produced weakly (+), strong HCN production was not observed. Karmel *et al*. (2014) showed strong production of HCN in *Pf1* and CPf5 by *Pseudomonas.* Maleki *et al.* (2010) reported the isolates of *Pseudomonas fluorescens* CV-6 strain which produced considerable amounts of siderophore, indole acetic acid and also showed positive reactions for HCN, catalase.

Table:2 Study of PGPR characters of all zinc solubilizing and potassium releasing bacterial and fungal isolates

S. No	Isolates	Antifungal activity			HCN production	Siderophore production
		Rhizoctonia solani	*Sclerotium rolfsii*	*Fusarium oxysporium*		
1.	ZnSB-1	-			-	+
2.	ZnSB-2	+	+	-	++	++
3.	ZnSB-3	-	-	-	-	+
4.	ZnSB-4	-	-	-	-	-
5.	ZnSB-5	-	-	-	-	-
6.	ZnSB-6	-	-	-	-	+
7.	ZnSB-7	-	-	-	-	-
8.	ZnSB-8	-	-	-	+	-
9.	ZnSB-9	-	-	-	-	-
10.	ZnSB-10	+	+	-	++	+
11.	ZnSF-1	-	-	-	-	-
12.	ZnSF-2	-	+	-	+	+
13.	ZnSF-3	-	-	-	-	-
14.	ZnSF-4	-	-	-	-	-
15.	ZnSF-5	-	-	-	-	-
16.	KSB-1	-	-	-	-	-
17.	KSB-2	+	+	-	+	++
18.	KSB-3	-	-	-	-	-
19.	KSB-4	-	-	-	-	-
20.	KSB-5	-	-	-	-	+
21.	KSF-1	-	-	-	-	-
22.	KSF-2	-	-	-	-	-

HCN- Hydrogen cyanide + Weak production ++ Moderate production
+++ Strong production – No production

Siderophore production

Out of fifteen bacterial and seven fungal isolates, seven bacterial and one fungal isolate produced siderophores. Among seven isolates, two isolates (ZnsSB-2 and KSB-2) produced moderately (++) and remaining six (ZnsSB-1, ZnsSB-3, ZnsSB-6, ZnsSB-10 and KSB-5) isolates were low (+) producers of siderophores (Table: 2). Tailor and Joshi (2012) isolated seven bacterial isolates from sugarcane rhizosphere. The isolates were found to produce more than 85% siderophore units. Amongst them S-11 was found be the most efficient siderophore producer (96 % SU). S-11 was further characterized and identified as *Pseudomonas fluorescens.* Physico-chemical parameters were evaluated for optimum production siderophores by *Pseudomonas fluorescens* strain.

CONCLUSION

PGPR colonize roots of plant and promote plant growth and development through a variety of mechanisms. The exact mechanism by which PGPR stimulate plant growth is not clearly known, although several mechanisms such as production of phytohormones, Induced systemic resistance in plants, suppression of deleterious organisms, antibiosis, activation of phosphate solubilization, Zn solubilization, Siderophore production and promotion of the mineral nutrient uptake are usually believed to be involved in plant growth promotion. PGPR inoculants can fulfil diverse beneficial interactions in plants leading to promising solutions for sustainable and environment-friendly agriculture. Recent progress of molecular biology and biotechnology in the understanding of rhizobacterial interactions with the nodules of crop plants will encourage a suitable area of research in PGPR mechanisms relating to rhizosphere colonization. In our study, we noticed the multiple role of PGPR and their ecological significance, it is helpful to study further in detailed in molecular level and exploring the genes concerned with multiple roles.

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