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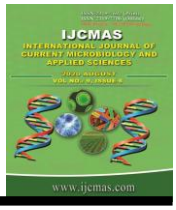

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Characterization of Plant Growth-Promoting Rhizobacteria Isolated from Chilli Rhizosphere of Southern Plateau and Hills Region

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A B S T R A C T

Plant Growth Promoting Rhizobacteria (PGPR) is a community of bacteria located in the rhizosphere of plant which are can suppress directly or indirectly plant diseases caused by different plant pathogens and also promote plant growth. Twenty three rhizospheric soil samples of chilli plants from Southern Plateau and Hills region agro-climatic zones of Karnataka and Andhra Pradesh states of India) were collected and isolated on four different types of media *viz.* TSA (Tryptone soya agar), NA (Nutrient Agar), CPG (Casamino peptone glucose) and Kings' B media in the present study. A total of thirty one bacterial isolates were isolated and screened their antagonistic against *Ralastonia solanacearum* UTT- 25 and plant growth promoting activities *in vitro* condition. Out of 31 isolates of rhizobacteria, 35.4 % rhizobacteria showed antagonistic ability to inhibit growth of *R. solanacearum. In vitro* screening of PGP activities rhizobacteria showed phosphate solubilization (64.2%), production of IAA (78.5%), production of ammonia (78.5%), production of HCN (35.7%), and siderophore production (50%). The rhizobacterial isolates showing plant growth promoting activities along with having biocontrol potentials were characterized using morphological, biochemical and physiological. These rhizobacteria are good potential to use as biopesticide and biofertilizers for improving crop health and growth.

Introduction

One of the most important commercial crops grown in India is chilli (*Capsicum annuum* L.). It is a tropical and subtropical crop, needing a warm humid climate and is an important condiment of Indian cuisine due to its pungence, colour, aroma and flavour. It is used in one form or the other for daily diet. India is the largest producer of chilies in the world, accounting for about 50 percent of production and about 20 percent of production exports (National Horticulture Board, 2018-19). Chili is grown in India over an area of 0.654 million hectares with a output of 1.039 million tonnes, with a productivity of 1588 kg / ha (FAO 2018). India accounts for an annual production of

approximately 1.1 million, followed by China (around 0.4 million tonnes), Mexico and Pakistan (around 0.3 million tonnes). The most important chilli states in India are Telangana (32.76%), Karnataka (25.01%) and Madhya Pradesh (23.51%) (National Horticulture Board, 2018-19).Chilli is known to be affected by as many as 83 various diseases, but major 26 diseases, among them 2 are serious disease which is caused by bacteria (Rangaswami, 1988). Between numerous diseases caused by fungi, bacteria and viruses, the bacterial wilt caused by *Ralstonia solanacearum* (Wicker *et al.,* 2007, Singh 2014) are considered to be one of the major important diseases causing losses of up to 90%. The disease is prevalent in Karnataka, Kerala, Maharashtra, Orissa and West Bengal in India causing heavy yield losses.

The worldwide use of pesticides rose by 4.4% annually in the 2000's (Zhang *et al.,* 2018). Every year, 2 million tons of active ingredient pesticides and fungicides are used worldwide, leading, of course, to further pollution. However, increased use of chemical inputs has led to many negative effects, i.e. a decline in soil biodiversity of soil microorganism; harmful effects on aquatic environments of pesticide runoff, disease resistance formed and adverse environmental impacts (Urban and Lebeda, 2004; Ma and Michailides, 2005).

Rising major safety issues, environmental risks and stringent legislation and limitations on the use of dangerous chemicals have led to a growing market for new, safer approaches to manage plant diseases. Biological control using microbes like rhizobia is an alternative method for management of the disease (PGPRs) (Kashyap *et al.,* 2017; Ranjan *et al.,* 2015; Singh *et al.,* 2012). Rhizobacteria act as an antagonistic agent to reduce disease incidence and overall improve crop health. Therefore, to satisfy the growing demand for chemical residue-free agricultural products, there is a need to look for more effective and efficient biocontrol agents. The population dynamics of the pathogen in rhizobacteria-treated chilli should be investigated, since they have proved to be resistance-inducing agents in host plants. The study will be helpful to find most potential antagonistic rhizobacteria having plant growth promoting ability. The present study was undertaken to find out potential rhizobacteria from rhizosphere of chilli plants from Southern Plateau and Hills region to have antagonistic and plant growth promoting activities under *in vitro* conditions and their characterizations using advanced molecular techniques.

Materials and Methods

Sample collection and isolation of rhizobacteria

The rhizospheric soil of chilli was collected from different agro-climatic regions of India such as Guntur, Warangal (Andhra Pradesh), Raichur, Gulbarga, Dharwad, Chintamani and Bangaluru (Karnataka) under southern plateau and hills. The rhizobacterial strains used in this study were isolated by serial dilution method on different growth medium such as TSA (Tryptone soya agar), NA (Nutrient Agar), CPG (Casamino peptone glucose) and Kings' B media at 28±1°C for 48-72h(Schaad *et al.,* 2001; Tan *et al.,* 2013).

The isolated bacteria showing irregular and creamy white morphology were maintained on YGCA (yeast glucose carbonate agar) medium. These cultures were store at 4ºC further use.

Characterization of antagonism of rhizobacteria against *R. solanacearum*

The antagonistic properties of 31 rhizobacterial isolates of chilli against *R. Solanacearum* (UTT25) were screened by dual culture method (Singh *et al.,* 2014).

Characterization of rhizobacteria for plant growth promoting traits

Phosphorus solubilization

Rapid screening of phosphate solubilization by rhizobacteria was performed on picovskaya media. 48 h old culture of the most potentially best antagonistic isolates from rhizobacterial isolates was inoculated in the picovskaya (PVK) broth medium and incubated at 280 C for 3-5 days. Then 1 ml of each culture was taken separately in the culture tube and 10 ml of ammonium molybdate was added to each bacterial culture and mixed thoroughly. The blue color intensity of the solution was measured by UV-VIS Spectrophotometer (Hitachi, U-2900) of 600 nm and the corresponding amount of soluble phosphorous was determined by the standard curve (Mehta and Nautiyal 2001).

Indole acetic acid (IAA) production

The production of IAA was estimated in accordance with the procedure (Vikram *et al.*, 2007). Twenty five ml of the supernatant of rhizobacteria were collected and the pH was adjusted to 2.8 with 1 N HCL in a 100 mL conical flask. Equal volume of diethyl ether was added and incubated in the dark for 4 h. Indole acetic acid extraction was performed at 4^{o}C in a separate funnel using diethyl ether.

The organic phase was discarded and the solvent was pooled and the IAA in the methanol extract was determined. To 0.5 mL of methanol extract, 1.5 mL of double distilled water and 4 mL of Sapler reagent (1 mL of 0.5 M $FeCl_3$ in 50 mL of 35% perchloric acid) were added and incubated in the dark for 1 h. The intensity of the pink color developed was read in a spectrophotometer at 535 nm.

Siderophore production

Siderophore production of rhizobacterial isolates was used as described by Singh *et al.*, 2012. Production of Siderophore was tested on Petri dishes containing CAS-agar. 48 h old cultures of rhizobacterial isolates were stabbed on CAS-agar plates using sterile toothpicks and incubated at 28^{o} C for two weeks in the dark. Colonies with orange zones were considered to be siderophore strains. Control plates of CAS-agar (uninoculated) were incubated under the same conditions and no color change in CAS-blue agar was observed after an incubation period of 1-14 days.

Ammonia production

Rhizobacterial isolates containing peptone water were inoculated; the tube was incubated at 30^{0}C for 4 days. Then 1 ml of Nessler's reagent was added to each tube. The presence of a faint yellow colour indicates a small amount of ammonia and a deep yellow to brownish color indicates the maximum production of ammonia.

HCN production

All isolated rhizobacteria were screened for hydrogen cyanide production following the method described by Bakker *et al.*, (1987). Each rhizobacterium was streaked on nutrient agar medium added with glycine (4.4 g/L). The agar was covered with a Whatman number 1 filter paper previously soaked in a specific solution (0.5% picric acid and 2% sodium carbonate w/v).

The dishes were sealed with Parafilm and incubated at 28 ^{o}C for 48 h. A change of colour of the filter paper from yellow to light brown, brown or reddish-brown was recorded as weak (+), moderate (++) or strong (+++) reaction respectively.

Morphological and biochemical characterization

Colony morphology, size, color, shape and growth pattern of 31 rhizobateria isolates were recorded after 24 h of growth on LB agar plates at 28 ± 1°C (Somasegaran and Hoben, 1994). Cell size and motility was observed by light microscopy. These isolates were biochemically characterized using various tests such as gelatin liquefaction, starch hydrolysis, H_2S, Arginine, citrate, KOH, Oxidase, arginine hydrolase, nitrate reduction and fermentation of various sugars as described by Schaad *et al.,* (2001).

Results and Discussion

Chili rhizosphere soil samples were collected from agro-climatic regions of Southern Plateau and Hills. Rhizobacterial colonies have been isolated on various microbiological media, Nutrient agar, Kings B, Tryptone soya agar and casamino peptone glucose media. Various bacterial colonies were observed in the respective media. Thirty one rhizobacterial isolates were isolated from different rhizospheric soil of chilli and out of these, 17 isolates of rhizobacteria from Karnataka, 14 isolates from Andhra Pradesh were selected and screened for their various morphological characteristics of colonies on the media such as texture, size, margin, shape and pigmentation (Table 1). All the microbes identified have a smooth-rough-slim texture and a color range between pure white-translucent-light brown-red-cream-yellow (Fig. 1). Rhizobacteria were characterized by using morphological and biochemical methods (Table 2). Out of 31 rhizobacteria isolates, 14 rhizobacteria were characterized, in which 11 isolates showed KOH test negative and gram reaction positive. Majority of the isolates showed rod shaped (9 isolates) and five isolates were coccus shaped. All rhizobacteria showed positive for utilization glucose as carbon source and 42.86 % isolates used galactose, and mannose as carbon sources. Xylose and sucrose were utilized by 78.57 % rhizobacteria. In biochemical test, all isolates showed positive in catalase test, where as 92.86 % isolates were positive in catalase test. Citrate utilization and H_2 S production were recorded 64.29 and 50.00 % isolates of rhizobacteria respectively, while 85.71 percent of rhizobacteria were positive for amylase production. It indicates that there are diverse group of bacteria present in the rhizosphere of chilli plants and they behave differently in utilization of carbon sources and production of different enzymes. As reported earlier that rhizospheric soil of chilli plant has a plenty of bacterial populations including antagonistic, plant growth stimulating bacteria and other bacteria. In these bacterial population, majority of them belong to *Bacillus* species and *Bacillus* derived genera isolated especially from the rhizospheric soil of wheat (Upadhyay *et al.,* 2009) and tomato (Tan *et al.,* 2013 Singh *et al.,* 2016) plants.

In Antagonistic assay, out of 31 isolates of rhizobacteria, 11 isolates showed positive result to inhibit the growth of *Ralstonia solanacearumin vitro* conditions. The presence of rhizobacterial isolates, isolate KA9 isolated from Karnataka formed highest inhibition zone (20.6 mm diameter) against *R. solanacearum* after 48h.

The inhibition zone formed by rhizobacteria was increased significantly by increasing the duration of culturing from 48 h to 144 h in broth culture (Fig. 2a and 2b). However, out 31 isolates, 64.52 % isolates did not show inhibitory effect on the growth of *R. solanacearum in vitro* conditions (Table 3).

Table.1 Morphological characterization of rhizobacterial colonies on isolates from rhizospheric soil of chilli from Karnataka and Andhra Pradesh states of India

Isolates	Size	Shape	Margin	Opacity	Elevation	Texture	Pigmentation
KA-1	Small	Irregular	Entire round	Opaque	Convex	Smooth	**Light yellow**
KA-2	Large	Round	Entire round	Opaque	Concave	Smooth	**Creamy-white**
KA-3	Medium	Round	Entire round	Transparent	Flat	Smooth	**Nil**
KA-4	Large	Regular	Entire round	Opaque	Convex	Smooth	**Greenish yellow**
KA-5	Medium	Round	thick ridges	Opaque	Convex	smooth, moist	**Gray-white**
KA-6	Large	Uniform	Undulate	Opaque	Flat	Rough	**White**
KA-7	Large	Regular	Entire round	Opaque	Convex	Smooth	**Greenish yellow**
KA-8	Small	Regular	Entire round	Opaque	Convex	Smooth	**Light red**
KA-9	Small	Irregular	Entire round	Opaque	Convex	Smooth	**Light yellow**
KA-10	Large	Round	Undulate	Semi- transparent	Flat	Slummy rough	**Off-white**
KA-11	Large	Irregular	Undulate	Opaque	Flat	Smooth	**Greenish yellow**
KA-12	Small	Round	Entire round	Opaque	Convex	Smooth	**White**
KA-13	Large	Irregular	Entire round	Opaque	Flat	Smooth	**Pale yellow**
KA-14	Small	Uniform	Entire round	Transparent	Convex	Smooth-slums	**Nil**
KA-15	Medium	Uniform	Entire round	Semi- transparent	Flat	Smooth	**Light brown**
KA-16	Very small	Uniform	Entire round	Opaque	Flat	Smooth	**Greenish yellow**
KA-17	Medium	Irregular	Undulate	Opaque	Flat	Irregular	**White**
AP-1	Medium	Uniform	Entire round	Opaque	Flat	Smooth-shiny	**Off-white**
AP-2	Large	Uniform	Entire round	Semi- transparent	Flat	Smooth-shiny	**Nil**
AP-3	Medium	Round	Entire round	Transparent	Flat	Smooth	**Nil**
AP-4	Medium	Round	thick ridges	Opaque	Convex	smooth, moist	**Gray-white**
AP-5	Very small	Uniform	Entire round	Opaque	Flat	Smooth	**White**
AP-6	Medium	Round	Entire round	Transparent	Flat	Smooth	**Nil**
AP-7	Small	Uniform	Entire round	Transparent	Convex	Smooth-slums	**Nil**
AP-8	Large	Regular	Entire round	Opaque	Convex	Smooth	**Off white**
AP-9	Large	Round	Entire round	Opaque	Convex	Smooth	**Off white**
AP-10	Small	Uniform	Entire round	Opaque	Convex	Smooth, Shiny	**Greenish yellow**
AP-11	Large	Regular	Entire round	Opaque	Convex	Smooth	**Off white**
AP-12	Small	Round	Entire round	Opaque	Convex	Smooth	**White**
AP-13	Very small	Uniform	Entire round	Opaque	Flat	Smooth	**White**
AP-14	**Small**	**Uniform**	**Entire round**	**Transparent**	**Convex**	**Smooth-slums**	**Nil**

Table.2 Morphological and biochemical characterization of rhizobacteria isolates isolated from rhizosphere of chilli from Karnataka and Andhra Pradesh states of India

Strains	KOH test	Gram reaction	Shape of bacteria	Glucose	Galactose	Lactose	Sorbitol	Mannose	Xylose	Sucrose	Citrate	H_2S	Arginine	Catalase	Amylase
KA2	-	+	Cocci	±	-	-	-	±	±	±	-	+	+	+	+
KA3	-	+	Cocci	+	+	+	-	-	+	+	+	+	+	+	+
KA5	-	+	Rod	±	-	-	+	+	±	+	+	-	+	+	+
KA-7	+	-	Rod	+	+	-	+	+	-	+	+	-	+	+	+
KA9	-	+	Rod	+	-	-	-	+	+	+	+	-	+	+	+
KA13	-	+	Rod	+	+	-	+	-	+	-	+	+	+	+	+
KA14	-	+	Rod	+	+	+	-	-	+	+	+	-	+	+	+
KA-15	+	-	Rod	+	-	+	+	-	+	-	-	+	-	+	+
KA-17	+	-	Rod	+	+	-	+	-	+	+	-	-	+	+	+
AP8	-	+	Rod	+	-	-	+	+	-	-	-	+	+	+	-
AP13	-	+	cocci	+	-	+	+	-	+	+	+	+	+	+	-
AP2	-	+	cocci	+	+	-	+	+	-	+	+	-	+	+	+
AP6	-	+	cocci	+	-	+	-	-	+	+	-	-	+	+	+
AP11	-	+	**Rod**	±	-	-	±	-	+	+	+	+	+	+	+

Table.3 Characterization of selected isolates of rhizobacteria for their plant growth promoting traits isolated from Southern Plateau and Hills region of India

Isolates of rhizobacteria	IAA Production	Phosphorus solubilization	Ammonia production	HCN Production	Siderophores Production
KA2	+	+	++	+	+
KA3	+	+	+	-	-
KA5	-	+	+	+	+
KA-7	+	-	+	-	-
KA9	+++	+	++	+	+
KA13	+	-	-	-	-
KA14	+	+	+	-	+
KA-15	-	+	-	+	+
KA-17	+	-	+	-	-
AP8	+	+	+	-	+
AP13	++	+	+	+	-
AP2	-	-	+	-	+
AP6	+	+	+	-	-
AP11	+	+	-	+	-

Fig.1 Representative photographs of rhizobacterial colonies on different media

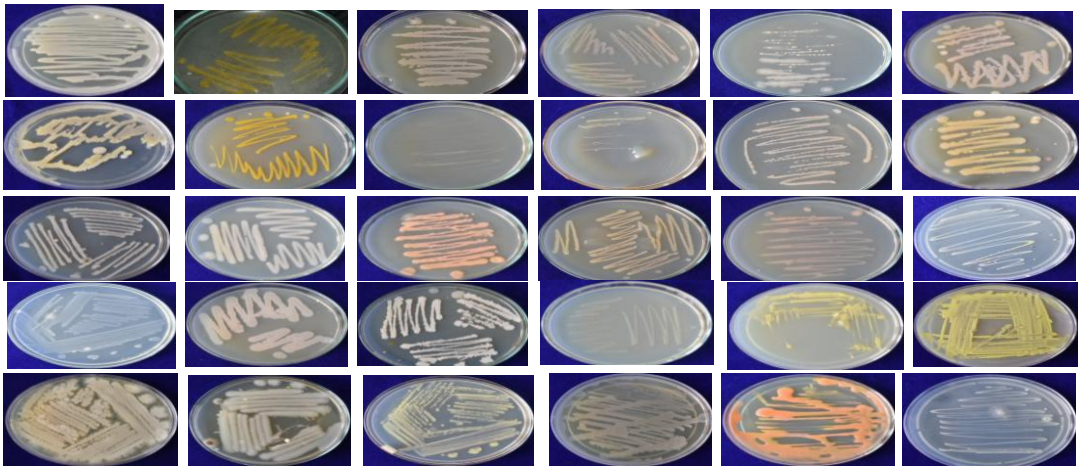


Fig.2a Showing antagonistic activity of rhizobacteria isolates against *Ralstonia solanacearum under in vitro* conditions

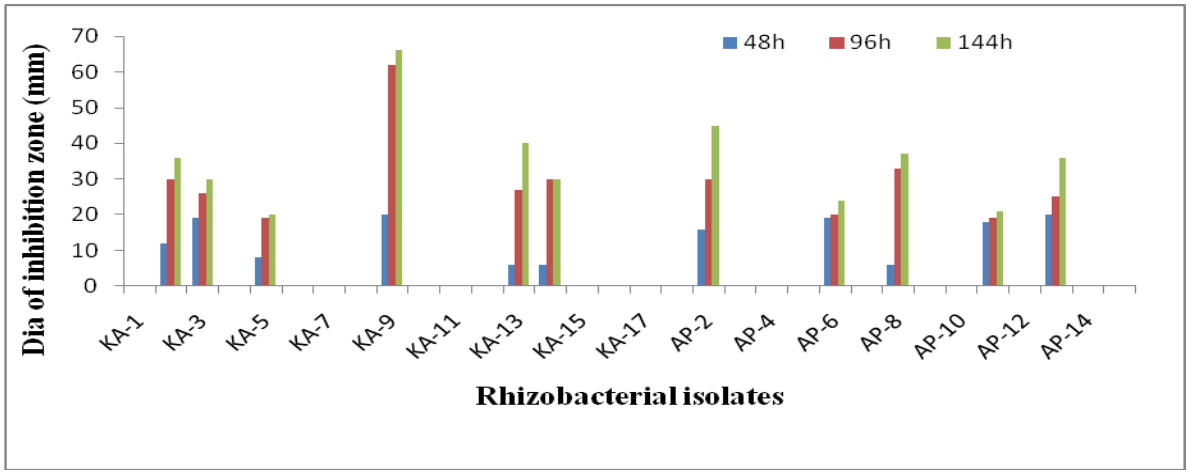


Fig.2b Antagonistic activity of rhizobacterial isolates against *Ralstonia solanacearum under in vitro* conditions

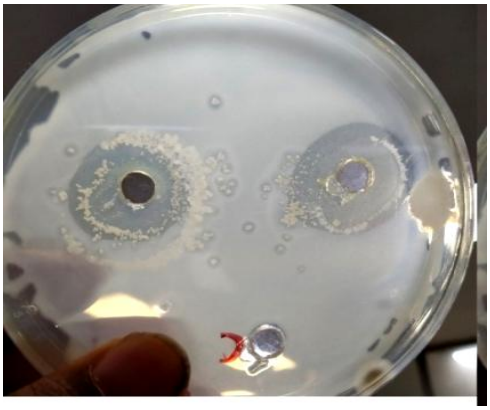
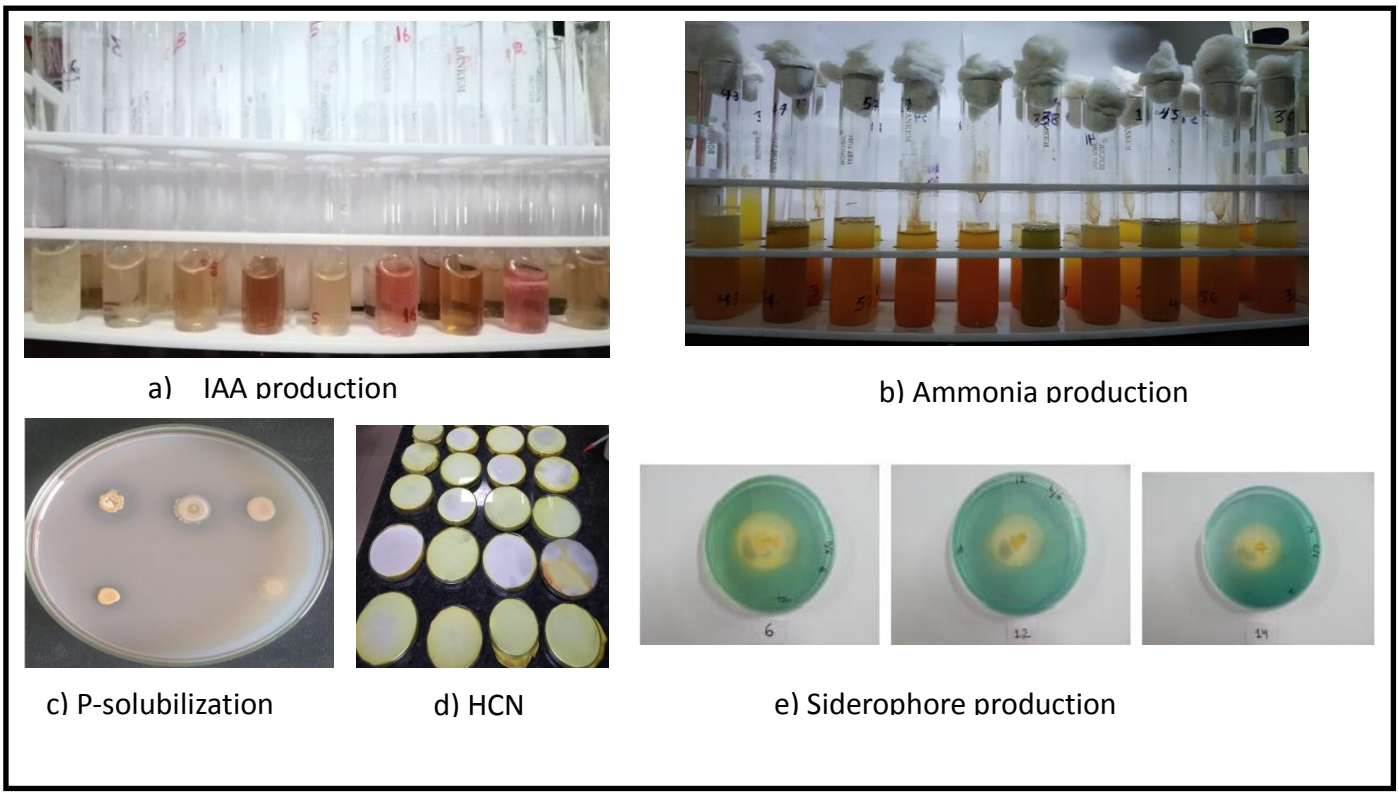


Fig.3 *In vitro* assay for plant growth promoting activities of rhizobacteria isolates isolated from rhizosphere of chilli



The rhozbacteria inhibited the growth o *R. solanacearum* on agar medium might be due to the production of secondary metabolites by the rhizobacteria (Gross and Vidaver, 1990) particularly antibiotic production (Aliya *et al.,* 2008; Singh *et al.,* 2013).

The eleven isolates of rhizobacteria having antagonist activity against *R. solanacearum* along with three more isolates those did not show antagonistic activity were selected for plant growth promoting such as production of indole acetic acid, siderophores, ammonia, Hydrogen cyanide and phosphate solubilization under *in vitro* conditions (Fig. 3). Table 2 reveals that out of 14 rhizobacteria isolates 78.57 % isolates showed positive in production of indole acetic acid and among them, isolate KA9 showed most potential for production of indole acetic acid followed by isolate AP13. Similar finding has also been reported in *B. amyloliquefaciens* FZB42, which produces IAA to promote the plant growth (Idris *et al.,* 2007), which is an important growth promoting hormones for the plant. 71.43 % isolates were able to solubilize phosphorus and 78.57 % isolates produced ammonia *in vitro* conditions. The phosphorus was solubilised by >70 % isolates of rhizobacteria which is an important major nutrient element for plant. Fifty percent isolates were able to produced siderophores while only 42.86 % isolates produced hydrogen cyanide. Siderophores, which may contribute as iron chelating and produces soluble complexes which is taken by plant or it make insoluble to phytopathogenic bacteria by binding the available form of iron in the soil (Kamnev and van der Lelie, 2000). Among these isolates, KA9 and KA2 isolates had ability to produce all pant growth promoting traits like production of indole

acetic acid, siderophores, ammonia, Hydrogen cyanide and phosphate solubilization (Fig 4, Table 2). The results indicate that all rhizobacteria did not possess ability to promote plant growth. Isolation of potential antagonistic bacteria from the soil is an important way to control plant disease successfully (Kohl *et al.,* 2015). However, This shows that KA9 KA2 and AP13isolates have a potential for plant growth promotions and in most studies it has been found that PGPR promotes the emergence of seedlings, crop yield, plant growth and plant protection against phytopathogens (Dey *et al.,* 2004; Herman *et al.,* 2008; Kloepper *et al.,* 2004; Kokalis *et al.,* 2006).

Based on morphological, Biochemical and PGP traits screening, we found three rhizobacteria isolates KA9, KA2 and AP13 showing most promising results in terms of biocontrol potential and plant growth promotions. Plant growth promoting (PGP) traits, morphological, and biochemical were done and found variable characteristic results in our studies. This type of research is important because it promotes the use of PGPR as biofertilizers or as bioinoculants as an effective approach to reducing the use of harmful pesticides and chemical fertilizers.

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