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Characterization of plant growth promoting rhizobacteria for compatibility with commonly used Agrochemicals

Bh. Sarvani*, R. Subhash Reddy and J. Sai Prasad

****Department of Agricultural Microbiology and Bioenergy,***
College of Agriculture, Rajendranagar, Hyderabad 500 030, Andhra Pradesh, India

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ABSTRACT

Modern Agriculture is heavily dependent on the application of chemical pesticides for disease control. Due to the concerns regarding both human health and environmental protection, viable alternatives to these chemicals are being sought. The interest in the use of biological approaches to replace hazardous pesticides in fertilizing soils or improve plant resistance against phytopathogens is steadily gaining worldwide acceptance. PGPR (Plant Growth Promoting Rhizobacteria) isolates having biocontrol activity are more effective when they are compatible with the plant protection inputs like pesticides, they can be freely used in the integrated crop protection practices. Therefore, the present investigation was undertaken to isolate and characterize bacterial isolates as *Rhizobium*, *Pseudomonas* and *Bacillus* isolates based on cultural, morphological and biochemical characters. The isolates were previously studied for PGP attributes including biocontrol activity. The PGP isolates having biocontrol activity were selected further to know the compatibility against commonly used agrochemicals like fungicides (Copper oxy chloride, Carbendazim, Thiram and Captan) insecticides (Phorate, Carbofuran, Imidachloprid and Chlorpyriphos) and herbicides (Alachlor, Butachlor, Pendimethalin and Oxy fluorofen) at their recommended and half the recommended dosages. Results revealed that, majority of the isolates found to be compatible with the agrochemicals used at their recommended and half the recommended dosages.

Key words *: Antagonistic property, Agrochemicals, Plant growth promoting Rhizobacteria (PGPR).*

Introduction

In Agriculture, the pesticides are recurrently applied for three major objectives- (i) To produce a larger yield (ii) to produce crops of high quality and (iii) to reduce the input of labor and energy in to crop production (Ayansina, 2009). Millions of tons of pesticides are applied annually; however, less than 5% of these products are estimated to reach the target organism, with the remainder being deposited on the soil and non-target organisms, as well as moving in to the atmosphere and water (Ahmed and Khan, 2010).

Previous studies reported mixed effects of the different classes of pesticides (Insecticides, Herbicides and Fungicides) on rhizobia. Published studies regarding pesticides' effect on free living or symbiotic rhizobia and done with few pesticides on a limited number of strains (Singh *et al*., 2002; Khan *et al.,* 2009; Srinivas *et al.,* 2008) or with a limited number of pesticides on different strains of the same genus (Santos *et al*., 2005; Kaur *et al.,* 2007). To our knowledge, no study investigated a combination of many pesticides with plant growth promoting

rhizobacterial isolates other than the *Rhizobium*, i.e. *Pseudomonas fluorescens* and *Bacillus* sp.

PGPR isolates having biocontrol activity are more effective when they are compatible with the plant protection inputs like pesticides, they can be freely used in the integrated crop protection practices. Biological control approach will be successful, if the biocontrol agents are compatible with the fungicides, insecticides and herbicides which are commonly used under field conditions.

The aim of the present study was to evaluate the compatibility of 40 PGPR isolates against 12 different pesticides (Herbicides, Insecticides and Fungicides). Although bacteria are more sensitive to pesticides in pure culture studies, the results reported in the present study will supply information that will be useful to predict the response of legumes to rhizobacterial inoculation in the presence of pesticides.

Materials and Methods

Isolation of bacterial isolates

The rhizospheric soil samples (20) were collected from fields growing Groundnut and Redgram crops from Rangareddy district, India. *Rhizobium* was isolated on Yeast Extract Mannitol Agar (Vincent, 1970), *Pseudomonas* on King's B agar (King *et al*., 1954) and *Bacillus* on Nutrient agar.

Identification of bacterial isolates

Cultural Characters: After incubation, Cultures were studied for their colony characters such as size, shape, margin, consistency, pigmentation etc. The colonies that were expected to be *Rhizobium*, *Pseudomonas* and *Bacillus* isolates were picked out and purified on respective media plates as mentioned.

Morphological Characters: Purified cultures were further studied for their cell morphology viz., Cell shape, Cell arrangement, response to the gram stain and for spore formation under 100X magnification of light microscope.

Biochemical Characters: The cultures were finally confirmed by following special procedures as described in Bergey's manual of systemic bacteriology and the confirmed pure isolates were maintained on the respective slants in refrigerator at 4 ^{o}C.

Screening for Antagonistic property

All the bacterial isolates were tested for antagonism against three soil borne pathogens such as *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium solani* by following dual culture technique (Skidmore and Dickinson, 1976).

The per cent growth inhibition over control was calculated by using the formula:

$$\text{Percent Inhibition} = \frac{\text{Growth of Pathogen in control (mm)} - \text{Growth of Pathogen in treatmnet (mm)}}{\text{Growth of Pathogen in control (mm)}} \times 100$$

Compatibility of efficient antagonists with commonly used agrochemicals

Compatibility of bacterial test isolates with commonly used agrochemicals like fungicides (Copper oxy chloride, Carbendazim, Thiram, Captan), insecticides (Phorate, Carbofuron, Imidacloprid, Chlorpyriphos) and herbicides (Alachlor, Butachlor, Pendimethalin, Oxy fluorofen) was tested by following inhibition zone technique (Nene and Thapliyal, 1993) at recommended and half recommended dosages (Table 1) by maintaining three replications.

5ml of water agar seeded with bacterial suspension was poured in to the petri plates containing 10ml of warm nutrient agar and rotated gently for uniform distribution. Sterilized filter paper discs of 6mm in diameter were dipped in different chemicals at different concentrations dried and placed over bacteria seeded nutrient agar medium plates. Respective control was maintained by dipping the filter paper discs in sterile distilled water and plates were incubated at 28 ± 2^{0}C for 48 to72 h. The inhibition zone (mm) around the disc was measured and calculated.

Results and Discussion

Isolation and Identification of bacterial isolates

A total of thirty bacterial isolates were obtained from twenty soil samples of groundnut and redgram crops and were identified as *Rhizobium* (10), *Pseudomonas* (15) and *Bacillus* (15) isolates based on their morphology, cultural (Table 2) and biochemical characteristics (Table 3).

Cultural characters: About ten isolates showed small-medium, milky translucent, raised, mucoid

colonies and formed non-spreading type of colonies. Fifteen of the total isolates took about 48h to establish their growth on *Pseudomonas* fluorescein agar. All the isolates developed small-medium, smooth and shiny colonies, of them about eight isolates showed yellowish green color colonies with green color pigmentation. Some of the isolates were fast growers and have taken 24h to show medium, off white, flat, rough and irregular colonies and formed spreading type of colonies.

Morphological characters: When these isolates were subjected to microscopic studies, twenty five of the isolates were found gram –ve, small, single, isolated rods without sporulation observed whereas, the remaining fifteen isolates were gram + ve, stout, isolated, single rods with sporulation were observed under microscope.

Biochemical characters: All the bacterial isolates were subjected to the above mentioned biochemical tests and were identified as *Rhizobium*, *Pseudomonas* and *Bacillus* isolates (Table 3). Mahalakshmi and Reetha (2009) isolated forty four bacterial isolates from the rhizosphere of tomato and were grouped into *Azospirillum* (18), *Azotobacter* (9) *Pseudomonas* (12) and *Bacillus* (5) based on their morphological and biochemical characteristics.

Screening for Antagonistic property

Majority of the isolates showed antagonism against one/more of the phyto pathogens *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium solani*. Some of the isolates showed antagonistic activity by the production of siderophores, while some of the isolates produced Hydrogen cyanide like substances, through which they have inhibited the mycelia growth. Interestingly, some of the isolates showed antagonism without producing either siderophores/ HCN, indicating that some other mechanisms like production of mycolytic enzymes which may be involved in the biocontrol mechanism (Table 4). Similar results were studied by Shaban and El-Bramaway (2011), in which the combined effect of both *Rhizobium* spp and *Trichoderma* sp were found to be beneficial in controlling the fungal diseases of legume crops caused by *Fusarium oxysporum*, *F. solani*, *Macrophomina phaseolina*, *Rhizoctonia solani* and *Sclerotium rolfsii*. Dev and Dawande (2010) evaluated the antagonistic property of *Pseudomonas fluorescens* against *Rhizoctonia solani*, which revealed that the mycolytic enzymes produced by these antagonists

Table 1. Recommended and Half the recommended dosages of the agrochemicals tested

Agrochemicals	Recommended dosage	Half the recommended dosage
FUNGICIDES		
1. Copper oxy chloride 50WP	3 g/l	1.5 g/l
2. Carbendazim 50WP	1 g/l	0.5 g/l
3. Thiram 50 WP	3 g/l	1.5 g/l
4. Captan 50 WP	3 g/l	1.5 g/l
INSECTICIDES		
1. Phorate 10G	25 g/l	12.5 g/l
2.Carbofuran 3G	50 g/l	25 g/l
3. Imidachloprid 17.8 SL	0.25 ml/l	0.125 ml/l
4. Chlorpyriphos 20EC	2.5 ml/l	1.25 ml/l
HERBICIDES		
1. Alachlor 50EC	4 ml/l	2 ml/l
2. Butachlor 50EC	4 ml/l	2 ml/l
3. Pendimethalin 30EC	6.6 ml/l	3.3 ml/l
4. Oxy fluorofen 1.6 EC	1.25 ml/l	0.625 ml/l

Table 2. Cultural and Morphological characters of the bacterial isolates

Isolates	No.	Size	Shape	Elevation	Colour	Pigment production	Spore production	Cell shape	Cell arrangement	Gram Reaction
Rhizobium	10	small	Round	Convex	Watery	No	No	Rod	Isolated	-ve
Pseudomonas	15	medium	Round	Convex	Greenish	Yes	No	Rod	Isolated	-ve
Bacillus sp.	15	medium	Irregular	Flat	Off white	No	Yes	Rod	Isolated	+ve

suppressed the growth of *Rhizoctonia solani.*

Testing for *in vitro* compatibility with Agrochemicals

In the present study, results revealed that majority of the isolates showed compatibility with the commonly used pesticides tested except few which were found sensitive (Table 4).

Compatibility with fungicides: None of the fungicides had any negative effect on the *Rhizobium* isolates. But some of the fungicides inhibited the growth of some *Pseudomonas* and *Bacillus* isolates. Only four of the fifteen *Pseudomonas* isolates, SFGP, SFRP, AGP and ARP were significantly inhibited by Thiram with maximum of 8mm, 5mm and with minimum of 5 mm, 3 mm zones at its recommended and half the recommended dosages.

In case of *Bacillus* isolates, three (COC, Thiram and Captan) of four fungicides inhibited the bacterial growth, whereas Carbendazim did not influence the growth of the isolates. Three isolates KGB, YGB and YRB were inhibited by COC with 2mm at recommended dosage and 1mm (MRB) at half the recommended dosage. Thirteen isolates except DRB and KGB were affected by Thiram with maximum inhibition of 8mm, 5mm (ARB, CFRB and MRB) and with minimum of 1mm, 0 (SFGB and SFRB) at its recommended and half the recommended dosages.

The growth of fourteen isolates except DRB was inhibited by Captan with maximum of 4mm (MRB) and with minimum inhibition of 2mm and 1mm at recommended and half the recommended dosages. Similar studies were found with survival of *Mesorhizobium ciceri* and phosphate solubilizing bacteria on chick pea seeds treated with fungicides Bavistin and Thiram and observed the viability of the bacteria which confirmed the compatibility (Sunitha *et al*., 2007). Madavi (2006) evaluated compatibility of different agrochemicals against *Pseudomonas fluorescens* strains *in vitro* and stated that Carbendazim was found to be compatible at their recommended and half the recommended dosages.

Kutcher *et al*. (2002) studied the effect of seed applied liquid or soil-applied granular *Rhizobium*

Table 3. Biochemical characterization of the bacterial isolates

Property		Bacterial isolates		
		Rhizobium sp.	*Pseudomonas fluorescens*	*Bacillus* sp.
1.	Indole production	+	+	+
2.	Methyl Red	–	–	–
3.	Voges Proskaur	–	–	–
4.	Citrate utilization	+	+	+
5.	Oxidase test	+	+	–
6.	Catalase test	+	+	+
7.	Hydrogen Sulfide	+	+	–
8.	Gelatin hydrolysis	–	–	+
9.	Ammonia Production	+	+	–
10.	Hofer's alkaline test	–	Not Done	Not Done
11.	Nitrate reduction	+	+	+
12.	Nodulation test	+	–	–
13.	Growth at 4^0C	+	+	–
14.	Growth at 41^0C	–	–	+

Table 4. Plant Growth promoting and Biocontrol activities of bacterial isolates

Bacterial Isolates	Plant Growth Promoting (PGP) characters				Antagonistic activity		
	Phosphate solubilization	IAA production	Siderophores	HCN	*R. solani*	*S. rolfsii*	*F. solani*
Rhizobium (10)	100%	100%	70%	100%	85%	85%	95%
Pseudomonas (15)	80%	80%	80%	40%	100%	100%	100%
Bacillus sp. (15)	33%	33%	33%	33%	100%	100%	100%

Note: the percent mentioned here in the study was regarding number of isolates showed positive for the growth promoting attributes.

(*Rhizobium leguminosarum* bv. *viciae*) inoculants and seed- applied fungicide treatments Thiram 75% WP and showed that there was compatibility between the seed-applied fungicide and seed-applied liquid or soil-applied granular *Rhizobium* inoculants. Naidu (2000) studied the effect of pre and post- inoculated seed treatment with chemicals on *Rhizobium* in Groundnut (*Arachis hypogeae*) and concluded that Thiram was non- toxic to *Rhizobium* in both pre- and post- inoculated seed treatments and gave more pod yield, whereas, post-seed treatment with Carbendazim, Benlate and Carbofuran was found to be beneficial and non- toxic.

Compatibility with Insecticides: All the bacterial isolates of *Rhizobium* (10), *Pseudomonas* (15) and *Bacillus* (15) were screened for their compatibility with the four insecticides Phorate, Carbofuran, Imidachloprid and Chlorpyriphos. Results revealed that, all the four insecticides did not inhibit the growth of PGPR (*Rhizobium*, *Pseudomonas* and *Bacillus*) isolates indicating their compatibility with the isolates used in the present study. Hence, any of these isolates can be used in combination with any of the above insecticides. Cheema *et al*. (2009), proved that insecticide Endosulfan @ 15 ml kg^{-1} and fungicide Captan were compatible with *Rhizobium* inoculants when applied as seed treatment for the control of termites in Chickpea (*Cicer arietinum* L.).

Ghosh *et al*. (2003) evaluated lethal concentrations of Chlorpyriphos, and Phorate in Green gram [*Vigna radiate*] and concluded that nitrogen fixation and grain yield were highest with *Rhizobium* + Chlorpyriphos and *Rhizobium* + Phorate treatments when *Rhizobium* was applied simultaneously or 24h after insecticide application, respectively.

Compatibility with Herbicides: When the bacterial isolates were tested with the four commonly used herbicides Alachlor, Butachlor, Pendimethalin and Oxy fluorofen. While the growth of the some of *Rhizobium* isolates was inhibited by the herbicides, none of the *Pseudomonas* and *Bacillus* isolates growth was inhibited by the herbicides.

Of the ten *Rhizobium* isolates tested, four isolates (SFRR, DGR, KGR and KRR) were affected by Alachlor with maximum inhibition of 3 mm and 1mm (DGR) and minimum inhibition of 1 mm (SFRR). Butachlor inhibited KGR and KRR isolates with maximum inhibition of 2 mm, 1 mm and minimum of 1mm respectively. Pendimethalin influenced seven isolates with highest inhibition zone of 3mm, 1 mm (AGR and CFGR) and minimum of 1mm (KGR). Oxy fluorofen also inhibited the growth of seven isolates, of which KRR recorded maximum of 3 mm and 2 mm at its recommended and half the recommended dosages, whereas SFGR recorded minimum inhibition of 1mm at its recommended dosage. When the four herbicides were evaluated for their compatibility with *Pseudomonas* and *Bacillus* isolates, no inhibition was found with any of the tested herbicides.

Since none of the isolates of *Pseudomonas*, *Bacillus* and *Rhizobium* isolates (DRR and SBGR) growth was not affected by the herbicides used in the present study. Hence, these isolates have the potential for their use as PGPR in combination with these herbicides. Jhala *et al.* (2005), reported that higher pod yield in Groundnut (*Arachis hypogaea*) was obtained significantly when four herbicides Fluchloralin,

Table 5. Number of bacterial isolates that are sensitive to different pesticides at Recommended (R) and half the recommended dosages (HR).

Type of chemical	Number of bacterial isolates that were sensitive to the agrochemicals					
	Rhizobium		*Pseudomonas*		*Bacillus* sp. (15)	
	H	HR	H	HR	H	HR
Fungicides						
Copper oxy chloride	0	0	0	0	3 (20%)	1 (6.6%)
Carbendazim	0	0	0	0	0	0
Thiram	0	0	4 (26.6%)	4 (26.6%)	13 (86.6%)	12 (80%)
Captan	0	0	0	0	14 (93.3%)	13 (86.6%)
Herbicides						
Alachlor	4 (40%)	3 (30%)	0	0	0	0
Butachlor	2 (20%)	1 (10%)	0	0	0	0
Pendimethalin	7 (70%)	4 (40%)	0	0	0	0
Oxy fluorofen	7 (70%)	‘5 (50%)	0	0	0	0

Note: The values given in the parenthesis indicates the percent of isolates that were sensitive to the chemical used.

Pendimethalin, Butachlor and Metalachlor were applied along with *Rhizobium* inoculation. Sarkar *et al.* (2005) tested the bio efficacy of Pendimethalin and Fluchloralin in mustard and concluded that the populations of fluorescent Pseudomonads were improved with the application of herbicides. Deshmukh *et al.* (2003) conducted field experiment to evaluate the effect of *Rhizobium japonicum* [*Bradyrhizobium japonicum*] with different herbicides (Alachlor, Pendimethalin, and Fluchloralin) and concluded that improved grain yield was obtained with application of *R. japonicum* + Pendimethalin.

Out of the forty isolates tested for their compatibility with the four each of the fungicides, insecticides and herbicides, and based on their PGPR attributes and antagonistic activity, the isolate *Rhizobium* SBGR, *Pseudomonas* isolate DGP, *Bacillus* isolate SBRB showed potential as PGPR. However, it is necessary to conduct the pot culture and field studies before using as bacterial inoculant for the purpose of improving crop growth and yield due to their PGPR attributes, biocontrol activity and compatibility with the fungicides, insecticides and herbicides.

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