Research Article

Isolation and evaluation of summer legumes Rhizobia as PGPR

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Abstract

Rhizobia are bacteria well known for biological nitrogen fixation through symbioses. These bacteria may also perform as plant growth promoting rhizobacteria (PGPR). In this study the role of rhizobia as PGPR was studied. Rhizobia was isolated from the root nodules of various summer legumes including *Glycine max* (Soybean), *Vigna radiata* (Mung bean), *Vigna unguiculata* (Cowpea), *Sesbania grandiflora* (Sesbania) and *Cymposistetra gonoloba* (Guar) grown at New Developmental Farm (NDF), the University of Agriculture Peshawar, Pakistan during summer 2011. Rhizobia were isolated from root nodules by using yeast minitol agar media, pure culture of each isolate was prepared and examined for various PGPR traits. Data revealed that 9, 83, 47 and 21% of tested strains were found to produce cyanide (HCN), siderophores, indole-acetic acid (IAA) and solubilized phosphorus respectively. Among the tested rhizobial isolates, rhizobia isolated from *Vigna radiata* showed highest PGPR activity followed by *Glycine max* while relatively lower PGPR activity was observed for rhizobial isolates of *Cymposistetra gonoloba* (Guar). All the tested isolates were gram negative. Soybean and cowpea isolates were found to be fast growing while mung bean, sesbania and guar were slow growing in nature. This study indicates that rhizobial isolates of *Vigna radiata* and *Glycine max* have the capability to be used as PGPR.

Key words: Rhizobia; PGPR; Legumes; Siderophores; *Vigna radiata*; *Glycine max*

Introduction

Rhizobia is a soil bacteria of family *Rhizobiaceae* [1] are Gram-negative, motile, non-sporulating rod shaped bacteria that can survive both in soil and inside the root nodules of host legumes [2]. Rhizobia fix

atmospheric nitrogen upon mutualism with legumes by the process called biological nitrogen fixation [3]. Nitrogen fixed from atmosphere symbiotically by the Rhizobium species and legume association is a renewable source of nitrogen for sustainable

agriculture [4]. Every year approximately 70 million tons of N_2 is fixed from atmosphere through biological nitrogen fixation [5].

Rhizobia which depends on soil organic matter without legume partners are known as native rhizobia. Farmers also add rhizobia as inoculants which are known as introduced rhizobia. The rhizobia that are exactly well-matched with a certain legume can cause nodulations [6]. This process has great economic importance for legume productivity because of which rhizobium inoculants are produced on large scale in different countries of the world. Inoculum is consisted of rhizobia obtained from plant nodules and cultured in the laboratory artificially on a special medium called yeast mannitol agar (YMA).

Modern research indicates that rhizobia can also be used as PGPR in cereals [7]. Researchers described growth promoting nature of rhizobia with certain non-legumes [8] by establishing internal associations with roots of rice [9], maize, wheat, barley, canola, and lettuce. Rhizobia promote growth and yield of cereals in numerous ways such as manufacture phytohormones IAA, gibberellins and cytokinins, siderophore [10], enzymes [11], increase supply of insoluble nutrients such as phosphorus [12] and iron through release of organic acids which enhance its solubility [13], execute pathogens by making antibiotics, HCN and exopolysaccharides, improve morphological characteristics of inoculated roots [14] which modify nutrient and water use efficiency, increase stress resistance and induce systemic stress and drought tolerance/resistance by producing substances like abscisic acid or lumichrome which decrease leaf stomatal opening and water losses from plants through the leaves by transpiration.

Isolation and characterization of rhizobia from root nodules of legume is essential for

preparation of inoculum for both legumes and cereals as it also act as PGPR, thus efficient use of the natural resources may take place for sustainable agriculture. This study was therefore carried out to isolate rhizobia from the root nodules of summer legumes such as soybean, mungbean, cowpea, guar, and sesbania grown in calcareous soil in Peshawar valley and to evaluate its potential as PGPR.

Materials and methods

An experiment was carried out at the University of Agriculture Peshawar Pakistan during winter 2011 to isolate rhizobia from root nodules of summer legumes and evaluate its potential as plant growth promoting rhizobia (PGPR). The following experimental protocol was used:

Sampling

Samples of nodulated roots were collected with the help of spade at pod fill stage for the isolation of rhizobia at proper soil moisture condition. During sampling the plants were carefully uprooted along with approximately 6 inches (15 cm) of rhizosphere soil to avoid detachment of nodules from roots. After collection, nodulated roots were kept in a bucket containing tap water for about 15 minutes. The soil was then carefully washed from the nodulated roots.

Washing and selection of nodules

The nodulated roots were properly washed with tap water to wash away adhered materials. Healthy, pink, unbroken and firm nodules were pointed in order to be isolated. The nodules were not removed from the roots, but roots were cut 0.5 cm each side of the nodule [15].

Surface sterilization of nodules

The surface of the nodules were sterilized by putting them step-wise for 4-5 minutes in each sterilized water, 95% ethanol (to break the surface tension and to remove air bubbles from the tissue), sterilized water,

0.1% mercuric chloride solution and finally again in sterilized water aseptically [15].

Isolation of Rhizobia from nodules

Nodules which were surface sterilized were immersed in sterilized water and were wrinkled with sterilized glass rod inside the glass tube. The crushed nodules suspension was taken with a sterilized loop and splashed

on YMA plates aseptically. The streak plates were properly sealed labeled and incubated at 28-30 °C [16].

Preparation of media

Yeast manitol agar (YMA) media was used for the growth of the rhizobia. Ingredients for one liter of YMA media were as follows:

Table 1. Composition of yeast manitol agar (YMA) media per liter

Ingredients	Quantity (g L-1)
K2HP04	0.50
MgS04.7H2O	0.20 .
NaCl	0.10
Mannitol	10.0
Yeast Extract	1.00
Agar	15-20
Congo Red 0.25 % solution.	10 ml
pH	6.8-7.0

All the above ingredients presented in table 1 were weighed for the preparation of 1000 ml media and dissolved by heating up to boiling in a can. The hot mixture was cotton filtered and poured in to 5 flasks about 200 ml per flask. The flasks were properly plugged and autoclaved at 121 °C and 15 psi pressure for 40 min. Few drops of Congo red were added into flask containing media. Congo red is applied to differentiate between rhizobia and other bacteria. The YMA was transferred to sterilized petri dishes and let to cool and solidify for few minutes. Then the plates were upturned so that condensed water may not fall on the surface of medium [15].

Growth of colonies

Several of rhizobium clusters were appeared within 3-4 days (fast growing) while some were seemed within 7-8 days (slow growing). These colonies were identified as rhizobium having milky color (mucoid),

round shape without picking the color of Congo red on YMA.

Obtaining the pure culture

The desired microbial growth was chosen with the help of pasteurized loop from the pre prepared plate, and streaked in sterilized glass tubes containing YMA media aseptically. The tubes were properly marked. Afterward streaking the loop was splashed with spirit and flamed for disinfection. The dishes were purified and incubated in incubator at 28-30 °C to obtain pure culture [17].

Bottle culturing of Rhizobia

Bottle culture of rhizobia was obtained by spreading or spraying pure culture suspension on solidified bottle culture media (Table 2-3) in cultural bottles aseptically. The bottles were properly marked and placed in the incubator at 28-30 °C for incubation in order to increase the biomass of pure culture.

Table 2. Composition of bottle culture media per liter

Ingredients	Quantity dissolved L ⁻¹
Agar	20 g
K ₂ HPO ₄	0.5 g
Sucrose	20 g
Mineral solution	25 mL
Yeast extract (10%)	100 mL
pH	6.8-7.0

Table 3. Mineral solution composition 500 mL⁻¹

Chemicals	Quantity (g/500 mL)
NaCl	2.0
CaSO ₄	1.0
MgSO ₄	2.0
FeSO ₄	0.1
MnSO ₄	0.1
K ₂ HPO ₄	1.0

All the above ingredients were weighed and dissolved in 1L by heating up to boiling in a cane. Then the hot mixture was cotton filtered and poured in to 5 flasks about 200 ml per flask. The flasks were properly plugged and autoclaved at 121 °C and 15 psi pressure for 40 minute [15].

Preparation of inoculum

Rhizobial inoculum were prepared from the already prepared bottle culture of rhizobia. The rhizobial colonies from the bottle culture were poured in pre sterilized carrier material that may either be peat soil or completely decomposed farm yard manure at the rate of 85-100 mL per 0.5 kg peat soil. The mixture was properly mixed, sealed, labeled aseptically and kept for incubation at 28-30 °C for specified period of time.

Preservation of inoculum

Rhizobial inoculum obtained was sealed, labeled and preserved in refrigerator at 4 °C.

Important characteristics of summer legume Rhizobia as PGPR

The isolated rhizobial strains were incubated at 28 °C and examined for various PGPR features on daily basis for up to 7 days. A rhizobia creating an orange halo on chrome azurol S agar plates was marked as siderophore manufacturer as described by Alexander and Zuberer [18]. A bacteria which changed their typical pink color to red while exposing to Salkowski reagent for 0.5–3 h [19] were identified as IAA generating bacteria. Rhizobial strains which changed their color from yellow to orange-brown on filter papers saturated with 0.5% picric acid–2% sodium carbonate according to Bakker and Schippers [20] were recognized as Cyanide (HCN) producers. The bacteria were considered as phosphate solubilizers by production of clarification halos on di calcium phosphate agar plates [21].

Results and discussion

Developmental characteristics of Rhizobial isolates

Data regarding morphological features of isolated rhizobial strains are presented in table 4. All the tested rhizobial isolates were gram negative. Rhizobia isolated from

cowpea and soybean were slow growing because it took 7-8 days for development of visible growth on Yeast Minitol Agar media while of susbnia and mungbean were fast growing because it took 2-3 days for producing best growth. Hungria *et al.* [22] also found susbnia and mungbean isolates as fast growing.

Table 4. Developmental characteristics of Rhizobia isolated from different summer legumes

Legumes rhizobial isolates	Growth nature	Gram reaction
<i>Glycine max</i> (Soybaen)	Slow growing	Negative
<i>Vigna radiata</i> (Mung bean)	Fast growing	Negative
<i>Vigna unguiculata</i> (Cowpea)	Slow growing	Negative
<i>Susbenia grandiflora</i> (Susbeenia)	Fast growing	Negative
<i>Cymposistetra gonoloba</i> (Guar)	Fast growing	Negative

PGPR characteristics of summer legumes Rhizobial isolates

PGPR features of the *Rhizobium* strains obtained from different summer legumes in this study are presented in Table 5. Upon descriptive analysis of data it was observed that 9% of tested rhizobial strains produced hydrogen cyanide (HCN), and cyanogens were not found in rhizobal species isolated from Sesbania. Relatively lower incidence of cyanogens was detected in rhizobia in this study. These finding are in line with de Britto Alvarez *et al.* [19] who also found that that of the 709 isolates tested from the rhizosphere of tomato less than 1% were

cyanogens. Most of the isolated rhizobia produced siderophores (67%) and around 58% of the isolates formed IAA (Table 5). Higher PGPR characteristics with respect to siderophores and IAA production is relatively more in *Vingaradiata* and *Glycine max* rhizobacteria than other de Britto Alvarez *et al.* [19] who found that 23- 38% of the isolates obtained from tomato roots were siderophores producers and 24– 42% produced IAA . In this study it was observed that 21% of the tested rhizobia were P-solubilizing bacteria (Table 5) while highest (35%) P solubilizer were found in *Vinga radiata*.

Table 5. Percentages of Rhizobial strain isolated from summer legumes exhibiting some PGPR characteristics

Legumes rhizobial isolates	Cyanide (HCN)	Siderophore	Indole acetic acid (IAA)	P solubilizers
		Producer (%)		
<i>Glycine max</i>	5	65	50	25
<i>Vigna radiata</i>	10	95	55	35
<i>Vigna unguiculata</i>	15	60	50	15
<i>Susbenia grandiflora</i>	5	80	45	20
<i>Cymposistetra gonoloba</i>	0	40	35	10
Mean (%)	9	68	47	21

Conclusion and recommendation

Rhizobia isolated from *Vigna radiata* and *Glycine max* have best potential to be used as PGPR for cereals and other non-leguminous crop. Further research is needed to examine the PGPR potential of other rhizobial isolates in existing soil and climatic conditions.

Authors' contributions

Conceived and designed the experiments: M Adnan & SRA Shah, Analyzed the data: N Saleem, A Basir & I Rahman. Contributed reagents/materials/analysis tools: H Ullah & M Ibrahim, Performed the experiments: M Adnan, Muhammad & A Khan, Wrote the paper: JA Shah & M Adnan.

References

1. Werner D (1992). *Symbiosis of Plants and Microbes*. Chapman and Hall, London.
2. Shamseldin A, Sadowsky MJ, EoSaadani M & An CS (2008). Molecular biodiversity and identification of free living rhizobium strains from diverse Egyptian soils as assessed by direct isolation without trap hosts. *American-Eurasian J Agric & Environ Sci* 4(5): 541-549.
3. FAO (1984). Legume inoculants and their use. Food and Agricultural Organization of United Nations. Rome, Italy, 1-63.
4. Peoples MB, Ladha JK & Herridge DF (1995). Enhancing legume N₂ fixation through plant and soil management. *Plant Soil* 174: 83-101.
5. Brockwell J, Bottomley PJ & Thies JE (1995). Manipulation of rhizobia micro flora for improving legume productivity and soil fertility: a critical assessment. *Plant Soil* 174: 143-180.
6. Abaidoo RC, Keyser HH, Singleton PW, Dashiell KE & Sangina N (2007). Population size, distribution and symbiotic characteristics of indigenous Bradyrhizobium spp. that nodulate soybean genotypes in Africa. *Applied Soil Ecology* 35: 57-67.
7. Yanni YG, Rizk RY, Corich V, Squartini A, Ninke K, Philip-Hollingsworth S, Orgambide G, de Bruijn F, Stoltzfus R, Buckley D, Schmidt T, Mateos PF, Ladha J. & Dazzo FB (1997). Natural endophytic association between *Rhizobium leguminosarum* bv. trifolii and rice roots and assessment of its potential to promote rice growth. *Plant and Soil* 194: 99-114.
8. Mehboob I, Naveed M & Zahir ZA (2009). Rhizobial Association with Non-Legumes: Mechanisms and Applications. *Critical Reviews in Plant Sci* 28(6): 432-456.
9. Biswas JC, Ladha JK & Dazzo FB (2000a). *Rhizobia* inoculation improves nutrient uptake and growth of lowland rice. *Soil Sci Soc America J* 64: 1644-50.
10. Meyer JM (2000). Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent *Pseudomonas* sp. *Arch Microbiol* 174: 135-142.
11. Yang SF & Hoffman NE (1984). Ethylene biosynthesis and its regulation in higher plants. *Annu. Rev Plant Physiol* 35: 155-189.
12. Fatima Z, Zia M & Chaudhary MF (2006). Effect of rhizobium strains and phosphorus on growth of soybean (*Glycine max*) and survival of *Rhizobium* and P solubilizing bacteria. *Pak. J. Bot.* 38: 459-464.
13. Biswas JC, Ladha JK, Dazzo FB, Yanni YG & Rolfe BG (2000b). Rhizobial inoculation influences seedling vigor and yield of rice. *Agron* 92: 880-6.
14. Biswas JC (1998). Effect of nitrogen fixing bacteria on growth promotion of lowland rice (*Oryza sativa* L.). Ph. D Thesis, Department of Soil Science University of Philippines, Los Banos.

15. Somasegaran P & Hoben HJ (1994). Handbook for rhizobia: Methods in legume *rhizobium* technology. Springer-Verlag, New York, Inc, 450.
16. Vincent JM (1970). A manual for the practical study of root nodule bacteria. IBP
17. Rao NSS (1999). Soil Microbiology. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi.
18. Alexander DB & Zuberer DA (1991). Use of chrome azurol S reagents to evaluate siderophore production by rhizosphere Bacteria. *Biol Fertil Soils* 12:39–45.
19. de Britto Alvarez MA, Gagné S & Antoun H (1995). Effect of compost on rhizosphere micro flora of the tomato and on the incidence of plant growth-promoting rhizobacteria. *Appl Environ Microbiol* 61: 194–199.
20. Bakker AW & Schippers M (1987). Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp.-mediated plant growth-stimulation. *Soil Biol Biochem* 19:451–457.
21. Goldstein AH (1986). Bacterial solubilization of mineral phosphates: historical perspective and future prospects. *Am J Altern Agric* 1: 51–57.
22. Hungria M, Andrade DS, Chueire LM Probanza A, Manero FJG, Megias M, Andrade DS & Chueire LM (2000). Isolation and characterization of new efficient and competitive bean (*Phaseolus vulgaris*) rhizobia from Brazil. *Soil Biol Biochem* 32: 1515-1528.