Bangladesh J. Bot. **48**(4): 1223-1229, 2019 (December)

MICROBIOLOGICAL STUDIES ON *RHIZOBIUM LEGUMINOSARUM* ISOLATED FROM PEA (*PISUM SATIVUM* L.)

FAROOQ SHAHZAD[1], MUHAMMAD KAMRAN TAJ*, FERHAT ABBAS, MUHAMMAD SHAFEE, SAEED AHMED ESSOTE[1], IMRAN TAJ AND ABDUL MANAN ACHAKZAI

Center for Advanced Studies in Vaccinology and Biotechnology, University of Baluchistan, Quetta, Pakistan

Keywords: Pesticide, Peas, *Rhizobium leguminosarum*

Abstract

Rhizobia are the true bacteria that establish symbiotic relationship leading to the development of new root nodules. This study has been designed to evaluate the microbiological aspects of *Rhizobium leguminosarum* in target area. A total of 1000 (200 from each site) roots were collected from five different agriculture fields (Quetta, Pishin, Killa Abdulla, Kuchlak and Hanna Urak) and screened through different standard microbiological procedures. Results revealed that 665/1000 (66.5%) roots samples were positive for *Rhizobium leguminosarum.* The highest percentage was from Pishin 180/200 (18%) and Killa Abdullah 160/200 (16%). A remarkable growth of *Rhizobium leguminosarum* was noted at 28 to 30^{o}C whereas, less growth was recorded at 24, 34 and 42^{o}C. Similarly, *Rhizobium leguminosarum* showed growth at pH 5 to 10, but superlative pH values for the growth of *Rhizobium leguminosarum* were from 6 to 8 pH. The PCR reconfirmed 1300 bp band of 16S rRNA gene of *Rhizobium leguminosarum.* The organism was further applied as biofertilizer and showed promising results in subjected plants. Medicinal plants application showed that *Rhizobium leguminosarum* was sensitive to different plants. However, the effects of insecticides showed that Cypermethrin exhibited least zone of inhibition 10 and 11 mm, while Chlorpyrifos showed least zone of inhibition 14 and 17 mm by using disc and well method with (1: 16) dilution. These findings ensure the devastation of microbiota in rhizosphere with rational use of these pesticides that may result in adverse effects over crop productions in the region.

Introduction

Legumes establish symbiotic relationship in response to nitrogen fixing bacteria that leads to the development of new nodules (Schultze *et al*. 1998). Soil bacteria play essential and vital role in crop production and different biogeochemical cycles. Plant-bacterial relationships are plant health and soil richness indicator in the rhizosphere. Similarly, free-living soil bacteria are also beneficial for plant growth (Hayat *et al*. 2010). Certain soil microorganisms are important for the soil life activity, responsible for various decomposition processes involving plants and animals (Gray and Smith 2005).

Many legumes have symbiotic bacteria such as, *Rhizobia* inside the root nodules (Rogers *et al*. 2007). *Rhizobia* is extensively used to enhance the capability of legume-*Rhizobium* synthesis to fix atmospheric nitrogen in agricultural systems. Pea is an annual plant with one-year life cycle that is cultivated in several countries with winter to early summer plantation that may slightly vary in different agro-ecological zones (Zahran 1999).

In natural environment temperature, pH, pesticides, medicinal plants are the most important factors that strongly affect many biological aspects of *Rhizobium leguminosarum.* Therefore, the present study was designed to study the different microbiological and physiological aspects of *Rhizobium leguminosarum* isolated from pea's plants of Quetta zone.

*Author for correspondence: <kamrancasvab@yahoo.com>. [1]Department of Zoology, University of Baluchistan, Pakistan.

Materials and Methods

The Quetta, capital of Baluchistan is located between 30°-03 and 30°-27-north latitude and 66°-44 and 67°-18 east longitudes. Total geographical area is about 2653 Km^2 with elevation from 1254 -3500 meters. It is known as fruit basket of the province. It experiences generally a dry to very cold weather in winter and mild hot in summer. It is out of Monsoon range with scanty rainfall (Islam *et al.* 2008).

About 1000 root samples of pea crops were collected from different agriculture fields of Quetta zone. The samples were collected aseptically in plastic bags and transported to microbiology laboratory, of Center for Advanced Studies in Vaccinology & Biotechnology (CASVAB), University of Baluchistan, Brewery Road, Quetta, Pakistan. These samples were processed for microbiological analysis according to the international standard protocol.

The nodules were washed under tap water to remove adhering mud and soil particles. After washing, treated carefully with 95% ethanol for surface sterilization. The nodules were crushed according to standard protocol. The crushed nodules were streaked on yeast extract mannitol agar (YEMA) medium and incubated at 28 ± 2ºC for 24 hrs (Shahzad *et al*. 2012).

Growth of colony culture was subjected to Gram staining and was further identified through various biochemical tests such as, oxidase, indole, catalase and voges proskauer (Shahzad *et al.* 2012).

The DNA was extracted from culture through DNA purification kit (Hiper® Bacterial Genomic DNA Extraction Kit). After isolation of template DNA, it was stored at –20 ^{0}C for further use. Primer of following sequence F: (5'AGAGTTTGATCCTGGCTCAG3') R: (5'ACGGCTA CCTTGTTACGACTT3') were designed to allow amplification of 1300 bp fragment of 16Sr RNA gene. For PCR amplification 25 µl volume reaction mixture was used containing 12 µl master mix (2x Amp Master™ Taq), 9 µl grade water, 1 µl of each primer (Forward, Reverse) and 2 µl template DNA. The PCR cycling conditions were, initial denaturation at 95°C for 5 min, denaturing at 94°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. The final PCR product was run on 1.5% agarose gel and observed under gel documentation system (Laguerre *et al.* 1996).

Experiments were conducted to evaluate *in vitro* effect of locally used pesticides (Chlorpyrifos and Cypermethrin) on the growth of *Rhizobium leguminosarum*. Different concentrations of pesticides were applied on *Rhizobium leguminosarum* by disc diffusion and well method (Singh *et al.* 2002).

The plant samples were dried and grinded into fine powder by using a blender (ANEX AG-179 GL). The samples were soaked in 50 ml methanol (LAB-SCAN ASIA Co., Ltd.) and shake agitated twice a day, placed for two weeks at room temperature (25^{0}C). After two weeks, the mixture was twice filtered by Wattman No. 4 filter. The methanol was completely evaporated by the help of rotary evaporator (Buchi Rotary-evaporator Model R-205). The semisolid extracts were stored at 8ºC. Agar diffusion method was used to determine the antimicrobial activity of medicinal plants and 10 µl extracted solution was poured in 6 mm agar wells in plates. After 24 hrs, the zone of inhibition was record as proposed by Begum *et al.* (2015).

To check the effect of different physiological aspects (temperature and pH) on *Rhizobium leguminosarum* growth. The *Rhizobium leguminosarum* was grown at different temperatures and pH (Rodrigues *et al.* 2006). For biofertilizer, trial culture of *Rhizobium leguminosarum* in yeast extract mannitol (YEM) medium was established in 250 ml of growth medium under aseptic conditions. Inoculated broth culture was incubated for 1 week at 28^o C. After one week incubation, the liquid biofertilizer were used directly (Datta *et al.* 2015). Quantitative test was performed using Plate Count Method (Vincent 1970).

In qualitative assessment of liquid biofertilizer, pH and visible contaminants were checked. Gram staining was done, also a loopfull of culture was streaked on Glucose-Peptone agar and the plates were incubated at 28 ± 2°**C** for 24 hrs and results were examined (Vincent 1970).

The growth enhancing potential and yield increasing efficacy of *Rhizobium leguminosarum* were evaluated in potted pea plants. Soil procured from nearby field (pH 7.8, Electrical conductivity. 2.3 dSm^{-1}) with organic matter 0.96% and total nitrogen 0.06% and were autoclaved. Seeds of *Pisum sativum* (pea) were surface sterilized using 0.1% mercuric chloride ($HgCl_2$) for 2 min in petri plates before sowing. Seeds were soaked in biofertilizers for 1 hr before sowing in pots keeping in laminar air flow chamber. Pots with sterile soil were filled up to one third of the pot height. The pots were arranged randomly at ambient light and temperature and examined periodically. The control, biofertilizer treated and inorganic fertilizer groups were also made for comparison (Datta *et al*. 2015).

Results and Discussion

Results revealed that 665/1000 (66.5%) roots samples were positive for *Rhizobium leguminosarum*, with highest from Pishin 180/200 (90%) followed by Killa Abdullah 160/200 (80%), Kuchlak 135/200 (67.5%), Hanna Urak, 110/200 (55%) and Quetta 80/200 (40%), respectively (Fig.1).

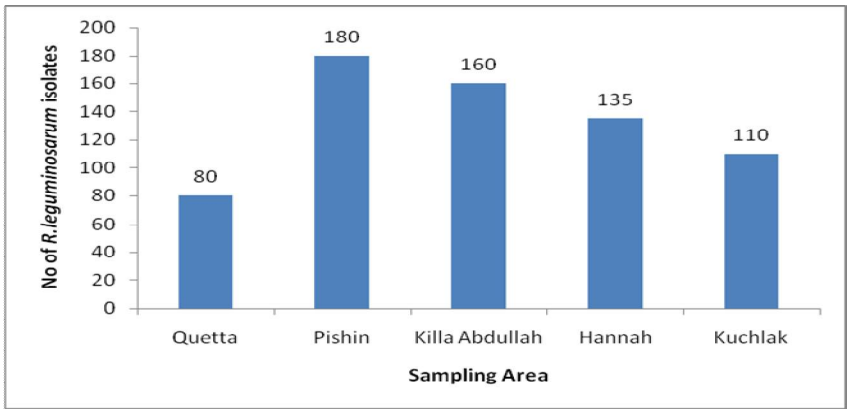

Fig. 1. Number of isolates found to be *Rhizobium leguminosarum* in different areas of Quetta zone, Pakistan.

Table 1 shows the results of biochemical test done for characterization of isolates.

Table 1. Biochemical characterization of *Rhizobium leguminosarum* isolates from Quetta, Pakistan.

Biochemical tests								
Motility	Indole	Citrate utilization	MR	VP	Urease	Catalase	Oxidase	
+	-	-	-	-	-	+	-	
			Sugar fermentation tests					
Glucose	Sucrose	Sorbitol	Trehalose	Lactose	Mannitol	Maltose	Xylose	Fructose
+	+	+	+	+	+	+	+	+

All the isolates of *Rhizobium leguminosarum* predicted 1300 base pair amplicons size with 16S rRNA gene as shown in Fig. 2.

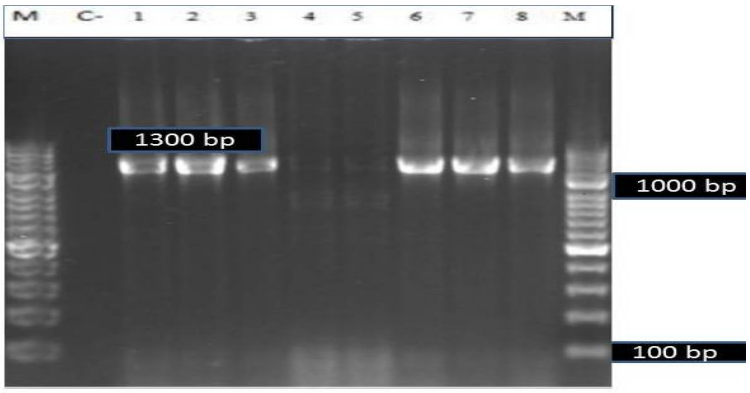

Fig. 2. Molecular identification of *Rhizobium leguminoserum*16S rRNA gene.
M: 100 bp plus DNA ladder, C-: Negative control, 1-3,6-8: Samples positive and 4-5, negative samples.

The pH and temperature are key environmental factors which directly affecting the growth and yield potency of both the crop and organism in biological system. The *Rhizobium leguminosarum* showed growth from 4 to 45^{0}C and pH result showed growth from 4 - 10 pH while, ideal temperature observed was 28 to 30^{0}C and pH 6 - 8 (Table 2).

Table 2. Growth of *Rhizobium leguminosarum* at different temperature and pH.

Sample name	Temperature (ºC)	Incubation period (hr)	Remarks
Rhizobium	4 - 45	24	Positive
leguminosarum	50	24	Negative

Growth on different pH

	pH	Incubation Temperature (^{0}C)	Incubation period/hr	Remarks
	2 - 3	28	24	No growth
	4 - 5	28	24	Growth
	6 - 8	28	24	Optimum growth
	9 - 10	28	24	Growth

Different dilutions of both pesticides (Chlorpyrifos and Cypermethrine) were evaluated by well and disc diffusion method. The Chlorpyrifos exhibited 14, 17 and 17 mm inhibitory zone up to dilution (1: 16), with the pesticide volume of 10, 20 and 30 µl using well method, respectively. Whereas no zone was seen in well method at (1: 32) dilution. Similarly, Cypermethrine showed 10 and 11 mm inhibitory zone at 1 : 16 dilution using both methods with the pesticides volume of 10, 20 and 30 µl (Table 3).

The antimicrobial activity of indigenous medicinal herbs was investigated by using agar well diffusion method against *Rhizobium leguminosarum.* In agar well diffusion method different medicinal plant trait showed that *Rhizobium leguminosarum* was sensitive to different medicinal plants as shown in Table 4.

Table 3. Effect of pesticides on *Rhizobium leguminosarum*.

Chlorpyrifos	Mean zone of inhibition (mm)									
Dilution No.	1 : 2		1 : 4		1 : 8		1 : 16		1 : 32	
µl	W	D	W	D	W	D	W	D	W	D
10	21	20	17	16	16	15	15	14	-	-
20	22	20	19	18	18	17	17	16	-	13
30	22	20	20	19	19	18	18	17	-	14
Cypermethrin	Mean zone of inhibition (mm)									
10	16	18	14	15	11	13	-	10		
20	18	19	16	15	13	14	-	-		
30	20	22	18	17	16	16	11	-		

Legend. W = Well method, D = Disc method

Table 4. Zone of inhibition caused by indigenous medicinal herbs against *Rhizobium leguminosarum*.

Botanical name	Common name	Zone of inhibition (mm)	Remarks
Cocculus pendulus	Zamur	29	Sensitive
Malva neglecta	Pochko	15	"
Rhazya stricta	Aeshark	11	"
Corchorus deprressus	Bandary	8	"
Salvia bucharica	Gul-e-kakar	16	"
Berberis baluchistanica	Badrah/zarch	17	"
Artemisa absinthium	Aftasen	13	"

The growth of *Pisum sativum* (pea) with *Rhizobium leguminosarum* biofertilizers was compared to the control and inorganic fertilizer groups after 50 - 75 days of sowing under normal growth conditions. The growth and yield indices such as germination days, plant height, fresh biomass, dry biomass and number of pods were significantly increased by biofertilizer applications in comparison to inorganic fertilizer (Table 5).

Table 5. Biofertilizer and inorganic fertilizer effect on peas crop production compares with control.

Parameters	Treatment		
	Control	Bio fertilizer treatment	Inorganic fertilizer
Germination days	8	5	6
Plant height (cm)	41.5	44.45	46,90
Fresh biomass (g)	22.5	24.37	27.26
Dry biomass (g)	6.2	8.69	9.07
Number of pods per plants	4	6	7

Total 1000 root samples were collected from different areas of Quetta, Pakistan, 66.5% has been found positive for *Rhizobium leguminosarum.* The area wise distributions were Quetta 8%, Pishin 18%, Killa Abdullah 16%, Hanna Urak 13.5% and Kuchlak 11%. *Rhizobium leguminosarum* was confirmed through routine microbiological tests. Gene specific PCR assay was used to detect *Rhizobium leguminosarum* that revealed 1300 base pair amplicons of 16Sr RNA gene. The present results were similar with the finding of Ismail *et al*. (2013). The different medicinal plant treatment showed that *Rhizobium leguminosarum* was sensitive to *Cocculus pendulus*, *Malva neglecta*, *Rhazya stricta*, *Corchorus deprressus*, *Salvia bucharica*, *Berberis baluchistanica* and *Artemisa absinthium*. Present finding was similar with the finding of Hasan *et al*. (2011).

The effects of different ranges of temperature and pH were evaluated which showed that *Rhizobium leguminosarum* growth was greatly temperature dependent. Temperature below 0^0C and over 45^0C did not show any growth. The *Rhizobium leguminosarum* grows from 4 to 45^0C while pH result showed growth from 4 - 10 pH. Similarly, Somasegaran and Hoben (2012), described that most rhizobium bacteria grew better at 28^0C. Maximum growth was reported at 28^0C. Present result corroborates with previous study who, stated that *Rhizobium* could grow within a wide range of pH and tolerate both moderate acidity and alkalinity with optimum pH 6 - 8 (Helemish *et al*. 1987).

Experiments were conducted to check the effect of locally used pesticides (Chlorofyrifos and Cypermethrine) on the growth of nitrogen-fixing pea rhizobia (*Rhizobium Leguminosarum) in vitro.* The significant effects were observed on the growth of *Rhizobium leguminosarum.* Similarly, Singh *et al.* (2002) also described that pesticides had adverse effect on *Rhizobia* under field conditions.

Results have revealed that biofertilizer performed significant improvement in plant productivity and yield. Significant positive results about the growth and yield parameters were observed i.e. germination days, plant height, dry biomass, fresh biomass and pod number after inoculating the plants with biofertilizer. Similarly, Naserirad *et al.* (2011) reported that biofertilizer increased plant growth and production of pea crop.

From the results, it may be concluded that *Rhizobium leguminosarum* is nitrogen-fixing bacteria that strongly establishes symbiotic relationship with pea plant for the higher yield of pea crop, but rational use of certain pesticides may lead to its destruction that will ultimately result in decreased production.

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(Manuscript received on 4 December, 2018; revised on 20 March, 2019)