PRELIMINARY SCREENING FOR PLANT DISEASE SUPPRESSION BY PLANT GROWTH PROMOTING RHIZOBACTERIA

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ABSTRACT

Fusarium wilt is one of the most important diseas which cause severe damages in lentil. In order to evaluate the effect of plant growth promoting rhizobacteria (PGPR) on disease suppression and stress inhibition in lentil three experiments were performed. A total of 11 rhizobacterial isolates obtained from lentil rhizosphere were characterized and found belonging to genera *Bacillus* (7) and *Pseudomonas* (4). The isolates were subjected to their morphological, cultural and biochemical characterization. Further the strains were screened for their disease-suppressing activity but only four isolates showed antagonism to *Fusarium oxysporum*. The antagonistic ability against *Fusarium oxysporum* ranged from 3.3–16.6%, maximum being recorded with P-1 (16.6%), followed by B-36 (13.3%) and B-40 (3.3%). Similar trend was recorded in liquid medium.

Keywords: Antagonism, *Bacillus*, *Fusarium oxysporum*, Lentil, *Pseudomonas*.

INTRODUCTION

Lentil (*Lens culinaris* Medikus), is a winter crop belonging to the family Leguminosae. Being major source of protein in the vegetarian diet system of the Indians, it has been an important component of agriculture since ancient times. In India, lentil is the second most important winter season pulse crop after chick pea with an annual production of 571 thousand tons (National spot exchange, 2008-2009). Its seed is rich in proteins, minerals and vitamins for human nutrition and the straw is a valuable animal feed. However low average yields are largely due to cultivation in neglected marginal lands, inadequate fertilizer inputs as well as diseases like vascular wilt of lentil, which is caused by *Fusarium oxysporum*. Biological control using PGPR (Plant Growth Promoting Rhizobacteria) is a desirable alternative to chemical control of vascular wilt. These are a group of bacteria that actively colonize plant roots and increase plant growth and yield (Wu *et al*., 2000; Banal, 2009). One of the various mechanisms by which PGPRs promote plant growth includes antagonism against phytopathogens (Bakker, Pieterse and van Loon, 2007) by production of siderophores, chitinases, antibiotics, etc., others being production of phytohormones (Karakurt, Aslantas, Ozkan and Guleryuz, 2009), asymbiotic nitrogen fixation, solubilization of mineral phosphates and other nutrients and production of ACC-deaminase (Liddycoat, Greenberg and Wolyn, 2009). Thus, plants are benefited in a number of ways, i.e. by suppression of fungal pathogens proliferation, improved N-fixation and prevention from environmental stress.

Keeping this in view, the present work was undertaken to isolate, characterize and evaluate PGPR from lentil rhizosphere for plant disease control and growth parameters under *in vitro* conditions.

MATERIALS AND METHODS

1. Procurement of reference cultures

The reference culture KB-133 was procured from Department of Microbiology, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand.

2. Isolation of rhizobacteria

1g of soil sample was suspended in 9 ml of sterile water and serial dilutions were made in sterile water blanks. Pour plating was done on Nutrient agar (NA) for *Bacillus* and on King's B (King *et al*., 1954) for *Pseudomonas*. The colonies showing whitish and pinkish coloration on NA plates and fluorescent yellow to green coloration on King's B were picked up and transferred to respective slants for further use.

3. Biochemical characterization of rhizobacteria

Biochemical characterization of bacterial isolates was done on the basis of gram reaction, catalase production, citrate utilization, nitrate reduction, starch hydrolysis and methyl red test were conducted as per the standard methods (Holt *et al*., 1994).

(a) Catalase production

A drop of 3% H_2O_2 was taken on glass slide and a small amount of bacterial culture was mixed with platinum inoculation loop. Rapid and sustained production of gas bubbles or effervescence constituted positive test.

(b) Nitrate reduction test

5ml nitrate broth was inoculated with pure culture of the test organism. It was incubated at 28°C for 48 hours. Equal volumes (0.5ml) of both reagents A and B were added. The development of red color within 30 seconds indicated positive test.

(c) Hydrolysis of starch

One gram of starch suspension in 10 ml of cold distilled water was added to 90 ml of nutrient agar, autoclaved at 120°C for 15 minutes and poured into sterile Petri plates. After inoculation and incubation at 28°C for 48 hours, the plates were flooded with grams iodine 20 solution. A clear zone surrounding colony indicated hydrolysis of starch.

(d) Methyl red (MR) test

A tube of GPPW (5 ml) was inoculated with pure culture of the test organism. It was incubated at 28°C for 48 hour, after this, 5 drops of the MR reagent was added directly to the broth.

4. Maintenance of bacterial cultures

Bacillus isolates were maintained on NA slants and *Pseudomonas* isolates on King's B slants, subcultured once in a month throughout the period of investigation, and slants were stored at 4°C in refrigerator. Gram staining was done to check the purity of the culture.

5. Intrinsic Antibiotic Resistance spectra

An antibiotic resistance spectrum of the isolates was studied by using different antibiotics. Filter paper discs (Hi Media) containing standard concentration of antibiotics viz. ampicillin (25µg/disc), chloramphenicol (25µg/disc), tetracycline (30µg/disc), streptomycin (25µg/disc), nalidixic acid (30µg/disc), carbenicillin (100µg/disc), gentamycin (30µg/disc) and kanamycin (30µg/disc) were used. Bacterial cultures were grown in their respective broth for 72 h and aliquot of 0.1 ml culture was poured on to the medium containing Petri plates. Then antibiotic discs of different concentrations were placed and incubated at 28°C for 72 h. The plates were observed for zone of inhibition around antibiotic discs.

6. Antagonistic effects of bacterial isolates against *Fusarium oxysporum*

Antagonistic activity of the bacterial isolate against *Fusarium oxysporum* was evaluated based on dual culture technique (Sakthivel and Gnanamanickam, 1987). Fifteen ml of PDA was poured into 9 mm sterile Petri dish. 5 mm mycelia plug of the respective fungus was inoculated at the centre of plate. Potential antagonistic bacteria were streaked 3 cm apart from the fungal inoculum and plates were incubated at 28°C for 3 days. Radial growth of the test fungus was measured and percentage growth inhibition was calculated using the formula:

$$\% \text{ Inhibition} = (R - r)/R \times 100$$

Where, r is the radius of the fungal colony opposite the bacterial colony and R, is the maximum radius of the fungal colony from the bacterial colony.

Quantitative evaluation of antagonistic potential was done in liquid medium by inoculating 1 ml of 24 h old bacterial culture and a disc of test fungus (5 mm) in 50 ml broth of potato dextrose medium in 250 ml conical flasks at 25°C on a rotary shaker. Broth inoculated with fungus served as control. The differences in dry weights between the fungus and the bacterium or the control cultures were recorded by passing 48 h grown dual cultures through pre-weighed Whatmann No.1 filter paper. The filter papers were dried for 24 h at 70°C and weighed. The % reduction of the test fungus was calculated using formula:

$$\% \text{ reduction in weight} = (w_1 - w_2)/ w_1 \times 100$$

Where, w_1 represents the weight of the test fungus in control flasks without w_2 with bacteria.

RESULTS AND DISCUSSION

A total of 11 isolates of rhizobacteria were isolated from soil samples taken from different locations. Out of these 4 isolates were selected from Kings B medium and 7 from Nutrient Agar. Most of the isolates from Kings B medium showed the characteristic fluorescent green pigmentation. The isolates from NA showed typical colony morphology which was predominantly off-white to creamish in colour.

Table 1: Cultural, morphological and biochemical characteristics of rhizobacterial isolates

Characteristic of test organism	*Pseudomonas*	*Bacillus*
Gram's reaction	-ve	+ve
Shape	Rods	Rods
Pigment	+	-
Pigment colour	Fluorescent green	White
Starch hydrolysis	+	+
Catalase production	+	+
Methyl red test	-	-
Citrate	+	+
Nitrate production	+	+

Table 2: Intrinsic Anibiotic Resistance spectrum of rhizobacterial isolates

Isolates	Gentamycin (30µg/ disc)	Kanamycin (30µg /disc)	Streptomycin (25µg /disc)	Tetracycline (30µg/disc)	Chloramphenicol (25 µg/disc)	Ampicillin (25µg /disc)	Carbenicillin (100µg/ disc)	Nalidixic Acid (30µg/ disc)	MAR Index
B-1	3.7	1.7	3.6	R	2.4	R	R	2.0	0.37
B-9	3.8	2.1	3.6	1.1	0.8	R	R	1.7	0.25
B-20	3.7	R	2.7	3.2	2.7	1.0	1.0	2.5	0.00
B-23	2.9	1.7	3.0	1.75	2.2	R	1.2	1.5	0.12
B-36	4.2	3.0	4.5	3.5	3.0	R	R	2.4	0.25
B-37	3.0	0.9	3.4	1.9	2.0	R	1.2	2.5	0.12
B-40	3.0	1.8	3.5	1.6	2.6	1.0	1.5	0.9	0.00
P-1	2.1	2.5	2.0	2.2	3.6	1.0	R	2.5	0.12
P-13	R	2.4	2.6	1.3	1.6	R	2.2	1.1	0.25
P-16	2.1	R	1.1	2.2	2.5	2.3	2.6	2.4	0.12
P-27	3.4	2.2	3.2	1.1	3.0	2.3	2.1	1.8	0.00

R-Resistance

Values indicate the diameter of zones of sensitivity in cm.

Table 3: *In vitro* screening of PGPR strains against *Fusarium oxysporum*

Rhizobacterial isolates	Growth inhibition of *Fusarium oxysporum*	
	% inhibition on PDA plate	Mycelial growth inhibition %
B-1	------	------
B-9	------	------
B-20	------	23.22
B-23	------	------
B-36	13.33	57.77
B-37	------	------
B-40	3.33	40.30
P-1	16.66	62.82
P-13	------	------
P-16	------	------
P-27	------	------

On the basis of cultural and morphological appearance, these were tentatively assigned to genera *Pseudomonas* and *Bacillus*. The predominance of *Pseudomonas* and *Bacillus* spp. in legume rhizosphere has been reported by many workers.

Joseph *et al*., (2007) reported that populations of *Pseudomonas*, *Bacillus* and *Azotobacter* predominantly colonized the rhizosphere and rhizoplane of healthy chickpea plants. They found that bacterial population ranged from 0.5- 2.1×106 of *Bacillus* spp., 1.1-2.1×106 of *Pseudomonas* spp. and 0.3-1.7×106 of *Azotobacter* spp. Similarly, Kundu *et al*., (2009) found that isolates from chickpea rhizosphere mostly belonged to genera *Pseudomonas*, *Aeromonas* and *Enterobacter*. Woyessa and Assefa (2011) also reported that various bacteria including species of *Burkholderia*, *Bacillus* and *Pseudomonas* are commonly found in the rhizosphere. The 11 isolates of rhizobacteria obtained were evaluated in detail for their cultural, morphological and biochemical characteristics as given in Bergey's manual of systematic bacteriology. The isolates were assessed for gram reaction and differences in colony morphology. 36.3% of the isolates isolated from lentil rhizosphere showed rapid growth on King's B medium at 28°C with production of yellow to green pigment. All of these were gram negative rods. The isolates were indole, catalase and citrate positive and methyl red negative (Table 1). They were also able to reduce nitrate and a few were able to hydrolyze starch. Seven of the isolates were found to be gram-positive, rod shaped bacteria. These isolates also showed profuse growth on NA medium at 28°C and were indole, catalase and citrate positive and methyl red negative and were able to reduce nitrate. A few of them were positive for starch hydrolysis. On the basis of these tests, the isolates were tentatively placed into three genera, *Bacillus* (7) and *Pseudomonas* (3).

It is clear from the data in Table 2 that 71.4% of the *Bacillus* isolates were resistant to ampicillin and 42.8% were resistant to carbenicillin, whereas different *Pseudomonas* isolates showed resistance to different antibiotics, viz. P-1 (carbenicillin), P-13 (gentamycin, ampicillin) and P-16 (kanamycin). Similar observations have also been reported by many workers. Siddiqui *et al*., (2006) found that *Pseudomonas* are resistant to ampicillin, penicillin and nalidixic acid. Suneesh (2004) also reported that most of the fluorescent pseudomonads of moist deciduous forests of Western Ghats were resistant to spectinomycin, ampicillin, nalidixic acid, chloramphenicol and kanamycin. The antibiotics produced by bacterial biocontrol agents and their role in microbial interaction, were reviewed by Raaijmakers *et al*., (2002).

Antagonistic potential of 4 of the rhizospheric isolates (B-20, B-36, B-40 and P-1), was tested against *Fusarium oxysporum* in dual culture under *in vitro* conditions. The growth of the fungus was lesser as compared to the control plate. The *Pseudomonas* isolate P-1 exhibited greater inhibition i.e. 16.6% followed by *Bacillus* isolates B-36 (13.3%) (Table 3). The four rhizobacterial isolates were also evaluated for their ability to inhibit mycelial proliferation of *Fusarium oxysporum* sp. *ciceris* in liquid potato dextrose medium. All the four isolates showed different capability to inhibit mycelial growth of the fungus and a notable reduction in mycelial biomass production was observed as compared to control. The percent inhibition on dry weight basis recorded after 3 days of incubation was 62.82 (P-1), 53.77 (B-36), 40.30 (B-40) and 23.22 (B-20). These results are in corroboration with Hassanein *et al*., (2009), who reported that reduction in dry weight of *Fusarium oxysporum* was 75% by *Pseudomonas aeruginosa*.

These studies concluded that the isolates which were positive for both the parameters could be further tested under field conditions. These are the preliminary studies for the selection of effective PGPR strains for consequent use as bioagents. The chemical pesticides which are used for the different pathogens for the management of diseases are very expensive besides being hazardous to environment and human health. Hence, the isolates of PGPR obtained in the present study can be further tested for toxicological aspects and mass produced, which might be useful for development of ecologically sustainable biocontrol strategy for the management of several plant pathogens and insect pests, simultaneously in a sustainable manner, so that it can reach the end users.

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