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Full Length Research Paper

Antifungal activity of plant growth-promoting rhizobacteria isolates against *Rhizoctonia solani* in wheat

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Seven plant growth-promoting rhizobacterial (PGPR) strains were isolated from the rhizoplane and rhizosphere of wheat from four different sites of Pakistan. These strains were analyzed for production of indole acetic acid (IAA), phosphorous solublization capability and inhibition of *Rhizoctonia solani* on rye agar medium. Strains WPR-51, WPR-42 and WM-30 were selected to test *in planta* antagonistic activity on two wheat verities infected with *R. solani*. These three strains belonging to *Azotobacter* and *Azospirillum* produced IAA ranging from 19.4 to 30.2 ug/ml and possessed phosphorus solublization capability. Out of these three strains WPR-51 and mixture of all three strains showed maximum inhibition of *R. solani* growth. These strains positively affected the germination of wheat as well as increased biomass and root shoot length by inhibiting *R. solani* growth when tested in pot experiments. PGPRs can be used as biocontrol agents that inhibit root rot and also strengthen the plant for better growth.

Key word: Biological control, plant growth-promoting rhizobacterial, *Rhizoctonia solani*, antifungal activity, wheat.

INTRODUCTION

The beneficial plant-microbes interactions in the rhizospere are determinants of plant health and soil fertility (Jeffries et al., 2003). For sustainable agriculture production, these interactions play a pivotal role in transformation, mobilization, solubilization, e.t.c. from a limited nutrient pool in the soil and subsequent uptake of essential plant nutrients by the plants to realize full genetic potential of the crops. In the biogeochemical cycles of both inorganic and organic nutrients in the soil and in the maintenance of soil health and quality, soil microorganisms are very important (Jeffries et al., 2003). Thus, it is necessary to improve the efficiency of the meager amount of external inputs by employing the best combinations of beneficial microbes. Soil bacterial isolates from rhizosphere which have been shown to improve plant health or increase yield, are usually referred to as plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978; Suslow and Schroth, 1982). The beneficial effects of PGPR have been observed in many crops including horticultural, oilseed crops etc. However in wheat, reports are scantly especially in biocontrol aspects. The world population is growing by 160 people per min and wheat is predicted to be the most important cereal crop in the world to feed the ever increasing world population (Hoisington et al., 1999). To meet future cereal production demands it is imperative to increase crop yield.

Biological control of plant diseases is gaining attention due to increased pollution concerns because of pesticides use for crop protection and development of pathogen resistance (Wisniiewski and Wilson, 1992). The use of environmental friendly microorganisms has proved useful in plant-growth promotion and disease control in modern agriculture (Weller, 1988). Kravchenko et al.

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(2002) averred that PGPR inoculation is a promising agricultural approach, that plays a vital role in crop protection, growth promotion or biological disease control (Dilantha et al., 2006).

Rhizoctonia solani, a soil borne plant pathogen causes root rot, and damages a wide range of host plants. 68 hosts plants for *R. solani* have been reported from Pakistan (Ghaffar, 1988) including wheat root rot disease. The pathogen reduces plant growth by rotting the roots and thus reducing the ability of the plants to take up water and nutrients (Wallwork, 1996). However, the nature of these problems is not fully understood and little research has been conducted in Pakistan in context of broader wheat cropping system. To overcome this problem to some extent we aimed (i) to isolate, identify and select bacterial strains showing phytohormone production in pure culture, (ii) identification and quantification of phytohormones produce by rhizobia associated with wheat, and their beneficial effects on plant growth and (iii) evaluation of PGPRs strains as potential biocontrol agents of soil borne disease, that is, *R. solani* of wheat in Pakistan. Here, seven rhizobacterial isolates were obtained from wheat rhizosphere and their effects on fungal growth suppression assessed *in vitro* as well as *in planta* in growth chamber.

MATERIALS AND METHODS

Isolation and characterization of PGPR from wheat rhizosphere

Soil samples were collected from wheat crop rhizosphere from Kala Shah Kaku, Faisalabad district and NARC field Islamabad Pakistan. The samples were collect in aseptic begs and immediately transported to lab under cold condition (4°C) for further process. 1 g sample was suspended in 5 ml autoclaved distilled water separately. After sedimentation of solid particles, dilutions were made up to 10 - 8. On pre-prepared nutrient agar plates, 0.1 ml of each dilution was spread by L-shape glass rod. After 3 - 4 days of incubation at 26 - 28°C morphology and texture of each colony was recorded. On the basis of literature (colony morphology) many colonies were randomly selected and further purified by streaking. Each strain was characterized by gram staining, hormone production and phosphorus solubilizing ability.

Extraction and identification of IAA

Bacterial isolates were grown on NFM (nonfat milk) broth medium containing tryptophan (1.0 mg/L) and NH_4Cl (1 g/L). The cultures were grown for 72 h at 26 - 28°C in a water bath shaker. The bacterial cells were separated by centrifugation at 10,000 rpm for 15 min. The pH of supernatant was adjusted at 2.8 with HCl and then extracted 3 times with equal volume of ethyl acetate (Tien et al., 1979). The extract was evaporated to dryness and residue was re-suspended in 2 ml of ethanol. The samples were analyzed on HPLC using UV-detector and Tech sphere 5-ODS C-18 column. The methanol : acetic acid : water (30:1:70) mixture was used as mobile phase with flow rate 1.5 ml/ min. For identification, 20 µl sample, filtered through a 0.45 µm filter, was injected into the column. The growth hormone was identified on the basis of retention time of the standard IAA by using a refractive index detector (RI). The concentration was calculated on the basis of peak height and peak area in comparison with standard.

Phosphorus solubilization by PGPRs

The BKSB broth, containing 5 gl^{-1} of Ca_3PO_4 was inoculated with 1% (v/v) cell suspension (10^8 cfu per ml) and incubated at 120 rpm at 30°C for six days. The water soluble phosphate was determined calorimetrically by the method of John (1970).

In vitro antifungal activity of PGPR

The *in vitro* inhibitions of mycelium growth of *R. Solani* by the PGPR were tested on rye media (Caten and Jinks, 1968). For each bacterial isolates 1 ml (10^8 cfu/ml) PGPR suspension was poured on the margin of rye media plates and a 6 mm agar disc of *R. solani* from fresh PDA culture was placed at the other marginal side and incubated at 25 ± 2°C for seven days. The radii of the fungal colony towards and away from the bacterial colony were noted. The percentage growth inhibition was calculated using the following calculation:

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

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

Among seven isolates, maximum inhibition of mycelial growth was found by WPR-51 (*Azotobacter*) and mixture of three isolates than WM-3 (*Azospirillum*) and WPR-42 (*Azotobacter*).

Determination of disease severity caused by *R. Solani* and colonization of fungus in roots (pathogenicity test)

To check the pathogenicity caused by *R. solani*, seed were grown in Petri plates for one week and then transferred to 30 ml vials containing 1 ml *R. solani* broth. After 15 days, roots were thoroughly washed under running tap water for 10 -15 min. The roots were thoroughly washed under running tap water for 10 -15 min. Washed roots were cut into pieces, immersed in 1% chlorox for 1 min and rinsed three times in sterilized distilled water. Roots were dried on sterile blotting paper and placed on potato dextrose agar. The plates were incubated at 25 ± 2°C for 7days. The colonization was observed physiologically and microscopically.

In Planta antagonistic activity of PGPR

Surface sterilized wheat seeds of two varieties WAFAQ- 2001 (A) and GA 2002 (B) obtained from NARC were soaked in PGPR (WPR-42, WPR-51, WM-3 and their mixture, 10^8 cfu/ml) suspension for 1 h with occasional shaking to ensure uniform coating on the surface under aseptic conditions. Seeds of each variety soaked in sterilized distilled water were treated as control. The seed were allowed to grow in petri plates having autoclave filter paper, at 20°C for 6 days in growth cabinet. One–week-old seedlings were then transplanted in plastic pots containing sterilized sand. Plants were watered with 1/4th Hoagland solution when required. Four plants were maintained in each pot and placed in a growth chamber under standard conditions (18 h light, 25 ± 2°C and 60% relative humidity). After one week of transplant 1 ml broths of *R. solani* and 1 ml of each PGPR and their mixture were applied to all plants to check the efficiency against the *Rhizoctonia*. Some of the control plants were contaminated only with *R. solani* treated as negative control. Plants containing neither pathogen nor PGPR were treated as positive control.

Plants were harvested after six weeks and disease resistance assessment and growth parameters, that is, root and shoot length and weight were recorded.

Table 1. Morphological, physiological and cultural characteristic of PGPR bacterial strains isolated from wheat.

PGPR isolates	Gram stain	Shape of bacteria	Colony color on nutrient agar	Colony size/ shape on N.A	Phosphate solubilization ability	IAA (ug/ml)	Strain
WPR-42	+ ve	rod	Off white	Regular size with crenate boarders	+ ve	19.4	*Azospirillum*
WPR-51	- ve	Short Rods	Light green	Irregular size with swarming growth	+ ve	30.6	*Azotobacter*
WPR-32	- ve	Short rods	Light pink	Irregular size with wrinkled surface	- ve	25.6	*Azotobacter*
WPR-61	-ve	Cocci shape	White	Shiny colony	+ ve	5.5	*Pseudomonas*
WM-1	- ve	Short rods of different sizes	Off white shiny	Shiny	- ve	10.5	*Azotobacter*
WM-2	- ve	Short rods of different sizes	Off white shin	Shiny	- ve	12.4	*Azotobacter*
WM-3	- ve	Curved rods J shape	Light green	Shiny	+ ve	30.2	*Azospirillum*

Disease resistance assessment

After harvesting, infection severity on roots was rated by visual scaling ranging from 0 to 5. A rating of 0 means no evidence of infection, and rating of 1, 2, 3, 4 and 5 reflected an infected surface area of appropriately 5, 25, 50, 75, and 99 -100% respectively.

Statistical analysis

All the experiments were performed in triplicate, while all treatments were in a factorial design and arranged as a randomized complete block (RBC) with four replications of 4 plants were maintained in each pots. All data were subjected to ANOVA procedure of the SAS statistical Package. An LSD test was applied and Duncan's Multiple Range (DMRT) test at 5% ($p < 0.05$) level of significance.

RESULTS

Isolation, characterization and purification of bacterial isolates

Seven bacterial strains from wheat were isolates (Table 1). All seven strains were Gram negative bacteria. These bacterial isolates were predominantly rod-shaped, though a few of them were slightly curved while one isolate (WPR-61) was cocci-bacilli. The colony color of isolates varied from white/off-white to slight/dark pink; whereas one isolate (WPR-51) was slightly green in color. The colony shape in most of the cases was irregular with wrinkled/ rough surface. Some of the colonies had swarming growth. Two isolates (WPR-42 and WPR-32) had regular size; however the former had crenate and lateral had circular borders.

IAA production and phosphorus solubilization

All the isolates showed significant production of IAA (Table 1) that ranged from 5.5 to 30.6 µg/ml. The isolates WPR-51 and WM-3 produced highest concentration of IAA that was equivalent to 30 µg/ml. Four out of seven bacterial isolates also showed phosphate solubilizing ability that was desirable character in this study.

In vitro disease index/resistance assessment

The results *in vitro* inhibition of mycelium growth of *R. Solani* by the PGPR strains viz, WPR-32, WPR-42, WPR-51, WPR-61, WM-1, WM-2 and WM-3 tested on rye media are presented in Table 2. Among seven isolates, maximum inhibition of *R. solani* mycelial growth was found by WPR-51 (*Azotobacter*) than WM-3 (Azospirillum sp.) and WPR-42 (*Azotobacter*). Control plates not treated with the PGPR isolates were completely covered by the phytopathogen showing no inhibition. The WPR-42 and WM-3 treated plates showed mycelium inhibition 55 and 75% respectively while WPR-51 inhibited mycelium growth (99%), in other words almost fully inhibition of fungal growth.

Determination of disease severity caused by *R. Solani* and colonization of fungus in roots (pathogenicity test)

The incidence of root rot in wheat by *R. solani* was observed in one week old plants. It was observed that the isolated fungal strain strongly affected the wheat root, retarding the growth and ultimately causes death of plants. This severity and antifungal potential of PGPRs provided a clue for further pot experiments. However, depending upon PGPR characteristics, three isolates were selected for in *planta* antagonistic activity. These three strains were tested individually and as mixture (co-inoculation) for analysis.

Table 2. Disease index of root rot of wheat in both *in planta* as well as *In vitro* experiment against three PGPRs strain.

Treatment	***In Planta* root rot infection rate (0 - 5)**		***In vitro* inhibition of *R. solani mycelium* on rye agar media (% mycelium inhibition)**
	Variety A	**Variety B**	
Control	0 - 1 (5%)	0 - 1 (5%)	----
Pathogen	0 - 5 (100%)	0 - 5 (100%)	0.00%
WPR -42	0 - 3 (50%)	0 - 3 (50%)	55%
WPR -51	0 - 1(5%)	0 - 1(5%)	99%
WM – 3	0 - 3 (50%)	0 - 2 (25%)	75%
Mixture	0 - 0 (0%)	0 - 0 (0%)	99%

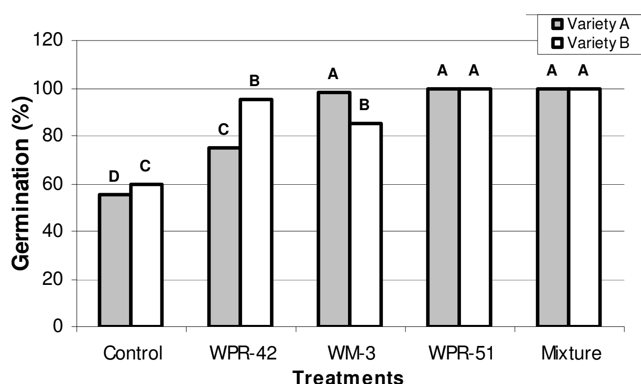

Figure 1. Effect of PGPRs on wheat germination (%).

In Planta antagonistic activity

The germination test in petri plates showed that all PGPR strains and their mixture significantly increased germination of both wheat varieties (Figure 1). The WPR-51 and mixture of all PGPRs treated seeds of both the wheat varieties showed 100% germination. The WPR-42 and WM-3 treated seeds of variety A and B showed 75 and 95%, and 98 and 85% germination, respectively. The untreated seeds showed 55 and 60% germination of both varieties after three days of inoculation.

The data of root and shoot length of six week old plants (Figure 2) showed significant differences ($P < 0.05$) between treatments. The *R. solani* inoculation (control Pathogen) severely retarded the growth as compares to non-inoculated control. The average root and shoot length of both varieties in *R. solani* treatment was 20.05 and 6.25 cm plant^{-1} respectively. The root and shoot of both wheat varieties was significantly increased by inoculation of PGPR isolates. Maximum root length was observed due to WPR-51 treatment (26.6 cm/plant) and mixture of three isolates (27.8 cm/plant). While shoot length was visa verse. A length of 39.4 cm per plant was observed due to treatment of WPR-51 while due to mixture shoot length was 36.1 cm plant^{-1}. The WPR-42 and WM-3 treatments were not found so effective to increase root and shoot length as compared to control (Figure 2).

The *R. solani* severely retarded root and shoot growth of both wheat varieties. The average root and shoot biomass of both varieties in this treatment ranged 0.05 - 0.2 g Pot^{-1} (Figure 3). All the PGPRs were effective in antagonizing *R. solani* and as a result increased root/ shoot biomass. The highest root/shoot biomass was recorded in WPR-51 (1.23 - 1.31 g Pot^{-1}in var A and 1.03 - 1.40 g Pot^{-1} in var B). However this was not significantly different than that of mixture of all PGPRs. WPR-42 and WM-3 also significantly improved root and shoot biomass of both varieties that on average ranged 0.46 - 0.55 and 0.25 - 0.43 g Pot^{-1}, respectively (Figure 3).

Antagonistic activity of PGPRs

In vivo results of antagonistic activity against *R. solani* have shown (Table 2) significant effect to control root rot disease. The inoculation by *R. solani* (without PGPR) showed that all the roots were fully infected (100%). Application of PGPR strains with *R. solani* showed that the PGPR controlled the infection/ disease at various degrees. Two PGPR strains, that were WR-42 and WM-3 controlled almost 50% infection in both wheat varieties. The strain WPR-51 controlled 99% of the infection; whereas mixture of all three isolates fully controlled the disease (100%) in both wheat varieties.

DISCUSSION

Seven plant growth promoting rhizobacteria were isolated and tested for antifungal potential against *R. solani*. Out of seven three isolates were selected on the basis of antifungal potential, IAA production and phosphorus solubilization and were examined for *in planta* antagonistic activity in wheat root rot. The PGPRs promote plant growth through more than one mechanism that include secretion of variety of growth stimulating hormones and suppression of plant growth retarding agents, that are pathogens. The *in vitro* test of this study showed that all the seven PGPR isolates from wheat rhizosphere produce growth promoting hormone IAA ranging 5.5 - 30.6 µgL^{-1}. Production of growth hormone such as IAA by PGPRs has also been reported by Dilfuza (2008). Among

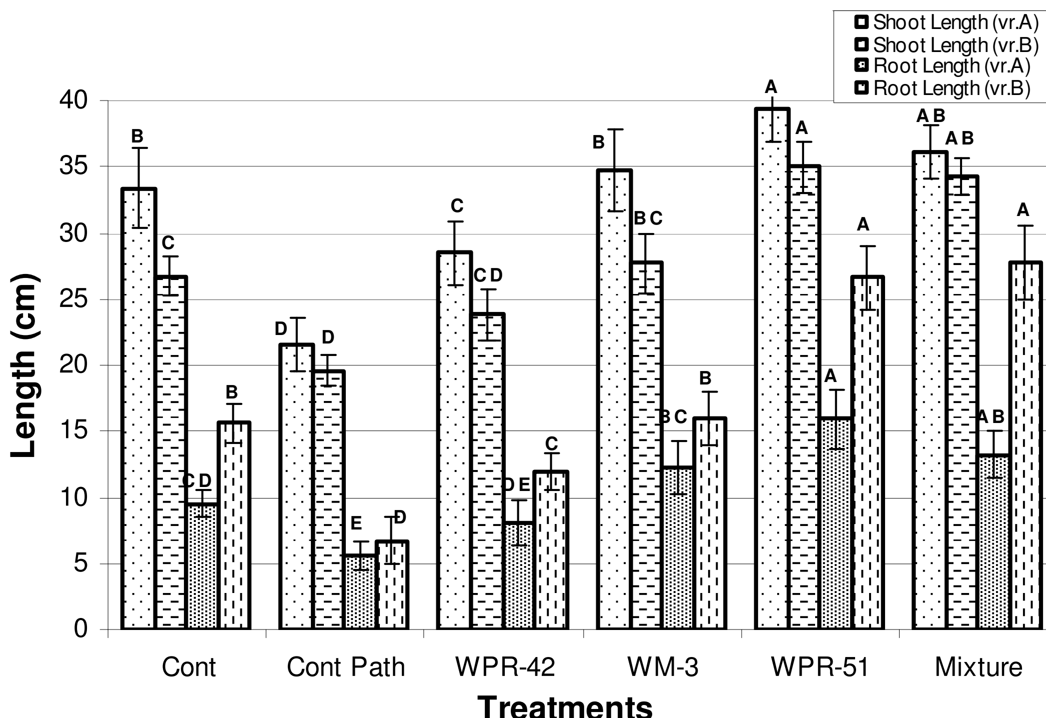

Figure 2. Antagonistic activity of PGPRs against *Rhizoctonia solani* as shown by wheat root/shoot length.

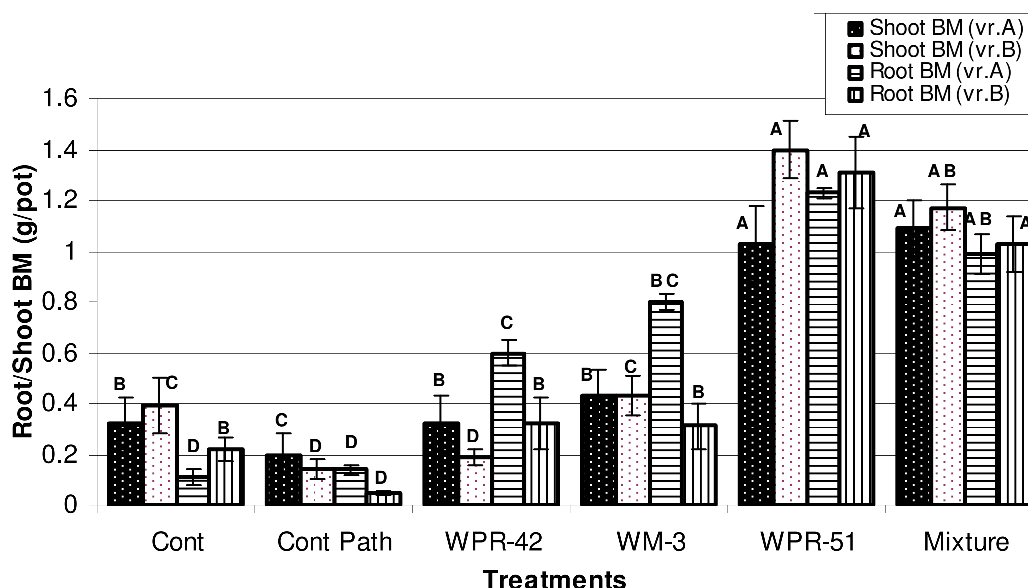

Figure 3. Antagonistic activity of PGPRs against *R. solani* as shown by wheat root/shoot biomass.

seven isolates four showed ability to convert insoluble P to soluble P usable for plants. Two isolates were identified as *Azospirillum* (WPR-42, WM-3), four *Azotobacter* (WPR-51, WPR-32, WM-1, and WM-2) and one *Pseudomonas* (WPR-61). WPR-32, WM-1 and WM-2 were unable to solubilize phosphate. These results supported by Kumar and Narula (1999, 2006) who isolated PGPRs from wheat rhizosphere that had ability to produce IAA and to solubilize phosphate.

Some bacterial isolates were found to be highly inhibitory of *R. solani* growth whereas others showed mild activity or no activity at all. This suggests that the mode

of action exerted and the type of antifungal metabolites produced by the isolates vary (Williams and Asher, 1996). Reduction of fungal growth by certain PGPR and formation of inhibition zones were presumably due to the materials (antifungal substances and/or cell wall degrading enzymes) released by the bacteria into the culture medium. WPR-51 and mixture of three isolates inhibited *R. solani* mycelium growth (99%) as compared to the other isolates. It has previously been reported that application of mixture of isolates inhibits pathogen growth more efficiently than single isolate (Marjan et al., 2003). The reason why application of single isolate does not control disease in better way might be related to insufficient root colonization. Therefore, these mechanisms by applying a mixture of the isolates lead to more effective or at least more reliable biocontrol of root rot of wheat.

Coating of PGPR strains either singly or mixture positively influenced on wheat germinations. The WPR-51 and the mixture of all three PGPRs improved wheat seed germination up to 100% in less time period compared to control. Ryu et al. (2003) also observed that PGPR treatment increase germination rate and root/ shoot growth in way similar to IAA, cytokinin and gibberellins treatments while Dal-Bello et al. (2002) observed that seed bacterization proved a successful method for enhancing biological control of plant disease.

Plant growth promoting activity and suppression of *R. solani* infection *in Planta* was observed in wheat by isolates WPR-42, WPR-51, WM-3 and by their mixture. All infected roots were characterized by dark brown to black coloration and rotting. The leaves of infected seedling were pale green and plants were stunted. Results demonstrate that individual PGPR treatments as well as their mixture induced significant disease protection against *R. solani* and on wheat growth parameters. Among three isolates, WPR-51 and their mixture significantly increased fresh and dry weight as compared to negative control pathogen treated plants.

The ranking order for disease suppression and wheat root rot by these PGPRs was WPR-51 > WM-3 > WPR-42. *Azotobacter* (WPR-42) has previously been reported as better plant protectant against root rot infection (Neyra at al., 1999). This contradiction may be due to plant species, survival rate of rhizobacteria and environmental conditions. Beneficial effects of PGPR and fungal bioprotectants on plants have been reviewed (Harman, 1991; Kloepper, 1991, 1993; Luz, 1993, 1996). Some other mechanism such as hydrocyanic acid, siderophores and induction of resistance may also play a role in the action of PGPR. So that rhizobacterial agents will probably be one of the most significant strategies for disease management (Luz, 1996). Therefore, the PGPR used in our study were promising as plant growth stimulator and biocontrol against wheat root rot disease.

Application of mixture of three PGPR isolates viz, WPR-42, WPR-51 (*Azotobacter*) and WM-3 (*Azospirilum*) has resulted in much more intensive plant growth promotion and disease reduction when compared to strains tested singly accept WPR-51. This might be due to different mode of action for PGPR strains (Raupach and Kloepper, 1998) and efficiency and reliability of biocontrol (Duffy and Weller, 1995; Kleopper, 2003). It can be concluded that PGPR isolated from wheat rhizosphere has potential to be used successfully for biological control of soil-borne plant pathogen (root rot caused by *R. solani*) in wheat especially in the fields where wheat is cultivated following potato crop.

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