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RESEARCH ARTICLE

Assessment of Antifungal Activity of PGPR (Plant Growth-Promoting Rhizobacterial) Isolates Against *Rhizoctonia solani* in Wheat (*Triticum aestivum* L.)

POOJA SINGH, PRASHANT SINGH AND M.P. SINGH
Department of Botany Udai Pratap (Autonomous) College, Varanasi - 221002

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Corresponding Author

PRASHANT SINGH
Prashantsingh12350@gmail.com

Abstract

Soil adhering to root is defined as the rhizosphere. 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). Seven plant growth-promoting rhizobacterial strains ($PGPR_S$) were isolated from the rhizoplane and rhizosphere of wheat from four different sites of Varanasi. These strains were analyzed for inhibition of *Rhizoctonia solani* on Waksman media. Strain WR-1, WR-3 and WR-5 were selected to test plant antagonistic activity on wheat infected with *Rhizoctonia solani*. Out of these strains WR-7 showed maximum inhibition of *R. solani* growth. Rhizobacterial isolates were tested in this study as biological control agent, positively affected the germination of wheat as well as increased biomass and root shoot length by inhibiting *R. solani* growth when tested in pot experiment.

INTRODUCTION

The region in the vicinity of roots can be distinguished into many microhabitats. The term 'rhizosphere' was introduced in 1904 by the German scientist Hiltner to denote that region of the soil which is subject to the influence of plant roots. Rhizosphere is characterized by greater microbiological activity than the soil away from plant roots. Soil adhering to root is defined as the rhizosphere. In addition, plants may develop a dense 'rhizosheath', which is a strongly adhering layer of root hairs, mucoid material, microorganisms and soil particles (Curl and Truelove, 1986; Sørensen, 1997).

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), were isolated from rhizoplane and rhizosphere of wheat. The term 'rhizoplane' denotes the root epidermis and outer cortex where soil particles, bacterial and fungal hyphae adhere. (Singer; 2006; Sylvia 2005). Thus, it is necessary to improve the efficiency of the major amount of external inputs by employing the best combinations of beneficial microbes.

The beneficial effects of PGPR have been observed in many crops including horticultural, oil, seed, 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). 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).

Rhizoctonia solani, a soil borne plant pathogen causes root rot, and damages a wide range of host plants (Figure1). 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 context of broader wheat cropping system.

Pathogenic microorganisms cause various plant diseases that usually weaken or destroy plant tissues and reduce crop yields varying from 25-100% (Frisvad and Samson, 1991). Root diseases are estimated to cause 10-15% yield losses annually in the world (Bajoria et al., 2008). Fungicide bavistin sprays are usually done for controlling the disease. But indiscriminate use of agrochemicals for disease and pest control has resulted into considerable pollution of soil, water and air. Moreover, wide spread use of agrochemicals also have undesirable effects on non-target organisms and possible carcinogenicity effects. Thus, their extensive use is environmentally unsafe and also uneconomical. Therefore, it is imperative to develop some alternate strategies for controlling plant diseases. Biological control using antagonistic microorganisms offers a low cost ecofriendly technology that reduces the number and activity of plant pathogens (Glick et al., 1999; Sindhu et al., 2009; Yang et al., 2014).

Fig 1. Root of Wheat infected with *Rhizoctonia solani* (Root Rot Disease).

Rhizosphere bacteria (rhizobacteria) suppress/control the plant diseases by various mechanisms viz., production of antibiotics (Keel et al., 1992; Saraf et al., 2014, production of hydrolytic enzymes (Sindhu and Dadarwal, 2001), hydrocyanic acid (Sarhan and Shehata, 2014), stimulation of phytoalexins or flavonoid-like compounds in roots (Goel et al., 2001) or by production of siderophores, which chelate metal cations rendering them unavailable for pathogenic forms (Raaijmakers et al., 1995; Sahu and Sindhu, 2011). Certain microbial strains protect the plants against pathogens through induced systemic resistance (Kaiser and Hannan, 1989). Some rhizosphere bacteria possess the enzyme ACC (1-aminocyclopropane-1-carboxylate) deaminase that reduces the level of stress hormone ethylene production. Enzyme ACC deaminase has been reported in many soil microorganisms (Khandelwal and Sindhu, 2012; Glick, 2014). These ACC deaminase-containing bacterial strains were also found more effective biocontrol strains (Glick, 2004; Wang et al., 2000). Thus, use of biocontrol agents isolated from plants and soils holds a great promise to establish them in the rhizosphere to control various plant diseases without disrupting the ecological balance (Weller, 2007).

MATERIALS AND METHODS:

Isolation and characterization of PGPR from wheat rhizosphere:

Rhizospheric soil samples were collected from different fields of wheat grown in agricultural field of Udai Pratap (Autonomous) College, Varanasi at 45 to 60 days of plant growth. The samples were collected in aseptic bag

and immediately transferred to lab under deep freeze condition (4^0C) for further process. The serial dilution of the rhizosphere soil samples (up to 10^{-4}) were plated on King's B agar medium. After 3-4 days of incubation at 26-28^{0}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.

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

The antagonistic interactions of rhizobacterial isolates with phytopathogenic fungus *R. solani* were studied by the spot test method on Potato Dextrose Agar (PDA) medium plates (Sindhu et al., 1999). *Rhizoctonia solani* was grown on PDA slants for 4 days and spore suspension was harvested in 3 ml sterilized water. Fungal spore suspension (3.0 ml) was added into sterilized Waksman media, mixed uniformly and plated. Growth suspension (5 µl) of 48 h old rhizobacterial cultures was spotted on spore suspension-containing plates. The inhibition of growth of *R. solani* by the spotted rhizobacterial isolates was recorded after 4 days of incubation at 28±2.

In Plant antagonistic activity of PGPR

Sterilized seeds were soaked in PGPR (WR-1, WR-3 and WR-7) suspension for 1hr with occasional shaking to ensure uniform coating on the surface under aseptic conditions. The seed were allowed to grow in Petri plates for 6 days in growth cabinets. One week old seedlings were then transplanted in plastic pots containing sterilized sand. Four plants were maintained in each pot and placed in a growth chamber under standard conditions (18 h light, 25 ± 20C 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 lengths were recorded.

Screening of rhizobacterial isolates for antagonistic activity against fungal pathogen (*R. solani)* under in vitro condition

Antagonistic activity of all the seven rhizobacterial isolates was studied by observing the zone of inhibition of fungal growth on Waksman media. The zone of fungal growth inhibition varied with different isolates tested. 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 show antagonistic activity, out of seven by observing inhibition zone of fungal growth on Waksman media.

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.

RESULTS

Seven bacterial isolates representing *Azotobacter, Azospirullum*, Pseudomonas based on (morphological and gram staining characteristics) were selected from rhizosphere soil after 6 day germination of wheat (Table1). Out of seven strains five were Gram positive bacteria and two were Gram negative. These bacterial isolates were predominantly rod-shaped, though a few of them were slightly curved while one isolate (WR-3) was cocci-bacilli. The colony colour of isolates varied from off-white to slight/dark pink; whereas one isolate (WR-7) was slightly green in colour. The colony shape in most of the cases was irregular with wrinkled/ rough surface. Some of the colonies had swarming growth.

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

PGPR isolates	**Gram stain**	**Shape of bacteria**	**Colony colour on nutrient agar**	**Colony size/shape on N.A**	**Antagonistic activity**	**Strain**
WR-1	+ve	Rods	Off white	Regular size with crenate borders	Positive	*Azospirillum*
WR-2	-ve	Short rods	Light green	Irregular size with rough surface	Negative	*Azotobacter*
WR-3	-ve	Cocci	White	shiny	Positive	*Azotobacter*
WR-4	-ve	Short rods	Light pink	Irregular size with wrinkled surface	Negative	*Pseudomonas*
WR-5	-ve	Short rods of different size	Shiny white	Irregular shiny surface	Positive	*Azotobacter*
WR-6	+ve	Rods	Off white	Regular size, uneven border	Positive	*Azospirillum*
WR-7	-ve	Curved rods ‘J shape’	Light green	Shiny	Positive	*Azotobacter*

In vitro disease index/resistance assessment

The results in vitro inhibition of mycelium growth of *R.solani* by the PGPR strains viz, WR-1, WR-2, WR-3, WR-4, WR-5, WR-6 and WR-7 tested on Waksman media are presented in Table 2. Among seven isolates, maximum inhibition of *R. solani* mycelial growth was found by WR-7 (*Azotobacter*) than WR-3 (*Azotobacter*) and WR-1 (*Azospirillum* sp). Control plates not treated with the PGPR isolates were completely covered by the phytopathogen showing no inhibition. The WR-1 and WR-3 treated plates showed mycelium inhibition 55 and 75% respectively while WR-7 inhibited mycelium growth (99%), in other words almost fully inhibition of fungal growth (Figure 2).

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

S.N.	**Treatment**	**In Plant root rot infection rate (0 - 5)**	**In vitro inhibition of *R. solani* mycelium on PDA media (% mycelium inhibition)**
1.	Control	0 - 1 (5%)	---
2.	Pathogen	0 - 5 (100%)	0.00%
3.	WR -1	0 - 3 (50%)	55%
4.	WR -3	0 - 1(5%)	75%
5.	WR -7	0 - 3 (50%)	99%

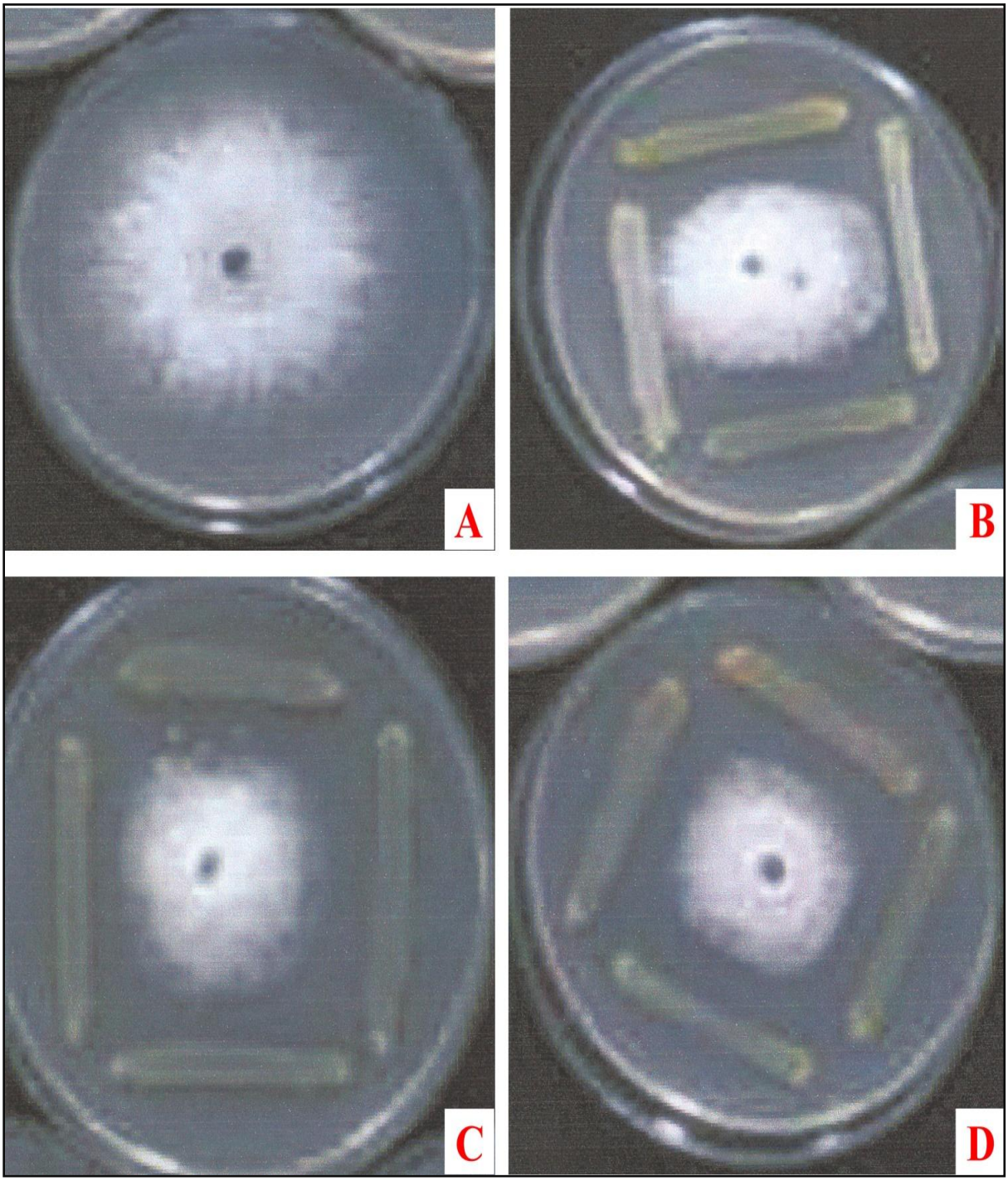

Fig 2. Rhizobacterial isolates showing antifungal activity against *Rhizoctonia solani* on different strains. (A. Control, B. WR-1, C. WR-3, D. WR-7).

Growth inhibition of *R.solani* by rhizobacterial isolates

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 plant antagonistic activity. These three strains were tested individually and as mixture (coinoculation) for analysis.

In Plant antagonistic activity

The germination test in controlled condition shows that all PGPR strains significantly increased germination of plants (Figure 3).The WR-1 treated seeds showed 65%, WR-3 showed 85% and WR-7 shows maximum 100% germination. The untreated seeds showed 40% germination after 3 days of inoculation. The data of root and shoot length of six week old plants 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 *R. solani* treatment was 20.05 and 6.25 cm plant^{-1} respectively. The root and shoot of wheat was significantly increased by inoculation of PGPR isolates. Maximum root and shoot length was observed in WR-7 (38.2,25.4 cm/plant respectively) and then in WR-3 while WR-1 was not found so effective to increase root and shoot length as compared to control (Figure 4).

The *R.solani* severely retarded root and shoot growth of wheat. All the PGPRs were effective in antagonizing *R.solani* and as a result increased root/shoot biomass. The highest shoot/root biomass was recorded in WR-7(1.32-1.54g Pot^{-1}). However WR-1 andWR-3 also significantly improved shoot and root biomass that on average ranged 0.48-0.65 and 0.28-0.51g Pot-1 respectively (Figure 5).

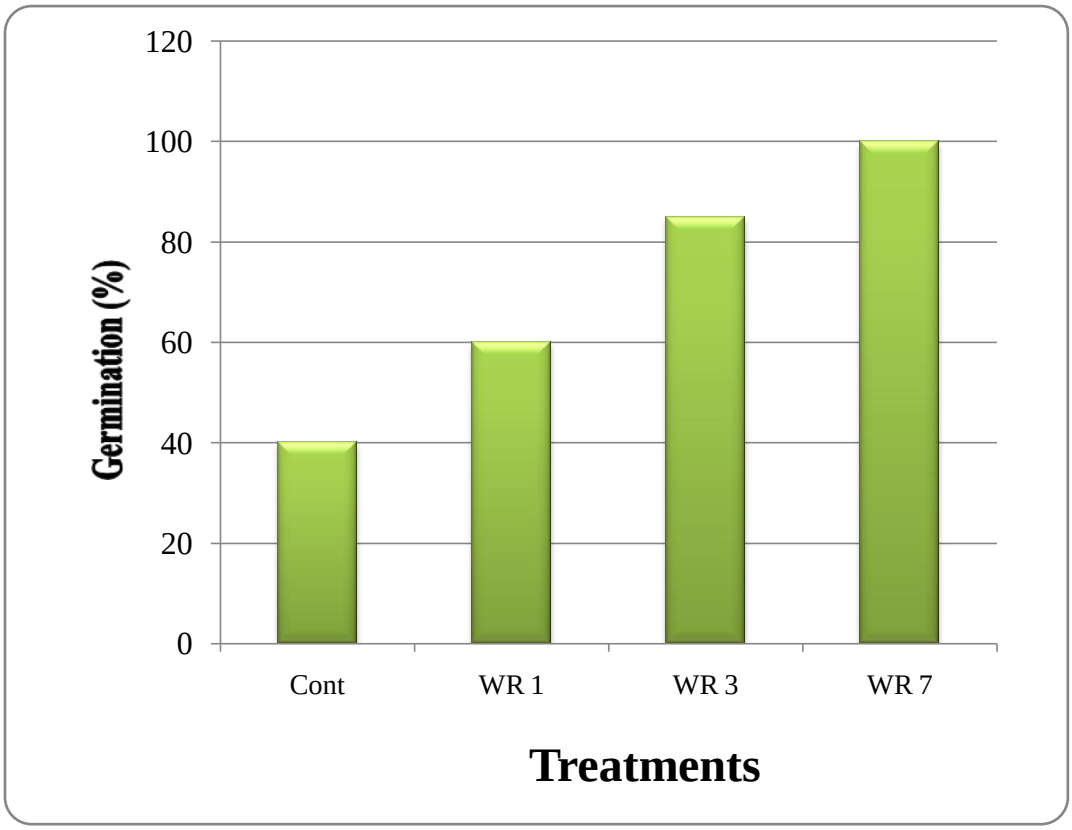

Fig 3. Effect of PGPRs on wheat germination (%).

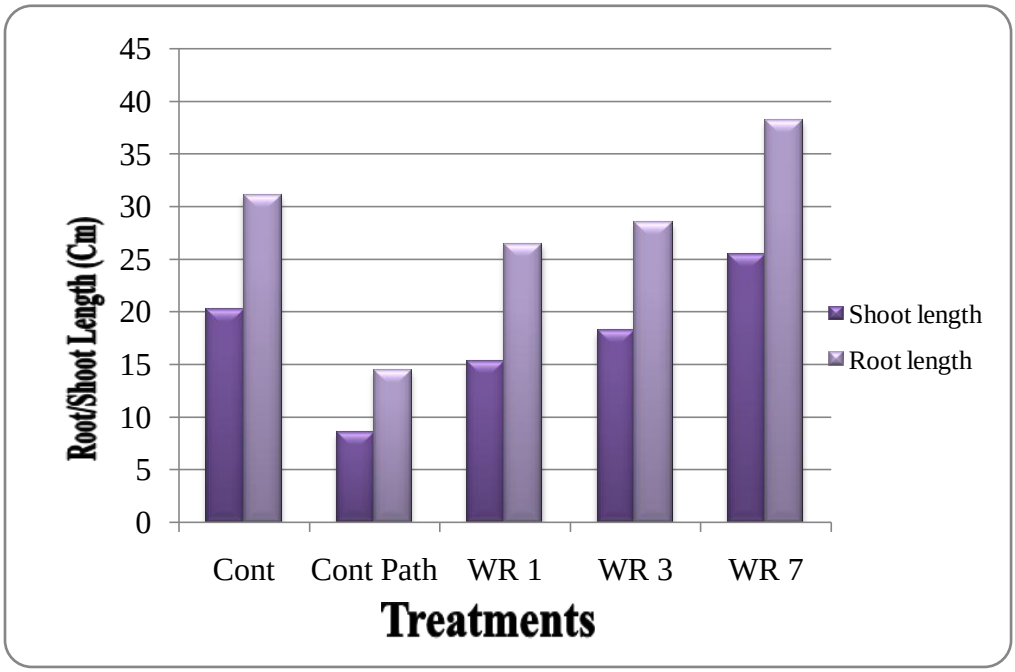

Fig 4. Antagonistic activity of PGPRs against *Rhizoctonia solani* as shown by wheat root/shoot length.

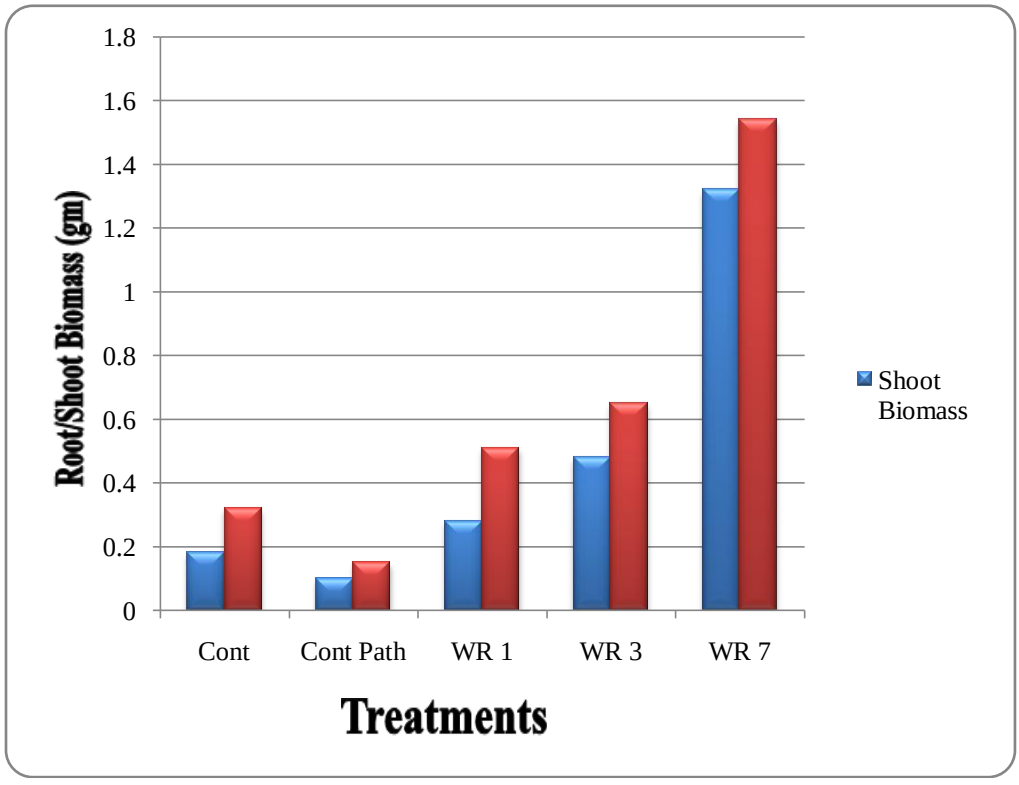

Fig 5. Antagonistic activity of PGPRs against *R.solani* as shown by wheat root/shoot biomass**.**

DISCUSSION

Previous studies have established that some rhizobacterial strains could serve as a useful biofertilizer and biocontrol agents for various crops (Sindhu et al., 2010). Antagonistic bacteria have also been found to promote the growth of different crops and termed as plant growth-promoting rhizobacteria. Seven plant growth-promoting rhizobacterial strains ($PGPR_S$) were isolated from the rhizoplane and rhizosphere of wheat and tested for antifungal potential against *R.solani*. Out of seven three isolates were selected on the basis of antifungal potential. The PGPRs promote plant growth hormones through more than one mechanism that include secretion of variety of growth stimulating and suppression of plant growth retarding agents, that are pathogens. 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. WR-7 inhibited *R. solani* mycelium growth (99%) as compared to the other isolates.

Coating of PGPR strains positively influenced on wheat germinations. The WR-7 PGPR 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 Plant* was observed in wheat by isolates WR-7, WR-3 and WR-1. 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 PGPR treatments induced significant disease protection against *R. solani* and on wheat growth parameters. Among three isolates, WR-7 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 WR-7 > WR-3 > WR-1. *Azotobacter* (WR-1) has previously been reported as better plant protection 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.

The different mode of action for PGPR strains (Raupach and Kloepper, 1998) efficiency and reliability of biocontrol (Duffy and Weller, 1995; Kleopper, 2003) 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.

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