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Integrated effect of plant growth-promoting rhizobacteria and phosphate-solubilizing microorganisms on growth of wheat (*Triticum aestivum* L.) under rainfed condition

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Abstract

Background: Biofertilizers are now considered to be the only alternative of chemical fertilizers which apart from their soaring cost are enhancing in pollution hazards of our environment. Increasing and extending the role of biofertilizers would reduce the need for chemical fertilizers and consequently decrease adverse environmental effects.

Results: After morphological and physiological characterization, 7 isolates out of 63 were selected as PGPR and seven as phosphate-solubilizing microbes (PSM). All seven PGPR exhibited indole acidic acid (IAA) production, whereas five isolates produced gibberellic acid (GA) ranging from 5.5 to 30.6 and 10.0 to 14.8 mg L^{-1}, respectively, isolate WPR-51 have highest concentration of IAA and GA. Two isolates (WM-1 and WM-2) did not showed GA production in culture solution. Phosphate solubilizing index (SI) of seven isolates was recorded for 6 days in an incubation study. The P solubilization was also quantified through spectrophotometry and was found to range from 25 to 130.1 µg mL^{-1}. These two isolates were further studied for their organic acid production (oxalic acid, citric acid and gluconic acids) through HPLC. The isolates PSM 202 showed higher acid production as compared to PSM-305. After biochemical screening, three PGPR (WRP-32, WRP-42 and WRP-51) and one PSM-202 were used in eight different combinations to test their effect on seed germination, seed vigor and root length in a 6-day Petri plates study. After laboratory study, a pot study was carried out, to verify the results of incubational experiment. Data were collected on root shoot length and root shoot biomass after 8 weeks of transplantation. Statistical analysis showed that among eight treatments, mixture or co-inoculation treatment (T_8) (WPR-42 + WPR-51 + WPR-32 + PSM) ranked as first followed by treatment (T_5) (WPR-51 + PSM) that significantly increased root shoot length and biomass as compared to un-inoculated treatment. Three PGPR isolates (WPR-42, WPR-51, WM-1) were also tested for their antifungal activity on seed germination of two wheat varieties and confirmed their antifungal activity against *Rhizoctonia solani*. The isolate WPR-51 and mixture of 3 isolates completely neutralized the harmful effects of *Rhizoctonia solani* as 100% of the seeds of both varieties germinated in these treatments.

Conclusion: The integrated effect of co-inoculation treatment of selected PGPRs (WRP-32, WRP-42 and WRP-51) and PSM-202 were found better in promoting crop growth and controlling disease as compared to all others treatments.

Keywords: PGPR, PSM, Wheat

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Background

Regardless of a record increase in agricultural productivity during the twentieth century, the world faces insecurity in global food security. The most critical issue is the predicted increase in world population that is expected to go up from the current 6.8 billion to 9.0 billion people by 2050. The pressure of feeding these extra people will be felt most severely by developing countries, whose populations are expected to grow to 7.9 billion [30].

The beneficial plant–microbes interactions in the rhizosphere are determinants of plant health, soil fertility recycling of nutrients [1]. For sustainable agriculture production, these interactions play a pivotal role in transformation, mobilization, solubilization, etc. 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 [12]. 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).

It has been reported by many scientists that a large number of phosphate-solubilizing bacterial genera secret indole acetic acid (IAA) which is absorbed by rhizospheric roots resulting increase in existing pool of plant IAA [2, 3, 19, 23]. It has been also reported that optimum level of IAA has positive effect, whereas excess IAA concentration has negative effect on root growth [10].

The beneficial effects of PGPR have been observed in many crops, i.e., for cereals [6, 17, 22] legumes and oilseed crops [12, 15, 25, 29, 31] also reported better growth in sunflower under saline condition inoculated with PGPR having ACC-deaminase activity as compared to un-inoculated.

Combined inoculation with PGPR and P-solubilizing bacteria were more effective than single microorganisms for providing a more balanced nutrition for plants [4, 9, 27]. But integrated effects of both PGPR and PSM on wheat reports are scanty especially in yield and biocontrol aspects. The interactions between these PGPR and PSM may be synergistic or antagonistic and beneficial effects of such interaction may be exploited for enhancing the biological nitrogen fixation, phosphate solubilization and biocontrol aspect against fungal diseases for better crop growth and yield.

To overcome this problem research was conducted in Pakistan in context of broader wheat cropping system. We firstly isolate, characterized, identify and select PGPR and PSM bacterial strains. Secondly, quantification of phytohormones (IAA, GA, organic acids produced) by PGPR and PSM. Thirdly, evaluation of selected isolates was made as potential growth promoters of wheat in vitro condition.

Methods

Isolation and characterization of PGPR from wheat

Wheat soil samples were collected from multiple locations, i.e., NARC, Potowar, Kala Shah Kaku and Faisalabad. The samples were collected in aseptic bags and stored at 4 °C till further process.

Bacterial isolation was carried out by following standard procedure of dilution plate technique. After 3–4 days of incubation at 26–28 °C morphology and texture of each colony was recorded.

Physiologically and biochemically identification was carried out by QTS-24 miniaturize identification system (DSTO Laboratories, Karachi, Pakistan). Twenty-four-hour-old PGPR strains were used to inoculate QTS kits. These PGPR strains were preserved for further testing/evaluation.

Extraction process and identification of growth hormones by PGPR through HPLC

Bacterial isolates were grown on NFM broth medium containing tryptophan (1.0 mg L^{-1}) and NH_4Cl (1 g L^{-1}). Tryptophan was added as precursor of IAA biosynthesis. The cells were grown for 72 h at 26–28 °C in a water bath shaker. The bacterial cells were collected by centrifugation at 10,000 rpm for 15 min. The pH of supernatant was adjusted at 2.8 with HCl and then extracted three times with equal volume of ethyl acetate [25].

The extract was evaporated to dryness and re-suspended in 2 mL of ethanol. The samples were analyzed on HPLC using UV-detector and Tech sphere 5-ODS C-18 column [18]. This column is used for elution of growth hormones. The methanol/acetic acid/water (30:1:70) mixture was used as mobile phase at the rate of 1.5 mL min^{-1}.

For identification, 20 µL samples, filtered through a 0.45 µm filter, were injected into the column. The growth hormones were identified on the basis of retention time of the standard IAA by using a refractive index detector (RI). The concentrations of each acid were calculated on the basis of peak height and peak area in comparison with standard.

Isolation and characterization of PSM from wheat

Isolation of phosphate-solubilizing microorganism (PSM) was carried out from the same samples of four sites of wheat. Dilutions were made by the same procedure as mentioned above. For PSM isolation, Pikovskaia [24] media was used. PSM colony was identified by

a transparent clearing zone around the colony. As this zone increased, it means bacterial phosphate-solubilizing activity is increased. PSM needs more incubation than PGPR strains. Colonies were picked and purified by streaking. Each strain was characterized by Gram staining microscopy, IAA spot test and nitrogen fixing ability. Twenty-four-hour-old PSM strains were used to inoculate QTS kits for identification. These PSM strains were stored for further testing/evaluation.

Quantification of P solubilized by PSM

Quantification of available phosphorus solubilized by PSM was done by phospho-molybdate method through spectrophotometry at 882 nm [20].

Quantification of organic acid produced by PSM

Organic acid produced by PSM strains in broth medium was analyzed by high-performance liquid chromatography (HPLC). Supernatant of samples, centrifuged at 1500 rpm for 15 min, was taken. Samples were passed through 0.45 µm non-sterile 4-mm syringe filters and injected with 20-µL injection loop into the column. These were determined by Bio-Rad ion-exchange column of Aminex 97-H (25 × 4.6 mm), mobile phase 0.001 N H_2SO_4 at the flow rate of 0.6 mL min^{-1}. The column was set at 45 °C temperature. The samples were detected at refractive index (RI) detector. The RI impulse was read at Turbochrom Navigator Programme in g L^{-1} after running standards of organic [16].

Seed germination in Petri dishes

Surface-sterilized wheat seed of variety GA-2002 obtained from NARC thoroughly soaked in PGPR (WPR-32, WPR-42, WPR-51, PSM-202 and their mixture @10^8 cfu mL^{-1}) suspension for 1 h to ensure uniform coating on the surface in aseptic conditions. Seeds soaked in sterilized distilled water were treated as control. The seeds were allowed to grow in Petri plates having autoclave filter paper, at 20 °C for 6 days in growth cabinet. Germination was observed on third day.

Pot experiment

One-week-old seedlings from the Petri plates were then transplanted in plastic pots containing autoclave sterilized sand. The experiment was laid out in completely randomized design (CRD). There were eight treatments with three replicates. Treatments were as follow:

Treatments
T1 = Un-inoculated (Control)
T2 = WPR-32 + PSM
T3 = WPR-42 + PSM
T4 = WPR-51 + PSM
T5 = WPR-32 + 42 + PSM
T6 = WPR-32 + 51 + PSM
T7 = WPR-42 + 51 + PSM
T8 = WPR-32 + 42 + 51 + PSM

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) for 6 weeks. After 2 weeks of transplant, inoculum was given again @ 1 mL broths of each strain applied to all plants. Plants were harvested after 6 weeks; growth parameters, i.e., root and shoot length, and weights were recorded.

Statistical analysis

All data were subjected to ANOVA procedure of the SAS statistical package. An LSD test and Duncan's Multiple Range (DMRT) test at 5% ($p < 0.05$) level of significance [28] were performed.

Results and discussion

Isolation, characterization and purification of PGPR and PSM bacterial isolates

Seven promising PGPR and two PSM isolates were selected for laboratory and pot study (Table 1). All were Gram-negative bacteria, whereas one PSM (PSM-202) was Gram-positive bacteria. These bacterial isolates were predominantly rod-shaped, though a few of them were slightly curved. Two isolates (WM-3 and PSM-305) were cocci-bacilli/oval-shaped. The colony color of isolates varied from white to off-white and transparent colonies, whereas one isolate (WM-1) had light pink color. The colony shape/size in most of the cases was regular with smooth surface. Some of the colonies had swarming growth. The genius identification of PGPR isolates was carried out through QTS-24 kit. Three isolates identified as *Azospirillum* spp. (WPR-42, WPR-51,WM-3), two *Azotobacter* spp. (WPR-32,WRP-61) and one *Pseudomonas* (*WM-3*) and one *Entobactor* spp. (WM-1), whereas PSM isolates, one as *Bacillus* spp. (PSM-202) and one as *Pseudomonas* spp. (PSM-305).

Extraction process and identification of growth hormones IAA and GA by HPLC

The IAA and GA both is growth promoter and stimulate growth of the plants that ultimately results in higher crop yields. Seven PGPR, which were analyzed for phytohormone production by HPLC technique [29]. All the isolates showed significant production of indole acetic acid (IAA) and gibberellic acid (GA) (Fig. 1). The IAA production from these isolates ranged 11.1–30.1 µg mL^{-1} in culture medium. The isolates WPR-51 (*Azospirillum*) and WPR-61 (*Bacillus*) had highest concentration of IAA.

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

Sr. no.	Strain	Gram stain	Shape of bacteria	Colony color on N.A.	Colony size/shape on N.A.	Tentative identification by QTS-24 kit
Plant growth-promoting rhizobacteria (PGPR)						
1.	WPR-32	−ve	Medium sized rod	Off-white	Regular size with crenate boarders	*Azotobacter* spp.
2.	WPR-42	−ve	Curved rod (pleomorphic)	White (semi-transparent)	Irregular size with rough surface	*Azospirillum* spp.
3.	WPR-51	−ve	J shaped rod	Off-white	Irregular size with empty centered	*Azospirillum* spp.
4.	WPR-61	−ve	Short rods	Off-white	Regular size/circular	*Azotobacter* spp.
5.	WM-1	−ve	Slightly curved rods	Light pink	Irregular size with wrinkled surface	*Enterobacter* spp.
6.	WM-2	−ve	Slightly curved rods	Off-white	Irregular size with entire edges	*Azospirillum* spp.
7.	WM-3	−ve	Cocci-bacilli	White	Regular size with rough surface	*Pseudomonas* spp.
Phosphate-solubilizing microorganisms (PSM)						
1.	PSM-202	+ve	Long rods	White/transparent	Regular size/circular with shiny surface	*Bacillus* spp.
2.	PSM-305	−ve	Cocci-bacilli	White	Regular size with rough surface	*Pseudomonas* spp.

N.A. nutrient agar

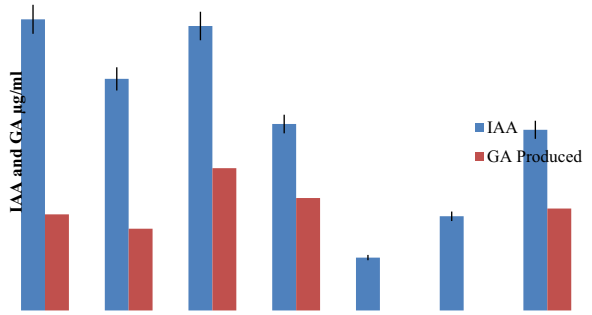

Fig. 1 Production of indole acetic (IAA µg mL^{-1}) and gibberellic acid (GA) from PGPR isolates measure through HPLC technique

The GA production from these PGPR isolates ranged 10.0–14.8 µg mL^{-1} of culture solution. The isolate WPR-51 (*Azospirillum*) produced the highest concentration of GA. In isolates WM-1 (*Pseudomonas*) and WM-2 (*Azospirillum*) GA was not detected in culture solution.

Solubilization and quantification of P

The zone diameter of seven PSM strains ranged from 21 to 83 mm and solubilization index (SI) ranged 1.63–3.29. The isolate PSM-305 showed maximum SI, whereas WM-1 indicated minimum SI (Fig. 2). The P solubilization was quantified by spectrophotometry and was found to range from 25 to 130.1 µg mL^{-1} (Table 2). Among seven isolates, five were PSB, whereas two were PGPR having the additional quality of P solubilization. The maximum P, i.e., 130.1 and 122 µg mL^{-1}, was solubilized by PSM-202 (*Pseudomonas*) and PSM-305 (*Pseudomonas*), respectively, and they differ non-significantly in P solubilization. High-performance liquid chromatography (HPLC) results showed that oxalic and citric acids were produced in larger concentrations, while gluconic acid was in smaller concentrations (Fig. 3).

Seed germination in Petri dishes

The germination test in Petri plates has shown that all combination of PGPR and PSM strains significantly increased germination of wheat as compared to control and also germination started earlier in inoculated treatments as compared to control.

The root and shoot lengths increased by the application of PGPR and PSM strains (Fig. 2). The root length in control was 1.4 cm that increased to 2.9–3.0 cm inoculated treatments. The maximum root length (i.e., 4.8 cm) was obtained in case of application of mixture of all three PGPR and PSM isolates.

The shoot length also increased by the PGPR isolates in a similar way but to slightly less extent. The shoot length in case of control was 1.6 cm that increased to 2.0–3.0 cm inoculated treatments. Again, the maximum shoot length was in case of application of mixture of all three PGPR + PSM isolates (Fig. 4).

Pot experiment (growth chamber study)

All the PGPR and PSM isolates improved vigor of wheat seedlings. Healthy plants were observed in treatments with inoculum as compared to control (no inoculum).

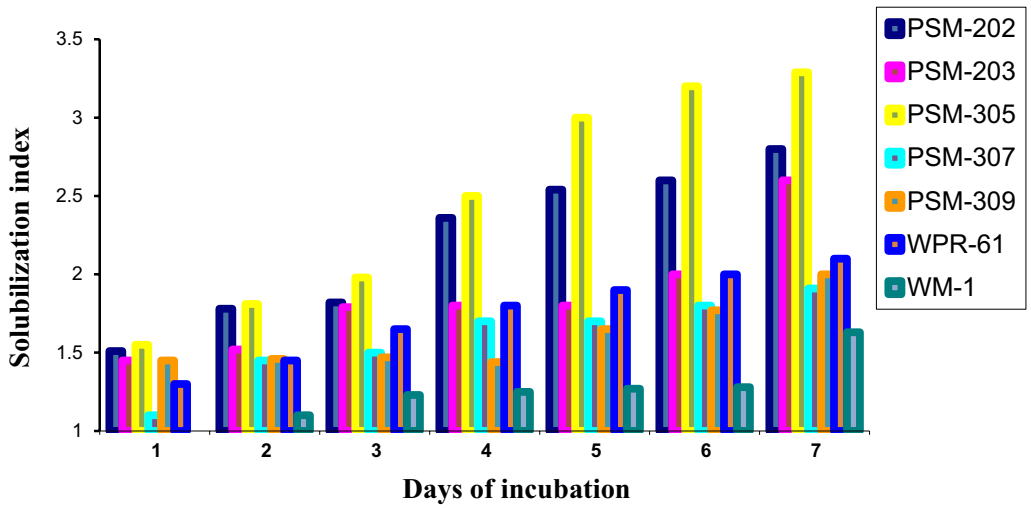

Fig. 2 Solubilization index of P-solubilizing isolates during 7 days of incubation (grown on Pikovskaya agar plates)

Table 2 Quantification of available phosphorus solubilized by P-solubilizing bacterial strains after 7 days of incubation (optical density noted at 882 nm)

Bacterial isolate	Optical density	pH	Available P (μg mL^{-1})
Control	0.8	6.8	21.5 g
PSM-202 (*Pseudomonas*)	4.1	3.6	130.1 a
PSM-203 (*Pseudomonas*)	2.4	5.5	78.6 c
PSM-305 (*Pseudomonas*)	3.8	4.0	122.0 ab
PSM-307 (*Bacillus*)	1.5	5.4	28.5 de
PSM-309 (*Bacillus*)	1.8	5.7	30.2 d
WPR-61 (*Bacillus*)	1.8	5.6	32.5 d
WM-1 (*Pseudomonas*)	1.0	6.0	25.2 f

Means bearing similar letter(s) do not differ statistically at $p < 0.05$

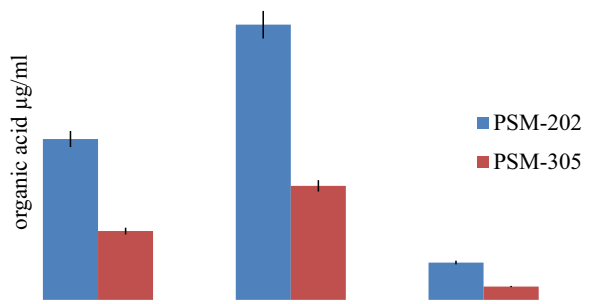

Fig. 3 Production of organic acid (μg mL^{-1}) from PSM isolates by HPLC technique

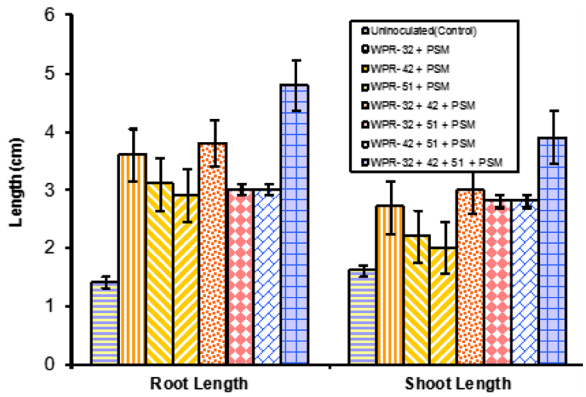

Fig. 4 Integrated effect of PGPR and PSB on root and shoot length of wheat in Petri plates (6 days after sowing in Petri plates)

Root and shoot length

Wheat plant growth showed the difference among the treatments. Extended root growth was indicated by the data. Maximum root length (27.8 cm) was observed in co-inoculation of mixture of PGPR and PSB. Minimum root length (8.1 cm) was recorded in control (un-inoculated). Among three PGPR with PSB strains, maximum shoot length (36.1 cm) was observed in co-inoculation of mixture of PGPR and PSM, which was 18% higher than control (Fig. 5).

Root and shoot biomass

Plants treated with PGPR + PSM isolates significantly increased root shoot biomass than control. However, mixture of three PGPR (WPR-32 + 42 + 51) with PSM gave the highest value (1.8 g). The PGPR isolate, WPR-32, performed best in axenic conditions. The ranking of treatment combination for increasing root length and shoot length was as T_8 followed by T_6, whereas in case of root/shoot biomass was T_8 followed by T_7. Mixture treatment of Three PGPR and PSM performed

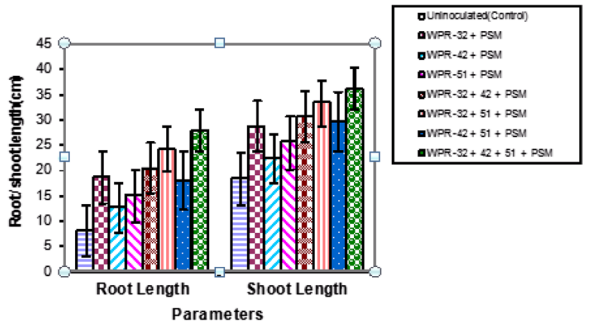

Fig. 5 Integrated effect of PGPR and PSM on root length and shoot length of wheat in pot under axenic conditions (6 week after sowing in pots)

better in all cases as compared to all other combination (Fig. 6).

Biocontrol aspects of PGPR

Three PGPR were evaluated for their antifungal activity in vitro. Three isolates, WPR-42, WPR-51 and WM-3, were found to be biocontrol agent.

Petri plate assay

The *Rhizoctonia solani* inoculation was done on one side of Petri plates containing fungal growth media, and PGPR isolate cultures were added on other side of the Petri plates. After 1 week of incubation, the disease rating was done on 1–5 scale on measuring the diameter of fungal pathogen growth. Also the disease inhibition by PGPR isolates was calculated by retardation of fungal growth toward PGPR isolate inoculation sites. In control fungal pathogen treatment, all the plates were covered with fungal mycelium, meaning the highest disease rating of 1–5 scale.

Seed germination %

The seeds of wheat were soaked in cultures of three PGPR isolates (WPR-42, WPR-51 and WM-3 and their mixture in Petri plates. The culture media without inocula of PGPR was treated as control. The culture of *Rhizoctonia solani* was added to the Petri plates. In the control treatment having only fungal pathogen, the germination was severely retarded. Only 55 and 60% of the seeds of both varieties A and B, respectively, germinated (Fig. 7). All the PGPR isolates reduced the harmful effects of *Rhizoctonia solani* on germination. The isolate WPR-42 had higher germination than control since 75 and 95% seeds of varieties A and B germinated. Whereas isolate WM-3 showed greater positive effect on germination of both varities A and B. Resulting 98 and 85% higher germination was observed as compared to control. The isolate WPR-51 and mixture of three isolates completely neutralized the harmful effect of *Rhizoctonia solani* as 100% of the seeds of both varieties germinated in these treatments. The ranking order for disease suppression and wheat root rot by these PGPRs was WPR-51 > WM-3 > WPR-42.

Discussion

The plant growth-promoting rhizobacteria play an active role in soil through their natural ability to provide important but scarce nutrients to the plants. Among the plant nutrients, N and P are the two key plant nutrients provided by these organisms under natural field conditions. In this context, the inoculation effects of PGPR and PSB including a symbiotic N_2 fixation [5, 13], phytohormone production [8], phosphate solubilization [14] and resistance against pathogen [32] are receiving increased attention for their use to develop microbial inoculants in order to improve crop productivity.

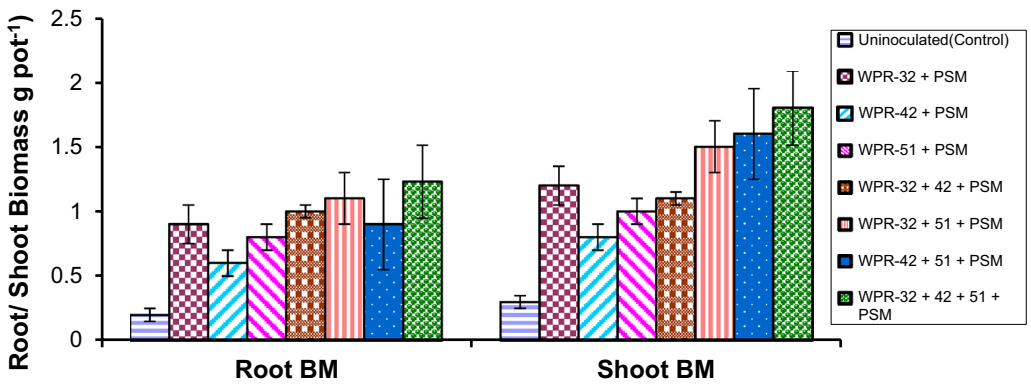

Fig. 6 Integrated effect of PGPR and PSB on root and shoot biomass of wheat in pot under axenic conditions (6 week after sowing in pots)

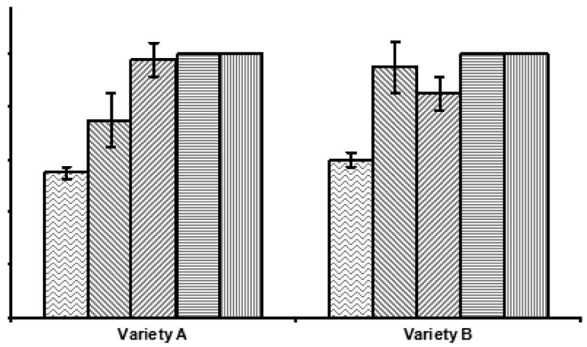

Fig. 7 Antagonistic activity of three PGPR isolates against *Rhizoctonia solani* shown by wheat germination (%)

Integrated effect of PGPR and PSM was studied for plant growth. The first evaluation was carried out in growth chamber by studying growth promotion under controlled condition. The in vitro test of this study showed that all the seven PGPR isolates from wheat rhizosphere and rhizoplane produced growth-promoting hormones IAA and GA. Production of growth hormone such as IAA by PGPRs has also been confirmed in other studies [21]. PSM isolates showed varying ability to convert insoluble to soluble P usable for plants. Coating of PGPR strains either singly or mixture has positive influence on wheat seed germinations. Among all treatments, isolates of WPR-51 and mixture of three isolates along with PSM significantly increased root shoot length and biomass and % seed germination as compared to control. These results supported by [12] that application of PGPR along with phosphate-solubilizing bacteria is highly effective for improving yield in Maize crop. PGPR treatment increased germination rate and root/shoot growth in way similar to indole acetic acid (IAA), cytokinin and gibberellins treatments [11]. The IAA hormones producing PGPRs stimulate/promote growth of the plants. Inoculated seed showed better seed germination because of antifungal activity of these isolates along with other factors. All three PGPR isolates were also found biocontrol agent. Similar finding regarding antifungal activity of *Pseudomonas* spp. in rice and sugarcane rhizosphere were reported by Reddy et al. [26]. Bacterization of seed proved a successful method for enhancing biological control of plant disease [7].

Conclusion and recommendation

It is concluded from this study that integrated application of PGPR and PSM isolated from wheat rhizosphere has potential to be used successfully for significant improvement in wheat growth because of more availability of phosphorus solubilization, phytohormone production and biological control of soil-borne plant pathogen. Rhizobacterial agents will probably be one of the most significant strategies for disease management. Therefore, the PGPR used in our study were promising as potential plant growth promotion and biocontrol against root rot wheat diseases. Therefore, it is recommended the potential of these PGPRs should be further investigated under pot and field condition to confirm their beneficial role.

Abbreviations

PGPR: plant growth-promoting rhizobacteria; PSB: phosphate-solubilizing bacteria; PSM: phosphate-solubilizing microbes; IAA: indole-3-acetic acid; GA: gibberellic acid; ANOVA: analysis of variance.

Authors' contributions

MS conducted research and statistically analyzed data. MZK prepared and wrote the manuscript. TS supervised the research work, AZ reviewed the paper and SM managed review of literature. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of supporting data

Supporting data is available.

Consent for publication

All authors agreed to published data.

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