Multitrait *Pseudomonas* spp. Isolated from Monocropped Wheat (*Triticum aestivum*) Suppress *Fusarium* Root and Crown Rot

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Accepted for publication 26 November 2019.

ABSTRACT

Fusarium root and crown rot is the most common disease of wheat, especially wheat grown in arid zones where drought is a common issue. The development of environmentally safe approaches to manage diseases of food crops is important for humans. The monocropping system recruits beneficial bacteria that promote plant growth through nutrient solubilization and pathogen suppression. In this study, a field where wheat was monocropped for 5 successive years under rainfed conditions was identified. A total of 29 bacterial isolates were obtained from the rhizosphere, endosphere, and phyllosphere of wheat at its harvesting stage. The Gram-negative bacteria were less prevalent (41%) but the majority (75%) exhibited plant growth-promoting traits. The ability of strains to solubilize nutrients (solubilization index = 2.3 to 4), inhibit pathogenic fungi (25 to 56%), and produce antifungal compounds was highly variable. The

rhizobacteria significantly promoted the growth and disease resistance of wheat varieties such as Pirsbak-2015 and Galaxy-2013 by inducing antioxidant enzyme activity (0.2- to 2.1-fold). The bacterial strains were identified as *Ochrobactrum* spp., *Acinetobacter* spp., and *Pseudomonas mediterranea* by 16S *rRNA* and *rpoD* sequence analysis. The endophytic bacterium *P. mediterranea* HU-9 exhibited maximum biocontrol efficacy against wheat root and crown rot diseases with a disease score/disease index from 1.8 to 3.1. The monocropping systems of rainfed agriculture are an ideal source of beneficial bacteria to use as bioinoculants for different crops.

Keywords: disease control and pest management, *Fusarium* root and crown rot, monocropping system, mycology, plant growth-promoting rhizobacteria, wheat (*Triticum aestivum* L.)

Fusarium root and crown rot is the most common disease of wheat and occurs in most areas of the world (Moya-Elizondo 2013). The disease is caused by numerous *Fusarium* spp. and results in significant yield losses, especially in dryland wheat growing regions (Shikur Gebremariam et al. 2018; Volova et al. 2018). Changes in rainfall patterns and prolonged droughts as a result of climate change could increase the expansion of this disease and pose major threats to wheat growing countries, especially arid and semiarid zones where drought occurs for long time periods and wheat is monocropped to fulfill dietary requirements (Poole et al. 2013; Wildermuth et al. 1997). *Fusarium* root and crown rot is usually managed by different approaches such as chemical control, cultural practices, and biological control (Volova et al. 2018). Biological control is advantageous because of its eco-friendly nature.

Plant growth-promoting rhizobacteria (PGPR) have been successfully utilized in controlling wheat root and crown rot (Barnett et al. 2019; Harish et al. 2019; Winter et al. 2019). They are a heterogeneous group of bacteria living in plant roots and contribute to agricultural productivity through multifarious activities such as biofertilization, bioremediation, phytostimulation, and pathogen suppression (Hassan et al. 2010; Rais et al. 2018; Zia et al. 2019). Thus, PGPR are key factors for maintaining soil health and increasing crop yield. The survival of beneficial rhizobacteria in the rhizosphere until crop maturity is essential to exert beneficial traits on certain crops (Coy et al. 2019). Gram-positive bacteria can

survive for a long period as a result of their spore-forming ability, whereas Gram-negative bacteria must produce multiple secondary metabolites to compete with inhabitants and maintain their population in the rhizosphere (González and Marketon 2003). Moreover, Gram negative bacteria also have the ability to utilize diverse root exudates such as amino acids, carboxylic acids, and sugars (Cordovez et al. 2015; Kloepper et al. 1980).

The accumulation of rhizobacteria during successive monocropping patterns leads to the development of agriculturally productive soil. One example is disease-suppressive soil, in which disease does not persist or persists at very low levels even in the presence of pathogens and favorable conditions for disease development (Cordovez et al. 2015; Penton et al. 2014; Shen et al. 2015). Soil suppressiveness can be categorized as natural suppressiveness and induced suppressiveness (Larkin 2015; Siegel-Hertz et al. 2018). Natural suppressiveness is independent of cropping patterns, whereas induced suppressiveness is dependent on cropping patterns. Monocropping of susceptible crop varieties leads to the development of induced suppressive soils (Liu et al. 2019; Schroth and Hancock 1982). Induced suppressive soils can further be categorized as general and specific suppressive soils. General suppression is the collective competitive activity of total soil microbiota, whereas specific suppression is the antagonistic activity of a specific group of microorganisms against the plant pathogens of a specific crop (Raaijmakers and Mazzola 2016; Schlatter et al. 2017). General suppressiveness is nontransferable, whereas specific suppressiveness is transferable. General suppressive soils generally contain members of genera *Bacillus*, *Burkholderia*, *Chthonomonas*, *Pseudomonas*, and *Serratia* (Shen et al. 2015; van Bruggen and Semenov 2000). Monocropping results in the development of specific suppressiveness, which is also termed as disease decline in the plant rhizosphere by recruiting specific groups of microbes through plant root exudates (Andrade et al. 2011; Cha et al. 2016; Dasgupta et al. 2015; Raza et al. 2015; Zhou et al. 2015). Disease-suppressive soils emerge as a major research domain owing to the establishment of organic and sustainable agriculture, minimization of the use of chemical fertilizers, and

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Funding: This work was supported by the Higher Education Commission, Pakistan (research grant 20-2991).

*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure and one supplementary table are published online.

The author(s) declare no conflict of interest.

control of plant pathogens with no or little farming efforts (Cha et al. 2016). Studies revealed that monocropping of vanilla in China resulted in a low incidence of Fusarium wilt (Kloepper et al. 1980; Shen et al. 2015; Xiong et al. 2017; Yin et al. 2013). In addition, wheat monocropping led to induced take-all decline against *Gaeumannomyces graminis* var. *tritici* (Chng et al. 2015) and almost eliminated Rhizoctonia root rot disease in Australia after successive monocropping for 7 to 9 years (Yin et al. 2013). These facts necessitate that researchers explore the microbes associated with monocultured crops for their beneficial use. This study aimed to isolate rhizobacteria from the rhizosphere/endosphere of the wheat monocropping system in an arid/semiarid agriculture zone and to harness the effective indigenous strains for the biocontrol of wheat root and crown rot caused by *Fusarium* spp.

MATERIALS AND METHODS

Field identification and sampling. A wheat field under monocropping system practiced for 5 successive years was identified in Tammah, Islamabad, Pakistan. Wheat plants were sampled at the harvesting stage. Plants with variable height, leaves (color and area), and panicles (size and number of grains) were randomly selected from the different corners of the field. The plants were gently uprooted, enclosed in paper bags, and transported to the Applied Microbiology and Biotechnology Laboratory at COMSATS University Islamabad. The plants were placed at 4°C for further processing.

Bacterial isolation. Bacteria were isolated from the rhizosphere and endosphere of representative wheat plants using the serial dilution method (Hassan et al. 2010; Rais et al. 2016; Shakeel et al. 2015). The composite rhizospheric samples were made by gently removing the soil that adhered to different plant roots and mixing them homogenously, whereas composite endospheric samples were made by pooling each part (e.g., roots, stems, leaves, and panicles) of different plants separately. Each endospheric sample was surface sterilized by dipping in 0.1% mercuric chloride (HgCl₂) for 1 to 2 min and washing twice with sterile distilled water. The parts were dried in layers of sterilized filter paper and crushed in a sterilized mortar and pestle. One gram of rhizospheric soil or fully crushed parts was used to make serial dilutions (10⁻¹ to 10⁻⁹) and each dilution (100 µl) was spread onto Luria Bertani (LB) agar. All procedures were performed in a laminar flow unit to avoid contamination. Moreover, the solutions and sterile materials used in the experiment were also spread on the LB agar as a sterility control. The inoculated LB agar plates were placed at 37°C overnight (the temperature prevailing in the field at the time of crop maturity). A single bacterial colony was picked from the LB agar and preserved in 20% glycerol at -20°C.

Differentiation of Gram-negative bacteria and their plant growth-promoting traits. Gram-negative bacteria were screened by Gram staining and grown on Gould S1 medium (Gould et al. 1985). Gram-negative bacteria were grown on LB agar and observed for their colony morphology, including size, shape, margins, elevations, texture, consistency, and opacity. Plant growth-promoting (PGP) traits of Gram-negative bacteria, such as nutrient solubilization (phosphate, potassium, and zinc), fungal antagonism (*F. moniliforme*, *F. oxysporum*, *Macrophomina phaseolina*, and *Pyricularia oryzae*), and production of antifungal compounds (cellulases, proteases, and siderophores), were detected in vitro.

Nutrient solubilization assay. The ability of the Gram-negative bacteria to solubilize phosphorous, potassium, and zinc was detected on modified Pikovskaya agar (Nautiyal 1999), Aleksandrova agar (Zhang and Kong 2014), and Bunt and Rovira agar (Bunt and Rovira 1955), respectively. The tested strains were grown overnight on LB broth and inoculated by placing a drop of 2 µL on the respective agar on the plate. The inoculated agar plates were incubated at 37°C for 4 to 7 days. The appearance of a halo zone around a bacterial colony on the respective agar indicated their

ability of that bacteria to solubilize the respective nutrient. The nutrient solubilizing index was measured in millimeters and computed using the following formula (Rashid et al. 2004):

$$\text{Solubilizing index} = \frac{\text{Halo zone diameter} + \text{Colony diameter}}{\text{Colony diameter}}$$

The experiment was repeated thrice.

Fungal antagonism assay. Phytopathogenic fungi *F. oxysporum*, *P. oryzae*, *F. moniliforme*, and *M. phaseolina* were obtained from the Applied Microbiology and Biotechnology Laboratory at COMSATS University. The antagonistic activity of the bacteria was assessed by the dual culture assay (Hassan et al. 2010; Rais et al. 2016). Briefly, a 5-mm mycelium plug of each actively growing fungus was placed at the center of a potato dextrose agar plate and bacterial strains cultured in LB broth overnight were spotted at equal distances from the fungus. Sterile LB without bacteria was used as the control. The plates were placed at 25 to 28°C for 7 to 15 days and observed for the inhibition of fungal growth. The percentage of inhibition was calculated as follows (Shakeel et al. 2015): Inhibition (%) = [(C - T) × 100]/C, where C is the growth of the fungal mycelium (in centimeters) toward the control and T is the growth of the fungal mycelium (in centimeters) toward the bacterial colony. The experiment was repeated thrice.

Antifungal traits. The ability of Gram-negative bacteria to produce cellulases, proteases, and siderophores was detected on carboxymethyl cellulase agar, skim milk agar (Dinesh et al. 2015), and chrome azurol S agar (Schwyn and Neilands 1987), respectively. A spot of 2 µl of freshly grown culture was made on the agar media and placed in an incubator at 37°C for 72 h. Halo zone formation transparent for protease and cellulase, which appeared after staining/destaining (Dinesh et al. 2015) while orange for siderophores, depicted the secretion of respective enzymes and siderophores. Production of each antifungal compound was expressed in terms of the substrate solubilization/discoloration index, which was computed as described in the previous section on nutrient solubilization. The experiment was performed in triplicate and repeated thrice.

Scoring of bacteria based on PGP traits. Bacteria were scored on the basis of their PGP characteristics. A criterion was developed for scoring on their individual PGPR trait (Supplementary Table S1). The strains with a maximum PGPR score were evaluated in planta.

In planta experiments. The effects of potential PGPR HU-8 and HU-9 were assessed on two wheat varieties, Pirsabak-2015 and Galaxy-2013, against the challenge inoculation of *F. oxysporum* and *F. moniliforme* in petri plate experiments and pot experiments. Four independent experiments (two petri plate and two pot experiments) were laid out as a complete randomized design. In each experiment, there were four treatments with three replications per treatment ($n = 3$) for each wheat variety as follows: T1 = pathogen (*F. moniliforme* or *F. oxysporum*), T2 = fungicide (thiophanate methyl), T3 = HU-8 plus pathogen, and T4 = HU-9 plus pathogen. The sterilized seeds of both varieties were dipped in a bacterial suspension consisting of 5×10^9 CFU/ml for 2 to 3 h and sown in petri plates filled with Murashige and Skoog (MS) agar as well as in pots filled with sterilized soil (structure, clay loam; total nitrogen, 0.03 to 0.05%; phosphorous, 12 to 14 mg/kg; pH, 7.7 to 7.9; and organic matter, 0.6 to 0.8%). The fungicide was also applied as seed dressing by following the dose recommended by the manufacturer. The nitrogen, phosphorous, and potassium fertilizer was applied at the rate of 120, 100, and 60 kg/ha, respectively, in the form of urea, single super phosphate, and sulfate of potash (Ramzani et al. 2016). The petri plate experiments were repeated thrice but the pot experiments were repeated twice.

Fungal inoculation. In the petri plate experiments, a 5-mm mycelium plug of freshly grown fungi (*F. oxysporum* or *F. moniliforme*)

was placed at the center of each plate and incubated at $25 \pm 3^\circ\text{C}$. In the pot experiments, pathogens (*F. moniliforme* or *F. oxysporum*) were inoculated by the soil drench method. Five milliliters of spore suspension consisting of approximately 10^5 spores/ml was drenched in soil near the roots of emerging seedlings (2 weeks old) as described in earlier studies (Jaber 2018; Motallebi et al. 2015). The inoculated pots were placed in a net house under natural conditions.

Antioxidant enzyme activity. Antioxidant enzyme activity was determined at 24 h after fungal inoculation. Ten plants were harvested and a composite sample was made by grinding the leaves and stalk of the plants. One gram of composite sample was processed to compute the activity of polyphenol oxidase (PPO), peroxidase (PO), and phenylalanine ammonia lyase (PAL) enzymes. PPO, PO, and PAL enzymes were extracted by mixing the wheat tissues (1 g) in different buffers, such as sodium phosphate buffer (pH 6.5 for PPO), potassium phosphate buffer (pH 6.0 for PO), and borate buffer (pH 8.8 for PAL). Enzyme activity was measured spectrophotometrically (Hassan et al. 2015). Enzyme activity was expressed as changes in the absorbance in minutes per gram of fresh weight of tissue.

Disease severity index and growth parameters. Disease was assessed visually at 14 to 15 days after fungal inoculation when the symptoms were clearly visible in control plants. Ten to twelve plants per treatment were randomly selected and disease was scored using the following 4-point scale, which is reported in numerous studies (Jaber 2018; Motallebi et al. 2015): 0, symptomless; 1, mild browning on roots/stem; 2, moderate browning (25 to 50%); 3, severe browning (>50%); and 4, severe browning with completely rotten or dead plant tissues. The disease severity index (DI) was calculated as described by Lounaci et al. (2016): $DI = [(0 \times F_0) + (1 \times F_1) + (2 \times F_2) + (3 \times F_3)]/N$, where F is the number of plants for each disease score and N is the total number of plants.

Plant growth parameters (e.g., root/shoot length) were measured with a scale, whereas fresh weight and dried weight were recorded on an electronic balance scale (Shimadzu).

Root colonization. The ability of inoculated bacterial strains to colonize wheat root rhizosphere grown in pots was determined at the time of disease assessment as described in our previous studies (Hassan et al. 2015; Rais et al. 2016). The composite samples of rhizosphere and endosphere were made by pooling the different plant parts per replication. The inoculated rhizobacteria were isolated by making the serial dilutions as described in the previous section on isolation of bacteria. The isolated colonies were counted and identified on the basis of their respective characteristics (i.e., nutrient solubilization and antagonism).

Molecular identification of PGPR. PGPR are identified at the genus level by sequence analysis of the 16S *rRNA* gene. The 16S *rRNA* gene was amplified by using universal primers P1 and P6 (Tan et al. 1997). The PCR products were purified using a PCR purification kit (Thermo Scientific) and were sent to Macrogen Inc. in Korea for sequencing. The reverse sequence was complemented at JustBio (<http://justbio.com>) and aligned with the forward

sequence at multiple sequence alignment (ClustalW). Both sequences were joined after removing the overlapping region and subjected to BLAST analysis using NCBI/EzTaxon (Altschul et al. 1990; Mekadim et al. 2018). The strains were identified on the basis of their closest type strains (Kim et al. 2012). The strains exhibiting maximum PGP traits and/or belonging to *Pseudomonas* spp. were further identified at the species level by sequencing the housekeeping gene (*rpoD*). The primers were designed by aligning the *rpoD* sequences of different *Pseudomonas* spp. About 20 to 25 consensus nucleotides were selected as forward (*rpoD* F: 5'-ATGTCCGG AAAAGCGCAACAGCA-3') and reverse (*rpoD* R: 5'-TCACTCGTC GAGGAAGGAGC-3') primers for the amplification of *rpoD*. PCR mixtures with a final volume of 50 μl consisted of 32.5 μl of sterile PCR water (Carl Roth), 5 μl of Taq buffer (10 $\times$), 4 μl of MgCl_2 (25 mM), 3 μl of dNTPs (10 mM), 2 μl of each primer (100 pM), 0.5 μl of DNA Taq polymerase (500 U; Thermo Fisher Scientific), and 3 μl of template DNA (30 ng/ μl). Amplifying conditions consisted of initial denaturation at 95°C for 5 min, annealing temperature at 65°C for 45 s, and final extension at 72°C for 11 min and 30 s for 25 cycles. The amplified genes were purified, sequenced, and analyzed as described above. The strains were identified by BLAST analysis of the gene sequences (Altschul et al. 1990; Mekadim et al. 2018).

RESULTS

Wheat rhizospheric and endospheric bacteria. Twenty-nine bacterial isolates were obtained from the rhizosphere and endosphere of wheat. Based on the isolate source, 16 bacteria were isolated from the rhizosphere and 13 from the endosphere. Among these isolates, 12 were Gram negative and 17 were Gram positive. Most of the bacterial isolates ($n = 16$) were found in rhizosphere, followed by the spike endosphere ($n = 8$), root endosphere ($n = 2$), and leaf endosphere ($n = 2$). Only a single isolate was found in the stem endosphere ($n = 1$). Eight Gram-negative bacteria were found in the rhizosphere, whereas there were four in the spike endosphere.

Multifarious traits of rhizobacteria. Out of 12 Gram-negative isolates, only seven exhibited one or more PGP traits.

Nutrient solubilization. Six of seven strains showed phosphate solubilizing ability. Maximum phosphate solubilization was shown by HU-5 (solubilization zone = 3.31 mm), followed by HU-8 (3.18 mm); HU-6 showed minimum solubilization (2.33 mm). Intermediate phosphate solubilization was shown by HU-1 (solubilization zone = 2.48 mm), HU-4 (2.51 mm), and HU-7 (2.6 mm) (Table 1). HU-9 did not solubilize the phosphate. Five of seven strains solubilized the zinc. Maximum zinc solubilization was performed by HU-4 (solubilization zone = 4.06 mm); the rest of the solubilizers showed an almost similar solubilization (HU-6, 2.68 mm; HU-5, 2.62 mm; HU-9, 2.47 mm; and HU-7, 2.42 mm) (Table 1). All of the isolates were unable to solubilize potassium.

Fungal antagonism. The isolated bacteria showed antagonistic effects against four phytopathogenic fungi: *F. moniliforme*, *F. oxysporum*, *P. oryzae*, and *M. phaseolina*. Four of seven bacterial

TABLE 1. Plant growth-promoting traits of wheat associated bacteria^y

Isolate code	Source	Phenotype ^z	Nutrient solubilization		Antifungal compound			Fungal antagonism	
			Phosphate	Zinc	Protease	Cellulase	Siderophore	<i>Fusarium oxysporum</i>	<i>F. moniliforme</i>
HU-1	Rhizosphere	Circular, moist, colorless	2.48 b	0.00 d	0.00 c	4.73 a	2.85 b	0.00 d	00.00 c
HU-4	Rhizosphere	Filamentous, filiform, colorless	2.51 b	4.05 a	2.41 a	3.74 b	0.00 c	28.33 c	0.00 c
HU-5	Rhizosphere	Circular, moist, yellow	3.31 a	2.62 b	0.00 c	0.00 e	0.00 c	0.00 d	0.00 c
HU-6	Rhizosphere	Circular, moist, colorless	2.33 b	2.68 b	0.00 c	3.10 c	0.00 c	0.00 d	0.00 c
HU-7	Rhizosphere	Irregular, dry, colorless	2.6 b	2.42 c	2.2 b	0.00 e	0.00 c	28.33 c	37.5 b
HU-8	Rhizosphere	Circular, moist, green	3.18 a	0.00 d	2.2 b	0.00 e	3.33 a	45.5 a	40.43 a
HU-9	Endosphere	Irregular, moist, orange	0.00 c	2.47 c	2.35 a	2.25 d	0.00 c	40.33 b	40.83 a

^y Values are the mean of three replicates and those bearing different letters in the same column are significantly different at $P \leq 0.05$.

^z Colony morphology was observed on Luria Bertani agar.

isolates inhibited the growth of *F. oxysporum*. Maximum inhibition was shown by HU-8 (45.45%), followed by HU-9 (40.39%); minimum inhibition was shown almost equally by HU-4 (28.27%) and HU-7 (27.8%), as shown in Table 1. Only three strains inhibited *F. moniliforme*. Maximum inhibition (40.83%) was shown by HU-8 and HU-9 followed by HU-7 (37.5%). Strain HU-8 also antagonized *P. oryzae* (43.2%) and *M. phaseolina* (25%), whereas strain HU-9 only antagonized *P. oryzae* (45.5%).

Production of antifungal traits. The bacterial isolates exhibited the major antifungal traits such as cellulases, proteases, and siderophores. Maximum cellulase was secreted by HU-1 (solubilization zone = 4.7 mm) followed by HU-5 (3.74 mm). Intermediate secretion was shown by HU-6 (3.1 mm), whereas minimum secretion was observed for HU-9 (2.25 mm) (Table 1). Three strains (e.g., HU-4, HU-7, and HU-8) did not secrete cellulase. Production of siderophores was shown by only two strains: HU-8 (discoloration zone = 3.32 mm) and HU-1 (2.85 mm). Protease production was observed for four strains, with the maximum by HU-4 (solubilization zone = 2.41 mm) followed by that of HU-9 (2.35 mm), HU-7, and HU-8 (2.2 mm each) (Table 1).

Selection of potential PGPR. On a scoring scale of 9 based on PGP activities, isolates HU-8 and HU-9 showed maximum scores of 6 to 7, whereas HU-1, HU-5, and HU-6 showed a minimum score of 3 (Supplementary Fig. S1).

Growth promotion and biocontrol activity on wheat. Isolates HU-8 and HU-9 significantly promoted the growth of wheat and reduced disease on both varieties (Pirsbak-2015 and Galaxy-2013). Strains HU-8 and HU-9 showed an increase in all plant parameters of wheat infected with *F. moniliforme* at par to that of fungicide. They increased the fresh weight by 75 to 77% and dry weight by 37 to 51% over the control in both experiments (Table 2). *F. oxysporum*-infected wheat plants inoculated with HU-8 and HU-9 showed a significant increase in root/leaf length and fresh/dry weight. The isolates showed enhancement in root length by 43 to 49% and 67 to 77%, leaf length by 33 to 41% and 22 to 39%, and fresh weight by 65 to 67% and 46 to

84% in the plate and pot experiments, respectively, compared with the control infected wheat plants. This effect was statistically similar to that of fungicide (Table 3).

Induction of antioxidant enzymes. Isolates HU-8 and HU-9 significantly induced antioxidant enzyme activity in wheat varieties Pirsbak-2015 and Galaxy-2013 challenged with phytopathogens *F. oxysporum* and *F. moniliforme* in the petri plate and pot experiments (Figs. 1 and 2).

PO activity. PGP strains significantly enhanced PO activity in both experiments. Higher PO activity in the pot experiments was observed for HU-8 (1.2-fold) against *F. oxysporum* in wheat variety Pirsbak-2015 followed by HU-9 (1-fold) against *F. moniliforme* in Pirsbak-2015, whereas the lowest PO activity was observed for HU-9 (0.2-fold) against *F. oxysporum* in variety Galaxy-2013. A similar trend was observed in the plate assay. However, the induction of PO enzyme was slightly higher in the pot experiments (0.2- to 1.2-fold) compared with the plate assay (0.3- to 0.9-fold). PO activity in fungicide-treated plants was slightly lower (0.17 to 0.7) than that of bacteria-treated plants (Figs. 1 and 2).

PPO activity. The PGP strains induced PPO activity in all experimental conditions. In the pot experiments, maximum PPO activity was observed for HU-9 (2.1-fold) against *F. moniliforme* in variety Galaxy-2013 followed by HU-8 and HU-9 (1.5-fold each) against *F. oxysporum* in Galaxy-2013 and *F. moniliforme* in variety Pirsbak-2015, respectively, and HU-8 (1.1-fold) against *F. oxysporum* in Galaxy-2013. Minimum PPO activity was observed for HU-9 (0.5-fold) against *F. moniliforme* in Pirsbak-2015 (Figs. 1 and 2). However, the induction of PPO enzyme was slightly higher in the pot experiments (0.5- to 2.1-fold) compared with the plate assay (0.6- to 1.9-fold). The PPO activity in fungicide-treated plants was either at par to that of the bacterial strains or slightly lower, as shown in Figures 1 and 2.

PAL activity. The PGP strains significantly induced PAL activity upon fungal challenge. In the pot experiments, maximum PAL activity was shown by HU-8 (1.8-fold) against *F. oxysporum* in

TABLE 2. Effect of rhizobacteria on the growth of wheat challenged with *Fusarium moniliforme*^z

Treatment	Root length (cm)		Shoot length (cm)		Leaf length (cm)		Fresh weight (g)		Dry weight (g)	
	Plate exp.	Pot exp.	Plate exp.	Pot exp.	Plate exp.	Pot exp.	Plate exp.	Pot exp.	Plate exp.	Pot exp.
Galaxy-2013										
T1	7.67 c	17.44 c	4.83 b	5.78 b	9.67 b	19.80 a	0.12 b	1.16 b	0.01 b	0.41 c
T2	12.05 a	31.70 a	5.94 a	10.43 a	15.22 a	22.9 a	0.17 ab	2.10 a	0.02 a	0.96 ab
T3	10.00 b	29.63 b	5.33 ab	10.17 a	15.17 a	23.32 a	0.15 b	2.16 a	0.02 b	0.84 b
T4	11.83 ab	31.77 a	5.67 ab	10.37 a	16.83 a	21.48 a	0.24 a	2.19 a	0.02 a	0.99 a
Pirsbak-2015										
T1	11.67 c	19.96 c	5.33 ab	6.43 b	10.67 b	16.74 b	0.11 b	0.81 b	0.01 b	0.55 b
T2	15.23 ab	29.56 ab	5.22 ab	8.77 a	13.01 ab	19.41 ab	0.15 ab	1.31 a	0.02 a	0.62 ab
T3	14.00 bc	32.37 a	5.67 a	8.59 a	14.16 a	20.76 a	0.16 a	1.67 a	0.02 a	0.68 a
T4	16.83 a	28.56 b	4.83 b	8.98 a	13.67 a	19.20 ab	0.17 a	1.41 a	0.02 a	0.62 ab

^z Values are the mean of three replicates and those bearing different letters in the same column are significantly different at $P \leq 0.05$. exp = experiment, T1 = pathogenic fungi (control), T2 = fungicide plus pathogenic fungi, T3 = HU-8 plus pathogenic fungi, and T4 = HU-9 plus pathogenic fungi.

TABLE 3. Effect of rhizobacteria on the growth of wheat challenged with *Fusarium oxysporum*^z

Treatment	Root length (cm)		Shoot length (cm)		Leaf length (cm)		Fresh weight (g)		Dry weight (g)	
	Plate exp.	Pot exp.	Plate exp.	Pot exp.	Plate exp.	Pot exp.	Plate exp.	Pot exp.	Plate exp.	Pot exp.
Galaxy-2013										
T1	6.67 b	14.32 b	5.73 a	5.80 c	11.00 b	16.89 c	0.08 b	1.25 b	0.014 b	0.43 c
T2	10.99 a	34.80 a	6.31 a	9.45 a	14.80 a	21.16 ab	0.13 a	1.46 ab	0.016 ab	0.63 ab
T3	10.67 a	32.59 a	5.17 a	8.167 b	15.17 a	20.67 b	0.14 a	1.58 a	0.015 ab	0.67 a
T4	11.00 a	27.83 a	5.83 a	8.89 ab	16.00 a	23.56 a	0.16 a	1.55 a	0.018 a	0.60 b
Pirsbak-2015										
T1	11.33 b	19.00 c	5.17 ab	5.89 b	11.17 b	17.28 a	0.12 b	0.67 b	0.007 b	0.47 b
T2	14.84 ab	29.85 a	5.46 ab	8.04 a	13.14 ab	18.83 a	0.16 ab	1.01 b	0.016 a	0.57 ab
T3	15.00 ab	29.33 ab	4.83 b	7.15 a	14.33 a	19.72 a	0.19 a	1.53 a	0.018 a	0.67 a
T4	15.67 a	26.18 b	5.83 a	6.18 b	15.17 a	19.50 a	0.18 a	0.84 b	0.018 a	0.567 ab

^z Values are the mean of three replicates and those bearing different letters in the same column (same variety) are significantly different at $P \leq 0.05$. exp = experiment, T1 = pathogenic fungi (control), T2 = fungicide plus pathogenic fungi, T3 = HU-8 plus pathogenic fungi, and T4 = HU-9 plus pathogenic fungi.

Galaxy-2013 followed by HU-9 (1.5-fold) against *F. moniliforme* in Galaxy-2013, whereas the lowest activity was shown by HU-8 (0.5-fold) against *F. oxysporum* in Pirsbak-2015.

A different trend was observed in the plate assay. Maximum PAL activity was observed for HU-9 (1.8-fold) against *F. moniliforme* in Galaxy-2013 followed by HU-9 (1.3-fold) against *F. oxysporum* in Galaxy-2013, whereas the lowest activity was observed for HU-8 (0.2-fold) against *F. oxysporum* in Pirsbak-2015. The fungicide also induced PAL activity compared with that of the control (Figs. 1 and 2).

DI and root colonization. The tested strains showed significantly less disease severity against *Fusarium* spp. compared with the control on both wheat varieties. Their efficacy was either statistically at par or even better than that of fungicide (Fig. 3). The minimum disease score was observed for HU-9 (1.7 in Galaxy-2013 and 2.3 in Pirsbak-2015) followed by HU-8 (2.3 in Galaxy-2013 and 2.7 in Pirsbak-2015), as shown in Figure 3. Strains HU-8 and HU-9 significantly colonized the wheat plants with a cell population of 6.6 to 8.5 log CFU/g of rhizospheric soil under the challenge inoculation of both fungi (Fig. 4). HU-9 was also an endophytic colonizer with a cell population of 4.5 log CFU/g of roots, whereas HU-8 was unable to colonize the roots endophytically.

Principal component analysis. The data were subjected to a principal component analysis (Figs. 5 and 6). The biplot clearly

demonstrated the correlation between different growth parameters, disease severity, and antioxidant enzyme activity of wheat with the inoculated strains.

Molecular identification of PGPR. *16S rRNA gene analysis.* Gram-negative bacteria associated with wheat and exhibiting PGPR traits were found to be members of different genera. For example, strains HU-1 (MF347446), HU-8 (MF347453), and HU-9 (MF347454) were identified as *Pseudomonas* spp.; strains HU-5 (MF347450), HU-6 (MF347451), and HU-7 (MF347452) were identified as *Ochrobactrum* spp.; and HU-4 (MF347449) was identified as *Acinetobacter* spp.

rpoD gene sequence analysis. The *Pseudomonas* species HU-1, HU-8, and HU-9 were further identified at the species level by sequencing the housekeeping gene, *rpoD*. Strain HU-1 was identified as *P. putida*, whereas HU-8 and HU-9 were identified as *P. mediterranea*. The nucleotide sequences of all three strains were submitted to Genbank under accession numbers MF580376 to MF580378.

DISCUSSION

PGPR play a vital role in improving plant health either through aiding in the acquisition of essential soil nutrients or suppressing pathogens (Kim and Anderson 2018). In this study, rhizobacteria isolated from the wheat rhizosphere grown in a monocropping

Petri-plate Experiment

Fig. 1. Rhizobacteria-mediated induction of antioxidant enzymes in wheat under the challenge inoculation of *Fusarium* spp. (petri plate experiment). Δ Abs = change in absorbance, FW = fresh weight, T1 = pathogenic fungi (control), T2 = fungicide plus pathogenic fungi, T3 = HU-8 plus pathogenic fungi, and T4 = HU-9 plus pathogenic fungi. The Pirsbak-2015 and Galaxy-2013 varieties are shown in the graphs on the left and right, respectively. Values are the means of three replicates and those bearing different letters within the same enzyme (peroxidase [PO], polyphenol oxidase [PPO], and phenylalanine ammonia lyase [PAL]) are significantly different. Bars represent the standard error.

system of an arid/semiarid agriculture zone exhibited multifarious traits and significantly controlled the root and/or crown rot of wheat. The population and diversity of rhizobacteria in certain crop rhizospheres/endospheres is highly dependent on multiple factors such as cultivar, cropping pattern, agriculture practices, and climate (Matthews et al. 2019; Na et al. 2019; Schlemper et al. 2018). In our findings, the culturable bacteria associated with wheat, cultivated in a monocropping system of arid zone agriculture, revealed a higher population in the root rhizosphere than that of the endosphere. These findings are supported by earlier reports showing that the plant rhizosphere/endosphere is the hotspot for microbial community (Truyens et al. 2015). The difference in population of endophytic and rhizospheric bacteria may be attributable to the potential of selective bacteria to effectively penetrate and adopt the host plant's tissues (Etesami et al. 2014; Mendes et al. 2007). The endophytic beneficial bacteria modulate the development and growth of the plants by eliciting different physiological changes (Van der Ent and Pieterse 2012). Low numbers of bacteria recovered from the leaf and spike endosphere may be attributable to many factors such as the challenges of habitat specificity and the limitations associated with culture-dependent isolation techniques (Truyens et al. 2015). The endophytic bacterial population densities are in concordance with earlier studies (Etesami et al. 2014; Mendes et al. 2007).

The high prevalence of Gram-negative PGPR in this study may be attributable to multiple reasons, such as (i) the harvesting stage of the wheat, where the only bacteria with predominant secondary metabolism can survive by tolerating the adverse environmental conditions, and (ii) the monocropping system, where wheat grown for successive years recruits the specific bacteria through exudation of specific compounds via roots (Cha et al. 2016). Root exudates favor the growth of selective microorganisms, resulting in their higher cell density in the rhizosphere (Cha et al. 2016; van der Voort et al. 2016). The PGPR prevailing in the wheat rhizosphere were less diverse. This is consistent with earlier reports showing that monocropping leads to changes in microbial communities (Zhao et al. 2018). Because the root exudate composition depends on the plant species, cultivar, and many environmental factors such as nutrient availability, temperature, pH, and humidity, the bacterial diversity also varies from plant to plant (Bainard et al. 2013; Zhou et al. 2015).

The bacteria exhibited multiple PGP traits. The majority of the isolates showed phosphate solubilization but none showed potassium solubilization, although zinc solubilization was shown by few bacterial isolates. It has been well described that PGPR solubilize nutrients by releasing organic acids such as citric acid, oxalic acid, lactic acid, and gluconic acid (Han and Lee 2005; Khan et al. 2007). These organic acids result in the lowering of soil pH,

Pot Experiment

Fig. 2. Rhizobacteria-mediated induction of antioxidant enzymes in wheat under the challenge inoculation of *Fusarium* spp. (pot experiment). ΔAbs = change in absorbance, FW = fresh weight, T1 = pathogenic fungi (control), T2 = fungicide plus pathogenic fungi, T3 = HU-8 plus pathogenic fungi, and T4 = HU-9 plus pathogenic fungi. The Pirsbak-2015 and Galaxy-2013 varieties are shown in the graphs on the left and right, respectively. Values are the mean of three replicates and those bearing same letter within same enzyme (peroxidase [PO], polyphenol oxidase [PPO], and phenylalanine ammonia lyase [PAL]) are significantly different. Bars represent standard error.

which favors the solubilization of phosphorous and zinc, whereas the maximum potassium solubilization occurs at neutral pH (de Werra et al. 2009; Lukkani and Reddy 2015; Parmar and Sindhu 2013). The possible reason for the inability of bacterial isolates to solubilize potassium is either the lowering of pH through secretion of organic acids or the physiological differences among isolates. However, this needs to be explored in further studies. The antagonistic activity of PGPR against four pathogenic fungi (*F. oxysporum*, *F. moniliforme*, *P. oryzae*, and *M. phaseolina*) was associated with the production of certain metabolites and/or compounds. The bacterial strains producing moderate levels of hydrolytic enzymes (e.g., cellulase, protease) and the maximum level of siderophores inhibited all of the tested phytopathogens, whereas the strains that produced the hydrolytic enzymes but not siderophores could not inhibit all pathogens. This result shows that the competition for the iron resource is a major antifungal mechanism, as reported earlier (Lambrese et al. 2018; Sharma and Johri 2003). The strains that produced only protease enzyme were able to moderately inhibit only two fungi (*F. oxysporum* and *F. moniliforme*) but strains that produced only cellulase were unable to inhibit any of the fungi. This revealed the limitation of hydrolytic enzyme in biocontrol activity owing to their substrate specificity

present in the targeted pathogen (Marin-Bruzos and Grayston 2019). These findings also predict that the antifungal compounds work synergistically to eliminate the fungal pathogens. However, their production and regulation is an interesting era of research under the “metabolic coregulation” domain. The bacterial strains exhibiting the best PGPR traits in vitro also significantly colonized the wheat rhizosphere and suppressed root and crown rot disease. These findings are inconsistent with earlier studies in which rhizobacteria successfully suppressed wheat root rot (Barnett et al. 2019; Shikur Gebremariam et al. 2018).

As depicted by the rhizobacterial effect on disease development, rhizobacteria could be an ideal substituent to the fungicides that pose severe environmental threats upon their irrational use (Upadhyay et al. 2018). This claim is supported by earlier reports in which PGPR effectively controlled economically important diseases like red rot caused by *C. falcatum* and blast disease caused by *P. oryzae* (Hassan et al. 2015; Rais et al. 2016). The root-colonizing ability of bacteria is strongly related to the production of their secondary metabolites by which they tolerate environmental stress (Bajpai and Johri 2019; Mehmood et al. 2019; Ramawat and Goyal 2019; Thomashow et al. 2019). Optimum population of bacterial cells (10^6 to 10^7 CFU/g of soil) is necessary for the beneficial plant–microbe interaction (Hassan et al.

Fig. 3. Disease suppression by the antagonistic bacteria. T1 = pathogenic fungi (control), T2 = fungicide plus pathogenic fungi, T3 = HU-8 plus pathogenic fungi, and T4 = HU-9 plus pathogenic fungi. Values are the mean of three replicates and those bearing different letters in the same column (same variety) are significantly different from each other at $P \leq 0.05$.

Fig. 4. Colonization of bacteria to wheat roots challenged with *Fusarium* spp. T3 = HU-8 plus pathogenic fungi and T4 = HU-9 plus pathogenic fungi. Values are the mean of three replicates and those bearing different letters in the same column (same variety) are significantly different from each other at $P \leq 0.05$.

F. moniliforme *F. moniliforme* *F. oxysporum* *F. oxysporum*

Galaxy- 2013

Pirsbak- 2015

Fig. 5. Principal component (PC) analysis between the rhizobacteria and their in planta plant growth-promoting traits (petri plate experiment). T1 = pathogenic fungi (control), T2 = fungicide plus pathogenic fungi, T3 = HU-8 plus pathogenic fungi, and T4 = HU-9 plus pathogenic fungi. The Galaxy-2013 and Pirsbak-2015 varieties are shown on the left and right, respectively. DW = dry weight, SL = shoot length, RL = root length, LL = leaf length, FW = fresh weight, PO = peroxidase, PPO = polyphenol oxidase, and PAL = phenylalanine ammonia lyase.

F. moniliforme *F. moniliforme* *F. oxysporum* *F. oxysporum*

Galaxy- 2013

Pirsbak- 2015

Fig. 6. Principal component (PC) analysis between the rhizobacteria and their in planta plant growth-promoting traits (pot experiment). T1 = pathogenic fungi (control), T2 = fungicide plus pathogenic fungi, T3 = HU-8 plus pathogenic fungi, and T4 = HU-9 plus pathogenic fungi. The Galaxy-2013 and Pirsbak-2015 varieties are shown on the left and right, respectively. LL = leaf length, SL = shoot length, RL = root length, DW = dry weight, FW = fresh weight, PO = peroxidase, PAL = phenylalanine ammonia lyase, and PPO = polyphenol oxidase.

2010; Rais et al. 2016; Zhang et al. 2019). Rhizobacteria significantly enhanced wheat plant immunity against *Fusarium* infection by activating the antioxidative defense system. Biocontrol activity of rhizobacteria against root and crown rot of wheat is well documented across the world (Jaber 2018; Lounaci et al. 2016; Winter et al. 2019). The indigenous antagonistic bacteria inhabiting the arid/semiarid agriculture zone are ideal candidates for the development of biopesticide against root/crown rot because of their adaptability with the host and environment. Their arid/semiarid habitat further strengthens their utilization based on drought-mediated aggravation of root/crown rot disease (Smiley 2019; Wakelin et al. 2018). Induction of antioxidant enzyme activity in wheat against *Fusarium* spp. depicts the adoption of the induced systemic resistance by the rhizobacteria investigated in this study. The role of these antioxidant enzymes, such as PAL, PPO, and PO, in minimizing the oxidative stress caused by phytopathogens is well reported in numerous studies (Hassan et al. 2015; Rais et al. 2017). We also observed increased activity of antioxidant enzymes in fungicide treatment, which is in accordance with earlier studies. This increased activity may be attributable to the fact that fungicides induce the synthesis of phytoalexins, cell wall lignification, and enzymes involved in the phenolic synthesis (Pantazopoulou and Dailianas 2007; Saladin et al. 2003).

The 16S *rRNA* gene sequence analysis revealed that the PGPR associated with the monocultured wheat belong to *Pseudomonas* spp., *Ochrobactrum* spp., and *Acinetobacter* spp. The *Ochrobactrum* spp. depicted high potential to solubilize nutrients, whereas *Pseudomonas* spp. were found to be potential biocontrol agents. *Pseudomonas* spp. have been extensively studied for their PGPR activity (Sivasakthi et al. 2014) and are considered as a potential ingredient of biofertilizer and biopesticide. The 16S *rRNA* gene is considered as a valuable tool to identify bacteria at the genus level and has limitations in distinguishing between the closely related species (Lv et al. 2016). However, *rpoD* is the best housekeeping gene to identify *Pseudomonas* at the species level (Martens et al. 2008; Mulet et al. 2009, 2010; Savli et al. 2003). *P. mediterranea* is associated with various cereal crops and its role as a biocontrol agent despite an unusual plant pathogen is well documented. This functional shift from a plant pathogen to a fungal biocontrol agent depicts its diversity in different habitats.

Conclusion. The indigenous Gram-negative antagonistic bacteria inhabiting the arid/semiarid agriculture zone are ideal candidates for the development of biopesticide against root/crown rot because of their adaptability with the host and environment. Their arid/semiarid habitat further strengthens their utilization based on the drought-mediated aggravation of root/crown rot disease. *RpoD* is the best housekeeping gene for identification of *Pseudomonas* species. Moreover, a monocropping system of wheat under arid/semiarid agriculture conditions could be an ideal source of beneficial biocontrol agents.

ACKNOWLEDGMENTS

We thank the National Agriculture Research Centre of Islamabad for providing the wheat seeds.

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