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RESEARCH ARTICLE

Rhizoplane microbiota of superior wheat varieties possess enhanced plant growth-promoting abilities

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BACKGROUND: Microbes affect the growth of plants. In this study, the diversity and plant growth-supporting activities of wheat rhizospheric bacteria were examined.
METHODS: Sampling was performed thrice at different phases of plant growth. Microbes associated with the rhizoplane of three wheat varieties (Seher, Lasani, and Faisalabad) were cultured and assessed for their plant growth-promoting abilities based on auxin production, hydrogen cyanide production, phosphate solubilization, and nitrogen fixation.
RESULTS: Bacterial load (CFU/mL) declined, and the succession of bacterial diversity occurred as the plants aged. Most auxin-producing bacteria and the highest concentrations of auxin (77 μg/mL) were observed during the second sampling point at the tillering stage. The Seher variety harbored the most auxin-producing as well as phosphate-solubilizing bacteria. Most of the bacteria belonged to *Bacillus* and *Pseudomonas*. *Planomicrobium*, *Serratia*, *Rhizobium*, *Brevundimonas*, *Stenotrophomonas*, and *Exiguobacterium* sp. were also found.
CONCLUSIONS: These results suggest that the rhizoplane microbiota associated with higher-yield plant varieties have better plant growth-promoting abilities as compared to the microbiota associated with lower-yield plant varieties.

Keywords wheat, microbiota, rhizoplane, auxin, phosphate solubilization

Introduction

Wheat is a cereal grass of the genus *Triticum* (family Poaceae). It is one of the oldest and most important edible crops, grown by 80% of farmers on around 40% of the total cultivated area, and constitutes a large portion of human food (Lægreid et al., 1999; Tilman et al., 2002). Given its worldwide caloric importance, increasing wheat yield by different means has always been an objective. Associations between plants and microorganisms can prove useful in this respect; these associations are very complex and useful and thus are the subject of increasing studies. Plant-microbe associations are most pronounced in the root–soil contact area called the "rhizosphere." Specifically, there are three regions between the root and the surrounding soil, i.e., the rhizosphere, rhizoplane, and root itself. The rhizoplane portion of the root includes the root epidermis and the outer cortex. Soil particles and various microorganisms adhere to this area. Plant roots secrete vast amounts of organic carbon, which promotes the establishment and growth of bacteria. These bacteria respond to the plant, interact with each other, and affect their environment, including both chemical and physical properties of the soil in and around the rhizosphere, which ultimately affects plants. There are more bacteria present on the rhizoplane than on the rhizosphere (Sylvia et al., 2005). However, most previous studies have focused on the rhizosphere, rather than the rhizoplane.

Bacteria present in the rhizoplane and rhizosphere can be beneficial to the plant (e.g., by pathogen suppression) as well as detrimental (e.g., by the depletion of nutrients). The microbial community that thrives in the rhizoplane/rhizosphere is dependent not only on the age and health of the plant, but also on its lineage. Moreover, the bacterial composition also differs between soil types and climates. Most importantly, microbial communities that flourish in the rhizosphere differ from those found on bulk soil. Hence, these communities exhibit what has been termed the "rhizosphere effect" by Curl and Truelove (1986). In studies on the rhizoplane of the plant *Oryza sativa,* neither fungi nor

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actinomycetes were observed on the root surface; rather, the entire surface of the basal region of the root was covered with bacteria. The bacteria found on the rhizoplane were largely distributed as aggregates. The most prevalent bacterial species were *Bacillus* and *Pseudomonas* (60%-40%, respectively) (Asanuma et al., 1979; Vessey, 2003). Comparisons of microbial diversity among plant varieties with different yields may reveal hidden roles and subsequent effects on plant yield (Berg, 2009).

In this study, beneficial culturable microbial diversity in the rhizoplane of three commercial varieties of wheat was examined. The purpose of the study was to assess whether relatively high-yield wheat varieties harbor beneficial microbiota that contribute to the increased yield, in addition to genetic factors.

Materials and methods

Selection of wheat varieties for bacterial isolation

Three different commercial wheat varieties (Seher, Faisalabad, and Lasani) were selected under the guidance of the Punjab Seed Corporation. These varieties varied with respect to various properties. Seeds were sown at the beginning of November in pots filled with sieved soil. The pots were irrigated regularly. Sampling was performed 3 times at intervals of 1.5 months. Lengths of roots and shoots were recorded.

The root (3 g) was cut and disinfected with bleach. Severed ends were covered with Parafilm to avoid contamination with histospheric microbiota. After washing 3–4 times with autoclaved distilled water, rhizoplane biomass was removed by vortexing in 30 mL of phosphate-buffered saline (NaCl 8.0 g, KCl 0.4 g, Na_2HPO_4 1.44 g, and KH_2PO_4 0.24 g per liter) using a horizontal vortex adapter (MoBio, Vancouver, Canada). Serial dilutions were prepared after 30 min of vigorous vortexing and plated on nutrient ager, followed by incubation at 37°C. The selected isolates were assigned codes to indicate the variety (Seher as SHR, Faisalabad as FDR, and Lasani as LAR).

Morphological characterization

Morphological characterization was performed for all bacterial isolates, and bacterial type was determined by Gram staining (Benson, 2005). Catalase (using 3% H_2O_2) and oxidase (using *p*-aminodimethylaniline oxalate) activities of the isolates were also checked (Cappuccino and Sherman, 2007).

Auxin quantification

Bacteria were cultured in nutrient broth supplemented with tryptophan (500 µg/mL). The cultures were inoculated and incubated for 7 days at 28°C with agitation at 120 r/min. After inoculation, supernatants were obtained and checked for the presence of auxin. The concentration of indole acetic acid (IAA) was determined colorimetrically with the Salkowski Reagent following the method reported by Gordon and Weber (1951). The standard curve for IAA was generated using 10, 25, 50, 75, 100, 125, 150, 175, and 200 µg/mL standard IAA.

Phosphate solubilization

Phosphate-solubilizing activity of the bacterial isolates was checked in Pikovskaya medium supplemented with 5 g/L tricalcium phosphate (Iqbal et al., 2010). Cultures were incubated at 28°C for 2–3 days. Following incubation, isolates were observed for clear zones of phosphate solubilization.

HCN production

Hydrogen cyanide (HCN) activity of bacterial cultures was determined as described by Lorck (1948). Bacterial isolates were cultured on glycine-supplemented (0.04 g/mL) nutrient agar medium. Whatman filter paper No. 1 soaked in 2% sodium carbonate (in 0.5% picric acid) was placed on the surface of the medium. Plates were sealed with Parafilm and incubated at 28°C for 4 days. Development of an orange to red color was recorded as a positive test result.

Nitrogen fixation

Bacterial isolates were cultured in nitrogen-free mannitol agar and incubated at 28°C for 2–3 days. The appearance of growth was considered a positive indicator of nitrogen fixation (Cappuccino and Sherman, 2007).

Phylogenetic analysis of selected isolates

16S rRNA gene sequencing was performed to identify the bacterial isolates. Bacterial isolates were sent to Macrogen (Seoul, Korea) for 16S rRNA gene sequencing using the primers 518F (CCAGCAGCCGCGGTAATACG) and 800R (TACCAGGGTATCTAATCC) (Porsby et al., 2008; Bernbom et al., 2011). Obtained sequences were checked using FinchTV (Geospiza, Seattle, WA, USA) and classified using NCBI BLAST searches against the 16S rRNA sequence database. Nearest homologs of the sequences were downloaded and aligned using ClustalW. Repeat sequences were omitted, and neighbor-joining phylogenetic trees were generated using MEGA 5.0 (Saitou and Nei, 1987; Tamura et al., 2011). Bootstrap tests with 100 replicates (Felsenstein, 1985) were performed to estimate the reliability of trees.

Results

Rhizosphere microbial diversity at the seedling stage

Numbers of diverse bacterial colonies from all three wheat

varieties were counted and recorded. The colony morphologies of the isolates obtained at sampling #1 are summarized in Table S1. The Lasani variety had the highest bacterial abundance (7.2×10^7 CFU/mL), while the Seher variety had the lowest abundance (6.2×10^6 CFU/mL). Sixteen diverse colonies were purified at the first sampling point.

Eight bacterial isolates (LAR1-4, LAR1-5, FDR1-4, SHR1-2, SHR1-3, SHR1-4, SHR1-5, and SHR1-9) were positive for phosphate solubilization, as evidenced by the production of hollow zones around the streak line. The remaining bacterial isolates did not solubilize phosphate. For HCN production, three bacterial isolates (SHR1-6, SHR1-8, and FDR1-1) tested positive based on the orange to red coloration on filter paper soaked in 0.5% picric acid, while the other bacterial isolates tested negative for HCN production. All bacterial isolates from the first sampling point showed growth on nitrogen-free mannitol agar medium (Table S2). Most of the isolates produced auxin ranging from 4 μg/mL (LAR1-4) to 60 μg/mL (LAR1-4). The number of auxin-producing bacterial isolates was highest for the Seher variety, and SHR1-8 produced the highest concentration of auxin (22 μg/mL). Bacterial isolate LAR1-4 from the Lasani variety produced the highest concentration overall (60 μg/mL), followed by FDR1-3 from the Faisalabad variety, which produced 52 μg/mL auxin (Fig. 1). The 16S rRNA gene sequences of the selected bacterial isolates were analyzed using NCBI BLAST. FDR1-3 and SHR1-3 were 99% homologous with *Bacillus stratosphericus* (GenBank accession numbers KT151930 and KT151936, respectively). LAR1-4 and FDR1-5 were 99% homologous with *Exiguobacterium aurantiacum* and *Rhizobium pusense,* respectively (GenBank accession numbers KT151943 and KT151931, respectively). FDR1-4 was 99% homologous with *Brevundimonas diminuta* (GenBank accession number KT151942).

Rhizosphere microbial diversity at the tillering stage

Diverse colonies were also obtained at sampling #2. The colony morphologies of the isolates obtained at sampling #2 are summarized in Table S3. At the second sampling point, the Faisalabad variety had the most abundant bacteria (4.3×10^7 CFU/mL), while the Lasani variety had the lowest bacterial abundance (9.6×10^5 CFU/mL). Eighteen different bacterial isolates were purified from the second sampling. Four bacterial isolates (SHR2-1, SHR2-4, SHR2-5, and SHR1-9) showed hollow zones around the streak line on Pikovskaya's medium. The remaining bacterial isolates did not solubilize phosphates. HCN was produced by four bacterial isolates (LAR2-2, SHR2-2, SHR2-5, and SHR2-7), and orange to red coloration on the filter paper was observed. All bacterial isolates from sampling 2 were able to grow on nitrogen-devoid mannitol agar and hence were nitrogen fixers, except SHR2-7 (Table S4).

Auxin production was observed for many bacterial isolates. The highest auxin concentration was observed for FDR2-4 (32 μg/mL) from the Faisalabad variety, followed by SHR2-3 from the Seher variety (19 μg/mL) (Fig. 2). Based on NCBI BLAST searches, isolates SHR2-4 and SHR2-7 were identified as *Serratia nematodiphila* (99.73% and 99.59% homology, GenBank accession numbers KT151945 and KT151938, respectively). SHR2-5 was 98% homologous with *Stenotrophomonas rhizophila* (GenBank accession number KT151937), and LAR2-3 and FDR2-4 showed 99% homology with *Pseudomonas hibiscicola* and *Bacillus*

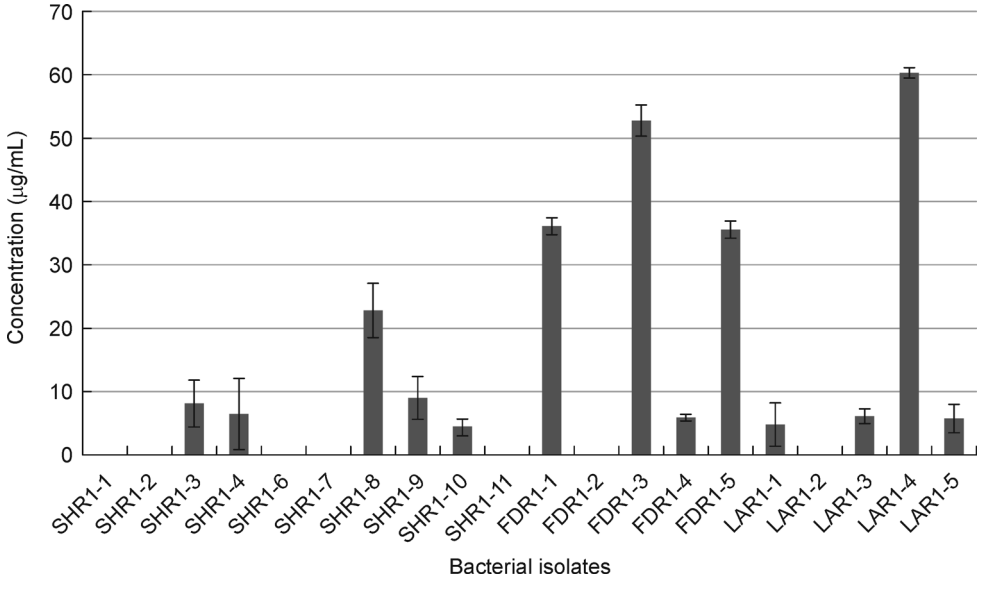

Figure 1 Auxin production by bacterial isolates of the rhizoplanes of three wheat varieties (Seher, Faisalabad, and Lasani) at the first sampling point. Most auxin producers were found in the rhizoplane of the Faisalabad variety. The highest auxin concentration was observed for LAR1-4 from the Lasani variety. (SHR = Seher, FDR = Faisalabad, LAR = Lasani)

cereus, respectively (GenBank accession numbers KT151933 and KT151932, respectively).

Rhizosphere microbial diversity at plant maturity

Colony morphologies of the isolates obtained at sampling #3 are summarized in Table S5. The Lasani variety had the most abundant bacteria at the third sampling point (2.6×10^6 CFU/mL), while the Seher variety had the least abundant bacteria (3.4×10^5 CFU/mL). The number of viable bacteria on the Faisalabad variety was 4.1×10^5 CFU/mL. Twenty-six diverse colonies were purified from the third sampling.

Eight bacterial isolates (FDR3-3, SHR3-1, FDR3-4, SHR3-2, SHR3-3, SHR3-10, SHR3-11, and SHR3-12) solubilized phosphates at the third sampling point. All bacterial isolates from sampling 3 were able to fix nitrogen, as evidenced by growth on nitrogen-free mannitol agar medium. At the third sampling point, HCN production by four bacterial isolates (LAR3-4, SHR3-6, SHR3-2, and SHR3-4) was observed. The remaining isolates did not produce HCN (Table S6).

SHR3-5 from the Seher variety produced auxin at the highest concentration (41 μg/mL), followed by SHR3-1 (32 μg/mL). LAR3-4 from the Lasani variety produced 24 μg/mL auxin (Fig. 3). FDR3-2 and LAR3-4 showed maximum homology with *Planomicrobium chinense* (98% and 97%, respectively) based on NCBI BLAST searches (GenBank accession numbers KT151944 and KT151935, respectively). SHR3-4 and SHR3-12 isolates showed 99% homology with *Staphylococcus hemolyticus* and *Bacillus marisflavi,* respectively (GenBank accession numbers KT151939 and KT151941, respectively). LAR3-1 showed 99% homology with *Bacillus subtilis* (GenBank accession number KT151934) and SHR3-1 was 99% homologous with *Bacillus safensis* (GenBank accession number KT151940). The identities of the bacteria were also confirmed by constructing a neighbor-joining tree based on the sequences along with their nearest homologs from the NCBI database. The results of the phylogenetic analysis were consistent with those obtained by the NCBI BLAST searches (Fig. 4).

Discussion

The microbiota is known to have both beneficial as well as harmful effects on plants. Microbiota may differ among different varieties within the same species and thus may have hidden roles in fruit yield associated with commercially important crop varieties. In this study, a substantial diversity of microbes was isolated from the rhizoplane of three different varieties of wheat (Seher, Faisalabad, and Lasani) at different stages of plant growth. Microbial diversity, microbial load, as well as plant growth-promoting abilities of microbiota (auxin production, phosphate solubilization, and HCN production) varied among the three wheat varieties, showing the importance and possible role of microbiota in the growth and fruit yield of the host varieties. These parameters also varied among the growth stages of plant varieties, suggesting the selection and succession of microbiota during plant growth.

The lengths of the roots and shoots of all three varieties were evaluated at each sampling point to clarify differences in

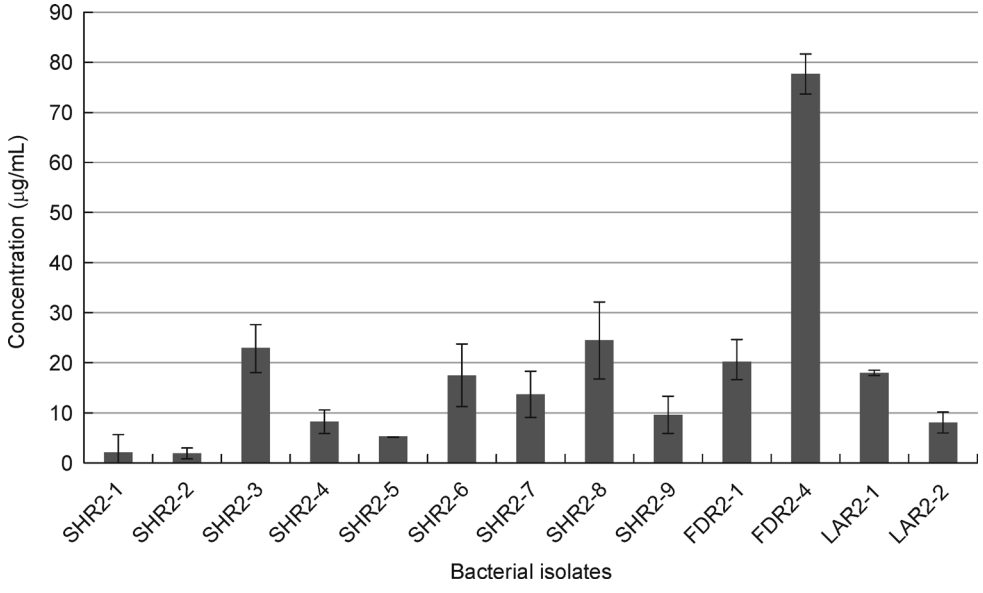

Figure 2 Auxin production by bacterial isolates of the rhizoplanes of three wheat varieties (Seher, Faisalabad, and Lasani) at the second sampling point. Most auxin producers were found in the rhizoplane of the Seher variety at the second sampling point, but highest auxin concentration was observed for FDR2-4 from the Faisalabad variety. (SHR = Seher, FDR = Faisalabad, LAR = Lasani)

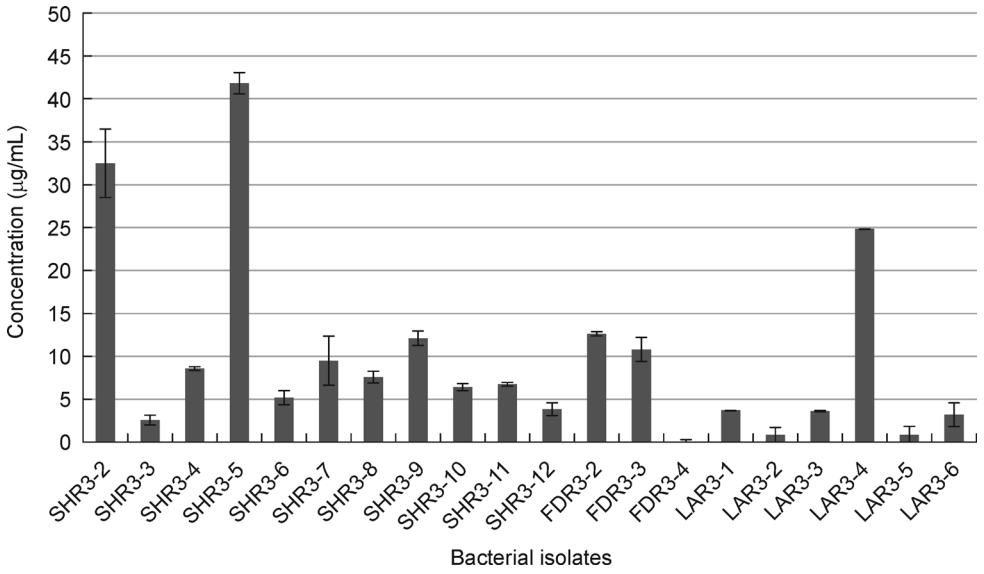

Figure 3 Auxin production by bacterial isolates of the rhizoplanes of three wheat varieties (Seher, Faisalabad, and Lasani) at the third sampling point. Most auxin producers were again detected in the rhizoplane of the Seher variety. The highest auxin concentration was observed for SHR3-5. (SHR = Seher, FDR = Faisalabad, LAR = Lasani)

growth rates. At the first sampling point, the shoot length of all varieties was almost the same. However, during the second and third sampling points, clear differences in shoot length were observed. The shoot length of the Seher variety was highest at the second and third sampling points, followed by the shoot lengths of the Faisalabad and Lasani varieties. At the second sampling point, the shoot lengths of the Seher, Faisalabad, and Lasani varieties were 25.19, 21.15, and 20.47 inches, respectively, whereas at the third sampling point, the shoot lengths of the Seher, Faisalabad, and Lasani varieties were 34, 32, and 31 inches, respectively. The greater shoot lengths of Seher at the start of the growing season indicated that the growth rate of the Seher variety was higher than those of the other two varieties.

Based on differences in bacterial abundance among varieties, the bacterial load associated with the Lasani variety was much higher than those of the Faisalabad and Seher varieties. The Seher variety carried the lowest bacterial load, but exhibited a greater diversity of bacterial colonies. This suggests that the genotype of the Seher variety favors associations with more diverse bacterial communities as compared to the genotypes of Faisalabad and Lasani. Moreover, because of competition among bacteria present on the Seher variety, a reduced CFU count was observed as compared to those of the other two varieties. Gram-positive (including bacilli, cocci, and coccobacilli) bacteria were predominant in the rhizoplane of all three wheat varieties as compared to gram-negative bacteria. CFU counts decreased as plant age increased, suggesting that the aging and drying of plants resulted in reduced nutrients to support the same bacterial load.

Bacteria from all three varieties at all three sampling times were purified and analyzed with respect to various parameters related to plant growth, such as auxin production, phosphate solubilization, and HCN production. Auxin-producing bacteria were present on all varieties at all sampling times. The highest concentration of auxin (77 μg/mL) was observed at the second sampling point (tillering) when root and shoot growth were more rapid, as compared to those at the other two sampling points. Moreover, the most auxin-producing bacteria were detected at the second sampling point. This suggests that, as this stage (2 months old) is characterized by rapid plant growth (i.e., stem extension), the number of auxin-producing bacteria was maximized to support the rapid plant growth. It is well known that auxin is responsible for the division, extension, and differentiation of plant cells and tissues (Teale et al., 2006). The Seher variety harbored the most auxin-producing isolates, suggesting that the higher growth rate of this variety could be explained by its microbiota. The phosphate-solubilizing ability of bacteria can also significantly enhance plant growth and fruit yield (Qureshi et al., 2012). All the bacteria isolated from the three wheat varieties were phosphate solubilizers. Bacteria present in the rhizosphere are known to solubilize phosphates, thus making insoluble phosphates available to plants (Goldstein, 1986). Most phosphate-solubilizing bacteria were found in the Seher variety. This property must contribute to the growth superiority of the Sehar variety as compared to other varieties. Lasani variety had the fewest phosphate-solubilizing bacteria, which may explain its comparatively lower plant growth and fruit yield. Soil is a rich source of inorganic nutrients such as nitrates and phosphates. Owing to the close contact of the rhizoplane with rhizospheric soil, the bacterial isolates showed good nitrogen fixation and phosphate solubilization.

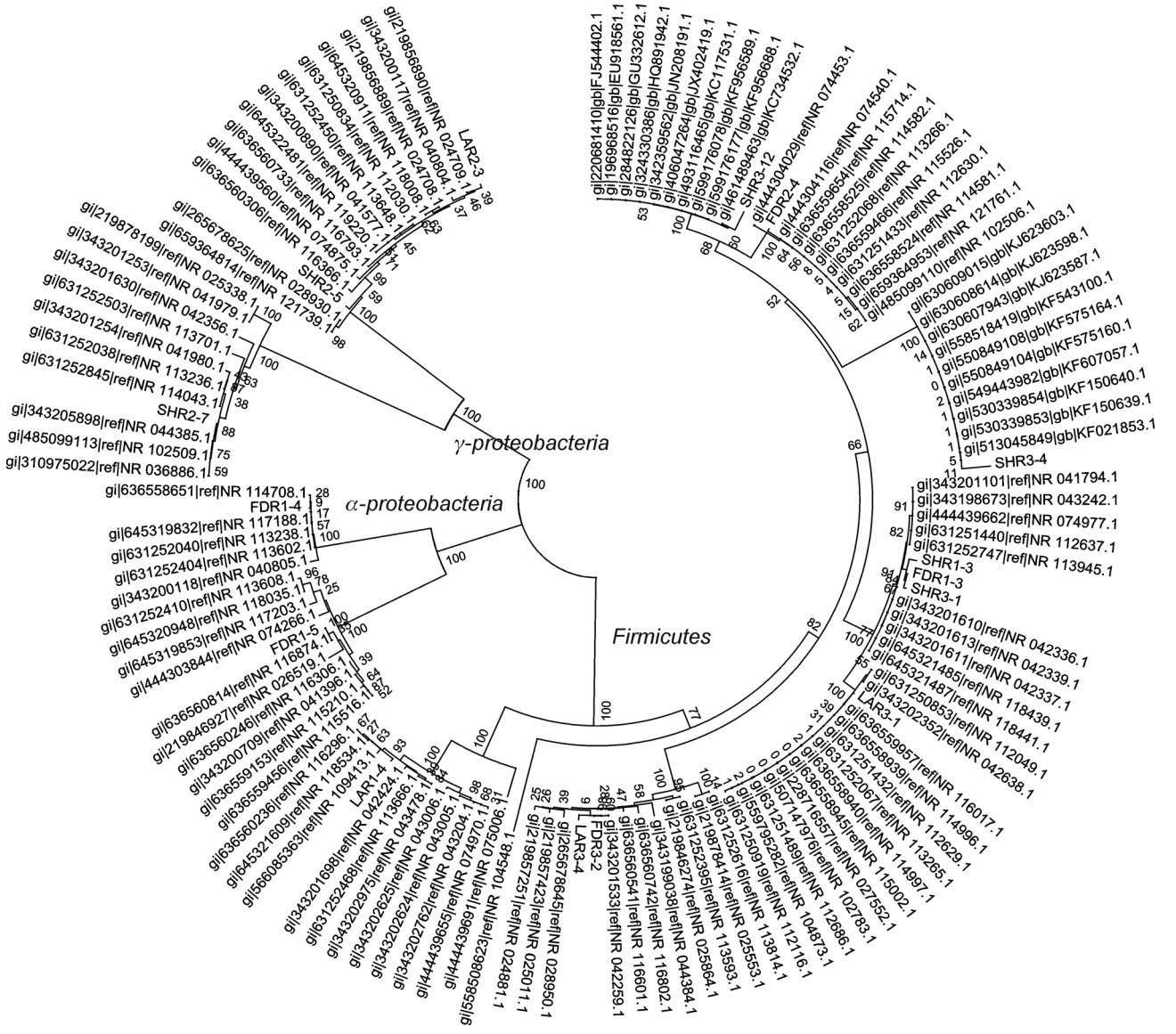

Figure 4 Neighbor-joining phylogenetic tree of the 16S rRNA gene sequences of the selected bacterial isolates. The tree was constructed by aligning the sequences and their nearest homologous sequences from NCBI using Mega 5. One hundred bootstrap replicates were used to measure reliability. Most of the bacterial isolates were identified as *Firmicutes.* (SHR = Seher, FDR = Faisalabad, LAR = Lasani)

This could contribute to the improved yield of commercially important wheat varieties. Hydrogen cyanide produced by bacteria has an important role in the control of weed growth (Khan et al., 2008). The Seher variety had the most HCN-producing bacteria as compared to the other two varieties. The pots of the Seher variety showed the fewest weeds as compared to the pots of the Faisalabad and Lasani varieties. This indicates a clear role of HCN-producing bacteria in the Seher variety in weed control. All bacterial isolates from all three wheat varieties were able to fix nitrogen, thus promoting plant growth. The 16S rRNA gene analysis revealed that most plant growth-promoting bacterial isolates in this study belonged to the genera *Bacillus (B. stratosphericus, B. cereus, B. subtilis, B. safensis,* and *B. marisflavi)* and *Pseudomonas (P. geniculata). Planomicrobium chinense, Serratia nematodiphila*, *Rhizobium pusense*, *Brevundimonas diminuta, Stenotrophomonas rhizophila*, and *Exiguobacterium aurantiacum* were also found. In various studies, bacteria belonging to the genera *Pseudomonas*, *Bacillus*, and *Rhizobium* have been reported to have a powerful role in phosphate solubilization (Rodríguez and Fraga, 1999). The results of this study show that higher yielding plant varieties carry a comparatively larger number of plant growth-promoting bacteria with comparatively better plant growth-promoting abilities as compared to the microbiota of low-yielding plant varieties. However, other plant varieties should

also be checked to confirm this hypothesis. Moreover, isolated bacteria need to be inoculated in low-yielding varieties in order to confirm their role in fruit yield.

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Compliance with ethics guidelines

The authors have no conflicts of interest regarding this work to report. The present study does not involve animal or human samples.

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