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High-yielding Wheat Varieties Harbour Superior Plant Growth Promoting-Bacterial Endophytes

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Abstract

Background and Objective: The purpose of this study was to compare the endophytic microbial flora of different wheat varieties to check whether a better yielding variety also harbours superior plant growth promoting bacteria. Such bacteria are helpful in food biotechnology as their application can enhance the yield of the crop.

Material and Methods: Three wheat varieties (Seher, Faisalabad and Lasani) were selected, Seher being the most superior variety. endophytic bacteria were isolated from the histosphere of the leaves and roots at different growth phases of the plants. The isolates were analyzed for plant growth promoting activities. Isolates giving best results were identified through 16S rRNA gene sequencing. Statistical analysis was done using Microsoft Excel 2013. All the experiments were conducted in triplicates.

Results and Conclusion: The endophytes of Seher variety showed maximum plant growth promoting abilities. Among the shoot endophytes, the highest auxin production was shown by Seher isolate SHHP1-3 up to 51.9µg ml^{-1}, whereas in the case of root endophytes, the highest auxin was produced by SHHR1-5 up to 36 µg ml^{-1}. The bacteria showing significant plant growth promoting abilities were identified by 16S rRNA sequencing. Bacillus, Proteobacteria and Actinobacteria species were the dominant bacteria showing all the traits of plant growth promotion. It can be concluded that Seher variety harbours superior plant growth promoting endophytes that must be one of the reasons for its better growth and yield as compared to the other two varieties. The investigated results support possible utilization of the selected isolates in wheat growth promotion with respect to increase in agro-productivity. The application of such bacteria could be useful to enhance wheat yield and can help in food biotechnology.

Conflict of interest: We declare that we have no conflicts of interests.

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1. Introduction

Wheat is one of the most important cereals in the world constitutes a large portion of human diet. As with any other plant, bacteria inhabit different parts of the wheat plant. The bacteria that live inside the plant are called 'endophytes', which means 'in the plant' [1]. This internal habitat is termed as histosphere. Endophytes can be pathogenic endophytic algae, parasitic endophytes, mutualistic endophytic bacteria, ectomycorrhizal helper bacteria, and endophytic bacteria in commensalistic symbioses [2-6]. The bacteria that are able to colonize the internal tissues of plants confer an ecological advantage over the bacteria that can only colonize epiphytically. More uniform and shielding environment is provided by the internal tissues of plants for the microbes as compared to the plant surfaces [7]. Endophytes play an important role in the host plant's growth and health by producing certain plant growth promoting factors.

There are several ways in which different plant growth promoting (PGP) bacteria directly facilitate the proliferation of their plant hosts. Endophytic nitrogen fixers (diazotrophs) have advantage over the root associated diazotrophs like Azospirillum and Azotobacter. Endophytes can more easily exploit carbon sources provided by the plants as they colonize inside the plant rather than on the surface [8,9]. They are often located in the dense plant tissues like stem nodes and xylem vessels, and so they have low O_2 environment, which is necessary for the functioning of nitrogenase [10,11]. Many species of Pseudomonas, Enterobacter, Staphylococcus and Azotobacter produce plant growth regulators, such as ethylene, auxins, or cytokinins [12,13]. Endophytes also benefit the plants indirectly by acting as biocontrol agents of phytopathogens in the root zone through production of bioactive secondary metabolites, antifungal or antibacterial agents, and induction of host immunity [6,14].

Researchers have been interested in discovering bacterial isolates with improved PGP competencies. The investigation of endophytic bacteria is a challenging field of research, from both fundamental and applied points of view, as more information about the biochemistry of the histosphere as well as the population dynamics is required. Many researchers have also reported the presence of endophytes in wheat varieties and their beneficial effects on the host plants [15,16].

As endophytes play a major role in the growth of host plants, there is a need to compare the endophytes of different wheat varieties. The present study evaluates the PGP-endophytic microbial flora of three commercial wheat varieties during different growth stages. PGP-endophytic bacteria of Seher Faisalabad and Lasani were analyzed to find out whether a better yielding variety also harbours distinct plant growth promoting microbial flora, which plays part in its being superior to the other two varieties.

2. Materials and Methods

2.1. Wheat varieties and sowing conditions

Different varieties of wheat (Seher, Lasani and Faisalabad) were selected. Seeds were obtained from Punjab Seed Corporation, Lahore. Sowing was done in the first week of November 2014. The seeds were sown 3.8 cm deep in sieved soil filled in pots 30 cm wide. Each variety was sown in 15 pots, 8 seeds per pot. The pots were labeled according to the wheat variety. Then they were kept in wire house and watered regularly with tap water. After germination, thinning was done leaving 5 seeds per pot [17]. All the experiments were proceeded in triplicates.

2.2. Harvesting

The plants were harvested three times during their growth. The first harvesting was done when the plants were a month old, the second harvesting was carried out when the plants were 2.5 months old, and the third harvesting was performed when the plants were 4 months old. To harvest the plants, the whole soil mass from the pot was removed, and the soil was washed off the roots carefully to keep the roots intact. Finally, the root and shoot lengths were measured.

2.3. Processing of samples

The plants were washed with the tap water. The roots and shoots were separated and cut into smaller pieces to weigh up to 3 g. The cut ends were sealed with parafilm. Surface sterilization was done by keeping the plant material in 70% v v^{-1} ethanol for 1 min, in NaClO for 3 min and in 70% v v^{-1} ethanol again for 30 s. After surface sterilization, the plants were washed with autoclaved water and crushed with sterile pester and mortar [18].

2.4. Isolation of endophytes

The crushed plant material was mixed in 30 ml autoclaved phosphate buffer saline in a sterile 50ml tube. The tubes were vortexed vigorously using horizontal vortex adapter (MoBio, USA) for 10 min. Serial dilutions were prepared and inoculums from 10^{-2}, 10^{-4} and 10^{-6} dilutions were cultured on the nutrient agar. The plates were incubated at 28°C for 48 h. After incubation, CFU g^{-1} (colony forming units) was calculated [19].

2.5. Characterization of isolates

Colonies from each sample that were morphologically different were selected and purified by several rounds of quadrant streaking on the N-agar. Microscopic examination was done using wet mount followed by Gram staining [20]. Catalase and oxidase tests were also performed.

2.6. Assays for detecting PGP-activities of endophytes

2.6.1. Nitrogen fixation

Nitrogen fixation ability was evaluated by streaking the 24 h fresh bacterial isolates on nitrogen free mannitol agar containing (g l^{-1}) Mannitol 15, K_2HPO_4 0.5, $MgSO_4$ 0.2, NaCl 0.2, $CaCO_3$ 5.0, $CaSO_4$ 0.1 and agar 15.0 [20]. The plates were incubated at 28° C for 2-3 days (Memmert, Germany). The appearance of growth was considered as positive test for nitrogen fixation.

2.6.2. Estimation of auxin production

In order to check auxin production by the bacterial isolates, 24 h fresh bacterial isolates were inoculated in autoclaved Luria-Bertani broth supplemented with 500 µg ml^{-1} filter sterilized L-tryptophan. All the tubes were incubated at 37° C for 72 h at 120 rpm. After incubation, the bacterial cultures were centrifuged at 17092 g for 10 min, and 100 µl of supernatant was mixed with 200 µl of Salkowski reagent (1ml 0.5 M $FeCl_3$ and 50 ml 35% v v^{-1} perchloric acid) [21] in a microtitre plate. The plate was incubated at room temperature in the dark for 1 h. After incubation, optical density was recorded at 535 nm in Epoch plate reader (BioTek, USA).

2.6.3. Hydrogen cyanide production

Hydrogen cyanide producing bacteria were screened by swabbing the 24 h fresh bacterial cultures on N-agar plates supplemented with 4 g l^{-1} glycine. Whatmann's filter paper no.1 was soaked in a solution of HCN reagent (2% $NaCO_3$ in 0.5% picric acid) and placed at the top of the plate over the swabbed area. The plates were incubated at 28° C for 4-5 days. After incubation, development of orange to red color was taken as a positive result for HCN production [22].

2.6.4. Phosphate solubilization

For assessing the ability of the isolates to solubilize phosphate, the 24 h fresh bacterial isolates were streaked on Pikovaskaya agar medium [23], and the plates were incubated at 28° C for 5-7 days. After incubation, the plates were observed for the presence of clear zones along the streak lines, an indication that phosphates have been solubilized.

2.6.5. Phylogenetic analysis

Isolates from each harvesting that showed remarkable PGP abilities were selected and identified through 16S rRNA gene sequencing from Macrogen (Korea) using the primers 518F (CCAGCAGCCGCGGTAATACG) and 800R (TACCAGGGTATCTAATCC) [24,25]. The obtained sequences were checked for quality of base calling using FinchTV (Geospiza-PerkinElmer, USA). Contigs were made using NCBI 2-sequences BLAST (Basic Local Alignment Search Tool). Then they were classified by aligning with the 16S ribosomal RNA sequences database of NCBI Nucleotide through BLAST. The nearest homologues were downloaded and aligned through ClustalW. The sequences were trimmed in FinchTV, and neighbour joining phylogenetic tree was made in Mega 5 software using bootstrap method for branch support.

2.6.6. Statistical Analysis

Statistical analysis was done using Microsoft Excel 2013. All the experiments were conducted in triplicates. Means and standard deviations were calculated and applied. Standard deviations are shown as error bars in the corresponding figures.

3. Results and Discussion

3.1. Analysis of PGP-endophytes of Seher variety

Seher root-endophytic samples showed less CFU g^{-1} during the first and second harvestings (2.4×10^6 and 1.6×10^6, respectively) but increased significantly at the time of the third harvesting (3.2×10^7). However, more diverse bacterial isolates were obtained during the second harvesting when the plants were growing rapidly. For the shoot-endophytic samples of Seher variety, the highest CFU g^{-1} was observed during the second harvesting (2.9×10^7). The diversity of shoot-endophytic samples did not vary much throughout the plant growth. The diversity of endophytic bacterial communities and the number of bacterial populations constituting those communities are plant-specific and vary over time, i.e. both temporal and spatial shifts in the microbial communities are found. Diversity of bacteria in a plant is affected by the plant age, environmental conditions and genetic variability of the plant [26,27].

3.2. PGP activities of Seher variety endophytes

All the selected isolates were tested for plant growth promoting abilities. Auxin production by the isolates was used as a major screening criterion. During the first harvesting, among the root-endophytes of Seher variety, SHHR1-5 produced the highest concentration of auxin (36 µg ml^{-1}). During the second harvesting, significant increase in auxin production per bacterial isolate was observed. SHHR2-2 and SHHR2-3 produced the highest concentrations of auxin. In the third harvesting, strain SHHR3-1 produced the highest concentration of auxin, and all the other strains showed moderate auxin production (Figure 1).

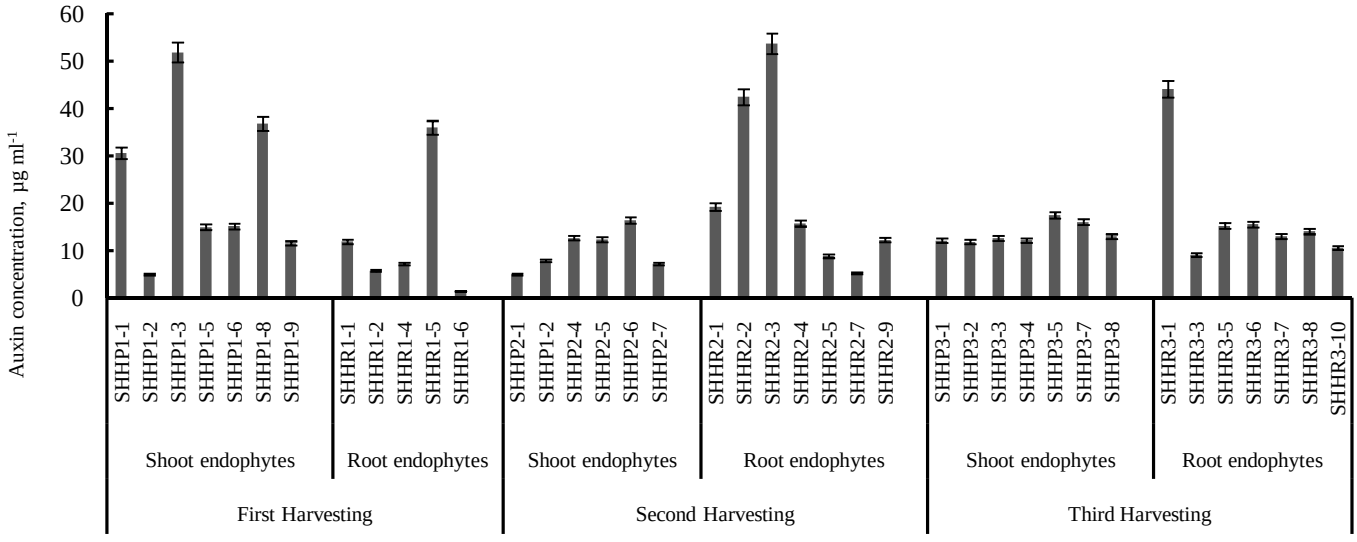

Figure 1. Auxin production by Seher variety shoot- and root-endophytes isolated during different growth stages of the plants.

Among the shoots-endophytic samples, the highest concentration of auxin per isolate was observed during the first harvesting. SHHP1-3 produced the highest auxin concentration (51.9 µg ml^{-1}). During the second harvesting, the concentration of auxin per bacterial isolate decreased considerably. Whereas in the third harvesting, all the isolates were observed to produce moderate concentrations of auxin, with SHHR3-1 producing the highest auxin concentration, 44.1 µg ml^{-1} (Figure 1). As maximum auxin production was observed in the second harvesting (for all the varieties) when the growth rate was highest, it can be concluded that the auxin producing microbes must have a strong influence on the rapid growth of the plant. Seher shoot endophytes showed higher auxin production as compared to the endophytes of the other two varieties. This could be one of the reasons behind the better growth of Seher variety. Phylloplane and rhizospheric bacteria of Seher variety are already known to show better PGP-activities with higher concentration of auxin production as compared to the other two varieties [28,29].

In case of HCN production, SHHP3-3 and SHHR3-1 of the shoot- and root-endophytic samples (second harvesting), respectively, showed positive results for HCN production. Phosphate solubilizers were also detected in the second harvesting: SHHP2-1 from the shoot-endophytic sample, and SHHR2-5 and SHHR2-10 from the root-endophytic sample. Phosphorus enables a plant to store and transfer energy, promotes root, flower and fruit development, and allows early maturity [30]. Some microbes have the ability to solubilize phosphorous into inorganic form and make it available to the plants. Most of the isolates from all the three harvesting periods were found to be nitrogen fixers (Table 1). Caballero et al. and Muangthong et al. have also reported presence of endophytic nitrogen fixing bacteria in different plant species [8,10].

Table 1. Salient features of Seher variety endophytes

Endophytic isolates	Staining properties	Nitrogen fixation	HCN production	Phosphate solubilization	Auxin production	Species Identified by 16S sequencing
FIRST HARVESTING						
Seher shoot endophytes						
SHHP1-1	GPB	+	-	-	++	
SHHP1-2	GPC	-	-	-	+	
SHHP1-3	GPB	+	-	-	+++	*Bacillus marisflavi*
SHHP1-5	GPC	+	-	-	+	
SHHP1-6	GPC	+	-	-	+	
SHHP1-8	GNB	+	-	-	++	
SHHP1-9	GPC	+	-	-	+	
Seher root endophytes						
SHHR1-1	GPB	+	-	-	+	
SHHR1-2	GNB	+	-	-	+	
SHHR1-4	GPB	-	-	-	+	
SHHR1-5	GPB	+	-	-	++	*Bacillus aerius*
SHHR1-6	GPC	+	-	-	+	
SECOND HARVESTING						
Seher shoot endophytes						
SHHP2-1	GNC	+	-	+	+	
SHHP2-2	GPB	+	-	-	+	
SHHP2-4	GPB	+	-	-	+	
SHHP2-5	GPC	+	-	-	+	
SHHP2-6	GPB	+	-	-	+	*Bacillus subtilis*
SHHP2-7	GPC	+	-	-	+	
Seher root endophytes						
SHHR2-1	GNB	+	-	-	+	
SHHR2-2	GPB	+	-	-	+++	*Brevibacillus brevis*
SHHR2-3	GPB	+	-	-	+++	*Brevibacillus parabrevis*
SHHR2-4	GNB	+	-	-	+	
SHHR2-5	GPB	+	-	+	+	*Bacillus. stratosphericus*
SHHR2-7	GNB	-	-	-	+	
SHHR2-9	GNB	+	-	-	+	
SHHR2-10	GPB	+	-	+	+	*Bacillus. subtilis*
THIRD HARVESTING						
Seher shoot endophytes						
SHHP3-1	GNC	+	-	-	+	*Acinetobacter baumannii*
SHHP3-2	GPC	+	-	-	+	
SHHP3-3	GPC	+	Weak +	-	+	
SHHP3-4	GPC	Weak +	-	-	+	
SHHP3-5	GPB	+	-	-	+	*Bacillus megaterium*
SHHP3-7	GPC	+	-	-	+	
SHHP3-8	GPC	-	-	-	+	
Seher root endophytes						
SHHR3-1	GPB	-	+	-	+++	*Exiguobacterium indicum*
SHHR3-3	GPB	+	-	-	+	
SHHR3-5	GPC	+	-	-	+	
SHHR3-6	GNB	Weak +	-	-	+	*Alcaligenes faecalis*
SHHR3-7	GNB	+	-	-	+	
SHHR3-8	GPB	+	-	-	+	
SHHR3-10	GPB	+	-	-	+	

GPB: Gram positive bacilli, GPC: Gram positive cocci, GNB: Gram negative bacilli, GNC: Gram negative cocci

3.3. Molecular Identification of Seher variety endophytes

The isolates showing the most promising results for plant growth promoting assays were identified by 16SrRNA gene sequencing. *Bacillus* sp. was dominant in both the root- and shoot-endophytic samples in all the growth stages of Seher variety. In the first and second harvestings, Firmicutes including *Brevibacillus* sp. and *Bacillus* sp. were dominant plant growth promoting bacteria, where as in the third harvesting, Proteobacteria (both γ and β) emerged as the dominant beneficial bacteria for the variety. The results were confirmed by making phylogenetic tree (Figure 2). The sequences were submitted in the GenBank under the accession numbers given in Table 2.

3.4. Analysis of PGP endophytes of Faisalabad variety

The CFU g^{-1} of the Faisalabad root-endophytic samples increased with the growth of the plants, i.e. the highest CFU g^{-1} was obtained during the third harvesting (1.4×10^7). However, the diversity of endophytic bacteria was highest during the second harvesting. It indicates that the young plants were more supportive to a diversity of bacteria; however, none of them were able to colonize in high numbers, perhaps due to competition with each other. On the other hand, as the plant growth progressed, few bacteria dominated over the others, and colonized in higher numbers, increasing the CFU g^{-1} and decreasing the diversity. In case of the shoot-endophytic samples of Faisalabad variety, the CFU g^{-1} decreased with the growth of the plants, i.e. the first harvesting gave the highest CFU g^{-1} (2.4×10^7). Whereas most varied endophytic bacteria were found during the third harvesting.

3.5. PGP activities of Faisalabad variety endophytes

In the first harvesting, three Faisalabad shoot endophytes (FDHP1-3, FDHP1-11 and FDHP1-9) produced auxin in concentrations slightly above 10 µg ml^{-1}. During the second harvesting, only FDHP2-3 produced significant concentration of auxin, i.e. 15.68 µg ml^{-1}. During the third harvesting, all the shoot endophytes produced auxin below 10 µg ml^{-1} (Figure 3).

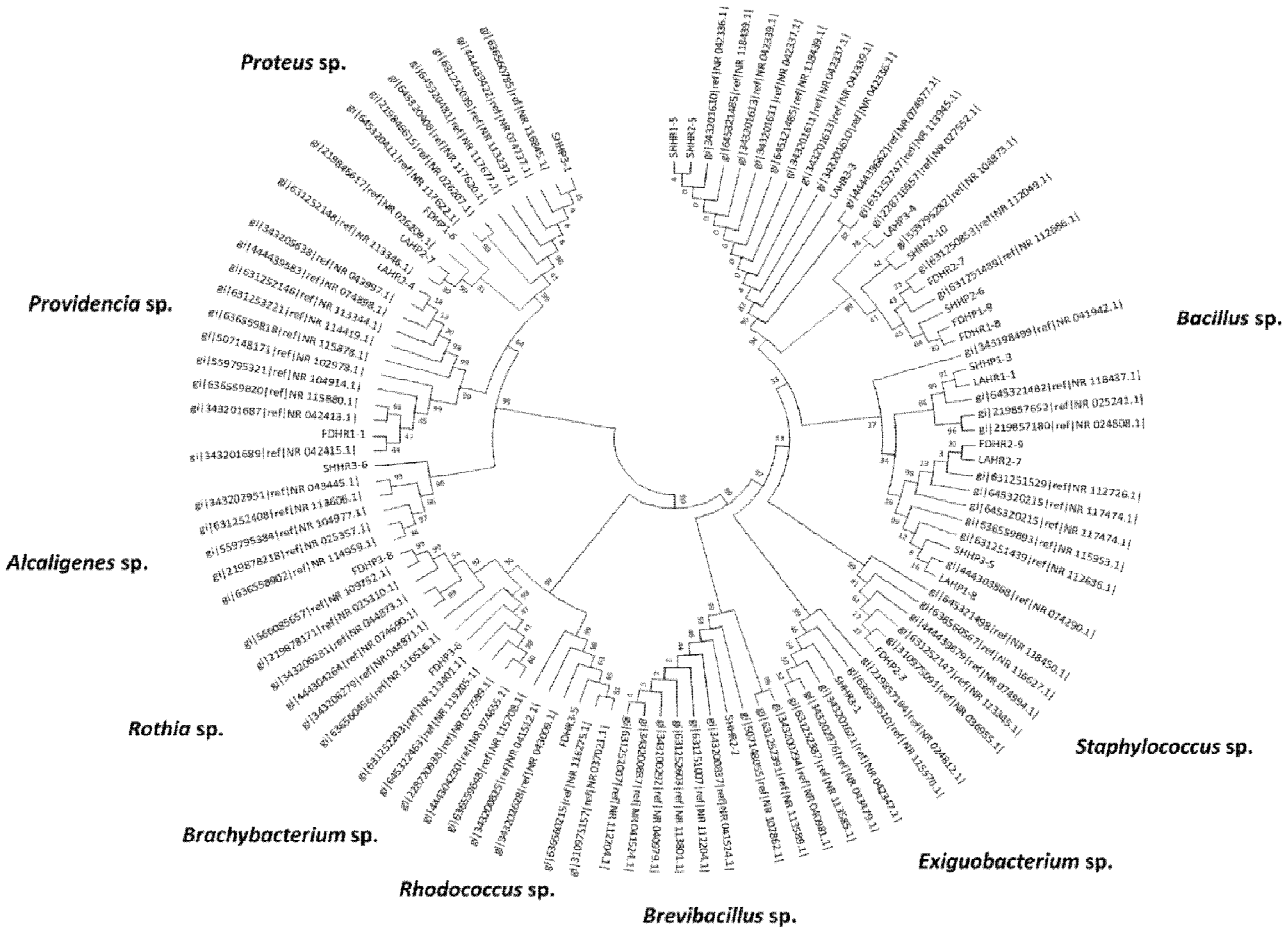

Figure 2. Neighbour-joining phylogenetic tree of 16S rRNA gene sequences of all the selected bacterial endophytes and their nearest homologues from NCBI 16S ribosomal RNA sequences database.

Table 2. Molecular identities and the GenBank accession numbers of PGP-endophytes

Serial No.	Isolate	Nearest NCBI homologue	% identity	GenBank accession number
1	FDHP1-6	*Acinetobacter calcoaceticus*	99.49	KT216578
2	FDHP1-9	*Bacillus subtilis*	99.39	KT216579
3	FDHR1-1	*Bacillus stuartii*	98.84	KT216580
4	FDHR1-8	*Bacillus subtilis*	99.32	KT216581
5	LAHP1-8	*Bacillus megaterium*	99.59	KT216582
6	LAHR1-1	*Bacillus marisflavi*	99.49	KT216583
7	SHHP1-3	*Bacillus marisflavi*	99.39	KT216584
8	SHHR1-5	*Bacillus aerius*	99.73	KT216585
9	FDHP2-3	*Staphylococcus haemolyticus*	100	KT216586
10	FDHR2-7	*Bacillus subtilis*	99.25	KT216587
11	FDHR2-9	*Bacillus simplex*	99.24	KT216588
12	LAHP2-7	*Acinetobacter lwoffii*	99.38	KT216589
13	LAHR2-4	*Proteus mirabilis*	99.45	KT216590
14	LAHR2-7	*Brevibacillus frigoritolerans*	99.31	KT216591
15	SHHP2-6	*Bacillus subtilis*	99.12	KT216592
16	SHHR2-2	*Brevibacillus brevis*	99.04	KT216593
17	SHHR2-3	*Brevibacillus parabrevis*	98.3	KT216594
18	SHHR2-5	*Bacillus stratosphericus*	100	KT216595
19	SHHR2-10	*Bacillus subtilis*	99.66	KT216596
20	FDHP3-6	*Brachybacterium paraconglomeratum*	98.66	KT216597
21	FDHP3-8	*Rothia.endophytica*	99.79	KT216598
22	FDHR3-5	*Rhodococcus yunnanensis*	98.81	KT216599
23	LAHP3-2	*Brevibacillus brevis*	98.9	KT216600
24	LAHP3-4	*Bacillus subtilis*	99.52	KT216601
25	LAHR3-3	*Bacillus aerius*	100	KT216602
26	SHHP3-1	*Acinetobacter baumannii*	98.41	KT216603
27	SHHP3-5	*Bacillus megaterium*	99.75	KT216604
28	SHHR3-1	*Exiguobacterium indicum*	99.79	KT216605
29	SHHR3-6	*Alcaligenes faecalis*	97	KT216606

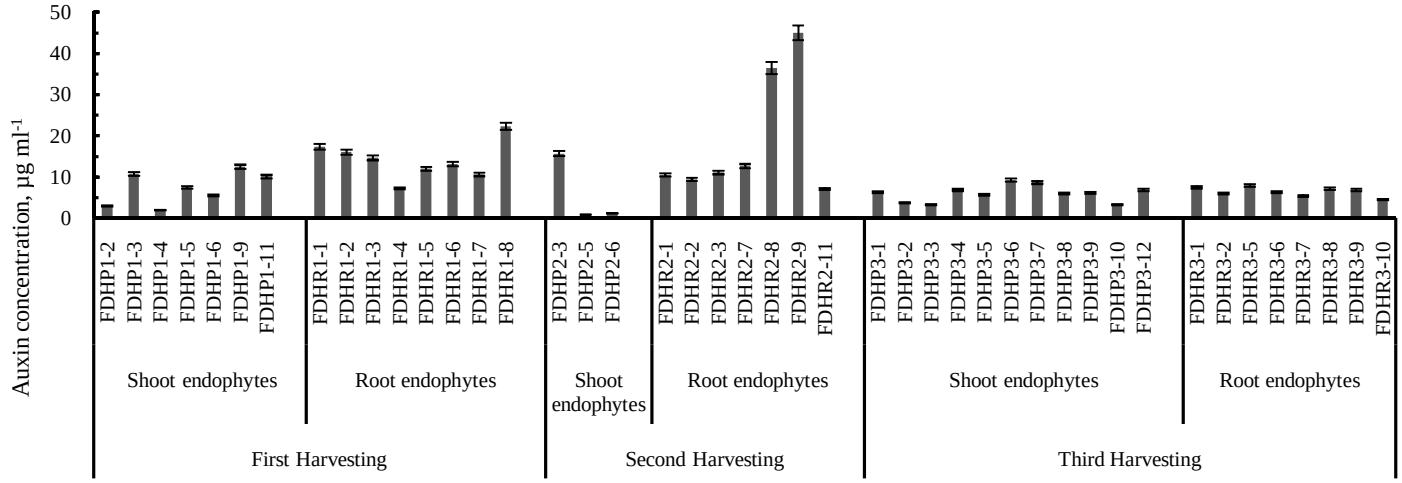

Figure 3. Auxin production by Faisalabad variety shoot- and root-endophytes isolated during different growth stages of the plants.

All the root-endophytes of Faisalabad variety, except FDHR1-4, showed the ability to produce significant concentrations of auxin (above 10 µg ml^{-1}) during the first harvesting. The highest auxin concentration was produced by FDHR1-8 (22.33 µg ml^{-1}). During the second harvesting, the root isolates of FDHR2-9 and FDHR2-8 produced the highest concentrations of auxin, i.e. 45 and 36.5 µg ml^{-1}, respectively. None of the endophytes from the third harvesting produced significant concentrations of auxin (Figure 3).

All of the endophytes from Faisalabad variety were found to be nitrogen fixers, except few isolates from the third harvesting. As the endophytes reside inside the plant, they are less exposed to the environmental oxygen, which is deleterious for the nitrogenase enzyme, so they can fix nitrogen more efficiently as compared to the rhizospheric or phyllospheric bacterial community [8]. FDHP1-6, an endophyte of shoot from the first harvesting, gave positive results for phosphate solubilization. During the first harvesting, none of the isolates showed positive results for HCN production. However, during the second harvesting, FDHP2-3 and FDHR2-7 from the shoot and root samples, respectively, indicated positive results for HCN production. During the third harvesting, FDHP3-9, an endophyte from the shoot sample, indicated positive result for HCN production (Table 3).

Table 3. Characteristics of the endopytes of Faisalabad variety

Endophytic isolates	Staining properties	Nitrogen fixation	HCN production	Phosphate solubilization	Auxin production	Species Identified by 16S sequencing
FIRST HARVESTING						
Faisalabad shoot endophytes						
FDHP1-2	GPB	+	-	-	+	
FDHP1-3	GPB	+	-	-	+	
FDHP1-4	GPC	+	-	-	+	
FDHP1-5	GPB	+	-	-	+	
FDHP1-6	GPC	+	-	+	+	*Acinetobacter calcoaceticus*
FDHP1-9	GPB	+	-	-	+	*Bacillus subtilis*
FDHP1-11	GPB	+	-	-	+	
Faisalabad root endophytes						
FDHR1-1	GNB	+	-	-	+	*Providencia stuartii*
FDHR1-2	GPB	-	-	-	+	
FDHR1-3	GPB	+	-	-	+	
FDHR1-4	GPC	+	-	-	+	
FDHR1-5	GPB	+	-	-	+	
FDHR1-6	GNB	+	-	-	+	
FDHR1-7	GNB	+	-	-	+	
FDHR1-8	GPB	+	-	-	++	*Bacillus subtilis*
SECOND HARVESTING						
Faisalabad shoot endophytes						
FDHP2-3	GPC	+	+	-	+	*Staphylococcus haemolyticus*
FDHP2-5	GNB	+	-	-	-	
FDHP2-6	GPB	+	-	-	+	
Faisalabad root endophytes						
FDHR2-1	GPC	+	-	-	+	
FDHR2-2	GPC	+	-	-	+	
FDHR2-3	GPB	+	-	-	+	
FDHR2-7	GPB	+	+	-	+	*Bacillus subtilis*
FDHR2-8	GNB	+	-	-	++	
FDHR2-9	GPB	+	-	-	+++	*Bacillus simplex*
FDHR2-10	GNB	+	-	-	+	
FDHR2-11	GNB	+	-	-	+	
THIRD HARVESTING						
Faisalabad shoot endophytes						
FDHP3-1	GNC	+	-	-	+	
FDHP3-2	GPC	+	-	-	+	
FDHP3-3	GNC	+	-	-	+	
FDHP3-4	GNB	-	-	-	+	
FDHP3-5	GPB	+	-	-	+	
FDHP3-6	GPB	+	-	-	+	*Brachybacterium paraconglomeratum*
FDHP3-7	GPC	+	-	-	+	
FDHP3-8	GPC	+	+	-	+	*Rothia endophytica*
FDHP3-9	GPC	+	-	-	+	
FDHP3-10	GPC	+	-	-	+	
FDHP3-12	GPB	-	-	-	+	
Faisalabad root endophytes						
FDHR3-1	GPC	-	-	-	+	
FDHR3-2	GNC	-	-	-	+	
FDHR3-5	GPC	+	-	-	+	*Rhodococcus yunnanensis*
FDHR3-6	GPB	+	-	-	+	
FDHR3-7	GNB	+	-	-	+	
FDHR3-8	GPC	+	-	-	+	
FDHR3-9	GNB	+	-	-	+	
FDHR3-10	GNB	+	-	-	+	

GPB: Gram positive bacilli, GPC: Gram positive cocci, GNB: Gram negative bacilli, GNC: Gram negative cocci

3.6. Molecular Identification of Faisalabad variety endophytes

Out of the 45 isolated endophytes of Faisalabad variety form all the three harvestings, 10 endophytes showing most promising PGP results were identified through 16S rRNA gene sequencing. In the first harvesting, γ-Proteobacteria were prominent including *Providencia stuartii* and *Acinetobacter calcoaceticus* in the root and shoot histospheres, respectively. Whereas in the second harvesting, Firmicutes dominated, including *Bacillus* sp. in the root histosphere and *Staphylococcus* sp. in the shoot histosphere. However, during the third harvesting, bacteria belonging to the class Actinobacteria became prominent PGP-bacteria, both in the root and shoot histospheres. The results were confirmed by making phylogenetic tree (Figure 2). The sequences were submitted in the GenBank under the accession numbers given in Table 2.

3.7 Analysis of the PGP endophytes of Lasani variety

The CFU g^{-1} of the Lasani variety samples increased with the age of the plant variety in both the root and shoot samples. The Highest CFU g^{-1} of the root and shoot samples, i.e. 3.5 $\times 10^7$ and 3.0 $\times 10^7$, respectively, was observed during the third harvesting. For the shoot samples, the diversity decreased with the increasing age of the plant variety. Whereas for the root samples, the third harvesting of Lasani variety gave the highest diversity of endophytes.

3.8. PGP activities of Lasani variety endophytes

In the first harvesting, three Lasani shoot endophytes (LAHP1-6, LAHP1-7 and LAHP1-8) produced auxin in concentrations above 10 µg ml^{-1}. During the second harvesting, LAHP2-1 and LAHP2-7 produced auxin in significant concentrations, i.e. 20.75 and 19.44 µg ml^{-1}, respectively. The highest concentration of auxin was produced by the endophytes of the third harvesting, especially by LAHP3-2, up to 42.88 µg ml^{-1} (Figure 4).

In the root-histosphere samples of Lasani variety, endophytes of the second harvesting produced higher concentrations of auxin as compared to the first and third harvestings. LAHR1-1 produced the highest concentration of auxin during the first harvesting, up to 31 µg ml^{-1}. During the second harvesting, the highest concentration of auxin was produced by LAHR2-4, up to 45.58 µg ml^{-1}. However, during the third harvesting, only one isolate (LAHR3-2) produced significant auxin concentration (10.94 µg ml^{-1}) (Figure 4). The high auxin production by Lasani root endophytes is again in accordance with the increased growth rate of Lasani root.

In case of phosphate solubilization, LAHP2-7 and LAHR2-7 from the second harvesting from the shoot and root samples, respectively, gave positive results. As the growth rate was highest during the second harvesting, more phosphate was needed by the plant, which presumably is helped by these isolates. None of the isolates of Lasani variety were found to produce HCN (Table 4). Díaz et al. [16] have also reported the presence of plant growth promoting endophytes in wheat.

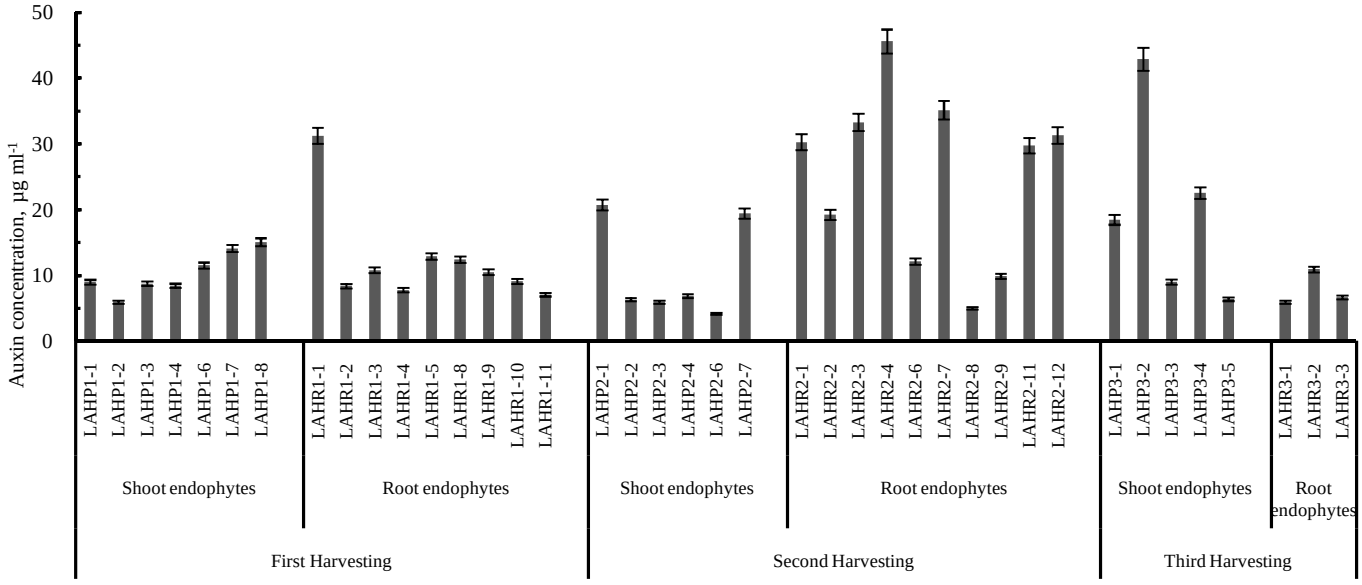

Figure 4. Auxin production by Lasani shoot- and root-endophytes isolated during different growth stages of the plants.

Table 4. Some of the major traits of Lasani variety endophytes

Endophytic isolates	Staining properties	Nitrogen fixation	HCN production	Phosphate solubilization	Auxin production	Species Identified by 16S sequencing
FIRST HARVESTING						
Lasani shoot endophytes						
LAHP1-1	GPB	+	-	-	+	
LAHP1-2	GPB	+	-	-	+	
LAHP1-3	GNB	+	-	-	+	
LAHP1-4	GPC	+	-	-	+	
LAHP1-6	GNB	+	-	-	+	
LAHP1-7	GPB	+	-	-	+	
LAHP1-8	GPB	+	-	-	+	*Bacillus megaterium*
Lasani root endophytes						
LAHR1-1	GPB	+	-	-	++	*Bacillus marisflavi*
LAHR1-2	GNB	+	-	-	+	
LAHR1-3	GNB	+	-	-	+	
LAHR1-4	GPC	+	-	-	+	
LAHR1-5	GNB	+	-	-	+	
LAHR1-8	GNB	+	-	-	+	
LAHR1-9	GPB	+	-	-	+	
LAHR1-10	GPB	+	-	-	+	
LAHR1-11	GPB	+	-	-	+	
SECOND HARVESTING						
Lasani shoot endophytes						
LAHP2-1	GPB	+	-	-	++	
LAHP2-2	GPB	+	-	-	+	
LAHP2-3	GNB	+	-	-	+	
LAHP2-4	GNB	+	-	-	+	
LAHP2-6	GPC	+	-	-	+	
LAHP2-7	GNB	-	-	+	+	*Acinetobacter lwoffii*
Lasani root endophytes						
LAHR2-1	GNB	+	-	-	++	
LAHR2-2	GNB	+	-	-	+	
LAHR2-3	GNB	+	-	-	++	
LAHR2-4	GPB	+	-	-	+++	*Proteus mirabilis*
LAHR2-6	GNB	+	-	-	+	
LAHR2-7	GPB	+	-	+	++	*Brevibacterium frigoritolerans*
LAHR2-8	GPB	-	-	-	+	
LAHR2-9	GNB	+	-	-	+	
LAHR2-11	GNB	+	-	-	++	
LAHR2-12	GPC	+	-	-	++	
THIRD HARVESTING						
Lasani shoot endophytes						
LAHP3-1	GNC	Weak +	-	-	+	
LAHP3-2	GPB	+	-	-	+++	*Bacillus brevis*
LAHP3-3	GNC	+	-	-	+	
LAHP3-4	GPB	+	-	-	++	*Bacillus subtilis*
LAHP3-5	GNB	+	-	-	+	
Lasani root endophytes						
LAHR3-1	GPB	-	-	-	+	
LAHR3-2	GNB	-	-	-	+	
LAHR3-3	GPB	+	-	-	+	*Bacillus aerius*

GPB: Gram positive bacilli, GPC: Gram positive cocci, GNB: Gram negative bacilli, GNC: Gram negative cocci

3.9. Molecular Identification of Lasani variety endophytes

Out of the 40 endophytic isolates of Lasani variety form all the three harvesting stages, endophytes showing the best PGP assays results were identified by 16S rRNA gene sequencing. During the first harvesting, *Bacillus* sp. (Firmicutes) were dominant in both the root and shoot histospheres. However, in the second harvesting, bacteria belonging to the classes γ-Proteobacteria and Actino-bacteria were dominant. In the third harvesting, Firmicutes were again dominant both in the root and shoot histospheres. The results were confirmed by making phylogenetic tree (Figure 2). The sequences were submitted in the GenBank under the accession numbers given in Table 2. Such shift in microbial communities, also called "Succession", is mainly dependent on the plant age and genetic variability, and occurs throughout the plant life

[31]. Bacteria best adaptable to a certain environmental condition replace other bacteria [32].

4. Conclusion

Many endophytes are exclusive for certain varieties, whereas few are able to colonize all the varieties. Moreover, shifts in the endophytic community also occur with time indicating the succession of microbial communities with environmental changes and the age of the plants. Seher wheat variety harbours most superior PGP endophytes as compared to the other two varieties. Therefore, it can be concluded that the superior growth and yield of Seher wheat variety is helped by the microbes present in it. These strains can be possibly used as inoculant to promote the growth and yield of other wheat varieties. However, this idea needs to be proved by doing further experiments on other plant varieties as well.

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6. Conflict of Interest

There is no conflict of interest among the authors regarding this research.

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گندم حاوی اندوفیت‌های باکتریایی افزایش دهنده برتر رشد گیاهی

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چکیده

سابقه و هدف: هدف از این مطالعه مقایسه فلور میکروبی اندوفیتیک ارقام گوناگون گندم است تا بررسی شود که آیا رقمی دارای راندمان بهتر و باکتری‌های افزایش دهنده برتر رشد گیاهی می‌باشد. چنین باکتری‌هایی در زیست فناوری غذایی به‌عنوان افزایش دهنده رشد محصول مفید می‌باشند.

مواد و روش‌ها: سه رقم گندم (Seher، Faisalabad و Lasani) انتخاب شدند، رقم Seher برترین رقم بود. باکتری‌های اندوفیتیک از هیستوسفیر برگ‌ها و ریشه‌ها در مراحل گوناگون رشد گیاه جدا شدند. باکتری‌های جدا شده از نظر فعالیت افزایش دهندگی رشد گیاه مورد بررسی قرار گرفتند. اغلب اندوفیت‌های تمام سه واریته تثبیت کننده ازت در مدت رشد گیاه بودند. بهترین نتیجه به‌دست آمده از باکتری‌های جدا شده از طریق توالی ژن 16S rRNA شناسایی شدند. آزمون آماری با استفاده از نرم‌افزار مایکروسافت اکسل 2013 انجام شد. تمام آزمون‌ها در سه تکرار انجام شد.

یافته‌ها و نتیجه‌گیری: اندوفیت‌های رقم Seher بیشترین توانایی در افزایش رشد گیاهی را نشان داد. در میان اندوفیت‌های جوانه، ایزوله Seher بیشترین میزان تولید آوکسین در ایزوله SHHP1-3 و تا میزان 51/9 میکروگرم در میلی‌لیتر را نشان داد. باکتری‌هایی که توانایی افزایش رشد قابل توجهی را نشان دادند، از طریق توالی 16S rRNA شناسایی شدند. گونه‌های باسیلوس، پروتئوباکترها و اکتینوباکترها باکتری‌های غالب بودند که تمام صفات افزایش رشد گیاه را از خود بروز دادند. نتیجه‌گیری می‌شود که یکی از دلایل رشد و راندمان بهتر رقم Seher در مقایسه با دو رقم دیگر، دارابودن اندوفیت های افزایش دهنده برتر رشد گیاهی می‌باشد. نتایج بررسی امکان کاربرد ایزوله‌های انتخابی برای افزایش رشد گندم و نیز افزایش بهره‌وری کشاورزی را تأیید می‌کند. کاربرد چنین باکتری‌هایی برای افزایش راندمان گندم می‌تواند مفید باشد و در زیست فناوری می‌تواند کمک کند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ تعارض منافعی وجود ندارد.

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- تولید HCN
- تثبیت ازت
- باکتری های PGP
- انحلال فسفات
- گندم

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