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# Secondary Metabolites Production and Plant Growth Promotion by *Pseudomonas chlororaphis* and *P. aurantiaca* Strains Isolated from Cactus, Cotton, and Para Grass S

**Izzah Shahid[1], Muhammad Rizwan[2], Deeba Noreen Baig[1], Rahman Shahzaib Saleem[3], Kauser A. Malik[1], and Samina Mehnaz[1]***

[1]*Department of Biological Sciences, Forman Christian College (A Chartered University), Lahore 54600, Pakistan*
[2]*Applied Chemistry Research Center, Pakistan Council of Scientific and Industrial Research, Lahore 54600, Pakistan*
[3]*Department of Chemistry, School of Science and Engineering, Lahore University of Management Sciences, Lahore 54792, Pakistan*

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**Corresponding author*
Phone: +92-42-99231581-88;
Fax: +92-42-99230703;
E-mail: saminamehnaz@fccollege.edu.pk

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Fluorescent pseudomonads have been isolated from halophytes, mesophytes, and xerophytes of Pakistan. Among these, eight isolates, GS-1, GS-3, GS-4, GS-6, GS-7, FS-2 (cactus), ARS-38 (cotton), and RP-4 (para grass), showed antifungal activity and were selected for detailed study. Based on biochemical tests and 16S rRNA gene sequences, these were identified as strains of *P. chlororaphis* subsp. *chlororaphis* and *aurantiaca*. Secondary metabolites of these strains were analyzed by LC-MS. Phenazine-1-carboxylic acid (PCA), 2-hydroxy-phenazine, Cyclic Lipopeptide (white line-inducing principle (WLIP)), and lahorenoic acid A were detected in variable amounts in these strains. *P. aurantiaca* PB-St2 was used as a reference as it is known for the production of these compounds. The *phzO* and *PCA* genes were amplified to assure that production of these compounds is not an artifact. Indole acetic acid production was confirmed and quantified by HPLC. HCN and siderophore production by all strains was observed by plate assays. These strains did not solubilize phosphate, but five strains were positive for zinc solubilization. Wheat seedlings were inoculated with these strains to observe their effect on plant growth. *P. aurantiaca* strains PB-St2 and GS-6 and *P. chlororaphis* RP-4 significantly increased both root and shoot dry weights, as compared with uninoculated plants. However, *P. aurantiaca* strains FS-2 and ARS-38 significantly increased root and shoot dry weights, respectively. All strains except PB-St2 and ARS-38 significantly increased the root length. This is the first report of the isolation of *P. aurantiaca* from cotton and cactus, *P. chlororaphis* from para grass, WLIP and lahorenoic acid A production by *P. chlororaphis*, and zinc solubilization by *P. chlororaphis* and *P. aurantiaca*.

**Keywords:** Phenazines, white line-inducing principle (WLIP), lahorenoic acid, zinc solubilization, *Pseudomonas aurantiaca*, *Pseudomonas chlororaphis*

## Introduction

Use of pesticides and chemical fertilizers has declined in several countries as growers return to organic farming. However, in Pakistan, the use of chemical pesticides is increasing day by day. Not only does it threaten public health and the environment, but it puts farmer's livelihood in jeopardy as well. Therefore, there is an immense need to develop other methods for adequate plant protection. The use of biopesticides can be a good alternative.

There has been a large body of literature describing the potential use of plant-associated bacteria as agents stimulating plant growth and managing soil and plant health [1–3]. These bacteria colonize the root surfaces and rhizosphere, live as endophytes in stems, leaves, tubers, and other organs, and can establish associations with host

plants without harming them [4, 5]. Despite their different ecological niches, rhizobacteria and endophytic bacteria use some common mechanisms to promote plant growth and control phytopathogens, such as production of inhibitory allelochemicals and induction of systemic resistance in host plants against a broad spectrum of pathogens [6–8], and consequently have the potential to provide an alternative to chemical fertilizers and fungicide/pesticides.

Among rhizobacteria, fluorescent pseudomonads have been described as effective biocontrol agents against plant diseases. Fluorescent pseudomonad species such as *Pseudomonas fluorescens*, *P. aeruginosa*, *P. aureofaciens*, *P. putida*, and *P. pyrrocinia* have been demonstrated to show antifungal activity with varying degrees of antagonism [9]. The antifungal and antibacterial activities of pseudomonads are due to the production of phenazines, 2-4-diacetyl phloroglucinol, pyrrolnitrin, pyoluteorin, cyclic lipopeptides [10, 11], and rhizoxin [12]. Phenazine-type antibiotics, heterocyclic nitrogen-containing brightly colored pigments, are especially active against lower fungi and most gram-positive and gram-negative bacteria, and play a vital role in biological control. Several *Pseudomonas* strains have already been marketed as commercial biocontrol products. For example, Blightban A506 (NuFarm Inc. USA), based on *Pseudomonas fluorescens* A506, provides protection to almond, apple, apricot, blueberry, cherry, peach, pear, strawberry, tomato, and potato against *Erwinia amylovora*, frost injury, and russet-inducing bacteria. Mycolytin is an antifungal biopesticide that contains *P. aurantiaca* M-518 [13]. Cedomon and Cerall (BioAgri AB, Sweden) are based on a *P. chlororaphis* strain providing protection against seedborne diseases and promote growth in wheat and barley. A group of researchers in Argentina are working on the commercial launch of a bioformulation through Laboratorios Biagro S.A. based on *P. aurantiaca* SR1, as a biofertilizer and biocontrol agent [14].

We have isolated the strains of fluorescent pseudomonads from a halophyte (para grass), mesophyte (cotton), and xerophyte (cactus). The objectives of this study included selection of fluorescent pseudomonads based on antifungal activity against local pathogens; characterization of the selected *Pseudomonas* isolates; search for those secondary metabolites that have been previously reported for fluorescent pseudomonads [15]; screen for HCN, siderophore production, and phosphate and zinc solubilization by using bioassays; and observation of the effect of these strains on wheat plant growth by inoculation. *P. chlororaphis* subspecies *aurantiaca* strain PB-St2 was previously isolated from sugarcane by our laboratory and its secondary metabolites were reported [16–18]. In this study, it was used as a reference/standard for phenazines, the cyclic lipopeptide white line-inducing principle (WLIP) and lahorenoic acid derivatives.

## Materials and Methods

### Selection of Fluorescent Pseudomonads Based on Antifungal Activity

Our research group has isolated more than 200 rhizobacteria from rice, wheat, cotton, sugarcane, *Kochia indica*, *Suaeda fruticosa*, para grass and cactus (deposited in FCC Bacterial Culture Bank). Based on the known abilities of "fluorescent pseudomonads" as plant growth promoters and biocontrol agents, only these were selected for this study and maintained on King's B medium [19]. The antagonistic activity of 17 fluorescent pseudomonads against fungal phytopathogens (*Fusarium solani, F. oxysporum, F. monoliforme, Rhizopus* sp., *Curvularia* sp., and *Aspergillus* sp.) was checked by using bioassays. A small plug cut from a fresh culture of target fungus was seeded at the center of malt extract agar medium. Bacterial cultures were spotted 2 cm away from the center (one isolate per plate) and incubated for 5–10 days at 25°C. Antifungal activity was observed by formation of an inhibition zone of mycelial growth.

### Biochemical and Molecular Characterization of Selected Isolates

Based on their antifungal activity, eight strains were selected. QTS-24 bacterial identification kits (DESTO Laboratories, Pakistan) were used for biochemical characterization of these strains. Bacterial DNA was isolated using a standard genomic DNA extraction protocol [20]. The 16S rRNA gene was amplified from genomic DNA of isolates RP-4, ARS-38, GS-1, GS-3, GS-4, GS-6, GS-7, and FS-2 by polymerase chain reaction (PCR). The forward primer FGPS 1509-153 (5′-AAGGAGGTGATCCAGCCGCA-3′) and reverse primer FGPS 4-281 (5′-AGAGTTTGATCCTGGCTCAG-3′) [21] were used for the amplification of all strains except FS-2. The denaturation temperature was 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 55°C for 50 sec, and 72°C for 90 sec, and a final extension at 72°C for 10 min. A reaction mixture of 50 µl was prepared by using Taq buffer 5 µl (10X), $MgCl_2$ 2 µl (25 mM), Taq polymerase (5 U) 2 µl, dNTPs 2 µl (2.5 mM), each of forward and reverse primer (20 pmol) 1 µl, $dH_2O$ 35 µl, and the template DNA 2 µl (>50 ng/µl). A large fragment of the 16S rRNA gene for FS-2 was amplified in a 50 µl reaction mixture using universal forward primer P1 (5′-CGggatccAGAGTTTGATCCTGGTCAGAACGAA CGCT-3′) and universal reverse primer P6 (5′-CGggatccTACG GCTACCTTGTTACGACTTCACCCC-3′) [22]. The denaturation temperature was 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min, and a final extension at 72°C for 10 min. PCR chemicals were purchased from Fermantas, USA. A PCR purification kit (Favorgen) was used for the purification of the PCR products. The 1.5 kb PCR products were sequenced with both reverse and forward primers by Eurofins, USA.

Sequences were compared with these on NCBI GenBank, using BLAST search tool. An alignment of nucleotide sequences of *Pseudomonas* isolates GS-1, GS-3, GS-4, GS-6, GS-7, FS-2, ARS-38, and RP-4 was carried out using Clustal W (ver. 1.83). *Klebsiella oxytoca* (Accession No. HF678365) was used as the outgroup. The evolutionary history was concluded using the neighbor-joining method. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were calculated using the maximum composite likelihood method and were in the units of number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset using the complete deletion option. There were a total of 1,423 positions in the final dataset. Phylogenetic analysis was conducted with MEGA4 software. These sequences were deposited in the GenBank database and accession numbers were obtained (*i.e.*, GS-1, KT888006; GS-3, LN898138; GS-4, KT888007; GS-6, KT888008; GS-7, KT888009; RP-4, KT888010; FS-2, LN898137; and ARS-38, KJ094432).

**Identification and Quantification of Secondary Metabolites by Liquid Chromatography Mass Spectrometry (LC-MS)**

Antimicrobial compounds were extracted individually, from 500 ml of 96-h-old bacterial cultures of PB-St2, FS-2, RP-4, ARS-38, GS-1, GS-3, GS-4, GS-6, and GS-7 grown in King's B medium. After the cultures were centrifuged at 3,376 ×*g* for 20 min, the supernatants were acidified to pH 2 with 1N HCl. Acidified supernatants were extracted twice with an equal volume of ethyl acetate (1:1 (v/v)). The organic layer was dehydrated with anhydrous sodium sulfate and evaporated to dryness. The residues of extracts from all bacterial strains were re-dissolved in methanol and used for analysis.

The extracts of all strains were subjected to liquid chromatography-electrospray ionization-mass spectrometry analysis (LC/ESI/MS) for the characterization of secondary metabolites. LC-ESI MS/MS runs were performed using a Thermo Finnegan HPLC system coupled to a LCQ Advantage Max ESI-Ion trap mass spectrometer (Thermo Electron, USA). LC-MS/MS data were acquired and processed using Xcalibur 2.0 software. Chromatographic separations were achieved on a Thermo Hypersil Gold C18 column (4.6 × 250 mm, 5 µm particle size). The temperature of the column compartment was kept at 25°C and 20 µl of sample was loaded on the column. A gradient used to separate the metabolites consisted of 0.1% formic acid in water (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B). The total LC-MS/MS run was of 55 min with a flow rate of 0.7 ml/min. The gradient was as follows: the initial mobile composition was 5% of solvent B and kept constant for 1 min, then increased to 95% in 36 min, maintained for 5 min and returned to the initial conditions (5% solvent B) in 3 min, and maintained for 10 min prior to the next run. A data-dependent protocol was used in ESI positive mode with a mass range of 150–1,500 amu and two scan events were employed. The first scan event was a full scan (150–1,500), and the second scan event was a dependent scan triggering $MS^2$ acquisition on the most abundant ion in the first scan event. Data were acquired at the normalized collision energy of 45 eV. The heated capillary was maintained at 350°C, and sheath and auxiliary/sweep gases were at 60 and 25 arbitrary units, respectively. The source voltage was set to 4.5 kV with 10 V capillary voltage. The ESI-mass spectra obtained were used to characterize the surfactant ionization behavior, and $[M + H]^+$ and $[M + Na]^+$ ions were monitored for phenazines (2-hydroxy-phenazine and phenazine-1-carboxylic acid (PCA)), lahorenoic acid A, and cyclic lipopeptide (WLIP). In addition, the ESI-MS/MS fragmentation behaviors of identified peaks were investigated to confirm the structure of these secondary metabolites.

Phenazine derivatives of *Pseudomonas* are colored compounds and they are extracted from late log phase supernatants. Using reference strain PB-St2, PCA and 2-hydroxy-phenazine were separated by TLC and determined quantitatively by HPLC and detected with the diode array detector. For detection of lahorenoic acid A and WLIP, PB-St2 purified fractions were considered as the reference [18]. These fractions were quantified through high-performance liquid chromatography (HPLC) and fractions of the other eight strains were quantified with the reference peaks of PB-St2.

**Amplification of *phzO* and *PCA* Genes from *Pseudomonas* Strains**

Amplification of the genes responsible for the production of 2-hydroxy phenazine and PCA was done using gene-specific primers. Primers used for these genes were *phzO* 10 (5′-AAGTGGCATGGC TCGAACAA-3′), *phzO* 30-84 (5′-AAGTCCAGATGCGAAAGAAC-3′) and *PCA2a*- (5′-TTGCCAAGCCTCGCTCCAAC-3′), *PCA3b*- (5′-CCGCGTTGTTCCTCGTTCAT-3′) [23]. PCR cycles were performed as described previously [23]. The PCR products were analyzed on 1% agarose gel. Respective bands were excised and purified using a FAVORGEN gel purification kit. Amplifications were conducted with positive control strain PB-St2 [16] to confirm PCR conditions. PCR products were sequenced (Eurofins) and analyzed using NCBI BLAST (BLASTn) and alignment tools.

**Identification and Quantification of Indole Acetic Acid (IAA) by HPLC**

Bacterial cultures were grown in King's B medium [19] supplemented with L-tryptophan (100 mg/l). After 7 days of growth, bacterial cells were centrifuged at 3,376 ×*g* for 15 min, the pH of the supernatants was adjusted to 2.8 with HCl, and the solution was extracted twice with an equal volume of ethyl-acetate [14]. Extracts were evaporated to dryness and resuspended in 1 ml of methanol. These samples were analyzed by HPLC (Shimadzu LC 9A) using a UV detector (280 nm) and ZORBAX SB-C 18 (5 µm, 4.6 × 254 mm) column with 20 µl injection volume. Methanol: acetic acid: water (30:1:70 (v/v/v)) was used as the mobile phase at the rate of 1.2 ml/min [24]. Indole-3-acetic acid (Sigma, USA) was used as the standard, and CSW32 software (DataApex, Czech Republic) was used for quantification purpose.

**Detection of HCN and Siderophore Production by Using Bioassays**

Qualitative determination of hydrocyanic acid (HCN) production was done by streaking each bacterial isolate on LB agar plates. A filter paper saturated with an alkaline picrate solution (g/l: picric acid, 2.5; $Na_2CO_3$, 12.5; pH 13) was placed into the lid of the petri plate [25]. After 3–4 days incubation at 28 ± 2°C, assessment of HCN production was made by the change in color of filter paper from yellow to brown/reddish brown.

Siderophore production was detected by using the O-CAS method [26]. Bacterial culture-containing plates were incubated at 28°C for 24 h. CAS medium [27] was overlaid on these cultures, and a change in color from blue to orange (hydroxamate-type siderophore) or purple (catechol-type siderophore) was considered as a positive result. *Pseudomonas aurantiaca* PB-St2 was used as a positive control [17]. Sterilized LB and water were used as the negative control.

**Phosphorous and Zinc Solubilization Assays**

Each bacterial culture was inoculated on NBRIP [28] and Pikovskaya agar [29] plates containing tricalcium phosphate as the insoluble phosphate source. Plates were incubated at 28 ± 2°C for 7–10 days and observed for the formation of halo zones around the bacterial colony. For the identification of zinc-solubilizing trait, Tris-minimal agar medium was supplemented with insoluble zinc compounds (zinc oxide, zinc sulfate, and zinc carbonate), individually, at a concentration of 0.1% zinc [30]. Fresh bacterial cultures were spot inoculated and the plates were incubated for 20–28 days. Halo zones around bacterial colonies indicated zinc solubilization efficiency.

**Effect of *Pseudomonas* Strains on the Growth of Wheat Plants**

Pot experiments were carried out in a climate control room to check the effectiveness of *Pseudomonas* isolates as bioinoculants. Plastic pots were filled with 300 g of autoclaved sand and provided with 50 ml of full-strength Hoagland's solution [31]. The local wheat variety FSD-Control-2008 was used for this study. Using 0.01 N sodium hypochlorite solution (Chlorex), wheat seeds were surface sterilized for 8–10 min, then washed with distilled water five times and dried on sterilized filter paper. Later on, seeds were transferred to 1% water agar plates. These plates were incubated at 28°C for a period of 3–5 days for the germination of seeds. All plants were inoculated with 1 ml of individual bacterial culture containing $1 \times 10^7$ cells/ml in 10 replicates for each treatment. Plants were kept in a climate control room at relative humidity of 60% with a 12 h photoperiod (200 $\mu M \cdot m^{-2} \cdot s^{-1}$ at pot heights with fluorescent lights, 15°/20°C). The experiment was set up in a completely randomized design. Plants were watered daily and harvested after 30 days. Roots were thoroughly washed to remove the sand, and the root length of individual plants of each treatment was noted. Roots and shoots were separated and dried in an oven for 72 h at 70°C and the dry weights were recorded. The effect of inoculation was determined by analysis of variance using IBM SPSS Statistics 23, and means were compared with application of the Tukey test at α 0.05.

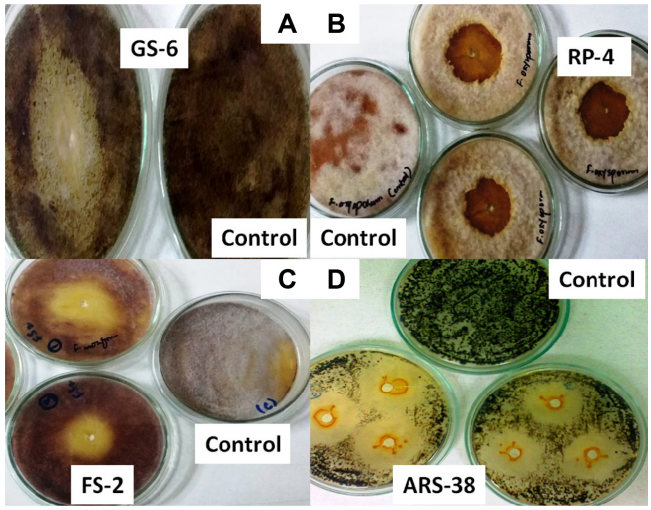


**Fig. 1.** Antifungal assay of *Pseudomonas* isolates with (**A**) *Aspergillus* sp., (**B**) *Fusarium oxysporum*, (**C**) *Fusarium moniliforme*, and (**D**) *Curvularia* sp.

## Results

**Fluorescent Pseudomonads Showed Antifungal Activity and Were Identified as Subspecies of *Pseudomonas chlororaphis***

Based on fluorescence under UV light, 17 strains were identified as fluorescent pseudomonads. Eight strains showed antifungal activity against pathogenic fungi and were selected for detailed study. These strains showed antifungal activity against all fungal pathogens used in this study (Fig. 1). The zone of inhibition ranged from 0.7 to 2.6 cm depending on the *Pseudomonas* strain and fungal pathogen. The maximum zone of inhibition by all strains, except GS-4, was shown against *Aspergillus* sp. FS-2, and ARS-38 showed the biggest zone of 1.8 cm against *F. moniliforme*. A maximum inhibition zone, 1.7 cm, against *F. oxysporum* was shown by FS-2 and GS-6, and 1.8 cm against *F. solani* by ARS-38. Against *Aspergillus* sp. and *Curvularia* sp., maximum antifungal activity was shown by ARS-38 (2.6 cm) and GS-4 (1.7 cm). For *Aspergillus* sp., seven strains showed 1.2 to 1.4 cm zones, and GS-3 and GS-1 showed smaller (0.7 and 0.9 cm, respectively) zones (Table S1).

These isolates were biochemically compared with each other for carbon source utilization and the presence/absence of specific enzymes with the help of QTS-24 identification kits (Table 1). All strains were positive for ortho-nitrophenyl-β-galactosidase; arginine dihydrolase enzyme; utilization of sodium citrate; acid production from sucrose, arabinose,

**Table 1.** Identification of *Pseudomonas* isolates by using QTS-24 identification kits.

| Host types | Mesophyte | Halophyte | Xerophyte | | | | | |
|---|---|---|---|---|---|---|---|---|
| Host plants | Cotton | Paragrass | Cactus | | | | | |
| Biochemical tests | ARS-38 | RP-4 | FS-2 | GS-1 | GS-3 | GS-4 | GS-6 | GS-7 |
| MALO | _ | + | + | + | + | + | + | + |
| VP | + | _ | _ | _ | _ | _ | _ | _ |
| GEL | + | + | _ | _ | _ | + | + | + |
| GLU | + | + | + | _ | + | + | + | + |
| $NO_3/N_2$ | -/- | -/+ | -/- | -/+ | -/+ | -/+ | -/+ | -/+ |
| MALT | + | + | + | + | - | + | + | + |
| ADO | + | + | + | + | _ | + | + | + |
| MEL | + | + | + | + | _ | + | + | + |
| RAFF | + | + | + | _ | _ | + | + | + |

Symbols: MALO, Sodium malonate; VP, Voges-Proskauer test (Acetoin); GEL, gelatin hydrolysis; GLU, acid from glucose; $NO_3$, nitrate reduction; MAL, acid from maltose; ADO, acid from adonitol; MEL, acid from melibiose; RAF, acid from raffinose.

All strains were positive for ONPG, ortho-nitrophenyl-β-galactoside; ADH, arginine dihydrolase; CIT, sodium citrate; SUC, acid from sucrose; MAN, acid from mannitol; ARA, acid from arabinose; RHA, acid from rhamnose; INO, acid from inositol; CO, cytochrome oxidase; catalase; and motility. All strains showed negative results for the production of LDC, lysine decarboxylase; ODC, ornithine decarboxylase; $H_2S$, $H_2S$ production; URE, urea; and TDA, tryptophane deaminase.

-, negative; +, positive; -/-, both test negative; -/+, one is negative and one is positive.

and mannose; catalase; cytochrome oxidase; and motility; and negative for lysine decarboxylase, ornithine decarboxylase, desulfhydrase, urease, and tryptophan deaminase; and acid production from sorbitol. Except for all strains could utilize sodium malonate. Gelatinase enzyme was detected in five strains. ARS-38 was positive for the Voges and Proskauer test, whereas the rest were negative. Except for GS-1, the other strains utilized glucose. Based on biochemical tests, all strains were identified as *Pseudomonas chlororaphis*.

For the 16S rRNA gene, a 1.5 kb PCR product was

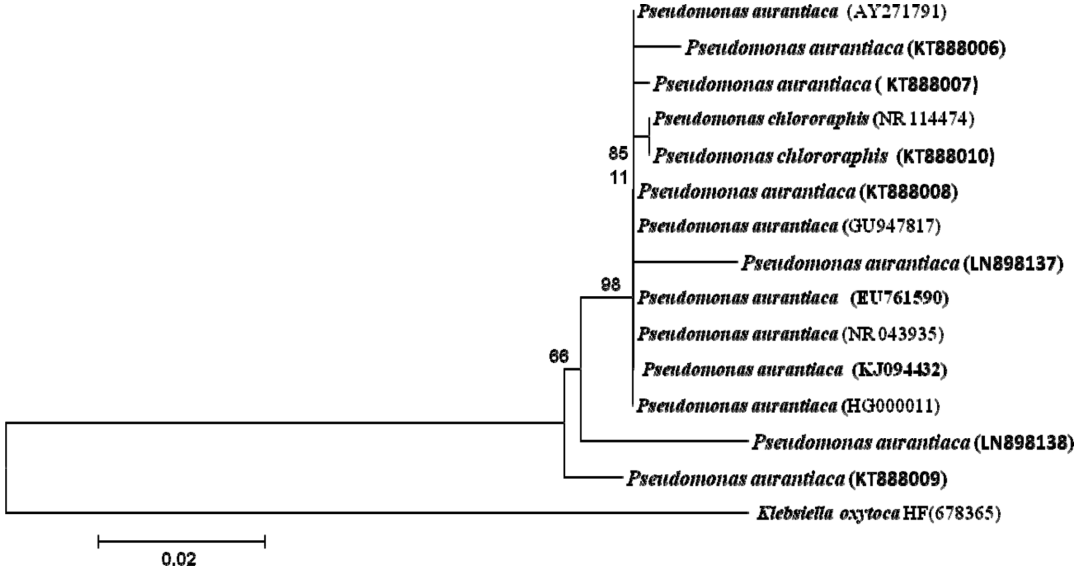


**Fig. 2.** Neighbor-joining tree of the 16S rRNA gene sequences of *Pseudomonas chlororaphis* subsp. *aurantiaca* and *Pseudomonas chlororaphis* subsp. *chlororaphis* strains.

Sequences of closest members were obtained from databases, and accession numbers are in parentheses. The isolates from para grass (RP-4) and cactus (GS-1, GS-3, GS-4, GS-6, GS-7, and FS-2) are in bold letters. There were 1,423 nucleotides in the final dataset. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances were computed using the maximum composite likelihood method and are in units of number of base substitutions per site.

obtained for each strain. Purified PCR products were sequenced and data were searched through NCBI BLAST. 16S rRNA gene sequence data confirmed that these are strains of *P. chlororaphis*. However, seven of these, ARS-38, GS-1, GS-3, GS-4, GS-6, GS-7, and FS-2, were identified as *Pseudomonas chlororaphis* subsp. *aurantiaca* and RP-4 as *Pseudomonas chlororaphis* subsp. *chlororaphis.* The phylogenetic tree based on 16S rRNA gene sequences showing homology of these strains with one another and closely related strains is shown in Fig. 2. All isolates showed 99% similarity with previously reported *P. aurantiaca* strain PB-St2 (Accession No. EU761590) and GS-1, GS-4, GS-6, and GS-7 also showed 99% similarity with *Pseudomonas chlororaphis* subsp. *aurantiaca* strain ARS-38 (Accession No. KJ094432). RP-4 showed 99% similarity (1,416/1,425 positions) with the sequence of *Pseudomonas chlororaphis* Type strain (Accession No. NR114474). FS-2 showed 99% homology (1,485/1,500 positions) with the sequence of *Pseudomonas chlororaphis* subsp. *aurantiaca* JD37 (Accession No. CP009290). The evolutionary distances were computed using the maximum composite likelihood method and are in units of number of base substitutions per site. The phylogenetic tree exhibited close relationship between *P. aurantiaca* strains ARS-38, GS-4, GS-6 and FS-2, and put them close to PB-St2 in the upper part of the tree, while RP-4 was very close to another *P. chlororaphis* strain. GS-1, GS-3, and GS-7 were comparatively less related to the rest of the strains and located at the lower end of the tree.

### *Pseudomonas* Strains Produced Secondary Metabolites (LC-MS Data)

The secondary metabolites lahorenoic acid A, PCA, 2-

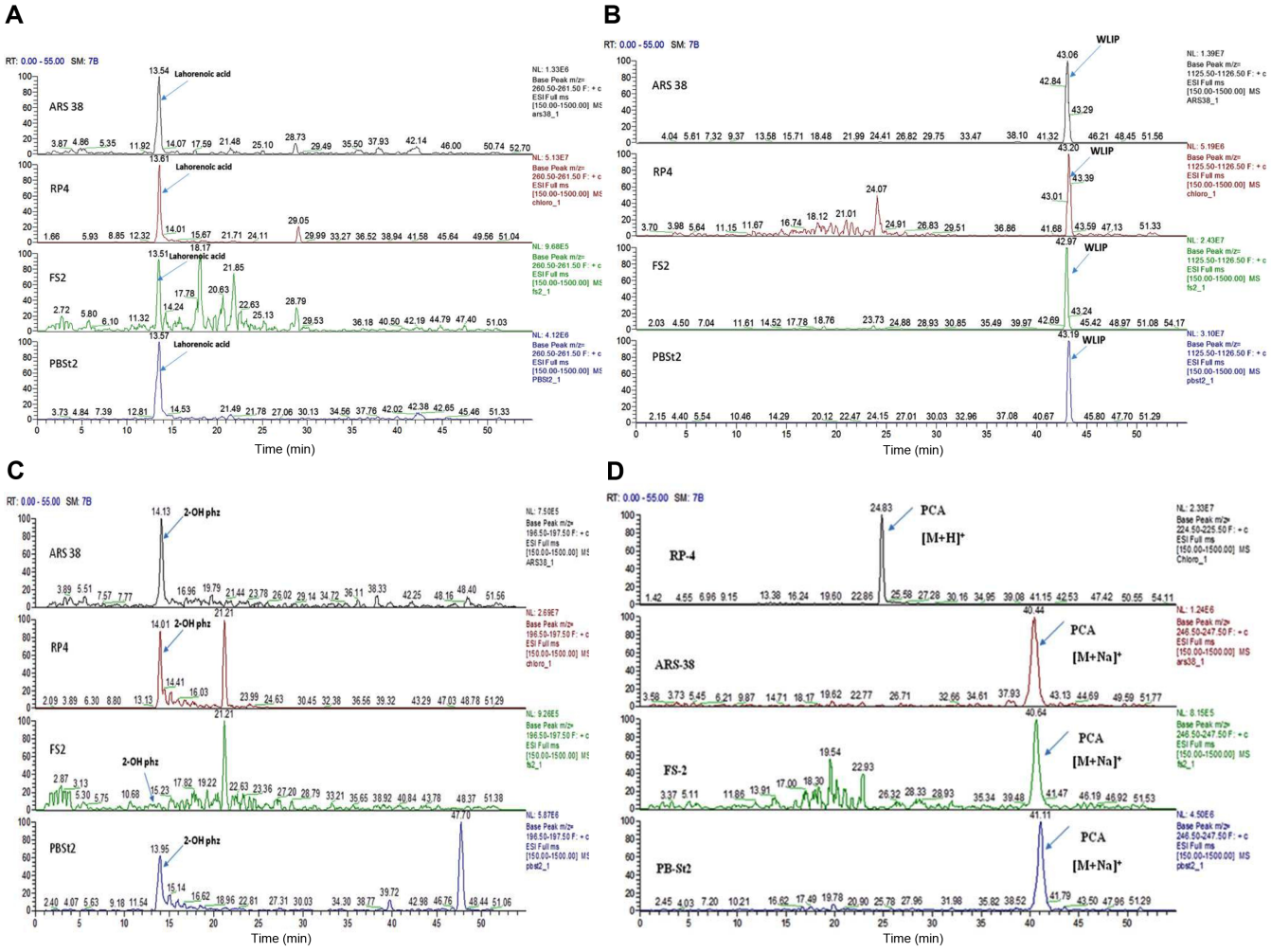


**Fig. 3.** Extracted ion current chromatograms for (**A**) lahorenoic acid A *m/z* 261 $[M+H]^+$, (**B**) white line-inducing principle *m/z* 1126 $[M+H]^+$, (**C**) 2-hydroxy-phenazine *m/z* 197 $[M+H]^+$, and (**D**) phenazine-1-carboxylic acid *m/z* 225 $[M+H]^+$ and *m/z* 247 $[M+Na]^+$ for PB-St2 (sugarcane reference strain), ARS-38 (cotton), RP-4 (para grass), and FS-2 (cactus).

hydroxy-phenazine and WLIP were analyzed through HPLC-ESI/MS in positive full-scan mode (*m/z* 150–1,500) (Fig. 3). PCA (retention time (RT) 24.83), 2-hydroxy-phenazine (RT 14.13), WLIP (RT 43.06), and lahorenoic acid A (RT 13.54) were characterized by intense peaks with $[M+H]^+$ values at 225, 197, 1,126, and 261, respectively (Figs. S1 to S4). The PCA sodium adduct eluted at retention time 40.44 was characterized with an intense peak with $[M+Na]^+$ value at 247. PCA was detected in all strains; WLIP was detected in all strains, except GS-7; and similarly production of lahorenoic acid A and 2-hydroxy-phenazine was confirmed in all strains except GS-6.

PB-St2 was used as a reference for the quantification of secondary metabolites produced by other strains. Among these strains, *P. chlororaphis* RP-4 produced the maximum amount of PCA, 2-hydroxy-phenazine, and lahorenoic acid A of 7.4, 8.5, and 16 μg/ml, respectively (Fig. 4). However, a minimum amount of WLIP was produced by this strain. The maximum amount of WLIP, 6.7 μg/ml, was produced by *P. aurantiaca* FS-2, but it was a little less than reference strain PB-St2. The rest of the strains produced WLIP as a dominant product but the amount was less than that from FS2 and ARS38. Among phenazines, PCA production was better than 2-hydroxy-phenazine for these strains (GS1 to GS7, FS-2 and ARS-38) but it was very low as compared with RP-4.

### *PCA* and *phzO* Genes of *Pseudomonas* Strains Were Amplified

The *phzO* and *PCA* genes were amplified using gene-specific primers. *Pseudomonas reactans* (PB-St3) was used as the negative control and *P. aurantiaca* PB-St2 was used as the positive control (Fig. 5a) for these amplifications. A 1.8 kb PCR product of *phzO* gene was detected in all strains except GS-6, and a 1.1 kb fragment for PCA was amplified for all strains (Fig. 5b).

### *Pseudomonas* Strains Produced IAA, HCN, and Siderophore, and Solubilized Zinc but Not Phosphate

All strains produced IAA in considerable amounts; 3.3 to 6.49 mg/l (Fig. 6). The highest IAA production (6.49 mg/l) was observed by *P. chlororaphis* subsp. *chlororaphis* RP-4,

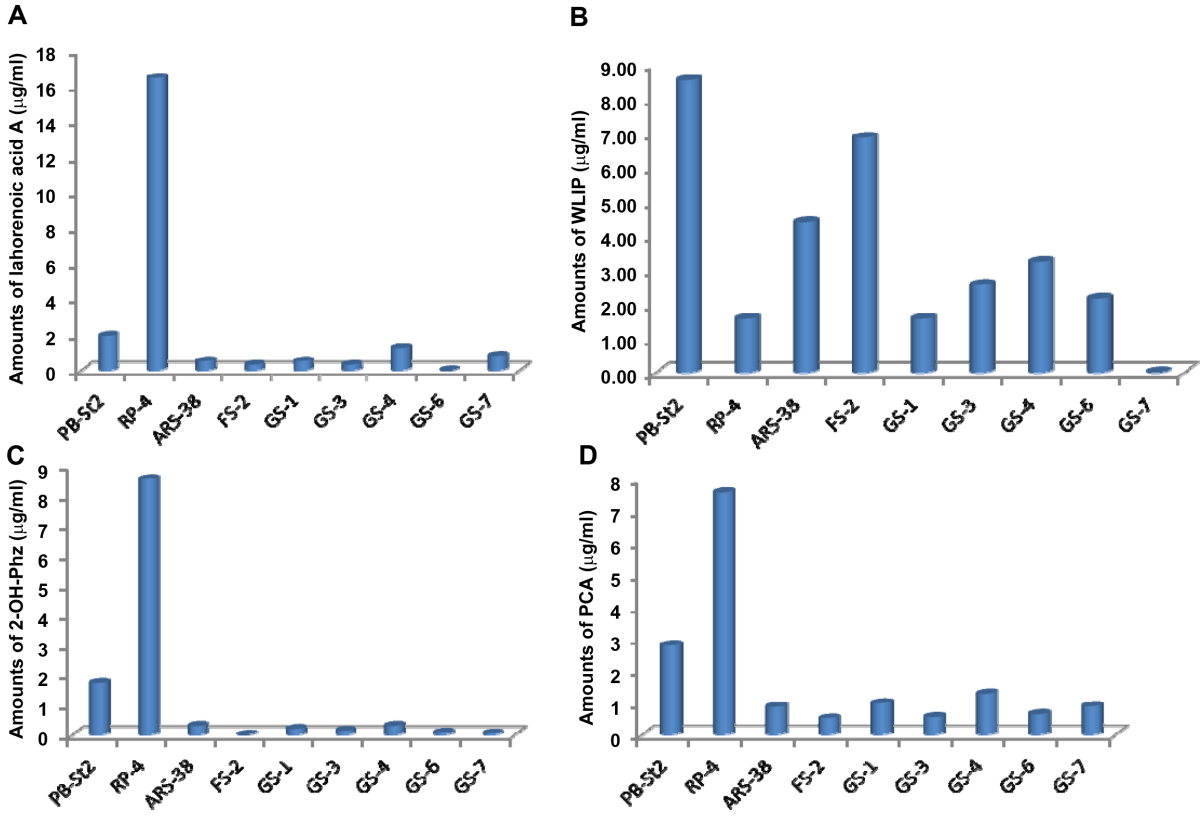


**Fig. 4.** Concentrations of (**A**) lahorenoic acid A, (**B**) white line-inducing principle (WLIP), (**C**) 2-hydroxy-phenazine (2-OH-Phz), and (**D**) phenazine-1-carboxylic acid (PCA) produced from different *Pseudomonas aurantiaca* and *P. chlororaphis* isolates in (μg/ml). PBSt2 is considered as the reference strain. Strains are shown on the X-axis and amounts are represented on the Y-axis.

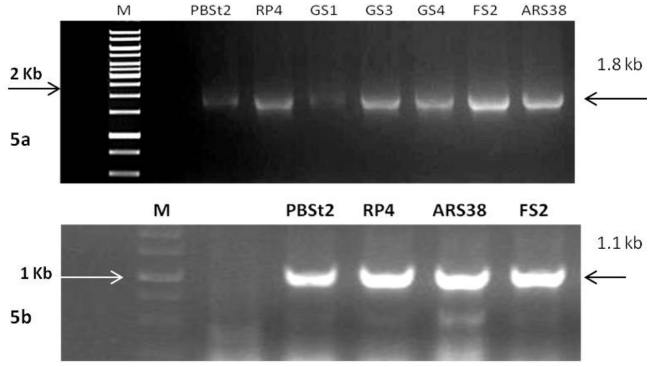


**Fig. 5.** Amplification of *phzO* gene (5a) and *PCA* gene (5b) showing bands of 1.8 and 1.1 kb, respectively.

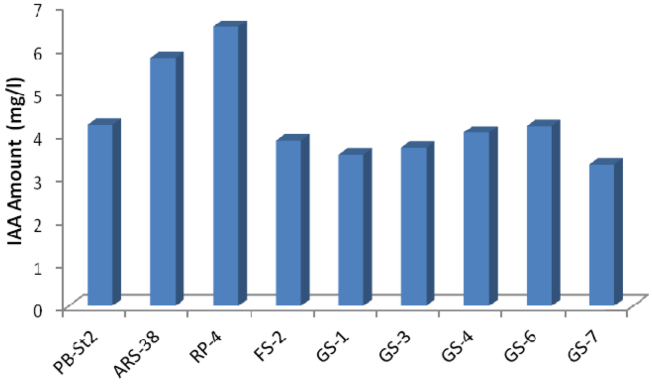


**Fig. 6.** Amount of indole-3-acetic acid (mg/l) produced by isolates of *P. aurantiaca* and *P. chlororaphis*.

followed by *P. chlororaphis* subsp. *aurantiaca* ARS-38 (5.77 mg/l). GS-1, GS-3, GS-7, and FS-2 produced 3.3–3.8 mg/l, and GS-4, GS-6, and PB-St2 produced 4.0–4.2 mg/l IAA.

All strains were positive for HCN and hydroxamate- type siderophore production. FS-2, RP-4, and GS-4, solubilized zinc carbonate and zinc oxide (Fig. S5). However, ARS-38 solubilized zinc oxide, and GS-1 solubilized zinc carbonate. GS-3, GS-6, GS-7, and PB-St2 did not solubilize zinc oxide or zinc carbonate. Zinc sulfate was not solubilized by these strains. Phosphate solubilization trait was not detected in any of these strains (Table S2).

### *Pseudomonas* Strains Promoted the Growth of Wheat Plants

The results manifested that most of these strains increased the dry weight of root and shoot and root lengths as compared with the uninoculated plants (Fig. 7). The dry shoot weights of the plants inoculated with *P. aurantiaca* strains GS-6, ARS-38, and PB-St2, and *P. chlororaphis* RP-4

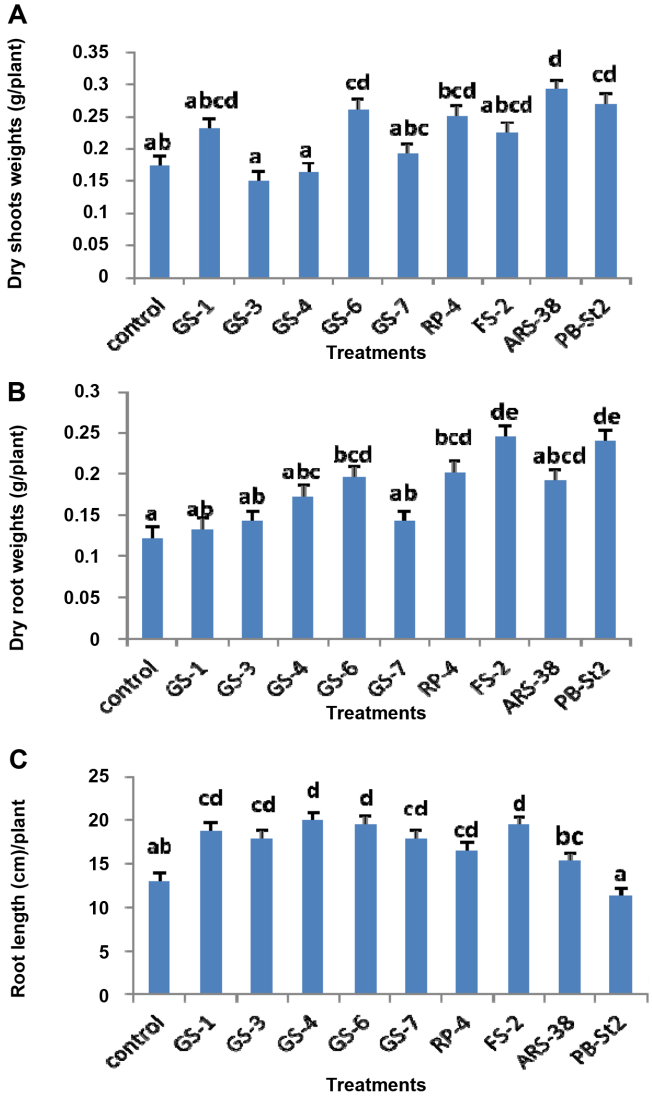


**Fig. 7.** Effect of bacterial inoculation on (**A**) dry weight of shoots, (**B**) dry weights of roots, and (**C**) root length of wheat plants in comparison with the control.

were significantly higher than those of plants inoculated with the rest of the strains and uninoculated. Although GS-1, GS-7, and FS2 also increased the shoot dry weights, the difference with control plants was nonsignificant. Plants inoculated with GS-3 and GS-4 and uninoculated showed almost similar shoot dry weights.

The root dry weight for all inoculated plants was higher than uninoculated plants; however, significant increase was observed for *P. aurantiaca* strains GS6, FS-2, PB-St2, and *P. chlororaphis* RP-4 inoculated plants. The maximum root dry weight was observed for FS-2 and PB-St2 inoculated plants. All strains except PB-St2 and ARS 38

significantly increased the root length as compared with uninoculated plants, and the maximum root length was recorded for GS-4, with inoculated plants and GS-6 and FS-2 following it with minor difference.

## Discussion

The Authors in this research group are working on bioformulations and therefore have isolated a large number of PGPR including fluorescent pseudomonads. Fluorescent pseudomonads are known for their plant growth promoting activities and their use as biocontrol agents. The main focus of this study was to identify and characterize the strains that have potential for use in future for both biofertilizers and biocontrol. Based on antifungal activity against local pathogens of economically important crops, eight isolates were selected. Seven of these were identified as strains of *P. chlororaphis* subsp. *aurantiaca* and one as a strain of *P. chlororaphis* subsp. *chlororaphis*. Pseudomonads are known to be isolated from exceptional habitats; however, *P. chlororaphis* subspecies are not among the common inhabitants of rhizosphere like other *Pseudomonas* species such as *P. putida* and *P. fluorescens*. There are only countable reports of *P. aurantiaca* strains isolated from different mesophytes, including sugarcane, soybean, and canola. It has not been reported earlier from cotton rhizosphere. Similarly, there is no report about its isolation from a drought-tolerant plant, cactus. Para grass is a halophyte and isolation of *P. chlororaphis* from this host is not recorded previously either. This study reports the extension in the host range of *P. aurantiaca* and *P. chlororaphis*, showing their adaptability to survive in diverse as well as extreme environments.

Pseudomonads produce a large number of secondary metabolites depending on the host, source of isolation, and environment. *P. chlororaphis* and *P. aurantiaca* are well-established biocontrol agents due to their production of phenazines and cyclic lipopetides as their secondary metabolites and their antagonistic activities. To date, around 14 compounds produced by different isolates of *P. aurantiaca* have been characterized [17]. However, the list of unidentified compounds is longer. Some of the known compounds are commercially available and can be used as standards, but these are expensive and getting them in Pakistan is not an easy task. Most of these compounds are produced by *P. aurantiaca* PB-St2 [18]; therefore, it had been used as a reference for secondary metabolites. Another reason to use this strain was to confirm that all local strains of *P. aurantiaca* produce the compounds reported earlier for PB-St2. Six compounds, including two phenazines (PCA and 2-hydroxy-phenazine), IAA, HCN, lahorenoic acid A, and WLIP were detected in our strains. It indicates that these compounds are produced in all three habitats; mesophytic, xerophytic, and halophytic. However, the amount produced by these strains varied depending on the requirement for a specific compound in a specific environment and host. RP-4 produced higher amount of 2-hydroxy-phenazine, PCA, and lahorenoic acid A, while WLIP was produced by PB-St2 in maximum amount. Other derivatives of lahorenoic acid (*i.e.*, B and C) were not detected in any strain.

*P. chlororaphis* subsp. *chlororaphis* and *aurantiaca* strains are reported to produce other phenazines, pyrrolnitrin, phenazine 1-carboxamide, and 2,4-DAPG commonly among identified compounds [18, 32–35]. We followed the protocols for the isolation of these compounds but they were not detected in these isolates. It can be due to the following reasons; (i) Our strains do not produce these compounds as these are not required under certain conditions; (ii) these compounds were produced but not as a major product and the level was below the detection limit of the equipment. We did search for 2,4-DAPG and pyrrolnitrin genes in these isolates but these had not been detected yet. More work is required to optimize the PCR conditions and MS analysis; a positive control will also be helpful. PCA and 2-hydroxy-phenazine genes were detected in our strains, confirming the production of these two compounds is not an artifact. We did not work on genes involved in production of lahorenoic acid and WLIP, as these clusters are not identified yet.

Phenazines are known to have antifungal and antibacterial activities. Among phenazines, PCA has been widely studied and produced by different *Pseudomonas* species, including *P. fluorescens, P. aeruginosa*, and *P. chlororaphis* [33]. It was shown to play a role in ecological fitness [33]. PCA was known under the name of tuberamycin for a long time owing to its antagonistic activity against *Mycobacterium tuberculosis*. Cezairliyan *et al*. [36] recently reported the anti-nematocidal activity of PCA, 1-hydroxy-phenazine and pyocyanin, killing *Cenorhabditis elegans*. 2-Hydroxy-phenazine was reported for the first time in *P. chlororaphis* subsp. *aureofaciens* [37] and later on in other subspecies [18]. In the current study, PCA and 2-hydroxy-phenazine were detected in all strains.

Cyclic lipopeptides are known for their role in motility, biofilms, antimicrobial, antitumor, immunosuppressant, and antisurfactant activities. Antibacterial and antifungal activity of WLIP is reported by Cantore *et al*. [38], and recently, it has been reported for antitumor/anticancer

activity [39]. Production of WLIP is known for *P. reactans*, *P. putida*, and *P. fluorescens* [40] but not for *P. chlororaphis*. In this study, cyclic lipopetide WLIP was detected in all strains of *P. chlororaphis* subsp. *aurantiaca* (except GS-7) and *P. chlororaphis* RP-4. Similarly, lahorenoic acid A was detected in all strains, except GS-6. Production of WLIP and lahorenoic acid by *P. aurantiaca* was first reported by Mehnaz *et al*. [18] and until now it has not been reported from other parts of the world. Other strains are reported from different hosts and different climates that might play a role in its production or these might be produced in small amounts and could not get detected. This is the first report of the production of WLIP and lahorenoic acid by *P. chlororaphis* subsp. *chlororaphis*.

IAA production is the characteristic feature of several species of *Pseudomonas*, including *P. putida*, *P. fluorescens*, and *P. chlororaphis* [16]. However, due to high phenazine production, colorimetric detection of this compound for *P. chlororaphis* strains is not an easy job. Therefore, authors used HPLC for its detection and quantification. All strains proved to be good producers with at least 3.0 mg/l IAA. *P. chlororaphis* subsp. *chlororaphis* was the highest producer among all strains with ~ 6.5 mg/l, and ARS-38 was the highest producer among *P. chlororaphis* subsp. *aurantiaca* with ~5.8 mg/l IAA. IAA production was reported earlier for two strains of *P. aurantiaca* [12, 32]. It might be produced by other strains as well but researchers working with *P. aurantiaca* and *P. chlororaphis* are more focused on its use as a biocontrol agent than growth promoter, therefore ignoring or not assessing their ability for IAA production.

HCN, siderophore, and antifungal activity are known for *P. chlororaphis* strains [15]. Based on these abilities, most of their strains are used as biocontrol agents. *P. aurantiaca* strains SR1, BL915, and S1 are HCN and siderophore producers [12, 32, 41]. PB-St2 was reported to show HCN and siderophore production and antifungal activity against more than 40 fungal pathogens [16–18]. Hu *et al*. [34] reported the antifungal activity of *P. aurantiaca* strain Pch010 against *F. graminearum* and used it as a biocontrol agent for wheat in field experiments. *P. aurantiaca* SR1 drastically inhibited the growth of *Macrophomina phaseolina* in vitro and in vivo when the soybean plants were inoculated with it [35]. Data obtained in the current study also support the production of these compounds by *P. aurantiaca* and *P. chlororaphis* strains.

Pseudomonads are known for phosphate solubilization. However, none of the strains used in this study has shown the ability to solubilize phosphate. *P. aurantiaca* PB-St2 strain used as a reference in this study was also reported as a non-solubilizer of phosphate [18]. *P. aurantiaca* SR1 is known to be a moderate phosphate solubilizer [42]. It indicates that *P. aurantiaca* strains show variability in this trait. *P. fluorescens*, *P. putida*, and *P. aeruginosa* are known for zinc solubilization [43, 44]. Di Simine *et al*. [44] reported the solubilization of zinc phosphate by *P. fluorescens*. Strains used in this study have solubilized zinc oxide and zinc carbonate in variable amounts but solubilization of oxide compound was higher than of carbonate. Similar trend for solubilization of zinc oxide and carbonate compounds is reported by Bapiri *et al*. [43] for fluorescent pseudomonads. To our best knowledge, this is the first report about zinc solubilization ability of *P. chlororaphis* subsp. *chlororaphis* and *aurantiaca*.

As these strains showed potential of being used as biofertilizer due to production of IAA and other traits that indirectly promote plant growth, wheat plants were inoculated and plant growth was observed. Our results suggested that *P. aurantiaca* GS-6 and FS-2 and *P. chlororaphis* RP-4 performed best, as significant increase in root, shoot dry weight, and root length was observed with these as compared with the rest of the *P. aurantiaca* strains and uninoculated plants. Rosas *et al*. [45] used *P. aurantiaca* SR1 as inoculums for wheat in a field experiment and observed significant increase in shoot and root lengths, root weight, and non-significant increase in shoot weight. They have also studied the effect of this strain on maize, soybean, and alfalfa, and reported growth enhancement in these crops. Among *P. aurantiaca* strains, SR1 had been extensively studied for plant growth promotion with different crops at the field level [32, 45–47]. There are several reports about the use of *P. putida* and *P. fluorescens* as inoculum for plant growth, but reports about *P. aurantiaca* and *P. chlororaphis* are rare. More information about their use as biocontrol agents on the commercial level and laboratory scale are available [13, 14].

In conclusion, all isolates used in this study have been identified as strains of *P. chlororaphis*. Seven of them belong to subspecies *aurantiaca* and one to *chlororaphis*, based on biochemical and molecular methods. Among secondary metabolites, two phenazines PCA and 2-hydroxy-phenazine, lahorenoic acid A, cyclic lipopetide WLIP, IAA, HCN, and siderophores were produced by these strains. Phosphate solubilization was not observed, but they did show the ability to solubilize zinc. These strains significantly promoted the root length, and dry weight of root and shoot, of wheat plants as compared with uninoculated ones. It showed their potential to be used as biofertilizers. The antifungal activity and secondary metabolites production by these

strains suggest that they can be used as biocontrol agents as well.

This is the first report of the production of WLIP and lahorenoic acid A by *P. chlororaphis* subsp. *chlororaphis*, zinc solubilization by both subspecies of *P. chlororaphis*, cactus and cotton as new hosts for *P. aurantiaca*, and paragrass as a new host for *P. chlororaphis*.

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