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Auxin Producing *Pseudomonas* Strains: Biological Candidates to Modulate the Growth of *Triticum aestivum* Beneficially

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ABSTRACT

The screening of plant growth promoting rhizobacteria is a crucial step for their utilization as beneficial input in improving the crop productivity. This study was carried out to screen and evaluate the auxin producing rhizospheric isolated *Pseudomonas* strains for their potential to improve growth of *Triticum aestivum* (wheat) plant under laboratory and natural conditions. Three strains PNS-4, PNS-6 and PNS-15 were evaluated for auxin production by Salkowski's method and further confirmed by high performance liquid chromatography (HPLC). The PNS-4, PNS-6 and PNS-15 strains were identified by I6S rRNA gene sequencing that showed maximum resemblance with *Pseudomonas mendocina* (99%), *Pseudomonas alcaliphila* (99%) and *Pseudomonas* sp. (99%) respectively. Selected strains were found to produce auxin with and without the amendment of exogenously applied L-tryptophan, a major precursor for auxin biosynthesis and an important constituent of plant root exudates. Efficacy of these strains on wheat plant growth was checked under laboratory and field conditions. All *Pseudomonas* species were found to improve the % seed germination and growth parameters (shoot length, root length, fresh weight and dry weight) of the wheat seedlings significantly ($P = 0.05$) as compared to the un-inoculated seedlings under laboratory condition. The biochemical parameters (total soluble protein content and endogenous auxin content) of the bacterial inoculated wheat seedling were also increased significantly than that of uninoculated ones. Under natural condition, seed bacterization also showed the significant effect ($P = 0.05$) on yield parameters (shoot length, number of tillers, spike length and weight of seeds in grams) of the wheat plants when compared with non-inoculated plants. Our results reported the three most promising *Pseudomonas* candidates and revealed the fact that experiments under laboratory and natural conditions may be helpful in selecting the best candidates as bio fertilizers for future agricultural practices.

Keywords: Auxin; *Pseudomonas*; *Triticum aestivum*; PGPR; Rhizobacteria

1. Introduction

Complex diversity of microbes interacts with plant roots continuously in rhizospheric soil region. These microbes can influence the plant growth in various ways that can be beneficial or detrimental to the plant development [1,2]. Beneficial rhizospheric microorganisms gained special attention due to their potential to enhance the plant growth by variety of mechanisms. Some mechanisms are involved in plant growth promotion directly *i.e.* phytohormone [3] and siderophore production [4], phosphate solublization [5], nitrogen fixation and denitrification [6,7] and 1-amino-cyclopropane-1-carboxylate deaminase production [8]. Whereas HCN (and antifungal metabolite and siderophore production are bacterial characteristics that are involved in plant growth promotion indirectly [9]. Beneficial rhizobacteria that increase the plant yield and productivity are considered as plant growth promoting rhizobacteria (PGPR) [10]. Phytohormone production has been studied as one of the main mechanisms by which PGPR may enhance the plant growth [11]. Several genera of *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Azospirillum*, *Klebsiella*, *and Enterobacter*, isolated from rhizospheric region of variety of crops, have been evaluated for plant growth promoting attributes and illustrated their synergistic effects on plant growth and development [12,13].

Auxin especially IAA production by rhizobacteria is involved in plant microbe signaling, which can lead the change in root morphology by proliferation and elongation of adventitious and lateral roots to the plant [14,15]. Therefore, facilitation of plant growth by rhizobacteria has been ascribed to the auxin production [16]. Barea *et al.* [17] reported that about 86% of the bacterial strains, isolated from the rhizosphere of various plants, produced auxins. IAA biosynthesis by these bacteria has been correlated with promoting of root proliferation [3,18,19]. Effect of IAA on plant growth mainly depends upon its concentration. Low concentration enhances the root length while high concentration retards the plant root length [20]. In order to select the most promising plant growth promoting bacteria, effective selection and screening procedures are considered very important [21]. This selection helps to assist the development of database of the microbes essential for plant growth that can be further used as bio fertilizers. Genera *Pseudomonas* especially *P. fluorescens* and *P. putida* are the most important kinds of PGPR. Production of auxin is one of the main reasons to promote plant yield with these Bacteria [22]. The main objective of the present study was to screen the most effective *Pseudomonas* species on the basis of auxin production and their further evaluation in promoting the growth and development of wheat plant under laboratory and field conditions.

2. Material and Methods

2.1. Isolation

The root adhering soil samples from the rhizosphere of *Lycopersicon esculentum*, *Vigna radiate* and *Corundum sativum* plants, grown in different location of Punjab, Pakistan were collected from the rhizosphere region in sterile bags, carried to the laboratory and stored at 4˚C for further processing. In order to screen the auxin producing rhizobacteria, one gram soil from each soil sample was homogenized in test tube containing 9 ml saline solution (0.85% NaCl) separately. The suspension was vortexed and dilutions of autoclaved water were made up to 10^{-6} by using the serial dilution method. The 0.1 ml of each dilution was spread on Luria Bertani medium plates. The plates were incubated at 28˚C ± 2˚C for 24 hours. After the development of growth, different colonies were selected on the basis of morphology and purified by further sub culturing [23].

2.2. Molecular Identification

In order to identify the selected strains, 16S rRNA gene sequence analysis was done by extraction and amplification of genomic DNA done by the method of Cui *et al.* [24]. DNA extraction was done by extraction kit (QIAGEN) and amplified by using universal primers forward primer 27f (5'AGAGTTTGATCCTGGCTCAG3') and reverse primer 1522r (5'AAGGAGATGATCCAGCC3'). The amplified product was purified and sent for sequencing at Cancer research Centre, University of Chicago. The obtained sequence was edited and submitted to BLAST to search phylogentically closely related bacteria already submitted in the GENBANK. The final sequence was submitted to GENBANK for accession numbers.

2.3. Auxin Production under *in Vitro* Condition

For auxin estimation, bacterial cell suspension adjusted to 10^6 to 10^7 CFU ml^{-1} was inoculated in autoclaved L-broth supplemented without and with a filter sterilized solution of 1000 µg L-tryptophan. Inoculated flasks were incubated at 28˚C for 72 hours. After incubation, bacterial cells were removed from culture medium by centrifugation at 14.000 rpm for 15 minutes. After centrifugation, auxin was detected by taking 1 ml of supernatant and 2 ml of Salkowski's reagent, mixed them properly and placed in dark for 30 minutes. After the color development, O.D was taken at 535 nm by spectrophotometer. A standard curve of synthetic auxin (Oxoid) with different concentration was drawn to quantify the auxin produced by bacteria [25]. The presence of IAA was further confirmed by thin layer chromatography and HPLC.

2.4. HPLC

Bacterial auxin was extracte by centifugation of the stationary phase cultue at 10.000 rpm for 20 minutes at 4˚C. The pH of the supernatent was adjusted to 2.5 with 1.0 M HCL and extracted three times with three volumes of ethyl acetate. Extracts were evaporated in rotary evaporator (Heidolph LABOROTA, Cole-Parmer, IL, USA) and dissolved in absolute methanol. For further confirmation of bacterial IAA, HPLC (Sykam Model 203) equipped with a sykam S1122 solvent delivery system and Sykam S 3210 uv/vis detector was used for extracted bacterial samples. Extracted sample (10 µl) dissolved in methanol was injected into Reverse-phase C 18 column (4.6 × 15 mm). The mobile phase was methanol: water 80:20 (v/v) at a flow rate of 1 ml/min. Peak was detected comparable to synthetic IAA.

2.5. Plant Microbe Experiment

2.5.1. Axenic/Laboratory Condition

Effect of auxin producing rhizobacteria was checked on plant growth by providing the natural system of soil under the controlled conditions in the laboratory. Effect of bacterial auxin on wheat plant was done by the me-

thod of Ali *et al.* [23]. Bacterial culture was grown in LB broth for 24 hour at 28˚C and centrifuged at 10.000 rpm for 10 minutes to get the pellet. Washing of bacterial pellet was done with I ml of phosphate buffer (PBS, 20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and suspended in the same buffer. Cell density of 10^7 CFU ml^{-1} was achieved by taking the O.D at 600 nm of suspension. Certified Seeds of wheat var Uqab-2000 were obtained from Punjab seed corporation Lahore Pakistan and surface sterilized with 0.1% $HgCl_2$ followed by several washings (7 times) of water. After incubating the seeds in bacterial inoculum for 30 minutes, they were sown in plastic pots having 200 gm autoclaved soil, at the depth of 2 - 2 cm. Same process was done with the seeds dipped in un-inoculated broth for control setup. The whole experiment was set in five replicates. Soil was moistened with autoclaved water equally in all experimental and control pots respectively. The pots were placed at 22˚C in growth chamber with 16:8 daylight (with light intensity of 200 $\mu E \cdot m^{-2} \cdot s^{-1}$) regime. The percentage germination was calculated in treated and control plants soon after germination. After 15 days wheat seedlings were taken out and root length, shoot length, fresh weight, dry weight were measured. Bio chemical parameters were also calculated. Experiment was performed three times to check the validity of the results.

2.5.2. Natural Condition

In order to check the effect of auxin producing Pseudomonas strains on the yield of wheat plants, sterilized seeds treated with PGPR strains for 30 minutes as mentioned above were sown in large pots containing 10 kg of unfertilized garden soil. Initially, 15 sterilized seeds were inoculated in each pot in five replicates. After germination, seedlings were thinned to 10 per pot. After 6 weeks, further thinning was carried out by keeping 5 seedlings per pot, which were grown till maturity. All pots were arranged in a completely randomized design in the wire house of Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan. Experiment was conducted between December, 2009 to June, 2010 under ambient light and temperature. Plants were irrigated with tap water when required. At full maturity, growth parameters including shoot length, number of tillers, spike length, and grain yield were recorded. Experiments were repeated three times to check the validity of the results [16].

2.6. Statistical Analysis

The data obtained were evaluated statistically by using SPSS (version 16; SPSS Inc, Chicago, USA) software for windows. Data were analyzed by ANOVA and mean values of different strains were compared by Duncan's multiple test ($P < 0.05$).

3. Results and Discussion

Plant growth promoting bacteria have gained the world wide attention, as an alternative of chemical fertilizers that can improve the plant growth by direct or indirect ways. The rhizobacteria have great potential to enhance the growth promotion of wheat crop [26,27].

3.1. Identification

Three *Pseudomonas* strains PNS-4, PNS-6 and PNS-15 showed 99% homology to *P. mendocina*, *P. alcaliphila* and *Pseudomonas* sp. (already submitted sequences in GENBANK). The nucleotide sequences have been submitted in GENBANK under accession number of JF 905443.1, JF905444.1 and JQ218449 for PNS-4, PNS-6 and PNS-15 respectively **(Table 1)**.

3.2. Auxin Biosynthesis by *Pseudomonas* Isolates

In vitro auxin production and screening of rhizobacterial isolates for plant growth promotion under gontobiotic conditions are considered as useful approach for selecting PGPR [28]. In the present study, all strains were found to produce auxin significantly in LB medium which were increased by addition of precursor L-tryptophan. The result showed that auxin production was not the same among all rhizobacterial strains. PNS-6 produced the highest amount of IAA (15.6 µg/ml) followed by PNS-15 (13.3 µg/ml) and PNS-4 (12.6 µg/ml) in the absence of L-tryptophan. But this production was increased by the amendment of L-tryptophan, which were highest in PNS-15 (114 µg/ml) while production in PNS-6 and PNS-4 reached up to 110 µg/ml and 105 µg/ml respectively (**Figure 1**). The result showed that selected isolates produced auxin through tryptophan dependent pathway, which could be helpful while interaction with plants as plants exudates contains tryptophan that may assist the auxin production ability of the colonized bacteria [9,16, 29]. Our work was in accordance with Ali *et al.* [23] who isolated the auxin producing rhizobacteria and have beneficial impact on plant growth promotion.

3.3. Identification of Auxin by HPLC

HPLC is a useful tool to identify and confirm the metabolites that were screened by manually method and not very reliable [30]. All strains showed a peak comparable with the peak of standard IAA on HPLC system. That indicated the bacterial ability to synthesize the IAA (**Figure 2**).

Table 1. Isolation and molecular identification of *Pseudomonas* strains.

Strains	Plant source	Sequence length	% homology	Identified as	Accession number
PNS-4	*Lycopersicon esculentum*	1445	99	*Pseudomonas mendocina strain PNS*-4	JF905443.1
PNS-6	*Vigna radiata*	1457	99	*Pseudomonas alcaliphila strain PNS*-6	JF905444.1
PNS-15	*Coriandrum sativum*	1537	99	*Pseudomonas sp. PNS*-15	JQ218453

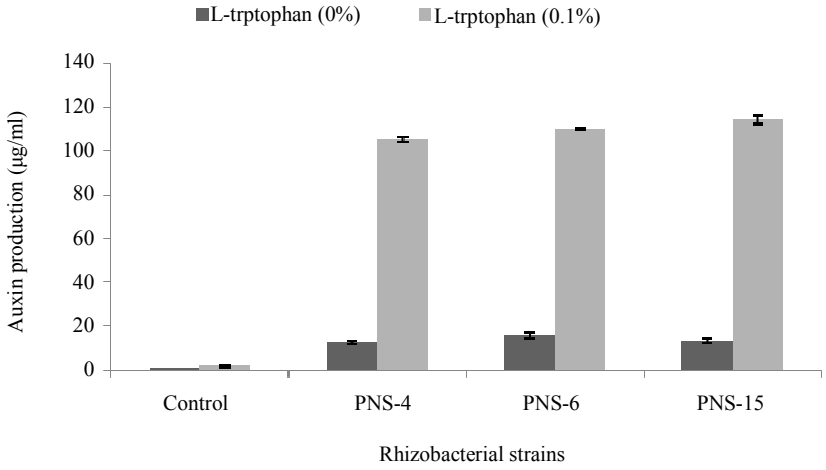

Figure 1. Auxin quantification of most effective strains by colorimetric method with and without the presence of L-tryptophan.

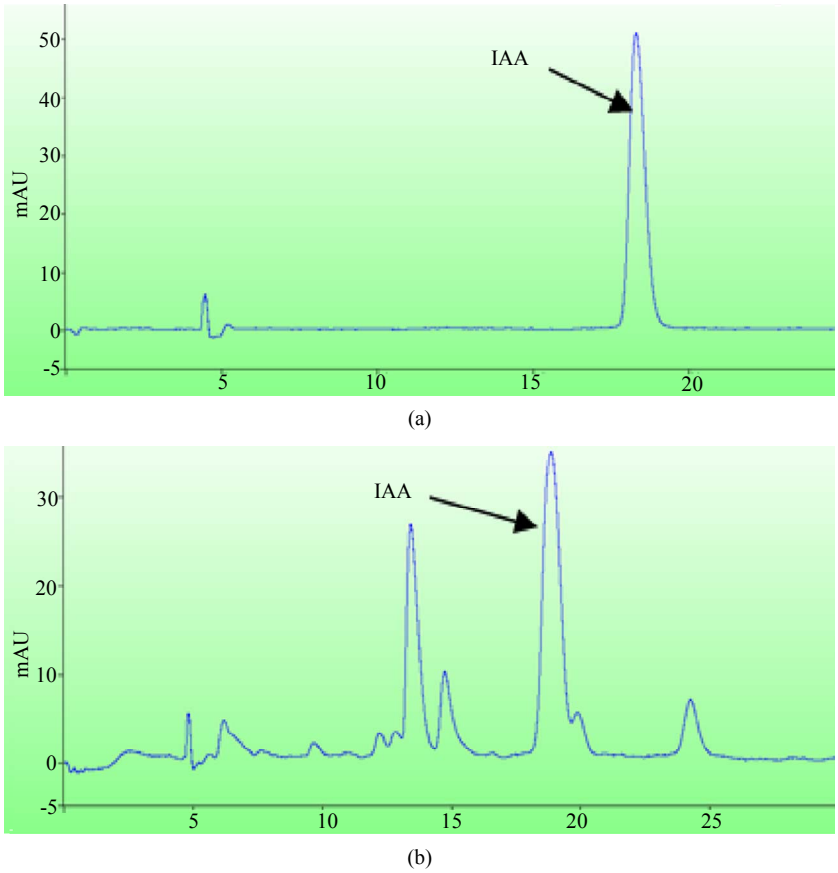

Figure 2. HPLC chromatogram of (a) standard IAA and (b) PNS-15 extract.

3.4. Effect on Growth Parameters under Laboratory Condition

Auxin producing strains were evaluated for their potential to enhance the wheat growth under laboratory condition. Induction in seed emergence has been considered as a very important step towards better growth promotion of plant, and the PGPRs are involved in plant growth promotion by enhancing the seed germination. All selected strains showed significant (P = 0.05) increase in percentage germination. PNS-15 showed maximum increase in % germination (42.8%) of wheat seedlings followed by PNS-6 (38%) and NS-4 (33.2%). The root length, shoot length, fresh weight and dry weight of the *Pseudomonas* inoculated seedlings were also increased significantly (P = 0.05) when compared than that of uninoculated ones **(Table 2)**. Strain PNS-15 showed the 45.8% increase in shoot length followed by PNS-4 and PNS-6 that showed the 26.5 and 20.3% increases over control. It was found that PNS-6 showed maximum promoting effect on root length (53.7%) followed by PNS-15 (53.1%) and PNS-4 (46.2%) as compared to control. Same was the case with fresh and dry weight of inoculated seedling biomass. Fresh and dry biomass of *Pseudomonas* inoculated wheat seedlings were significantly (P = 0.05) increased than that of non-inoculated plant seedlings. Strain PNS-15 showed increase in weight of fresh (77%) and dry (90%) biomass as compared to control **(Table 2)**. Whereas increase fresh and dry weight of PNS-6 inoculated seedlings were found 49.6 and 61.2% respectively. Our results were in agreement with Hameeda *et al.* [31], Cakmakci *et al.* [32] and Shaharoona *et al.* [33] who demonstrated that auxin producing rhizobacterial *Pseudomonas* have the ability to increase the biomass of wheat seedlings in laboratory conditions. The *Pseudomonas* strains also have significant (P = 0.05) beneficial effect on total soluble protein and endogenous auxin of wheat seedlings. PNS-15 strain was found to be most efficient that caused 85 and 12.2% increase in auxin and soluble protein content when compared to non-inoculated ones **(Figure 3)**. The results showed that bacterial auxin plays an important role in changing the endogenous auxin pool that is involved in root proliferation and more absorption of nutrients that may leads to better development of the plant.

Table 2. Auxin producing effect of rhizobacterial strains on bioactive parameters of wheat seedlings in pot experiment.

Rhizobacterial isolates	*Germination (%)*	*Root length (cm)*	*Shoot length (cm)*	*Fresh weight (mg/plant)*	*Dry weight (mg/plant)*
Control	70 ± 2.44 (a)	13.03 ± 0.84 (a)	15.06 ± 0.91 (a)	1350 ± 0.06 (a)	310 ± 0.009 (a)
PNS-4	94 ± 2.12 (b)	19.06 ± 0.97 (b)	19.06 ± 0.87 (c)	1970 ± 0.1 (b)	470 ± 0.006 (b)
PNS-6	98 ± 3.14 (c)	20.03 ± 0.98 (c)	18.13 ± 0.78 (b)	2020 ± 0.12 (b)	500 ± 0.01 (c)
PNS-15	100 ± 0.00 (d)	19.96 ± 0.95 (b)	21.96 ± 1.06 (d)	2390 ± 0.10 (c)	590 ± 0.05 (d)

Values shows mean of 25 replicates ± SE. The different letters showed significant differences between values of different strains by using Duncan's multiple range test (P = 0.05).

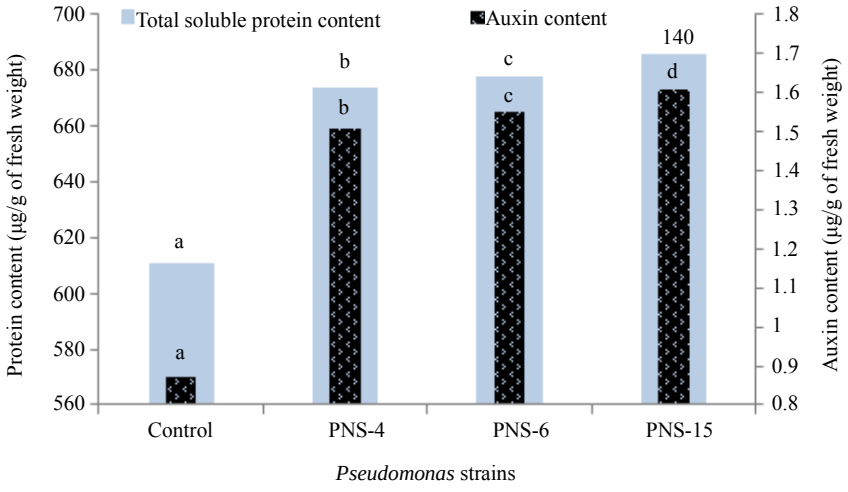

Figure 3. Effect of *Pseudomonas* strains on biochemical parameters (auxin content and total soluble protein content) of wheat seedlings under laboratory conditions. Values shows mean of 10 replicates ± SE. The different letters showed significant differences between values of different strains by using Duncan's multiple range test (P = 0.05).

Table 3. Impact of *Pseudomonas* inoculations on growth and yield of *T. aestivum* at full maturity under natural condition.

Rhizobacterial strain	Percentage germination	Shoot length (cm)	No. of tillers/plant	Spike length (cm)	weight of 100 seeds (g)
Control	86.63 ± 4.13 (a)	51.9 ± 0.78 (a)	2.2 ± 0.24 (a)	8.06 ± 0.72 (a)	3.04 ± 0.32 (a)
PNS-4	98.8 ± 4.76 (b)	60.2 ± 0.91 (c)	3.13 ± 0.22 (b)	9.36 ± 0.73 (b)	3.87 ± 0.19 (b)
PNS-6	98.8 ± 5.12 (b)	58.5 ± 0.76 (b)	3.16 ± 0.21 (c)	9.53 ± 0.89 (c)	3.91 ± 0.18 (c)
PNS-15	100 ± 5.14 (c)	65.1 ± 1.05 (d)	4.03 ± 0.15 (d)	10.7 ± 0.92 (d)	4.07 ± 0.24 (d)

Values shows mean of 25 replicates ± SE. The different letters showed significant differences between values of different strains by using Duncan's multiple range test (P = 0.05).

3.5. Effect on Growth Parameters under Natural Condition

The efficiency of PGPR inoculation in plant growth promotion depends upon its survival and propagation rate in diversified environmental conditions, including soil type, environmental conditions and Plant age [34]. The potential of bacterial strains may never be fruitful in natural condition as lot of environmental factors (indigenous microflora, survival rate, environmental conditions) are involved that may interfere their capability as plant growth promoters. After the laboratory trials, the strains were checked under natural environment. The results of bacterial effect on plant growth under natural condition revealed that PNS-4 PNS-15 and PNS-6 significantly (P = 0.05) increased all growth parameters in field condition. PNS-15 increased percentage up to 15.4%, shoot length up to 25.4% than that of uninoculated ones (**Table 3**). While PNS-4 exhibited 15.9% enhancement in the shoot length of the plant as compared to the control. Similarly number of tillers and spike length in PNS-15 inoculated strains were also increased up to 83.1% and 32.9% than that of non-inoculated ones (**Figure 4** and **Table 3**). PNS-15 efficiently increased the grain weight of 100 seeds (34%) followed by PNS-6 (28.6%) and PNS-4 (27.5%) as compared to control ones. studies done by Hussain and Hasnian [35] showed the increase in yield of the wheat plant by *Pseudomonas* strains. Similarly Abbaspoor *et al.* [36] showed that *Pseudomonas* species enhanced the wheat grain yield by 26%. Furthermore, *Pseudomonas* species have the ability to colonize the plant roots efficiently and causes the significant increase in the plant yield [34].

Figure 4. Effect of rhizobacterial inoculation on the spike length of wheat plants under natural conditions.

4. Conclusion

The selected three most promising *Pseudomonas* species were evaluated as PGPR on the basis of *in vitro* and laboratory screening procedures. All strains enhanced the plant growth in laboratory by utilizing phytohormones producing ability. The strains were able to retain their growth promoting trait under natural condition as well that can be further utilized in enhancing the wheat crop productivity as an alternative of chemical fertilizers.

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