Pure Appl. Chem., Vol. 82, No. 1, pp. 313–319, 2010.
doi:10.1351/PAC-CON-09-02-06

Auxin-producing *Bacillus* sp.: Auxin quantification and effect on the growth of *Solanum tuberosum**

Ambreen Ahmed[1,‡] and Shahida Hasnain[2]

[1]Department of Botany, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan; [2]Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-i-Azam Campus, Lahore 54590, Pakistan

Abstract: Plant-associated bacteria are known to improve plant growth and play a major role in the development of plants. The present study is concerned with the isolation of two auxin-producing plant growth-promoting bacteria (PGPB). On the basis of 16S rRNA sequencing, both of the strains are identified as *Bacillus* sp. Maximum auxin production was observed at 37 °C after 48 h of incubation. Increase in tryptophan concentration stimulated auxin production by the isolates. High-performance liquid chromatography analysis showed that the bacterial auxin exhibited similar retention time as the standard indole-3-acetic acid (IAA). Sprouts of *Solanum tuberosum* var. Desiree were inoculated with the isolates. Comparison of various growth parameters of inoculated plants with non-inoculated plants revealed the improvement of plant growth by bacterial inoculation. Almost 40 and 35 % increase in shoot length with P4 and S6 inoculation, respectively, was observed. Considerable improvement in root growth was observed with an increase in the number and length of roots. On the basis of the above findings, it is concluded that the plant growth-promoting *Bacillus* strains affect *S. tuberosum* beneficially, resulting in improved plant growth.

Keywords: auxin; *Bacillus* sp.; plant growth-promoting bacteria; sequencing; *Solanum tuberosum*.

INTRODUCTION

Plants coordinate and control their development by using chemical signals to regulate the growth of cells throughout the plant. These signals, called "phytohormones", have a profound effect on development at vanishingly low concentrations and have the ability to alter plant growth patterns. Plant-associated bacteria are involved in symbiotic and associative microbial activities. These are economical and safer source of nutrition for increasing agricultural production and improving soil fertility. Microorganisms get nutrients from the root exudates of plants by colonizing their roots. Suggested mechanisms for plant growth promotion include bacterial synthesis of phytohormones indole-3-acetic acid (IAA), cytokinins, gibberellins, increased mineral and nitrogen availability in the soil [5], and suppression of ethylene production by ACC (1-aminocyclopropane-1-carboxylate) deaminase activity, etc. Auxins are quantitatively the most abundant phytohormone secreted by most plant-associated bacteria [18], and mostly auxin production rather than nitrogen fixation is considered as the major factor re-

*Paper based on a presentation at the 13th International Biotechnology Symposium (IBS 2008): "Biotechnology for the Sustainability of Human Society", 12–17 October 2008, Dalian, China. Other presentations are published in this issue, pp. 1–347.

‡Corresponding author

sponsible for enhanced plant growth. Phytohormones producing bacteria may metabolize the precursors of hormones, or they may produce and secrete similar phytohormones. As the concentration of hormone signals is critical to the regulation of various physiological processes in plants, modification of phytohormone levels by microbes can lead to characteristic changes in plant growth and development. Phytohormones produced by the bacteria can increase root area, leading to higher water and other nutrients uptake from the soil, plant height, and grain yield, etc. [16]. The present work aims to isolate phytohormone-producing bacteria and study their effect on the growth of *Solanum tuberosum*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains were isolated from the rhizosphere of different plants. The isolated strains were purified and then screened for auxin production colorimetrically [19]. Two strains, P4 and S6, were found to produce auxin. These strains were routinely grown on L-agar at 37 °C for 24 h and used for further study.

Strains characterization

Isolated auxin-producing bacteria were characterized morphologically, biochemically, and physiologically following Gerhardt et al. [7]. To study morphology, color, shape, margin, elevation, and size of the colonies of isolates were recorded while cell morphology was studied by observing Gram-staining, motility, cell shape, and spore-staining of the bacteria. The effect of different environmental factors such as temperatures and pH on the growth of bacteria was studied. Growth of isolates in the presence of various antibiotics and metals was also recorded. Growth behavior of bacterial strains with increasing time of incubation was also determined.

16S rRNA gene sequence analysis

To identify the isolated auxin-producing strains, 16S rRNA gene sequence analysis was carried out. The genomic DNA was isolated from 24 h incubated culture at 37 °C in L-broth using genomic DNA purification kit (Promega; cat. no. A1125). The purified DNA was then used as template for polymerase chain reaction (PCR). Amplification of the isolated DNA was carried out using primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1522r (5'-AAGGAGGTGATCCA(AG)CCGCA-3'). The reaction conditions were as follows: heating the mixture for 5 min first and then 30 cycles of denaturation at 94 °C for 20 s, primer annealing at 50 °C for 20 s, and extension at 72 °C for 2 min followed by final extention at 72 °C for 5 min. The amplified DNA was resolved on 1 % agarose gel along with the standard DNA marker and was then purified using Jet Quick Gel Extraction Spin Kit (cat. no. 420050) according to the manufacturer's instructions. The extracted DNA was sequenced using Automated Sequencer (Applied Biosystem; Model 3100). Homology of the obtained sequences was searched through BLAST using the National Center for Biotechnology information database (<www.ncbi.nlm.nih.gov/BLAST>).

Auxin quantification

Auxin production by the isolated bacteria was initially determined through colorimetric analysis using Salkowski reagent [19]. Bacterial auxin production was also quantified by high-performance liquid chromatography (HPLC) (Sykam Model 203) equipped with a reverse-phase C_{18} column (5 µm; 4.6 × 15 mm) following Patten and Glick [15]. Eluates were detected at 220 nm. The flow rate was adjusted

to 1 ml min^{-1}. Synthetic IAA was taken as standard. IAA production by each strain was measured in triplicate.

Plant growth experiment

Certified seed tubers of *S. tuberosum* var. Desiree were procured from Punjab Seed Corporation, Lahore, Pakistan. The sprouts were disinfected with 0.1 % $HgCl_2$ and then inoculated with bacterial suspension adjusted to the same optical density (10^8 ml^{-1} cfu) at 600 nm for 30 min. Control seeds were treated with sterilized distilled water for the same period of time. Both treated and untreated sprouts were then sown in pots containing 120 g sieved soil each at the rate of four sprouts per pot and allowed to grow at 25 ± 2 °C. After 25 days, the plants were harvested and different growth parameters were measured. Biochemical analysis was carried out by estimating auxin content [13] and chlorophyll content of the plants following Lichtenthaler and Wellburn [11]. For determining the effect of the isolates on the root growth of *S. tuberosum*, the inoculated and non-inoculated sprouts were transferred aseptically to sterile moistened filter paper (Whatman No. 1) in petri dishes and incubated at 25 ± 2 °C. After 10 days, number of roots and root lengths were measured. The experiment was conducted in triplicate.

RESULTS

For morphological characterization of the auxin-producing bacterial strains P4 and S6, cell and colony morphology was recorded. Both the strains were Gram-positive, motile rods arranged in pairs or small chains. Biochemical characterization was carried out by performing different biochemical tests. Both the strains were positive for oxidase, catalase, nitrate, glucose, mannitol, xylose, ortho-nitrophenyl-β-galactosidase (ONPG), VP, citrate, malonate, sorbitol, rhamnose, sucrose, lactose, arabinose, adonitol, raffinose, and arginine tests while ornithine, H_2S, indole, urease, tryptophan deaminase (TDA), gelatin, inositol tests gave negative results for both the strains. For the lysine test, P4 showed negative response while S6 was positive. Similarly for salicin, P4 gave positive result, whereas S6 showed negative result. The impact of varying temperatures (25, 37, and 45 °C), pH (5, 6, 7, 8, 9) and presence of different antibiotics and metals on the growth of the bacteria recorded. The optimum temperature for the growth of both the strains was found to be 37 °C. Optimum growth of isolates P4 and S6 was observed at pH 5 and 6, respectively. When grown in the presence of various antibiotics, P4 was found to be resistant to lincomycin, streptomycin, cefixime, and trimethoprim but sensitive to neomycin, gentamycin, ampicillin, rifamycin, chloramphenicol, doxycyclin, cefuroxime, ceftizoxime, cefazolin, and clarithromycin. S6 showed resistance to cefixime and trimethoprim only but was unable to grow in the presence of the rest of the antibiotics. To study the effect of metals on the growth of the isolates, they were grown at varying concentration of different metals. It was observed that Cu and Ba do not affect growth of both the strains while higher concentrations (1000 μg ml^{-1}) of Cr, Mn, Co, Fe, Ni, and Zn inhibited bacterial growth. Hg was found to be the most toxic metal for both the strains, which were unable to grow at lower concentrations of Hg (100 μg ml^{-1}). When grown for varying time of incubation, both the strains showed maximum growth after 72 h of incubation after which decline was observed (Fig. 1a).

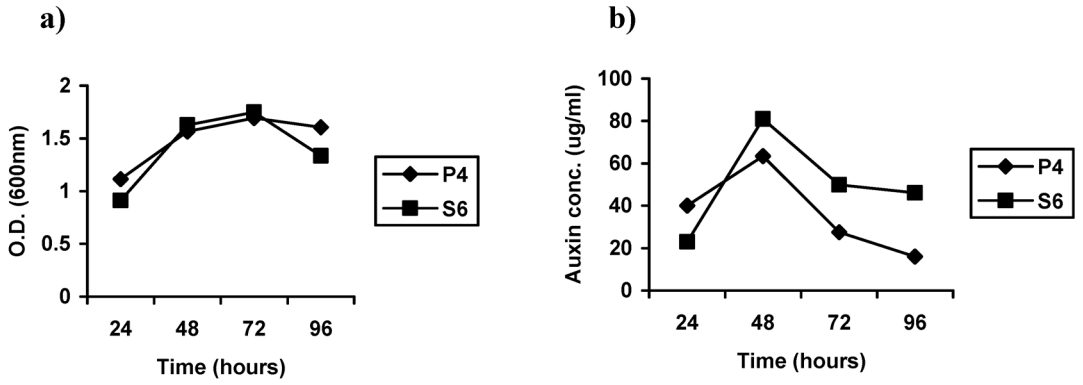

Fig. 1 Effect of different times of incubation (24, 48, 72, 96 h) (a) on the growth of the isolates (b) on auxin production of the isolates.

16S rRNA gene has been used to identify unknown bacteria. Gene sequence analysis of 16S rRNA was carried out. Sequence homology was searched through BLAST. The strain P4 was identified as *Bacillus flexus* while S6 was found to belong to the genus *Bacillus*. The sequences were submitted to GenBank under the accession numbers FJ356233 and FJ356234, respectively (Fig. 2).

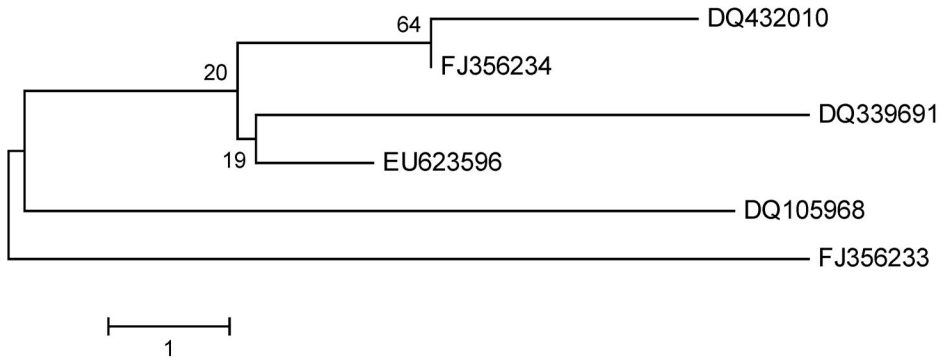

Fig. 2 Phylogenetic tree based on 16S rDNA gene sequencing analysis. Affiliations: FJ356233, *B. flexus* (Amb7); FJ356234, *Bacillus* sp. (Amb8); DQ432010, *B. aquimaris*; DQ339691, *Bacillus* sp.; EU623596, *B. pumilus*; DQ105968, *B.megatarium*.

Auxin production was determined following Torres-Rubio et al. [19]. Optimum temperature for auxin production was found to be 37 °C for both the strains. To investigate the effect of tryptophan on the auxin production, strains were grown in the presence of tryptophan ranging from 0.2 to 1 mg/ml. Increase in the concentration of tryptophan caused an increase in the amount of auxin produced. Auxin production was also determined by varying time of incubation of the strains. Both strains showed an increase in the amount of auxin production with increase in time of incubation until the early stationary phase, i.e., 48 h after which reduction in the concentration of auxin was recorded although the strains were showing constant increase in growth until 72 h of incubation (Fig. 1b). P4 and S6 were found to produce auxin in a concentration of 59 and 86 μg ml^{-1}, respectively.

Inoculation with the bacterial strains stimulated growth of *S. tuberosum*. Almost 40 and 35.5 % increase in the shoot length and 42 and 75 % increase in root length of the inoculated plants was observed with P4 and S6 inoculation, respectively, as compared to non-inoculated treatments. Both isolates improved plant growth by increase in the number of leaves over the control treatment, i.e., 67 and

33 % for P4 and S6, respectively. Both the strains enhanced auxin content of inoculated plants up to 71.4 and 433 %, respectively as compared to non-inoculated plants. Chlorophyll content of the plants was found to be affected by bacterial inoculation. Both the strains caused increment in chlorophyll "*a*" almost 6 and 26 % for P4 and S6, respectively, in comparison with the non-inoculated plants. Similarly, increase in the concentration of chlorophyll "*b*" was observed when compared with control, i.e., 55 and 82 % for P4 and S6, respectively. Increase in carotenoid content was recorded with P4 inoculation (213 %), whereas S6 has no effect on carotenoid concentration of the plants (Table 1).

Table 1 Effect of bacterial inoculation on growth parameters, auxin content, and chlorophyll content of *Solanum tuberosum*.

Treatment	Shoot length (cm)	Root length (cm)	No. of leaves	Auxin content (μg g^{-1})	Chlorophyll "*a*" (μg g^{-1})	Chlorophyll "*b*" (μg g^{-1})	Carotenoids (μg g^{-1})
Control	5.9 ± 0.38	4.0 ± 0.22	3 ± 0.12	10.5 ± 0.96	8.43 ± 0.45	2.56 ± 0.19	2.72 ± 0.13
P4	8.3 ± 0.50	5.7 ± 0.45	5 ± 0.14	18.0 ± 1.20	8.98 ± 0.56	3.99 ± 0.22	8.54 ± 0.55
S6	8.0 ± 0.40	7.0 ± 0.52	4 ± 0.26	56.0 ± 4.92	10. 63 ± 0.68	4.68 ± 0.25	2.74 ± 0.22
LSD at 0.05	2.85	1.58	4.96	15.4	0.65	0.78	0.27

Inoculation of sprouts with the isolates stimulated the growth of the roots in *S. tuberosum*. P4 and S6 caused 100 and 130 % increase respectively, in the number of the roots when compared with the control. Bacterial inoculation also affected the root length. Approximately 40 % increase in the root length was observed when inoculated with P4, whereas S6 caused 50 % increase in the length of roots as compared to the non-inoculated treatment (Table 2).

Table 2 Effect of bacterial inoculation on the number and length of roots of *Solanum tuberosum* sprouts.

S. no.	Treatment	No. of roots/sprouts	Root length (cm)
1	Control	3 ± 0.25	0.50 ± 0.02
2	P4	6 ± 0.46	0.70 ± 0.04
3	S6	6 ± 0.42	0.75 ± 0.05
	LSD at 0.05	2.55	0.27

DISCUSSION

The isolated strains were purified and screened for auxin production. Two of the isolates, P4 and S6, were found to produce auxins which were used for further study. Morphological characterization showed that both isolates were Gram-positive, motile, spore-forming rods. Both strains were catalase-positive and were able to ferment xylose, mannitol, sorbitol, arabinose, sucrose, and raffinose. The presence of metals affects bacterial growth. Both strains were unable to grow in the presence of Hg. According to Afrasayab et al. [1], Hg is highly toxic and exerts growth inhibitory effects on bacteria even at lower concentrations. The isolates were grown in the presence of various antibiotics. P4 was found to be resistant to lincomycin, streptomycin, cefixime, and trimethoprim, whereas S6 was resistant to cefixime and trimethoprim only. The optimum temperature for the growth of both of the isolates was found to be 37 °C. Decline in bacterial growth was observed with temperature. High temperature has been reported to adversely affect *Bacillus* growth due to variation in membrane lipids as well as im-

balance in cellular protein synthesis. Nonpermissive temperature causes thermal denaturation, which results in cell death [2]. Both of the strains grew over a pH range from 5 to 9 with an optimum at 5 and 6 for P4 and S6, respectively.

In cells, rRNA is the least variable gene, therefore it has been widely used to identify living organisms as well as to estimate species divergence [9]. Phylogenetic trees based on the whole genomic analysis have been reported to be closely similar to the trees constructed on the basis of 16S rRNA gene sequencing, justifying the extensive use of 16S rRNA sequencing for accurate identification of unknown species [3]. 16S rRNA gene sequencing of the isolated auxin-producing strains was carried out, and homology of the obtained sequences was searched through BLAST. The strain P4 was identified as *B. flexus* and S6 as *Bacillus sp*. Phylogenetic tree was generated using the neighbor-joining method (Fig. 2), indicating the relatedness of the isolates with other *Bacillus* sp.

One of the mechanisms involved in growth promotion by PGPB is by encoding the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which degrades ACC, resulting in the reduction of ethylene levels [21]. Other factors such as nitrogen fixation, suppression of phytopathogens, and induction of disease resistance may also be involved in growth promotion by PGPB, but the major factor considered to be responsible for growth promotion by plant growth-promoting bacteria is auxin production [10]. *Bacillus* sp. has been widely studied as plant growth-promoting rhizobacteria-producing phytohormones [8]. The effect of different environmental factors on the auxin production of the isolates was investigated by growing the strains at varying temperatures, times of incubation, and in the presence of tryptophan concentrations. Maximum amount of auxin was recorded at 37 °C after 48 h of incubation. *Pseudomonas putida* was found to produce higher levels of auxin after 42 h of incubation [15]. Increase in growth of both the isolates was recorded with increasing time of incubation up to 72 h, but the isolates showed a steady increase in auxin production 48 h after which reduction in auxin concentration was observed (Fig. 1b). When strains were grown with varying tryptophan concentration, it was observed that increase in tryptophan concentration stimulated auxin production by the isolates. Patten and Glick [15] also reported increase in auxin production in the presence of higher tryptophan concentrations. Auxin production was quantified by comparison of the peak areas of the bacterial auxin with standard IAA. P4 and S6 were found to produce auxin in a concentration of approximately 59 and 86 $\mu g\ ml^{-1}$ respectively.

Effect of the bacterial strains on the growth of the plants was studied by inoculating *S. tuberosum* sprouts and comparing various growth parameters of inoculated plants with non-inoculated treatments. Bacterial inoculation improved the plant growth by increasing shoot length approximately 40 and 35.5 % and root length up to 42 and 75 % for P4 and S6, respectively, as compared to the control plants. Enhanced mineral and nutrient uptake ability due to bacterial inoculation promotes plant growth. It is speculated that tryptophan is supplied by the root exudates to the bacteria which plays a vital role in microbial IAA synthesis [20]. Approximately 66 and 33 % respective increase in the number of leaves for P4 and S6 inoculation was observed as compared to control. The biochemical analysis of treated and untreated plants revealed that bacterial inoculation promoted auxin production by the plants as well, which is manifested by the higher auxin content values, i.e., 71 and 433 % increase for P4 and S6 inoculation, respectively, over the control treatment. *Bravebacterium* is also reported to enhance auxin content of *Helianthus annus* [6]. Pigment analysis of the inoculated and control plants showed increment in the production of chlorophyll and carotenoids in treated plants.

Bacterial inoculation has a pronounced effect on the roots of the plants. P4 and S6 caused 100 and 130 % increase in the number of roots, whereas 40 and 50 % increase in root length was observed for P4 and S6 inoculation, respectively. Auxins act as long-distance signals controlling many developmental processes of the plants either directly or indirectly. Stimulatory effects of auxin-producing bacteria on root morphogenesis and development have been reported in various studies, which results in enhanced root surface area and increased root elongation. Increase in root length and surface area stimulates efficient water and nutrient uptake, which in turns effects the overall development and growth of the plants. *Azospirillum*, a plant growth-promoting rhizobacteria, has been reported to stimulate root

growth in wheat and maize [14]. Selvakumar et al. [17] also reported plant growth promotion by *Bacillus* sp. in wheat and maize.

CONCLUSION

In conclusion, we can say that the auxin-producing *Bacillus* sp. exert stimulatory effects on the growth of plants. The beneficial effects of these plant growth-promoting bacteria are mostly related to the changes in auxin concentration. Modification of phytohormone levels by microbes can lead to characteristic changes in plant growth development such as phytohormones produced by the bacteria, which can increase root area, leading to higher water and other nutrients uptake from soil. These bacteria, therefore, can be effectively used for plant growth improvement. Further investigations about the mechanisms involved would help to improve the understanding of plant growth promotion by microorganisms.

REFERENCES

1. S. Afrasayab, A. Yasmin, S. Hasnain. *Pak. J. Biol. Sci*. **5**, 792 (2002).
2. A. Ahmed, A. N. Sabri, S. Hasnain. *Afri. J. Biotechnol.* **7**, 1505 (2008).
3. J. E. Clarridge. *Clin. Microbiol. Rev*. **17**, 840 (2004).
4. S. Compant, B. Duffy, J. Nowak, C. Clement, E. A. Barka. *Appl. Environ. Microbiol*. **71**, 4951 (2005).
5. J. T. Coombs, C. M. M. Franco. *Appl. Env. Microbiol*. **69**, 5603 (2003).
6. M. Faisal, S. Hasnain. *Res. J. Bot*. **1**, 24 (2006).
7. P. Gerhardt, R. G. E. Murray, W. A. Wood, N. R. Kreig. In *Methods for General and Molecular Bacteriology*, American Society for Microbiology, Washington, DC (1994).
8. A. Karadeniz, S. F. Topcuoglu, S. Inan. *World J. Microbiol Biotechnol*. **22**, 1061 (2006).
9. P. Kiratisin, L. Li, P. R. Murray, S. H. Fischer. *Eur. J. Clin. Microbiol*. **22**, 628 (2003).
10. S. Lee, M. Flores-Encarnacion, M. Contreras-Zentella, L. Garcia-Flores, J. E. Escamilla, C. Kennedy. *J. Bacteriol.* **186**, 5384 (2004).
11. H. K. Lichenthaler, A. R. Wellburn. *Biochem. Soc*. **11**, 591 (1983).
12. H. H. Long, D. D. Schmidt, I. T. Baldwin. *PLoSOne.* **3**, e2702 (2008).
13. A. Mahadevan. In *Growth Regulators, Microorganisms and Diseased Plants*, p. 31, Oxford and IBH Publishing, India (1984).
14. S. Mantelin, B. Touraine. *J. Exp. Bot.* **55**, 27 (2004).
15. C. L. Patten, B. R. Glick. *Appl. Environ. Microbiol.* **68**, 3795 (2002).
16. C.-M. Ryu, M. A. Farag, C.-H. Hu, M. S. Reddy H.-X. Wei, P. W. Pare, J. W. Kloepper. *Plant Biol.* **100**, 4927 (2003).
17. G. Selvakumar, S. Kundu, A. D. Gupta, Y. S. Shouche, H. S. Gupta. *Curr. Microbiol.* **56**, 134 (2007).
18. S. Spaepen, W. Versees, D. Gocke, M. Pohl, J. Steyaert, J. Vanderleyden. *J. Bacteriol*. **189**, 7626 (2007).
19. M. G. Torres-Robio, S. A. Valencia-Plata, J. Bernal-Castillo, P. Martinez-Nieto. *Rev. Latinoamer. Microbiol.* **42**, 171 (2000).
20. E. A. Tsavkelova, T. A. Cherdyntseva, S. G. Botina, A. I. Netrusov. *Microbiol. Res.* **162**, 69 (2007).
21. H. Youai, T. C. Charles, B. R. Glick. *Can. J. Microbiol*. **53**, 1291 (2007).

