Auxin production by phyllospheric bacteria and their growth promoting effects on *Cicer arietinum* L

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A B S T R A C T

The main objective of present study was to evaluate the phyllospheric bacteria for their auxin production and plant growth promoting potential under axenic conditions. Bacterial strains were isolated from the phyllosphere of different plants and screened for *in vitro* auxin production in the presence of precursor L-tryptophan. Finally, 7 isolates (BjP-2, BjP-5, CsP-1, CsP-6, ZmP-3, ZmP-4, SpP-6) that released maximum auxin levels were selected to evaluate the phytostimulatory effect on *Cicer arietinum* L. under axenic conditions. Antibiotic susceptibility pattern of strains was evaluated by using different antibiotic discs. Maximum auxin production was recorded with strain CsP-1 (85 µg ml^{-1}) when medium was supplemented with 250 µg ml^{-1} L-tryptophan. Antibiotic susceptibility pattern of phyllobacterial strains was also evaluated by using six different types of antibiotic discs. Results showed that strains BjP-2, CsP-1, CsP-6, ArP-1, ZmP-3, ZmP-4, BjP-4 and SpP-5 were resistant to erythromycin (15 µg) ampicillin (10 µg) and oxytetracyclin (30 µg). In pot trials, majority of the treatments showed significant improvements in different growth parameters amended with 250 µg g^{-1} of soil. It was obesrved that strain CsP-1, ZmP-4 and SpP-6 showed the most promising results for shoot length, root length and plant biomass. Strain CsP-1 showed up to 190%, 160%, 50% and 32% increases for shoot length, root length, fresh and dry biomass, respectively, over control. It can be concluded that phyllosphere of plants can be screened to select agriculturally important bacteria on the basis of their auxin production.

Keywords: Phyllobacteria, phytostimulatory effects, plant growth promotion, Cicer arietinum, plant growth promoting rhizobacteria.

INTRODUCTION

Plants are host to a variety of microorganisms that includes bacteria, fungi and yeasts. Bacteria that colonize aerial surfaces of plants including leaves, flowers, stems and fruits are termed as phyllospheric bacteria. Phyllosphere is one of the largest habitats for terrestrial microorganisms that may be deleterious or beneficial to plants. Phyllosphere is colonized by complex and highly diverse bacterial communities and there is great variability in bacterial composition on different plants. Among microbial inhabitants bacteria are considered the most diverse and abundant members of phyllosphere microbial community that colonize leaves at density up to 10^8 cells m^{-2} (Lindow and Brandle, 2003; Stapleton and Simmons, 2006; Meyer and Leveau, 2012). Different phyllospheric bacterial genera have the ability to colonize aerial plant surfaces that includes *Burkholderia, Acinetobacter, Bacillus, Paenibacillus, Pantoea, Xanthomonas, Photobacterium* and *Pseudomonas* (Kaur and Singh, 2001; Izhaki et al., 2013; Leff and Fierer, 2013).

Aerial plant surfaces represent a niche with great agricultural and environmental significance. There is a growing evidence for important interactions of phyllosphere microbial inhabitants that may affect the fitness of natural plant populations and the productivity of agronomic crops (Lindow and Brandle, 2003; Whipps et al., 2008). Phyllosphereic bacteria competitively colonize the roots of plant and can be used as biofertilizers or biopesticides to enhance the growth and productivity (Kumar et al., 2009). These bacteria can promote plant growth by suppressing the colonization and infection of tissues by plant pathogens. Commercial formulations of bacteria antagonistic to phytopathogens has been used as an environmentally safe method to control plant diseases (Lindow and Leveau, 2002; Rasche et al., 2006). The aim of the current study was to isolate and screen effective auxin producing phyllospheric bacteria to enhance growth of *Cicer arietinum.* For this purpose, bacterial

strains were isolated from the phylloplane of different crops i.e *Spinacia oleracea, Zea mays, Lactuca sativa, Armoracia rusticana, Coriandrum sativum and Brassica juncea* and evaluated for their *in vitro* auxin production ability. Hence, auxin production and plant growth promoting potential of bacteria colonizing the aerial plant surfaces of crops was reported in present study.

MATERIALS AND METHODS

Isolation of bacteria from phyllosphere

Isolation of bacteria from the phyllosphere of *Spinacia oleracea, Zea mays, Lactuca sativa, Armoracia rusticana, Coriandrum sativum and Brassica juncea* was carried out from leaf samples. One gram of leaves of each plant were collected, weighed and surface sterilized in 0.1% solution of $HgCl_2$. The samples were washed with autoclaved distilled water, then finely crushed in a sterilized pastel and mortar. Serial dilutions of different samples were made separately by mixing paste in 9 ml of autoclaved distilled water. One ml of this suspension was taken and added in 9 ml of autoclaved distilled water to prepare 10^{-1}, 10^{-2} and so on. About 50 μl of each dilution was plated on Luria-Bertani agar (L-agar) and plates were incubated at $37^{\circ}C$ for 24 h. Bacterial strains showing prolific growth were selected and purified by streaking several times on LB-agar plates (Cappuccino and Sherman, 2002).

Characterization of bacterial strains

Initially, thirty five bacterial strains were selected from the phyllosphere of different plants. Finally, eleven bacterial strains were selected on the basis of *in vitro* auxin production. Bacterial strains were characterized morphologically by recording colony characteristics such as color, size, form, gram staining, cell shape, margin, elevation and consistency. Strains were also evaluated for biochemical tests including indole production, urease, citrate, catalase, cytochrome oxidase and nitrate reduction (Cappuccino and Sherman, 2002).

Antibiotic susceptibility pattern of bacterial strains

Antibiotic sensitivity of strains was evaluated by Kirby-Bauer method (1966). Mueller-Hinton agar plates were prepared and susceptibility of the isolates was determined by using six antibiotic discs of erythromycin (15 μg), ampicillin (10 μg), carbenicillin (100 μg), gentamycin (100 μg), penicillin (10 μg) and oxytetracyclin (30 μg) (Bioanalyse Co., Ltd, Turkey). Antibiotic susceptibility pattern was evaluated by measuring the zone of inhibition in mm. Each disc was placed at equal distance from each other and incubated at $37^{\circ}C$ for 24 h. After incubation, zone of inhibition in the form of clear zone around the antibiotic disc was recorded (mm) and compared with the standard chart as mentioned in Cappuccino and Sherman (2002).

Bacterial auxin production

Bacterial auxin production was evaluated by colorimetric method using Salkowski reagent (Tang and Borner, 1979). About 25 ml L-broth medium was supplemented with 0, 100, 150, 200 and 250 μg ml^{-1} of L-tryptophan in 50 ml Erlenmeyer flask. Broth was inoculated with 100 μl of bacterial culture (in triplicate) and incubated at $37^{\circ}C$ for 72 h on shaker at 150 rpm. Control treatments without L-tryptophan were also kept for comparison. After incubation, 1.5 ml of bacterial culture was centrifuged at 5000 *g* for 15 min and one ml of supernatant was taken in a test tube and mixed with 2 ml of Salkowski reagent. Test tubes were kept in dark for 25 min for the development of pink color. The intensity of pink color was measured with the help of spectrophotometer (CECIL 7200) at 535 nm wavelength. Finally, the concentration of auxin produced by bacteria was determined by standard curve constructed by using different concentrations of standard auxin (Sigma).

Thin layer chromatography

L-broth medium (50 ml) was supplemented with filter sterilized solution of L-tryptophan and inoculated with bacterial strains as mentioned above. After incubation at $37^{\circ}C$ for 72 h, cultures were centrifuged at 5000 *g* for 20 min to obtain cell free supernatant. After adjusting pH to 2.5 with 1N HCl, 20 ml of cell free supernatant was extracted thrice with equal amount of ethyl acetate in 250 ml separating funnel. Ethyl acetate fraction was separated in 100 ml beaker and evaporated at $45^{\circ}C$. The concentrated extracts were re-dissolved in 5 ml methanol and placed on water bath till the volume reached to 2 ml. Thin layer chromatography (TLC) was carried out on 0.5 mm-thick silica coated aluminium plates (Merck). About 5 μl of bacterial extracts and standard auxin were spotted on TLC plate and allowed to dry for 10 min. LB broth medium was also spotted as negative control. TLC plate was placed in a glass tank containing a solvent, a mixture (v/v) of isopropanol, ammonium hydroxide and water (10:1:1) to separate auxin. Presence of auxin was detected by spraying with Salkowski reagent and observed the development of pink band which is an indication of auxin.

Pot trials with Cicer arietinum

Procurement of seeds was accomplished by taking healthy seeds of *Cicer arietinum* (Bittal-98) from Punjab seed corporation Lahore, Pakistan. Healthy and uniform seeds were selected and surface sterilized with 0.1% $HgCl_2$ for 5 min

followed by repeated washings 3-4 times with autoclaved distilled water. Bacterial inoculum preparation was performed by selecting seven most efficient auxin producing strains (BjP-2, BjP-5, CsP-1, CsP-6, ZmP-3, ZmP-4, SpP-6). Strains were streaked on L-agar medium and incubated at 37ºC for 24 h. After incubation, a loopful of bacterial cells were taken and suspended in autoclaved and distilled water. Optical density of bacterial suspension was adjusted to a final concentration of 10^7 cells per ml with spectrophotometer (CECIL 7200). Surface sterilized seeds were inoculated with bacterial culture for 25 min and control seeds were soaked in sterilized distilled water for the same duration. Finally, pot experiments under axenic conditions were conducted to evaluate the phytostimulatory effect of bacterial strains on *Cicer arietinum*. For experiment, pots (6×6 cm) were filled with 90 g of autoclaved, dried soil and amended with L-tryptophan to final concentrations of 100 and 250 μg g^{-1} of soil as source of precursor for auxin biosynthesis within plant roots. The soil had a pH 7.1, electrical conductivity (EC) 42 ds m^{-1}, and 0.65% organic content. Five seeds were sown in pots in triplicate and experiment was repeated twice. Pots were incubated at 25±1ºC under photoperiod of 16 h. Sterile conditions were maintained in Versatile Environmental Test Chamber (MRL 350H, Sanyo, Japan) throughout the growth period of plants. After 2 weeks, all the plants were harvested and different growth parameters such as root length, number of roots, shoot length, numbers of leaves were recorded.

Statistical analysis

Data was subjected to analysis of variance (ANOVA) using SPSS 16 program and means separated using Duncan's multiple range test (P = 0.05).

RESULTS AND DISCUSSION

Results
Characterization of bacterial strains

Morphological and biochemical characteristics of bacterial strains are given in Table 1. Most of the strains showed circular colonies with entire margins; however, small sized colonies were with convex elevation and off-white color. But some also showed color variations like orange (ArP-1) and yellow (BjP-4). Majority of the strains were gram negative rods (BjP-2, SpP-7, CsP-1, CsP-6, ZmP-3, ZmP-4, BjP-4 and SpP-6). A few strains were also gram positive rods (SpP-5, BjP-5, CsP-6). For further characterization, biochemical tests were also performed (Table 1). All the strains (BjP-2, SpP-7, CsP-1, CsP-6, ArP-1, ZmP-3, ZmP-4, BjP-4, BjP-5, SpP-5 and SpP-6) were catalase positive. Majority of the strains including BjP-2, SpP-7, CsP-6, ArP-1, ZmP-3, ZmP-4, BjP-4, BjP-5, SpP-5 and SpP-6 gave positive results for cytochrome oxidase. The production of acidic and non-acidic end products was evaluated by change in color of methyl red turned to yellow which is an indicative of negative result that was observed with CsP-1. The strains having the ability to degrade urea produced deep pink color in the medium that was recorded for BjP-4, BjP-5, SpP-5 and ZmP-3. While the strains BjP-2, SpP-7, CsP-1, CsP-6, ArP-1, ZmP-4, and SpP-6 were urease negative. Majority of the strains also showed positive results for citrate utilization that was indicated by change in color of the medium from green to blue. For motility testing stabbing was done with a single streak on SIM Agar, strains that are motile show smeared growth whereas non-motile strains showed a single line of growth. The presence of indole is determined by the addition of Kovac's reagent and cherry red layer appeared on the surface of medium. Cherry red color is the indication of indole positive test that was shown by ArP-1, ZmP-3, ZmP-4, BjP-4 and BjP-5.

Table 1. Morphological and biochemical characteristics of bacterial strains

characteristics	Bacterial strains										
	BjP -5	BjP -2	BjP-4	SpP-5	SpP-6	SpP-7	CsP -1	CsP -6	ArP-1	ZmP-3	ZmP-4
Colony color	Y	OF	OF	OF	OF	Cir	Cir	Cir	Irr	Cir	Cir
Colony size	S	S	S	M	M	W	Y	C	O	OF	Y
Colony form	Cir	Cir	R	Cir	Irr	Ent	Ent	Ent	Und	Ent	Und
Gram staining	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Cell shape	Cocci	cocci	Rod	Rod	Rod	Rod	Rods	Rod	CB	Rod	Rods
Colony margin	E	E	F	E	E	Co	Co	Flat	Flat	Flat	Ra
Colony elevation	Co	Ra	Ra	Co	Flat	M	S	S	M	S	S
Colony consistency	Dry	Mu	Mu	sticky	sticky	Dry	Dry	sticky	sticky	Dry	Mu
Indole production	+ve	-ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve
Urease test	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve	+ve	-ve
citrate	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	-ve	+ve	+ve
catalase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve
cytochrome oxidase	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve
IAA production	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Nitrate reduction	+ve	-ve	-ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve

Affiliations: Cir, circular; Irr, irregular; OF, off-white; Y, yellow; W, white; C, creamy, O, orange; Co, convex; S, Small; Mu, Mucoid; M, Medium; R, Rhizoidal; F, Filamentous; E, Entire; Und, Undulate; Ra: raised ; CB, Cocco- bacillus

Antibiotic susceptibility pattern of bacterial strains

Six antibiotic discs of erythromycin (15 μg), ampicillin (10 μg), carbenicillin (100 μg), gentamycin (100 μg), penicillin (10 μg) and oxytetracyclin (30 μg) were used to evaluate the antibiotic susceptibility pattern of bacterial strains. Strains (BjP-2, CsP-1, CsP-6, ArP-1, ZmP-3, ZmP-4, BjP-4, SpP-5) were resistant to erythromycin, ampicillin and oxytetracyclin with zone of inhibition less than 3 mm (Table 3). On the other hand, BjP-5, SpP-7 and SpP-6 were sensitive to erythromycin with zone of inhibition 14, 16 and 12 mm, respectively. For ampicillin, majority of the strains were sensitive while BjP-4, CsP-6, ZmP-3, ZmP-4 were resistant. Susceptibility against gentamycin was shown by BjP-4, ArP-1, SpP-5, ZmP-4 that respectively recorded zone of inhibition of 22, 14, 16 and 11 mm. For oxytetracyclin, majority of the strains where resistant, however, SpP-6, SpP-7 and CsP-1 were sensitive. In case of penicillin, BjP-2, BjP-4, Bjp-5, CsP-6 and ZmP-4 were sensitive. For carbenicillin, bacterial strains SpP-7, CsP-1 and CsP-6 were susceptible with zone of inhibition 14, 13 and 18 mm, respectively.

Table 2. L-tryptophan dependent auxin production by phyllospheric bacteria

| Strains | L-tryptophan (μg ml^{-1}) | | | | |
| | 0 | 100 | 150 | 200 | 250 |
	Auxin (μg ml^{-1})				
BjP-5	10 (ab)	21 (c)	34 (c)	45 (d)	58 (d)
BjP -2	4 (a)	13 (ab)	16 (ab)	20 (b)	34 (b)
SpP-7	2 (a)	3 (a)	7 (a)	11 (a)	15 (a)
CsP -6	7 (b)	11 (b)	12 (b)	15 (a)	21 (ab)
ArP-1	5 (a)	8 (b)	10 (b)	13 (a)	17 (a)
CsP -1	31 (f)	54 (f)	58 (e)	63 (g)	85 (f)
ZmP-4	31 (f)	54 (f)	13 (b)	14 (a)	16 (a)
BjP -4	32 (f)	34 (d)	39 (c)	50 (e)	32 (b)
ZmP-3	16 (c)	51 (f)	55 (e)	58 (f)	63 (e)
SpP-5	22 (d)	31 (d)	34 (c)	38 (c)	42 (c)
SpP-6	29 (e)	37 (e)	44 (d)	58 (f)	66 (e)

Mean of 3 replicates. Different letters within same column indicates significant difference between treatments using Duncan's multiple range test (P = 0.05)

| Strains | Antibiotics | | | | | | | | | | | |
| | Ery (15 μg) | | Amp (15 μg) | | Gen (15 μg) | | Oxy (15 μg) | | Pen (15 μg) | | Car (15 μg) | |
	Zone Size (mm)	Sensitivity	Zone Size (mm)	Sensitivity	Zone Size (mm)	Sensitivity	Zone Size (mm)	Sensitivity	Zone Size (mm)	Sensitivity	Zone Size (mm)	Sensitivity
BjP-2	2	R	5	R	5	R	1	R	11	S	4	R
BjP-4	0	R	0	R	22	S	5	R	13	S	7	I
BjP-5	14	S	11	R	8	I	7	I	14	S	0	R
ArP-1	4	R	25	S	14	S	1	R	1	R	1	R
SpP-5	0	R	26	S	16	S	4	R	7	I	1	R
SpP-6	12	S	19	S	3	R	17	S	2	R	6	I
SpP-7	16	S	18	S	2	R	7	I	2	R	14	S
CsP-1	2	R	18	S	4	R	6	I	6	I	13	S
CsP-6	0	R	0	S	4	R	1	R	13	S	18	S
ZmP-3	7	I	0	R	1	R	3	R	1	R	7	I
ZmP-4	0	R	0	R	11	S	0	R	15	S	0	R

Abbreviations: Ery, Erythromycin; Amp, Ampicillin; Gen, Gentamicin; Oxy, Oxytetracyclin; Pen, Penicillin; Car, Carbenicillin; S, Sensitive; R, Resistant; I, Intermediate

Auxin production by bacterial strains

Production of auxin in bacterial culture supernatant was quantified by colorimetric method in the absence and presence of L-tryptophan. Strain Bjp-4 was the most effective to produce highest level of auxin (32 μg ml^{-1}) in the absence of L-tryptophan. However, increasing precursor concentrations enhanced auxin production several folds. For instance, bacterial strains Csp-1, SpP-6, ZmP-3, BjP-5 and SpP-5 released 85, 66, 63, 58 and 42 μg ml^{-1} auxin, respectively, when medium was amended with 250 μg ml^{-1} L-tryptophan (Table 2). The confirmation for auxin production ability of bacterial strains was further carried out by TLC. TLC plates showed pink colored bands of auxin at similar distance in comparison with standard (Sigma). On comparing the R$_f$ of standard IAA with bacterial extracts, it was observed that the R$_f$ values of samples were very close to that of the standard IAA.

Growth promotion of Cicer arietinum

Pot experiments under laboratory conditions were carried out in the presence of 100 and 250 μg ml^{-1} L-tryptophan to evaluate the phytostimulatory effect of phyllospheric bacteria. Overall, significant increases in growth parameters were recorded when soil was amended with 250 μg ml^{-1} L-tryptophan (Table 4). For shoot length, significant improvements were recorded with CsP-1 (190%), SpP-6 (172%), ZmP-4 (163%) and BjP-2 (136%), over water treated control. Significant increases for shoot length were recorded with SpP-6 (170%) CsP-1 (160%) and ZmP-4 (150%). In case of fresh weight, significant increases of 110 and 50 and 42, respectively, were recorded for SpP-6, CsP-1 and ZmP-4. For dry weight, ZmP-4 (42%) and CsP-1 (34%) were the most effective, over control.

Table 4. Effect of bacterial inoculation on growth of *C. arientinum* in L-tryptophan amended soil.

Strains	L-tryptophan (μg g^{-1} soil)	Shoot Length (cm)	Root Length (cm)	Fresh Weight (g)	Dry Weight (g)
Control	000	11 (a)	10 (a)	0.211 (ab)	0.159 (b)
BjP-2	100	22 (c)	15 (ab)	0.225 (ab)	0.161 (ab)
BjP-2	250	26 (c)	18 (c)	0.280 (d)	0.157 (b)
BjP-5	100	15 (b)	10 (a)	0.205 (a)	0.164 (ab)
BjP-5	250	19 (b)	14 (ab)	0.206 (a)	0.155 (b)
CsP-1	100	28 (cd)	22 (c)	0.312 (e)	0.213 (c)
CsP-1	250	32 (e)	26 (cd)	0.315 (e)	0.211 (c)
CsP-6	100	16 (b)	12 (a)	0.220 (ab)	0.160 (ab)
CsP-6	250	18 (b)	14 (ab)	0.231 (c)	0.150 (b)
ZmP-4	100	23 (c)	22 (c)	0.271 (d)	0.206 (c)
ZmP-4	250	29 (cd)	25 (cd)	0.301 (e)	0.225 (c)
ZmP-3	100	17 (b)	13 (a)	0.188 (a)	0.145 (b)
ZmP-3	250	23 (c)	18 (c)	0.265 (d)	0.175 (ab)
SpP-6	100	25 (c)	21 (cd)	0.287 (d)	0.111 (a)
SpP-6	250	30 (e)	27 (cd)	0.44 (f)	0.123 (a)

Mean of 30 plants. Different letters within same column indicates significant difference between treatments using Duncan's multiple range test (P = 0.05)

Discussion

Present study demonstrated the auxin production potential of phyllospheric bacteria isolated from different plants. Bacterial strains were evaluated for their phytostimulatory effect by inoculating *C. arietinum* plants in pot trials. It was observed in our study that phyllospheric bacteria produced auxin as secondary metabolite in culture supernatant. L-tryptophan has been identified as main precursor for auxin biosynthesis in bacteria. *In vitro* studies have demonstrated that some microbial cultures can produce small amounts of auxin in the absence of precursor. However, in the presence of L- tryptophan, microbes often released much greater quantities of auxin (Ali et al., 2009). Efficacy of bacterial auxin on in vitro growth of *Brassica oleracea* was also reported by (Ali and Hasnain, 2007). In our study, phytostimulatory effect of phyllospheric bacterial strains was evaluated after amending soil with 100 and 250 μg g^{-1} of soil. Bacterial strains CsP-1, ZmP-4 and SpP-6 showed the best results for enhancing shoot length, root length and plant biomass. Phyllosphere bacteria can promote plant growth and suppress the colonization and infection of tissues by plant pathogens (Leff and Fierer, 2013). It has been proposed that these microbial originating phytohormones, especially IAA, would be responsible for the root development changes induced by rhizospheric bacteria. Plant-exuded tryptophan would enter the IAA biosynthesis pathways of bacteria living in the rhizosphere, and in return, a significant part of the bacterial IAA would be delivered to the plant root (Spaepen et al., 2007, 2008). It has been reported that the inoculation with the phyllobacterial STM196 strain resulted in increased lateral root length in plants grown under gnotobiotic conditions (Contesto et al., 2010).

Finally, it can be concluded that phyllospheric bacterial strains have the potential to produce variable levels of auxin in the presence of different L-tryptophan concentrations. Strains also showed variation in their susceptibility pattern against different antibiotics. In pot trials, strains CsP-1, ZmP-4 and SpP-6 were the most effective in enhancing different growth parameters. Overall, phyllosphere of plants offer good opportunity for the isolation and screening of agriculturally important bacteria.

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