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Original research article

Plant Growth-Promoting Endophyte *Serratia marcescens* AL2-16 Enhances the Growth of *Achyranthes aspera* L., a Medicinal Plant

Khaidem Aruna Devi,[1] Piyush Pandey,[1*] Gauri Dutt Sharma[2]

[1] *Department of Microbiology, Assam University, Silchar, Assam, India.*
[2] *Bilaspur University, Bilaspur, Chattisgarh, India.*

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ABSTRACT

An endophytic bacterium, AL2-16, was isolated from *Achyranthes aspera* L. It was characterized and identified as *Serratia* sp. AL2-16 and was experimented for the presence of plant growth-promoting properties. AL2-16 produced siderophore in iron-deficient conditions. The quantitative estimation of siderophore production unit of AL2-16 was maximum after 48 hours of incubation (83.488%) in the presence of 1 μM of ferric chloride. The fructose followed by glucose and sucrose were proved to be the best carbon sources resulting in appreciable amount of siderophore production, i.e. 77.223%, 73.584%, and 65.363% respectively. AL2-16 also has the ability to produce indole acetic acid in medium supplemented with L-tryptophan. The highest amount of indole acetic acid, in the presence of 1.0% L-tryptophan, was 123.2 μg/mL after 144 hours. This isolate solubilized inorganic phosphate and also gave positive result for ammonia production. Colonization and pot trial experiments were conducted on *A. aspera* L. plant. The population of AL2-16 increased from 16.2×10^6 to 11.2×10^8 colony forming unit/g between 3rd and 5th days after inoculation. It significantly ($p \leq 0.05$) increased shoot length by 95.52%, fresh shoot weight by 602.38%, fresh root weight by 438%, and area of leaves by 127.2% when inoculated with AL2-16, as compared with uninoculated control.

1. Introduction

Achyranthes aspera L. is an important medicinal plant belonging to the family *Amaranthaceae* and commonly known as *latjeera*. This plant has medicinal importance as is used for the dilation of the blood vessels, lowering of the blood pressure, depression of the heart, and increase in the rate and amplitude of respiration (Neogi *et al.* 1970). *A. aspera* L. is known to have bioactive chemicals like ecdysterone, n-hexacos-17-enoic acid, spinasterol, achyranthine, betaine, pentatriaontane, hexatriacontane, tritriacontane, hydroquinone, p-benzoquinone, spathulenol, nerol, asarone, and essential fatty acids that are known for pharmacological activities like spermicidal, antiallergic, cardiovascular, nephroprotective, antiparasitic, anti-inflammatory, hypoglycemic, analgesic, hepatoprotective potency, and inhibit leukocyte infiltration (particularly eosinophils and neutrophils), antiperiodic, antimicrobial, purgative, antipyretic, and are used in various types of gastric disorders (Praveen 2014). Considering the medicinal importance of *A. aspera* L., it is pertinent to understand the role of endophytic bacteria on its growth and other properties.

The existence of endophytic bacterial communities has been recognized for more than a hundred years (Hardoim *et al.* 2008). Initially, these microorganisms were considered to be neutral with regard to their effects on host plants; more recently, however, their positive impact has been verified in a broad range of crops (Ryan *et al.* 2008), in which they may contribute directly to plant growth by promoting nutrient availability, biological nitrogen fixation, and the production of phytohormones (Shishido *et al.* 1999; Kim *et al.* 2011). Indirectly, they may also reduce microbial populations that are harmful to the plant, acting as agents of biological control through competition, antibiosis, or systemic resistance induction (Sturz *et al.* 2000; Ramamoorthy *et al.* 2001). *Serratia marcescens* has been described to be an important rice endophyte (Gyaneshwar *et al.* 2001), and it has also been isolated from flowers of summer squash (Selvakumar *et al.* 2008a,b), healthy tissue of edible cactus plants (Li *et al.* 2011), and from medicinal plant, *Centella asiatica* (Nongkhlaw and Joshi 2014). Many studies have shown the potential of *S. marcescens* that induces plant growth by

* Corresponding author.
E-mail address: piyushddn@gmail.com (P. Pandey).
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stimulating phytohormone production and phosphate solubilization (Chen *et al.* 2006; Selvakumar *et al.* 2008a,b) along with improvement of nitrogen supply in nonsymbiotic associations (Islam *et al.* 2010). In the present study, endophytic *S. marcescens* isolated from the aerial part of *A. aspera* L. was investigated for its plant growth-promoting potential.

2. Materials and Methods

2.1. Isolation of bacterial endophytes

Individual plants of *A. aspera* L. were randomly collected from different parts of Manipur, India. Briefly, the leaves were surface sterilized (70% ethanol, 3 minutes; 0.1% $HgCl_2$, 2 minutes) and added with phosphate buffer and homogenized with mortar and pestle. About 0.1 mL of approximately 10^{-3}–10^{-6} dilution of tissue was plated on yeast extract mannitol agar (YEMA). Plates were incubated at 28°C for 3 days to isolate bacteria. Morphologically distinct colonies were selected by colony characters, subcultured, purified, and used for further studies.

2.2. Morphological, biochemical, and molecular identification of bacteria

The isolate was characterized for colony morphology, Gram staining, and biochemical analysis. Isolate was also tested for catalase, oxidase, and carbohydrate fermentation. The identification of AL2-16 was done on the basis of 16S rRNA gene sequence homology using MEGA6 software by neighbor-joining method with 1000 bootstrap replicates (Tamura *et al.* 2011).

2.3. IAA production assay

Isolate was cultivated at 28°C for 10 days in YEM broth supplemented with different L-tryptophan concentration (0%, 0.2%, 0.4%, 0.6%, 0.8%, and 1.0%). It was harvested through centrifugation (8000 rpm, 28°C) after every 24-hour interval. Supernatant (1 mL) was mixed with 2 mL of Salkowski reagent (50 mL of 35% perchloric acid with 1 mL of 0.5 M ferric chloride [$FeCl_3$]) (Gordon and Weber 1951). The optical density (OD) of solution was measured at 530 nm after 30 minutes of incubation, and the amount of indole acetic acid (IAA) produced was calculated by comparing with the standard curve prepared with pure IAA.

2.4. Siderophore production assay

Siderophore production was monitored by formation of orange halos around bacterial colonies on chrome azurol S (CAS) agar plates kept incubated at 28°C for 48 hours (Schwyn and Neilands 1987). The cultures were inoculated in iron-deficient medium containing (g/L): K_2HPO_4 (6.0), KH_2PO_4 (3.0), $MgSO_4 \cdot 7H_2O$ (0.2), $(NH_4)_2SO_4$ (1.0), and succinic acid (4.0) at 30°C on a rotary shaker at 120 revolutions per minute. Quantitative estimation of siderophores was done by CAS-shuttle assay. About 1 mL of culture supernatant was mixed with 1 mL of CAS reagent (10 mM of hexadecyltrimethylammonium, 1 mM of $FeCl_3$ solution, and 2 mM of CAS solution), and absorbance was measured at 630 nm against a reference, having 1 mL of uninoculated broth and 1 mL of CAS reagent (Payne 1994). The activity was recorded in percentage siderophore units (SUs) calculated as $[(Ar-As) \times Ar^{-1}) \times 100]$, where Ar = absorbance of reference at 630 nm (uninoculated media + CAS reagent) and As = absorbance of sample at 630 nm (culture supernatant + CAS reagent).

2.4.1. Effect of growth medium on siderophore production

Production of siderophore was investigated on different media viz. succinic medium (SM) (g/L): K_2HPO_4 (6.0), KH_2PO_4 (3.0), $MgSO_4 \cdot 7H_2O$ (0.2), $(NH_4)_2SO_4$ (1.0), and succinic acid (4.0); nutrient broth (g/L): peptone (5.0), NaCl (5.0), beef extract (1.5), and yeast extract (1.5); luria broth (g/L): casein enzymichydrolysate (10.0), yeast extract (5.0), and NaCl (10.0); yeast extract mannitol broth (g/L): yeast extract (1.0), mannitol (10.0), K_2HPO_4 (0.5), $MgSO_4 \cdot 7H_2O$ (0.2), and NaCl (0.1). A loopful of culture from YEMA slants was separately inoculated in 100 mL of different media and incubated at 28°C, on a rotary shaker at 220 rpm. Siderophore production was monitored every 24 hours.

2.4.2. Influence of sugars, nitrogen sources, and organic acids

The influence of different carbon sources such as glucose, sucrose, mannitol, maltose, and fructose was studied on siderophore production by the isolate. All carbon sources were added externally at concentration 1% (w/v) to the SM (Sayyed *et al.* 2005). In case of nitrogen sources, influence of urea and sodium nitrate was studied by replacing ammonium sulphate in SM. Siderophore production in these media was compared with that of SM containing ammonium sulphate. To examine the influence of different organic acids on siderophore production, SM was supplemented with 0.4% (w/v) each of malic acid, oxalic acid, and citric acid.

2.4.3. Effect of iron concentration

To determine the threshold level of iron for siderophore production, iron content of SM was varied by the addition of $FeCl_3$ in the range of 0–30 μM concentration. Bacterial strain was inoculated and kept for incubation at 29°C at 120 rpm, and siderophore content was estimated.

2.5. Phosphate solubilization

The isolate was screened for phosphate solubilization using modified Pikovskaya medium (g/L): glucose (10), $(Ca_3)_2PO_4$ (5), $(NH_4)_2SO_4$ (0.5), NaCl (0.2), $MgSO_4 \cdot 7H_2O$ (0.1), KCl (0.2), $FeSO_4 \cdot 7H_2O$ (0.002), yeast extract (0.5), $MnSO_4 \cdot 2H_2O$ (0.002), agar (20), and pH 7 containing bromophenol blue (2.4 mg/mL) (Nautiyal 1999). The medium was inoculated and incubated for 48 hours and observed for the formation of zone around the colony. Quantitative estimation of P content in the supernatant was estimated using the vanadomolybdate colorimetric method (Koenig and Johnson 1942).

2.6. Ammonia production and nitrogen fixation

The ability of bacterial strains to produce ammonia was assessed as described by Marques *et al.* (2010). In this method, 20 μL of an overnight grown test culture was inoculated into 5 mL of 1% proteose peptone broth and incubated at 30°C in a shaking water bath. After 48 hours, 0.5 mL of Nesseler's reagent was added to the culture, and the color change was noted; a yellow coloration indicates positive result. Nitrogenase activity was determined by acetylene reduction assay to confirm nitrogen fixation ability of isolate by using gas chromatography-flame ionization detector (GC-FID) (Hardy *et al.* 1971).

2.7. *In vitro* propagation of *A. aspera* L. in bacteria-free condition

Micropropagated plantlets of *A. aspera* were raised from the surface-sterilized seeds on half-strength Murashige and Skoog (MS) medium. The seeds of *A. aspera* L. were placed in the petri dishes containing 25 mL of sterilized half-strength MS medium. The plates were incubated under humidity (60%), temperature (24 ± 2°C), and light (1000 lux, 16 hours light and 8 hours dark). After 4 weeks, seedlings having cotyledons and roots were transferred to freshly prepared MS medium and allowed them to grow. After development of extensive root system and six leaflets, the plantlets were gradually acclimatized to natural environment and finally planted in sterile soil under greenhouse conditions (26 ± 2°C and 70% relative humidity).

2.8. Inoculation of plants with endophytic bacteria

Bacteria were grown to the mid-log phase, pelleted by centrifugation (6000 rpm, 10 minutes, 24°C), washed twice, and suspended in sterile double-distilled water. It was maintained at an OD_{600} of 1. To confirm inoculation density and purity, an aliquot of culture was serially diluted in sterile double-distilled water and plated on YEM agar medium. The plants were inoculated in triplicate when a minimum height of 7.5 cm was reached, and the stalks were at least 0.5 cm in diameter, which corresponded to 75–80 days after seed germination. A 26-gauge needle attached to a tuberculin syringe containing a bacterial suspension was passed horizontally through the stem just above the first cotyledon leaves of the plant. A 10 μL droplet of suspension was formed at the tip of the needle, which was withdrawn through the plant stem.

2.9. Population density of AL2-16 from experimentally inoculated plants and effect on plant growth in pot trial experiment

The bacterial multiplication in the stem and leaves of plants was determined at 3 and 5 days after inoculation (DAI). Lower parts of stems and first leaves were collected from three replications. Each stem and leaves sample were weighed and surface sterilized for 15 seconds in 70% ethanol. They were rinsed with double-distilled water and macerated with 1 mL of sterile double-distilled water. After 20 minutes, the macerated tissues were settled down, and the supernatant was serially diluted. Supernatant was plated on YEM agar and incubated at 28°C for 48 hours. As a control for the inoculation studies to test for the presence of indigenous endophytic bacteria, the control plants were inoculated with sterile double-distilled water by using the same methods used for the experimental plants. All bacterial endophytes recovered were compared morphologically with control plants to distinguish growth of introduced bacteria.

Furthermore, the pots were kept at randomized block design and compared with the control. The plants were harvested after 150 days, and different growth parameters, such as shoot length, root length, number of leaves, fresh leaf weight, fresh shoot weight, fresh root weight, dry leaf weight, dry shoot weight, dry root weight, and area of the leaf, were measured.

2.10. Analysis of availability of N, P, and K contents and N, P, and K uptakes in soil

The available N content in soil was determined using the boric acid method (Subbiah and Asija 1956), P content by ascorbic acid method (Bray and Kurtz 1945), and K content by flame photometer (Hanway and Heidel 1952). Nitrogen uptake in *A. aspera* L. plants was determined by micro-Kjeldahl digestion method (Jackson 1958) and total P and K contents by dry ashing method (Chapman and Pratt 1961).

2.11. Experimental design and statistical analysis

The experiment has two treatments (with and without bacterial inoculation), each with five plants and arranged in a completely randomized design. All data were subjected to one-way analysis of variance followed by independent t test at $p \leq 0.05$ using the SPSS 16 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Isolation and characterization of endophytic AL2-16 isolate

About 73 isolates were isolated from different areas of Manipur as endophytic bacteria from the leaves and stems of *A. aspera* L. One of the isolate, AL2-16, was selected for this study. AL2-16 was gram negative, catalase and oxidase positive, small rod, forming reddish, circular, and smooth colonies on YEMA. It was able to ferment glucose, fructose, mannitol, sucrose, and maltose but found negative for lignin and cellulose. It had indole negative, methyl red negative, Voges-Proskauer negative, and Simmon citrate positive characteristics. The results of the BLAST search of the 16S rRNA gene sequences indicated AL2-16 isolate as closely related to *S. marcescens*. Based on the phylogenetic tree constructed with the 16S rDNA similarity (%), it was identified as *S. marcescens*, maximum similarity was observed with isolate KREDT/AB061685 (Figure 1). Strain AL2-16 clustered with *S. marcescens* subsp. *sakuensis* KREDT/AB061685 and *S. marcescens* subsp. *marcescens* DSM30121T/AJ233431.

3.2. Determination of IAA production

The IAA production by AL2-16 was in the range of 0.8–133.2 μg/mL. About 1.0% concentration of L-tryptophan was found to be optimum for IAA production by this isolate. IAA production decreased at higher concentrations of tryptophan (Figure 2). The amount of 1AA production was found to be maximum after 144 hours of incubation for all the concentrations of tryptophan (133.2 μg/mL).

3.3. Siderophore production assay

Formation of orange halo zone in CAS medium inoculated with AL2-16 was observed after 24 hours, which indicated the production of siderophore. Zone of siderophore produced at 96 hours was found to be 30% higher than that of zone produced at 24 hours. Siderophore release was further confirmed by quantitative CAS test where instant decolorization of CAS reagent from blue to orange was observed. About 74.931% unit of siderophore was recorded for AL2-16 in succinate broth. In accordance to the reports from *S. marcescens* AL2-16, the siderophore production was found to start after 24 hours of incubation in SM broth, and maximum siderophore was released after 48 hours of incubation (74.931 units) (Figure 3). It was evident that the siderophore production was high at late log phase, and amount of siderophore release was in accordance with the growth profile of AL2-16 isolate.

3.3.1. Effect of growth medium

SM was found to be the best medium among all other media with maximum siderophore release (74.931%), followed by nutrient broth medium (70.176%). Amount of siderophore was lower in other two media tested. Results are given in Table 1. Based on these results, SM was selected as the base medium for subsequent experiments.

3.3.2. Influence of sugars, nitrogen sources, and organic acids on siderophore production

S. marcescens AL2-16 was grown in SM supplemented with different sugars—glucose, sucrose, mannitol, fructose, and maltose one at a time. Fructose followed by glucose and sucrose were proved to be the best carbon sources (Table 1) resulting in appreciable amount of siderophore production, i.e. 77.223%, 73.584%, and 65.363%. Furthermore, amendment of SM with urea or sodium nitrate as sole source of nitrogen (0.1% w/v) resulted in variation in siderophore production. Siderophore production (71.024%) was obtained with urea as nitrogen source followed by sodium nitrate (22.146% SUs) (Table 1). Influence of different organic acids on siderophore production was experimented. Maximum siderophore production was found with oxalic acid (86.927%) followed by citric acid (83.423%) and succinic acid (Table 1).

3.3.3. Effect of iron concentration

Siderophore production was considerably affected by the presence of iron in medium. Initial increase in iron concentration

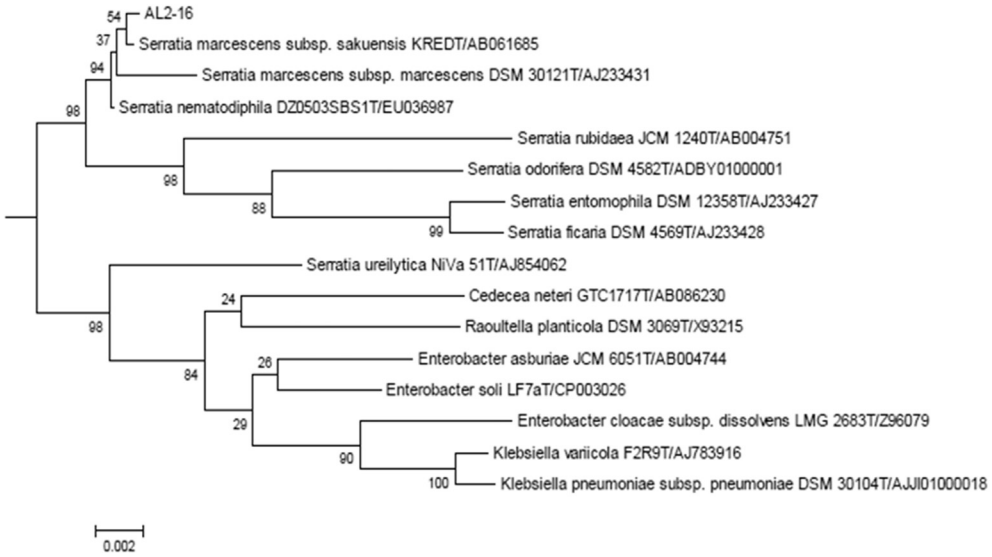

Figure 1. Phylogenetic analysis of 16S rRNA sequence of AL2-16 isolated from *Achyranthes aspera* L. The analysis was conducted with MEGA6 using neighbor-joining method.

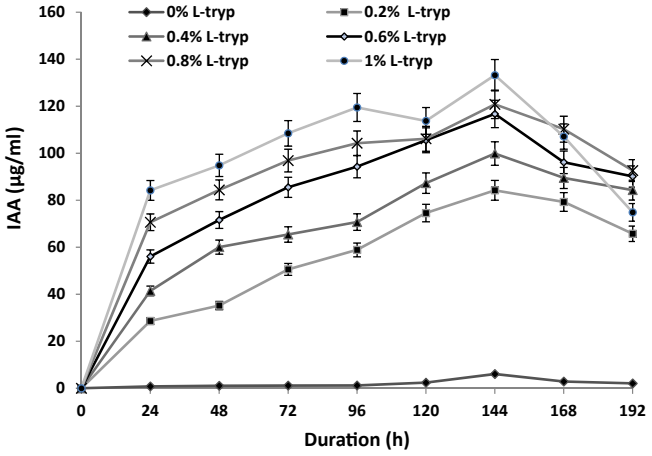

Figure 2. Quantitative estimation of indole acetic acid (IAA) produced by AL2-16 at different L-tryptophan (L-tryp) concentrations.

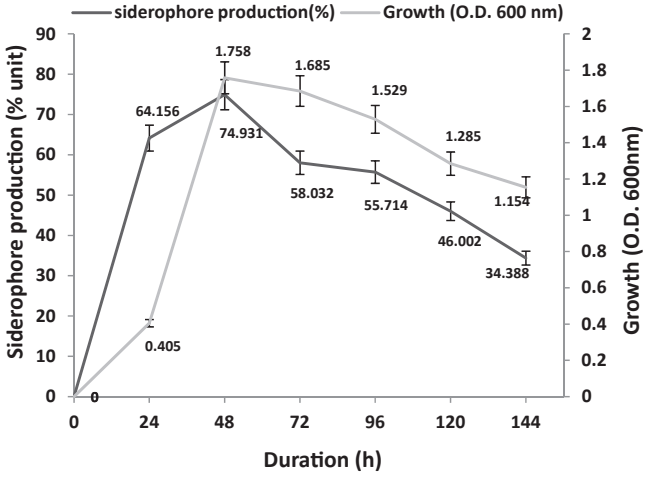

Figure 3. Quantitative estimation of siderophore release by *Serratia marcescens* AL2-16 against growth of bacteria. OD = optical density.

induced siderophore production. Increase in iron concentration resulted in successive decrease of siderophore production by *S. marcescens* AL2-16. Maximum siderophore release was recorded at 1 µM concentration of iron (83.488%). Siderophore production decreased to 24.271 SU at 30 µM of iron tested. Result is given in Figure 4.

3.4. Phosphate solubilization

AL2-16 solubilized tricalcium phosphate in Pikovskaya's agar, forming a clear halo around the colony. The phosphate solubilization index of the isolate from 24 to 96 hours was found to be in the range of 2.5–4.5. The pH of the medium decreases with the increase in the amount of free phosphate released, showing maximum P solubilization at pH 4.9 after 72 hours of incubation (259 µg/mL) (Figure 5).

3.5. Determination of ammonia production and nitrogen fixation

The AL2-16 isolates produced ammonia as evidenced by the change in the color of the inoculated broth to yellow when Nessler's reagent was added. Furthermore, the isolate was found to have nitrogen fixation ability. Nitrogenase activity was quantified, and it was found to be 2.523 nmol ethylene/µg/protein/hour as detected using GC-FID technique.

3.6. *In vitro* propagation of *A. aspera* in germ-free condition

About 29% of seeds were able to germinate in half-strength MS medium compared with full-strength MS medium, which germinated 22% of seeds. Seeds without husk help in easy germination compared with husk-coated seeds. The germinated seeds were transferred to MS medium to get proper nutrient for growth of the plant (Figure 6A–C).

3.7. Colonization studies with endophytic bacteria

AL2-16 was reisolated from infected seedlings. In addition, the morphological and physiological characteristics of the endophytic bacterium recovered after experimental inoculations were indistinguishable from the colony morphologies of the inoculated organisms. This was further confirmed by 16S rRNA sequence similarity of inoculated and recovered bacteria (AL2-16). About

Table 1. Production of siderophore by AL2-16 at different nutrient requirements

Parameters against SUs (%)							
Medium	SU	Sugars (10 g/L)	SU	Nitrogen sources (1 g/L)	SU	Organic acids (1 g/L)	SU
SM	74.931	Glucose	73.584	Control*	74.931	Control*	74.931
NB	70.176	Sucrose	65.363	Urea	71.024	Malate	66.172
LB	44.935	Mannitol	56.603	$NaNO_3$	22.146	Oxalate	86.927
YEMB	28.148	Fructose	77.223			Citrate	83.423
		Maltose	57.142				

LB = luria broth; NB = nutrient broth; SM = succinic medium; SU = siderophore unit; YEMB = yeast extract mannitol broth.
* SM was used for control. Other details described in Materials and Methods section.

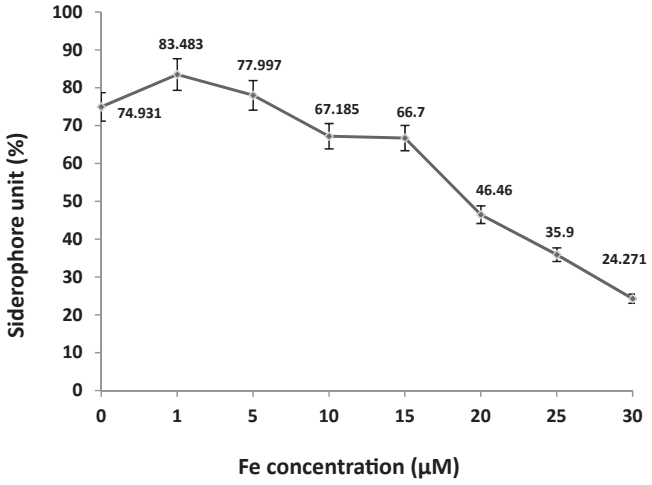

Figure 4. Effect of iron concentration on siderophore production.

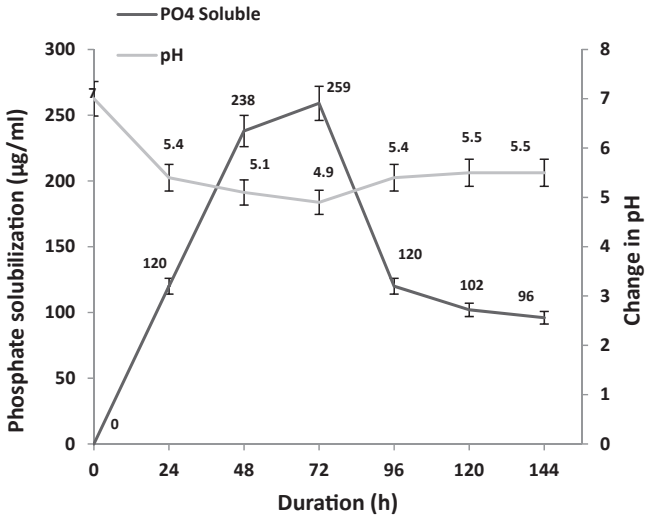

Figure 5. Phosphate solubilization by AL2-16 after different time intervals. Soluble-free phosphate (PO_4) concentration is given against primary Y-axis, whereas variation of pH in the culture medium is given at secondary Y-axis. Standard deviation is showed as bars.

10 µL of 1 OD_{600} AL2-16, which corresponds to 12.45×10^5, was inoculated into the homologous plant hosts and grown in the greenhouse. The population of AL2-16 increased from 16.2×10^6 to 11.2×10^8 colony forming unit (CFU)/g (fresh weight) between 3rd and 5th DAI (Table 2). Furthermore, AL2-16 was not detected in leaves till 3rd DAI; however, after 5th DAI, AL2-16 was recovered from leaves of bacteria-treated *A. aspera* L. plants. The representative control trials yielded no indigenous bacteria.

3.8. Pot trial experiment

The endophytic bacteria AL2-16 treatment increased all growth parameters of *A. aspera* L. It significantly ($p \leq 0.05$) increased shoot length by 95.52%, fresh shoot weight by 602.38%, dry shoot weight by 107.33%, fresh root weight by 438%, dry root weight by 675%, and area of leaves by 127.2% (Figure 6D and E; Table 3).

3.9. Effect of endophytes in NPK content

The NPK concentration, i.e. the availability was estimated in the soil of *A. aspera* L. at the 1st and after 30th day of the treatments. It showed that the NPK concentration in soil at the 1st day was 42, 35.42, and 80.5 mg/kg, respectively, which was found to decrease when inoculated with AL2-16. The NPK content in soil inoculated with AL2-16 decreased up to 38, 34.75, and 40.95 mg/kg, respectively. This decrease was due to uptake by the plants after 30 days.

Again the uptake of NPK was analyzed by estimating their concentration in the leaves. In the case of control plant (without inoculation), the NPK content in leaves was found to be 29,400, 2300.85, and 48,550 mg/kg, respectively. The higher concentration of NPK was recorded in the leaves of *A. aspera* L. inoculated with AL2-16. The NPK concentration in leaves with AL2-16 was recorded at 32,480, 2501.75, and 52,850 mg/kg, respectively.

4. Discussion

Endophytes have been defined as microbes that settle living interior tissues of plants without causing any instant and exert negative effects (Bacon and White 2000). Endophytic bacteria in a single plant host are not restricted to a single species but comprise several genera and species (Ryan *et al.* 2008). Currently, endophytic bacteria associated with medicinal plants have attracted the attention of several investigators. Isolation of *S. marcescens* from the rhizoplane of *A. aspera* L. for phosphate solubilization has been already reported (Misra *et al.* 2012). This study, however, is the first report to demonstrate the plant growth activities of endophytic *S. marcescens* in *A. aspera* L. Earlier, Misra *et al.* (2012) reported the production of IAA by *S. marcescens* isolated from the rhizoplane of *A. aspera* L., which produced 65.5 µg/mL. Our results showed that the endophytic *S. marcescens* AL2-16 isolated from the leaves of *A. aspera* L. produced 123.2 µg/mL of IAA with the supplement of 1% L-tryptophan. Selvakumar *et al.* (2008a,b) reported that the amount of IAA production by endophytic *S. marcescens* strain SRM was 11.1 µg/mL after 48 hours of incubation with the addition of 100 µg/mL of L-tryptophan concentration. The property of synthesizing IAA is considered essential for selecting favorable microorganisms as there have been reports suggesting that IAA-producing bacteria have reflective effects on plant growth (Khan *et al.* 2016; Yu *et al.* 2016). Different ranges of IAA release have

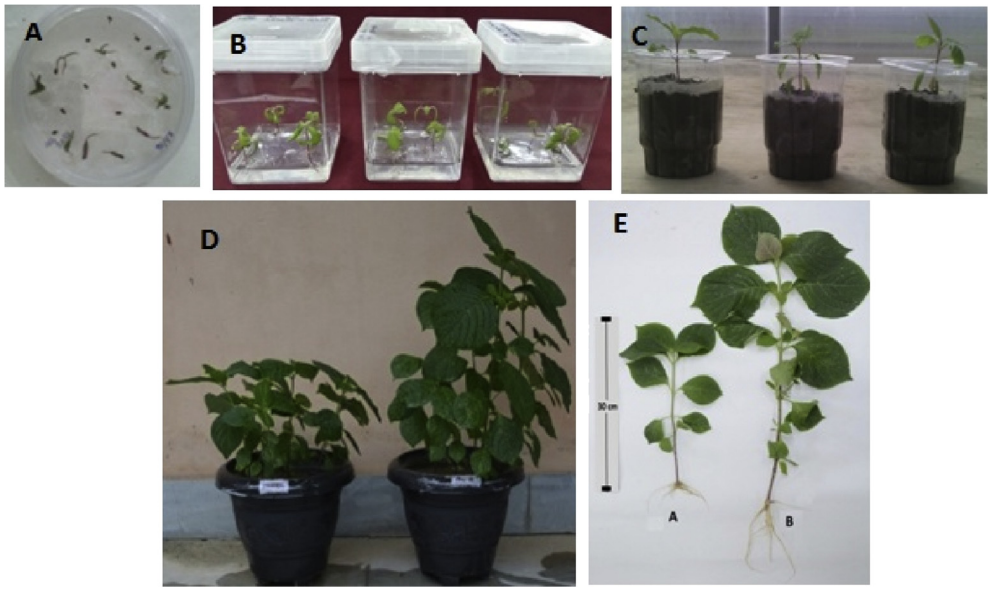

Figure 6. (A) Seed germination of *Achyranthes aspera* in half-strength MS. (B) Transfer the germinated seed into MS media. (C) Acclimatization of plantlets in green house. (D and E) Effect of *Serratia marcescens* AL2-16 on growth promotion of *A. aspera* L. MS = Murashige and Skoog.

Table 2. Population of endophytic bacteria from *Achyranthes aspera* L. grown in greenhouse condition

Bacteria	Initial population of bacteria as inoculated		Final population of bacteria CFU/g (fresh weight)			
			3 DAI		5 DAI	
AL2-16	Stem 12.45×10^5	Leaf Nil	Stem 16.2×10^6	Leaf Nil	Stem 93.4×10^3	Leaf 18.9×10^4

CFU = colony forming unit; DAI, days after inoculation.

been reported from different plant growth-enhancing bacteria. Khamna *et al.* (2010) reported that the amount of IAA production by *Streptomyces* CMU-H009 was 143.95 μg/mL after 3 days of incubation with the addition of 2 mg/mL of L-tryptophan concentration, whereas *Klebsiella* strain K8 produced 22.7 mg/L of IAA under optimum conditions at 72 hours of incubation (Sachdeva *et al.* 2009). Akbari *et al.* (2007) reported that *Azospirillum* strain 118-I produced 285.51 mg/L of IAA.

Siderophores play an essential role in the microbial interactions, enhancing the growth of plants and yield of agricultural crops in iron-limiting conditions. Khilyas *et al.* (2016) reported that two strains of *S. marcescens* SM6 and SR41-8000 started to produce siderophore after 12 hours of incubation, reached its maximum at 30 hours of growth, and remained constant on further incubation. Our results showed that *S. marcescens* AL2-16 produced

Table 3. Effect of *Serratia marcescens* AL2-16 on the growth characteristics of the *Achyranthes aspera* L.

Parameters	Control	Inoculated with AL2-16
Shoot length (cm)	20.54 ± 2.5 (a)	40.16 ± 0.59 (b)
Root length (cm)	10.20 ± 2.28 (a)	21.31 ± 0.72 (a)
No. of leaves	9.60 ± 0.89 (a)	20.00 ± 1.00 (a)
Fresh leaves weight (g)	0.26 ± 0.12 (a)	0.63 ± 0.13 (a)
Dry leaves weight (g)	0.05 ± 0.023 (a)	0.14 ± 0.018 (a)
Fresh shoot weight (g)	0.84 ± 0.14 (a)	5.90 ± 0.82 (b)
Dry shoot weight (g)	0.15 ± 0.02 (a)	1.76 ± 0.25 (b)
Fresh root weight (g)	0.21 ± 0.35 (a)	1.13 ± 0.25 (b)
Dry root weight (g)	0.04 ± 0.005 (a)	0.31 ± 0.163 (b)
Area of leaves	24.77 ± 2.11 (a)	56.28 ± 4.95 (b)

Each value is the mean of five plants. Values with the same letter within a row are not significant at $p \leq 0.05$.

83.827% unit of siderophore in succinate broth after 48 hours of incubation and decreased slowly. Percent of SU is correlated with growth of the cell. The impact of different iron concentrations, carbon sources, organic acids, and nitrogen sources on siderophore production unit was conducted. The most suitable iron concentration for siderophore production unit was 1 μM for the isolate. Sayyed *et al.* (2005) reported that *Pseudomonas fluorescens* NCIM 5096 produced 72% of SU in SM, which was similar to this work on *S. marcescens*. Among nitrogen source, urea gave 87% of SU and 54% by glucose in sugar source. Results showed that *S. marcescens* AL2-16 produced 77.223% of SU by fructose followed by glucose, sucrose, maltose, and mannitol among the carbon sources. Oxalic acid (86.927%) resulted in maximum production of SU among organic acids followed by citric acid, succinic acid, and malic acid. Among nitrogen sources, urea gave more siderophore production unit compared with sodium nitrate. Afzali *et al.* (2015) reported that endophytic *S. marcescens* MOSEL-W2, isolated from *Cannabis sativa*, can also produce siderophore.

The isolate *S. marcescens* AL2-16 formed halo zone when inoculated on the Pikovskaya's solid medium containing tricalcium phosphate. The pH of the medium decreased with the increase in the amount of free phosphate released, showing maximum P solubilization at pH 4.9 after 72 hours of incubation (259 μg/mL). This result was in accordance to the findings of George *et al.* (2013) where it was reported that *S. marcescens* KiSII isolated from the rhizospheric soil of coconut solubilize 216 μg/mL of inorganic phosphate after 72 hours of incubation. Ji *et al.* (2014) isolated endophytic *Klebsiella* sp. from leaves, stem, and roots of rice plants with phosphate solubilization activity ranging from 1.3 to 3.3 μg/mL after 72 hours of incubation.

Endophytic *S. marcescens* AL2-16 was found to have 2.523 nmol ethylene/μg/protein/hour of nitrogenase activity, as accessed by acetylene reduction assay using GC-FID technique, which is an important attribute for endophytic plant growth-promoting rhizobacteria. N-fixing endophytic bacteria are better than their rhizospheric and rhizoplanic counterparts as they provide fixed nitrogen directly to their host (Cocking 2003). Moreover, endophytic bacteria are less vulnerable to competition with other soil microbes for scarce resources and remain protected to various abiotic and biotic stresses (Reinhold-Hurek and Hurek 1998).

Earlier, Gyaneshwar *et al.* (2001) and Balachandar *et al.* (2006) had reported N-fixing endophytic *S. marcescens* from rice plants. The results obtained establish that the activities of IAA production, siderophore production, phosphate solubilization, and nitrogen fixation are critical to attain maximum growth promotion through the activity of plant growth-promoting bacteria.

The colonization of the bacteria in the host tissues also varied with the plant parts. *S. marcescens* AL2-16 was isolated from stems only till 3 DAI, where bacterial counts increased by 1.49×10^2 CFU/g (fresh weight). However, after 5 DAI, the introduced bacteria were found both in stem (93.4×10^3 CFU/g [fresh weight]) and leaves (18.9×10^4 CFU/g [fresh weight]). It seems that the bacteria were translocated from stem to leaves. This hypothesis is supported by Compant *et al.* (2005) where he reported that the systematic spread of an endophytic *Burkholderia* strain to aerial parts of *Vitis vinifera* seems to be through the transpiration stream. Singh and Jha (2016) demonstrated the efficiency of *S. marcescens* CDP-13 to colonized wheat plant. He found that the colonized bacterium after 15 DAI detected associative bacteria in the range of 1.1×10^2 CFU/g of the root. Rangel de Souza *et al.* (2015) demonstrated the endophytic colonization of *Arabidopsis thaliana* by *Gluconacetobacter diazotrophicus* and found out that bacterial population within roots was 1.5×10^6, 3.1×10^6, 21.1×10^5 CFU/g at 14, 28, and 50 DAI, respectively; however, there was no bacterial population in leaves at 14 and 50 dpi but only detected at 28 dpi, at very low concentration. Application of *S. marcescens* AL2-16 increased the root and shoot lengths, fresh and dry leaves weight, fresh and dry shoot weight, and number of leaves. There was an increase in leaf area in inoculated plant. Earlier, Chakraborty *et al.* (2010) also reported that the application of *S. marcescens* TRS-1 promoted growth in tea seedlings as evidenced by increase in height, new leaves, and leaves biomass. However, here we have reported *S. marcescens* as an endophyte that promote growth of *A. aspera.*

In conclusion, *S. marcescens* AL2-16 produced good quantity of siderophore, IAA, as well as can fix atmospheric nitrogen and solubilized inorganic phosphate. It enhances the growth of the host plant. Therefore, AL2-16 isolate has potential to use as a plant biofertilizer or bioenhancer for plant growth improvement of *A. aspera* L. Because endophytic organisms are an environmentally friendly alternative to chemical fertilizers and pesticides, AL2-16 has potential to be exploited as a growth-promoting agent.

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