HAYATI Journal of Biosciences xxx (2017) 1–8

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Contents lists available at ScienceDirect

HAYATI Journal of Biosciences

journal homepage: http://www.journals.elsevier.com/hayati-journal-of-biosciences

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.*

ARTICLE INFO

Article history:
Received 29 October 2016
Received in revised form
17 December 2016
Accepted 21 December 2016
Available online xxx

KEYWORDS:
Achyranthes aspera L.,
indole acetic acid,
phosphate solubilization,
pot trial,
Serratia sp.,
siderophore

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.

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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).
Peer review under responsibility of Institut Pertanian Bogor.

http://dx.doi.org/10.1016/j.hjb.2016.12.006
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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).

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