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IAA production and other plant growth promoting traits of endophytic bacteria from apple tree

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All plants in nature harbor a diverse community of endophytic bacteria. They can produce a wide range of various compounds, which can positively affect the host plant growth. Endophytes can participate in providing plant with nutrients, competing with plants pathogens or directly effecting the plant growth by synthesis of phytohormones.

In this study, endophytic bacteria associated with apple tree buds were isolated, characterized and tested for their ability to produce the plant hormone IAA (indole 3 acetic acid). Nine isolates were shown to produce IAA. Amounts of IAA produced in culture varied between 0.12–0.24 micrograms per milligram of protein. Several bacterial endophytes were shown to produce siderophores and substances which inhibited the growth of the test strain. We also screened the isolates for other PGP traits, as abilities to solubilize phosphate and fix nitrogen. Our results suggest that IAA production and other PGB traits are common among apple tree endophytic bacteria.

Key words: Bacterial endophytes, plant growth promoting bacteria, indole-3 acetic acid production, *Malus domestica*

INTRODUCTION

Microorganisms that live in plant tissues without causing disease symptoms are termed as endophytes. Each plant is a host of endophytes, though only a small part of them is so far described. According to Rosenblueth [1], endophytes belong to different phylogenetic groups, such as proteobacteria, actinobacteria, firmicutes.

As Doty & Weyens state [2], endophytes are a promising source for application in biotechnology; they can participate in plant bioremediation and are considered as a potential source of antibiotics and other bioactive compounds [3].

According to Ryan [4], endophytes promote plant growth by several mechanisms. These include: plant hormone synthesis, siderophore production, phosphate solubilization, nitrogen fixation.

In this study, were isolated endophytic bacteria from apple tree buds, screened the isolates for plant growth promoting properties and estimated the amounts of produced indole 3 acetic acid.

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MATERIALS AND METHODS

Isolation of endophytic bacteria

Buds were collected from the Institute of Horticulture collection of apple trees. The buds were sterilised as described in Hata [5]. In brief, apple tree buds were incubated in 70% of ethanol for 1 min, in 15% hydrogen peroxide for 15 min, again in 70% of ethanol for 1 min and washed with sterile distilled water 5 times. Then buds were mechanically homogenized, plated on the nutrient media (LB agar or actinomycete agar) and grown for 24–48 hours. As the control of sterility, intact buds were plated on the nutrient medium.

The gram staining, catalase, oxidase, nitrate reduction tests were performed according to Johnson [6].

Methylotrophy

Methylotrophy was detected by ability to use methanol as a single carbon source. Methanol minimal salts medium consisted of (per liter of water): K_2HPO_4, 1.20 g; KH_2PO_4, 0.62 g. $CaCl_2 \cdot 6H_2O$ 0.05 g; $MgSO_4 \cdot 7H_2O$ 0.20; NaCl, 0.10 g; $FeCl_3 \cdot 6H_2O$ 1.0 mg; $(NH_4)2SO_4$, 0.5 mg; $CuSO_4 \cdot 5H_2O$, 5.0 mg; $MnSO_4 \cdot 5H_2O$, 10 mg; $Na_2MoO_4 \cdot 2H_2O$, 10.0 mg;

H_3BO_5, 10 mg; $ZnSO_4 \cdot 7H_2O$ 70.0 mg; $CoCl_2 \cdot 6H_2O$, 5 mg, pH 7.0. According to Bergey [7] 0.5% methanol was added after autoclaving.

Nitrogen fixation

We used semisolid nitrogen free Rennie [8] medium for screening of the bacteria capable to fix nitrogen. Medium was prepared as described by Elbeltagy [9] and consisted of (per liter): 0.8 g of K_2HPO_4, 0.2 g of KH_2PO_4, 0.1 g of NaCl, 28 mg of $Na_2FeEDTA$, 25 mg of $Na_2MoO_4 \cdot 2H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.06 g of $CaCl_2 \cdot 2H_2O$, 100 mg of yeast extract, 3.0 g of mannitol, 5.0 g of sucrose, 0.5 ml of 60% (vol / vol) sodium lactate, 2.0 g of sodium malate, 2.0 g agar, pH 7.0. After autoclaving, filter-sterilized biotin and *para*-aminobenzoic acid were added to final concentrations of 5 and 10 μg per liter.

Siderophore production

Siderophore production was detected by growth on CAS medium. Chrome azurol S plates were prepared as in Vellore [10]. To prepare 100 ml of CAS indicator solution, 60.5 mg of chrome azurol S was dissolved in 50 ml of distilled water. To this solution, 10 ml of iron III solution was added. (Iron III solution was prepared by adding 27 mg of FeCl3 · 6H2O and 83.3 μl of concentrated HCl in 100 ml of distilled water). Then the solution, 72 mg of hexadecyltrimethyl ammonium bromide (HDTMA) dissolved in 40 ml of water, was added. To prepare 100 ml of basal agar medium, 3 g of 3-(N-morpholino) propane sulfonic acid (MOPS), 0.05 g of NaCl, 0.03 g KH_2PO_4, 0.01 g of NH_4Cl, 0.5 g of L-asparagine were dissolved in 83 ml of distilled water. Finally, 1.5 g of agar was added to the above solution. To the autoclaved basal agar medium 2 ml of the 50% glucose solution and 10 ml of the CAS indicator solution was added.

Phosphate solubilization

The ability to solubilize calcium phosphate was tested according to Mehta [11]. In brief: the isolates were incubated in LB medium for 24 hours, then 100 μl of culture was transferred to NBRIP medium containing (per liter): glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g, $(NH_4)_2SO_4$, 0.1 g, and Bromo phenol blue, 0.025 g. Phosphate solubilisation was detected by the colour change.

Antibiotic properties

Bacterial isolates were grown at 25 °C for 48 hours, 1.5 ml of suspension centrifuged, supernatant sterilized via 0.2 μm filter. The supernatant on the test cultures was detected using either the agar well method [12] or sterile discs. Using agar well method, 100 mm LB agar dishes were filled with 20 ml agar, and then wells (~5 mm deep) were formed, using a glass tube to remove the agar. The wells were filled with 30 μl of sterilized supernatant. In the second application, sterile cellulose discs were placed on agar surface and soaked by dropping 30 μl of supernatant on them. The plates were incubated for 48 hours. If an inhibition zone around wells or discs with supernatant appeared, its semi-diameter was measured. As test strains *Pseudomonas aeruginosa* (PAO) and *Salmonella typhimurium* (SL 1344) were used.

Estimation of IAA

As Gordon & Weber [13] have reported, the synthesis of IAA was estimated colorimetrically using ferric chloride – perchloric acid reagent ($FeCl_3$-$HClO_4$). The measurements were performed as in Husen [14]. The medium contained (per liter) 5 g NaCl, 10 g peptone and 10 g beef extract. According to Frankengerger & Poth [15] after overnight incubation, 100 μl of culture was inoculated to 10 ml minimal salt (MS) medium amended with 5 nM L – tryptophan and grown again for 48 hours on the shaker. The MS medium contained (per liter) 1.36 g KH_2PO_4, 2.13 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$ and trace elements. The pH of MS medium was adjusted to 7.0 before autoclaving. L – tryptophan solution was prepared as stock solution containing (in 100 ml distilled water) 10 g glucose, 1 g L – tryptophan, and 0.1 g yeast extract. The stock solution was filtered through a sterile 0.2 μm membrane filter.

To measure the amount of the IAA produced, 1.5 ml bacterial broth culture was centrifuged at 12,000 rpm for 5 minutes. 1 ml of the supernatant was added to 2 ml $FeCl_3$ – $HClO_4$ reagent. After 25 minutes, the mixture was read in spectrophotometer at 530 nm absorbance. The amount of IAA was estimated using a standard curve. As Sozren [16] noted, the quantitative amounts of protein in bacteria suspension were estimated by copper (II)–neocuproine method.

RESULTS

The results of gram staining, endospore formation, catalase, oxidase activity and the nitrate reduction are presented in Table 1. All isolates were catalase positive. None of them, except 5Da5.1 (which was able to produce nitrite) could reduce nitrate. Almost all isolates investigated (except 6G2.1) were able to use methanol as a single carbon source (Table 1).

Plant growth promoting properties

12 of 18 isolates tested could grow on nitrogen free medium (Table 2). Each property of phosphate solubilization, siderophore or IAA production was harboured by about a half of screened isolates.

Three isolates 5Da5.1, 5Da6.1, 6O1.1, excreted to growth medium compounds, inhibited the growth of *Salmonella typhimurium* (Fig. 1). Only one of them, 5Da6.1 was a siderophore producer. None of the isolates showed a similar effect to *Pseudomonas aeruginosa*.

Table 1. **Biochemical and morphological traits of apple bud endophytes**

Isolate	Gram stain	Cell shape	Catalase test	Nitrate reduction	Oxidase test	Methylotrophy	Endospore
1Aa1.2	+	bacilli	+	–	+	+	
1O1.1	+	bacilli	+	–	+	+	
2D2.1	–	cocci	+	–	–	+	+
2D3.1	+	bacilli	+	–	–	+	
2O1.2	+	bacilli	+	–	+	+	–
5Da2.1	–	cocci	+	–	+	+	
5Da2.3	+	bacilli	+	–	–	+	–
5Da5.1	–	cocci	+	+	–	+	+
5Da6.1	+	bacilli	+	–	–	+	+
6A1.1	+	bacilli	+	–	–	+	+
6D1.1	+	bacilli	+	–	+	+	+
6D3.1	+	bacilli	+	–	+	+	–
6D4.1	+	bacilli	+	–	–	+	+
6G2.1	+	bacilli	+	–	–	–	
6G4.1	–	cocci	+	–	+	+	
6O1.1	+	bacilli	+	–	–	+	+
6O4.1	+	bacilli	+	–	+	+	
6O5.1	+	bacilli	+	–	+	+	+

Table 2. **Plant growth promoting properties of isolates**

Isolate	Nitrogen fixation	Siderophore production	Phosphate solubilization	IAA production
1Aa1.2	+	+	+	+
1O1.1	+	+	+	+
2D2.1	+	–	+	–
2D3.1	+	+	+	–
2O1.2	+	+	+	–
5Da2.1	–	+	–	+
5Da2.3	–	–	–	–
5Da5.1	+	–	–	+
5Da6.1	–	+	–	+
6A1.1	+	+	+	+
6D1.1	–	+	–	–
6D3.1	+	–	–	+
6D4.1	–	–	–	–
6G2.1	+	–	+	–
6G4.1	–	+	+	–
6O1.1	+	–	+	+
6O4.1	+	–	–	–
6O5.1	–	+	+	–

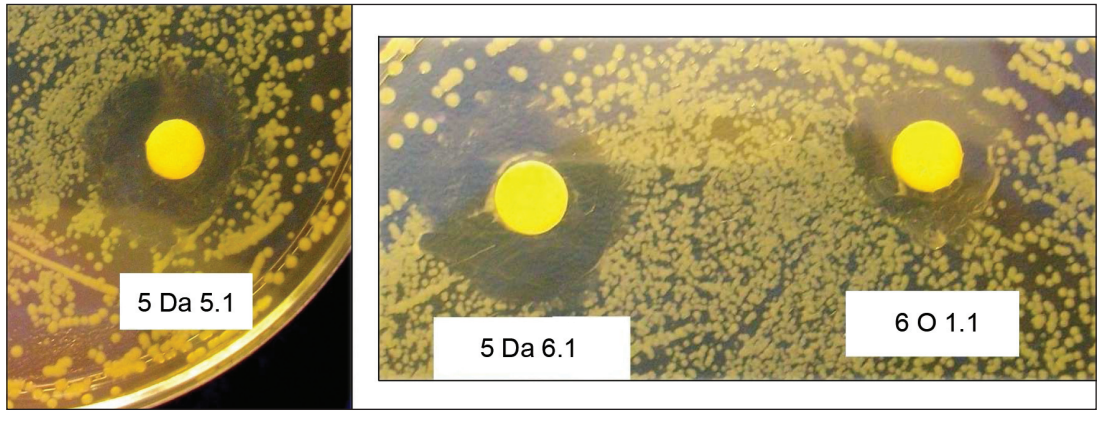

Fig. 1. Growth inhibition zones formed by endophytic bacterial isolates – apple endophyte 5Da5.1; 5Da6.1; 601.1. Test strain *Salmonella typhimurium* (SL 1344)

Table 3. **Amounts of IAA produced by apple tree endophytes. Means and ± standard deviations of five independent experiments**

Isolate	IAA, µg / ml		IAA, µg / mg of protein	
	mean	SD	mean	SD
1Aa1.2	42.16	± 8.79	0.202	± 0.095
1O1.1	30.68	± 5.04	0.176	± 0.108
5Da2.1	27.33	± 4.18	0.13	± 0.034
5Da5.1	34.41	± 3.56	0.13	± 0.012
5Da6.1	34.95	± 2.31	0.143	± 0.04
6D1.1	34.64	± 3.34	0.125	± 0.017
6D3.1	26.55	± 6.51	0.236	± 0.093
6O1.1	26.62	± 3.38	0.133	± 0.03

IAA quantitative measurements

In suspension, concentration of produced IAA varied between 27–42 micrograms of IAA per millilitre between all IAA positive isolates and from 0.12 to 0.24 micrograms per milligram of bacterial protein (Table 3).

DISCUSSION

Our results show that methylotrophy is a common trait of apple tree endophytes. Most of the isolates had more than one of the PGP traits tested (phosphate solubilization, diazotrophy, siderophore production, IAA synthesis). The isolates 5Da5.1, 6D3.1 and 6O1.1 were able to fix nitrogen and produce IAA, both abilities potentially conditioning a direct plant growth promotion. The isolates 1Aa1.2, 1O1.1, 6A1.1 showed all of PGP traits tested. We consider these isolates to be an attractive choice for plant – endophyte interaction studies.

Our IAA measurement results correspond to IAA production by plant associated bacteria from various sources (in the presence of tryptophan): according to Lata [17] 18.8 µg/ml by *Pseudomonas stutzeri* (from *Echinacea*), according to Merzaevra & Shirokikh [21] 77–83 µg/ml by actinomycetes, 94–95 µg/ml by coryneforms (winter rye), according to Omer [18] 6–13.3 µg/ml *Methylobacterium* (red and white clover), according to Long [19] 1,1–154 µg/ml by various endophytes (*Solanum nigrum*), according to Raddadi [20] 1.53–9.71 µg/ml by *Bacillus thuringiensis*.

Though IAA positive endophytes originated from different apple trees and were different by their colony morphology and sets of metabolic properties (Tables 1 and 2), however, the levels of IAA were relatively similar among all *Malus* endophytic isolates (Fig. 2).

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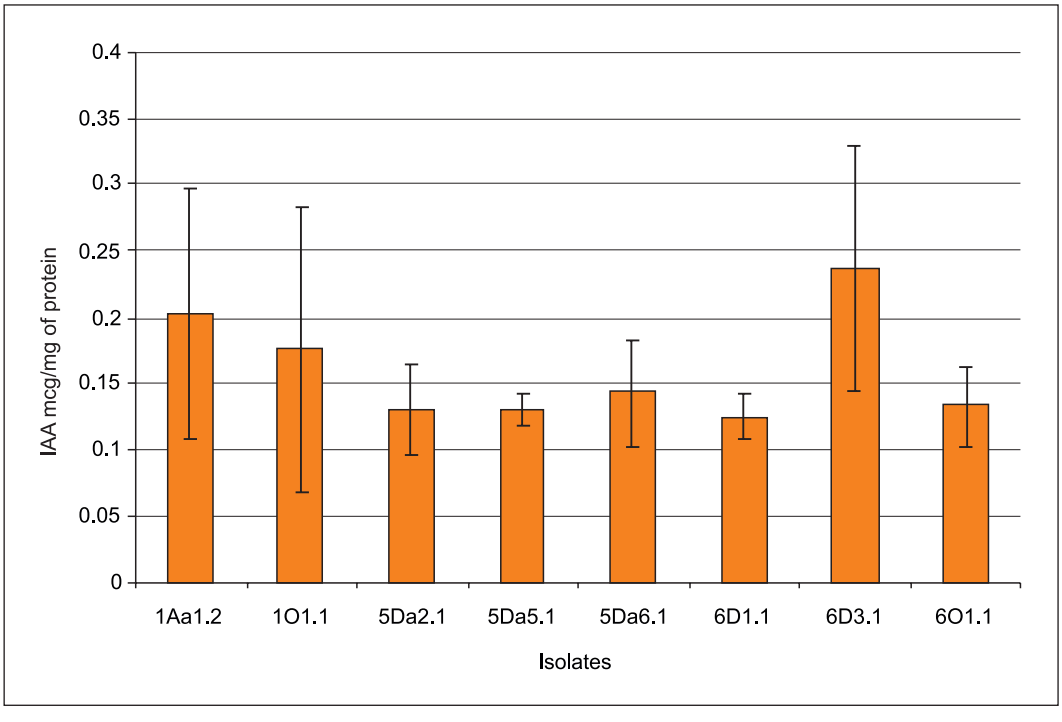

Fig. 2. Amounts of IAA produced by apple tree endophytes.
Means and ± standard deviations of five independent experiments

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OBELŲ ENDOFITINIŲ BAKTERIJŲ GEBĖJIMAS GAMINTI INDOLIL-3-ACTO RŪGŠTĮ IR KITOS SU AUGALŲ AUGIMO SKATINIMU SUSIJUSIOS JŲ SAVYBĖS

Santrauka

Daugelio augalų audiniuose esantys endofitiniai mikroorganizmai gali paveikti augalų augimą. Toks tiesioginis skatinimas yra susijęs su augalų augimo hormonų gamyba bei jos reguliavimu; atmosferos azoto fiksavimu, netirpių fosfatų tirpdymu (padidėja augalams prieinamo azoto ir fosforo kiekis). Netiesiogiai augalų augimą gali skatinti endofitinių mikroorganizmų konkurencija su patogeniniais augalų mikroorganizmais – sideroforų išsiskyrimas mažina patogenams prieinamą geležies kiekį, kitas mikroorganizmų augimą slopinančias medžiagas.

Šių tyrimų metu buvo išskirta 18 endofitinių obelų pumpurų grynų kultūrų ir nustatytos augalų augimą skatinančios jų savybės: atmosferos azoto fiksavimas, antibakterinių medžiagų išskyrimas, netirpių fosfatų tirpdymas, sideroforų išskyrimas, indolil-3-acto rūgšties (IAR) gamyba. Nustatyta, kad 17 kultūrų iš 18 yra metilotrofinės; 5Da5.1, 5Da6.1 ir 6O1.1 kultūros išskyrė medžiagas, slopinančias *Salmonella typhimurium* augimą. Dalis tirtų kultūrų tirpdė fosfatą ir išskyrė sideroforus. Devynios kultūros gamino IAR. Tirtų obelų endofitai pagamino 0,12–0,24 µg IAR miligramui baltymo.

Raktažodžiai: bakteriniai endofitai, augalų augimą skatinančios bakterijos, indolil-3-acto rūgšties gamyba, naminė obelis (*Malus domestica*)