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Plant growth promoting properties of culturable phylloepiphytic pine bacteria

Viktorija Vaštakaitė,

Odeta Buzaitė*

Vytautas Magnus University, Department of Biochemistry and Biotechnologies, Kaunas, Lithuania

Plant-associated bacteria can affect plants in different ways, including plant growth promoting. Beneficial effects on plant growth can be conditioned directly, by providing plants with nutrients – nitrogen, iron or soluble phosphate or by producing plant hormones. Indirect plant growth promotion way is conditioned by the inhibition of growth of pathogenic microorganisms. The surface of plant leaf – phylloplane along to other epiphytic bacterial habitats, is a promising source of microorganisms with plant growth affecting abilities, including those with biotechnological potential.

Ability to synthesize indole-3- acetic acid was frequently found among isolates (17 of 19 isolates tested). 13 of the isolates were able to produce siderophores, 10 – solubilize phosphates and 10 were diazotrophs. Isolates J41.1, J41.2 J4a3, J4a4 showed a beneficial effect on pine seedling.

Key words: Plant-associated bacteria, plant growth promotion, *Pinus sylvestris*

INTRODUCTION

Phylloplane, the surface of leaves, is microhabitat populated with microorganism communities. Bacteria of the phylloplane are diverse and include many different genera and fungi. They live on the leaf surface as solitary cells, or form microcolonies and biofilms [1]. Habitat provides specific conditions with regard to the availability of nutrients, presence of volatile plant compounds.

Epiphytic microorganisms can affect plant growth in various ways. Some effects can improve plant growth, increase resistance to biotic and abiotic stress, thus making epiphytes an attractive object for biotechnology research. It was shown that bacterial inoculations can stimulate seedling growth of conifers [2–4].

In this study we isolated bacteria from the surface of Scots pine (*Pinus sylvestris*) needles and screened 19 isolates for properties which can determine plant growth promotion. Those included siderophore and auxin (indole -3-acetic acid) production, phosphate solubilization, nitrogen fixation. We also estimated the effect of bacteria suspension on pine seed germination, root and hypocotyls growth.

* Corresponding author. E-mail: o.buzaite@gmf.vdu.lt

MATERIALS AND METHODS

Isolation of bacteria. Pine needles were collected aseptically, 3–5 needles incubated in 1ml of 0.9% NaCl with shaking for 30 min. Solution plated on LB and actinomycete agar plates and incubated at 25 °C for 48 hours.

Gram staining, catalase, oxidase, nitrate reduction tests were performed according [5].

Methylotrophy. Methylotrophy was detected by the 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. 0.5% methanol added after autoclaving [6].

Nitrogen fixation. We used semisolid nitrogen free Rennie [7] medium for screening of bacteria capable fix nitrogen. The medium was prepared as described in [8]. Medium 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, biotin 5 µg, *para*-aminobenzoic acid 10 µg, pH 7.0.

Siderophore production. Siderophore production was detected by growth on CAS medium. Chrome azurol S plates were prepared as in [9]. 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 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 K_2HPO_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 accompanied by stirring. To 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 [10]. In brief: isolates were incubated in LB medium for 24 hours, then 100 µl of culture 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 solubilization was detected by colour change.

Estimation of IAA. Synthesis of IAA was estimated colorimetrically using ferric chloride – perchloric acid reagent ($FeCl_3$-$HClO_4$) [11]. Measurements were performed as in [12]. The medium contained (per liter) 5 g NaCl, 10 g peptone, and 10 g beef extract. After overnight incubation, 100 µl of culture was inoculated to 10 ml minimal salt (MS) medium amended with 5 nM L – tryptophan [13] 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 a 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 sterile 0.2 µm membrane filter.

To measure the amount of 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 FeCl3 – HClO4 reagent. After 25 minutes, the mixture was read in a spectrophotometer at 530 nm absorbance. The amount of IAA was estimated using a standard curve.

Effect on pine seedling. 10 bacterial isolates were used for the seed germination experiment. Surface disinfected (3 min in 15% H_2O_2) pine seeds (30 for each bacterial sample) were incubated in bacteria suspension in distilled water (10^7 cfu/ml) for 24 hours, then plated in Petri dishes with soaked paper. Control seeds were incubated in sterile distilled water. Samples were incubated for 14 days, then the germination rate was estimated and lengths of the roots and hypocotyls were measured. The vigor index (VI) was calculated using the formula VI = (mean root length + mean hypocotyl length) × % germination [14].

Table. **Properties of isolates**

Isolate	Catalase test	Ability to use methanol as a single carbon source	Nitrogen fixation	Siderophore production	Phosphate solubilization	IAA production
2.1.1	+	+	+	+	+	+
2.1.2	+	+	+	+	+	+
5.3.1	+	+	+	+	+	+
8.4.5	+	+	+	+	+	+
12.1.1	+	+	+	+	+	+
J4a1.1	+	+	–	–	–	–
J4a1.2	+	+	–	–	–	+
J4a3	+	+	+	–	–	+
J4a4	+	+	+	–	–	+
J4a5	+	+	–	–	+	+
J5a8	+	+	+	+	–	+
J5a9	+	+	+	+	+	+
J5a10	+	+	nd	+	+	+
M2a2	+	+	nd	+	+	+
M4.1	+	+	+	+	–	–
M4.5	+	+	–	+	–	+
M5.4	+	+	–	+	+	+
M6.1	+	+	–	–	–	+
Vp2a1	+	+	nd	+	–	+

"nd" – not detected.

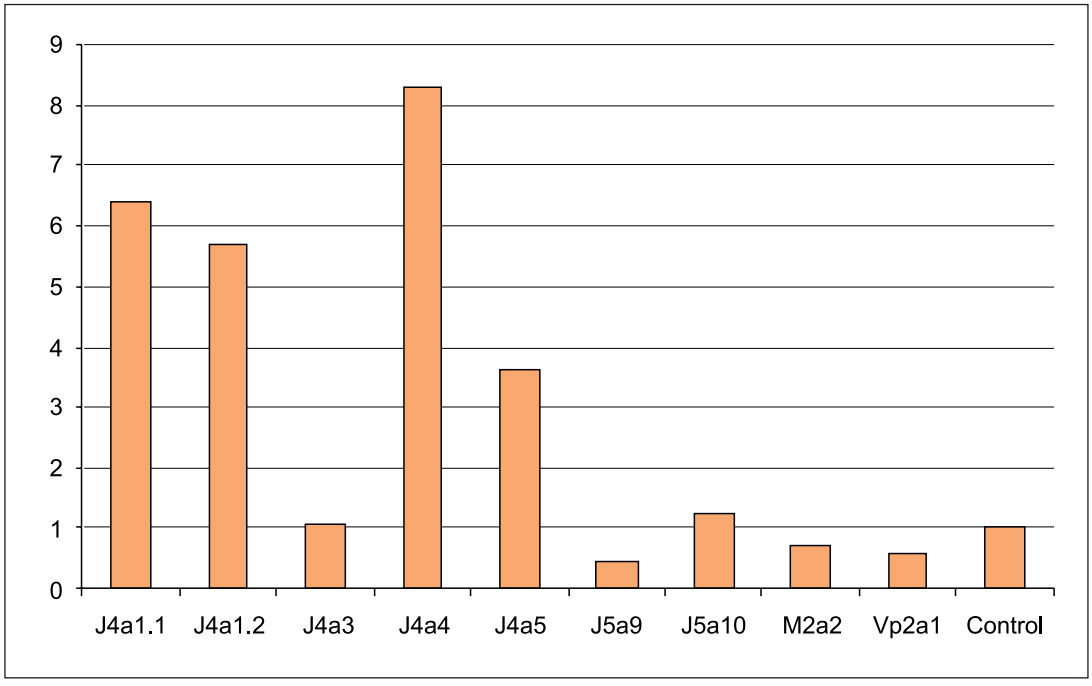

Fig. The effect of bacteria on pine seed vigor index

RESULTS AND DISCUSSION

All isolates investigated were able to use methanol as a single carbon source (Table). All were catalase positive. Ability to synthesize indole-3-acetic acid was found in 17 from 19 isolates tested. 13 of the isolates were able to produce siderophores, 10 – solubilized phosphates and 10 were diazotrophs.

In germination assay, inoculation with isolates J5a8, J5a10, Vp2a1, J41.2, J4a3, J4a4 has increased the root length by 20–75%, most of these isolates (except J5a8) have also enlarged the hypocotyl length; J41.2, J4a3, J4a4 have increased the germination rate by 11–77%. Samples, incubated with J41.1, J41.2 J4a3, J4a4, showed the highest vigor index. The ratio of vigor index versus control is shown in Figure.

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Viktorija Vaštakaitė, Odeta Buzaitė

AUGALŲ AUGIMĄ SKATINANČIOS PUŠŲ SPYGLIŲ EPIFITINIŲ BAKTERIJŲ SAVYBĖS

Santrauka

Su augalais susijusios bakterijos gali įvairiai paveikti augalus, kartu ir skatinti jų augimą. Teigiamas bakterijų poveikis augalų augimui gali būti tiesioginis, kai bakterijų dėka augalas geriau aprūpinamas azotu, geležimi, tirpiais fosfatais arba kai augalą veikia bakterijų išskiriami fitohormonai, ir netiesioginis, kai bakterijos slopina kitų patogeninių augalų mikroorganizmų augimą. Lapo paviršius – filoplanas, kaip ir kitos epifitinių bakterijų augavietės, yra galimas mikroorganizmų, pasižyminčių augalų augimą veikiančiomis savybėmis, šaltinis.

Daugelis mūsų išskirtų pušų epifitinių bakterijų kultūrų (17 iš 19 tirtų) gamino indolil-3 acto rūgštį, 13 kultūrų gamino sideroforus, 10 tirpdė fosfatus ir 10 buvo diazotrofai. J41.1, J41.2 J4a3, J4a4 kultūros pasižymėjo teigiamu poveikiu pušų sėklų dygimui.

Raktažodžiai: su augalais susijusios bakterijos, augalų augimo skatinimas, *Pinus sylvestris*