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Stimulatory effect of phosphate-solubilizing bacteria on plant growth, stevioside and rebaudioside-A contents of *Stevia rebaudiana* Bertoni

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ABSTRACT

The effect of four phosphate-solubilizing bacteria (PSB), (*Burkholderia gladioli* 10216, *Burkholderia gladioli* 10217, *Enterobacter aerogenes* 10208 and *Serratia marcescens* 10238) as identified on the basis of 16S rRNA gene sequencing was evaluated on plant growth and commercially important glycosides, stevioside (ST) and rebaudioside-A (R-A) of *Stevia rebaudiana* in pots containing tricalcium phosphate (TCP) supplemented soil. The PSB were isolated from the rhizosphere of *S. rebaudiana* plants and tested for P-solubilization ability, biocompatibility, indole acetic acid (IAA) and siderophore production. In greenhouse study, treatment of either individual PSB or a consortium (of PSB) resulted in increased plant growth, ST and R-A contents. The stimulatory effect was observed with consortium treatment in plant growth parameters (shoot length, 22.5%; root length, 14.7%; leaf dry weight, 89.0%; stem dry weight, 76.3% and shoot biomass, 82.5%) and glycoside contents (ST, 150% plant^{-1} and R-A, 555% plant^{-1}) as compared to the un-inoculated plants. Among individual PSB treatments, *B. gladioli* 10216 showed most promising response in majority of the parameters studied. The root colonization potential of PSB, assayed by RAPD technique, showed the colonization of all PSB isolates, though their extent of colonization varied.

1. Introduction

Stevia rebaudiana is a perennial shrub of *Asteraceae* (*Compositae*) family native to certain regions of South America. Its leaves produce zero-calorie ent-kaurene glycosides – stevioside and rebaudiosides which are in demand as non-nutritive and high potency sweetener in food and beverages by the persons suffering from diabetes and obesity. The amount of active principles depends on total biomass, which further depends on climatic features, agro-techniques, water management and fertilizer applications. Recently, the integrated application of microbial inoculants to agro-technologies for the cultivation of medicinal plants is being promoted for improving their productivity in terms of biomass and biochemical constituents (Leithy et al., 2006; Jaleel et al., 2007). PSB are well known to promote plant growth because of their ability to convert insoluble form of P to soluble form that can be readily taken up by the plant roots. Usually the soils are supplemented with inorganic P in the form of chemical fertilizers. A large proportion of the applied P gets fixed in the soil as phosphates of iron, aluminum and calcium (Altomare et al., 1999). This fixed form of P is not efficiently taken up by the plants and known to cause many environmental problems like eutrophication and soil salinity (Del Campillo et al., 1999). The use of PSB as biofertilizers could decrease the environmental problems associated with conventional chemical fertilizers.

In addition to P-solubilization, PSB may also improve the plant productivity by producing other secondary metabolites. Several evidence related to plant growth promotion by PSB through the production of IAA (Patten and Glick, 2002; Shahab et al., 2009) and siderophore (Koo and Cho, 2009) make the PSB more suitable as biofertilizers.

The effect of PSB on medicinal plants is gaining momentum, as evidenced by an increase in the number of publications. There are limited reports (Earanna, 2007; Das et al., 2008) related to the effect of PSB on the growth of *S. rebaudiana* and no report has been found on the yield enhancement of stevioside and rebaudiosides by PSB inoculation. The present study was carried out to isolate the PSB from the rhizosphere of *Stevia* plants and examine their effect on the plant growth, availability of P in soil, P uptake by plants and yield of ST and R-A of *S. rebaudiana.*

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2. Materials and methods

2.1. Isolation of PSB

Rhizospheric soil samples of five *S. rebaudiana* plants growing in organic farms (loamy soil, without any input of chemical fertilizers) were collected in sterile plastic bags. Samples were processed on the same day. One gram soil of each sample was suspended separately in 9.0 ml of phosphate buffer saline (PBS) of pH 7.2. The serial dilutions (1:10) were made and spread on Pikovskaya's (PVK) agar plates containing 0.5% TCP and incubated at 30 °C. Colonies showing the zone of solubilization, which indicates microbial P-solubilizing ability, were streaked on nutrient agar plates to check their purity and stored for further studies.

2.2. Screening of PSB

2.2.1. Quantitative estimation of phosphate solubilization

P-solubilization was estimated in a liquid medium amended with 0.5% TCP. The isolates were grown in a 100 ml liquid medium at 30 °C on a rotary shaker (130 rev min^{-1}). The composition of the medium was (g l^{-1}): yeast extract, 0.50; dextrose, 10.0; TCP, 5.0; $(NH_4)_2SO_4$, 0.50; KCl, 0.20; $Mg_2SO_4{\cdot}7H_2O$, 0.10; $Mn_2SO_4{\cdot}H_2O$, 0.0001; $Fe_2SO_4{\cdot}7H_2O$, 0.0001, pH adjusted to 7.0. The 5.0 ml culture was taken out at regular intervals of 24 h, for 7 days, centrifuged (Sigma 103456 cooling centrifuge, Germany at 10,000 × *g* for 10 min) and soluble-P content of culture supernatant was estimated by colorimetric chlorostannous reduced molybdophosphoric acid blue method (Jackson, 1973). The final values were calculated with the help of a standard curve obtained using 0–2 mg l^{-1} KH_2PO_4.

2.2.2. Biocompatibility assay

In order to prepare a consortium of PSB, it is essential that all microbes present in a consortium should be biocompatible. Biocompatibility among PSB isolates was determined by streaking one PSB isolate across the middle of the PVK agar plate. Other isolates were streaked perpendicular to the above isolate and incubated (30 °C, 48 h). Zone of inhibition of growth at the junction of cultures was noted (Mittal et al., 2008).

2.2.3. Quantitative estimation of indole acetic acid (IAA)

IAA was assayed by the colorimetric method using ferric chloride–perchloric acid reagent ($FeCl_3$–$HClO_4$) (Gordon and Paleg, 1957). PSB were inoculated in the minimal medium (g l^{-1}): KH_2PO_4, 1.36; Na_2HPO_4, 2.13; $MgSO_4{\cdot}7H_2O$, 0.2, pH 7.0, amended with 5.0 mM L-tryptophan solution (g 100 ml^{-1}: glucose, 10; L-tryptophan, 1.0; yeast extract 0.1; filtered through sterile 0.2 μm Millipore membrane filter) (Frankenberger and Poth, 1988). Flasks were incubated at 30 °C on a rotary shaker (130 rev min^{-1}). Cultures were withdrawn after 48 h intervals and centrifuged (10,000 × *g* for 10 min). The 2.0 ml of Salper's reagent was added dropwise to 1.0 ml of culture supernatant, and samples were incubated in the dark for 30 min. Development of pink color was assayed with a spectrophotometer at 530 nm. The concentration of IAA in μg ml^{-1} was determined from a standard curve of IAA (0–10 μg ml^{-1}).

2.2.4. Quantitative estimation of siderophore production

PSB were grown in an iron deficient medium containing (g l^{-1}): K_2HPO_4, 0.1; KH_2PO_4, 3.0; $MgSO_4{\cdot}7H_2O$, 0.2; $(NH_4)_2SO_4$, 1.0; succinic acid, 4.0 (Bharbaya and Rao, 1985) at 30 °C on a rotary shaker at 130 rev min^{-1} for 48 h. The 0.5 ml of cell free supernatant was mixed with 0.5 ml of Chrome Azurol Sulfonate (CAS) assay solution (1.5 ml of 1 mM Fe stock solution + 7.5 ml of 2 mM CAS stock solution added to 0.003 mM hexadecyltrimethylammonium (HDTMA) + 30 ml of 1.5 mM Piperazine buffer) along with 10 μl of shuttle solution (0.2 M 5-sulfosalicylic acid) (Schwyn and Neilands, 1987). Absorbance was read at 630 nm for the loss of blue color. The activity was recorded in percentage siderophore units calculated as $[((Ar - As) \times Ar^{-1}) \times 100]$, where 'Ar' is defined as absorbance of reference (un-inoculated media + CAS solution) and 'As' is absorbance of test (culture supernatant + CAS solution).

2.3. Physiological characterization of PSB

PSB isolates were characterized based on colony morphology, Gram staining and biochemical testing: catalase (Graham and Parker, 1964), oxidase (Kovaks, 1956) and lactose fermentation (Ronald and James, 2006). The guanosine + cytosine content (mol% G + C) of the genomic DNA was determined by thermal denaturation method (Marmur and Doty, 1962).

2.4. Molecular identification of PSB

The genomic DNA of PSB isolates were extracted using Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA). The primers fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (3′-ACGGCTACCTTGTTACGACTT-5′) were used for amplification of 16S rRNA gene (Weisburg et al., 1991). The total PCR reaction mixture was 50.0 μl, comprising 200 μM dNTPs, 50 μM each primer, 1× PCR buffer, 3 U *Taq* polymerase, and 100 ng genomic DNA. The thermocycler conditions involved an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min and final extension at 72 °C for 8 min. The 16S rRNA gene was purified from the gel and was ligated to pGEM-T easy vector (Promega, Madison) and transformed in *E. coli* JM109. The sequences of the insert were determined using a Big-Dye Terminator Cycle Sequencer and an ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA). The RNA gene sequences were analyzed using the gapped BLASTn (http://www.ncbi.nlm.nih.gov) search algorithm and aligned to their nearest neighbors. The evolutionary distances among phosphate-solubilizing isolates and their related taxa were calculated using TREECON software and Kimura's two-parameter model, after aligning the sequences with ClustalW.

2.5. Site of pot experiments

Experiments were conducted in a greenhouse (uncontrolled conditions) during March–June, 2008, in the Department of Botany, Panjab University, Chandigarh, India.

2.5.1. Preparation of inoculum

Each PSB was grown separately in the nutrient broth at 30 °C in an orbital shaker (150 rev min^{-1}) for 24 h. The cultures were centrifuged in 50 ml sterile plastic tubes at 6000 × *g* for 15 min. The pellets were re-suspended in PBS and optical density (OD) was adjusted to have a final concentration of colony forming units, i.e. 10^8 CFU ml^{-1}. This liquid culture of each PSB was used for the individual inoculation in pot experiments. For making a PSB consortium inoculum, all individual cultures of PSB of equal cell density, i.e. 10^8 CFU ml^{-1} were mixed together into a 250 ml sterilized flask. This mixture was used as a consortium inoculum.

2.5.2. Soil conditions and sowing of plantlets

Unsterile loamy soil (pH, 7.8; available N, 46.9 mg kg^{-1}; available P, 5.0 mg kg^{-1}; available K, 14.9 mg kg^{-1}; total Ca, 6.0 mequiv. kg^{-1}; total Mg, 1.2 mequiv. kg^{-1}; total organic carbon, 0.10%) was thoroughly mixed and passed through 2 mm sieve to remove large particulate matter and kept under sunlight for 7 days. Ethanol disinfected plastic pots (10.2 cm × 25.4 cm), were filled with 3.5 kg of soil. Roots of tissue culture plantlets were surface-sterilized by dipping in 2% NaOCl solution for 10 min and