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Plant growth promoting potential of *Pontibacter niistensis* in cowpea (*Vigna unguiculata* (L.) Walp.)

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ABSTRACT

During the past couple of decades, understanding of rhizosphere biology has progressed with the discovery of a special group of microorganisms known as plant growth promoting rhizobacteria (PGPR) and its application for sustainable agriculture has increased tremendously in various parts of the world. The search for microorganisms that improve soil fertility and enhance plant nutrition has continued to attract attention due to the increasing cost of fertilizers and some of their negative environmental impacts. In this study we demonstrated, a novel bacterial species *Pontibacter niistensis* NII-0905 isolated from forest soil in Western *ghat* forest soil with potential plant growth promoting ability (PGP) such as phosphate solubilization, indole acetic acid (IAA), siderophore and hydrogen cyanide (HCN) production. The activity varies with different growth temperatures, strain solubilize 28.5 ± 0.9, 48.02 ± 1.9 and 65.07 ± 2.1 $\mu g\,mL^{-1}$ at 4, 15 and 30 °C respectively and produced 24.8 $\mu g\,mL^{-1}\,day^{-1}$ of indole acetic acid (IAA) in tryptophan amended media. Qualitative detection of siderophore production and HCN were also detected at all temperature tested. At a lower temperature (4 °C) strain NII-0905 retained all the plant growth promotion attributes. A significant increase in the growth of cow pea was recorded with inoculations of strain NII-0905 in pot experiments. Scanning electron microscopic study revealed the root colonization on cow pea seedlings against the untreated one. These results demonstrate that, the isolate NII-0905 has the promising PGPR attributes for both in cold as well as in humid condition. It has potential as a biofertilizer to enhance soil fertility and promote the plant growth.

1. Introduction

Modern agriculture is heavily depending on the application of chemical inputs particularly, fertilizers and pesticides. Fertilizers are become essential components of modern agriculture which provides essential plant nutrients. However, overuse of fertilizers can cause unanticipated environmental impacts. Many concerns regarding human health and environmental protection with agriculture are focused on reduction in the use of chemical pesticides and inorganic fertilizers, which compelling the search for alternatives that enhance soil and environmental quality (Haggag, 2002). One potential way to decrease negative environmental impacts resulting from continued use of chemical fertilizers is inoculation with plant growth promoting rhizobacteria (PGPR), these bacteria exert beneficial effects on plant growth and development (Bakker et al., 2007). One of the important mechanisms for these beneficial effects is PGPR-elicited enhanced nutrient availability and nutrient use efficiency. In a recent review, Glick et al. (2007) observed that some PGPR may influence plant growth by synthesizing plant hormones or facilitating uptake of nutrients from the soil through different direct mechanisms such as atmospheric nitrogen (N) fixation, solubilization of phosphorus (P), and synthesis of siderophores for iron sequestration making nutrients more available for plants. Microbial inoculants have showed some promising outcome in increasing nutrient availability. For example, previous reports have suggested positive impacts of microbes on N uptake involving nonlegume biological fixation (Wu et al., 2005; Aseri et al., 2008). And also, inoculation with microbes, including arbuscular mycorrhiza fungi (AMF) resulted in P-solubilization and enhanced plant uptake of fixed soil phosphorous and resulting in higher crop yield (Canbolat et al., 2006; Aseri et al., 2008). The main mechanism resulting in increased availability of inorganic phosphorous appears to be through the action of organic acids synthesized by inoculants (Pratibha and Arvind, 2009), because essential plant nutrients are taken up from the soil by roots, root growth is considered a prerequisite for enhanced plant development. Many PGPR systems cause stimulation of root growth, sometimes via production of phytohormones by the plant or by the bacteria (Shaharooma et al., 2008). If promotion of root growth by PGPR could be achieved

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with high frequency in the field, PGPR may be potential tools for increasing nutrient uptake in the plants.

The management of soil organic matter requires inputs of composts, crop residues, green manure and other organic wastes (Beri et al., 2003; Manici et al., 2004). Beneficial plant–microbe interactions in the rhizosphere are the determinants of plant health and soil fertility (Jeffries et al., 2003). In the era of sustainable agricultural production, the interactions in the rhizosphere play a pivotal role in transformation, mobilization and solubilization, etc., from a limited nutrient pool in the soil and subsequent uptake of essential plant nutrients by the crop plants to realize full genetic potential of the crop. Soil microorganisms are very important in the biogeochemical cycles of both inorganic and organic nutrients in the soil and also in the maintenance of soil quality and health (Jeffries et al., 2003; George et al., 2005; Diby and Sarma, 2006). Thus, the need of the hour is to enhance the efficiency of the meager amount of external inputs by employing the best combinations of beneficial microbes for sustainable agricultural production. In this study an attempt was made to look for potential plant growth promoting ability of novel isolate *Pontibacter niistensis* reported from Western *ghat* dense forest in cowpea.

2. Materials and methods

2.1. Isolation, characterization and identification of bacteria

P. niistensis NII-0905 used in the present investigation was isolated in this laboratory. The soil used for bacterial isolation was collected from a root-free soil of Western *ghat* in west coast of India, located at an altitude of 900 m above mean sea level [GPS coordinates for the sample site are 74°52′E, 8°18′N] located in the Nilgiri Hills, Palakkad district, Kerala, in South India, from soil sample collected from Western *ghat* forest soil in South India. The cultural condition, characterization and taxonomical assignment using 16S rRNA gene sequencing and phylogenetic analysis of the strain were as described earlier (Dastager et al., 2010). Strain NII-0905 was then screened for traits that might be associated with ability to functions as PGPR, such as P-solubilization, indole acetic acid production (IAA), siderophore and HCN activity. Each test was performed in triplicate.

2.2. Quantitative estimation of phosphate solubilization

Initial qualitative estimation of the P-solubilizing activity of the isolate was carried out on Pikovskaya agar (Pikovskaya, 1948). Quantitative estimation of P solubilization was carried out as per standard methodology, by inoculating 1 mL of bacterial suspension (3×10^7 cells mL^{-1}) in 50 mL of National Botanical Research Institute Phosphate NBRIP broth (Mehta and Nautiyal, 2001), in Erlenmeyer flasks (150 mL), and incubating the flasks for 7 days. At the end of the incubation period the cell suspension was centrifuged at 10,000 rpm min^{-1} for 10 min and the P content in the supernatant was spectrophotometrically estimated by the ascorbic acid method (Murphy and Riley, 1962), pH of the medium was recorded with a pH meter equipped with glass electrode.

For the analysis of organic acids, bacterial cultures were filtrated through 0.2 mm filter (Millipore, GTBP) and 20 μL of filtrates were injected to HPLC (Shimadzu Corporation, Kyoto, Japan) equipped with Photo Diode Array detector. The organic acid separation was carried out on C-18 column (Bio-Rad Laboratories, Inc.) with 10.8% acetonitrile in 0.0035 M H_2SO_4 as mobile phase. Retention time of each signal was recorded at a wavelength of 210 nm and compared with the standard acids. Estimation of indole acetic acid (IAA) was done by inoculation of 200 μL of bacterial suspension (3×10^7 cells mL^{-1}) in 10 mL Luria Bertani (LB) broth amended with L-tryptophan (100 μg mL^{-1}) and incubating it at 28 °C for 48 h. The IAA content in the culture suspension was estimated by the standard procedure (Gordon and Weber, 1951). All the studies were repeated on three independent dates to confirm the results.

2.3. Extraction of IAA and ACC-deaminase activity

Single bacterial colonies of isolates were inoculated in 200 mL of nutrient broth amended with 1 or 5 mg/mL of tryptophan and incubated at 28 ± 2 °C for 3–5 days on a shaker incubator. Bacterial cells were separated from the supernatant by centrifugation at 10,000 rpm for 30 min. The supernatant was acidified to pH 2.5–3.0 with 1 N HCl and extracted twice with ethyl acetate at double the volume of the supernatant. Extracted ethyl acetate fraction was evaporated to dryness in a rotator evaporator at 40 °C. The extract was dissolved in 300 mL of methanol and kept at −20 °C. Methanol extract was quantified by reverse phase HPLC (Shimadzu Corporation, Kyoto, Japan). The mobile phase was methanol/water/acetic acid (36:64:1) at a flow rate of 1 mL/min. Elutes were detected at 220 nm and IAA was quantified by integrating the areas under the peaks. Authentic IAA was used as a standard.

ACC-deaminase activity (1-aminocyclopropane-1-carboxylate deaminase) of the strain NII-0905 was detected on plates with DF minimal medium containing 1-aminocyclopropane-1-carboxylate (ACC) as the sole source of nitrogen (Jacobson et al., 1994). Germinating of seed bioassay for ethylene reduction due to ACC deaminase activity was performed. Measurement of the root length of cow pea treated with 48-h-old *Pontibacter* strain NII-0905 culture [OD_{600} = 1.0–1 × 10^9 colony forming unit (cfu) mL^{-1}] for 1 h in nutrient broth was compared with uninoculated controls after 5 days of incubation at 30 °C in Petri plates as described earlier (Dey et al., 2004).

2.4. Qualitative measurement of siderophore and hydrocyanic acid (HCN) production

Siderophore production was detected by the standard Chrome Azurol-S (CAS) assay (Pankaj et al., 2007) in 110 mm Petri dishes, and the diameter of the clearing zone was measured. HCN production was inferred by the qualitative method of Bakker and Schipper (1987). The change in the color of the filter paper previously dipped in 2% sodium carbonate prepared in 0.05% picric acid, from yellow to dark brown was rated visually depending on the intensity of the color change.

2.5. Bioassay-based plant growth promotion ability in cow pea (*Vigna unguiculata*)

A bioassay-based determination of the plant growth promotion ability of the isolate was conducted using cow pea seedlings in pots under glass house conditions. The cowpea seeds were sterilized in 70% ethanol for 2 min and in 2% sodium hypochlorite for 2 min and followed ten times washing in sterile tap water. For this experiment, pure cultures were grown in nutrient broth at 28 °C and diluted to a final concentration of 10^8 colony-forming units (cfu) mL^{-1} in sterile saline water (0.85%). The surface sterile seeds were inoculated by immersion in the appropriate PGPR suspension (ca. 10^8 cfu mL^{-1}) for 45 min on a rotary shaker (140 rev min^{-1}), air-dried, and sown immediately. The cell densities in the suspension were adjusted to a final density of approximately 10^8 cfu $seed^{-1}$. Control seeds were treated with sterile distilled water. Seeds were sown in plastic pots (15-cm diameter) containing 1 kg of sterile soil (pH – 7.2, organic carbon – 2.6%, available P – 537.5 kg ha^{-1}, available K – 448 kg ha^{-1}, iron – 40 mg kg^{-1}) and placed in a temperature controlled growth chamber at 28 ± 2 °C. Thinning of seedlings was