Isolation, molecular characterization and plant growth promoting traits of Neoasaia Chiangmaiensis (KD) from banana

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doi: 10.6088/ijes.2014050100027

ABSTRACT

Aim: To study plant growth promoting properties of Neoasaia chiangmaiensis, an endophyte from banana fruit, a novel habitat

Methods: The endophytes from banana were characterized with respect to cultural, morphological, biochemical and plant growth promoting traits. One of the potent isolates was identified by 16S rDNA sequencing.

Results: Of the twelve isolates, the isolate, designated as KD was identified as Neoasaia chiangmaiensis KD based on 16S rDNA analysis. The plant growth promoting traits such as phosphate solubilization (256.3µg/ml) and indole acetic acid production (35µg/ml) were maximum amongst all isolates. It has been found that the ability of this organism to produce higher amounts of IAA resulted in increased tolerance to stress. Besides, this isolate also exhibited antifungal activity against Fusarium oxysporum, a wilt causing fungus. Inoculation studies with maize indicated significant increase in root and shoot biomass.

Conclusions: Thus, the natural inhabitance of Neoasaia chiangmaiensis in banana fruits and its plant growth promoting traits is being reported for the first time. The banana fruit represents novel habitat of N. chiangmaiensis in banana fruit.

Keywords: Neoasaia chiangmaiensis, 16S rDNA, Banana, Phosphate solubilization, Stress tolerance

1. Introduction

Endophytic bacteria colonize the internal tissues of the plant showing no external sign of infection or negative effect on their host. Nearly 3,00,000 species of plants exist on the earth. Each of them is host of one or more endophytes. However, very few of them have been completely studied for endophytic biology. Therefore, there is considerable opportunity to find new and beneficial endophytic microorganisms among the great diversity of plants in different ecosystems. Many bacterial species are reported to promote plant growth in different ways that has made them valuable for agriculture in improving crop performance (Muthukumarasamy et al. 2002). The mechanism by which they promote plant growth include the ability to fix nitrogen (Salantur et al. 2006), production of phytohormones (Costacurta and Vanderleyden 1995), production of siderophores that decrease heavy metal toxicity in plants (Burd et al. 2000) and solubilization of mineral phosphates and other
nutrients (Pandey et al. 2006). They may increase the resistance of plants to phytopathogens, thus becoming ideal candidates for biological control (Madhaiyan et al. 2004).

The genus Neoasaia was introduced as the ninth genus in the family Acetobacteriaceae with a single species, Neoasaia chiangmaiensis by Yukphan et al. (2005). As an endophyte, it was first isolated from a flower of red ginger collected in Chiang Mai, Thailand. Literature search indicated lack of report on isolation of N. chiangmaiensis from banana as well as its plant growth promoting traits. Considering this, the present investigation was conducted to demonstrate the natural occurrence of N. chiangmaiensis in banana fruits and its plant growth promoting nature.

2. Materials and methods

2.1 Isolation

Fruit of different varieties of banana was thoroughly washed under tap water. It was then disinfected using 70% (v/v) alcohol for 1 min and 0.1% (w/v) HgCl₂ for 2-3 min and peeled off under aseptic condition. One gram of the fresh pulp was separately added to 9 ml sterile saline and macerated using pestle and mortar and utilized for serial dilutions. Tubes with 10 ml semisolid LGI medium (Cavalcante and Dobereiner 1988) were then inoculated with 0.1 ml of each dilution and incubated at 30ºC for 48 hours. Tubes were observed for thick white pellicles. A loopful of each pellicle was streaked onto LGI agar plates and incubated at 30ºC for 48 hours. Total twelve isolates were obtained from different varieties of banana. They were maintained on LGI agar slants at 4ºC and in 20% glycerol at -80ºC.

2.2 Identification and characterization of the bacterial isolates

Genus-level identification was carried out by subjecting the isolates to cultural, morphological (colony morphology and pigmentation), microscopic (Gram staining and motility), biochemical (utilization of carbon sources and enzymatic activity) and physiological characteristics (temperature, pH, salt and sugar tolerance) following standard procedures (Kastura et al. 2001; Yamada et al. 2000 and Yukphan et al. 2005). Oxidation of acetate and lactate, production of acetic acid on ethanol-CaCO₃ medium and the growth on 21% sucrose (w/v) and 0.35% acetic acid were also tested.

2.3 Bacterial identification using 16S rRNA gene sequences

Total genomic DNA was isolated using GeneElute Genomic DNA isolation kit (Sigma, USA) as per the manufacturer’s instructions and used as template for PCR. Each reaction mixture contained approximately 10 ng of DNA; 2.5 mM MgCl₂; 1x PCR buffer (Bangalore Genei, Bangalore, India); 200 µM each dCTP, dGTP, dATP and dTTP; 2 pmol of each, forward and reverse primer; and 1 U of Taq DNA polymerase (Bangalore Genei, Bangalore, India) in a final volume of 20 µl. FDD2 and RPP2 primers were used to amplify almost entire 16S rRNA gene, as described previously (Rawlings 1995). The PCR was performed using the Eppendorf Gradient Mastercycler system with a cycle of 94ºC for 5 min; 30 cycles of 94º, 60º and 72ºC for 1 min each and final extension at 72ºC for 10 min and the mixture was held at 4ºC. The PCR product was precipitated using polyethylene glycol (PEG 6000, 8.5%) washed thrice using 70% ethanol and dissolved in Tris-HCl (10 mM, pH 8.0). The ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Caliph) was used for the sequencing of the PCR product. The sequencing reaction and template preparation were performed and purified in accordance with the directions of the
manufacturer (Applied Biosystems). Samples were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sequencing output was analyzed using the accompanying DNA Sequence Analyzer computer software (Applied Biosystems). The sequence was compared with National Center for Biotechnology Information GenBank entries by using the BLAST algorithm.

2.4 Phosphate solubilization

Phosphate solubilization ability of the isolates was tested using petridish assays on Pikovskaya agar containing 0.5% (w/v) tricalcium phosphate (Pikovskaya 1948). The plates in triplicate were spot inoculated (5 µl with ~2 x 10^6 CFU/ml) and incubated at 30ºC for 6 days. The zone of solubilization around the bacterial colony and colony diameter were measured. Phosphate solubilization index was calculated by determining the ratio of the total diameter (colony + halo zone) to the colony diameter. Quantitative estimation of tricalcium phosphate solubilization in broth was carried out at 30ºC using Erlenmeyer flasks (250 ml) containing 100 ml Pikovskaya broth (triplicate) inoculated each with 100 µl of bacterial suspension (5 x 10^6 CFU/ml). The growth medium was sampled aseptically at 2-days intervals from each flask and centrifuged at 10,000 rpm for 20 min. The supernatant was analyzed for P_2O_5 content using the method suggested by Katewa and Katyare (2003). The pH of the supernatant was measured in each case. The phosphate solubilization in presence of different levels of D-glucose was also studied. The ability of organism to produce phosphatase was detected by using plate based assay (Gerhardt et al. 1994). In order to study the relationship between P solubilization and the production of organic acids, culture supernatant was filtered, purified and analyzed by HPLC.

2.5 Qualitative and quantitative estimation of IAA production

The production of IAA in broth culture was assayed by Salkowsky’s colorimetric method (Glickmann and Dessaux 1995; Mayer 1958). Production of IAA was studied in 1.5% tryptone water (w/v). Erlenmeyer flask (250 ml) containing 100 ml tryptone water was inoculated with 1 ml of bacterial suspension (9 x 10^6 CFU/ml) and incubated in the dark at 30ºC for 7 days on shaker at 200 rpm. The cultures were sampled aseptically every day from each flask and centrifuged at 10,000 rpm for 20 min and the production of IAA was quantitatively estimated.

2.6 Stress tests

To determine if the ability to produce high levels of IAA conferred increased tolerance to stress, the isolates were exposed to various adverse conditions including antibiotic resistance, osmotic, heat and cold shock, UV irradiation and oxidative stress. The isolate, designated as GN, that did not produce IAA was used as a negative control. The cells were grown in 1.5% tryptone water (w/v) at 30ºC on shaker at 200 rpm. Maximum IAA production was observed on the third day of observation. The cells were harvested, washed and then exposed to different stress tests (Khan and Doty 2009). For antibiotic resistance, the cells were spread plated on LGI agar medium and exposed to the following antibiotics by disk diffusion method (µg/disc): vancomycin (VA) (30), ampicillin (Amp) (10), chloramphenicol © (30), azithromycin (AT) (15) and penicillin (P) (10 units/disc). Growth was observed after 48 h incubation at 30ºC. For osmotic shock, the cells were incubated with 1 and 2 M sodium chloride for up to 2 h at 30ºC. For heat shock, cells were exposed to 60ºC for 5 and 10 min in a water bath. UV irradiation was carried out in the sterile chamber exposing the cells to UV light for 1 and 2 h. For oxidative stress, cells were treated with 3% H_2O_2 and grown for 1 and
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2 h at 30°C. For cold stress, the cells were inoculated on LGI agar plates and incubated at 4°C for 1 and 2 w.

2.7 Antagonistic properties

For examining the antagonism produced by the isolate KD, fungal cultures of test fungi were individually inoculated on antifungal assay agar plates. Culture supernatant of N. chiangmaiensis KD, previously inoculated in Pikovskaya’s broth was added to the same plate by well method. The plates were incubated at 30°C for 5 d and observed for the zone of inhibition. The antifungal activity was tested against Fusarium graminearum (causing head blight of wheat and barley), Fusarium oxysporum (causing wilting of banana, cotton and wheat), Aspergillus parasiticus (causing seed infection of peanut and maize) and Phoma glomerata (causing phoma spot on wheat). The production of other antifungal compounds was also detected following standard procedures.

2.8 Plant response to inoculation

Period for growth and media for propagation of banana were the logistic problems using banana as the test plant. The effect of inoculation of N. chiangmaiensis KD on plant growth was studied on maize because it is one of the economical agricultural crops. Before this bioassay, an endophytic colonization of this isolate in maize was tested. It was found that this organism colonized in large numbers within vascular system. To study the effect of inoculation on plant growth, seeds of maize (Zea mays) were surface sterilized with 95% ethanol for 5 min and washed with sterile water. They were grown in a plastic container (10 cm x 7 cm) containing sterile soil. Two days, after seed germination 2 ml of bacterial culture (~10^9 cells/ml) was added to the soil, around each seedling. Two treatments were carried out: seeds treated with the suspension culture of the bacterium (test) and seeds sown without any inoculation (control). One plant was raised in each plastic container filled with the sterile local soil. For each treatment, 30 containers were used. Ten randomly selected plants from each treatment were uprooted 30 days after sowing the seeds. Fresh weight measurements of roots were seemed to be affected by the laboratory environment. Environmental variables cause negligible variability to dry weight values of root and shoot biomass (Bashan and de-Bashan 2005). Therefore, only dry weight of roots and shoots were recorded. The results were pooled for one-way ANOVAs test.

3.1 Results

3.1 Isolation and identification

On LGI agar plates all isolates produced pink, shiny, smooth and raised colonies with entire margin. Twelve culturable bacterial isolates of N. chiangmaiensis were obtained from different varieties of banana. They were Gram-negative, aerobic, rod shaped and non-motile. They produced acetic acid from ethanol. They could grow in the presence of 0.35% acetic acid (w/v). Oxidation of acetate and lactate was negative. They produced from sucrose, D-xylose, D-fructose, D-mannitol and glycerol. The isolates showed vigorous growth on vitamin-free medium indicating a lack of requirement for growth factor. None of the isolates produced brown colonies on yeast extract-glucose-CaCO3 agar. All of the isolates belonged to the genus Neoasaia on the basis of reported characteristics (Kastura et al. 2001 and Yukphan et al. 2005). The isolate designated as KD (accession number – FJ887939) showed 97% identity with corresponding N. chiangmaiensis deposited under NCBI GenBank accession number AB208549.1.
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The phylogenetic dendrogram based on 16S rRNA sequence analysis showing the relationships of the isolates with the most closely related strains and with each other (using MEGA-5.0 software) are shown in Figure 1.

![Phylogenetic dendrogram](image)

**Figure 1:** Phylogenetic dendrogram based on 16S rDNA gene sequence showing the relationship of isolates M6, KD, SP, SC and GV with the most closely related strains and with each other. Bootstrap values (percentage of 1000 replications) are shown at the nodes.

### 3.2 Phosphate solubilization

The bacteria isolated from banana fruits demonstrated a wide range (21-256 µg/ml) of Ca$_3$(PO$_4$)$_2$ solubilization after 3 days. The isolate, KD with highest phosphate solubilizing efficiency was selected for further study. It showed high rates of solubilization of Ca$_3$(PO$_4$)$_2$ (256.3µg/ml) at 6% D-glucose (w/v) after 3 days of incubation. A steady increase in biomass was observed up to third day after which the microbial population remained static. The pH of Pikovskaya’s broth decreased after Day 1 which is followed by a gradual decline (Figure 2). N. chiangmaiensis KD did not produced acid phosphatase. Two major peaks in analysis of organic acids by HPLC were identified as formic acid (365 mg/lit) and acetic acid (389.6 mg/lit). Lactic acid (0.99 mg/lit), oxalic acid (1.7 mg/lit) and citric acid (0.56 mg/lit) were also found to be produced by N. chiangmaiensis KD.
4. IAA production

Growth media from most of the isolates contained substantial amount of indole acetic acid. Higher amount of IAA was produced in 1.5% tryptone water (w/v) than in 1% tryptone water (w/v). Isolate KD produced highest amount of IAA (35 µg/ml), as against VW (26.7 µg/ml), KB (21.6 µg/ml), however VS produced least IAA (2.5 µg/ml). The isolate GN did not produce any IAA. It has been found that higher amounts of IAA resulted in increased tolerance to stress. The isolate KD producing highest amount of IAA was compared with the isolate GN that did not produce any IAA. Both were exposed to various adverse conditions. Isolate KD could grow well in the presence of antibiotics including vancomycin, ampicillin, penicillin and azithromycin except chloramphenicol whereas GN did not tolerate the tested antibiotics except penicillin and azithromycin. The data presented in Table 1 reveal that IAA producing endophyte (KD) was more resistant (more % survival) to osmotic shock, UV irradiation, oxidative stress, heat and cold shock as compared to the endophyte (GN) that did not produce IAA.

Table 1: Effect of various stress conditions on the survival of N. chiangmaiensis isolates

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>% survival of isolates</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>KD</td>
</tr>
<tr>
<td>Vancomycin (30µg/disc)</td>
<td>21.6 ± 3</td>
</tr>
<tr>
<td>Ampicillin (10µg/disc)</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>Chloramphenicol (30µg/disc)</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Azithromycin (5µg/disc)</td>
<td>42.3 ± 5</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment group 1</th>
<th>Treatment group 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin (10 units/disc)</td>
<td>36.2 ± 4</td>
<td>35.4 ± 2</td>
</tr>
<tr>
<td>Osmotic shock (2 M NaCl)</td>
<td>83 ± 2</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>Oxidative stress (3% w/v H₂O₂)</td>
<td>76 ± 6</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>Cold shock (4°C for 15 days)</td>
<td>81 ± 7</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>Heat shock (60°C for 5 min)</td>
<td>18.6 ± 1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>UV irradiation (100 J/m² for 2 hr)</td>
<td>87 ± 2</td>
<td>56 ± 4</td>
</tr>
</tbody>
</table>

*IAA producing N. chiangmaiensis isolate (test), IAA non-producing N. chiangmaiensis isolate (control). The values are means of three replicates ± standard deviation.

4.1 Antagonistic properties

The isolate KD exhibited several properties associated with biocontrol. The bacterium inhibited the growth of the phytopathogenic fungus, Fusarium oxysporum IACC 284, a causative agent of the wilting of wheat (Figure 3). The bacterium was found to produce protease, pectinase and siderophore. A color change of CAS agar plate from blue to orange indicated the presence of a hydroxamate-type siderophore (Schwyn and Neilands 1987). It did not produce chitinase and HCN.

![Figure 3: Growth inhibition of Fusarium oxysporum IACC 284 by the culture supernatant of N. chiangmaiensis KD. A: 50 µl of the culture supernatant of N. chiangmaiensis KD grown in Pikovskaya’s broth with 6% D-glucose (w/v); B: 50 µl of the supernatant of uninoculated Pikovskaya’s broth (medium blank)](image)

4.2 Plant response to inoculation

Roots of plants showed a striking growth response when maize seedlings were inoculated with the isolate KD. Roots were densely covered by root hairs. Very few were developed on uninoculated control plants. Both root hairs and the number of roots were denser on plants grown with this isolate. Also roots and root hairs were longer on plants grown with this isolate. The bacterial inoculation significantly increased root and shoot biomass of maize after 30 days of growth (Table 2).
Table 2: Effect of N. chiangmaiensis KD on growth of maize

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dry weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Root biomass</td>
<td>0.32 ± 0.06</td>
</tr>
<tr>
<td>Shoot biomass</td>
<td>0.77 ± 0.1</td>
</tr>
</tbody>
</table>

* Significant at 5%, ** Significant at 1%, Each value represents mean of replicate (n = 10) ± SD

5. Discussion

Plant genotypes differ in the capacity to convert nonavailable forms of nutrients to available forms and to take them up. Factors underlying the differential capacities of plant genotypes to access soil nutrients include differences in the surface area of contact between roots and soil, in the composition and amount of exudates and rhizosphere microflora, resulting in differences in the chemistry and biology of the rhizosphere (Rengel and Marschner 2005). This could influence the endophytic microbiota of different plant genotype. Twelve bacterial isolates were obtained from different genotypes of banana plant. Based on colony morphology, microscopic observations, cultural, biochemical and physiological properties, the isolates were closely matching with the genus Neoasaia. The sequence of the 16S rRNA fragment of the isolate KD had 97% identity with Neoasaia chiangmaiensis that belongs to the family Acetobacteriaceae. Recently, acetic acid bacteria are intensively studied from the viewpoint of microbial diversity and for their contribution to plant growth promotion. At present, this family has been divided into eleven genera: Acetobacter, Gluconobacter, Acidomonas, Gluconacetobacter, Asaia, Kozakia, Swaminathania, Saccharibacter, Neoasaia, Granulibacter and Tanticharoenia (Kommanee et al 2009). Among them, only four genera include plant growth promoting species, viz. Acetobacter, Gluconacetobacter, Swaminathania and Asaia (Pedraza 2007 and Weber et al. 2004). The present study demonstrating the inhabitance of Neoasaia chiangmaiensis in banana fruits and its plant growth promoting activities is first of its kind.

Phosphate is one of the major essential macronutrients for biological growth and development. Most of the inorganic phosphates applied to soil as fertilizer are rapidly immobilized after application and become unavailable to plants. Therefore, slow release of insoluble and fixed forms of phosphorus is important for increasing soil phosphorus availability (Mehta and Nautiyal 2001 and Xiufan et al. 2006). In both plants and microorganisms, the primary mechanisms of P solubilization are H⁺ excretion, organic acid production and acid phosphatase biosynthesis (Bianco and Defez 2009). Most of the isolates from banana demonstrated phosphate solubilizing ability. The isolate KD was assayed for organic acid production by virtue of which it would solubilize insoluble inorganic phosphate. Organic acids can form complexes with the iron or aluminum in ferric and aluminum phosphates, thus releasing plant-available phosphate into the soil (Gyaneshwar et al 2002).

Indole acetic acid is a phytohormone which is known to be involved in root initiation, cell division and cell enlargement. This hormone is very commonly produced by PGPR (Boiero et al 2007). IAA-producing bacteria are believed to increase root growth and root length, resulting in greater root surface area which enables the plant to access more nutrients from soil. Some of the isolates of banana synthesized IAA. The isolate KD, producing highest
amount of IAA showed a plant growth promoting effects when inoculated into maize plants. This was resulted into significant increase in seedling growth variables. The present study supports the hypothesis that the banana endophyte may cross inoculate, colonize and contribute to the growth of other plants.

Abiotic stresses are the major limiting factor for plant growth. The protective response of plants to both biotic and abiotic stresses is primarily regulated by phytohormones, such as IAA, salicylic acid (SA) and abscisic acid (ABA) (Bianco and Defez 2009). IAA induces an increased level of protection against external stress conditions by coordinately enhancing different cellular defence systems (Bianco et al. 2006). It has been demonstrated that rhizobia able to overproduce IAA might be selected in order to increase plant yield in extreme environments (Bianco and Defez 2010). The results of present findings are in accordance with the results of Bianco et al. (2006) who reported the effects of IAA treatment on the tolerance of E. coli cells to a variety of adverse conditions. The IAA producer (KD) showed more tolerance to various stress conditions under study as compared to the IAA non-producer (GN). We thus speculate that N. chiangmaiensis KD, able to produce higher amount of IAA than other isolates might be selected in order to increase plant yield in extreme environments.

Most of the PGPR exhibit an antagonistic effect on fungal pathogens of plants by either production of antimicrobial compound or siderophore. The isolate KD exhibited several properties associated with biocontrol. It was found to produce several organic acids, protease and hydroxamate-type siderophore. It demonstrated antifungal activity against F. oxysporum which is the causative agent of wilting disease in cotton and wheat. Finally, N. chiangmaiensis KD with properties such as nitrogen fixation, phosphate solubilization, higher IAA production levels, resistant to several adverse conditions and disease control potential would seem a good and ideal candidate for selection as a suitable bioinoculant.

Acknowledgements

This work is part of the Ph.D. thesis of Patil N. B. We are very thankful to University Grant Commission, New Delhi, India for funding this research work.

6. References


22. Pikovskaya, R. I., (1948), Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. Mikrobiologiya, 17, pp 362-370


