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Research Article

Isolation, characterization and *in vitro* efficacy of banana phyllospheric bacteria

Rahul Dewada, M. D. Khunt, D. H. Tandel, Harish Suthar, H. D. Bhimani

Abstract

Phyllospheric bacteria can help in plant-growth promotion by various direct and indirect mechanisms. A total of ten healthy leaf samples were collected randomly from the banana farms located at Navsari Agricultural University, Navsari, Gujarat. A total of 28 morphologically distinct bacterial isolates were obtained in pure culture from collected samples and screened for their ability to plant growth-promoting parameters under *in vitro* conditions. Out of these, 14.28%, 14.28%, 28.57%, and 82.14% showed positive biological nitrogen fixation ability, phosphate solubilization, potassium mobilization, and zinc solubilization, respectively. Indole-3-acetic acid (IAA) production was detected in 28.57% of isolates and 21.42% isolates were found positive for siderophore production. All (100%) isolates showed antagonism against *Fusarium oxysporum*. Further, 78.57%, 39.28%, and 32.14% of isolates showed positive protease, lipase, and amylase secretion, respectively. Based on *in vitro* screening, the two most potent isolates with multiple plant-growth promoting (PGP) traits were identified by16S rRNA sequencing method, as *Priestia megaterium* and *Pantoea cypripedii*. Further, these isolates can be used in banana plant growth promotion as an alternative to synthetic chemicals for sustainable agriculture practices.

Keywords antagonism, phosphate solubilization, phytohormone, plant growth promotion

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Authors:
R. Dewada, M. D. Khunt ✉,
Department of Agricultural Microbiology, N. M. College of Agriculture, Navsari Agricultural University, India

D. H. Tandel
Department of Plant Pathology, N. M. College of Agriculture, Navsari Agricultural University, Navsari, India

H. Suthar
Department of Post-Harvest Technology, ASPEE College of Horticulture, Navsari Agricultural University, Navsari, India

H. D. Bhimani
ASPEE Shakilam Biotechnology Institute, Navsari Agricultural University, Surat, India

✉ khunt_mrugesh@yahoo.com

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Introduction

Banana is a widely cultivated fruit crop with numerous economic, nutritional, and cultural values. However, the production and productivity of the banana is largely compromised due to various biotic and abiotic stresses that pertain to the field. These stresses can be effectively managed in an eco-friendly manner by applying plant-beneficial bacteria. The phyllosphere is the habitat of well-diversified plant-beneficial microbial communities, encompassing bacteria, fungi, algae, and protozoans [1]. Among these microbes, bacteria make up the majority of the total microbial community and can be found in the tune of $1x10^2$ to $1x10^{12}$ colony forming unit (cfu) per g of a leaf [2].

Leaf physiology and the microenvironment support the distribution and abundance of microorganisms on the leaf surface is described by the arrangement of leaf epidermal cells [3]. Due to variable microenvironments, phyllospheric microbial communities are often exposed to ever-changing environmental conditions, leading to their impact on the diversity and composition of phyllospheric microbial communities. Phyllospheric bacteria could enhance plant growth and yield by secreting natural growth regulators such as IAA, gibberellic acids, cytokines, etc., and have the ability to fix nitrogen and solubilize/mobilize other macro as well as micronutrients [4].

Foliar application of phyllospheric bacteria can promote plant growth and increase their ability to withstand adverse environmental stresses.

Methodology

Collection of leaf samples and isolation of phyllospheric bacteria

Banana leaf samples were randomly collected from farms of Navsari Agricultural University, Navsari, Gujarat (20.9248° N, 72.9079° E). Altogether, total of ten leaf samples were taken from healthy, developing plants around the banana orchard by using sterile forceps. The collected leaves were washed with sterilized distilled water separately to remove the dirt present on the leaf surface under aseptic conditions. Isolation of phyllospheric bacteria was performed by leaf imprinting method [5]. Using the four-sector streaking approach, well-isolated colonies with characteristic colonial shapes were carefully transferred on fresh N-agar plates in order to purify phyllospheric bacteria. Pure cultures of the isolates were preserved on N-agar slants at refrigerated temperature for further studies.

Screening of phyllospheric bacteria

Based on various plant growth-promoting factors, isolated phyllospheric bacteria were evaluated for their *in vitro* plant growth-promoting performance. The parameters are listed below:

Nitrogen fixation: The nitrogen fixation potential of isolates was judged on a nitrogen-deficient Jensen's medium [6]. Following three days of incubation at 28±2ºC, the existence of a yellow-colored zone surrounding the colonies was regarded as proof of positive BNF.

Phosphate solubilization: The rate of P solubilization was checked by spot inoculation of bacteria on Pikovskaya's medium [7] amended with 0.5% (w/v) tri-calcium phosphate. Pure cultures of isolates were inoculated in the medium and incubated at 28±2ºC for 3 days. Positive P solubilization was shown by a distinct zone of tri-calcium phosphate solubilization surrounding the colonies. The zone ratio was calculated by following the formula.

$$\text{Zone ratio} = \text{Zone diameter (mm)}/\text{Colony diameter (mm)}$$

Potash mobilization: On a glucose, yeast extract, and calcium carbonate (GYC) medium, the rate of K mobilization was monitored [8]. The development of a halo zone surrounding the bacterial colony after incubation indicated a positive test for K mobilization. The zone ratio was worked out as discussed earlier.

Zinc solubilization: The Zn solubilization potential of the isolate was investigated in Bunt and Rovira's medium containing 0.1% insoluble ZnO [9]. Isolates were spot inoculated on the medium followed by incubation at 28±2ºC for 3 days. A clear zone around the bacterial colony indicated the solubilization of insoluble ZnO and the zone ratio was calculated to find the efficiency of isolates.

Extracellular enzyme secretion: Various extracellular enzymes such as Proteases [10], amylases [11], and lipases [12] were determined on medium amended with specific substrates. The zone ratio was worked out after incubation as discussed earlier.

Biological control (Antagonistic potential): The biocontrol efficiency of isolates was checked against *Fusarium oxysporum* by dual culture method on N-agar medium. The efficiency of isolates was judged by percent growth inhibition (PGI) [13].

IAA production: We employed the Salkowski approach for the IAA estimate [14]. Bacterial cultures were inoculated in a minimal medium amended with tryptophan and after incubation; 2-3 drops of ortho-phosphoric acid+salkowski reagents were added into the broth. The development of the pink color was

considered evidence of IAA production.

Siderophore test: Siderophore production of the isolate was determined using chrome azurol S (CAS) agar [15] medium. Isolates were spot inoculated on CAS agar and incubated at 28±2ºC for 7 days in the dark. Positive siderophore production is shown by the yellow or orange halo zone surrounding the colony on CAS agar on a blue background.

Identification of isolates

Potent PGPB isolates were identified based on16S rRNA sequencing method. Total DNA was extracted from cultures followed by PCR amplification of 16s rDNA gene with primer 27F using BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic Analyzer. The gene sequence was used to carry out BLAST with the database of NCBI Genbank database. Sequences of isolates were deposited in the NCBI gene bank with successful accession numbers.

Results and Discussion

Isolation of phyllospheric bacteria

Banana leaf samples were randomly collected from the farms of Navsari Agricultural University Navsari, Gujarat for the isolation of native candidate phyllospheric bacteria having the potential for plant growth promotion. A total of ten different samples from healthy, developing plants around the banana orchard were taken for the experiment. The leaves were carefully kept in sterilized polythene bags and were brought to the laboratory in a 4°C ice box. Collected samples were washed with sterilized distilled water separately to remove the dirt present on the leaf surface. Further, samples were processed under a laminar airflow cabinet while being cut with sterilized scissors by avoiding direct touch. Phyllospheric bacteria were isolated from the leaf surface by the leaf imprinting method. After 24 hours of incubation at 28±2°C, well-isolated colonies showing distinct colonial characters were purified on a fresh N-agar plate by the four-sector method. Again after incubation, purity of isolates was confirmed, and a total of 28 morphologically diverse phyllospheric bacterial isolates were preserved on the N-agar slant for further studies.

In vitro screening of isolated bacteria

Plant growth promotion may be observed due to different direct and indirect mechanisms such as an increase in nutrient availability, phytohormone production, macronutrients biodegradation, disease management, etc. Thus, *in vitro* screening was performed to determine the lab efficacy of all the isolates that could provide tolerance to growing plants against biotic stress during different phases of plant growth.

Following isolation, several bacteria were tested for their ability to promote plant growth *in vitro* (Table 1, Figure 1). Data from experiments showed that all the isolates possess variable abilities in plant growth promotion (PGP) under *in vitro* conditions. The majority of the isolates showed multiple PGP characters under the lab conditions. Overall, out of a total of 28 phyllospheric isolates, 4(14.28%), 4(14.28%), 8(28.57%), and 23(82.14%) showed positive biological nitrogen fixation ability, phosphate solubilization, potassium mobilization, and zinc solubilization capacity, respectively under *in vitro* conditions. Many researchers have already reported the role of plant-growth promoting bacteria in increasing nutrient availability under lab conditions [16-17]. Plant-associated bacteria can induce plant development by exerting plant-beneficial effects such as ammonia production, IAA secretion, P solubilization, siderophore production, etc. [16-17]. *In vitro* screening data indicated that out of 28 banana phyllospheric isolates, IAA production was detected in 8(28.57%) isolates, and 6(21.42%) were found positive for siderophore production on CAS medium. Plant disease management by beneficial bacteria may be the cost-effective and eco-friendly way to increase crop production. In the present study, all (100%) isolates displayed antagonistic potential against *Fusarium oxysporum*, PGI ranging from 43.75% to 65.00%. Out of 28 isolates 21 bacterial isolates showed the PGI above 50%. Munoz et al., [18] revealed that phyllospheric bacteria displayed good antagonistic potential against plant pathogens and showed a plant growth promotion effect. Beneficial bacteria produce extracellular enzymes like proteases,

Table 1. In vitro screening of different banana phyllospheric isolates

S.N.	Bacterial Isolates	N_2 Fixation	P Solubilization*	K Mobilization*	Zn Solubilization*	Protease Activity*	Amylase Activity*	Lipase Activity*	Antagonistic Effect (%)	IAA Production	Siderophore Production
1	R1B1	-	-	-	3.3	2.2	1.6	-	52.50	-	-
2	R1B2	-	-	1.2	-	2.5	-	-	58.75	+	-
3	R1B3	-	-	3.4	2.3	4.2	2.1	-	50.00	+	+
4	R1B4	-	-	-	1.5	6.3	-	-	56.25	-	-
5	R1B5	+	2.0	1.3	3.0	-	-	1.6	43.75	+	+
6	R1B6	+	2.0	2.3	3.6	-	-	1.3	50.00	+	+
7	R1B7	+	-	3.3	-	4.4	2.1	-	50.00	+	+
8	R1B8	-	-	-	1.3	7.0	-	2.8	60.00	-	-
9	R1B9	-	-	-	1.5	3.1	2.8	2.2	61.25	-	-
10	R1B10	-	-	-	1.4	7.0	-	-	60.00	-	-
11	R1B11	-	-	-	3.0	5.5	-	-	53.75	-	-
12	R1B12	-	-	-	2.2	6.6	-	-	58.75	-	-
13	R1B13	-	-	-	-	2.2	1.2	-	56.25	-	-
14	R1B14	-	-	-	2.2	5.2	-	-	56.25	-	-
15	R1B15	-	-	-	3.0	6.3	-	2.8	56.25	-	-
16	R1B16	-	-	-	1.4	7.0	-	2.8	62.50	-	-
17	R1B17	-	-	-	1.4	-	3.8	-	65.00	-	-
18	R1B18	-	-	-	2.3	5.3	-	-	62.50	-	-
19	R1B19	-	-	-	1.5	-	1.6	-	58.75	-	-
20	R1B20	-	3.3	3.9	-	-	-	2.5	43.75	+	+
21	R1B21	+	1.2	3.2	3.6	-	-	-	53.75	+	+
22	R1B22	-	-	-	1.5	6.6	-	3.0	60.00	-	-
23	R1B23	-	-	-	3.6	9.0	-	-	50.00	-	-
24	R1B24	-	-	3.1	1.3	4.2	-	2.0	50.00	+	-
25	R1B25	-	-	-	-	2.4	2.2	3.5	62.50	-	-
26	R1B26	-	-	-	2.2	6.0	-	2.6	58.75	-	-
27	R1B27	-	-	-	3.0	6.3	-	-	56.25	-	-
28	R1B28	-	-	-	1.5	3.4	1.8	-	57.50	-	-

*** is zone ratio (zone diameter/colony diameter); + and – sign indicates positive and negative test respectively (Data represented as mean of 3 repetitions)**

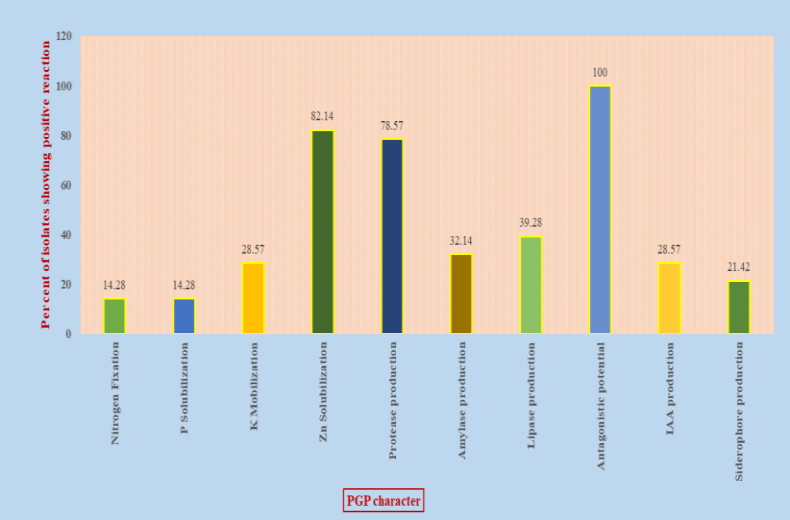

Figure 1. Percent of isolates showing positive reaction for PGP character

amylases, and lipases [19-20] that exert plant growth promotion effects. In the present study, isolates exhibited the variable potential of hydrolytic enzyme production, among them 22(78.57%), 11(39.28%), and 9(32.14%) isolates showed positive results for the protease, lipase, and amylase secretion, respectively. Many of the isolates in the study exhibit various PGP characters and were found more potent under *in vitro* conditions. Two isolates out of all the others demonstrated the most characteristics and were the most potent in terms of individual reactions. The isolate R1B3 showed positive IAA activity, K mobilization, Zn solubilization and siderophore production with 50% antagonism against *Fusarium oxysporum* under *in vitro* conditions. Isolate R1B20 also showed the highest P solubilization, K mobilization with good IAA production, and siderophore production characters. Therefore, by considering the multifaceted potential of R1B3 and R1B20 in the different PGP characters tested, both were further identified based on16S r-RNA sequencing method.

Molecular identification

The 16S r-RNA sequencing technique was used to identify the two most powerful isolates. Data indicated that R1B3 was *Priestia megaterium* (Gene bank accession number OR018926) (Figure 2) and R1B20 was identified as *Pantoea cypripedii* (accession number OR018927) (Figure 3).

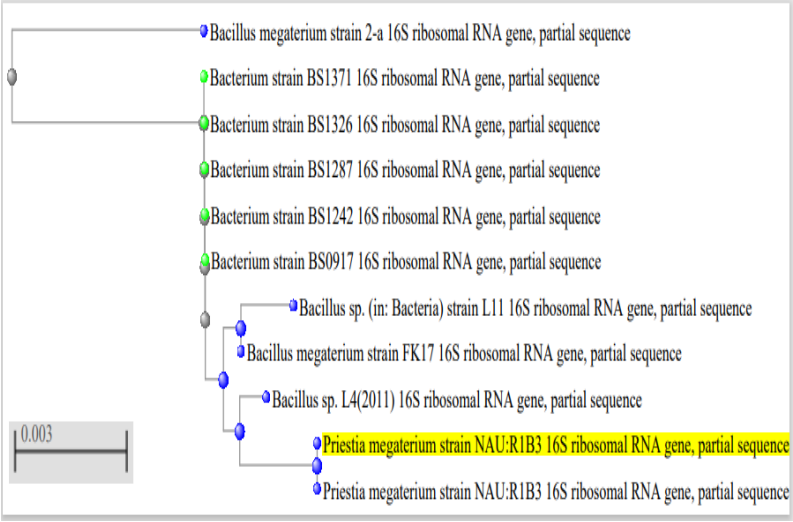

Figure 2. Phylogenetic tree of *Priestia megaterium* R1B3 (Accession No. OR018926)

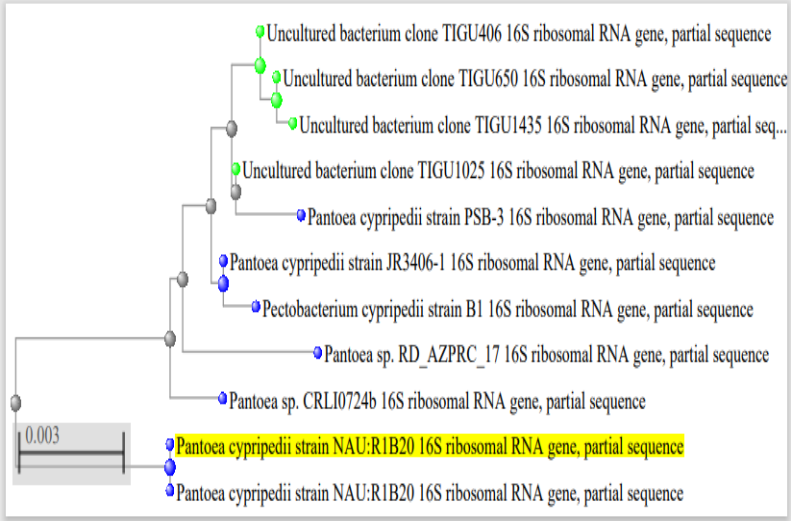

Figure 3. Phylogenetic tree of *Pantoea cypripedii* R1B20 (Accession No. OR018927)

Conclusion

The phyllospheric microbiome of any crop playes an important role in the overall growth and development of that plant. The plant suffers from various biotic and abiotic stresses during the growth phase and their productivity is comprised under such stresses. This study indicated that banana phyllospheric bacterial isolates can be used for formulating bacterial biofertilizers or spray solutions for increasing the production without affecting the soil property and environment as bacterial isolates increase the soil fertility and maintain its property and ecology without damaging it. The use of these bacteria is a cost-effective and noble sustainable approach to agriculture and provides an alternative to artificial chemicals without any hazardous effects on soil and human health. This can be a noble approach to solving food insecurity across the globe.

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