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Isolation and Identification of Shoot-Tip Associated Endophytic Bacteria from Banana cv. Grand Naine and Testing for Antagonistic Activity against *Fusarium oxysporum* f. sp. *cubense*

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Abstract

Endophytic bacteria colonizing the shoot-tips of banana cv. Grand Naine were isolated and tested for the antagonistic activity against the Panama wilt pathogen *Fusarium oxysporum* f. sp. *cubense* (*Foc*). Pre-isolation, the suckers were given extensive disinfection treatments and the homogenate from the excised shoot-tip portion was plated on nutrient agar (NA) and trypticase soy agar (TSA). This yielded altogether 47 isolates: 26 on NA and 21 on TSA, respectively, from the 10 suckers collected during August to February. The number of bacterial isolates obtained per sucker varied from one to 15 based on colony characteristics registering up to 10 distinct species per shoot-tip based on 16S rRNA sequence analysis. The 47 isolates belonged to 19 genera and 25 species under the phylogenetic classes of Actinobacteria, α- and γ-Proteobacteria and Firmicutes. Actinobacteria constituted the predominant phylum (55% isolates) with the constituent genera of *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Curtobacterium*, *Kocuria*, *Kytococcus*, *Micrococcus*, *Naumanella*, *Rothia* and *Tessaracoccus* spp. and an unidentified isolate belonging to the family Frankiaceae. Proteobacteria constituted the second major phylum (*Brevundimonas*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia* and *Sphingomonas* spp.) followed by Firmicutes (*Bacillus* and *Staphylococcus* spp.). Antagonistic activity of the endophytes against *Foc* was tested through agar plate assays (pit and spot applications on fungal lawn) employing potato dextrose agar and NA. Endophytic *Pseudomonas aeruginosa* (isolate GNS.13.2a) which was associated with a single sucker showed significant growth inhibition effect on *Foc* while *Klebsiella variicola* (GNS.13.3a) and *Enterobacter*

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***cloacae* (GNS13.4a) exhibited moderate inhibition. The study brings out considerable sucker to sucker variation in the associated cultivable endophytic bacteria in "Grand Naine" banana and identifies a few bacterial endophytes with biocontrol potential against the devastating *Foc* pathogen.**

Keywords

Bacterial Endophytes, Bacterial-Fungal Antagonism, Biological Control, *Fusarium oxysporum* f. sp. *cubense*, *Musa* sp., Plantains, *Pseudomonas aeruginosa*

1. Introduction

The interactions between endophytic microorganisms and host plants and the relevance of such integrally associated microbes in crop production and protection form emerging areas of research in plant sciences [1] [2]. Endophytic bacteria have major roles in plant growth including growth promotion [3], biocontrol of pathogens [4] and pests [5]. Earlier cultivation-based studies have indicated endophytic bacteria, as predominantly root colonizers often present in fewer numbers [6]. Application of cultivation independent molecular techniques has brought out the prevalence of considerable amount of bacterial endophytes in different plant species, mostly in a non-cultivable form [2] [7]. Cultivation-based previous studies in banana cv. Grand Naine revealed the presence of a wide range of endophytes both in field shoot-tip region and in micropropagated *in vitro* stocks [8] [9]. Recent studies employing different banana cultivars have revealed the extensive inhabitation of shoot tissues including the intracellular niches by normally non-cultivable bacterial endophytes. This included colonization in the cytoplasm and in the cell wall-plasma membrane peri-space with the designation of the organisms in the above two niches as "Cytobacts" and "Peribacts" respectively [10] [11]. Although a vast share of endophytes exist in a non-cultivable form, cultivation-based studies and community analysis are warranted for further exploitation of organisms in crop production and protection.

Banana wilt caused by the fungal pathogen *Fusarium oxysporum* Schlect f. sp. *cubense* (E. F. Smith) Snyd. & Hans. (*Foc*), widely referred to as Panama disease, is a devastating disease of bananas and plantains (*Musa* sp.) throughout the world [12]-[14]. The first description of *Fusarium* wilt in banana and plantains came from Australia [13] [15]. The disease is now recognized as the most destructive one limiting banana cultivation all over the world [13] [14] [16]. The pathogen is soil-borne invading the roots and obstructing the water and nutrient flow through vascular colonization. Consequently, leaves turn yellow with the oldest ones succumbing first. The plants eventually wilt and collapse, thus causing serious crop losses [13] [15]. The pathogen is broadly categorised into four races, of which races 1, 2 and 4 are pathogenic to banana, with race 1 infecting most banana cultivars excluding "Cavendish group" [13] [14] [17]. In India, the disease is prevalent in all banana growing areas with race 1 forming the most common one followed by race 2 [16] [18], while race 4 has been reported in certain pockets [19].

Conventional measures to control the pathogen include crop rotation, stringent adoption of phytosanitary measures and the use of resistant varieties [13] [14] [16]. Chemical control measures are often impractical and ineffective. Biological control of plant pathogens employing antagonistic microorganisms is considered as an environment-friendly and cost effective approach. *Fusarium* wilt in banana is reported to be controlled by *Pseudomonas fluorescence* [18], *Bacillus subtilis* [20] and the endophytic *Burkholderia cenocepacia* [21]. Endophytes colonizing the vascular system which constitutes the same niche as *Foc* form potential candidates for developing biocontrol strategies. This study was undertaken with the aim of isolating the bacterial endophytes associated with field-derived banana suckers and evaluating them for the antagonistic activity against *Foc*.

2. Materials and Methods

2.1. Nutrient Media

Two commonly employed bacteriological media, namely nutrient agar (NA: bacteriological peptone 5 $g \cdot l^{-1}$, NaCl 5 $g \cdot l^{-1}$, beef extract 3 $g \cdot l^{-1}$, agar 15 $g \cdot l^{-1}$, pH 6.8 ± 0.2) and trypticase soy agar (TSA: peptone from casein 17 $g \cdot l^{-1}$,

peptone from soymeal 3 $g \cdot l^{-1}$, dextrose 2.5 $g \cdot l^{-1}$, NaCl 5 $g \cdot l^{-1}$, K_2HPO_4 2.5 $g \cdot l^{-1}$, agar 15 $g \cdot l^{-1}$, pH 7.2 ± 0.2) were used for the bacterial isolation and further characterization. The nutrient plates were monitored prior to use for 4 - 7 days to ensure freedom from all microbial contaminants [7] [8]. Potato dextrose agar (PDA: potato infusion from 200 $g \cdot l^{-1}$, dextrose 20 $g \cdot l^{-1}$, agar 15 $g \cdot l^{-1}$) and NA were employed for fungal-bacterial dual culture experiments while PDA was used for normal fungal culturing.

2.2. Surface Sterilization and Preparation of Plant Material

Suckers of banana cv. Grand Naine (2 - 3 months old) were collected from the institute (IIHR) farm. The suckers were disinfected as per as per Thomas *et al*. [7]. Briefly, the rhizome and pseudostem parts were chopped down and the central 5 - 6 cm tissue was incubated in 0.5% Bavistin® (Carbendazim 50% WP) overnight at 150 rpm in a rotary shaker. After a rinse in sterile water, the sucker was trimmed down to 3 - 4 cm and treated with 0.05% cetrimide for 1 h. All subsequent steps were performed in a laminar air flow (LAF) cabinet fitted with an Ultra-Low Penetration Air filter (Esco Biotechnology Equipment Division, Singapore). After removing the external leaf sheaths, the shoot-tip part was treated with NaOCl (4% available chlorine) for 20 min followed by 8 rinses in sterile water. The last two wash solutions were plated on NA and TSA to confirm the effectiveness of disinfection treatments. Shoot-tips of 1 - 1.5 cm were collected after removing the external oxidized tissue and were used for bacterial isolation soon after.

2.3. Tissue Homogenization and Plating

Ten suckers were used for the bacterial isolation during August 2013 to February 2014 in five batches of two suckers each. The excised 1 - 1.5 cm shoot-tips comprising of half pseudostem and corm tissue were cut longitudinally one half of which was used for bacterial isolation. The tissue was ground in a mortar employing sterile peptone salt (1 $g \cdot l^{-1}$ each of peptone and NaCl) [22]. The homogenate was allowed to stand for 20 - 30 min at 4˚C and the supernatant (10^0) and three decimal serial dilutions (10^1 and 10^3) were spread on NA and TSA through the spotting and tilt spreading (SATS) method [22] employing three replications per dilution. The NA pates were incubated at 37˚C and TSA plates at 30˚C for a week and observed for the bacterial colony growth. Thereafter the plates were monitored at room temperature up to one month.

2.4. Identification of Bacterial Isolates

Distinct colony types were picked up from NA and TSA plates for each sucker and were purified through three rounds of streaking and single colony selection on the same medium. The purified cultures were grown together on either media by single colony spotting to identify the distinct colony types, thus to avoid duplications. The identity of the organisms was established through 16S rRNA gene sequence based homology analysis. For this, bacterial DNA was isolated employing the AxyPrep Bacterial Genomic DNA Miniprep kit (Axygen Biosciences, USA) and 16S rRNA gene amplification was performed through PCR using universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R-Y (5'-GGYTACCTTGTTACGACTT-3'; Y = C/T) as described elsewhere [7]. The thermocycling conditions included initial one denaturation step of 94˚C for 5 min followed by 35 amplification cycles of 94˚C for 30 s, 55˚C for 40 s, 72˚C for 40 s followed by a final extension at 72˚C for 5 min [7] [9]. After confirming PCR amplification in a 1% agarose gel and column purification of the PCR product (Macherey-Nagel GmbH, Germany), the 16SrRNA gene was single end-sequenced (Xcelris Genomics, Ahmedabad, India) using 27F primer. The identity of the organisms was determined by megablast analysis at the NCBI Gen Bank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and was validated through Seqmatch search at the Ribosomal Database Project (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

2.5. Confirming the Identity of Fungal Pathogen

The culture of pathogen (*Foc*, race 1), supplied by Dr. T. R. Usharani of this institute (IIHR), was purified through three rounds of hyphal tip culturing in PDA at room temperature (26˚C - 28˚C). Mycelia from PDA culture were used for DNA isolation employing MOBIO microbial DNA isolation kit. The ITS region was amplified in PCR with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [23] employing the thermocycling conditions: 95˚C for 5 min followed by 35 cycles of 94˚C for 30 sec, 53˚C for 40 sec, 72˚C for 30 sec and a final extension of 72˚C for 10 min as per our optimization. PCR products were

column purified as above and sequenced with the ITS1 primer. The identity was established through megablast analysis at the NCBI GenBank as detailed earlier.

2.6. Fungal Antagonistic Assays

The endophytic isolates were tested for the potential antagonistic activity against the pathogen through agar plate assay. In the initial trial, 0.1 OD suspension of overnight culture of *Foc* in potato dextrose broth (PDB) was plated onto PDA and NA in 10 cm diameter plates (Hi Media Biosciences, Mumbai). After surface drying, pits of 5 - 6 mm diameter were prepared with the distal end of 200 µl sterile pipette tips and 50 µl of 1.0 OD suspensions of different bacterial isolates were applied singly followed by surface-drying in the LAF. The plates were incubated at room temperature and observed for fungal lawn formation and inhibition zone development for 2 - 7 days. The experiment was repeated twice. As an alternate approach, adding the test bacterial suspensions as 10 µl spots on the previously *Foc*-spread PDA and NA plates was tried.

A pathogen-antagonist confrontation assay was performed with the selected bacterial isolates. For this, PDA, NA and NA + PDA (1:1) plates were spotted at the edges with 1.0 OD suspension of four test isolates per plate and an agar plug (5 mm diameter) cut from an actively growing PDA culture of *Foc* was placed at the centre. Control plates contained the mycelial plug placed similarly at the centre without bacterial cultures. The plates were observed for up to 2 weeks for the extent of fungal growth.

2.7. Accession Numbers

The 16SrRNA gene sequence data of the endophytic bacterial isolates identified in this study have been deposited with the NCBI GenBank with the acc. nos. KP798811 to KP798857 (**Table 1**).

3. Results

3.1. Endophytic Bacterial Population in Banana Shoot-Tips

The ten suckers showed considerable variation in the population of cultivable bacteria (CFU in the range of 3.0 × 10^1 to 5.1 × 10^4 g^{-1} tissue fresh weight) with the number of distinct isolates per sucker varying from one to 15 (**Figure 1**). Altogether, 47 isolates were obtained which included 26 from NA and 21 from TSA. Once revived, all of the isolates showed growth on either media. NA was employed as the choice medium during further bacterial culturing works. The wash solutions upon plating did not yield any bacterial growth confirming the effectiveness of surface sterilization treatments.

3.2. Bacterial Identification

The PCR products were single-end sequenced yielding > 500 bp 5'-16S rRNA nucleotide data in most instances. The identity of the isolates was established through megablast analysis at the NCBI database which was further

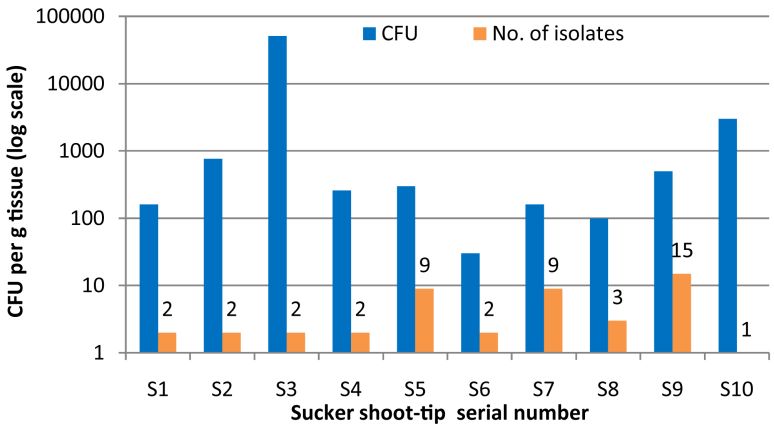

Figure 1. Endophytic bacterial CFU·g^{-1} shoot-tip tissue in individual suckers (S) of banana cv. Grand Naine and the number of bacterial isolates per sucker.

Table 1. Endophytic bacteria isolated from banana cv. Grand Naine identified based on partial 16S rRNA sequence homology analysis through megablast at NCBI GenBank and Seqmatch at RDP database.

S. No.	Isolate ID	16SrRNA seq. (bp)	NCBI acc. no.	Closest species match in NCBI GenBank with acc. no. and homology	Closest species match in RDP with acc. no. and similarity score	Suggested identification and phylogeny
1	GNS13.1a	615	KP798811	*Micrococcus endophyticus* (AB931118; 99%)	*Micrococcus endophyticus* (S000893772, 0.977)	*M. endophyticus;* Actinobacteria
2	GNS13.1b	754	KP798812	*Bacillus safensis* (LC015558; 100%)	*Bacillus safensis* (S000458519, 1.000)	*B. safensis*; Firmicutes
3	GNS13.2a	760	KP798813	*Pseudomonas aeruginosa* (CP010555; 100%)	*Pseudomonas aeruginosa* (S003288366, 1.000)	*P. aeruginosa;* γ Proteobacteria
4	GNS13.2b	633	KP798814	*Kytococcus sedentarius* (EU443746; 99%)	*Kytococcus sedentarius* (S000007341, 0.974)	*K. sedentarius*; Actinobacteria
5	GNS13.3a	751	KP798815	*Klebsiella variicola* (KC853303; 100%)	*Klebsiella variicola* (S000324392, 0.990)	*K. variicola;* γ Proteobacteria
6	GNS13.3b	760	KP798816	*Pseudomonas putida* (HQ236534; 100%)	*Pseudomonas taiwanensis* (S001095516, 0.999)	*P. putida;* γ Proteobacteria
7	GNS13.4a	751	KP798817	*Enterobacter cloacae* (KM077045; 100%)	*Enterobacter cloacae* (S000021184, 0.990)	*E. cloacae;* γ Proteobacteria
8	GNS13.4b	771	KP798818	*Staphylococcus pasteuri* (KJ767309; 100%)	*Staphylococcus pasteuri* (S000381991, 1.000)	*S. pasteuri;* Firmicutes
9	GNS13.5a	876	KP798819	*Sphingomonas mucosissima* (KF924237; 100%)	*Sphingomonas aurantiaca* (S000145110, 0.946)	*S. mucosissima;* α Proteobacteria
10	GNS13.5b	423	KP798820	*Micrococcus luteus* (KF891348; 100%)	*Micrococcus luteus* (S000110436, 0.857)	*M. luteus;* Actinobacteria
11	GNS13.5c	885	KP798821	*Brevundimonas vesicularis* (KC494336; 99%)	*Brevundimonas nasdae* (S000334613, 0.954) *Brevundimonas vesicularis* (S000112396, 0.952)	*B. vesicularis;* α Proteobacteria
12	GNS13.5d	451	KP798822	*Kocuria palustris* (KF424691; 98%)	*Kocuria palustris* (S000014978, 0.875)	*K. palustris;* Actinobacteria
13	GNS13.5e	681	KP798823	*Sphingomonas panni* (HQ739092; 99%)	*Sphingomonas panni* (S000471092, 0.949)	*S. panni;* α Proteobacteria
14	GNS13.5f	601	KP798824	*Micrococcus yunnanensis* (KF424616; 98%)	*Micrococcus yunnanensis* (S001241135, 0.857)	*M. yunnanensis;* Actinobacteria
15	GNS13.5g	611	KP798825	*Micrococcus luteus* (KF358260; 98%)	*Micrococcus endophyticus* (S000893772, 0.861)	*Micrococcus* sp.; Actinobacteria
16	GNS13.5h	691	KP798826	*Staphylococcus epidermidis* (KP053611; 99%)	*Staphylococcus epidermidis* (S000413964, 0.993)	*S. epidermidis*; Firmicutes
17	GNS13.5i	641	KP798827	*Sphingomonas mucosissima* (KM272391; 100%)	*Sphingomonas aurantiaca* (S000145110, 0.949)	*S. mucosissima;* α Proteobacteria
18	GNS13.6a	618	KP798828	*Bacillus safensis* (LC015558; 100%)	*Bacillus safensis* (S000458519, 1.000)	*B. safensis;* Firmicutes
19	GNS13.6b	561	KP798829	*Serratia marcescens* (KF624754; 100%)	*Serratia nematodiphila* (S000903064, 1.000)	*S. marcescens;* γ Proteobacteria
20	GNS13.7a	556	KP798830	*Arthrobacter chlorophenolicus* (JQ958834; 100%) *Arthrobacter globiformis* (FN178398; 100%)	*Arthrobacter equi* (S002033220, 0.981)	*Arthrobacter* sp.; Actinobacteria
21	GNS13.7b	666	KP798831	*Curtobacterium oceanosedimentum* (KM019700; 99%)	*Curtobacterium pusillum* (S000367091, 0.981)	*C. oceanosedimentum;* Actinobacteria
22	GNS13.7c	529	KP798832	*Brevibacterium pityocampae* (JQ388726; 97%)	*Brevibacterium pityocampae* (S001016069, 0.753)	*Brevibacterium* sp.; Actinobacteria

Continued

23	GNS13.7d	521	KP798833	*Corynebacterium lipophiloflavum* (KF777371; 99%)	*Corynebacterium lipophiloflavum* (S000011673, 0.901)	*C. lipophiloflavum;* Actinobacteria
24	GNS13.7e	600	KP798834	*Kytococcus sedentarius* (EU443746; 99%)	*Kytococcus sedentarius* (S000007341, 0.967)	*K. sedentarius;* Actinobacteria
25	GNS13.7g	503	KP798835	*Serratia nematodiphila* (KJ535365; 99%)	*Serratia nematodiphila* (S000903064 0.949)	*S. nematodiphila*; *γ* Proteobacteria
26	GNS13.7h	641	KP798836	*Serratia marcescens* (KF624754; 100%)	*Serratia marcescens* (S000116423 0.990)	*S. marcescens;* *γ* Proteobacteria
27	GNS13.7j	616	KP798837	*Brevibacterium pityocampae* (NR116332; 98%)	*Brevibacterium pityocampae* (S001016069, 0.848)	*Brevibacterium* sp.; Actinobacteria
28	GNS13.7k	610	KP798838	*Brevibacterium pityocampae* (NR116332; 98%)	*Brevibacterium pityocampae* (S001016069, 0.837)	*Brevibacterium* sp.; Actinobacteria
29	GNS13.8a	661	KP798839	*Kocuria rhizophila* (KC429605; 99%)	*Kocuria rhizophila* (S000008149, 0.978)	*K. rhizophila*; Actinobacteria
30	GNS13.8b	599	KP798840	*Jatrophihabitans endophyticus* (NR109586; 95%); *Streptomyces aomiensis* (NR112998; 95%); *Streptomyces catbensis;* (NR125457; 93%)	*Actinokineospora cibodasensis* (S001151982, 0.708)	Actinobacteria; Frankiaceae
31	GNS13.8c	606	KP798841	*Tessaracoccus flavescens* (JQ897419; 96%)	*Tessaracoccus lubricantis* (S001156636, 0.758)	*Tessaracoccus* sp.*;* Actinobacteria
32	GNS13.9a	660	KP798842	*Klebsiella pneumoniae* (JF489150; 100%)	*Klebsiella pneumoniae* (S000021704, 0.991)	*K. pneumoniae;* *γ* Proteobacteria
33	GNS13.9b	661	KP798843	*Rothia terrae* (KM577655; 99%)	*Rothia terrae* (S000722242, 0.972)	*R. terrae;* Actinobacteria
34	GNS13.9c	546	KP798844	*Rothia terrae* (KM577655; 99%)	*Rothia terrae* (S000722242, 0.964)	*R. terrae*; Actinobacteria
35	GNS13.9d	656	KP798845	*Rothia amarae* (FR682692; 100%)	*Rothia amarae* (S000484536, 1.000)	*R. terrae*; Actinobacteria
36	GNS13.9e	712	KP798846	*Staphylococcus pasteuri* (KJ767309; 100%)	*Staphylococcus pasteuri* (S000381991, 1.000)	*S. pasteuri*; Firmicutes
37	GNS13.9f	668	KP798847	*Staphylococcus pasteuri* (KP261076; 99%)	*Staphylococcus pasteuri* (S000381991, 0.992)	*S. pasteuri*; Firmicutes
38	GNS13.9g	712	KP798848	*Rothia amarae* (FR682692; 100%)	*Rothia amarae* (S000484536 1.000)	*R. amarae*; Actinobacteria
39	GNS13.9h	650	KP798849	*Staphyloccoccus warneri* (KP261060; 99%)	*Staphylococcus warneri* (S000414717, 0.986)	*S. warneri*; Firmicutes
40	GNS13.9i	604	KP798850	*Rothia terrae* (KM577655; 100%)	*Rothia terrae* (S000722242, 0.990)	*R. terrae;* Actinobacteria
41	GNS13.9j	519	KP798851	*Rothia terrae* (HQ908710; 99%)	*Rothia terrae* (S000722242, 0.927)	*R. terrae*; Actinobacteria
42	GNS13.9k	686	KP798852	*Rothia amarae* (FR682692; 100%)	*Rothia amarae* (S000484536, 1.000)	*R. terrae*; Actinobacteria
43	GNS13.9l	693	KP798853	*Bacillus okhensis* (NR043484; 99%)	*Bacillus okhensis* (S000653122, 0.994)	*B. okhensis;* Firmicutes
44	GNS13.9n	696	KP798854	*Naumannella halotolerans* (FR832426; 100%)	*Auraticoccus monumenti* (S002032927, 0.773)	*N. halotolerans*; Actinobacteria
45	GNS13.9o	691	KP798855	*Micrococcus terreus* (HQ908754; 100%)	*Micrococcus terreus* (S001241593, 0.967)	*M. terreus;* Actinobacteria
46	GNS13.9p	601	KP798856	*Kytococcus sedentarius* (KJ732936; 99%)	*Kytococcus sedentarius* (S000007341, 0.974)	*K. sedentarius*; Actinobacteria
47	GNS13.10a	639	KP798857	*Staphylococcus hominis* (KM603639; 100%)	*Staphylococcus hominis* (S000016099 0.989)	*S. hominis*; Firmicutes

validated through Seqmatch at RDP (Table 1). Wherever any differences in the species identity appeared between the two databases, the identity was fixed based on the more number of homologous matches at NCBI or higher scores towards a particular species at RDP. The identity was established to the species level in 85% instances and to genus level in 13% cases, but not for one isolate, GNS.13.8b, which showed the maximum homology score of 95% to *Jatrophihabitans endophyticus* and *Streptomyces aomiensis* (95%) in the NCBI database followed by *Streptomyces catbensis* (93%). The maximum homology in RDP database was to *Actinokineospora cibodasensis* (score 0.708). Therefore, this isolate could be assigned only up to the family level, namely, Frankiaceae. The isolate GNS.13.8c also displayed a low homology to the closest match in NCBI (*Tessaracoccus flavescens*; 96%) and low RDP score (*Tessaracoccus lubricantis*; 0.758) but could be identified to the genus level. Thus, the two isolates GNS.13.8b and GNS.13.8c appeared to be novel organisms which demands additional inputs for their proper characterization.

The 47 endophytic isolates belonged to the phylogenetic classes of Actinobacteria, α- and γ-Proteobacteria and spore-forming and non-spore-forming Firmicutes (Figure 2). Actinobacteria constituted the predominant class, also displaying the highest diversity. These included *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Curtobacterium*, *Kocuria*, *Kytococcus*, *Micrococcus*, *Naumanella*, *Rothia*, *Tessaracoccus* spp. and the unidentified GNS.13.8b isolate (Figure 3). Proteobacteria group included *Sphingomonas mucosissimas*, *S. panni* and *Brevundimonas vesicularis* from α-subclass, and *Pseudomonas aeruginosa*, *P. putida*, *Klebsiella variicola*, *K. pneumonia*, *Enterobacter cloacae*, *Serratia marcescens* and *S. nematodiphila* from γ-subclass. Spore-forming Firmicutes included *Bacillus safensis* and the uncommon *B. okhensis*. Non-sporulating Firmicutes included *Staphylococcus epidermidis*, *S. pasteuri*, *S. hominis* and *S. warneri.* Thus, several uncommon organisms were obtained as endophytic bacterial associates from "Grand Naine" shoot-tips.

3.3. Identification of Fungal Pathogen

PCR with ITS1 and ITS4 primers gave an amplicon of about 500 bp. Sequencing of the purified PCR product with primer ITS1 yielded 511 bp data. The megablast analysis at the NCBI GenBank showed 100% homology to *Fusarium oxysporum* f. sp. *cubense*.

3.4. Fungal Antagonistic Assay

A uniform lawn of *Foc* was obtained on PDA and NA within 4 - 7 days of incubation at room temperature. Among the 47 bacterial isolates tested, only one organism, namely *Pseudomonas aeruginosa* (GNS.13.2a) showed significant antagonistic activity against the pathogen in pit method of screening on both NA and PDA (Figure 4). Two other organisms, namely *Klebsiella variicola* (GNS13.3a) and *Enterobacter cloacae* (GNS13.4a) showed relatively mild zone of inhibition. Similar results were obtained in the spot method of application (Figure 5). In confrontation assay, which was undertaken on NA+PDA medium, *P. aeruginosa* showed clear inhibition of fungal growth while the effect was less prominent for the other two (Figure 6).

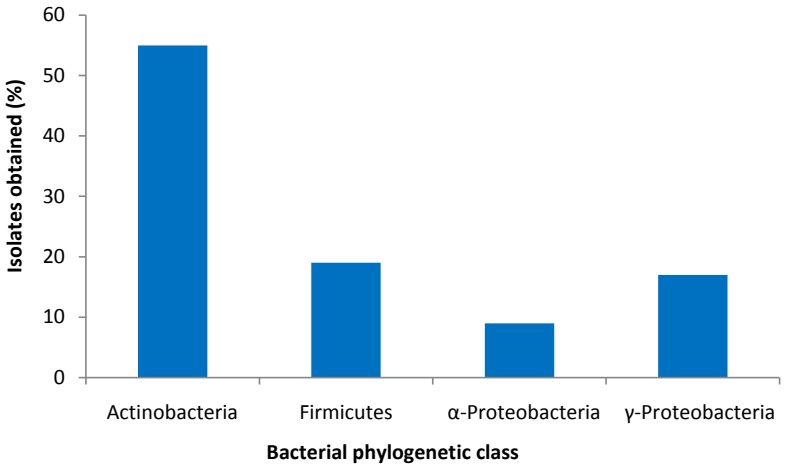

Figure 2. Frequency distribution of endophytic bacterial isolates from shoot-tip tissue of banana based phylogenetic groups.

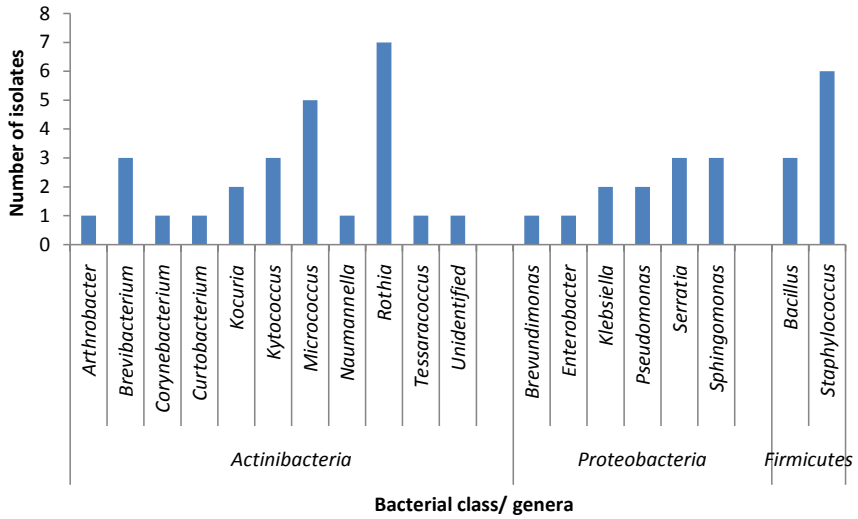

Figure 3. Diversity of endophytic bacteria isolated from shoot-tip tissue of banana cv. Grand Naine.

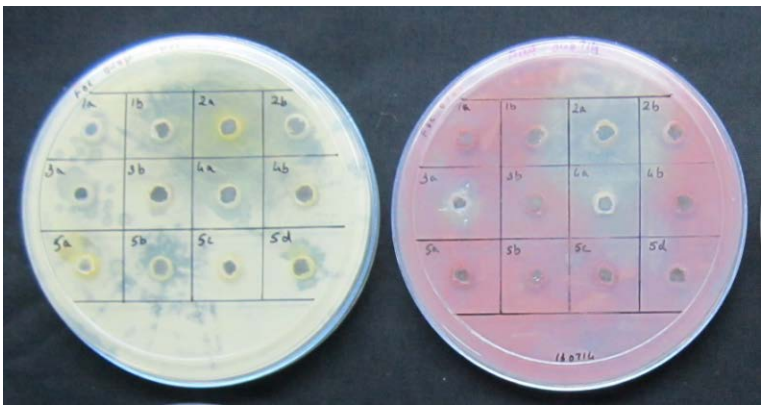

Figure 4. Screening of banana bacterial endophytes for antagonistic activity against *Fusarium oxysporum* f. sp. *cubense* on NA (left*)* and PDA (right) through pit-inoculation approach: 2a, 3a and 4a showing inhibition zone correspond to *Pseudomonas aeruginosa*, *Klebsiella variicola* and *Enterobacter cloacae*, respectively.

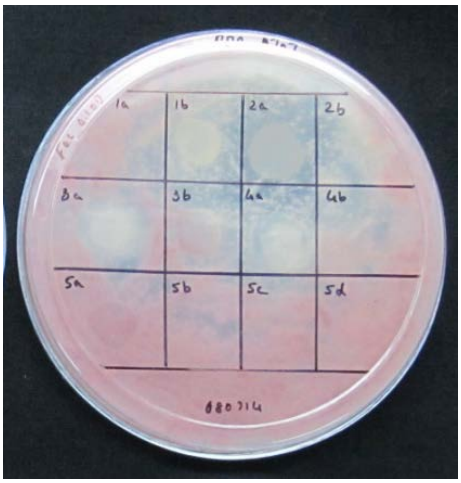

Figure 5. Screening of banana bacterial endophytes for antagonistic activity against *Fusarium oxysporum* f. sp. *cubense* on PDA employing spot-inoculation method: 2a, 3a and 4a showing inhibition zone correspond to *Pseudomonas aeruginosa*, *Klebsiella variicola* and *Enterobacter cloacae*, respectively.

Figure 6. Confrontation assay between *Fusarium oxysporum* f. sp. *cubense* and the endophytic isolates GNS13.1a, 1b, 2a and 2b on NA + PDA 1:1 medium with GNS13.2a (*Pseudomonas aeruginosa*) displaying antagonistic activity against the pathogen.

4. Discussion

The present study was taken up with the objectives of isolating the bacterial endophytes associated with field-derived banana suckers and evaluating them for the antagonistic activity against the Panama wilt pathogen, *F. oxysporum* f. sp. *cubense*. In continuation of the previous studies on banana which revealed the ubiquitous association of bacterial endophytes in the shoot-tips of cv. Grand Naine [7]-[9] and the intracellular colonization by the endophytic bacteria [10] [11], the current study confirms the association of diverse bacterial endophytes in the healthy field derived shoot-tips in banana suckers. The majority of bacterial isolates identified in this study belonged to Actinobacteria class which included organisms under 9 genera (*Arthrobacter, Brevibacterium*, *Corynebacterium*, *Curtobacterium*, *Kocuria, Kytococcus*, *Micrococcus*, *Naumanella*, *Rothia*, *Tessaracoccus* spp.) besides an unidentified bacterium. This is at variance from the earlier reports which indicated Proteobacteria as the predominant endophytes [8] [9]. In this study, Proteobacteria formed the second major class with *Pseudomonas*, *Klebsiella* and *Enterobacter* spp. from γ-subclass and *Sphingomonas*, *Brevundimonas* and *Serratia* spp. from *α*-subclass. *Bacillus* and *Staphylococcus* spp. constituted the Firmicutes. Considerable variation from sucker to sucker in the CFU per unit tissue weight and the associated species has been observed. In an earlier study, the season of sucker-collection appeared to play a major role with more CFU and cultivable endophytes isolated during the rainy spell than summer months [7]. In the present study, no correlation was observed between the extent of colonization as indicated by CFU g^{-1} tissue and the number of isolates retrieved per sucker.

Diverse endophytic bacterial species have been isolated from banana in the previous studies which include *Pantoea agglomerans*, *Citrobacter* sp., *Klebsiella pneumoniae* [24], *Cellulomonas fermentans*, *Acinetobacter lwoffii*, *Micrococcus luteus*, *Bacillus cereus*, *Paracoccus* sp., *Kocuria kristinae*, *Pseudacidovorax* sp. [8], *K. variicola*, *Enterobacter cloacae*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *S. arlettae*, *Brevundimonas* sp., *Alcaligenes faecalis*, *Ralstonia mannitolilytica*, *Corynebacterium amycolatum*, *Pseudomonas stutzeri*, *Microbacterium testaceum*, *Methylobacterium hispanicum*, *Brachybacterium* sp. and *Brevibacterium* sp. [9]. A series of root associated bacterial endophytes were isolated from cv. Prata Anã in Brazil which belonged to 10 genera (*Agrobacterium*, *Aneurinibacillus*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Lysinibacillus*, *Micrococcus*, *Paenibacillus*, *Rhizobium* and *Sporolactobacillus*) constituting 15 species [25].

Endophytes, perhaps share a mutualistic association with the host [6] [26]. They draw the benefits of residency and nutritional support from the host. The host plant in turn is benefitted in many ways such as enhanced nutrient absorption leading to the improved vegetative growth and vigour of the host plant [3] [27]. Conferred resistance to plant pathogens is another beneficial effect which is accomplished through competitive root colonization, synthesis of bacterial allelochemicals and through induced systemic resistance [2] [4] [28].

Antagonism assays against fungi usually employ PDA or cornmeal agar on which the fungal culture and one or two test organisms are grown in a plate [29] [30]. This allows the testing of only one or two organisms per plate whereas spot-inoculation and pit-inoculation methods presented in this study permitted the testing of up to 12 organisms per plate. In this study, testing the 47 endophytic isolates for antagonistic activity against *Foc*

showed three organisms displaying an inhibition effect towards the pathogen of which one organism (*P. aeruginosa*) exhibited good inhibition zone in confrontation assay too.

F. oxysporum f. sp. *cubense* is a devastating soil-borne pathogen which is very difficult to control. Biocontrol agents active against *Foc* such as *P. fluorescens* have been reported in the earlier studies [18] [31]. *P. aeruginosa* isolated from banana rhizosphere showed effective antagonistic activity towards *Foc* and also displayed remarkable growth-promoting characteristics [32]. The identification of *P. aeruginosa* as an antagonist in this study opens the scope for engaging an endophytic organism as a potential biocontrol agent against *Foc*. Endophytes are able to colonize the plants internally and they share an intimate association with the host. The commonality of vascular niche colonization by the pathogen and endophytes offer great potential for the utilization of such antagonistic endophytes as biocontrol agents. Further efforts are now warranted towards testing this isolate as potential biocontrol agent against *Foc*.

Pseudomonas aeruginosa has been isolated as an endophyte from several other plant species and demonstrated as a biocontrol/growth promoting agent in different crop plants [33] [34]. *P. aeruginosa* is also known to be a human pathogen [34] [35]. However it is possible that the isolates associated with animal and plant systems are perhaps different. This aspect needs further investigations. The low proportion of endophytes with antagonistic activity against *Foc* suggests the limited availability of such choice organisms. In the present study, *P. aeruginosa* was associated with only one of the 10 suckers explored. It would now be possible to introduce this organism in other suckers/plants through fortification at the time of field planting or at the hardening phase in the case of micropropagated plants. Banana being a long-duration crop of 10 - 12 months, it would need more time and efforts to test the organisms as potential biocontrol agents and to evolve a biocontrol strategy which is in our next plan of action.

5. Conclusion

The present study has facilitated the isolation and identification of endophytic bacteria associated with the shoot-tip region in banana cv. Grand Naine yielding diverse organisms belonging to Actinobacteria, Proteobacteria and Firmicutes with Actinobacteria forming the commonest phylum. Testing the isolates for potential antagonistic activity against the banana wilt pathogen *F. oxysporum* f. sp. *cubense* brings out *Pseudomonas aeruginosa* as a promising antagonistic agent. Further studies are warranted to test the efficacy of this organism in biocontrol and the feasibility of introducing it in susceptible cultivars through inoculation of micropropagated plants or at field planting of suckers.

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