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Endophytic Bacterial and Fungi Associated to Banana Leaves (*Musa* spp.) Cultivated Under Organic Management

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Abstract

Banana as a domesticated plant has a long evolutionary history of cultivation and has become one of the most important fruit in a world widely market, devoted to its high nutritional characteristics. However, its biology and association with microbes are poorly understood. Then the objective of the present study was isolate the cultivable endophytic bacterial and fungal community associated to leaves of an organic banana plantation in the Brazilian Amazon state of Roraima. A total of 24 fungi and 27 bacteria isolates were selected. The taxonomical classification showed that the cultivable endophytic fungi community is affiliated to the following 11 genera: *Aspergillus*, *Peniophora*, *Meyerozyma*, *Saccharicola*, *Hypocreales*, *Nigrospora*, *Byssochlamys*, *Periconia*, *Myrothecium*, *Acrocalymma* and *Peroneutypa*. Regarding the bacterial isolates 13 genera were found: *Serratia*, *Pantoea*, *Streptococcus*, *Neisseria*, *Bacillus*, *Arsenicicoccus*, *Sphingobacterium*, *Herbaspirillum*, *Lactococcus*, *Variovorax*, *Pseudorhodoferax*, *Stenotrophomonas* and *Brevibacterium*. Comparing the endophytic microorganisms isolated in the present research with previous studies already published, some new genera and species were detected. This could be due to use of organic cultivated bananas without the utilization of fertilizers and other chemical products. This could provide the isolation of bacteria and fungi which are benefic to their hosts but not yet previous found in banana.

Keywords: agriculture, Musaceae, organic plantation, Brazilian Amazon

1. Introduction

Banana is one of the main fruits cultivated in the world, comprising a fruit largely exported and consumed in many tropical countries. Brazil is characterized as a gross producer being the fourth country in a scale of area planted (FAO, 2015). The expansion of banana cultivation mainly due to the high nutritional characteristics related to vitamins and minerals, and its production are mostly related to a great number of producers, however, in many cases is the most important source of income from small to big producers. Opposed to this high production, the development of banana in field has undergone several difficulties caused by attack from insect pests, microbial diseases that drive the lack of plants of good quality (Pereira et al., 1999; Su et al., 2017; Souza et al., 2013; Zakaria & Rahman, 2011; Kavino & Manoranitham, 2018).

The first to describe no pathogenic fungi living inside plants was De Bary (1866) which named them as endophytes. After that, bacteria and other microorganisms were also found practically in all host plants studied. From about 40 years ago the endophytic microbiota started to be considered as beneficial to their plant hosts protecting them against insect pests and also diseases caused by pathogenic bacteria, fungi and nematodes (Kavino & Manoranjithan, 2018; Su et al., 2017; Souza et al., 2014; Thangalevu & Gopi, 2015).

Other beneficial properties were also added as endophytes producing growth hormones, able to fix atmospheric nitrogen and causing phosphate solubilization (Karthik et al., 2017; Souza et al., 2013; Souza et al., 2017; Muthuri et al., 2012; Ting et al., 2008; Andrade et al., 2014; Benzon et al., 2014). In Brazil, the Roraima state located in the Amazon North region of the country is expanding the production of banana. The average

production in this region is lower than that found in other parts of the country. The low productivity demands also use of agrochemicals which increase production costs. One alternative is the use of banana endophytes which could act as biological controllers and increase growth production as a sustainable and environmental safe practice to improve banana growth. As far as we know, related papers isolating endophytes from banana all over the world, utilize cultures subject to the use of fertilizers, insecticides, fungicides, nematicides and other antimicrobials in general.

In the present research we isolated endophytic, both bacteria and fungi, from organic cultures of banana. This probably provide ways to avoid the reduction in number and genera of isolated microorganisms increasing the probability to found beneficial ones which could increase productivity with reduction of economic costs and helping the ecological environmental conditions.

2. Material and Methods

2.1 Plant Material and Sampling Procedure

Banana leaves were collected in November 2017 from the São Pedro farm located in state of Roraima, city of Boa Vista, Amazonas region, Brazil 3°20′42.3″ N 60°34′39.9″ W. The rainfall during the month of collection was 00 mm, average temperature was 33.5 °C and the relative medium humidity was 44.0%.

2.2 Fungi Assessment and Culturing Conditions

The procedure described by Araujo et al. (2014), with modifications adapted to banana was used. Surface of 20 leaves, about 85 cm length and 20 cm wide with veins, were fragmented in smaller pieces and sterilized by immersion in 70% ethanol for 1 minute, sodium hypochlorite 3% for 4 minutes, and finally ethanol 70% for 30 sec. After surface sterilization the fragments were rinsed two times in autoclaved distilled water. The effectiveness of the method was verified by plating 0.1 mm of the final rinse in Petri dishes containing potato dextrose agar medium (BDA) supplemented with tetracycline to prevent bacterial growth. Only considered surface sterilized fragments were used for next fungal endophytic isolation. Smaller fragments of about 0.5 cm were then prepared from the previous fragments and placed on dish plates containing BDA medium as mentioned before. Five fragments were inoculated to each dish in a total of 250 fragments. The plates were incubated from 7 to 12 days at 28 °C. The colonization frequency was determined by the ratio between the number of fragments colonized by fungi and the total number of fragments used. Purification of isolates was performed using morphological characterization of distinct forms and their frequency. Single purified colonies were used for molecular identification. Both this procedure 2.2 and item 2.3 had as main objective to eliminate the microorganisms that live on the surface of the banana leaves, because the focus of this study and the line of research of this work were the endophytic microorganisms, which live inside the leaves that protect them against pathogenic invaders in general.

2.3 Bacterial Assessment and Culturing Conditions

The bacterial isolation was performed according to Araujo et al. (2014). The total of 5 g of leaves were macerated and placed in flasks containing 90 mL of phosphate buffer solution (PBS) containing NaCl, 9.0 g; KCl, 0.2 g; Na_2HPO_4, 1.44 g and KH_2PO_4, 0.24 g distilled water 1000 mL, pH 7.4). Samples were incubated under agitation (150 rpm) for 30 minutes at 28 °C. Aliquots of 0.1 mL of three-fold serial dilutions were inoculated in duplicate onto petri dishes plates with 20 mL 10% triptone soy agar medium (TSA) including 50 $\mu g.mL^{-1}$ benomil to avoid fungal growth. The plates were incubated at 28 °C and colonies were counted starting from 48 hours incubation. Colonies were selected considering their color and shape, and purified being preserved in 15% glycerol at -80 °C to be used for molecular identification analysis.

2.4 DNA Isolation

The bacterial strains were cultured in 5 mL TSA 10% liquid for 24 h at 28 °C under 150 rpm stirring. 5 mL of the culture were centrifuged for 5 min at 14,000 g and the resuspended cells in 500 µL TE (10 mM Tris-HCl pH 8.0) were centrifuged and resuspended again in 500 µL TE with the addition of 0.5 g of glass beads (0.1 mm in diameter-Sigma) and 15 µL of 20% SDS. Cells were shaken in a homogenizer (Mine-Beadbeater™, Biospec Products) for 30 sec at 3500 bpm. To the cell lysate were added 500 µL of phenol, homogenized by inversion and centrifuged for 5 min at 14,000 g. The aqueous phase was extracted once with phenol-chloroform (1:1) and once with chloroform, then the DNA was precipitated with 1/10 volume of 5 M NaCl and 0.6 volume of isopropanol (3 minutes at room temperature) and collected by centrifugation (10 minutes at 14,000 g). The DNA precipitate was washed with 70% ethanol, dried at 37 °C and resuspended in 50 µL of sterilized milli-Q water. Total DNA was analyzed by agarose gel electrophoresis (0.8% w/v) in 1x TrisAcetateEDTA buffer (TAE) buffer (40 mM de

Tris-acetate; 1 mM EDTA) stained with ethidium bromide (EB) (0.5 μg mL^{-1}), according to Kuklinsky et al. (2004).

The fungi strain were cultured onto fifth mL of the liquid medium BDA (200 g potato broth and 20 g dextrose in 1 L water, [pH 6.0]) for 5 to 7 days at 28 °C. After this period of culture growth and multiplication, the whole content was centrifuged at 20,000 g for 10 minutes to remove the excess culture medium. The precipitated was subjected to a filtration process for water elimination. Subsequently, approximately 100 mg was triturated in liquid nitrogen. The isolate was submitted to a DNA extraction using the Genomic Wizard complete DNA purification kit (Promega Corporation, Wisconsin, USA), following the manufacturer's instructions. The DNA extracted, as well as its quantification was determined by 1.2% agarose gel electrophoresis medium (w/v), the gel stained with an EB solution and visualized in ultraviolet light (DNr Bio-Imaging Systems Minibis pro 16 mm).

2.5 Amplification and Sequencing of the 16S rRNA Gene and ITS Region of the Endophytic Strains

A sample of 27 bacteria and 24 fungal strains were selected for a partial sequencing of the 16S rRNA gene and the ITS region, respectively. The amplification of the bacterial fragments was performed in a 25 μL final volume containing 1 μL (0.5-10.0 ng) of total DNA, 0.2 mM of P27F primer (5'-GAGAGTTTGATCCTGGCTCAG-3'), 0.2 mM of 1492R primer (5'-TACGGYTACCTTGTTACGACT-3') (Welsburg et al., 1991), 0.2 mM of each dNTP, 0.02 mg mL^{-1} BSA, 3.75 mM $MgCl_2$ and 0.05 U of Taq DNA polymerase (Fermentas). The reaction was subjected to a temperature-controlled thermal cycler performing an initial denaturation at 94 °C for 4 minutes, 35 additional cycles of denaturation at 94 °C for 30 sec each, annealing at 63 °C for 1 minute and primer extension at 72 °C for 1 minute, followed by a final extension at 72 °C for 10 minutes. After amplification, the PCR products were visualized by agarose gel electrophoresis (1.5% w/v) in 1x TAE buffer.

The ITS-DNA hypervariable region of the strain was amplified using primers ITS-1(5'-TCCGTAGGTGAAC CTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') (WHITE et al., 1990). In a final volume of 50 μL containing 1x Buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4); (3.7 mM $MgCl_2$, 1 mM dNTP, 0.05 U μL^{-1} Taq-Invitrogen DNA polymerase), 0.2 μM ITS-1 primer, 0.2 μM ITS-4 primer and, approximately 5 ng of DNA. The reactions were performed in a thermocycler (Veriti® Thermocycler, Applied Biosystems, Waltham, USA), programmed to an initial denaturation step of 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec and a final extension at 72 °C for 7 minutes.

The amplified fragment (approximately 600 pb), was visualized onto 1.2% agarose gel electrophoresis. The gel was stained in ethidium bromide solution and photodocumented under UV light. Subsequently, the amplified fragments were purified with the PCR kit GFX (Amersham Pharmacia Biotech) and Sanger sequenced.

The PCR products were purified using a Super Charger Switch Kit and Sanger sequenced using the 1387R primer (Heuer et al., 1997) and primer ITS4 (White et al., 1990). Analyses of sequences were performed with the basic sequence alignment BLAST program, which was run against the database on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST).

3. Results

3.1 Amplification and Sequencing of Fungal Endophytes

After purification, twenty-four colonies were selected based on morphology and microscopic examination and further sequenced. The obtained results are presented in Table 1. Only similarities of 96 to 100% were included as identified isolates. The isolated and identified fungi belonged to the following 11 genera: *Aspergillus*, *Peniophora*, *Meyerozyma*, *Saccharicola*, *Hypocreales*, *Nigrospora*, *Byssochlamys*, *Periconia*, *Myrothecium*, *Acrocalymma* and *Peroneutypa*. The species *Aspergillus versicolor* was the most frequently found.

Table 1. Genetic characteristics of the endophytic fungi strains isolated from banana leaves and sequenced of the ITS region

Strain Code	ITS region		
	NCBI Best BLAST Hits	**Identity (%)**	**Ac. Number NCBI**
FFB01	*Aspergillus versicolor*	97%	MH453585
FFB02	*Aspergillus versicolor*	99%	MH453586
FFB03	*Aspergillus versicolor*	98%	MH453587
FFB04	*Aspergillus versicolor*	99%	MH453588
FFB05	*Aspergillus versicolor*	99%	MH453589
FFB06	*Aspergillus versicolor*	99%	MH453590
FFB07	*Aspergillus* sp.	99%	MH453591
FFB08	*Peniophora crassitunicata*	98%	MH453592
FFB09	*Peniophora crassitunicata*	97%	MH453593
FFB10	*Meyerozyma guilliermondii*	99%	MH453594
FFB11	*Aspergillus versicolor*	99%	MH453595
FFB12	*Peniophora crassitunicata*	99%	MH453596
FFB13	*Peniophora* sp.	99%	MH453597
FFB14	*Saccharicola* sp	99%	MH453598
FFB15	*Hypocreales* sp.	100%	MH453599
FFB16	*Peniophora crassitunicata*	100%	MH453600
FFB17	*Nigrospora zimmermanii*	99%	MH453601
FFB18	*Byssochlamys spectabilis*	99%	MH453602
FFB19	*Periconia* sp.	98%	MH453603
FFB20	*Myrothecium* sp.	98%	MH453604
FFB21	*Acrocalymma vagum*	98%	MH453605
FFB22	*Byssochlamys spectabilis*	96%	MH453606
FFB23	*Peniophora* sp.	99%	MH453607
FFB24	*Peroneutypa scoparia*	99%	MH453608

3.2 Amplification and Sequencing of Bacterial Endophytes

After plating diluted aliquots from endophytic bacteria obtained from macerated leaves of banana trees a total of 9.4×10^3 colonies.g^{-1} of fresh weight leaves tissues were obtained. From all plates based on morphological colony features bacteria representing about 2% of total bacteria were chosen, based mainly on shape, margin and colony color. Chosen colonies were further purified and stocked cultures were maintained at -80 °C in glycerol. Isolates were taken for DNA isolation and sequencing. The results are shown in Table 2. Only similarities of 97 to 99% were included as identified isolates. From 27 identified bacteria, 13 following genera were encountered: *Serratia*, *Pantoea*, *Streptococcus*, *Neisseria*, *Bacillus*, *Arsenicicoccus*, *Sphingobacterium*, *Herbaspirillum*, *Lactococcus*, *Variovorax*, *Pseudorhodoferax*, *Stenotrophomonas*, *Brevibacterium* the most frequently genera found were *Herbaspirillum*, *Serratia* and *Bacillus*.

Table 2. Genetic characteristics of the endophytic bacteria strains isolated from banana leaves and sequenced of the 16S rRNA gene

Strain Code	16S rRNA gene		
	NCBI Best BLAST Hits	Identity (%)	Ac. Number NCBI
BFB01	*Serratia marcescens*	98%	MH447310
BFB02	*Pantoea cypripedii*	99%	MH447302
BFB03	*Serratia marcescens*	99%	MH447309
BFB04	*Serratia marcescens*	98%	MH447308
BFB05	*Streptococcus* sp.	98%	MH447328
BFB06	*Neisseria* sp.	99%	MH447327
BFB07	*Bacillus* sp.	98%	MH447326
BFB08	*Arsenicicoccus bolidensis*	99%	MH447325
BFB09	*Bacillus circulans*	99%	MH447324
BFB10	*Arsenicicoccus*_sp.	97%	MH447307
BFB11	*Sphingobacterium multivorum*	99%	MH447323
BFB13	*Bacillus cereus*	99%	MH447322
BFB14	*Bacillus circulans*	99%	MH447321
BFB15	*Herbaspirillum* sp.	99%	MH447320
BFB16	*Lactococcus lactis* subsp. *lactis*	99%	MH447319
BFB17	*Herbaspirillum* sp.	99%	MH447318
BFB18	*Neisseria* sp.	99%	MH447317
BFB19	*Herbaspirillum* sp.	99%	MH447316
BFB20	*Pseudorhodoferax_aquiterrae*	98%	MH447306
BFB21	*Variovorax* sp.	97%	MH447305
BFB22	*Pseudorhodoferax aquiterrae*	99%	MH447315
BFB23	*Herbaspirillum* sp.	98%	MH447304
BFB24	*Herbaspirillum* sp.	99%	MH447314
BFB25	*Herbaspirillum* sp.	99%	MH447313
BFB26	*Herbaspirillum* sp.	99%	MH447303
BFB27	*Stenotrophomonas maltophilia*	99%	MH447312
BFB28	*Brevibacterium permense*	99%	MH447311

4. Discussion

Endophytic microbiota is found practically in all plant hosts studied (Azevedo & Araujo, 2007). Many endophytic bacteria and fungi are nowadays considered beneficial to their hosts. Several papers are already published showing endophytic microorganisms from bananas (Tables 3 and 4). Isolated endophytic fungi and bacteria from banana produced increase growth promotion (Andrade et al., 2014; Kartick et al., 2017; Muthuri et al., 2012; Souza et al., 2017; Thomas & Soly, 2009), protect plants against prejudicial bacterial, fungal, nematodes and insect pests (Su et al., 2017; Souza et al., 2014; Thangalevu & Gopi, 2015; Zakaria & Rahman, 2011). Most of the studies related to isolation of endophytes from plant hosts are performed in cultures submitted to use of fertilizers and chemical products aiming increasing production and reduction of diseases and pests. As far as we know, the present research may be the first one to isolate endophytic fungi and bacteria from organic cultivated bananas without the use of fertilizers and other chemical products. This could provide the isolation of microorganisms which are benefic to their hosts but not yet isolated due to inhibition by chemical fungicides and other products.

Table 3. Studies describing the main diversity of fungal genus strains isolated from banana leaves (*Musa* spp.)

Most common fungi genus isolated from Banana leaves	Management	Country	References
Alternaria, *Aspergillus*, *Cordana*, *Curvularia*, *Dreshlera*, *Epicoccum*, *Fusarium*, *Glomerella*, *Humicola*, *Nigrospora*, *Periconia*, *Phomopsis*, *Phylosticta*, *Trichoderma*, *Xylaria*	Convetional	Brazil[a]	Pereira et al. (1999)
Xylaria, *Guignardia*, *Colletotrichum*, *Deightoniella*, *Pyriculariopsis*, *Dactylaria*	Convetional	Thailand[a]	Photita et al. (2001)
Gloeosporium, *Myxosporium*,*Deightoniella*, *Alternaria*, *Sphaceloma*, *Aureobasidium*, *Melida*, *Uncinula*, *Penicillium*, *Aspergillus*, *Sarcinella*, *Cladosporium*, *Cephalosporium*, *Paecilomyces*, *Fusarium*, *Spicaria*, *Meliola*	Convetional	China[a]	Cao et al. (2002)
Fusarium	Convetional	Malaysi[b]	Ting et al. (2008)
Trichoderma	Convetional	India[b]	Thangavelu & Gopi (2015)
Fusarium	Convetional	Malaysi[b]	Zakaria & Rahman (2011)

Note. [a]Fungal Diversity; [b]Fusarium control.

Table 4. Studies describing the main diversity of bacteria genus strains isolated from banana leaves (*Musa* spp.)

Most common fungi genus isolated from Banana leaves	Management	Country	References
Enterobacter, *Klebsiella*, *Rhizobium*	Conventional	Mexico	Martinez et al. (2003)
Serratia	Wild	Malaysia	Ting et al. (2008)
Agrobacterium, *Klebsiella*, *Pseudacidovorax*	Conventional	India	Thomas & Soly (2009)
Pseudomonas, *Bacillus*, *Streptomyces*	Conventional	China	Su et al. (2017)
Pantoea, *Pseudomonas*, *Serratia*, *Klebsiella*, *Rhizobium*, *Microbacterium*, *rhodococcus*, *Bacillus*	Conventional	India	Karthik et al. (2017)
Agrobacterium, *Rhizobium*, *Aneurinebacillus*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Lysinibacillus*, *Micrococcus*, *Paenibacillus*, *Sporolacto bacillus*	Conventional	Brazil	Souza et al. (2013)
Bacillus	Conventional	Brazil	Souza et al. (2017)
Arthrobacter, *Brevibacterium*, *Corynebacterium*, *Curtobacterium*, *Kocuria*,*Kytococcus*, *Micrococcus*, *Naumanella*, *Rothia*, *Brevundimonas*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, *Sphingomonas*, *Bacillus*, *Staphylococcus*	Conventional	India	Sekhar & Thomas (2015)
Serratia, *Pseudomonas*, *Rahnella*, *Enterobacter*, *Klebsiella*, *Yersinia. Evingella*	Conventional	Kenya	Muthari et al. (2012)

In the present research several microorganisms were not yet described as banana endophytes by other authors. Of course, this could also be the result from the use of distinct banana cultivars, different climates and regions and distinct use of plant organs and tissues as leaves and, root tips among others. However, the distinct differences encountered as being due to the lack of chemical products and processes inhibiting or reducing the microbial diversity could not be discarded. Among the isolated endophytic fungi and bacteria from our results using the organic bananas, several genera and species were recorded for the first time both in fungi (Tables 1 and 3) and in bacteria (Tables 2 and 4). Of course, future studies must be conducted with these isolated microorganisms in an attempt to detected plant growth promotion, reduction of diseases and pests and other beneficial uses as production of antimicrobials substances, enzymes and compounds of biotechnological uses. However, among the newly isolated genera and species from the organic bananas used in the present research, some are related to beneficial and biotechnological uses as for instance the bacterium genus *Herbaspirillum*, the most frequently detected in our research, known as including species capable of nitrogen fixation and promoting growth of plant hosts (Ebeltagy et al., 2011).

Other bacteria genera also frequently found in our study as *Serratia* and *Bacillus* which were already isolated from banana and other plant hosts and proved to be capable of plant growth promotion (Table 4). Other bacteria genera isolated as *Pantoea* and *Variovorax* are also important for biotechnological purposes as plant growth promoters, enzymes production and degradation of pollutants (Quecine et al., 2012; Satola et al., 2013). Among the isolated fungi from organic bananas the most frequently one and for the first time recorded in bananas was *Aspergillus versicolor* which is known to produce antibacterial, fungicidal and insecticidal properties besides enzymes production (Domsch, 2001).

Other fungal isolates as the genus *Hypocreales* are also cited as possessing biological controller properties. Other found genera as *Periconia* are related as pathogenic to several plant hosts. These, isolated as endophytic by us may be no pathogenic forms and could be used as controllers of pathogenic forms as related in endophytic

Fusarium able to control *Fusarium oxysporum* pathogenic forms in banana (Table 3). Future studies may be conducted to test the properties of the fungi and bacteria isolated in the present study as growth hormones and production, phosphate solubilization, ability to fix nitrogen, as well detection of possible prejudicial properties which could impair their use in growth promotion and biological control among others.

5. Conclusion

Isolation of endophytic fungi and bacteria from banana plant plantation using organic practices, resulted in the presence of endophytic genera and species not yet reported from this host as compared to previous studies using conventional practices. The newly isolated endophytes could be tested for biotechnological approaches aiming increase in plant growth promotion, protection against pests and diseases. If successful, these endophytes would help increase production reducing the use of fertilizers and other chemical compounds with cost reduction and environmental ecological benefits.

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