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Title: Etiological agents of crown rot disease affecting organic bananas in Dominican Republic

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Keywords: Colletotrichum musae; Fusarium spp.; Lasiodiplodia theobromae; Cavendish AAA; Postharvest disease.

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Abstract: Crown rot is a postharvest disease with a great negative impact on banana fruits quality. The infections occur at harvest, but the symptoms appear after overseas transportation of fruits. Different fungal pathogens are involved in crown rot, varying according to farming area. To the best of our knowledge this is the first study on organic bananas as it's the first covering Dominican Republic which is one of the leading exporters of organic bananas. Five organic farms and their corresponding packing stations located in Valverde province were investigated. Over a period of three years, 558 banana hands were collected and a total of 5000 fungal colonies were obtained from the crown tissues and 518 representative colonies were purified, characterized and identified using morphological and molecular methods. Fungi were found in all the analyzed samples from field to packing house and belonged to 11 genera. The fungal community was dominated by Fusarium, the most frequent genus (55%) found in more than 80% of all analyzed samples. It was represented by nine species; F. incarnatum 53%, F. verticillioides 12%, F. sacchari 12%, F. proliferatum 7%, and F. solani 6%. Strains belonging to eight less frequent genera were represented by Colletotrichum musae 7% and found in 13% of all samples; Lasiodiplodia theobromae 4% and L. pseudotheobromae 1%, both found in 7% of all samples; Nigrospora sp. 11%, Alternaria spp. 6%, Phoma spp. 2%, Pestalotiopsis sp. 2%, Curvularia spp. 1% and Microdochium sp. 1%, and some other known as saprophyte.

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***Cover Letter**

Date: 04/02/2016

To: Editorial Team of postharvest biology and technology Journal.

Dear Sir

We are submitting to your journal our manuscript entitled "**Etiological agents of crown rot disease affecting organic bananas in Dominican Republic**". As the first study conducted to cover Dominican Republic region and organic farming, we focused on isolation and identification of etiological agents associated with crown rot disease affecting organic production of bananas in Dominican Republic area, which is famous for the cultivation of tropical organic fruits. We finally compared our results with others obtained in different regions. This is our original research manuscript that has not been previously published and is not under consideration in the same or substantially similar form in any other journal. It is our pleasure to inform you that all those listed as authors are qualified for authorship and they were contributed significantly into this work. Finally, all authors have agreed and approved the current version of the manuscript.

Highlights

- It's the first study on crown rot disease of organic bananas in Dominican Republic.
- *Fusarium* was the most frequent genus among all isolated fungi.
- *F. incarnatum, C. musae, F. sacchari, F. verticillioides,*and *L. theobromae* are pathogens.
- Fungi were found in all the analyzed samples from field to packing house.

Etiological agents of crown rot disease affecting organic bananas in Dominican Republic.

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Abstract

Crown rot is a postharvest disease with a great negative impact on banana fruits quality. The infections occur at harvest, but the symptoms appear after overseas transportation of fruits. Different fungal pathogens are involved in crown rot, varying according to farming area. To the best of our knowledge this is the first study on organic bananas as it's the first covering Dominican Republic which is one of the leading exporters of organic bananas. Five organic farms and their corresponding packing stations located in Valverde province were investigated. Over a period of three years, 558 banana hands were collected and a total of 5000 fungal colonies were obtained from the crown tissues and 518 representative colonies were purified, characterized and identified using morphological and molecular methods. Fungi were found in all the analyzed samples from field to packing house and belonged to 11 genera. The fungal community was dominated by *Fusarium*, the most frequent genus (55%) found in more than 80% of all analyzed samples. It was represented by nine species; *F. incarnatum* 53%, *F. verticillioides* 12%, *F. sacchari* 12%, *F. proliferatum* 7%, and *F. solani* 6%. Strains belonging to eight less frequent genera were represented by *Colletotrichum musae* 7% and found in 13% of all samples; *Lasiodiplodia theobromae* 4% and *L. pseudotheobromae* 1%, both found in 7% of all samples; *Nigrospora* sp. 11%, *Alternaria* spp. 6%, *Phoma* spp. 2%, *Pestalotiopsis* sp. 2%, *Curvularia* spp. 1% and *Microdochium* sp. 1%, and some other known as saprophyte.

Keywords: *Colletotrichum musae*, *Fusarium* spp., *Lasiodiplodia theobromae*, Cavendish AAA, Postharvest disease.

1. Introduction

Banana is one of the most important world tropical crop cultivated in more than 100 countries (Lassois et al., 2010a), with a total production in 2012 of 133.3 million tons (FAOSTAT, 2015). Dominican Republic is the biggest exporter of organic banana to Europe (FAOSTAT, 2015). Bananas are affected by several diseases, and crown rot is considered one of the most important postharvest disease, causing a great negative impact on fruit quality (Umana-Rojas and Garcia, 2011a). Fungi are the causal agents of crown rot, and genera and species varied according to farming area (Alvindia and Natsuaki, 2007; Anthony et al., 2004; Eckert and Ogawa, 1985; Goos and Tschirsch, 1962; Greene and Goos, 1963; Griffee, 1976; Joas and Malisart, 2001; Khan et al., 2001; Knight, 1982; Krauss and Johanson, 2000; Lassois et al., 2008; Meredith, 1962; Mulvena et al., 1969; Reyes et al., 1998; Thompson, 2010). The disease has seasonal variation determined by many pre-harvest factors (Ewane et al., 2013). Infections start at harvesting and continued during packaging processes (deBellaire and Mourichon, 1997) and flowers are the main inoculum source for *Fusarium* and for *Colletotrichum*. Furthermore, fungal inoculum present on banana stalks is knife transferred onto the cut crown surface at dehanding (Finlay et al., 1992) or when clusters are cleaned in contaminated water (Shillingford, 1977). Symptoms appeared after overseas transportation as blackening and mold on the surface of the crown area and fruits resulted unmarketable.

To our knowledge there are no data available for crown rot etiological agents in organic farmed banana, and no data for Dominican Republic, therefore our research is the first that focuses on identification of fungi associated to organic bananas in this country, at various processing stages, from field to packing house, and assessment of their pathogenicity.

2. Materials and methods

Five different banana (*Musa* AAA, Cavendish) plantations, covering approximately 750 hectares, and their corresponding packing stations located in Valverde province in Dominican Republic were investigated.

2.1. Sampling

Symptomatic banana fruits were delivered to the laboratory of Plant Pathology, Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Italy, between February and March 2013. Additional samples were collected from fields and packing stations as symptomless crowns during different periods over three years (June and October 2013; March, June and September 2014; and March and July 2015). The sampling covered all the different stages of cultivation and post-harvest processing of bananas from field to shipping. Flower and crown parts were randomly sampled at deflowering and at harvest time. Hands of bananas were randomly sampled from each step of processing at the packinghouse: dehanding, delatexing tank, clustering and trimming, second washing tank, post-treatment and fruit packaging. Additional sampling of cut crown debris was done at clustering and trimming step. Each hand was considered a separate sample and 3 samples were collected for each processing-step, and maintained in paper bag.

2.2. Isolation

Fungi were isolated from small part of each crown sample following surface disinfection with sodium hypochlorite 5% for 2 min and subsequent rinsing in sterile water. Five pieces of about 5 mm^2, were taken aseptically by cutting the crown tissue to different depth after removing the outer layer, and placed to dry on sterile filter paper under a sterile air flow and then cut in 5 pieces transferred into Petri dishes, 9 cm in diameter, on the surface of Potato Dextrose Agar, (PDA) (Difco Laboratories, USA) added with Nalidixic acid, Novobiocin, and Streptomycin sulfate, 25 mg/L each, to inhibit bacterial growth. The plates

were incubated for 6 days at 24°C.

2.3. Purification and preservation

The plates were periodically observed using an optical microscope in order to count and identify the fungi developed. Representative colonies were selected and purified on PDA and Malt Extract Agar (malt extract 20 g, agar 15 g, glucose 20 g, peptone 1 g, and water 1000 mL; pH 6.5 ± 0.5) (MEA), and Czapek Solution Agar (Difco Laboratories, USA) amended with 0.5% yeast extract (CYA). Pure cultures were obtained by excising hyphal tips from colony margins emerging from crown tissue onto fresh PDA and incubated at the same conditions as above. Pure cultures were stored at 4°C on PDA slants.

2.4. Identification based on morphological and cultural characters:

Morphology and cultural characters of individual samples were studied on PDA, MEA and CYA. Fungal cultures were incubated at 24°C for 7 days and three cultures of every isolate were investigated. After 7 days, colony size, color of the conidial masses and zonation were recorded (Than et al., 2008). Average increase in diameter was calculated by measuring the average daily growth. Isolates were grouped on the basis of growth rate, mycelium texture and morphology. Mycelium morphology and reproductive structures were observed using an optical microscope Orthoplan (Leitz, Germany) equipped with a digital camera Coolpix 4700 (Nikon, Japan). The sizes of reproductive structures and the shapes were recorded. Representative isolates were identified at genus level using different identification keys (Von Arx, 1974; Barnett and Hunter, 1998; Hanlin, 1998). Isolates were grouped at genus level and representative isolate were submitted to molecular identification.

2.5. Identification based on molecular methods.

Representative isolates were grown on cellophane (Discocell PT60 - CELSA, Italy) placed onto PDA in Petri dishes to facilitate collection of the mycelium. After seven days of incubation at 24°C the mycelium was collected and transferred to Eppendorf tubes, frozen at

-25 °C, lyophilized and ground to a powder as previously described (Rocchi et al., 2010).

The DNA was extracted from lyophilized mycelium following the (CTAB) method (Kelly et al., 1998), and modified by adding an RNase treatment of the aqueous phase before the second addition of cetyltrimethylammonium bromide (CTAB) hot buffer (Rocchi et al., 2010). The concentration of nucleic acid extracts was estimated by Qbit analyser (Invitrogen, USA) and brought to a concentration of approximately 5 mg/ml in sterile water. The primers ITS1 and ITS4 (White et al., 1990) were used to amplify ITS-1 - 5.8S - ITS-2 region of the nuclear rDNA. PCR was performed in 30 µl volume containing 1xGoTaq Buffer (Promega, USA), 0.1 mM of each dNTP, 0.1 µM of each primer, 0.9 units of GoTaq DNA polymerase (Promega, USA), and at least 1 ng of genomic DNA. For the ITS region, PCR reaction was performed in a Gene Cycler (Biorad, USA) using an initial cycle of denaturation at 95°C for 2 min, 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 10 min. The amplification of the region of the β-tubulin was carried out using specific primers that bind in the sequences of the introns (Glass and Donaldson, 1995). For *Fusarium* and *Lasiodiplodia*, primer pairs BT1a, BT1b and BT2a, BT2b were used (Hyde et al., 2014). PCR was performed in 30 µl volume using the same conditions and reactions as for ITS primers. The translation elongation factor 1-α gene was used for PCR amplification of *Fusarium* samples using primer pair TEF1T and TEF2T (Geiser et al., 2004; Hyde et al., 2014; Nitschke et al., 2009; O'Donnell et al., 1998; Sampietro et al., 2010). PCR reactions were performed in a total volume of 25 µl containing 1xGoTaq Buffer (Promega, USA), 10 mM Tris–HCl (pH 9), 0.1% Triton X-100, 0.2 mM of each dNTP, 1mM $MgCl_2$, 0.4 µM of each primer, 0.8 unit of GoTaq polymerase (Promega, USA) and at least 1 ng of genomic DNA. TEF1T (ATG GGT AAG GAG GAC AAG AC) and TEF2T (GGA AGT ACC AGT GAT CAT GTT). The PCR reaction was done in a Gene Cycler (Biorad, USA) using the following program: initial denaturation at 94°C for 5 minutes,

followed by 35 cycles at 94°C for 30 seconds, annealing at 61°C for 45 seconds, and extension at 72°C for 1 minute. Final extension at 72°C for 5 min (Hoffman and Winston, 1987). For *Colletotrichum*, the amplification of intergenic region of apn2 and MAT1-2-1 genes was carried out using specific primers pares AMF (TCA TTC TAC GTA TGT GCC CG) and AMR (CCA GAA ATA CAC CGA ACT TGC) (Hyde et al., 2014; Sharma et al., 2013; Silva et al., 2012). PCR was performed in 30-µl volume containing 1xGoTaq Buffer (Promega, USA), 0.2 mM of each dNTP, 0.8 µM of each primer, 2 units of GoTaq DNA polymerase (Promega, USA), and at least 2 ng of genomic DNA. Reactions were performed in a Gene Cycler (Biorad, USA) using an initial cycle of denaturation of 3 min at 94°C followed by 30 cycles of 45 s at 94°C, 45 s at 62°C and 1 min at 72°C, with a final extension of 7 min at 72°C. The GPDE region in the genus *Curvularia* was amplified using primer pairs GPDEF (TCA TTC TAC GTA TGT GCC CG) and GPDER (CCA GAA ATA CAC CGA ACT TGC) (Berbee et al., 1999; Câmara et al., 2002; Hyde et al., 2014). The PCR was performed in 30-µl volume as for ITS primers described above. Reactions were performed in a Gene Cycler (Biorad, USA) using an initial cycle of denaturation of 2 min at 94°C followed by 35 cycles of 94°C for 30s, 57°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 3 min. The PCR products were electrophoresed in 1.5% agarose gel, and visualized by staining with ethidium bromide in a GEL DOC 2000 (Biorad, USA). After electrophoresis, the PCR products were purified by QIAquick purification kit (Qiagen, USA), and sequenced by Eurofins genomics (Vimodrone, Italy) with the primers used for their amplification. All DNA fragments were sequenced on both strands. The Chromas, version 1.45 software (McCarthy 1998) programme was used to assemble and edit the sequences. Nucleotide collection nr/nt database from BLAST GenBank online database was used to compare the sequences obtained with those deposited for prokaryotes and fungi, and the FUSARIUM-ID v. 1.0 sequence database for identifying some *Fusarium* strains. Sequence homology higher

than 98% was used to assume sequence identity and molecular identification.

2.6. Pathogenicity test

Koch's postulates were carried out for 24 representative strains of eight identified taxa. For each isolate, five-millimeter-diameter agar plug was removed from the edge of 7 day-old mycelium and placed upside-down into a same diameter wound, 3 mm deep, created on the surfaces of symptomless crown of green bananas previously wiped with 5% sodium hypochlorite and rinsed with sterile distilled water. Five single finger of banana (Musa AAA, Cavendish) were used as replicates. Controls were set using sterile agar plugs. After inoculation the bananas were packaged in plastic bags mimicking the packing process, and stored at 13 °C for 10 days.

The disease severity was assessed by measuring the depth of the rot at the inoculation point after cutting the crown longitudinally, using the crown rot index scale (Alvindia et al., 2004). Re-isolation of inoculated fungi was carried out from all infected crowns analyzed. The data were analyzed using One- Way ANOVA model with Minitab program (package 17), and averages were compared using Tukey pairwise comparisons at $P \leq 0.05$.

3. Results

We showed that, out of 558 hands of banana analyzed, a total of 5000 fungal colonies were obtained from crown tissues samples, and 518 representative fungal colonies were purified. Fungi were isolated both from field and packinghouse samples. More than one fungal colony was seldom isolated from one crown fragment. The fungal community was composed mostly by *Fusarium* spp., and other fungi belonging to eight less frequent genera (Table 1).

Table 1: Frequency of isolated fungal taxa associated with crown tissues sampled in Dominican Republic.

Taxa	Frequency %
Fusarium incarnatum	29
Fusarium verticillioides	7
Fusarium sacchari	7
Fusarium proliferatum	4
Fusarium solani	3
Fusarium oxysporum	3
Fusarium pseudocircinatum	2
Fusarium dimerum	1
Fusarium musae	1
Alternaria spp.	6
Nigrospora sp.	11
Colletotrichum musae	7
Lasiodiplodia theobromae	4
Phoma sp.	2
Pestalotiopsis sp.	2

Curvularia spp.	1
Lasiodiplodia pseudotheobromae	1
Microdochium sp.	1
Others	9

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178 *Fusarium* was the most frequent genus, equal to 55% of all isolated fungi, and it was
179 found in more than 80% of all analyzed samples. The 285 *Fusarium* isolates representative of
180 nine species were dominated by *F. incarnatum-equiseti* species complex (53%), followed by
181 *F. verticillioides* (Sacc.) (12%) and *F. sacchari* (E.J. Butler & Hafiz Khan) (12%), then by *F.*
182 *proliferatum* (Matsush.) (7%), and *F. solani* (Mart.) (6%) (Table 2).

183 Table 2: Identification of Fusarium strains based on TEF gene nucleotide sequence.

Code	Fusarium-ID database			NCBI-blast database		
	Identification	**similarity**	**Accession n°**	**Identification**	**similarity**	**Accession n°**
D181	*F. incarnatum-equiseti*	100%	FD_01664_EF-1a	*F. incarnatum*	100%	KJ126171.1
H5	*F. incarnatum-equiseti*	100%	FD_01664_EF-1a	*F. equiseti*	100%	KM886212.1
E 3	*F. incarnatum-equiseti*	99%	FD_01639_EF-1a	*F. equiseti*	99%	JF508173.1
F20				*F. equiseti*	100%	KP336404.1
D51				*F. equiseti*	100%	KM886212.1
B12	*F. incarnatum-equiseti*	100%	FD_01664_EF-1a	*F. equiseti*	100%	KM886212.1
A15	*F. incarnatum-equiseti*	100%	FD_01692_EF-1a	*F. equiseti*	100%	KM886212.1
H4	*F. incarnatum-equiseti*	96%	FD_01643_EF-1a	*F. incarnatum*	100%	JX971222.2
D41	*F. incarnatum-equiseti*	99%	FD_01635_EF-1a	*F. incarnatum*	99%	JF270304.1
E1	*F. incarnatum-equiseti*	99%	FD_01664_EF-1a	*F. equiseti*	100%	KM886212.1
D166	*F. incarnatum-equiseti*	98%	FD_01683_EF-1a	*F. incarnatum*	100%	KR003731.1
D20	*F. incarnatum-equiseti*	100%	FD_01683_EF-1a	*F. incarnatum*	100%	KR003731.1
I24	*F. incarnatum-equiseti*	100%	FD_01683_EF-1a	*F. incarnatum*	100%	JX268996.1
I28	*F. incarnatum-equiseti*	99%	FD_01664_EF-1a	*F. incarnatum*	99%	HM770723.1
D164	*Gibberella fujikuroi*	100%	FD_01770_EF-1a	*F. sacchari*	99%	DQ465945.1
F25	*Gibberella fujikuroi*	99,70%	FD_01770_EF-1a	*F. sacchari*	100%	DQ465942.1
D220	*Fusarium* sp.	100%	FD_01859_EF-1a	*F. sacchari*	99%	HM347125.1
D125	*Gibberella fujikuroi*	99,70%	FD_01770_EF-1a	*F. sacchari*	100%	DQ465942.1
D176	*Gibberella fujikuroi*	99,70%	FD_01770_EF-1a	*F. sacchari*	100%	DQ465942.1
C2-2	*Gibberella fujikuroi*	99.54%	FD_01770_EF-1a	*F. sacchari*	99%	DQ465942.1
C2-4	*Gibberella fujikuroi*	100%	FD_01770_EF-1a	*F. sacchari*	99%	DQ465945.1
C5-2	*Gibberella fujikuroi*	99,70%	FD_01770_EF-1a	*F. sacchari*	100%	DQ465942.1
F27	*Gibberella fujikuroi*	100%	FD_01770_EF-1a	*F. sacchari*	100%	DQ465945.1
D225	*F. proliferatum*	99%	FD_01378_EF-1a	*F. proliferatum*	99%	KP732085.1
C4-3	*F. proliferatum*	99,70%	FD_01378_EF-1a	*F. proliferatum*	100%	KR856505.1

D127	*F. proliferatum*	99%	FD_01378_EF-1a	*F. proliferatum*	99%	KP732085.1
D46	*F. proliferatum*	99%	FD_01378_EF-1a	*F. proliferatum*	99%	KP732085.1
C1-2	*F. proliferatum*	99%	FD_01378_EF-1a	*F. proliferatum*	99%	KP732085.1
F31	*Fusarium* sp.	98,80%	FD_01278_EF-1a	*F. musae*	99%	KC599241.1
D175	*Gibberella fujikuroi*	99,70%	FD_01185_EF-1a	*F. verticillioides*	99%	KP732012.1
E4	*F. verticillioides*	100%	FD_01387_EF-1a	*F. verticillioides*	100%	KM598774.1
D192	*F. verticillioides*	99,70%	FD_01387_EF-1a	*F. verticillioides*	100%	FN179337.1
D53	*Gibberella fujikuroi*	98%	FD_01767_EF-1a	*F. pseudocircinatum*	99%	JF740710.1
F33	*Gibberella fujikuroi*	99%	FD_01176_EF-1a	*F. pseudocircinatum*	99%	GU377298.1
D152	*Gibberella fujikuroi*	97,90%	FD_01145_EF-1a	*Fusarium* sp.	98%	AF160309.1
D11	*F. solani*	99,45%	FD_01598_EF-1a	*F. solani*	100%	KP761172.1
D187	*F. solani*	100%	FD_01415_EF-1a	*F. solani*	100%	LN827985.1
E08	*F. incarnatum-equiseti*	99%	FD_01692_EF-1a	*F. equiseti*	99%	KM886212.1
H09	*F. incarnatum-equiseti*	99%	FD_01664_EF-1a	*F. equiseti*	100%	KM886212.1
D44	*F. oxysporum*	99%	FD_01227_EF-1a	*F. oxysporum*	99%	LN828039.1
D71	*F. incarnatum-equiseti*	100%	FD_01664_EF-1a	*F. equiseti*	100%	KM886212.1
D210	*F. incarnatum-equiseti*	98%	FD_01647_EF-1a	*F. equiseti*	99%	AB674278.1
D137	*F. solani*	98%	FD_01051_EF-1a	*F. solani*	99%	DQ247674.1

The second most frequent genus was *Colletotrichum*, accounting for 7% of isolated strains, and found in 13% of all samples, and all strains were *C. musae* (Berk. & M.A. Curtis) Arx. *Colletotrichum musae* was isolated only from crown tissues and mainly from internal crown tissues. Six percent of strains belonged to *Lasiodiplodia* spp. (Ellis & Everh), and were found in 7% of all samples and were isolated mainly from crown's outer tissues. Strains were distributed in two species: *L. theobromae* (Pat.) Griffon & Maubl. and *L. pseudotheobromae* (A.J.L. Phillips, A. Alves & Crous) (Table 3). The other isolated fungi were: *Nigrospora* sp. (Zimm) 11%, *Alternaria* spp. (Nees) 6%, *Phoma* spp. (Sacc) 2%, *Pestalotiopsis* sp. (Steyaert) 2%, *Curvularia* spp. (Boedijn) 1% and *Microdochium* sp. (Syd. & P. Syd) 1% (Table 3). It is important to note that, we had similar fungal composition isolated from the five different area investigated.

Table 3: Strains identification based on ITS, β-tubulin, ApMat and GPDEF sequences.

Code	Primer	Identification	similarity	Accession n° in NCBI
D75	ITS	*L. pseudotheobromae*	100%	AB873040.1
	BT2		99%	KF254943.1
D76	ITS	*L. pseudotheobromae*	99%	JX914479.1
	BT2		99%	KP308523.1

D88	ITS	*L. pseudotheobromae*	100%	AB873041.1
D89	ITS	*L. pseudotheobromae*	100%	FJ904838.1
	BT2		100%	KM510360.1
D90	ITS	*L. pseudotheobromae*	100%	JX464075.1
	BT2		99%	KP308523.1
D91	ITS	*L. pseudotheobromae*	100%	JX464092.1
	BT2		99%	KP308523.1
A12	ITS	*L. theobromae*	99%	JX945583.1
	BT2		100%	KP721699.1
A13	ITS	*L. theobromae*	99%	JX868613.1
A05	ITS	*L. theobromae*	99%	JX275790.1
D255	ITS	*L. theobromae*	99%	JQ344356.1
	BT2		99%	KP721700.1
D66	ITS	*L. theobromae*	100%	KJ381073.1
	BT2		99%	KP721700.1
D73	ITS	*L. theobromae*	99%	JX275790.1
D74	ITS	*L. theobromae*	99%	JX275790.1
	BT2		100%	KP721699.1
D77	ITS	*L. theobromae*	100%	KJ381073.1
	BT2		100%	KP721699.1
D78	ITS	*L. theobromae*	100%	KJ381073.1
D79	ITS	*L. theobromae*	99%	JX275790.1
D80	ITS	*L. theobromae*	100%	KJ381073.1
	BT2		99%	KP721699.1
D81	ITS	*L. theobromae*	99%	HM346880.2
D94	ITS	*L. theobromae*	100%	JX275790.1
D96	ITS	*L. theobromae*	100%	JX275790.1
D97	ITS	*L. theobromae*	99%	KJ381073.1
D98	ITS	*L. theobromae*	99%	KJ381073.1
D99	ITS	*L. theobromae*	99%	KJ381073.1
D18	ITS	*Botryotinia fuckeliana*	99%	KF532975.1
D50	ITS	*Diaporthe phaseolorum*	77%	KF697689.1
D117	BT2	*Fusarium incarnatum*	99%	KJ020861.1
D124	ITS	*Curvularia hawaiiensis*	100%	KC999918.1
	GPDEF	*Curvularia dactyloctenii*	99%	KJ415401.1
D126	ITS	*Exserohilum rostratum*	100%	FJ949084.1
D131	BT2	*Penicillium* sp.	100%	KP691061.1
D132	BT2	*Phoma sorghina*	100%	FJ427183.1
D137	ITS	*Fusarium solani*	99%	KC341961.1
D254	ITS	*Microsphaeropsis arundinis*	99%	JQ344356.1
D200	ITS	*Corynespora cassiicola*	99%	KF928288.1
D232	BT2	*Phoma sorghina*	100%	FJ427175.1
D236	BT2	*Phoma sorghina*	99%	FJ427182.1
D234	BT2	*Verticillium dahliae*	95%	XM_009651338.1
D239	BT2	*Fusarium incarnatum*	99%	KJ020861.1
D253	BT2	*Fusarium incarnatum*	99%	KJ020861.1
D277	BT2	*Fusarium incarnatum*	99%	KJ020861.1
D263	BT2	*Phoma* sp.	94%	JN130386.1
D235	ITS	*Sordariomycetes*	97%	JQ761140.1
F41	GPDEF	*Curvularia aeria*	99%	HF565451.1
D135	GPDEF	*Curvularia hawaiiensis*	100%	HG779142.1
F22	ITS	*Pestalotiopsis* sp.	99%	GU723442.1
	BT2		99%	KJ623200.1

F14	BT2	*Pestalotiopsis* sp.	95%	KC247155.1
F37	BT2	*Pestalotiopsis* sp.	93%	JX399043.1
D100	ITS	*Nigrospora* sp.	99%	JN207248.1
F35	ITS	*Nigrospora* sp.	99%	JN207298.1
D134	ITS	*Phoma* sp.	99%	HQ630963.1
D113	ITS	*Alternaria alternata*	99%	KF193470.1
B9	ITS	*Alternaria tenuissima*	99%	JX156349.1
D128	ITS	*C. musae*	99%	AJ301904.1
C3-1	ITS	*C. musae*	99%	DQ453982.1
	ApMat		99%	KC790670.1
C4-2	ITS	*C. musae*	99%	DQ453982.1
D48	ITS	*C. musae*	99%	DQ453986.1
C2-1	ITS	*Colletotrichum* sp.	99%	HQ264183.1
	ApMat	*C. tropicale*	99%	JX145306.1
H30	ApMat	*C. musae*	100%	JQ899268.1
H28	ApMat	*C. musae*	98%	KC888926.1
355 in1	ApMat	*C. musae*	100%	KC888926.1
361 in1	ApMat	*C. musae*	99%	KC790670.1
974 in1	ApMat	*C. musae*	100%	JQ899268.1

The pathogenicity tests allowed us to reproduce the crown rot symptoms. All inoculated crown used showed crown rot symptoms which resulted no variance in disease incidence among the strains, but disease severity varied and clearly showed the importance of *Lasiodiplodia* spp. and *C. musae* as the most virulent species, followed by *F. sacchari, F. verticillioides*, *F. incarnatum* and *F. dimerum* (Table 4). *Fusarium incarnatum,* which is considered the main crown rot pathogen in many countries, showed strain-specific disease severity variability; severity ranged between 3 and 5 grade. *F. sacchari* and *F. verticillioides* were more virulent than *F. incarnatum*, but considering the total frequency of different species isolated from crown tissues, *F. incarnatum* might be the principal crown rot disease agent in Dominican Republic. No significant differences were observed between *L. theobromae* and *L. pseudotheobromae*. The same situation was observed in C. *musae* with no significant differences between strains. Re-isolation was carried out from all infected crowns, and the Koch's postulates were confirmed by re-isolating the same strains used for inoculation.

Table 4: Disease severity index assessed by experimental inoculation using representative

213 strains isolated from crown tissues.

Code	strain	Disease severity	Statistical report[1]
A13	*Lasiodiplodia theobromae*	7	a
C3-1	*Colletotrichum musae*	7	a b
D89	*Lasiodiplodia pseudotheobromae*	7	a b
D128	*Colletotrichum musae*	6	a b c
A07	*Microdochium* sp.	6	a b c d
C2-2	*Fusarium sacchari*	5	a b c d e
B11	*Fusarium incarnatum*	5	a b c d e f
B01	*Fusarium verticillioides*	4	a b c d e f g
F34	*Fusarium incarnatum*	4	b c d e f g h
D67	*Fusarium incarnatum*	4	c d e f g h
D100	*Nigrospora* sp.	4	c d e f g h
F30	*Fusarium dimerum*	4	c d e f g h i
A16	*Fusarium incarnatum*	3	d e f g h i
C2-1	*Colletotrichum tropicale*	3	d e f g h i
C4-4	*Fusarium proliferatum*	3	d e f g h i
D41	*Fusarium incarnatum*	3	d e f g h i
D187	*Fusarium solani*	3	e f g h i
D221	*Fusarium oxysporum*	3	e f g h i
F41	*Curvularia lunata*	3	e f g h i
D113	*Alternaria alternata*	2	f g h i
D134	*Phoma* sp.	2	g h i
F22	*Pestalotiopsis* sp.	2	g h i
F35	*Nigrospora* sp.	1	h i
B09	*Alternaria tenuissima*	1	i
	Control	1	i

214 [1]. The Tukey method with 95% confidence was used and means that do not share a letter are

215 significantly different.

4. Discussion

The composition of the fungal population isolated from various organic banana samples from Dominican Republic was similar to those found in other banana cultivation areas affected by crown rot disease (Alvindia and Natsuaki, 2008; Anthony et al., 2004; Ewane et al., 2013; Goos and Tschirsch, 1962; Johanson and Blazquez, 1992; Lassois et al., 2008; Lassois et al., 2010b) (Table 5). However, some taxa were specific of this area, e.g. *F. sacchari, F. musae, F. dimerum, F. proliferatum, F. pseudocircinatum, Alternaria* spp., *Curvularia* spp., and *Microdochium* sp., which were not previously reported in other studies. To the best of our knowledge, this is the first time these species were isolated from asymptomatic crown tissues. The identification of *F. musae*, was possible thanks also to the progress in identification approaches and the taxonomic revision or new description of some fungal taxa (Van Hove et al., 2011). Studies on fungal population composition that targeted the traditional bananas farming are numerically low. In Costa Rica the most frequent taxa found in diseased samples were *C. musae* and *F. subglutinans* (Umana-Rojas and Garcia, 2011a) but the two species had different frequency from what reported in the present study. In the same study other *Fusarium* species were reported, which were not found in Dominican Republic. In Ghana, *Botryodiplodia theobromae* Pat. (the former name of *L. theobromae*) was the most frequent fungal organism (Ocran et al., 2011) whereas in this study it represent only as 6% of the population.

236 Table 5: Fungi isolated from crown tissues sampled in Dominican Republic in comparison with other different production areas.

Countries	Dominican Republic	Honduras	Central and South America		Windward Islands (WI)									Jamaica	Sri Lanka	Nigeria	Somalia, WI, Guatemala	Philippines
References	Present study	15	1	4	16	7	6	12	11	9	8	13	3	10	14	5	2	17
C. musae	7%	3%	F	0-10%	23-33%	36%	24%	26-44%	I	X	I	I	I	11%	X	27%	38%	P
F. incarnatum	29%	80%	MF	4-50%		27%	18%	7-23%		X	I	I	I	F		3%	X	
F. verticillioides	7%	14%	X	0-28%		6%	3%	6-21%	I	X		I	I	X	X	10%	X	P
F. sporotrichoides																	20%	
F. oxysporum	3%						3%					I	I	X		4%		
F. solani	3%						2%									6%		
Lasiodiplodia spp.	6%	12%	R			2%	2%	3-9%		X		I	I	3%	X	26%		P
Musicillium theobromae		81%	F		5-7%	8%	3%	4-18%				I	I	13%		2%	2%	
Gliocladium roseum				0-30%	5%		2%	0-10%										
Nigrospora sphaerica	11%		R			<1%	<1%	24-26%				I	I	1%				
Acremonium sp.	R	93%		0-8%			2%	0-10%	I					3%			4%	
Penicillium sp.	11%			0-33%			<1%											
Alternaria sp.	6%																	
Curvularia sp.	1%																	
F. sacchari	7%																	
F. dimerum	1%																	
F. musae	1%																	
F. proliferatum	4%																	
F. pseudocircinatum	2%																	
Microdochium sp.	R																	
Pestalotiopsis sp.	R																	
Thielaviopsis paradoxa																		P

237 Percent values correspond to isolation frequencies when given by Lassois and Colleagues (2010b)
238 except in Dominican Rep. by the author. X: indicates that the pathogen was identified on the crown by
239 Lassois and Colleagues (2010b), but without providing any isolation frequency data. F: frequently
240 isolated; MF, most frequently isolated; R: rarely isolated. I: indicate that Lassois and Colleagues
241 (2010b) evaluated fungal pathogenicity without information about isolation frequencies. Information
242 about pathogenicity is shown when given by Lassois and Colleagues (2010b) and in Dominican Rep.
243 by the author. ■ = Highly pathogenic; ■ = medium pathogenicity; ■ = slightly or nonpathogenic.
244 The references cited in table 5, are as follows: 1- (Goos and Tschirsch, 1962). 2- (Meredith, 1962). 3-
245 (Greene and Goos, 1963). 4- (Lukezic et al., 1967). 5- (Mulvena et al., 1969). 6- (Stover, 1972). 7-
246 (Griffee, 1976). 8- (Knight, 1982). 9- (Eckert and Ogawa, 1985). 10- (Reyes et al., 1998). 11- (Krauss
247 and Johanson, 2000). 12- (Joas and Malisart, 2001). 13- (Khan et al., 2001). 14- (Anthony et al.,
248 2004). 15- (Lassois et al., 2008). 16- (Thompson, 2010). 17- (Alvindia, 2013).

5. Conclusions

Based in our results, the genus *Fusarium* was the most frequent genus among all isolated fungi, thus confirming its importance in the etiology of crown rot disease. The species can be ranked based on their frequency and pathogenicity as following: *F. incarnatum, C. musae, F. sacchari, F. verticillioides, L. theobromae* and *L. pseudotheobromae*. Our results are similar to the data obtained for Windward Island, where *C. musae* was the most virulent species (Finlay and Brown, 1993), and for Costa Rica, where a *Fusarium* spp. strain was the most virulent (Umana-Rojas and Garcia, 2011b), but different from those of Sri Lanka, where *L. theobromae* caused a crown rot epidemic (Gunasinghe and Karunaratne, 2009).

The strains we identified as belonging to taxa known as saprophytes or postharvest pathogens were retained for ongoing studies in which we investigate their role in pathogenicity following combined inoculations with known pathogens.

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