Epiphytic microorganisms associated with banana phyllosphere with potential antagonism to Black Sigatoka (*Pseudocercospora fijiensis*) in Los Ríos, Ecuador

Identificación de microorganismos epífitos asociados a la filósfera de banano con potencial antagonismo a Sigatoka Negra (*Pseudocercospora fijiensis*) en la provincia de Los Ríos, Ecuador

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Abstract

Black Sigatoka (*Pseudocercospora fijiensis)* is the most important leaf spot disease of bananas worldwide, particularly affecting Cavendish banana, the most exported variety. Additionally, this pathogen has developed resistance to some effective fungicides, making its management increasingly difficult. Epiphytic microorganisms with potential antagonism to *P. fijiensis* were identified in conventional banana farms in the province of Los Ríos. Sampling areas were determined through zoning processes and selecting the cantons of Mocache, Valencia, Baba and Pueblo Viejo. Leaf tissue samples were collected from three farms per zone. Microorganisms were isolated and morphologically and molecularly characterised in nine farms in the cantons of Valencia (63 bacteria), Baba (39 bacteria), Pueblo Viejo (8 bacteria) and 8 genera of fungi including 15 species. The isolated bacteria presented macroscopic and microscopic characteristics with different shapes, elevations, edges, consistencies and pigmentations. Taxonomically, they belonged to the genera *Bacillus* and *Cocos*, 81% Gram-negative and 19% Gram-positive. The analysis conducted for sampling-site selection allowed the identification of different microbial behaviours.

Keywords
microorganisms • fungi • bacteria • *Musa* spp.

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Resumen

Sigatoka negra es la enfermedad de la mancha foliar más importante del banano a nivel mundial. La variedad de banano Cavendish, es considerada la más común y la más exportada; sin embargo, presenta una alta susceptibilidad frente a la enfermedad. Existen fungicidas altamente efectivos para su control; sin embargo, el patógeno ha logrado generar resistencia a algunos de estos, lo que ha dificultado cada vez más su manejo. Se identificaron microorganismos epífitos con potencial antagonismo a *Pseudocercospora fijiensis* en fincas de banano convencionales de la provincia de Los Ríos. Las zonas de muestreo fueron determinadas a través de procesos de zonificación, seleccionando los cantones Mocache, Valencia, Baba y Pueblo Viejo. Se recolectó muestras de tejido foliar en tres fincas por zona. Se aislaron microorganismos y se caracterizaron morfológica y molecularmente en nueve fincas en los cantones de Valencia (63 bacterias), Baba (39 bacterias) y Pueblo Viejo (8 bacterias) y 8 géneros de hongos que incluyen 15 especies. Las bacterias aisladas presentaron características macroscópicas y microscópicas con diversas formas, elevaciones, bordes, consistencias y pigmentaciones, así como diversas taxonomías pertenecientes a los géneros *Bacillus* y Cocos, siendo 81% Gram negativas y 19% Gram positivas. El análisis realizado para la selección de los sitios de muestreo fue apropiado ya que se observó un comportamiento diferencial de los microorganismos en estas zonas.

Palabras claves
microorganismos • hongos • bacterias • *Musa* spp.

Introduction

Musaceae is a family of monocotyledonous plants that include bananas and plantains, often called giant herbs (31). These plants belong to the genus *Musa,* cultivated in tropical and subtropical regions (36).

The banana sector in Ecuador has 167,893 hectares, with a productivity of 6,684,916 tons. Los Ríos province has the highest participation in the national production of fresh fruit with 38.47% (2 571 356 t), with a contribution of 1 328 537 964.98 US dollars (45).

Investment in production and related industries (goods and services needed for banana production) and the current banana export process created jobs for more than one million households in Ecuador, benefiting around 2.5 million people in nine provinces heavily dependent on the banana industry. Compared to other non-oil sectors in the country, this sector is the backbone of economic activity, generating higher incomes and providing more employment opportunities (16).

Black Sigatoka is the most economically important leaf spot disease of *Musaceae*, affecting many plantations and resulting in forced early harvesting (27). This disease is caused by the fungus *Pseudocercospora fijiensis*, exclusive of banana foliage with sexual and asexual reproduction. It infects the plants, hindering photosynthesis and causing gradual leaf necrosis and death. Disease severity is determined by the Stover scale modified by Benavides-López (2019), Gauhl (1994) and Muimba-Kankolongo (2018).

Black Sigatoka is mainly controlled by technical management and appropriate fungicide rotation. However, given climatic variability, the disease shows different behaviours around the country. Los Ríos province is the most affected, with 74% of production losses. Twenty-two to 29 annual aerial spraying cycles are used to fight the disease, representing costs between $430 and $800 (8, 9). In addition, surgical practices like excision of mottled areas and leaf removal are carried out (9, 17).

International markets for plant protection products are dominated by synthetic pesticides (30). These chemical substances seriously affect the ecosystem and induce resistance, altering ecological equilibriums (28). Therefore, searching for alternative control strategies is relevant worldwide (24).

The search for antagonistic microorganisms for biological control of pathogens in economically important crops has aroused particular interest due to their potentialities (4, 54). Microorganisms of agricultural importance represent a key ecological strategy

towards the integrated development of practices such as nutrient, disease and pest management, reducing chemicals and improving crop yield (42). Several microorganisms showing beneficial effects on plants may constitute potential biocontrol agents (3, 41) and important actors in sustainable agriculture (51).

Biological control of Black Sigatoka has received relatively little attention due to the availability of highly effective fungicides. However, the emergence of pathogen isolates resistant to systemic fungicides and the need for cleaner production technologies have increased interest in biological control (22).

The search for effective biological products against this disease has studied different microorganisms associated with these crops (12). Therefore, this research aimed to collect, isolate and characterise microorganisms from the phyllosphere of *Musaceae.*

Materials and methods

Study area

This research was conducted at the Pichilingue Tropical Experimental Station (EETP) of the National Institute of Agricultural Research (INIAP) with samples obtained from conventional banana farms of Cavendish Williams cultivar in the cantons of Mocache, Valencia, Baba and Pueblo Viejo, Los Ríos province.

Field methodology

Sampling sites were chosen by zoning with cartographic charts from the IGM (Military Geographic Institute) database. The climate micro-zonation map was generated by satellite images with ArcGIS 10.8, at a scale of 1:25 000 for geo pedological conditions considering soil pH, organic matter and surface texture, with 1:50 000 scale, considering geopedology, geomorphology, CUT (soil usage capacity) and isotherms, including climatic zones, temperature and cover use (figure 1, page XXX).

Ten subsamples were collected from each farm, constituting one composite sample. In selected plants, the third and fourth leaves were identified for tissue to be obtained from the central third, both on the right and left side of the midrib. Samples were identified by recording origin and date (41).

Fungal identification from leaf tissue was conducted in Mocache, Baba and Pueblo Viejo. Bacteria were identified from leaves in Valencia, Baba and Pueblo Viejo.

Microorganism isolation

For the isolation of bacteria, the samples obtained were processed according to Intriago Mendoza (2010). Agar culture medium was prepared in flasks, sterilised in autoclave for 30 minutes and distributed in petri dishes. Twenty-five g of leaf tissue were washed in 100 mL of sterile distilled water (SDW). Product water was used for serial dilutions up to -3. One ml of each dilution was seeded by Digralsky loop, and plates were incubated at room temperature for 5 days for growth evaluation.

In order to isolate fungi, plant tissue samples were washed with distilled water, cut into small portions of tissue (3 to 5 mm) and immersed in a 1-3% hypochlorite solution for one minute, followed by rinsing with sterile water. Tissue portions were seeded in Petri dishes with PDA (potato dextrose agar) + chloramphenicol medium and incubated at 28°C for 5 days. Isolates were purified and preserved at 5°C (20).

Morphological characterization

After biochemical Gram staining and catalase tests, macroscopic and microscopic characterisation was carried out on the isolated microorganisms, described by their shape, colour, edges, elevation and consistency (52),

Number of Colonies:

Number of colonies on the plates is expressed as CFU/ml (Colony Forming Units) according to Casas *et al.* (2017).

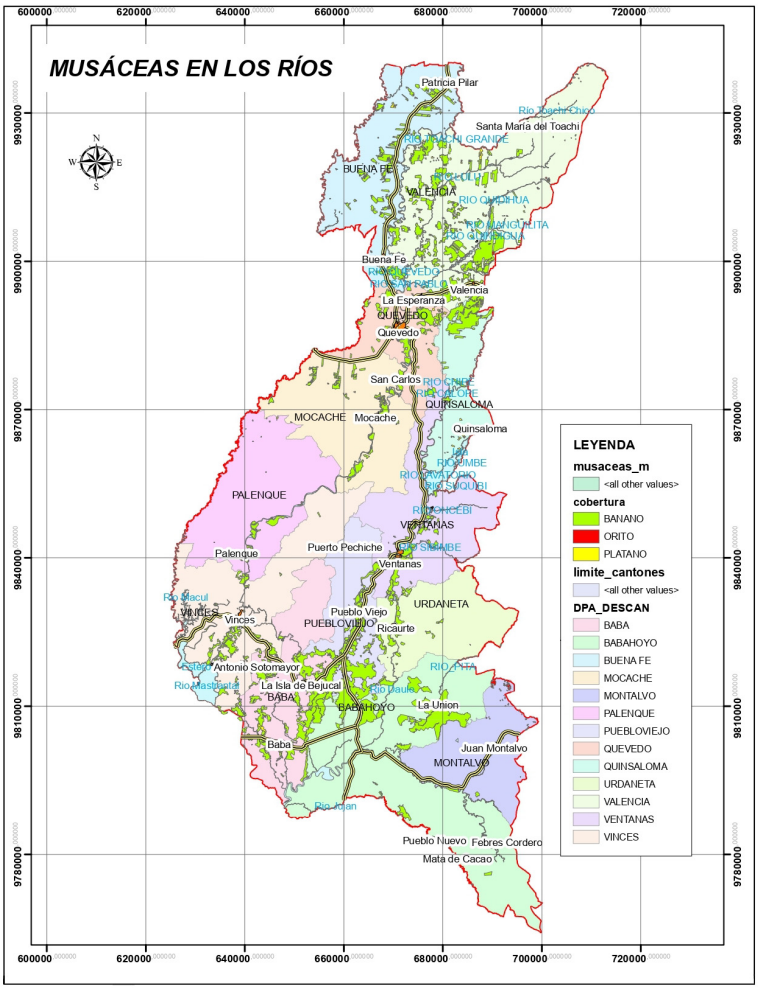

Figure 1. Climate micro-zonation map for sampling sites in the Province of Los Ríos, generated by ArcGIS 10.8 software.

Figura 1. Mapa de microzonificación climática para sitios de muestreo en la Provincia de Los Ríos, generado con el software ArcGIS 10.8.

Extraction of fungal genomic DNA

According to Doyle & Doyle (1987) modified by Faleiro *et al.* (2002), samples were split into two boxes per sample with 14 daysqold mycelium and triturated with liquid nitrogen. The homogenate was mixed with 800 µL extraction buffer (7% cetyltrimethylammonium bromide [CTAB], 5 M NaCl, 0.5 mM ethylenediaminetetraacetic acid [EDTA], 1 M Tris-HCl pH = 8, Polyvinylpyrrolidone (PVP-40), 1% (v/v) ß-mercaptoethanol and milliQ water). Five µL of proteinase K (concentration 20 mg/uL) was added to the homogenate and incubated at 65°C for 1 hour in a water bath. Then, it was centrifuged at 14 000 rpm for 15 minutes and the supernatant was collected into 2 mL tubes, added 55 µL of 7% CTAB and 700 µL chloroform: isoamyl alcohol (25: 1; v: v), mixed with inversion and vortexed until an emulsion was formed and centrifuged at 14 000 rpm for 16 minutes. Again, the supernatant was extracted to 2 mL tubes by adding 700 µL chloroform: isoamyl alcohol (25: 1; v: v), mixed and centrifuged. The supernatant was recovered in 1.5 mL tubes and 700 µL (2/3 of the tube) was added with ice-cold absolute ethanol (-20°C), for storage at -20°C for 1 to 2 hours. Centrifugation was performed at 14 000 rpm for 5 minutes, obtaining a white pellet and the supernatant was removed by washing the pellet 3 to 4 times with 70% ethanol at -2°C. (500 µL). Finally, the pellet was dried in a thermoblock at 55°C and DNA was resuspended in 100 µL of TE with RNAsa (concentration 20 mg/ml).

PCR Amplification of Ribosomal ITS Region

To verify the extracted DNA, amplification was performed with markers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATATGC) and the amplification cocktail proposed by Morillo & Miño (2011). All samples were amplified on the Applied Biosystems thermal cycler in a total reaction volume of 25 µl, including 2.50 µL of 5x Green GoTaq® Flexi Buffer (Promega), 1.5µL of MgCl2 (25 mM), 0.50 µL of dNTPs (5mM), 2 µL of each primer (5 µmol), 0.50 µL of DNA polymerase (Thermo Scientific DreamTaq) (5 U/ µl), 1 µL of genomic DNA (5 ng/ µl) and 15 µL of ultrapure water. PCR included initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, hybridisation at 55°C for 2 min, elongation at 72°C for 1 min and a final elongation step at 70°C for 10 min.

DNA amplicons were analysed on a 1.5% (w/v) agarose gel using Syber Safe for 30 minutes at 100V. Amplicon sizes were estimated by comparison with a TrackIt™ 1 Kb Plus DNA Ladder molecular weight marker and visualised in a photodocumenter.

Sequence analysis

PCR products were shipped to the research laboratories of the Universidad de las Américas (UDLA), according to the university guidelines, which consisted of 10 µL of PCR product, 2 µL of each primer (ITS 1/ ITS4) (2 µM concentration) per sample and cold chain storage at 4°C or below. Sequence editing was performed using the Unipro UGENE software and the BLAST programme at the Centro Nacional de Información Biotecnológica (http://www.ncbi.nlm.nih.gov/) to obtain consensus sequences. Sequence alignment was performed by MUSCLE algorithm with MegaX software. The matrix obtained was used to assemble the phylogenetic tree based on the Maximum-Likelihood (ML) algorithm, with genetic distance calculated by the Jukes-Cantor model, Bootstrap-100 and Uniform Rates.

Results

Colony count

Considering the three farms evaluated in each canton, in Valencia, the Bellavista farm had the highest number of colonies with $9.18x10^4$ CFU; in Baba the Soledad farm had the highest number of colonies with $3.468x10^4$ CFU and in Pueblo Viejo, Valdivia and Viviana farms had $6.12x10^3$ CFU, obtaining a total of 63 bacteria in Valencia, 39 bacteria in Baba and 8 bacteria in Pueblo Viejo.

Estate	Colony Forming Units
Bellavista	$9.18x10^4$ CFU
El Zapote	$4.08x10^4$ CFU
Buenos Aires 2	$6.528x10^4$ CFU
San Luis	$1.224x10^4$ CFU
Carolina	$2.244x10^4$ CFU
Soledad	$3.468x10^4$ CFU
Carolina	$4.08x10^3$ CFU
Valdivia	$6.12x10^3$ CFU
Viviana	$6.12x10^3$ CFU

Strain characterisation

Colonial morphological characterisation, microscopic morphology and biochemical tests were performed on the bacteria isolated in the province of Los Ríos (table 1, page XXX).

Table 1. Characterisation of microorganisms associated with the banana phyllosphere, Los Ríos province.
Tabla 1. Caracterización de microorganismos asociados a la filósfera de banano, provincia de Los Ríos.

Cantons	Estate	Strain	Colonial morphology					Microscopic morphology	Biochemical Test
			Shape	Color	Borders	Elevation	Consistency	Gram stain	Catalase
Valencia	Bellavista	VF1C5	circular	Orange	whole	elevated	Viscous	coconuts (+)	(+)
	Bellavista	VF1C6	irregular	Yellow	wavy	elevated	Viscose	coconuts (+)	(+)
	Bellavista	VF1C9	irregular	Milky	wavy	Flat	Viscose	coconuts (+)	(+)
	Bellavista	VF1C15	irregular	Orange	wavy	Flat	Viscose	coconuts (-)	(+)
	Bellavista	VF1C16	irregular	Red	whole	Flat	Viscose	coconuts (+)	(+)
	Bellavista	VF1C17	circular	Red	whole	convex	Viscose	coconuts (-)	(+)
	Bellavista	VF1C24	irregular	Yellow	wavy	convex	Viscose	coconuts (-)	(+)
	Bellavista	VF1C29	circular	Yellow	whole	convex	Viscose	coconuts (+)	(+)
	El Zapote	VF2C3	circular	Red	wavy	convex	Viscose	coconuts (-)	(+)
	El Zapote	VF2C5	irregular	Yellow	lobed	Flat	Viscose	bacillus (-)	(+)
	El Zapote	VF2C8	circular	Orange	whole	convex	Viscose	bacillus (-)	(-)
	El Zapote	VF2C10	circular	Orange	whole	Flat	Viscose	coconuts (-)	(-)
	El Zapote	VF2C12	circular	Orange	whole	convex	Viscose	coconuts (-)	(+)
	Buenos Aires 2	VF3C13	circular	Pink	whole	convex	Viscose	coconuts (-)	(-)
	Buenos Aires 2	VF3C18	circular	White	wavy	Flat	Viscose	coconuts (-)	(+)
	Buenos Aires 2	VF3C24	circular	pink	whole	elevated	Viscose	coconuts (-)	(+)
	Buenos Aires 2	VF3C31	circular	milky	wavy	convex	Viscose	coconuts (-)	(+)
Baba	San Luis	BF1C2	circular	milky	Wavy	Flat	Viscose	bacillus (-)	(-)
	San Luis	BF1C3	irregular	white	Wavy	Flat	dry	cocobacillus (+)	(+)
	San Luis	BF1C4	circular	pink	Wavy	convex	Viscose	bacillus (-)	(+)
	San Luis	BF1C5	circular	milky	Wavy	elevated	Viscose	bacillus (-)	(+)
	San Luis	BF1C8	circular	yellow	Wavy	convex	Viscose	bacillus (-)	(+)
	Carolina	BF2C3	punctate	yellow	Wavy	Flat	Viscose	coconuts (+)	(+)
	Carolina	BF2C7	irregular	white	Wavy	Lisa	Viscose	bacillus (-)	(+)
	Carolina	BF2C8	irregular	milky	Irregular	Lisa	Viscose	bacillus (-)	(+)
	Carolina	BF2C9	circular	yellow	Lobed	Flat	Viscose	bacillus (-)	(+)
	Carolina	BF2C15	irregular	white	Irregular	Flat	Viscose	streptobacillus (-)	(+)
	Carolina	BF2C18	irregular	orange	Lobed	elevated	Viscose	bacillus (-)	(+)
	Soledad	BF3C1	circular	milky	Whole	convex	Viscose	coconuts (-)	(+)
	Soledad	BF3C2	spindle	white	Whole	Flat	Viscose	coconuts (-)	(+)
	Soledad	BF3C3	circular	white	Whole	convex	Viscose	coconuts (-)	(+)
	Soledad	BF3C5	circular	white	Whole	Flat	Viscose	coconuts (-)	(+)
	Soledad	BF3C9	irregular	milky	Lobed	Flat	Viscose	bacillus (-)	(+)
	Soledad	BF3C12	punctate	milky	Wavy	Flat	Viscose	coconuts (-)	(+)
Pueblo Viejo	Viviana	PvF1C1	circular	milky	whole	elevated	Viscose	coconuts (-)	(+)
	Viviana	PvF1C2	circular	milky	filamentous	Flat	Viscose	bacillus (-)	(+)
	Viviana	PvF1C3	circular	milky	whole	convex	Viscose	cocobacillus (-)	(+)
	Valdivia	PvF2C1	circular	milky	whole	convex	Viscose	cocobacillus (-)	(+)
	Valdivia	PvF2C2	circular	milky	whole	convex	Viscose	cocobacillus (-)	(+)
	Valdivia	PvF2C3	circular	milky	whole	convex	Viscose	cocobacillus (+)	(+)
	Valdivia	PvF2C4	circular	milky	wavy	elevated	Viscose	cocobacillus (-)	(+)
	Carolina	PvF3C1	circular	milky	lobed	Flat	Viscose	coconuts (-)	(+)

Eight fungal strains were isolated in Mocache, 5 in Baba and 13 in Pueblo Viejo. Eight PCR products were amplified from Mocache, 5 from Baba and 13 from Pueblo Viejo. Twenty-six sequences were obtained. Similarity percentages were 90-100 % with NCBI homologous sequences (table 2).

Table 2. Collection and ITS rDNA sequences of fungi isolated in the cantons of Mocache, Baba and Pueblo Viejo.

Tabla 2. Detalles de la colección y secuencias ITS del ADNr de hongos aislados en los cantones de Mocache, Baba y Pueblo Viejo.

Canton	Estate	Identity in GenBank		Identity
		Accession	Description	
Mocache	INIAP Lot 1	MN173821.1	*Colletotrichum fructicola*	100%
	INIAP Lot 1	MT982177.1	*Fusarium oxysporum*	99.41%
	INIAP Lot 1	KM519998.1	*Colletotrichum gloeosporioides*	100%
	INIAP Lot 1	KU350718.1	*Nectria haematococca*	98.66%
	INIAP Lot 2	JN649339.1	*Cymatoderma dendriticum*	98.34%
	INIAP Lot 2	MH483999.1	*Miyakei microdiplody*	99.61%
	INIAP Lot 2	MK041519.1	*Colletotrichum fructicola*	98.89%
	INIAP Lot 2	HM855208.1	*Nodulisporium sp.*	99.27%
Baba	San Luis	KY488352.1	*Cladosporium uredinicola*	99.41%
	San Luis	KT240141.1	*Cladosporium cladosporioides*	99.22%
	San Luis	MN515070.1	*Nigrospora oryzae*	98.80%
	Carolina	MW016510.1	*Fusarium subglutinans*	99.21%
	Carolina	MT645782.1	*Nigrospora osmanthi*	98.64%
Pueblo viejo	Viviana	MH864880.1	*Diatrypella vulgaris*	99.27%
	Viviana	KY488352.1	*Cladosporium uredinicola*	99.22%
	Viviana	KY488352.1	*Cladosporium uredinicola*	99.41%
	Viviana	MH864880.1	*Diatrypella vulgaris*	99.45%
	Viviana	MH270558.1	*Cladosporium tenuissimum*	99.22%
	Viviana	MN704703.1	*Cladosporium cladosporioides*	99.60%
	Viviana	MH270558.1	*Cladosporium tenuissimum*	99.42%
	Viviana	MW723859.1	*Cladosporium cladosporioides*	93.79%
	Valdivia	KY488352.1	*Cladosporium uredinicola*	98.83%
	Valdivia	KY488352.1	*Cladosporium uredinicola*	99.41%
	Valdivia	MW624421.1	*Nigrospora sphaerica*	98.84%
	Carolina	MW113408.1	*Cladosporium cladosporioides*	99.80%
	Carolina	MN959990.1	*Fusarium oxysporum*	99.21%

The identified fungi were from the genera *Fusarium* sp., *Colletotrichum* sp., *Diatrypella* sp., *Nodulisporium* sp., *Nigrospora* sp., *Microdiplodia* sp., *Cymatoderma* sp. and *Cladosporium* sp. *Fusarium* sp. is a candidate biological control agent against *Pseudocercospora fijiensis. Nodulisporium* sp. is an endophyte capable of producing insecticidal nodilosporic acids and volatile antifungal substances (Suwannarach *et al.*, 2013). *Nigrospora* sp. produce bioactive secondary metabolites with antifungal activity (23) (figure 2).

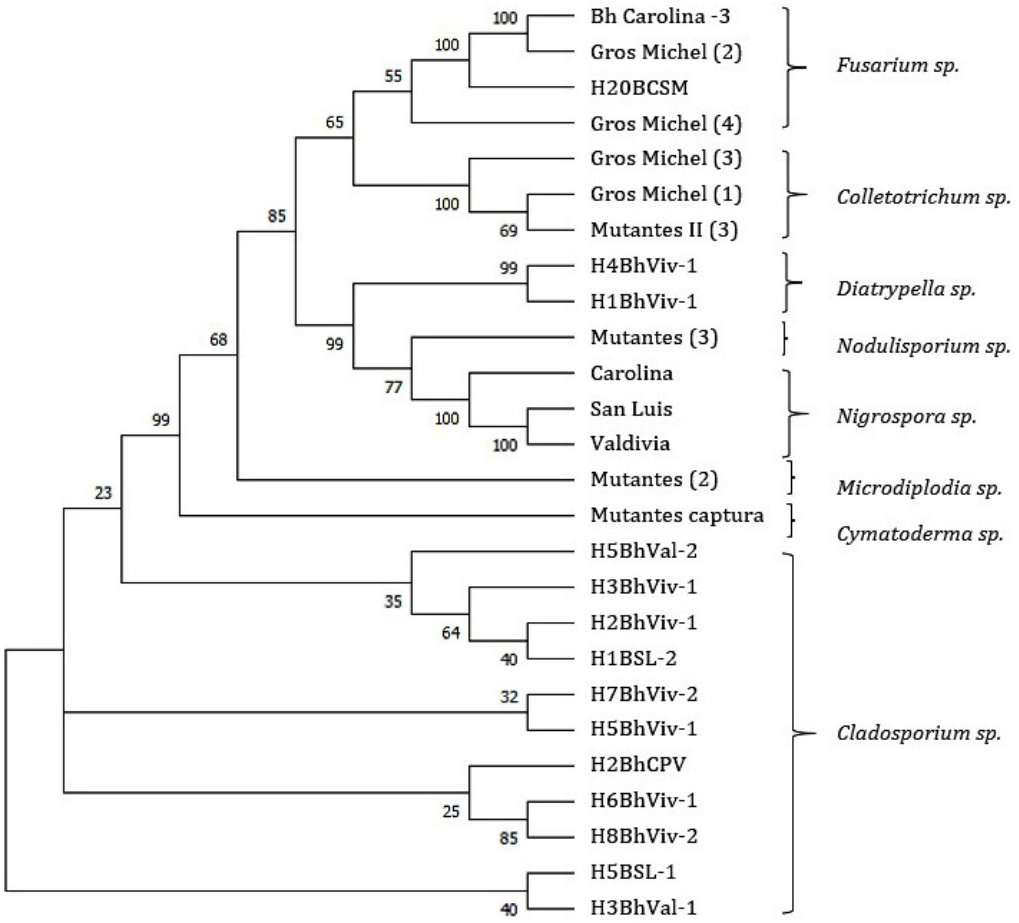

Figure 2. Phylogenetic tree of fungal isolates associated with banana phyllosphere with potential antagonism to Black Sigatoka in the cantons of Mocache, Baba and Pueblo Viejo. Phylogenetic construction by *Maximum- Likelihood* method, Jukes-Cantor model, Bootstrap-100 and Uniform Rates.

Figura 2. Árbol filogenético de aislamientos de hongos asociados a la filósfera de banano con potencial antagonismo a Sigatoka Negra en los cantones de Mocache, Baba y Pueblo Viejo. Construcción filogenética por el método Maximum-Likelihood, Jukes-Cantor model, Bootstrap-100 y Uniform Rates.

Discussion

Fungal disease control mainly relies on the application of agrochemicals. However, this practice causes pathogen resistance after prolonged application, generating public concern about the effects of toxic residues on human health and the environment.

This research identified *Bacillus* bacteria. According to Cruz-Martín *et al.* (2018) *Bacillus pumilus* CCIBP-C5 decreases fungal biomass, induces phytodefense mechanisms in the plant, and may constitute a potential biological control agent against *P. fijiensis*. Based on this finding, *B. pumilus* CCIBP-C5 constitutes a guideline for further research.

Contrary to other studies, proportions of Gram-negative bacteria (81%) exceeded that of Gram-positive bacteria (19%). Previously, Ceballos *et al.* (2012) found higher proportions of Gram-positive bacteria (67%) than Gram-negative bacteria from three banana and plantain cultivars in Urabá (Northwest Colombia). Regarding the inhibitory capacity of the isolated microorganisms, the results showed that *Bacillus* bacteria have antagonistic activity against the fungus *P. fijiensis*, as seen by Villegas-Escobar *et al.* (2013) when isolating 649 strains of aerobic endospore-forming bacteria. The strain *Bacillus subtilis* showed the highest inhibition (89±1%), proving its bioactive potential against *P. fijiensis*.

After isolation, colonies showed different shapes (circular, pointed, irregular, spindle), elevations (flat, convex, smooth, raised), edges (entire, wavy, lobulated, irregular, filamentous), consistencies (viscous, dry) and pigmentations (orange, yellow, red, pink, milky, white), as reported by Alfaro (2013) when isolating and quantifying epiphytic bacteria from the banana phylloplane *Musa* AAA cv. Grande Naine.

Considering the 26 isolated fungi, 25 belonged to Ascomycota and one to Basidiomycota. Ascomycota fungi grow in subtropical conditions, as bananas (44).

Fungal molecular identification is frequently assessed with the internal transcribed spacer (ITS) region (47). The primers ITS1 and ITS4 have broad utility and presence in universal databases, with successful amplification rates of fungal lineages (53). Based on in silico analysis (49) the primer ITS1 represents 73.8% of Ascomycota and 85.6% of Basidiomycota in the SSU region, while the primer ITS4 represents 97.6% in Ascomycota and 96.9% in Basidiomycota in the LSU region. This means ITS primers allowed amplifying sequences from both Ascomycota and Basidiomycota fungi.

In a study of pathogenic taxa in wild banana (*Musa acuminata*), Brown *et al.* (1998) identified potential endophytic pathogen genera and species that may remain dormant, such as *Colletotrichum* sp. and *Nigrospora* sp. Zakaria & Aziz (2018) isolated fungi of the genus *Nigrospora* sp., *Fusarium* sp. and *Colletotrichum* sp. on bananas. Very similar results were obtained by Horra (2014) and in the present study, including *Diatrypella* sp., *Nodulisporium* sp., *Microdiplodia* sp., *Cymatoderma* sp. and *Cladosporium* sp.

Fusarium oxysporum is present among the rhizosphere microflora, and some strains cause wilting or total root rot of banana plants (19). It should be noted that all *F. oxysporum* strains are saprophytes, surviving for long periods in both soil organic matter and the rhizosphere. This possibly explains their latent presence in leaf tissue and the sampled areas. We also isolated *Nectria haematococca* (sexual morph of *F. solani*), a filamentous type of fungus. *F. solani* is part of a complex with 60 phylogenetic species (46). These two *Fusarium* sp. strains could constitute candidate biological control agents against *P. fijiensis* (2), encouraging future antagonistic tests for evaluations against black Sigatoka.

Colletotrichum sp. predominates in the tropics and subtropics with heavy rainfall and high relative humidity (43). Two species were found in this study, *C. gloeosporioides* and *C. frutícola*. The former causes banana anthracnose and leaf spot (38), while *C.* frutícola has been identified on mango plants (29). One of the main characteristics of *Colletotrichum* sp. infection in bananas is the difficulty in detecting the disease before fruit generation, given latency (37).

Diatrypaceae members like the genus *Diatrypella* are saprobes and pathogens associated with different hosts in both terrestrial and aquatic environments (14). Species of this genus are separated according to their entostrophic morphology and the number of ascospores they present (50). Fungal pathogenicity is moderate for this genus. This study firstly reports *Diatrypella vulgaris*, in banana. However, it is commonly isolated from diseased *Vitis vinifera*, causing necrotic lesions on the plant (40).

The fungal isolate *Nodulisporium* sp. produces nodilosporic acids with insecticidal properties and volatile antifungal substances used against other microorganisms through mycofumigation (48).

Fungi of *Nigrospora* sp. are host-specific phytopathogenic, endophytic and saprophytic species that produce bioactive secondary metabolites with antifungal activity (23). During isolations, *N. sphaerica* and *N. osmanthi* were isolated from the environment. *N. sphaerica* exhibits a violent spore-discharging mechanism that projects spores over long distances (57). Similarly, *Nigrospora oryzae* and *N. sphaerica* were identified from banana leaves (58). This study also firstly reports *N. osmanthi* in bananas.

Isolated fungi of the genus *Microdiplodia* sp. and *Cymatoderma* sp. have not previously been recorded in bananas. However, Pinheiro da Costa *et al.* (2021) detail the presence of *Microdiplodia* sp. with antifungal activity on Brugmansia suaveolens and *Cymatoderma* sp. as the only Basidiomycota reported in tropical rainforest (1).

Finally, the genus *Cladosporium* sp. comprises more than forty species, including pathogenic species causing leaf spot and saprophytic species acting on vegetation and soil (35). Isolated *C. cladosporioides* confirms this fungus as an endophyte isolated from foliar culture tissues of banana plants (58). In addition, *C. uredinicola*, also isolated in this study, presents small conidia formed with branched chains that facilitate its propagation over long distances (6), explaining its higher prevalence over other fungi. Another species identified, *C. tenuissimum* is an abundant saprobe in the tropics (56). Moubasher *et al.* (2016) state that the frequency of *Cladosporium* sp. isolation is moderate and peaks during winter, when humidity benefits banana development.

Conclusions

Considering the differential behavior of microorganisms and the number of strains found in the studied sites, the analysis focused on selecting sampling sites was appropriate.

The isolated microorganisms presented macroscopic and microscopic characteristics with different shapes, elevations, borders, consistencies and pigmentations. Different taxonomies belonged to the *Bacillus* and Coccus genera, 81% being Gram-negative and 19% Gram-positive.

References

1. Agudelo, M. B.; Calderón, M.; Betancourt, O.; Gallejo, Á. S. 2007. Hongos Macromycetes en dos rellictos de bosque húmedo tropical montano bajo de la vereda La Cuchilla. Marmato, Caldas. Museo de História Natural. 11: 19-31.
2. Alfaro, F. 2013. Aislamiento y cuantificación de bacterias epífitas del filoplano de banano (Musa AAA cv. Grande Naine) y selección de cepas quitinolíticas y glucanolíticas como potenciales antagonistas de *Mycosphaerella fijiensis*, agente causal de la Sigatoka negra.
3. Ayala, D.; Acevedo, C.; Enrique, B.; Aguilar, F.; Orlandoni, G. 2018. Seguimiento de la actividad PGPR de microorganismos inoculados en plantas de Sacha inchi (*Plukenetia volubilis*) bajo condiciones de vivero. Archivo digital de prácticas, Microbiología Industrial. Universidad de Santander UDES. 1-14. https://doi.org/10.13140/RG.2.2.25676.36489
4. Barrera, S.; Sarango, S.; Montenegro, S. 2019. The phyllosphere microbiome and its potential application in horticultural crops. A review. Revista Colombiana de Ciencias Hortícolas. https://revistas.uptc.edu.co/index.php/ciencias_horticolas/article/view/8405
5. Benavides-López, L. 2019. Cuantificación temprana de *Pseudocercospora fijiensis* por medio de qPCR en modelos predictivos de Sigatoka negra en plantas de banano (Musa AAA). 9-162.
6. Bensch, K.; Braun, U.; Groenewald, J. Z.; Crous, P. W. 2012. The genus Cladosporium. Studies in Mycology. 72(1): 1. https://doi.org/10.3114/SIM0003
7. Brown, K.; Hyde, K.; Guest, D. 1998. Preliminary studies on endophytic fungal communities of *Musa acuminata* species complex in Hong Kong and Australia. https://www. fungaldiversity.org/fdp/sfdp/FD_1_27-51.pdf
8. Carrión Abad, B. A. C. 2015. Análisis microbiológico del complejo orgánico activfol para determinar la efectividad en el control del hongo (*Mycosphaerella fijiensis*) causante de la Sigatoka negra en el banano.
9. Carrion Ramon, J. J. C. 2020. Monitoreo y control especial de la Sigatoka negra (*Mycosphaerella Fijiensis* M), en el cultivo de banano. UTMACH. Unidad Académica de Ciencias Agropecuarias. Machala. Ecuador. http://repositorio.utmachala.edu.ec/handle/48000/16135
10. Casas, J.; López, M.; Salinas, M.; Gisbert, J.; Giménez, E.; García, F.; Sánchez, S.; Lacalle, A.; Cortés, A.; Moyano, F. 2017. Guía para la realización de un estudio ambiental: El caso de la cuenca del río Adra (J. Casas, Ed.; Vol. 11). Universidad de Almería. 3qk3vTP
11. Ceballos, I.; Mosquera, S.; Angulo, M.; Mira, J.; Argel, L.; Uribe-Velez, D.; Romero-Tabarez, M.; Orduz-Peralta, S.; Villegas, V. 2012. Cultivable bacteria populations associated with leaves of banana and plantain plants and their antagonistic activity against *Mycosphaerella fijiensis*. Microbial Ecology. 64(3): 641-653. https://doi.org/10.1007/s00248-012-0052-8
12. Cruz-Martín, M.; Acosta-Suárez, M.; Roque, B.; Pichardo, T.; Castro, R. 2016. Diversidad de cepas bacterianas de la filosfera de Musa spp. con actividad antifúngica frente a *Mycosphaerella fijiensis* Morelet. Biotecnología Vegetal. 16(1): 53-60.

13. Cruz-Martín, M.; Alvarado, Y.; Mena, E.; Acosta, M.; Roque, B.; Pichardo, T. 2018. Cepas bacterianas con potencial para el manejo de la Sigatoka negra. Anales de la Academia de Ciencias de Cuba. 8(1). http://www.revistaccuba.sld.cu/index.php/revacc/article/view/374/373
14. Dissanayake, L. S.; Wijayawardene, N. N.; Dayarathne, M. C.; Samarakoon, M. C.; Dai, D. Q.; Hyde, K. D.; Kang, J. C. 2021. *Paraeutypella guizhouensis* gen. et sp. nov. and *Diatrypella longiasca* sp. nov. (Diatrypaceae) from China. Biodiversity Data Journal 9: e63864. https://doi.org/10.3897/BDJ.9.E63864
15. Doyle, J. J.; Doyle, J. L. 1987. A rapid DNA isolation procedure for samal quantities of fresh leaf tissue. Phytochemical Bulletin. 19: 11-15.
16. Ecuador, M. de C. E. del. 2017. Informe Sector Bananero Ecuatoriano. Ministerio de Comercio Exterior. 53(9): 1689-1699. https://doi.org/10.1017/CBO9781107415324.004
17. Etebu, E.; Young-Harry, W. 2011. Control of black Sigatoka disease: Challenges and prospects. African Journal of Agricultural Research. 6(3): 508-514. https://doi.org/10.5897/AJAR10.223
18. Faleiro, F. G.; Santos, I. S.; Bahía, R.; Santos, R. F.; Anhert, D. 2002. Otimização da extração e amplificação de DNA de *Theobroma cacao* L. visando obtenção de marcadores RAPD. 31-34.
19. Fravel, D.; Olivain, C.; Alabouvette, C. 2003. *Fusarium oxysporum* and its biocontrol. New Phytologist. 157(3): 493-502. https://doi.org/10.1046/J.1469-8137.2003.00700.X
20. García, F.; Pachacama, S.; Jarrín, D.; Iza, M. 2020. Guía andina para el diagnóstico de Fusarium Raza 4 Tropical (R4T) *Fusarium oxysporum f.sp. Cubense* (syn. *Fusarium odoratissimum*) agente causal de la marchitez por *Fusarium* en musáceas (plátanos y bananos). www.comunidadandina.org
21. Gauhl, F. 1994. Epidemiology and ecology of Black Sigatoka on plantain and banana (Musa spp.) in Costa Rica, Centro América. Ph.D. Thesis of the Systematisch Geobotanische-Institut der Georg-August-Universität Göttingen and Institut für Pflanzenpathologie und Pflanzenchutz der Georg-August-Universität Göttingen. INIBAP.
22. Guzmán, M. 2012. Control biológico y cultural de la sigatoka-negra. Tropical Plant Pathology. 37 (Suplemento). Agosto. https://doi.org/10.13140/2.1.2927.7442
23. Hao, Y.; Aluthmuhandiram, J. V. S.; Chethana, K. W. T.; Manawasinghe, I. S.; Li, X.; Liu, M.; Hyde, K. D.; Phillips, A. J. L.; Zhang, W. 2020. Nigrospora species associated with various hosts from Shandong Peninsula, China. Mycobiology. 48(3): 169-183. https://doi.org/10.1080/1229 8093.2020.1761747
24. Hernández-Montiel, L. G.; Larralde-Corona, C. P.; Vero, S.; López-Aburto, M. G.; Ochoa, J. L.; Ascencio-Valle, F. 2010. Caracterización de levaduras *Debaryomyces hansenii* para el control biológico de la podredumbre azul del limón mexicano. CYTA-Journal of Food. 8(1): 49-56. https://doi.org/10.1080/19476330903080592
25. Horra, M. L. P. 2014. Evaluación *in vitro* de la capacidad de extractos orgánicos de biodiversidad ecuatoriana para inhibir al patógeno *Mycosphaerella fijiensis* (Morelet) causante de la Sigatoka Negra en banano. http://repositorio.puce.edu.ec/bitstream/handle/22000/10936/4.42.000892.pdf?sequence=4
26. Intriago Mendoza, L. 2010. Identificación y evolución en laboratorio e invernadero de microorganismos antagonistas de Sigatoka negra *(Mycosphaerella fijiensis Morelet)* en musáceas en el Litoral Ecuatoriano. Tesis de Grado. Universidad Técnica de Manabí. Ecuador. 95 p.
27. Israeli, Y.; Lahav, E. 2016. Banana. In Encyclopedia of Applied Plant Sciences. Elsevier. 3: 363-381. https://doi.org/10.1016/B978-0-12-394807-6.00072-1
28. Izzeddin, A. N.; Medina, T. L. 2011. Efecto del control biológico por antagonistas sobre fitopatógenos en vegetales de consumo humano. Salus. 15(3): 8-18.
29. Lima, N. B.; Marcus, M. V.; Morais, M. A. D.; Barbosa, M. A. G.; Michereff, S. J.; Hyde, K. D.; Câmara, M. P. S. 2013. Five Colletotrichum species are responsible for mango anthracnose in northeastern Brazil. Fungal Diversity. 61(1): 75-88. https://doi.org/10.1007/s13225-013-0237-6
30. López, S. 2019. Bacillus un género que alberga especies que cumplen diversos roles biológicos. Infive-Conicet UNLP. 1-26.
31. Martínez, G.; Pargas, R.; Manzanilla, E. 2012. Orden Zingiberales: Las musáceas y su relación con plantas afines. Scielo. http://ve.scielo.org/scielo.php?script=sci_arttext&pid=S0002-192X2012000100014
32. Morillo, E.; Miño, G. 2011. Marcadores moleculares en biotecnología agrícola: manual de técnicas y procedimientos en INIAP. https://repositorio.iniap.gob.ec/bitstream/41000/848/4/iniapscm91.pdf
33. Moubasher, A. H.; Moharram, A. M.; Ismail, M. A.; Abdel-Hafeez, M. H. 2016. Aeromycobiota over banana plantations in Assiut Governorate and enzymatic producing potential of the most common species. Journal of Basic & Applied Mycology (Egypt). 7: 9-18.
34. Muimba-Kankolongo, A. 2018. Fruit Production. In Food crop production by smallholder farmers in Southern Africa (p. 275-312). Elsevier. https://doi.org/10.1016/B978-0-12-814383-4.00012-8

35. Nam, M. H.; Park, M. S.; Kim, H. S.; Kim, T. I.; Kim, H. G. 2015. *Cladosporium cladosporioides* and *C. tenuissimum* cause blossom blight in strawberry in Korea. Mycobiology. 43(3): 354. https://doi.org/10.5941/MYCO.2015.43.3.354
36. Novák, P.; Hřibová, E.; Neumann, P.; Koblížková, A.; Doležel, J.; Macas, J. 2014. Genome-wide analysis of repeat diversity across the family musaceae. PLoS ONE. 9(6). https://doi.org/10.1371/journal.pone.0098918
37. Nuangmek, W.; McKenzie, E. H. C.; Lumyong, S. 2008. Endophytic fungi from wild banana (*Musa acuminata* Colla) works against anthracnose disease caused by *Colletotrichum musae*. Research Journal of Microbiology. 3(5): 368-374. https://doi. org/10.3923/JM.2008.368.374
38. Photita, W.; Lumyong, S.; Lumyong, P.; Mckenzie, E. H. C.; Hyde, K. D.; Photita, W.; Lumyong, S.; Lumyong, P.; Hyde, M. E. H. C. 2004. Fungal diversity are some endophytes of *Musa acuminata* latent pathogens? Fungal Diversity. 16: 131-140.
39. Pinheiro da Costa, S.; Schuenck-Rodrigues, R.; da Silva Cardoso, V.; Valverde, S.; Vermelho, A.; Ricci-Júnior, E. 2021. Actividad antimicrobiana de hongos endofíticos aislados de *Brugmansia suaveolens* Bercht.; Presl, J. Research, Society and Development. 10(14): e113101421646. https://doi. org/10.33448/rsd-v10i14.21646
40. Pitt, W. M.; Trouillas, F. P.; Gubler, W. D.; Savocchia, S.; Sosnowski, M. R. 2013. Pathogenicity of Diatrypaceous Fungi on grapevines in Australia. Plant disease. 97(6): 749-756. https://doi.org/10.1094/PDIS-10-12-0954-RE
41. Poveda, I.; Cruz-Martín, M.; Sánchez-García, C.; Acosta-Suárez, M.; Leiva-Mora, M.; Roque, B.; Alvarado-Capó, Y. 2010. Caracterización de cepas bacterianas aisladas de la filosfera de Musa spp. con actividad antifúngica *in vitro* frente a *Mycosphaerella fijiensis*. Biotecnología Vegetal. 10(1): 57-61.
42. Ramírez, M.; Rodriguez, T.; Peniche, C.; Alfonso, L. 2010. Chitin and its derivatives as biopolymers with potential agricultural applications. Biotecnología Aplicada. 27: 270-276.
43. Riera, N. J. 2015. Caracterización molecular y de patogenicidad de *Colletotrichum* spp, en bananas var Cavendish y pruebas de antagonismo con *Trichoderma* spp., recolectadas en fincas bananeras de la región costa del Ecuador. Universidad San Francisco de Quito. 98.
44. Ruiz-Boyer, A.; Rodríguez-González, A. 2020. Preliminary list of fungi (Ascomycota and basidiomycota) and myxomycetes (myxomycota) of Isla del Coco, Puntarenas, Costa Rica. Revista de Biología Tropical. 68(S1): S33-S56. https://doi.org/10.15517/RBT.V68IS1.41165
45. SIPA. 2021. Ficha del cultivo de banano (*Musa paradisiaca* AAA). https://sipa.agricultura.gob.ec/index.php/bananos
46. Šišić, A.; Baćanović-Šišić, J.; Al-Hatmi, A. M. S.; Karlovsky, P.; Ahmed, S. A.; Maier, W.; Hoog, G. S. D.; Finckh, M. R. 2018. The 'forma specialis' issue in *Fusarium*: A case study in *Fusarium solani* f. Sp. Pisi. Scientific Reports 2018 8:1, 8(1), 1-17. https://doi.org/10.1038/s41598-018-19779-z
47. Sun, X.; Guo, L. D. 2012. Endophytic fungal diversity: Review of traditional and molecular techniques. Mycology. 3(1): 65-76. https://doi.org/10.1080/21501203.2012.656724
48. Suwannarach, N.; Kumla, J.; Bussaban, B.; Nuangmek, W.; Matsui, K.; Lumyong, S. 2013. Biofumigation with the endophytic fungus Nodulisporium spp. CMU-UPE34 to control postharvest decay of citrus fruit. Crop Protection. 45: 63-70. https://doi.org/10.1016/J.CROPRO.2012.11.015
49. Toju, H.; Tanabe, A. S.; Yamamoto, S.; Sato, H. 2012. High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples. PLoS ONE. 7(7): 40863. https://doi.org/10.1371/JOURNAL.PONE.0040863
50. Trouillas, F. P.; Úrbez-Torres, J. R.; Gubler, W. D. 2017. Diversity of diatrypaceous fungi associated with grapevine canker diseases in California. 102(2): 319-336. https://doi.org/10.3852/08-185
51. Vázquez, J.; González, J.; Castillo, J.; Álvarez, M. 2019. Microorganismos benéficos MOBs obtenidos de plantas, como promotores en la germinación de semillas. Dominio de Las Ciencias. 5: 615-628. http://dx.doi.org/10.23857/dc.v5i1.1064 Cie
52. Velasco, R.; Tapia, R. 2014. Curso práctico de Microbiología. Universidad Autónoma Metropolitana.
53. Vilgalys, R. 2003. Taxonomic misidentification in public DNA databases. New Phytologist. 160(1): 4-5. https://doi.org/10.1046/J.1469-8137.2003.00894.X
54. Villamil Carvajal, J. E. V.; Viteri Rosero, S. E. V.; Villegas Orozco, W. L. V. 2015. Aplicación de antagonistas microbianos para el control biológico de *Moniliophthora roreri* Cif & Par en *Theobroma cacao* L. bajo condiciones de campo. Revista Facultad Nacional de Agronomía Medellín. 68(1): 7441-7450. https://doi.org/10.15446/rfnam.v68n1.47830
55. Villegas-Escobar, V.; Ceballos, I.; Mira, J. J.; Argel, L. E.; Peralta, S. O.; Romero-Tabarez, M. 2013. Fengycin C produced by *Bacillus subtilis* EA-CB0015. Journal of Natural Products. 76(4): 503-509. https://doi.org/10.1021/np300574v
56. Walker, C.; Muniz, M. F. B.; Rolim, J. M.; Martins, R. R. O.; Rosenthal, V. C.; Maciel, C. G.; Mezzomo, R.; Reiniger, L. R. S. 2016. Morphological and molecular characterization of *Cladosporium cladosporioides* species complex causing pecan tree leaf spot. Genetics and molecular research: GMR. 15(3). https://doi.org/10.4238/GMR.15038714

57. Wang, M.; Liu, F.; Crous, P. W.; Cai, L. 2017. Phylogenetic reassessment of Nigrospora: Ubiquitous endophytes, plant and human pathogens. Persoonia: Molecular Phylogeny and Evolution of Fungi. 39(December). 118-142. https://doi.org/10.3767/persoonia.2017.39.06
58. Zakaria, L.; Aziz, W. N. W. 2018. Molecular Identification of endophytic fungi from banana leaves (Musa spp.). Tropical Life Sciences Research. 29(2): 201. https://doi.org/10.21315/TLSR2018.29.2.14

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