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Article *in* Journal of Plant Protection Research · January 2016

DOI: 10.1515/jppr-2016-0020

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Co-occurrence of pathogenic and non-pathogenic *Fusarium decemcellulare* and *Lasiodiplodia theobromae* isolates in cushion galls disease of cacao (*Theobroma cacao* L.)

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Received: October 31, 2015
Accepted: April 21, 2016

Abstract: Flowery cushion gall of cacao is a disease complex with six types. *Fusarium decemcellulare* have been isolated from both flowery and green point galls and recognized as the etiological agent of the disease. In the present work we: i) identified by ITS-rDNA sequencing and/or taxonomy the cultivable fungal species or Operative Taxonomic Units (OTUs) associated with the five symptoms of cushion galls in cacao from Venezuela, and ii) determined the gall inducing capacity on cacao peeled seeds after 45 days of inoculation with suspensions of mycelia/spores from distinct isolate types. The whole isolate collection rendered an abundance of 113 isolates with a richness of 39 OTUs (27) and eight identified at the species or genera levels, respectively, and in unidentified fungi. The dominant recovered species (≈36%) were *F. decemcellulare* and *Lasiodiplodia theobromae*. Some isolates of *F. decemcellulare, L. theobromae, F. equiseti, Fusarium* spp., *F. solani, F. incarnatum, Rhizoctonia solani* and *Penicillium* sp. were pathogenic. Some other isolates of the first six mentioned taxa behave as non-pathogenic. Furthermore, pathogenic and non-pathogenic isolates can also co-occur within a single plant and gall type. Moreover, 2–5 species within a single gall symptom in a single tree were identified (not necessarily at the same point in the tree), indicating a broad diversity of co-occurring taxa.

Key words: endophytic fungi, *Fusarium decemcellulare*, gall disease complex, *Lasiodiplodia theobromae*, mycobiota, *Theobroma cacao*

Introduction

Fungal diseases are major threats to cacao (*Theobroma cacao* L.) production. The cushion gall disease complex corresponds to abnormalities that occur on the trunk and branches, developing on and distorting the growth of flower cushions and affecting normal fruiting. Cushion galls have usually been considered a minor problem, but can be potentially serious in cacao (Gregory 1977; Ploetz 2007a). The cushion gall disease complex affected 90% of trees in 12 months in Costa Rica (Hutchins *et al*. 1959) and up to 15.5% of farms were infected with cushion galls in Venezuela in the 70ś (Parra *et al*. 2009). The cushion gall disease complex is most frequently associated with *Albonectria rigidiuscula* (Berk. & Broome) Rossman & Samuels (*Fusarium decemcellulare*, anamorph) (Hansen 1963; Hansen and Capriles 1963; Sabah Department of Agriculture 1972; Ploetz 2007b).

Five types of cushion galls have been described in cacao: flowery gall (FLG), green point gall (GPG), fan gall (FG), knob gall (KG), and a hard flat or disc gall (DG) (Brenes and Enriquez 1982; Phillips-Mora and Cerda 2009). In Venezuela, a lobular gall (LG) was also described, similar in its rounded appearance to the GPG, but its base is ridge-shaped or lobulated (Brenes and Enríquez 1982). The LG form no buds or flowers, since from the beginning they show woody tissue. Pictures available showing symptoms of the complex are mostly of GP galls. Figure 1 shows five out of those six gall types described. On the other hand, not all gall types are present or reported in all places. Thus, DG was not described in a recent catalog of cacao diseases in Central America (Phillips-Mora and Cerda 2009) and, according to Ploetz (2007b) it seems that all cushion galls are GPG. Cushion gall symptoms are mainly FLG (Hutchins *et al*. 1959) or GPG (Ploetz 2007b; Phillips-Mora and Cerda 2009). *Fusarium decemcellulare* is the species most often isolated from GPG (Bourret and Ford 1965; Ploetz 2007b; Pérez *et al.* 2012), FG (Matlick *et al.* 1999) and FLG (Bourret and Ford 1965), although some authors considered that the causal agent of FLG is not defined (Frison and Feliu 1989). *Fusarium decemcellulare* also behave as a saprophyte on cacao pods

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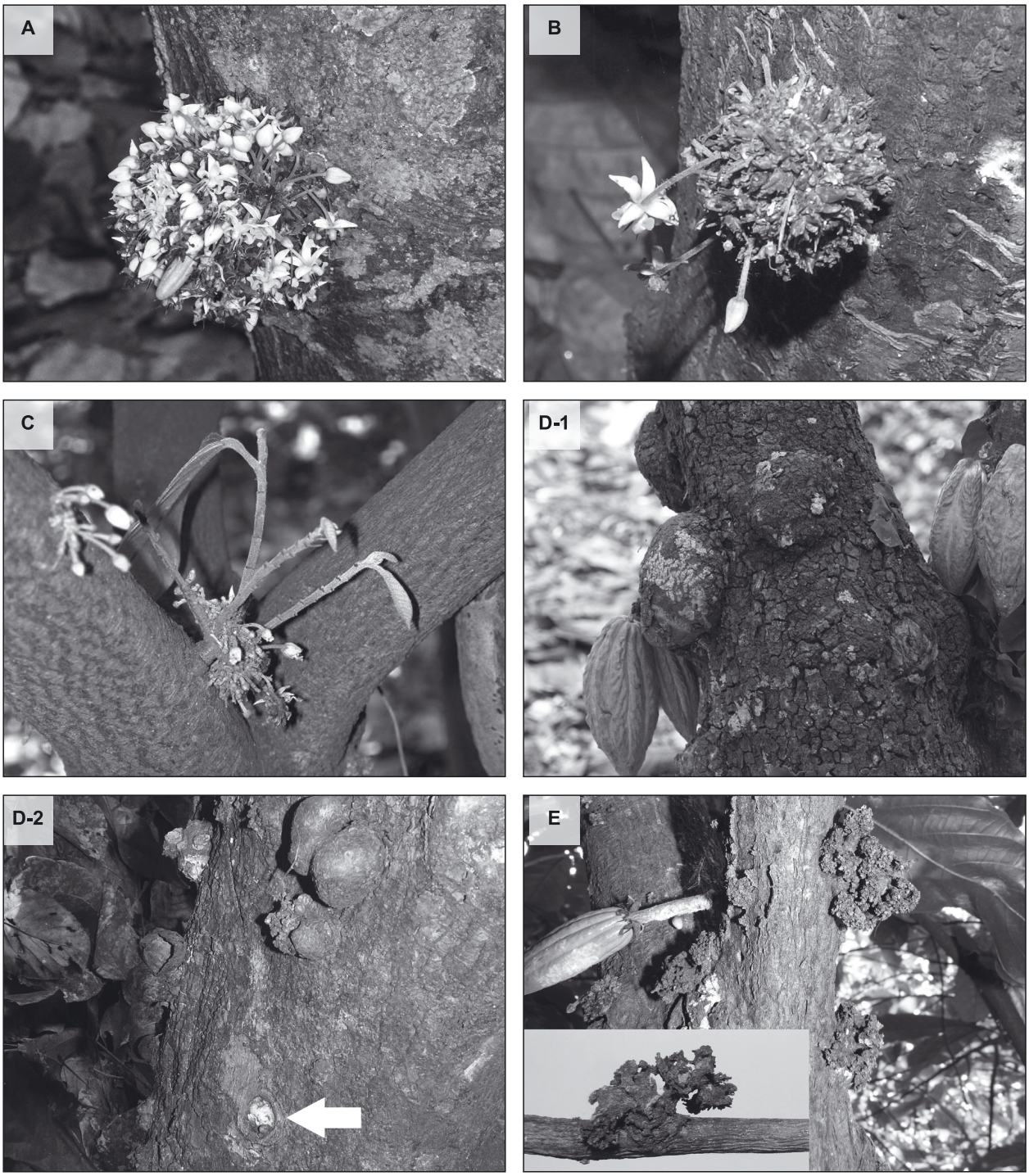

Fig. 1. Cacao cushion gall symptoms observed in field collections in Venezuela: flowery gall (A), green point gall (B), fan gall (notice nodes close together) (C), knob galls (D-1, hard to remove and D-2, easily removable) and lobular gall (E)

(Owen 1956) or as an endophyte in flowers, stems or pods (Alexander and Cook 1965; Evans *et al*. 2003; Mejía *et al*. 2008). Additionally, *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl., was occasionally isolated from gall-tissue during early investigations in Ghana (Brunt and Wharton 1960), and more recently in Cuba (Pérez *et al*. 2012).

Weak or opportunistic fungal pathogens, such as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *L. theobromae* or *F. decemcellulare* were isolated from non-symptomatic tissue (Evans *et al*. 2003; Rubini *et al*. 2005) and some isolates of *Colletotrichum*, *Xylaria*, and *Fusarium/Nectria* have been recognized as endophytes, and played a major role in protecting cacao trees from pathogens (Arnold *et al*. 2003). This points towards the interconversion of one fungal lifestyle into another while interacting with the plant system, meaning that the endophyte may become a parasite or *vice versa* (Rai and Agarkar 2014). *Fusarium decemcellulare* inhabits areas with no report of gall diseases on a Venezuelan mango plantation (Morales-Rondón and Rodríguez-González 2006). Therefore, this species as an endophyte may become a source of primary inoculum for disease dissemination. In this paper we: i) identified, by sequencing the internal transcribed spacers (ITS) and/or through morphological traits, the cultivable fungal community isolated from the cushion gall complex from a Venezuelan cacao plantation, and ii) determined the relationship of gall-inducing capacity with the isolate identity.

Materials and Methods

Symptoms survey and fungal isolations

Cushion galls from mature cacao (*T. cacao*) trees were collected from November 2006 to November 2008. Localities (17) and trees were chosen following a convenience sampling strategy. Galls were stored in a plastic box with ice until processed in a laboratory. Most isolates came from farms in Aragua and Miranda (Central Region) and Zulia ("Sur del Lago de Maracaibo" Region) states (Table 1). Within 24–48 h of sample harvest, a protocol described for endophytic isolation (Arnold *et al*. 2000) was generally followed. Each gall+stem piece was peeled (cortex elimination) with a disinfected and surface-flamed blade and/or knife. In order to reduce saprophytic and bacterial contamination, 2 mm of woody tissue segments of the internal part of each gall were surface-sterilized with 0.52% sodium hypochlorite solution for 3 min. After washing twice in sterile distilled water for 3 min, these fragments were dried with sterile Whatman No. 1 filter paper. Four pieces of each gall were arbitrarily selected and each one was placed on a separate Petri dish containing 2% malt agar. Then they were incubated in the dark at room temperature (24±2°C). Each segment was assessed for fungal growth daily for 7 days. Hyphal tips from visually distinct colonies emerging from each plate were sub-cultured on Potato Dextrose Agar (PDA) plates to obtain pure colonies (isolates). According to this isolation procedure, pathogenic and endophytic isolates were presumed to be within the isolate collection. A total of 113 isolates from cushion galls from trunks and branches were obtained (see supplementary archive for details) and screened for their morphologic characteristics. Isolates were conserved in saline solution (0.9% NaCl) and stored at room temperature until use. Living vouchers are conserved at the Phytopathology Lab at the Advanced Studies Institute (IDEA) in Caracas.

Isolate identification

Macro and microscopic morphological traits and/or molecular identification were determined on all conserved isolates (see supplementary archive for details). For this purpose, isolates were grown in the dark at 26±2°C for 7 days. Morphological traits of spore, colony (hyphal height and depth, form, surface texture, margin characters, growth rings, color of mycelium exudate), color of the pigment released into the media and growth rate were evaluated. Representative isolates of morphological groups were identified on PDA and SNA (Spezieller Närstoffarmer Agar).

DNA extraction

Fungi cultures were grown in a shaking incubator at 27°C for 3–5 days in 250 ml conical flasks containing 50 ml of culture media (7.0 g malt extract, 1.0 g Bacto peptone, 0.5 g yeast extract, in 1 l dH_20). Mycelia were harvested by filtration (Whatman filter paper), washed with sterile distilled water, freeze-dried and ground. Total genomic DNA was extracted from these mycelia according to Naranjo *et al*. (2007). The obtained DNA pellet was kept in 50 µl TE buffer at –20°C.

Amplification and sequencing of fungal internal transcribed spacers (ITSs)

ITS amplifications for sequencing were carried out by polymerase chain reaction (PCR) in a Mastercycler Eppendorf thermal cycler using ITS1 and ITS4 universal primers (White *et al*. 1990). The amplified fragments include ITS1, 5.8S and ITS2 regions of rDNA. Each PCR was performed in a 40 µl reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1,5 mM $MgCl_2$, 0.2 mM dNTPs, 0,25 µM primers, 1U GoTaq® Flexi Polimerasa, and 40 ng of DNA. Amplifications were made 2–3 times in order to assess the reproducibility of the method. The thermal cycling program was as follows: initial denaturation at 94°C for 2 min, followed by 35 amplification cycles (denaturation at 94°C for 40 s, primer annealing at 53°C for 40 s and extension at 72°C for 40 s), and final extension at 72°C for 10 min. PCR products were purified with AccuPrep®PCR Purification Kit (BioNEER). Direct amplicon sequencing was performed on an ABI 3100 automated sequencer following manufacturer's instructions (Applied Biosystems, Inc.).

Table 1. Origin of the isolates from cushion gall types on mature trees of *Theobroma cacao* in Venezuela

Gall types	Regions			Total general
	Central	Oriental	Maracaibo	
Fan G (FG)	4	–	43	48
Green Point G (GPG)	12	–	15	27
Unidentified G	8	3	2	12
Knob G (KG)	4	–	6	10
Flowery G (FG)	11	–	–	10
Disc G (DG)	3	–	–	3
WB* on cushion	2	–	–	2
Total	44	3	66	113

*Witches' Broom

Table 2. Fungal Operative Taxonomic Units (OTUs) isolated from cacao cushion galls and identified by morphological observations or similarities to ITS1-5.8S-ITS2 sequences at Genbank

No.	Isolate	Genbank	Pathogenicity	Collection date	Operative Taxonomic Unit	Gall type
1	C218 +	KU377469	–	Aug-2008	*Fusarium decemcellulare*	disc
2	C064 * +	KU377518	–	Aug-2007	*F. solani*	disc
3	C060 *	–	–	Jul-2007	*Fusarium* sp.	disc
4	D055 * +	KU377447	doubtful	May-2008	*F. solani*	fan
5	D053 +	KU377490	doubtful	May-2008	*Lasiodiplodia theobromae*	fan
6	D073 +	KU377495	doubtful	May-2008	*L. theobromae*	fan
7	D080 +	KU377499	doubtful	May-2008	*F. decemcellulare*	fan
8	D066 * +	KU377446	doubtful	May-2008	*F. decemcellulare*	fan
9	D074 * +	KU377452	not pathogenic	May-2008	*F. decemcellulare*	fan
10	D076 * +	KU377453	not pathogenic	May-2008	*F. decemcellulare*	fan
11	D025 * +	KU377454	not pathogenic	May-2008	*F. incarnatum*	fan
12	D061 * +	KU377456	not pathogenic	May-2008	*Clonostachys rosea f. catenulata*	fan
13	D006 +	KU377474	not pathogenic	May-2008	*Diaporthe phaseolorum*	fan
14	D008 +	KU377475	not pathogenic	May-2008	*L. theobromae*	fan
15	D009 +	KU377476	not pathogenic	May-2008	*Phomopsis sp.*	fan
16	D030 +	KU377485	not pathogenic	May-2008	*Cylindrocladium quinqueseptatum*	fan
17	D033 +	KU377486	not pathogenic	May-2008	*Fusarium* sp. 4	fan
18	D065 * +	KU377442	not pathogenic	May-2008	*L. theobromae*	fan
19	D007 +	KU377487	not pathogenic	May-2008	*Botryosphaeria mamane*	fan
20	D043 +	KU377488	not pathogenic	May-2008	*L. theobromae*	fan
21	D077 +	KU377496	not pathogenic	May-2008	*Fusarium* sp. 4	fan
22	D078 +	KU377497	not pathogenic	May-2008	*F. equiseti*	fan
23	D052 +	KU377524	not pathogenic	May-2008	*F. decemcellulare*	fan
24	D072 * +	KU377455	pathogenic	May-2008	*F. incarnatum*	fan
25	D054 * +	KU377458	pathogenic	May-2008	*Rhizoctonia solani*	fan
26	D064 * +	KU377459	pathogenic	May-2008	*R. solani*	fan
27	D063 +	KU377493	pathogenic	May-2008	*L. theobromae*	fan
28	D062 +	KU377525	pathogenic	May-2008	*Calonectria sulawesiensis*	fan
29	D026 *	–	pathogenic	May-2008	*R. solani*	fan
30	D045 *	–	pathogenic	May-2008	*F. decemcellulare*	fan
31	D047 *	–	pathogenic	May-2008	*R. solani*	fan
32	D056 *	–	pathogenic	May-2008	*R. solani*	fan
33	D069 *	–	pathogenic	May-2008	*R. solani*	fan
34	D100 *	–	pathogenic	Oct-2008	*Penicillium* sp.	fan
35	D101 * +	KU377448	–	Oct-2008	*Cladosporium cladosporioides*	fan
36	D075 * +	KU377450	–	May-2008	*F. camptoceras*	fan
37	C219 +	KU377470	–	Aug-2008	*F. solani*	fan
38	D002 +	KU377473	–	May-2008	*Diaporthe phaseolorum*	fan
39	D028 +	KU377484	–	May-2008	*F. equiseti*	fan
40	D046 +	KU377489	–	May-2008	*F. decemcellulare*	fan
41	D068 +	KU377494	–	May-2008	*L. pseudotheobromae*	fan
42	D079 +	KU377498	–	May-2008	*F. equiseti*	fan
43	D004 +	KU377516	–	May-2008	*L. theobromae*	fan
44	D032 +	KU377522	–	May-2008	*Calonectria sulawesiensis*	fan
45	D031 *	–	–	May-2008	*Cylindricladium* sp.	fan
46	D003 *	–	–	May-2008	*Diaporthe* sp.	fan
47	D001 *	–	–	May-2008	*F. decemcellulare*	fan
48	C232 *	–	–	Oct-2008	*Phlebiopsis flavidoalba*	fan
49	D071 *	–	–	May-2008	*R. solani*	fan
50	D040 *	–	–	May-2008	*L. theobromae*	fan
51	D082 +	KU377500	doubtful	Aug-2008	*F. equiseti*	flowery
52	C013 * +	KU377460	not pathogenic	Mar-2007	*L. theobromae*	flowery
53	C239 +	KU377471	not pathogenic	Nov-2008	*F. solani*	flowery
54	C073 * +	KU377451	pathogenic	Aug-2007	*F. decemcellulare*	flowery
55	D081 * +	KU377440	pathogenic	Aug-2008	*F. decemcellulare*	flowery
56	C173 +	KU377467	–	Aug-2008	*Arthrinium* sp.	flowery
57	C208 +	KU377468	–	Aug-2008	*F. decemcellulare*	flowery

Table 2. *Continuation*

No.	Isolate	Genbank	Pathogenicity	Collection date	Operative Taxonomic Unit	Gall type
58	CT005 +	KU377472	–	Sep-2007	*Trichoderma reesei*	flowery
59	D094 * +	KU377443	–	Aug-2008	*F. decemcellulare*	flowery
60	D095 *	–	–	Aug-2008	*F. solani*	flowery
61	C052 *	–	–	Jun-2007	*Fusarium* sp.	flowery
62	D092 * +	KU377438	doubtful	Aug-2008	*F. solani*	green point
63	D051 * +	KU377445	doubtful	May-2008	*Fusarium* sp. 2	green point
64	D018 +	KU377481	not pathogenic	May-2008	*Fusarium* sp. 4	green point
65	D058 +	KU377492	not pathogenic	May-2008	*F. decemcellulare*	green point
66	D111 +	KU377509	not pathogenic	Oct-2008	*L. theobromae*	green point
67	D109 *	–	not pathogenic	Oct-2008	*F. decemcellulare*	green point
68	D048 +	KU377523	pathogenic	May-2008	*L. theobromae*	green point
69	D021 * +	KU377449	–	May-2008	*L. theobromae*	green point
70	C051 +	KU377461	–	Jun-2007	*Fusarium oxysporum*	green point
71	C059 +	KU377462	–	Jul-2007	*Fusarium* sp. 1	green point
72	D016 +	KU377480	–	May-2008	*F. camptoceras*	green point
73	D020 +	KU377482	–	May-2008	*L. theobromae*	green point
74	D022 +	KU377483	–	May-2008	*L. theobromae*	green point
75	D057 +	KU377491	–	May-2008	*Fusarium solani*	green point
76	D090 * +	KU377503	–	Aug-2008	*L. theobromae*	green point
77	D091 * +	KU377504	–	Aug-2008	*Ceratocystis paradoxa*	green point
78	D049 * +	KU377444	–	May-2008	*F. decemcellulare*	green point
79	D102 +	KU377508	–	Oct-2008	*Pestalotiopsis* spp.	green point
80	D113 +	KU377510	–	Oct-2008	*F. solani*	green point
81	V302 +	KU377515	–	Nov-2008	*F. decemcellulare*	green point
82	D017 * +	KU377519	–	May-2008	*Fusarium* sp. 4	green point
83	D019 +	KU377520	–	May-2008	*Fusarium* sp. 3	green point
84	D023 +	KU377521	–	May-2008	*Fusarium* sp. 4	green point
85	D060 *	–	–	May-2008	*F. solani*	green point
86	D112 *	–	–	Oct-2008	*F. solani*	green point
87	D114 *	–	–	Oct-2008	*F. solani*	green point
88	D110 *	–	–	Oct-2008	*Phytohthora palmivora*	green point
89	D012 +	KU377477	not pathogenic	May-2008	*L. theobromae*	knob
90	D015 +	KU377479	not pathogenic	May-2008	*Ceratobasidium* sp.	knob
91	D014 +	KU377478	pathogenic	May-2008	*F. equiseti*	knob
92	C128 +	KU377464	–	jan-2008	*T. harzianum*	knob
93	C157 +	KU377465	–	Aug-2008	*Fungal* sp.	knob
94	D085 +	KU377501	–	Aug-2008	*L. theobromae*	knob
95	C234 * +	KU377517	–	Nov-2008	*Clonostachys rosea f. catenulata*	knob
96	V010 *	–	–	Nov-2006	*Aspergillus* sp.	knob
97	D013 *	–	–	May-2008	*F. decemcellulare*	knob
98	D010 *	–	–	May-2008	*Fusarium* sp.	knob
99	D088 +	KU377502	doubtful	Aug-2008	*F. decemcellulare*	unidentified
100	D099 +	KU377507	not pathogenic	Aug-2008	*Colletotrichum gloeosporioides*	unidentified
101	D096 * +	KU377441	pathogenic	Aug-2008	*F. decemcellulare*	unidentified
102	D089 * +	KU377439	–	Aug-2008	*Fusarium* sp. 4	unidentified
103	C009 +	KU377457	–	mar-2007	*L. theobromae*	unidentified
104	D097 +	KU377505	–	Aug-2008	*L. theobromae*	unidentified
105	D098 * +	KU377506	–	Aug-2008	*Ceratocystis paradoxa*	unidentified
106	I037 * +	KU377511	–	Apr-2007	*C. gloeosporioides*	unidentified
107	I038 +	KU377512	–	Apr-2007	*Podospora austroamericana*	unidentified
108	I039 +	KU377513	–	Apr-2007	*Diaporthe phaseolorum*	unidentified
109	V114 * +	KU377514	–	Apr-2007	*L. theobromae*	unidentified
110	C062 *	–	–	Jul-2007	*Cylindrocladium scoparium*	unidentified
111	V112 *	–	–	Apr-2007	*Lasiodiplodia theobromae*	unidentified
112	C118 +	KU377463	–	Nov-2007	*Fusarium proliferatum*	witches´broom on cushion
113	C163 +	KU377466	–	Aug-2008	*Annulohypoxylon stygium*	witches´broom on cushion

* identity of some OTUs were determined by morphological analysis of the taxonomist Rafael Castañeda (INIFAT, Cuba)

+only 88 out of 113 isolates were sequenced and submitted to GenBank. The name was assigned according to the closest hit in Genbank

*+ the priority to assign a name to the OTU was morphology over GenBank identification

DNA sequence assembly and alignment

The ITS1-5.8S-ITS2 sequences were edited with Chromas Pro version 1.41 y BioEdit (Sequence Alignment Editor). Alignments were inspected and manually adjusted when necessary. Similarity was inspected for each sequence against the non-redundant database maintained by the National Center for Biotechnology Information using the BLAST algorithm (http://www.ncbi.nlm.nih.gov). GenBank accession numbers for 88 nucleotide sequences are KU377438-KU377525 (Table 2).

Gall-inducing capacity

Mycelia/spores of the 53 grown isolates were inoculated on seeds (Hansen 1963) of cacao clone IMC-67xOC-61 from the clonal garden of INIA-Miranda. Inoculations were carried out by immersing whole, peeled, scarified cacao seeds in a 5×10^6 cfu $\cdot$ ml^{-1} solution for 24 h. Inocula were prepared by scraping two plates of each isolate (PDA, 7–10 day, darkness) after sowing with a flamed scalpel and washing with 20 ml of sterile distilled water. After inoculation, the seeds were sowed in a disinfected (water at 80°C) mix of organic matter and soil contained in plastic bags, which were then placed in a greenhouse under approximately 60% shade. Five plants per isolate were used and weekly observations were recorded for gall development for two months after sowing. Then, the gall-inducing capacity of each isolate was screened as early as 21 days (Hansen 1963) and 45 days after inoculation.

Results

Cushion galls in the field

All five of the main symptoms of cushion galls (Brenes and Enríquez 1982; Phillips-Mora and Cerda 2009) were seen in the field. Figure 1 shows the observed symptoms of naturally infected cacao trees. Although disease incidence was not recorded, in general, FLG and GPG were more widely distributed and more common in cacao plantations in Venezuela. In the same way, we found that the severity and distribution of the symptoms varied from one locality to another. Knop gall was widely distributed, but less frequent than FLG and GPG. Fan gall was common within the Central and "Sur del Lago" Regions. Disc gall occurred only in Miranda state. Two types of KG were observed (Fig. 1): D-1 type (hard to remove), and D-2 type (easily removable). Nevertheless, almost 30% of the trees on a farm in Trincheras (located in Carabobo state, Central Region) were infected with KG, an unususally large quantity of diseased trees. The farmer referred to these trees as "male trees". From LG (Fig. 1E) only an isolate of *Fusarium* was purified, but it was lost in the meantime.

Identified species

From cushion galls, 113 isolates were purified, from which 28 isolates were identified at the species level, nine at the genera level and four as fungal endophytes in Genbank. In summary, an abundance of 113 and a richness of 39 OTU (Table 2). *Fusarium* spp. and *Lasiodiplodia* spp. with 58 and 22 isolates, respectively, were the dominant genera. The dominant species were *F. decemcellulare* and *L. theobromae* (Table 2), representing about 36% of the total isolates. There were high proportions of singletons (OTU that appeared only once), nearly 21%. The two samples of galls with broom-like growths on cushions (Table 1) yielded a *Fusarium proliferatum* isolate (C118) from Carabobo state and a *Annulohypoxylon stygium* (C163) from Miranda state, both in the Central Region (Table 2).

Diversity of cultivable fungal species within galls

Fan gall and GPG showed the highest isolate abundance and taxa richness per gall symptom (Fig. 2), presumably because they were the most gall types sampled. From 47 fungal isolates of FG, about 20 OTU were differentiated (Fig. 2, Table 2), within GPG the proportion was 27/9, in

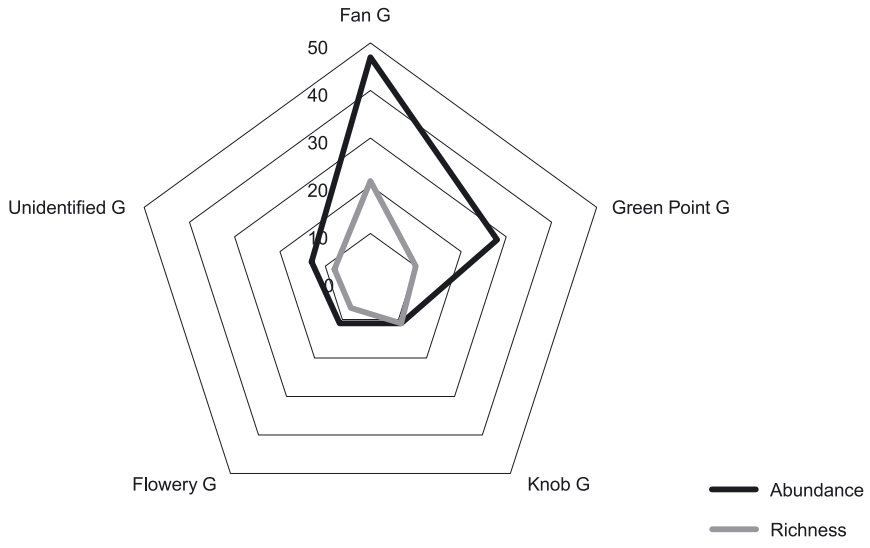

Fig. 2. Abundance (number of isolates) and richness (number of taxa) of 113 cultivable fungal isolates from different cushion galls (G) types of cacao in Venezuela. Unidentified galls, means galls that were not classified in the sampling

Table 3. Species identified and pathogenicity of isolates from single different gall types and plants of *Theobroma cacao*

Operative Taxonomic Unit	Plants with fan gall*							Plants with green point gall				
	1**	2	3	4	5	6	7	1	2	3	4	5
Calonectria sulawesiensis	–	–	–	–	D62	–	–	–	–	–	–	–
Fusarium camptoceras	–	–	–	–	–	–	●	●	–	–	–	–
F. decemcellulare	–	D45***	D52	–	–	D66	D74, D76, D80****	–	–	●	D58	–
F. equiseti	●	–	–	–	–	–	D78	–	–	–	–	–
F. incarnatum	D25	–	–	–	–	–	D72	–	–	–	–	–
F. solani	–	–	–	D55	–	–	–	–	–	–	●	D92
Clonostachys rosea f. *catenulata*	–	–	–	–	D61	–	–	–	–	–	–	–
Lasidioplodia pseudotheobromae	–	–	–	–	–	●	–	–	–	–	–	–
L. theobromae s.l.	–	●	D53		D63, D65****	–	D73	–	●	D48	–	●
Rhizocthonia solani	D26	D47	–	D54, D56	D64	D69	–	–	–	–	–	–
Ceratocystis paradoxa	–	–	–	–	–	–	–	–	–	–	–	●

*most plants were collected in a single day from Zulia state (at 01.05.08 in Moralitos, Colón); but plant 5 with green point gall from Aragua state (at 11.08.08 in Ocumare de la Costa)

**from each plant, all isolates listed from a single symptom type, but not necessary the same gall, were identified

***results from pathogenicity tests. Isolates number underlined indicate an isolate with gall inducing capacity in seedlings of 45 days old; not underlined – non-pathogenic. Other isolates were isolated but not tested (●)

****indicate co-ocurrence of pathogenic and non-pathogenic isolates of the same species in the same plant

KG, 10/9, in FLG 11/7 in DG 3/3. Table 3 shows more details on diversity, when the origin of some isolates was traced back to the same symptom and single plants. Up to five species within a single gall symptom in a single plant were identified, indicating a broad diversity of taxa that co-occurred within a single gall symptom. For example, in FG from plant 7, at least 5 different species were observed: *F. decemcellulare* (isolates D74, D76 and D80), *F. camptoceras* Wollenw. & Reinking (D75), *F. incarnatum* (Desm.) Sacc. (D72), *L. theobromae* (D73) and *Fusarium* sp. (D77).

Pathogenicity test

Fifteen out of 53 tested isolates showed gall inducing capacity in 3–5 evaluated seedlings as early as 21 days after inoculation (Fig. 3, Tables 2 and 4). The other nine isolates induced galls in only 1–2 plants out of 5, and were recorded as doubtful as gall inducers. Most of the inoculated isolates (about 59%) showed no symptoms on any of the seedlings at 45 days after seed inoculations. *Fusarium decemcellulare, L. theobromae, F. equiseti* (Corda) Sacc., *Fusarium* spp., *F. solani* (Mart.) Sacc., *F. incarnatum, Rhizocthonia solani* J.G. Kühn and *Penicillium* sp. were all capable of inducing galls in cacao seedlings (Table 4, Fig. 3). Apart from the pathogenic isolates, there were also non-pathogenic isolates within these taxa (Table 4). A closer look showed that pathogenic and non-pathogenic isolates co-occurred in the same gall symptom of a single plant (Table 3). From isolates D74 and D80 of *F. decemcellulare* from plant 7, only D74 induced galls on seedlings after 45 days of inoculation. The same happened for two isolates of *L. theobromae* (D63 pathogenic and D65 non-pathogenic) from plant 5. *Rhizoctonia solani* is a pathogen not usually associated with cacao, but peculiarly it was found in our study, since its six isolates were pathogenic (Table 4).

Discussion

On healthy *T. cacao* plants, foliar endophyte assemblages (different fungal OTU at very fine scales within the matrix) were demonstrated on the basis of species composition, leaf age and host species (Arnold and Herre 2003; Herre *et al*. 2007), although 1–3 species predominated (5.8% of the morphotaxons corresponding to 60% of total isolates). The most frequent species varies between sites of study: *Colletotrichum gloeosporioides* s.l. (*C. tropicale*) in Panamá (Mejía *et al*. 2008); *Trichoderma, Pestalotiopsis* and *Fusarium* in Brazil (Rubini *et al*. 2005; Hanada *et al*. 2010). *Coprinellus* sp. was the most common in a collection of isolates from Latin America and West Africa (Crozier *et al*. 2006). Our results, from diseased plants, and limited to cacao cushions galls, showed two dominant species which represent 5.1% of the total OTU and about 36% of the total isolates (Table 2). Thus, our data on diseased plants resemble the broad picture of healthy cacao plants in nature, with a high number of uncommon species and a few dominant ones.

We also observed that within the most common species (*F. decemcellulare* and *L. theobromae*) some isolates induced galls and other did not, even in the same gall type from the same plant (Tables 3 and 4). Thus, other species distinct from the traditional *F. decemcellulare* could be

Table 4. Gall inducing capacity* of *Fusarium decemcellulare*, *Lasidioplodia theobromae* and other cultivable fungi isolated from gall symptoms on cacao seedlings

Species/Genus	Non-pathogenic	Pathogenic	Doubtful	Total
Fusarium decemcellulare	5	4	3	12
Lasidioplodia theobromae s.l.	9	2	2	13
F. equiseti	2	1	1	4
Fusarium spp.	3	–	1	4
Rhizoctonia solani	–	6	–	6
F. solani	1	–	2	3
F. incarnatum	1	1	–	2
Penicillium sp.	–	1	–	1
Botryosphaeria mamane	1	–	–	1
Ceratobasidium sp.	1	–	–	1
Colletotrichum gloesporioides s.l.	1	–	–	1
Cylindrocladium quinqueseptatum	1	–	–	1
Diaporthe phaseolorum	1	–	–	1
Clonostachys rosea f. catenulata	1	–	–	1
Calonectria sulawesiensis	–	1	–	1
Phomopsis sp.	1	–	–	1
Total	**29**	**15**	**9**	**53**

*pathogenic – isolate that induce galls in at 3–5 seedling at 45 days after seeds inoculations; doubtful – induced gall only in 1–2 seedling; non-pathogenic – no symptoms at all

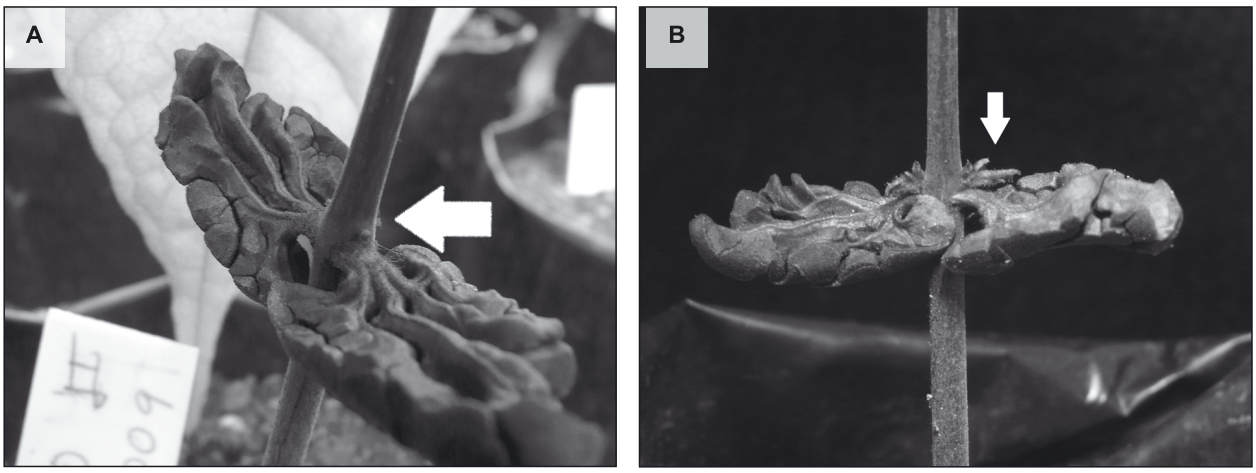

Fig. 3. Seedlings of *Theobroma cacao* inoculated with water showing no bud swelling (A), and with a pathogenic isolate (B) showing early gall at 21 days after seed inoculation

responsible for the variety of symptoms in the cushion gall complex, mainly *L. theobromae*. In previous research, *F. decemcellulare* and *L. theobromae* were isolated together in cacao galls in Cuba, but the latter was considered non-pathogenic, since humid chamber isolation yields only the former species (Pérez *et al*. 2012). Furthermore, no significant association of *L. theobromae* with cushion gall disease was reported (Alexander and Cook 1965; Brenes and Enríquez 1982; Ploetz 2007b) until the present work.

In particular cases, fan galls are similar in appearance to very small witches' brooms, caused by *Moniliophthora perniciosa* (Stahel) Aime & Phillips-Mora (Frison and Feliu 1989; Pérez *et al*. 2012), but these primarily develop from terminal or leaf axillary buds. None of the two galls with broom-like growths (Table 1) sampled in our study yielded *M. perniciosa*, thus it seems that they were fan galls. The tissues colonized by this species lead to swelling and the formation of brooms within flower cushions, thus distinguishing between witches' broom and FG may be difficult (Matlick *et al*. 1999; Pérez *et al*. 2012).

One of the aims of phytopathology is the identification of etiological agents for specific diseases. Our approach did not follow the classic way to determine the etiological agent of each gall symptom. Instead, we tried to isolate all cultivable fungi associated with each symptom. Additionally, we inoculated not only the more frequent OTU, but also rare and renewed saprophytic specimens. On these bases, we also found that rare species in

cacao, such as *F. equiseti*, *F. solani*, *F. incarnatum*, *R. solani* and *Penicillium* sp. could have a pathogenic behavior in this host. *F. equiseti* have been reported in Malaysia; *F. incarnatum* in Nicaragua, Papua New Guinea and Tanzania; and *F. solani* in Ecuador, Malaysia and Tanzania (Farr and Rossman 2015), all of them in *T. cacao*. *Rhizoctonia solani* is an uncommon species reported in cacao, although it has been previously isolated from affected cushions of cacao in Malaysia (Sabah Department of Agriculture 1972), but it has not been further investigated. This fungus has also been isolated from leaf necrosis of the same host species in Costa Rica (Salas 1962), as Kochś postulates demonstrated. This fungus species has also been reported in a check list of pathogens in Ghana and Papua New Guinea (Farr and Rossman 2015).

Little progress in the knowledge of cushion gall disease in cacao has been achieved since the intensive 60–70ś reports. The co-occurrence of i) several species and ii) pathogenic and non-pathogenic isolates within cushion galls, even from the same species, plus iii) the endophytic behavior of *F. decemcellulare* and *L. theobromae* in cacao raises many questions about the complexity of host-endophyte-pathogen interactions. Knowledge gained from molecular and biochemical studies of plant-pathogen interactions need to be coupled with new results of endophyte-plant interactions (Mejía *et al*. 2014). In a broad sense, classical plant pathology, with over a century of experience, should start contextualizing knowledge about the "disease triangle" (Agrios 2005) together with advances in knowledge about endophytic microbial communities in plant ecology. The presence of endophytic fungi plays a role in host adaptation to the environment (Arnold *et al*. 2000; Mejía *et al*. 2008; Rodriguez *et al*. 2009; Kembel and Mueller 2014). What once was analyzed as only a plant, is now conceived as a plant + endophyte (Saikkonen *et al*. 1998; Herre *et al*. 2007). Herre *et al*. (2007) argued that if the fungal endophytic effects on their hosts turn out to be large (*sensu* host defense), then a ''plant'' response is more than a plant alone.

The combined ITS1, 5.8S, and ITS2 regions of the genomic rRNA gene has been applied efficiently for identification of some species (Jamali and Nasimi 2014), but some limitations to resolve identities at the species level has also been reported (Arzanlou *et al*. 2015). However, our data with this technique showed enough species richness to think that there is more than *F. decemcellulare* in cushion galls of cacao. The present study describes co-occurrence of pathogenic and non-pathogenic isolates from several fungus species within a single cushion gall of cacao. Furthermore, it is a challenge to understand how endophytic and pathogenic behavior of the same species influences the response of cacao plants to the infections (gall type) with regard to *F. decemcellulare*, *L. theobromae* and some other taxa. This study had a more "ecological" approach than "phytopathological". Taking into consideration our findings, and the fact that the cushion gall complex seems to be a group of similar diseases or one disease with varied symptoms (Matlick *et al*. 1999), a complete etiological description of the cushion gall complex of cacao remains to be made.

Acknowledgements

This research was funded by Science, Technology and Intermediate Industries Ministry of Venezuela as part of the Route of the Chocolate Project No. 200500898. Last author thanks PROMETEO-SENESCYT Program of Ecuador for financial support during the writing of this article. We are grateful to the taxonomist Rafael Castañeda (INIFAT, Habana, Cuba) for identify some isolates, to Raisa Rumbos for pictures of galls, to Bárbara Gutiérrez for providing isolates from Sucre state, and to María A. Istúriz, Jorge Sarmiento, Tomás D. Fariñas, José Lara, Diamaris Domínguez, Diego Quito and Robert Álvarez for field and laboratory assistance. We are also grateful to an anonymous reviewer for his suggestions.

References

Agrios G.N. 2005. Plant Pathology. Elsevier, Academic Press, USA, 952 pp.

Alexander J.V., Cook R.J. 1965. Especies de *Fusarium* y *Calonectria* asociadas con la buda del cacao en Centro América. [*Fusarium* species and *Calonectria* associated with cushion gall of cacao in Central America]. Cacao (Costa Rica) 10 (1): 16–17. (in Spanish)

Arnold A.E., Herre E.A. 2003. Canopy cover and leaf age affect colonization by tropical fungal endophytes: Ecological pattern and process in *Theobroma cacao* (Malvaceae). Mycologia 95 (3): 388–398.

Arnold A.E., Maynard Z., Gilbert G.S., Coley P.D., Kursar T.A. 2000. Are tropical fungal endophytes hyperdiverse? Ecology Letters 3 (4): 267–274.

Arnold A.E., Mejía L.C., Kyllo D., Rojas E.I., Maynard Z., Robbins N., Herre E.A. 2003. Fungal endophytes limit pathogen damage in a tropical tree. Proceedings of the National Academy of Sciences of the United States of America 100 (26): 15649–15654.

Arzanlou M., Bakhshi M., Karimi K., Torbati M. 2015. Multigene phylogeny reveals three new records of *Colletotrichum* spp. and several new host records for the mycobiota of Iran. Journal of Plant Protection Research 55 (2): 198–211.

Bourret J.A., Ford E.J. 1965. Distribución de la *Calonectria rigidiuscula* y otras especies de Fusarium relacionadas con la buba del cacao. [Distribution of *Calonectria rigidiuscula* and other *Fusarium* species in relations to cushion gall of cacao]. Cacao (Costa Rica) 10: 13–15. (in Spanish)

Brenes O.E., Enríquez G.A. 1982. Buba del cacao, antecedentes y datos de investigaciones en Turrialba, Costa Rica. [Buba of cacao, background and research data in Turrialba, Costa Rica]. El Cacaotero Colombiano 22: 29–38. (in Spanish)

Brunt A.A., Wharton A.L. 1960. Transmission of gall disease of cocoa. Nature 187 (4731): 80–81.

Crozier J., Thomas S.E., Aime M.C., Evans H.C., Holmes K.A. 2006. Molecular characterization of fungal endophytic morphospecies isolated from stems and pods of *Theobroma cacao*. Plant Pathology 55 (6): 783–791.

Evans H.C., Holmes K.A., Thomas S.E. 2003. Endophytes and mycoparasites associated with an indigenous forest tree, *Theobroma gileri*, in Ecuador and a preliminary assessment of their potential as biocontrol agents of cocoa diseases. Mycological Progress 2 (2): 149–160.

Farr D.F., Rossman A.Y. 2015. Fungal Databases. Systematic Mycology and Microbiology Laboratory, ARS, USDA. Available on: http://nt.ars-grin.gov/fungaldatabases/. [Accessed: October 30, 2015]

Frison E.A., Feliu E. 1989. FAO/IBPGR Technical Guidelines for the Safe Movement of Cocoa Germplasm. FAO-IBPGR, Rome, 29 pp.

Gregory P.H. 1977. Una vista de las enfermedades del cacao [A view of cocoa diseases]. In: Proceeding of 6ta Conferencia Internacional de Investigaciones del Cacao. [Proceedings of 6th International Cocoa Research Conference], Caracas, Venezuela, 6–12 November 1977, 20 pp. (in Spanish)

Hanada R.E., Pomella A.W.V., Costa H.S., Bezerra J.L., Loguercio L.L., Pereira J.O. 2010. Endophytic fungal diversity in *Theobroma cacao* (cacao) and *T. grandiflorum* (cupuacu) trees and their potential for growth promotion and biocontrol of black-pod disease. Fungal Biology 114 (11–12): 901–910.

Hansen A.J. 1963. The role of *Fusarium decemcellulare* and *Fusarium roseum* in the greenpoint cushion gall complex of cacao. Revista Turrialba 13 (2): 80–87.

Hansen A.J., Capriles de Reyes L. 1963. Agalla de puntos verdes en el cacao de Venezuela y Costa Rica. [Green point galls on cacao of Venezuela and Costa Rica]. Revista Turrialba 13 (2): 128–130. (in Spanish)

Herre E.A., Mejía L.C., Kyllo D.A., Rojas E., Maynard Z., Butler A., Bael S.A. van. 2007. Ecological implications of antipathogen effects of tropical fungal endophytes and mycorrhizae. Ecology 88 (3): 550–558.

Hutchins L.M., Siller L.R., Hansen A.J. 1959. Diseases and their control: Cushion gall. Research Annual Report of CATIE 1958. Cacao (Costa Rica) 4: 6–10.

Jamali S., Nasimi Z. 2014. First report of black-foot disease, caused by *Cylindrocarpon destructans*, on ornamental marigold (*Tagetes minuta*) in Iran. Journal of Plant Protection Research 54 (3): 139–143.

Kembel S.W., Mueller R.C. 2014. Plant traits and taxonomy drive host associations in tropical phyllosphere fungal communities. Botany 92 (4): 303–311.

Matlick B.K., Purdy L.H., Stevenson C. 1999. Technical Support to Haitian Cacao. Productive land use system (PLUS) Haití. South East Consortium for International Development (SECID) and Auburn University. SECID/Auburn PLUS Report No. 48. USAID/Haiti Economic Growth Office, 40 pp.

Mejía L.C., Herre E.A., Sparks J.P., Winter K., García M.N., Bael S.A. van, Stitt J., Shi Z., Zhang Y., Guiltinan M.J., Maximova S.N. 2014. Pervasive effects of a dominant foliar endophytic fungus on host genetic and phenotypic expression in a tropical tree. Frontiers in Microbiology 5 (479): 1–16.

Mejía L.C., Rojas E.I., Maynard Z., Bael S. van, Arnold A.E., Hebbar P., Samuels G.J., Robbins N., Herre E.A. 2008. Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. Biological Control 46 (1): 4–14.

Morales-Rondón V., Rodríguez-González M. 2006. Hongos endofitos en plantaciones de mango Haden' de la planicie de Maracaibo, Venezuela. [Endophytes fungi in mango 'Haden' orchards of Maracaibo plain, Venezuela]. Revista de la Facultad de Agronomía (LUZ) 23 (3): 273–283. (in Spanish)

Owen H. 1956. Further observations on the pathogenicity of *Calonectria rigidiuscula* (Berk. & Br.) Sacc. to *Thebroma cacao* L. Annals of Applied Biology 44 (2): 307–321.

Parra D., Pérez-Martínez S., Sosa D., Rumbos R., Gutiérrez-Cedeño B.J., Moya A. 2009. Avances en las investigaciones venezolanas sobre enfermedades del cacao. [Advances in venezuelan researches on cacao diseases]. Revista de Estudios Transdisciplinarios 1 (2): 56–75. (in Spanish)

Pérez L., Martínez E., Cantillo T. 2012. Primer reporte en Cuba de la agalla de punto verde del cacao causada por *Albonectria rigidiuscula* (*Fusarium decemcellulare* Brick). [First report in Cuba of green point gall of cocoa cushion caused by *Albonectria rigidiuscula* (*Fusarium decemcellulare* Brick)]. Fitosanidad 16 (1): 19–25. (in Spanish)

Phillips-Mora W., Cerda R. 2009. Catálogo de enfermedades del cacao en Centroamérica. [Catalog Cacao Diseases in Central America]. Technical series. Technical Manual no. 93. Tropical Agricultural Research and Higher Education Center (CATIE), Turrialba, Costa Rica, 24 pp. (in Spanish)

Ploetz R.C. 2007a. Diseases of tropical perennial crops: challenging problems in diverse environments. Plant Disease 91 (6): 644–663.

Ploetz R.C. 2007b. Cacao diseases: important threats to chocolate production worldwide. Phytopathology 97 (12): 1634–1639.

Rai M., Agarkar G. 2014. Plant-fungal interactions: what triggers the fungi to switch among lifestyles? Critical Reviews in Microbiology 42 (3): 428–438.

Rodriguez R.J., Jr White J.F., Arnold A.E., Redman R.S. 2009. Fungal endophytes: diversity and functional roles. New Phytologist 182 (2): 314–330.

Rubini M.R., Silva-Ribeiro R.T., Pomella A.W.V., Maki C.S., Araújo W.L., dos Santos D.R., Azevedo J.L. 2005. Diversity of endophytic fungal community of cacao (*Theobroma cacao* L.) and biological control of *Crinipellis perniciosa*, causal agent of Witches' Broom Disease. International Journal of Biological Sciences 1 (1): 24–33.

Sabah Department of Agriculture 1972. Annual Report of the Department of Agriculture, Sabah. For the year 1970. Kota Kinabalu, Sabah, Malasya, 142 pp.

Saikkonen K., Faeth S.H., Helander M., Sullivan T.J. 1998. Fungal endophytes:a continuum of interactions with host plants. Annual Review of Ecology and Systematics 29: 319–343.

Salas A. 1962. Manchas necróticas en hojas de plantas adultas de cacao (*Theobroma cacao* L.) causadas por *Rhizoctonia* sp. [Leaf necrotic spots on adult cacao plants (*Theobroma cacao* L.) caused by *Rhizoctonia* sp.]. Turrialba 12 (2): 93–95. (in Spanish)

White T.J., Bruns T., Lee S., Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. p. 315–322. In: "PCR Protocols: A Guide to Methods and Applications" (M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White, eds.). Academic Press, New York, USA, 482 pp.