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Diversidad de hongos y patogenicidad de *Fusarium oxysporum* asociados a la corchosis de la raíz del cafeto en México

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Originales: *Recepción:* 18/10/2017 - *Aceptación:* 01/06/2018

Abstract

The disease known as coffee corky-roots associated to the infection by the root-knot nematode *Meloidogyne paranaensis* is an important issue for coffee crop in several countries. In Mexico, particularly in the Veracruz state, considerable loses are recorded annually in *Coffea arabica* plantations by corky-root disease. Previous studies have revealed the presence of fungi in coffee corky-root tissues. However these fungi have not been yet identified. This work aimed to identify at species level the fungi associated to the coffee corky-root symptoms and determine their pathogenicity on coffee plants. Coffee roots with corky-root symptoms were collected in eight sites distributed through the major coffee growing region of Veracruz. Observations of inside cortical root tissues under scanning electron microscope revealed abundant mycelium and conidia in corky-root samples in contrast with absence of every fungi development in healthy roots. Forty-nine fungi strains from internal corky-root tissue were isolated and identified at species level by ITS sequences. *Fusarium oxysporum* was the most frequent species and the only present in all of the corky-root samples. These strains were selected for the pathogenicity test. All *F. oxysporum* strains colonize the vascular system of coffee plants although none caused wilting symptoms.

Keywords
Coffea arabica • root-knot nematodes • filamentous fungi

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Resumen

La corchosis de la raíz del café asociada a la infección del nematodo agallador de la raíz *Meloidogyne paranaensis* es un importante problema para el cultivo de café en varios países. En México, particularmente en el estado de Veracruz, se registran considerables pérdidas anuales en las plantaciones de *Coffea arabica* por esta enfermedad. Estudios anteriores han revelado la presencia de hongos en los tejidos afectados con corchosis de la raíz del café. Sin embargo, estos hongos aún no han sido identificados. El objetivo de este trabajo fue identificar a nivel de especie los hongos asociados a la corchosis de la raíz y determinar su patogenicidad en plantas de café. Se recolectaron raíces de cafetos con síntomas de corchosis en ocho sitios distribuidos a través de la principal región cafetalera de Veracruz. Las observaciones de los tejidos internos de las raíces bajo el microscopio electrónico de barrido revelaron abundante micelio y conidios en muestras de raíz con corchosis, en contraste con su ausencia en raíces sanas. Se aislaron 49 hongos de los tejidos internos afectados con corchosis y se identificaron a nivel de especie mediante secuencias de ITS. *Fusarium oxysporum* fue la especie más frecuente y la única presente en todos los sitios de muestreo, por lo que estas cepas fueron seleccionadas para la prueba de patogenicidad. Todas las cepas de *F. oxysporum* fueron capaces de colonizar el sistema vascular de las plantas de café, aunque ninguna causó síntomas de marchitez.

Palabras claves
Coffea arabica • nematodo agallador • hongos filamentosos

Introduction

Coffee is an important crop since it takes the second place among the most worldwide traded products (after oil) providing economic livelihood to more than 125 million people. During the coffee cycle 2015/2016 the coffee producing countries all together exported more than 110 million of 60 kg bags (22).

In Mexico, Arabica coffee plantations represents almost the 90% of the coffee production and still plays an important socio economic role in many rural areas of high level of poverty. Additionally, Arabica plantations provide many important ecosystemic services to the country, since this crop is predominantly grown in agroforestry systems and in ecologically sensible mountainous areas (20). However, Mexico coffee exportable production decreased almost constantly since the end of the 90's falling from around 4 million to less than half million of 60 kg bags for the last harvest 2015/2016 (22). This dramatic production decrease is caused by different reasons like the aging of most coffee plantations and biotic stresses especially the coffee leaf rust which affected Mexican coffee crop mainly during the last three years and plant-parasitic nematodes which have wide distribution in all coffee growing regions of Mexico (21). Actually, coffee leaf rust and plant-parasitic nematodes are the two major phytosanitary problems affecting Arabica coffee plantations through all Latin America (1, 42).

Nonetheless, while coffee leaf rust incidence and damage are determined by many factors such as, micro and macroclimate conditions and agronomic practices, plant-parasitic nematodes represent a continued and underlying threat for both Arabica and Robusta plantations with a high potential damage. Due to the lack of analysis for the detection of plant-parasitic nematodes in nurseries and the fact that seedlings show symptoms until high nematode densities are reached, field nematode infestations continue to expand.

Moreover plant-parasitic nematodes create a continued stress during the entire lifetime of the plantation by the lack of a complete eradication by control methods and the use of susceptible germplasm (41). The major nematode damages in Latin America are caused by root-knot nematodes (RKN), *Meloidogyne* spp. (42), particularly by two species associated with a devastating syndrome called coffee corky-root disease: *Meloidogyne arabicida*, only detected in Costa Rica to date (24) and *M. paranaensis*, with a wider distribution, in Brazil (11); in Guatemala (42), and Hawaii (11).

In Mexico, coffee corky-root disease has been detected since the 1960s in the state of Veracruz (the second national coffee producing state) and *M. paranaensis* has been confirmed by using specific SCAR molecular markers as the RKN species linked to coffee corky-root symptoms (25).

The affected coffee trees show a progressive decline, starting with chlorosis followed by fall of flowers, leaves and fruits until the death of plants which occurs in a period between two to four years depending on agro-ecological conditions and mainly when plant begin to produce (5).

The root system of infested plants shows numerous small elongated galls on young white roots and large swelling on older and more lignified roots with occurrence of large, deep and cracked cortical tissues remembering cork aspect (5). These corky symptoms can affect the primary roots including the taproot up to the plant crown and even sometimes reach the first centimeters of the stem as observed in this work. Cuttings of these corky root swellings reveal numerous *M. paranaensis* females with their egg masses (25).

In Mexico, *Fusarium oxysporum* has been associated to coffee plants with corky-root symptoms (16) along with other fungi like *Cylindrocladium* sp., *Fusarium solani*, *Trichoderma* sp. and *Verticillium* sp. (36).

Nevertheless, it is necessary to research those fungi directly associated with internal tissues of coffee roots damaged by nematodes, collected from different sites of coffee regions. Therefore, the objectives of this work were to: i) observe the presence of fungi in the affected tissues, ii) isolate and identify the fungal community associated with the coffee corky-root disease using molecular methods and iii) pathogenicity test of isolated fungi on coffee plants without the presence of the nematode *M. paranaensis.*

Materials and methods

Sampling

The sampling of coffee corky-roots was done on eight coffee plantations distributed in the main coffee cropping area of the Veracruz state, located between the eastern slope of the Mexican Trans Volcanic Belt and the southern slope of the Sierra Madre Oriental.

The sampled coffee plantations were selected based on field technicians information and on previous studies that registered the presence of the corky-root disease or spots in coffee plantations with roots affected and aerial symptoms such as, chlorosis, deficient growth, defoliation and premature death of plants. On each plantation, roots were taken from 8-9 coffee plants with corky-root symptoms to form one composite sample of each sampling site. In a previous study, it was determined that in all coffee corky-root collected samples the only present RKN was *M. paranaensis* (25).

Scanning electron microscope observations

For the scanning electron microscope (SEM) observations, 3 month aged seedlings of an *in vitro* propagated F1 intraspecific hybrid line of *Coffea arabica* (7) cultivated in 6 litres pots filled with previously sterilized substrate were infested with a population of *M. paranaensis* reared on tomato plants in greenhouse. This population was initially collected on coffee at one of the eight sampling sites of this study, Jilotepec site (table 1, page XXX-XXX).

The plants were kept in a greenhouse for one year to obtain numerous corky-roots. The roots were washed with tap water to remove the excess of soil. Longitudinal cuts of corky-roots were made with scalpel, and 1 mm thickness rectangular sections (2 mm x 5 mm) of inner tissues of the corky-root parts were collected and fixed in glutaraldehyde at 2% for 5 days to preserve the structural integrity. Subsequently, the samples were submitted to a dehydration process with increasing concentrations of ethanol (10, 30, 50, 70 and 90% from 15 to 25 min in each concentration) until conserving the tissue root sections in absolute alcohol.

The samples were placed in a filter paper bag and dehydrated in a critical point camera. Then the samples were mounted on aluminum cylindrical stubs and coated with gold-palladium for its further observation under SEM. Root sections of healthy plants of same age (15 months) were collected and processed in the same way as control for comparison.

Isolation and identification of fungi

For the fungi isolations, roots from the eight sampling sites, apparently with recently formed corky swelling, were selected to avoid saprophytic fungi that may be widely present in old corky-root formations. Roots were carefully washed with tap water to remove adhered soil, then disinfected by soaking it consecutively in 70% alcohol (during 1 minute), 3% NaClO (1 min), 96% alcohol (30 seconds); and then rinsed tree times with distillated sterile water. Longitudinal cuts of the corky-root tissues were made and fragments of the inner tissues were extracted and placed in Petri dishes prepared with potato dextrose agar (PDA) and chloramphenicol (1 mg mL^{-1}).

Fungi mycelia that grew from the extracted inner part corky-root tissue fragments were transferred to other Petri dishes with PDA, until pure cultures from each isolate were obtained. To identify the fungi at species level, DNA was extracted from 25 mg of mycelia of each strain using the extraction kit: Fungal/Bacterial DNA MiniPrep Zymo Research. A molecular marker of 500 bp, that encompasses the Internal Transcriber Spacer (ITS) 1, the 5.8 rDNA, and the ITS2 molecular markers, was used and amplified by PCR (34).

The PCR products were analyzed on a 1.2 % agarose gel; next the DNA was purified and sent to Macrogen INC for sequencing.

The obtained sequences were edited in the e-Biox program and compared by BLAST analysis to the database of the National Center for Biotechnology Information (NCBI).

Pathogenicity tests of fungi in coffee plants

The 27 *Fusarium oxysporum* strains previously isolated and identified were selected for pathogenicity test because were the only species founded in all sampling sites. The inoculum was prepared by culturing mycelium of each strain in flasks with oat-yeast extract (10 g L^{-1} and 1 g L^{-1}) liquid medium. The flasks were incubated in an orbital shaker at 150 rpm and 25°C during 5 days. The conidia concentration was determined with a Neubauer chamber and was adjusted to 1.10^6 spores per mL.

Ex vitro plantlets of *Coffea arabica* with two or three pairs of leaves of a F1 intra-specific hybrid H18 (ET06 wild Ethiopian accession x introgressed Cv. Naryelis) was used for this experiment. Besides the fact that *ex vitro* plantlets acclimated in horticultural trays filled with sterilized peat-moss allowed working with pathogen-free vegetal material this germplasm micropropagated by somatic embryogenesis provided strongly homogeneous material (8). Plantlets were extracted from horticultural trays and roots were carefully washed in distilled sterile water. Two different groups of plantlets were prepared for *F. oxysporum* inoculation.

The first group of plantlets was predisposed to the fungus infection by cutting the roots 1 cm from their apex with a sterile scalpel (13, 35). In the second group, plantlets were kept with intact roots. Each strain of *F. oxysporum* was inoculated on 5 plantlets of each of the two groups by submerging rootlets in 75 mL of a conidia suspension for 20 min (17).

In each case, a group of plantlets without *F. oxysporum* inoculation was used as control. Subsequently all coffee plantlets were sowed in 100 mL pots filled with a sterilized by twice autoclaving peat moss-sand 2:1 mix and placed in a greenhouse at 25 ± 2°C with a relative humidity of 80-90 % and a photoperiod of 12 hours. The experiment was arranged under a completely randomized design. The plantlets were manually watered each 72 hours with sterile water.

The plants were extracted from the pots, 45 days after the inoculation. Roots were washed with sterile distilled water to remove the substrate. Symptoms like lesions, root necrosis and wilt were annotated through a scale from 1 to 5 in order to determine the severity rate of disease according to Parke and Grau (1993) and Reis and Boiteux (2007) where: 1 = Plant without symptoms; 2 = Plants without wilting symptoms, but with light brown spots on the root; 3 = Plants with vascular necrosis symptoms and wilting symptoms, but without yellowing of the leaves; 4 = Generalized necrosis in the root, wilting and severe chlorosis; 5 = Dead plant.

To detect the vascular colonization of the different *F. oxysporum* strains along the root and the stem, three plants of each strain were selected in each group of plants. The surface of both parts of the plantlets was subsequently disinfected with 70% alcohol (during 1 min), 3% NaCIO (1 min) and 96% alcohol (30 seconds); then rinsed three times with sterile distilled water.

The first 3 mm next to the collar plant cutting were removed from root and stem parts.

Table 1. Geographic data of sampling sites and molecular identification of fungal species associated with coffee corky-root disease.

Tabla 1. Datos geográficos de los sitios de muestreo e identificación molecular de las especies de hongos asociadas a la corchosis de la raíz del cafeto.

Location	Municipality/ Locality	Altitude m a. s. l.	Host species, and cultivar	Isolate code	Molecular species diagnostic	% identity	NCBI accession number
N 19°54'13.1" W 97°13'28.4"	Atzalan, Napuala	689	*Coffea arabica*cv. Costa Rica 95	Na1	*Fusarium oxysporum*	99	KU847855
				Na2	*Purpureocillium lilacinum*	99	KC157741
				Na3	*Penicillium citrinum*	99	KX090324
				Na4	*Fusarium oxysporum*	99	KU847855
				Na5	*Stereum complicatum*	99	KJ140584
				Na6	*Fusarium oxysporum*	94	KY073258
N 19°51'50.8" W 97°10'06.8"	Atzalan, Chachalacas	664	*Coffea canephora* cv. unknown	Ch1	*Fusarium oxysporum*	99	LT571434
				Ch2	*Fusarium oxysporum*	99	KC304813
				Ch3	*Purpureocillium lilacinum*	100	KM458848
				Ch4	*Purpureocillium lilacinum*	99	KX347471
				Ch5	*Fusarium oxysporum*	100	KF718222
N 19°50'27.6" W 96°47'05.8"	Yecuatla, La Victoria	559	*Coffea arabica* cv. Typica	Ye1	*Beauveria bassiana*	99	KX219590
				Ye2	*Purpureocillium lilacinum*	99	FJ765023
				Ye3	*Fusarium oxysporum*	100	KX196809
				Ye4	*Alternaria longissima*	99	DQ865104
				Ye5	*Fusarium solani*	99	KU377471
				Ye6	*Alternaria longissima*	96	DQ865104
				Ye7	*Fusarium oxysporum*	100	KC304807
				Ye8	*Fusarium solani*	98	KU878142
N 19°35'42.2" W 96°53'01.4"	Jilotepec, Paso San Juan	996	*Coffea arabica* cv. Costa Rica 95	Ji1	*Penicillium citrinum*	100	KX090324
				Ji2	*Fusarium oxysporum*	100	KU680363
				Ji3	*Fusarium oxysporum*	100	KX196807
				Ji4	*Fusarium oxysporum*	100	KX058057
				Ji5	*Fusarium solani*	100	HQ176441
				Ji6	*Penicillium citrinum*	100	KX090324
				Ji7	*Penicillium citrinum*	100	KX090324

Table 1 (cont.). Geographic data of sampling sites and molecular identification of fungal species associated with coffee corky-root disease.

Tabla 1 (cont.). Datos geográficos de los sitios de muestreo e identificación molecular de las especies de hongos asociadas a la corchosis de la raíz del cafeto.

Location	Municipality/ Locality	Altitude m a. s. l.	Host species, and cultivar	Isolate code	Molecular species diagnostic	% identity	NCBI accession number
N 19°19'45.4" W 96°58'20.1"	Cosautlán, La Lagunilla	1152	*Coffea canephora* cv. unknown	Co1	*Fusarium oxysporum*	100	KT896661
				Co2	*Gliocladiopsis curvata*	99	JX500723
				Co3	*Fusarium oxysporum*	96	KC282839
				Co4	*Fusarium oxysporum*	100	KC304799
				Co5	*Fusarium oxysporum*	100	KC304807
N 19°11'44.5" W 96°56'38.1"	Sochiapa, Sochiapa	1361	*Coffea arabica* cv. Caturra	So1	*Fusarium oxysporum*	98	KY073257
				So2	*Fusarium oxysporum*	100	KJ699122
				So3	*Fusarium oxysporum*	100	KC304800
				So4	*Fusarium solani*	100	JQ676178
				So5	*Fusarium oxysporum*	100	KC787019
				So6	*Fusarium oxysporum*	98	XR001936475
				So7	*Fusarium oxysporum*	99	KU847855
N 19°03'16.5" W 96°55'41.3"	Ixhuatlán del Café, Moctezuma	1186	*Coffea arabica* cv. Garnica and Pacamara	Mo1	*Fusarium oxysporum*	100	EU715659
				Mo2	*Fusarium oxysporum*	100	KC304813
				Mo3	*Penicillium citrinum*	100	KX090324
				Mo4	*Penicillium citrinum*	100	KX090324
N 19°01'50.7" W 96°56'58.0"	Ixhuatlán del Café, Nevería	1219	*Coffea arabica* cv. Bourbon and Caturra	Ne1	*Fusarium solani*	99	JQ712137
				Ne2	*Purpureocillium lilacinum*	99	KX347471
				Ne3	*Fusarium oxysporum*	99	EF590327
				Ne4	*Fusarium oxysporum*	100	KX196809
				Ne5	*Fusarium oxysporum*	99	KC304802
				Ne6	*Pochonia chlamydosporia*	99	KT583165
				Ne7	*Fusarium oxysporum*	100	KT896661

The remaining roots and stems parts were cut over a 50 mm length from the base into 10 sections of equal length. Root and stem sections of each plantlet were placed horizontally clockwise orderly into 90 mm Petri dishes with PDA- Chloramphenicol.

The Petri dishes with root and stem sections were accommodated in the laboratory under a totally randomized design and were incubated at 25°C for 8 days and examined every day for outgrowths of the fungi from the vascular ring of each root or stem section. The mycelia that grew from the root or stem fragments was transferred to Petri dishes with PDA to obtain pure cultures and to identify them molecularly in accordance with the methodology described above. The depth or height reached by the fungus into the root or stem was determined from the re-isolation data for the root or stem sections for each plant (35).

The data of symptoms and development of the plants were submitted to a one-way ANOVA for each group of plants (root with lesions and healthy root). The data obtained from the frequency of re-isolation between the two groups of plants (healthy root and lesion root) and between plant compartment (root and stem), were used to build a distance matrix, calculating statistical distances with the Bray-Curtis method. To assess the effect of the groups of plant and plant compartment on the frequency of fungal re-isolation, the distance matrix was analyzed with a permutational analysis of variance (PERMANOVA). To compare the vascular colonization between each isolate, the data was evaluated as a function of the frequency of re-isolations in the stems and roots in the ten sections arranged from the base of the stem or the root every 5mm up to 50 mm. Percentage values of re-isolations were submitted to one-way ANOVA.

Results and discussion

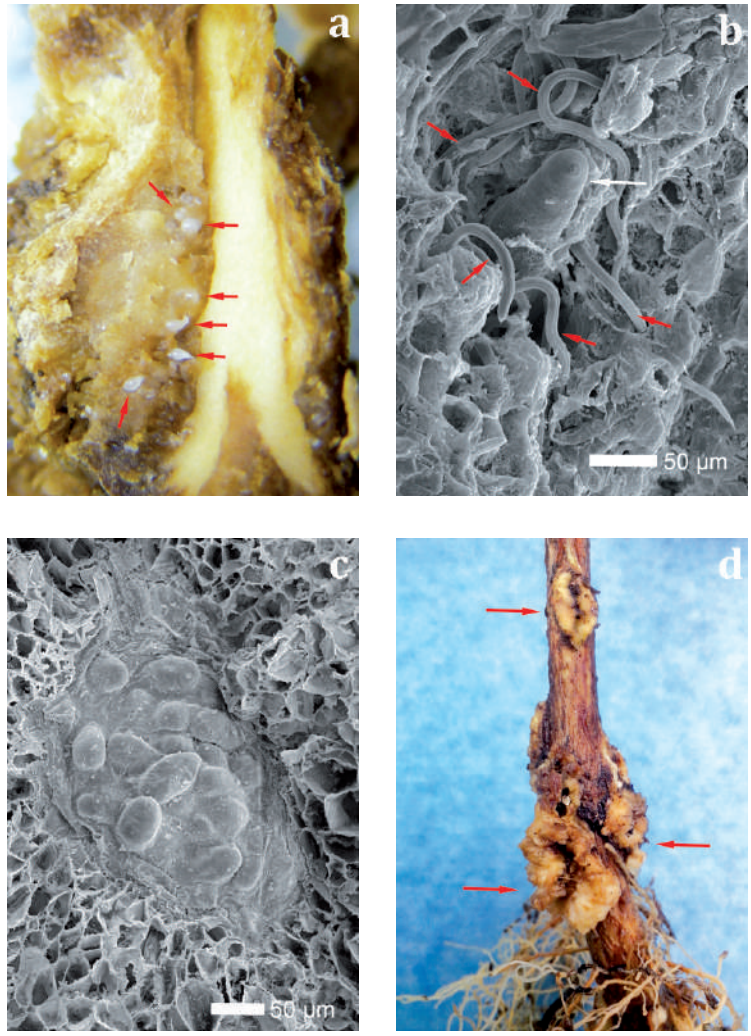
Coffee corky-root disease tissues observations

Many females, eggs and second juvenile stage (J2) of *M. paranaensis* were observed in the roots affected with the coffee corky-root disease (figure 1a, 1b and 1c, page XXX).

Cell lesions caused by the displacement of the J2 through the root tissues were also observed (figure 1b, page XXX). Corky protrusions with presence of numerous *M. paranaensis* individuals (females, J2 and egg masses) have been even observed on the stem up to about 5 cm above ground (figure 1d, page XXX).

As far we know, this the first report of RKN presence and symptoms at this high level in plant. The pericycle and cortical tissues of corky-roots and stem lower parts of infested coffee plants showed cell distortions and corrugations and also some cell wall thickenings (figures 1b, c and figures 2a, b, page XXX).

No change in cortical cell volumes was observed in corky-root tissues compare to healthy tissues but hyperplasia like process were observed such as much more cortical cell layers leading to a lateral expansion of the root or stem cortex. The presence of many conidia was observed in the cells of the infested tissues so as abundant mycelium crossing the cell walls, even in tissue area where nematodes were not observed (figure 2c, d, page XXX).

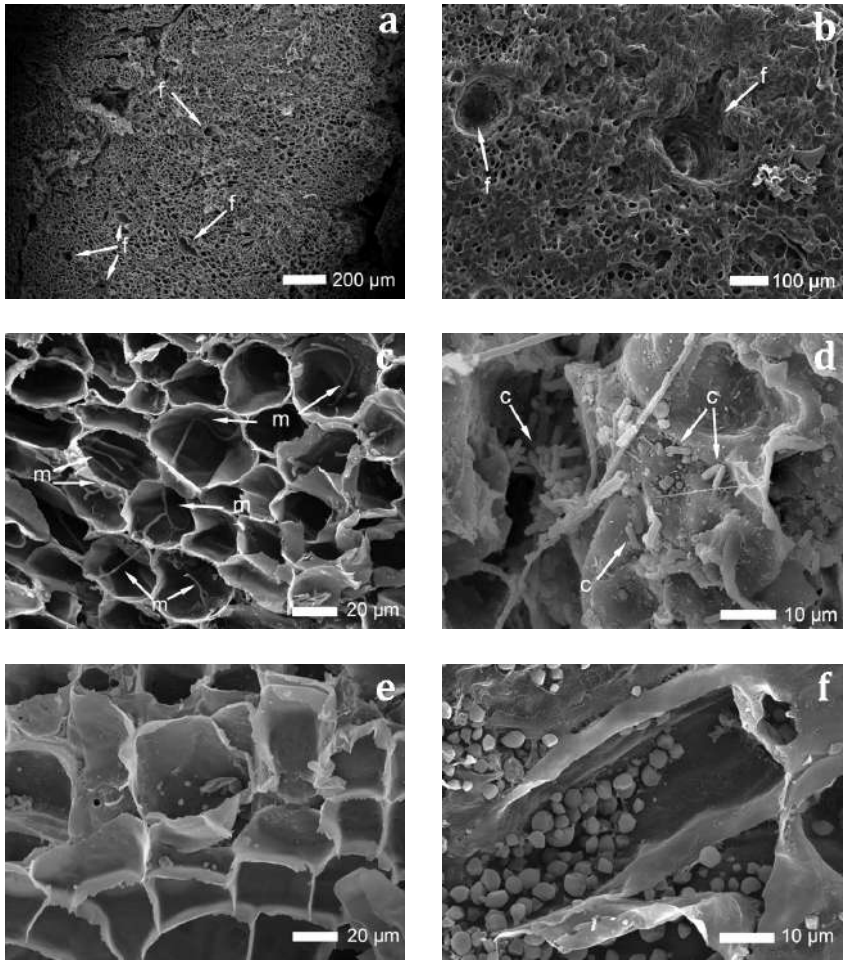


a) Longitudinal section of an infested root showing numerous *M. paranaensis* females (red arrows) inside the corky tissues 10X; **b)** Detail of a root inside corky tissue with a female (white arrow) and J2 stage juveniles (red arrows) of *M. paranaensis* 350X; **c)** Egg mass of *M. paranaensis* (center of the picture) with the eggs already wrapped by the gelatinous matrix and surrounded by distorted and corrugated tissues 270X; **d)** Corky-root symptoms on the stem base of a 12 month aged coffee seedling.

a) Sección longitudinal de una raíz infestada que muestra numerosas hembras de *M. paranaensis* (flechas rojas) en el interior del tejido afectado 10X; **b**) Detalle de una raíz dentro del tejido corchoso con una hembra (flecha blanca) y juveniles J2 (flechas rojas) de *M. paranaensis* 350X; **c)** Masa de huevos de *M. paranaensis* (centro del cuadro) con los huevos envueltos por la matriz gelatinosa y rodeados de tejidos distorsionados y corrugados 270X; **d)** Síntomas de corchosis en la base del tallo de una plántula de café de 12 meses.

Figure 1. Coffee corky-root disease symptoms associated to *Meloidogyne paranaensis* parasitism on *Coffea arabica*.

Figura 1. Síntomas de la corchosis de la raíz del cafeto asociada al parasitismo de *Meloidogyne paranaensis* en *Coffea arabica*.



a) Transversal section of a corky formation on the stem 60X; **b)** Transversal section of a corky-root 120X; **c)** Pericycle cells in an infected root with presence of mycelia 700X; **d)** Transversal section of the vascular system of a coffee corky-root with presence of mycelium and conidia 1600X; **e)** Pericycle cells in a healthy root 700X; **f)** Longitudinal section of a vascular system of a healthy coffee root with presence of numerous sap organic particles 1600X. f= lesions left by the growth of females; m= mycelium; c = conidia.

a) Sección transversal de una formación corchosa en el tallo 60X; **b)** Sección transversal de una raíz con corchosis 120X; **c)** Células del periciclo en una raíz infectada con presencia de micelio 700X; **d)** Sección transversal del sistema vascular de una raíz de café con corchosis con presencia de micelio y conidios 1600X; **e)** Células de periciclo en una raíz sana 700X; **f)** Sección longitudinal de un sistema vascular de una raíz de café sana con presencia de numerosas partículas orgánicas de savia 1600X. f = lesiones dejadas por el crecimiento de las hembras; m = micelio; c = conidios.

Figure 2. Sections of healthy and infested coffee roots and stems observed under scanning electron microscope.

Figura 2. Secciones de raíces y tallos de café sanos e infestados observados bajo microscopio electrónico de barrido.

In the vascular system of healthy roots, it was possible to observe the presence of many organic particles which nature was confirmed by energy-dispersive X-ray spectroscopy (72.1% C and 27.9% O) (figure 2e, f, page XXX), while this material was not observed in the vascular systems of diseased roots, revealing a dysfunction in the vascular nutrient transport. Numerous bacteria were observed in the corky root tissues (figure 3a, b) while no bacteria were observed in healthy tissues. To date no bacteria has been reported as associated to the coffee corky-root disease. However, by the observations it seems necessary to investigate if some of these bacteria detected in the inner corky-root tissues could be involved in the pathogenesis of the disease as being part of the corky-root pathobioma or if they just have an opportunistic role as saprophytes developing on decaying tissues. Studies realized in tomato indicates that the community of endophyte bacteria are significantly affected by the infection of the nematode *M. incognita* which brings some new groups of bacteria, particularly those that contributes to the nematode infection process by degrading the plant cellular walls in the feeding sites or allowing a mutualistic relation with the provision of nutrients (37).

Diversity of fungi associated to coffee corky root disease

Forty-nine fungi strains were obtained from the coffee corky-root inner tissues. According to the molecular identification, 55% of the isolates correspond to *Fusarium oxysporum*; 12% to *Penicillium citrinum*; 10% to *F. solani;* 10% to *Purpureocillium lilacinum;* 4% to *Alternaria longissima* and the remaining 8% to the following species: *Baeuveria bassiana, Gliocladiopsis curvata, Pochonia chlamydosporia* and *Stereum complicatum*.

The isolation of every fungus in each sampling sites is summarized in the table 1 (page XXX-XXX). Except *F. oxysporum* and *F. solani*, all species of fungi found in this work are registered for the first time on coffee corky-roots. *Alternaria* sp., *B. bassiana, F. oxysporum* and *P. citrinum* have been registered as endophyte of healthy coffee plant roots (30, 39, 40).

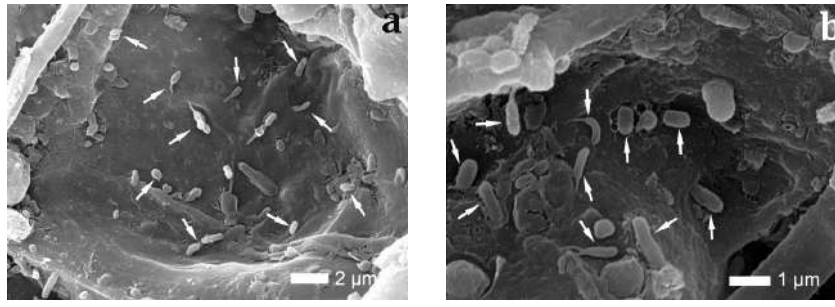


Figure 3. Bacteria inside cells of coffee corky-root tissues: **a)** 6000 and **b)** 13000X.

Figura 3. Bacterias dentro de las células de los tejidos de una raíz de café con corchosis: **a)** 6000 y **b)** 13000X.

G. curvata is a fungus previously isolated from soil and plants debris, although its ecology or role as potential pathogen of plants is less known (23). *S. complicatum* is a saprophytic fungus commonly found in decaying wood tissues (3).

P. lilacinus and *P. chlamydosporia* are fungi commonly associated to nematodes and they may be found parasitizing *M. paranaensis* (19).

F. oxysporum was the only species found in all sampling sites of this study. In Costa Rica, the simultaneous role of *F. oxysporum* and the RKN, *M. arabicida* as causal agents of a similar coffee corky-root disease was demonstrated (4). In Puerto Rico, strains identified as *F. oxysporum* f. sp. *coffeae,* have been registered as pathogen, causing vascular wilting in coffee plants infested with the RKN, *M. incognita*, without corky-root symptoms observation (27).

In Brazil, this same *F. oxysporum* f. sp. *coffeae* was reported as causing vascular wilting without pointing the presence of nematodes (10). Also it had been registered in abundance in the rhizosphere of coffee plants infected with the RKN *M. exigua* without causing any symptoms of corky-root or vascular disease, and it has been checked that some of this strains could have nematicide activity (15).

On the other hand, though *F. solani* has been only detected on four sampling sites, this fungus has also been previously detected in coffee corky-roots in the State of Veracruz (36).

However, the only report of *F. solani* as a confirmed causal agent of a coffee disease is from Kenya causing a coffee root rot (2). *F. oxysporum* and *F. solani* are considered separately as a complex of species which includes: numerous plant pathogenic strains referred as special forms related to some host plant(s); opportunistic strains that cause infections in humans and animals; saprophytic populations that are found commonly in soil, roots in senescence and vegetal debris (12, 38).

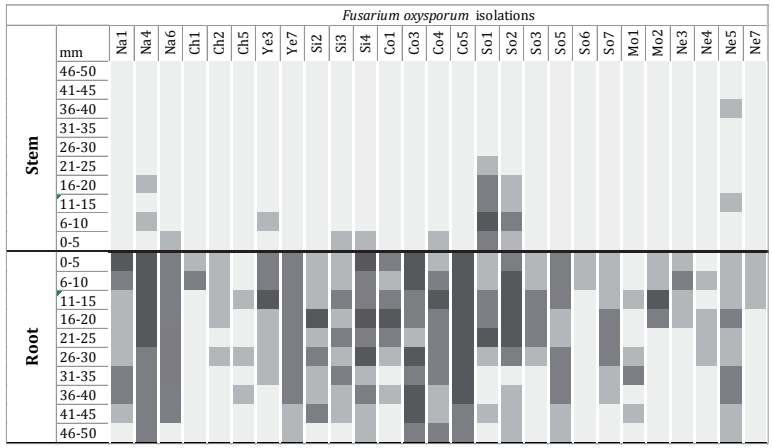
Pathogenicity and vascular colonization of *Fusarium oxysporum* on *Coffea arabica* plants.

Six weeks after the inoculation, the plants with the healthy and wounded roots were kept without symptoms of wilting. Of the 1680 fragments examined in each group of plants, 354 re-isolations were achieved of *F. oxysporum* in plants with healthy roots, 288 in the plants with injured roots (0.06868, p = 0.001). No re-isolate was obtained from control plants. In both groups of plants, the fungi strains colonized the root, but not all the stems (0.11666 p = 0.001) and the re-isolation of the strains was discontinuous (figure 4 and 5, page XXX).

In the healthy roots, all the strains of *F. oxysporum* were re-isolated, 330 (93.2%) re-isolations correspond to root and 24 (6.8%) to stems.

The strains Co5, Na4 and Co3 presented the highest (F = 7.8995, p = 0.00) frequency with 93, 83 and 80% respectively along the root. Ten strains of *F. oxysporum* were re-isolated from the stem of the plants with healthy roots.

The strain So1 presented the highest (F = 3.0639, P = 0.00) frequency of re-isolations in the stem (33%) to 30 mm high, although the NE 5 strain was isolated at 40 mm from the stem base. In the plants with injured roots 25 of the 27 strains of *F. oxysporum* were re-isolated from the roots, 270 (93.75%) re-isolations correspond to the root and 18 (6.25%) to stems.

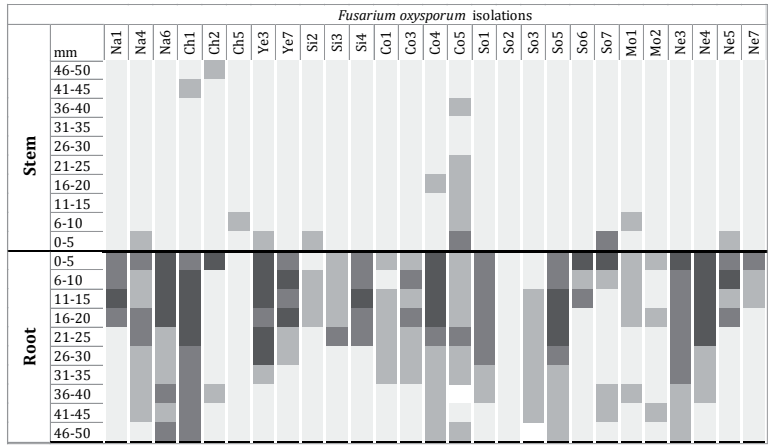


The colors on the bars indicate the frequency of re-isolation in each longitudinal section of the stem or root: 0= , 1= , 2= , 3=

Los colores en las barras indican la frecuencia de reaislamientos en cada sección longitudinal del tallo o de la raíz: 0= , 1= , 2= , 3=

Figure 4. Vascular colonization in root and stem of *Fusarium oxysporum* strains 45 days after inoculation in *Coffea arabica* plants with roots without wounds.

Figura 4. Colonización vascular en raíz y tallo de las cepas de *Fusarium oxysporum* 45 días después de la inoculación en plantas de *Coffea arabica* con raíces sin heridas.



The colors on the bars indicate the frequency of re-isolation in each longitudinal section of the stem or root: 0= , 1= , 2= , 3=

Los colores en las barras indican la frecuencia de reaislamientos en cada sección longitudinal del tallo o de la raíz: 0= , 1= , 2= , 3=

Figure 5. Vascular colonization in root and stem of *Fusarium oxysporum* strains 45 days after inoculation in *Coffea arabica* plants with wounded roots.

Figura 5. Colonización vascular en raíz y tallo de las cepas de *Fusarium oxysporum* 45 días después de la inoculación en plantas de *Coffea arabica* con raíces con heridas.

The strains Ch1 and Na6 presented the highest (F=3.8618, P= 0.000) percentage of re-isolations in root with 80 and 67% respectively. 11 strains of *F. oxysporum* were re-isolated from the stems of this group of plants with the injured root, the strain Co5 presented the highest percentage of the re isolation (23%) to the height of 40 cm (F = 3.2517, P = 0.00). Although the highest height was recorded in the strains Ch1 and Ch2 which were found at 45 and 50 mm from the stem base respectively.

In different studies is mentioned that the phytopathogenic fungi that causes the withering enter into the roots by mechanic wounds, like caused by nematode penetration (14) and in the case the RKN by the wounds caused by the expulsion of the eggs masses (18). Nevertheless with our results it is proven that this wounds are not necessary for *F. oxysporum* to colonize the vascular systems of coffee plants, since the isolations of *F. oxysporum* isolated from the corky root disease, colonize coffee plants roots with wounds and without them.

All the strains studied here colonized the root but only some get to the stem. The movement of the fungus to the stem is considered as an indicator of pathogenicity, nevertheless in this work none of the isolations of *F. oxysporum* caused symptoms of vascular withering (9, 26, 32).

Some studies suggest that the phytopathogenic fungi can colonize their host and behave as endophytes long before presenting any symptoms of disease, which are expressed when the host plant goes into stress (33).

In the coffee plants is possible that the strains of *F. oxysporum* presents in the roots with the corky root disease have enter into the plant as endophytes and remain without causing any symptoms while the plant does not enter into stress due to the damage caused by the nematodes in the root or any abiotic stress (29) however this hypothesis must be studied.

Conclusions

According to the observations, in the tissues of coffee corky-root diseases are being interacting nematodes, fungi and bacteria. *F. oxysporum* is a major fungus associated with coffee corky-root disease.

F. oxysporum strains isolated from corky-root disease do not cause wilting symptoms in coffee plants in the absence of nematode *M. paranaensis*. Nevertheless, there are necessary more studies *in vivo* and at molecular level for detect pathogenicity genes and determine if these strains found like latent pathogens or like saprophytes in coffee roots before to be affected by nematodes.

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Aknowledgements

The authors of this study acknowledge to Greta Hanako Rosas Saito for the coffee root observations under the scanning electron microscope. We also thank Magda Gomez Columna for their technical support in the isolation and maintenance of fungal strains.
This work was supported by the Consejo Nacional de Ciencia y Tecnología (grant number 231215).