Article

Fungi associated with the regressive death of citrus fruits in Nuevo Leon and Tamaulipas, Mexico

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Abstract

The cultivation of citrus fruits is affected by several phytopathogenic fungi, which can cause diseases and reduce their production. The fungus *Lasiodiplodia* spp. causes regressive death and other symptoms in citrus and other crops in various countries, including Mexico. The objective of this work was to identify isolated fungal strains of citrus trees with symptoms of regressive death and to evaluate their pathogenicity in greenhouse conditions. The isolated fungi were identified based on their morphological characteristics such as *Lasiodiplodia theobromae*, *Fomitopsis meliae* and *Eutypella citricola*, confirmed with PCR amplification and sequencing of the ITS region when compared to the GenBank sequences. In the greenhouse bioassay, it was found that *L. theobromae* and *F. meliae* cause symptoms of wilting, descending death and necrotic lesions in the inoculated areas from the fourth day after inoculation in plants of sweet orange variety Valencia. *F. meliae* + *L. theobromae* caused more severe damage, causing necrotic lesions of 22 to 27 cm in length, wilt and regressive death and both were reisolated from the lesions produced. *E. citricola* only produced necrosis around the inoculated area. According to the results of the pathogenicity test, fungi *L. theobromae* and *F. meliae* cause regressive citrus death.

Keywords: *Lasiodiplodia*, pathogenicity, phytopathogenic fungi.

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Introduction

Citrus cultivation is affected by several phytopathogenic fungi, which can cause various diseases and reduce their production. These pathogens cause lesions on leaves, cankers, necrosis, root rot, peduncles and fruits, wilting, branch death and regressive death (McBride *et al.*, 2010; Adesemoye *et al*., 2014). The fungus *Lasiodiplodia* spp. it has been reported as a cause of regressive death and other symptoms in citrus fruits and in a wide range of crops (Adesemoye *et al*., 2014; Rodríguez *et al*., 2016).

In Mexico, peduncle rot and branch dieback were observed in mango trees (*Mangifera indica* L.) in the states of Guerrero and Michoacán. The pathogens identified were *L. theobromae*, *L. pseudotheobromae* and *Neofusicoccum parvum* (Sandoval *et al*., 2013). In Guerrero, the *L. theobromae* species was reported in sapote mamey trees (*Pouteria zapota* [Jacq] HE Moore and Stearn) causing wilting, death of the apical bud and consequently descending death with gradual drying at 22 days after grafting (Tovar *et al*., 2013). In Nayarit, regressive death has been observed in guanaban orchards, beginning in the branches, which continued drying from the tip to the base and *L. theobromae* could be identified as the causative agent (Hernández *et al*., 2013).

The pathogenicity of *Lasiodiplodia* spp. has been studied in different crops, Al-Sadi *et al*. (2014) isolated *L. theobromae* from lime trees (*Citrus aurantifolia* S.), inoculated the fungus and produced symptoms of regressive death in 40% of the inoculated plants from which it was subsequently reisolated. In the same way, Cedeño and Palacios (1992) demonstrated through the postulates of Koch that *L. theobromae* produced gummosis and lesions in the inoculated citrus plants, symptoms similar to those observed in the field. At the same time, the morphological characteristics and dimensions of the reisolated conidia allowed to associate *L. theobromae* as the cause of the disease.

At the beginning of 2015, citrus farmers in the state of Sinaloa reported the regressive death of Persian lemon trees (*Citrus latifolia* Tan). In that same year, producers of Nuevo Leon reported the death of branches in descending direction to the main trunk in Persian lemon and sweet orange (*Citrus sinensis* [L.] Osbeck) variety Valencia grafted on sour orange (*Citrus aurantium* L.); later, similar symptoms were also observed in citrus orchards in the state of Tamaulipas. In several of the cases, *Lasiodiplodia* spp. was identified and identified as the possible causative agent; however, there are no publications in this regard. Due to the above, the objective of this work was to identify isolated fungal strains of citrus trees with symptoms of regressive death and to evaluate their pathogenicity under greenhouse conditions.

Materials and methods

Plant sampling and fungal isolation

Sampling was directed towards sweet orange trees (*Citrus sinensis* L.) with symptoms of regressive death and necrosis in the trunk and branches, collected in orchards of the municipalities of General Teran and Montemorelos, Nuevo León, and Llera de Canales, Tamaulipas.

Affected tissues with symptoms of necrosis and regressive death were cut into sections of approximately 1 cm in the transition zone between the necrotic part and the apparently healthy part, disinfected with 10% commercial bleach for 1 min, then washed with water two-distilled, were planted in Petri dishes with potato dextrose agar (PDA) and incubated at 25 ±2 °C with a photoperiod of 12 h light and 12 h of darkness (Marques *et al*., 2013). The isolated fungi were reseeded by the hypha tip method on water agar and incubated under the same conditions of light and temperature for 4 weeks, in order to obtain pure strains (Twizeyimana *et al*., 2013).

Morphological and molecular identification of fungi

For the morphological identification of the isolated fungi, cultural characteristics were taken into account, such as growth, color, type and shape of the colony, as well as the growth rate (Urbaez *et al*., 2013) observed with the microscope in preparations of the fungi stained with lactophenol. The identification of fungal species was carried out using the keys of Phillips *et al*. (2013).

For molecular identification, the strains were grown in PDA at 25 ±2 °C for 7 days. DNA extraction was performed using the DNeasy Plant Mini Kit^{TM} (Qiagen, Inc.) with slight modifications to the manufacturer's instructions and the DNA was quantified using a Take3^{MR} spectrophotometer (Bioselec).

The amplification reactions were carried out in volumes of 25 µL. The ITS-1 region, 5.8S ribosomal gene and ITS-2 were amplified using the oligonucleotides ITS-1fu 5'-tccgtaggtgaacctgcgg-3' and ITS-4 5'tcctccgcttatttgatatgc-3' (White *et al*., 1990).

For the reactions, 5 µL of 5X PCR buffer, 2 µL of $MgCl_2$ (25 µM), 2 µL of dNTP's (2.5 mM), 0.2 µL of go-Taq enzyme (5U μL^{-1}) (Promega^{MR}), 9.8 µL of Mili-Q grade water, 2 µL of each primer (10 pmol μL^{-1}) and 2 µL of DNA were used. The PCR consisted of an initial denaturation cycle at 94 °C for 2 min, followed by 35 cycles at 94 °C for 20 s, 55 °C for 25 s and 72 °C for 50 s, followed by a final elongation step for 4 min. The PCR products were visualized on 1% agarose gels, previously stained with 0.5 ng μL^{-1} of ethidium bromide and compared with a molecular weight marker (ladder-100^{MR}, Axygen).

The obtained PCR products were sequenced in the company Macrogen (EE. UU.) and compared with GenBank sequences to confirm the identity of the fungi at the species level.

Evaluation of the pathogenicity of fungi isolated

To determine the pathogenicity of the isolated strains, a bioassay was conducted under greenhouse conditions at the UANL Faculty of Agronomy located in Gral. Escobedo, NL. This municipality is located at 25° 47' 5.98" north latitude and 100° 17' 12.12" west longitude at a height of 481 meters above sea level.

For the evaluation of the pathogenicity plants were used sweet orange (*C. sinensis*) Valencia variety of one year of grafted, which measured 60 to 75 cm in height and were obtained from a commercial nursery. The inoculum was obtained by cultivating the fungi in PDA for 6 days at 25 ±2 °C and 50% relative humidity.

A completely randomized design with 9 treatments was established. Three treatments with the fungi individually, three treatments where the different strains were combined in pairs, a treatment with the combination of the three strains, a control (plants inoculated with sterile PDA) and an absolute control of plants in which only the cut (Table 1). For each treatment, three repetitions were used.

Table 1. Description of the treatments used in the evaluation of the pathogenicity test of isolated fungi.

Treatments	Number
Absolute control (cut)	1
Control (sterile PDA)	2
Fomitopsis meliae (F)	3
Lasiodiplodia theobromae (L)	4
Eutypella citricola (E)	5
F+L	6
F+E	7
L+E	8
F+L+E	9

For the inoculation of the fungi, the plants were subjected to longitudinal wounds of 1 cm using sterile knives. The bark was detached slightly as a flange, causing a wound by plant. In the wound fungi were inoculated by placing mycelial discs of 5 mm in diameter taken from the margin of mycelial growth, then the flange was closed and covered with parafilm to avoid drying the fungus and the plant. Periodic observations were made at seven-day intervals to measure the length of the lesions. Of the plants inoculated with symptoms of necrosis and regressive death, we proceeded to re-isolate and identify the phytopathogenic fungi following the previously described method.

Statistical analysis

An analysis of variance (Anova) was performed to determine the average effectiveness of each treatment and a multiple comparison of means with the Tukey method ($p \leq 0.05$) with the statistical program SPSS 22 (IBM Corp., 2013).

Results

Isolation, purification and identification of fungi

In the cultures carried out, between 2 and 5 days, several fungi grew, which were purified by hyphae tip and identified by their morphological characteristics as *L. theobromae*, which in PDA medium initially showed a whitening colony coloration, becoming gray and darkening as the days passed, the conidia initially oval in shape, hyaline and without septa, but as the crop grew older they became septate and acquired a brown coloration. *Fomitopsis* sp. was identified by its gray-black aerial mycelium and hyaline, unicellular, ovoid and ellipsoidal conidia. *E. citricola*

was identified by its colonial growth of white color, with aerial mycelium and when mature it formed dark and scattered picnidia. The present conidia were hyaline, filiform, unicellular and some curved.

With respect to molecular identification, DNA extraction of the three species of fungi isolated from symptomatic samples of sweet orange was successful. In the 3 cases, an average of 30 ng μL^{-1} of DNA with an absorbance ratio of 260/280 between 1.8 and 2 indicating good quality was observed.

In the PCR reactions, the DNA amplification of the 3 fungal species was achieved using the universal primers for fungi ITS-1fu/ITS-4. The consensus sequence of the PCR products was 515 bp for the ITS-1, 5.8S and ITS-2 regions, in addition to partial sequences of 18S and 28S RNA. When comparing the DNA sequences of the isolates with GenBank sequences, in all three cases 99% similarity coincided with *L. theobromae* (accession JN048466.1), *F. meliae* (HQ248221.1) and *E. citricola* (KM396616.1).

Pathogenicity test

Four days after the inoculation, the plants inoculated with *F. meliae* (treatment 3), showed symptoms of necrosis, wilting and death of branches in descending direction. After 32 days the external necrotic lesions measured between 9.5 and 29 cm long, this fungus being the cause of more aggressive symptoms of descending death (Table 2, Figure 1). At the end of the bioassay, longitudinal cuts were made of the plants with wilt symptoms, and brown spots on the vascular tissues could be observed in them.

Table 2. Average length of stems lesions in inoculation treatments with fungi in sweet orange trees (*C. sinensis*).

	Treatments	Lesion length[1] (cm)	
3	*Fomitopsis meliae* (F)	25 ±1.52	a
6	F + L	22 ±8.11	a
9	F + L + E	3 ±1.33	b
7	F + E	3 ±0.96	b
4	*Lasiodiplodia theobromae* (L)	2 ±0.4	b
8	L + E	2 ±0.43	b
5	*Eutypella citricola* (E)	1.56 ±0.51	b
1	Absolute control (cut)	0	b
2	Control (sterile PDA)	0	b

[1]= Average of three repetitions ±2 standard deviations 32 days after inoculation. Treatments with the same letter have no significant difference at the 0.05 level of significance.

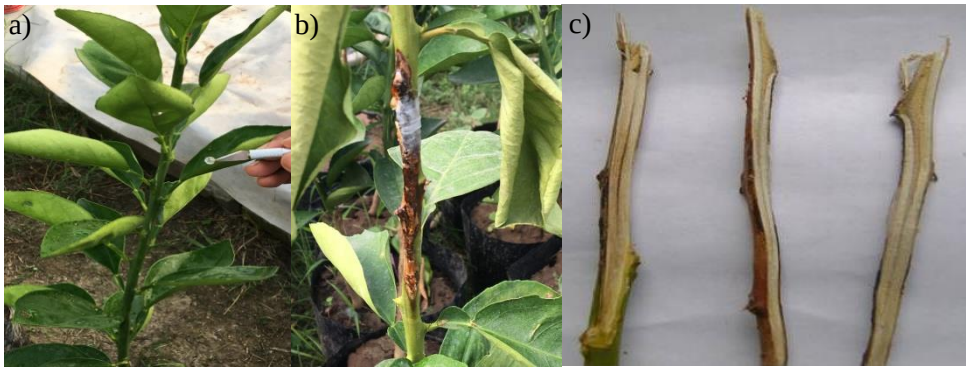

Figure 1. Pathogenicity test of fungi causing regressive death in sweet orange (*C. sinensis* L.). a) inoculation with mycelium discs; b) symptoms of regressive death; and c) vascular beams of the stem.

In the treatments in which *E. citricola* was used as inoculum, the symptoms were less aggressive, since only 1 to 4 cm necrosis was observed around the inoculated area, but they did not express symptoms of wilt.

From all inoculated plants, direct lamellar assemblies and replantings were made in PDA. The morphological and colonial growth characteristics were similar to those previously described for the strains of *F. meliae* and *L. theobromae* used for the inoculation. The DNA sequencing of the PCR products coincided in 99% only for *L. theobromae* and *F. meliae*, for the re-isolation of *E. citricola* from the inoculated plants, PCR amplification was not possible and therefore neither DNA sequencing

Discussion

It is reported that the genus *Lasiodiplodia* spp. and other genera belonging to the Botryosphaereacea family cause regressive death in various woody species (Adesemoye *et al*., 2014). Other common symptoms are gummosis, canker, peduncle rot and fruits (McDonald and Eskalen, 2011). In Mexico there are reports of *L. theobromae* as a cause of regressive death in mango, zapote and guanabana (Sandoval *et al*., 2013; Tovar *et al*., 2013; Hernández *et al*., 2013), but there is no report in citrus. In the present work it was possible to isolate *L. theobromae* and its pathogenicity was checked. The study of this species affecting citrus trees, where previously they had not been detected is of great interest, since some factors are possibly influencing them to produce the disease. One of these factors may be drought stress (Mullen *et al*., 1991), since some of these species may be present in crops as saprophytes and not cause any symptoms (Trakunyingcharoen *et al*., 2015) until favorable conditions arise.

With respect to the identification of *F. meliae* causing this problem in citrus, these results coincide with that reported by Roccotelli *et al*. (2014), who demonstrated through pathogenicity tests that by inoculating *F. meliae* on lemon branches (*Citrus lemon*), it can quickly colonize the inoculated branches, indicating that it is a potential citrus pathogen. Our results also coincide with that reported by Mayorquin *et al*. (2016), who point out that *Eutypella* sp. caused cankers and necrotic lesions on the inoculated citrus branches. Although *F. meliae* is an aggressive pathogen (Roccotelli *et al*., 2014), its pathogenicity decreased when it was combined with *E. citricola*.

The length of the lesions varied depending on the inoculated fungus. This difference in the length of the lesions can be compared with that reported by Chen *et al*. (2014), where *Lasiodiplodia citricola* produced canker and decay in walnut branches (*Juglans nigra*) when they were inoculated by a spore suspension, unlike inoculation with mycelial discs. However, the method of inoculation used for *Lasiodiplodia* spp. has shown that it is an aggressive pathogen, because it causes necrosis, vascular discoloration, crown wilt and death regress (Adesemoye *et al.*, 2014; Linaldeddu *et al*., 2014).

The fact of being able to re-isolate the fungi of the inoculated plants, shows that the infection was achieved because penetration of the hyphae of the pathogens and colonization of the vascular system of the plants was registered (Figure 1).

Conclusions

The isolation of 3 phytopathogenic fungi from citrus trees with symptoms of regressive death was achieved in the states of Nuevo Leon and Tamaulipas, Mexico. The fungi were identified as *L. theobromae*, *F. meliae* and *E. citricola* by cultural, morphological, molecular and pathogenic characteristics. Of the species identified only *L. theobromae* and *F. meliae*, they reproduced the symptoms of the disease in greenhouse conditions, which were re-isolated and their identification was corroborated.

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