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First report of *Phytopythium vexans* causing the "Avocado sadness" in Michoacan, Mexico

Hernández Pérez A[1], E Cerna Chávez[2], JC Delgado Ortiz[3], M Beltrán Beache[4], LM Tapia Vargas[5], YM Ochoa Fuentes[2]

Abstract. Mexico is the main producer, consumer and exporter of avocado in the world, being Michoacan the main producer state contributing more than 80% of the national production. There are phytopathogens that decimate the production causing the death of the tree. Root samples were collected in avocado trees that showed the characteristic symptomatology of the disease known as avocado sadness, the sampling was carried out in four of the main avocado producing towns, in the state of Michoacan, Mexico. The isolation consisted in sowing root tissue in Petri dishes with V8®-PARPH culture medium, subsequently they were identified morphologically and for species level it was determined by molecular biology, with the PCR-ITS technique. Pathogenicity tests were performed in triplicate with avocado seedlings with more than six leaves. After 24 hours, the inoculated plants expressed decay in the apical part, after 120 hours the leaves showed yellowing and after 15 days there was a generalized wilt on the stem and leaves, re-isolating the phytopathogen Phytopythium vexans.

This study confirms the first report of the oomycete *P. vexans* affecting avocado trees in the most important producing region of the Mexican Republic.

Keywords: *Molecular identification; Pathogenicity; Phytopythium vexans; Avocado.*

INTRODUCTION

The world production of avocado is of 4,700,000 tons, of which the American continent contributes 70.3% of the production (FAOSTAT, 2013). Mexico is considered the center of origin of this crop, standing out as the main producer, consumer and exporter of avocado in the world with an established area of 203,732 ha and a production that exceeds 1.8 million tons, of which, the 49% goes to the international market (SIAP, 2016).

In this sense, Michoacan is the main producer, contributing 8 out of every 10 tons produced by the Mexican Republic (SAGARPA, 2017). However, there are different phytosanitary limitations, highlighting the oomycete *Phytophthora cinnamomi* as the most important economically, this phytopathogen is the cause of the rot of the root system and in the aerial part of the tree a wilt known as avocado sadness (Zentmyer et al., 1994; Pérez, 2008). Until a few years ago only *P. cinnamomi* was mentioned as the only pathogen responsible for this symptomatology, however, some authors report new species causing wilt in this crop such as: *Cylindrocladium parasiticum*, *Cylindrocarpon liriodendri*, *Nectria liriodendri*, *Ilyonectria macrodidyma* (Dann et al., 2011; Vitale et al., 2012) and *Phytopythium vexans* isolated from avocado roots in the Canary Islands and showing a more severe pathogenicity than *P. cinnamomi* (Rodríguez et al., 2014). Therefore, the objective of the present investigation was to identify and determine the pathogenicity of *Phytopythium vexans* in avocado.

MATERIALS AND METHODS

Root samples were collected from avocado trees (*Persea americana* Mill. Hass var.) from Auguste to September 2014 , they showed typical symptoms of avocado wilt disease ("Avocado sadness"), the trees had necrotic roots and brittle, yellowing in leaves and a descending death. They were sampled 40 orchards from Peribán, Uruapan, Tancítaro and San Juan Nuevo Parangaricutiro, being the main producers of avocado. The area per orchard was variable 3-15 ha and was directed in trees with the characteristic symptomatology

[1] Estudiante del postgrado Universidad Autónoma Agraria Antonio Narro, Departamento de Parasitología, Buenavista, Saltillo, Coahuila, México CP 25315.
[2] Universidad Autónoma Agraria Antonio Narro, Departamento de Parasitología, Buenavista, Saltillo, Coahuila, México CP 25315.
[3] Catedrático CONACYT-UAAAN. Universidad Autónoma Agraria Antonio Narro, Departamento de Parasitología, Buenavista, Saltillo, Coahuila, México CP 25315.
[4] CULTA S.A. de C.V., Investigación y Desarrollo, Altavista, El Mante, Tamaulipas, México CP 898800.
[5] INIFAP, Campo Experimental Uruapan. Av. Latinoamericana No. 1101, Col. Revolución CP. 60500, Uruapan, Michoacán, México.
Address correspondence to: YM Ochoa Fuentes, *e-mail:* yisa8a@yahoo.com

of the disease "Avocado sadness". The isolation procedure began by eliminating soil excess from the roots with water and making longitudinal cuts measuring less than 0.5 cm, only on dark brown brittle roots taken from the infection's leading edge. The root cuts were disinfected with sodium hypochlorite at 3% during 3 min, followed by three sterile distilled water washes, before being seeded in petri dishes with selective medium (for oomycetes) PARPH (Pimaricin 10 µg/mL, Ampicillin 250 µg/mL, Rifampicin 10 µg/mL, PCNB 100 µg/mL and Hymexazol 50 µg/mL) proposed by Davison & Ribeiro (1996). The plates were incubated at 28 ºC for 4 days and a mycelia disc of 0.5 cm in diameter was transferred to petri dishes with V8®-Agar cultural media, where it grew as pure isolate. These obtained colonies were identified up to the gender level with taxonomic keys proposed by De Cock & Lévesque (2004) and Bala *et al*. (2010). Molecular identification was done through PCR-ITS. DNA extraction was performed using the method Doyle and Doyle (1990), in which 0.2 g of mycelium was grinded with liquid nitrogen and resuspended in 500 µL of extraction buffer (Tris-HCl pH 8.0, 100 mM; EDTA pH 8.5, 50 mM; NaCl 50mM & 2% SDS) the DNA was visualized on a 2% agarose gel. PCR was used to amplify internal transcript regions with ITS1 primers (5´- TCCGTAGGTGAACCTGCGG-3´) and ITS4 (5´- TCCTCCGCTTATTGATATGC-3´). The PCR reaction conditions were: 1 cycle of initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 95 °C for 10 seconds, 30 cycles of alignment at 57 °C for 30 seconds, 30 cycles of extension to 72 °C for 2 min and 1 cycle of final extension at 72 °C for 5 min (Ochoa *et al*., 2012). Results from the reaction were seen through electrophoresis in agarose gel at 2% stained with GelRed (GenScript®). The PCR products of the synthesized chain were sequenced in both directions by Macrogen USA. The sequences were compared in NCBI Gene Bank data base (National Center for Biotechnology Information www.ncbi.nlm.nih.gov/). Pathogenicity tests were replicated three times using avocado seedlings with 6 true leaves, 3 control plants were used, treated with sterile distilled water, 3 positive control inoculated with *P. cinnamomi* and the inoculation technique described by Van den Berg *et al*. (2007) and Engelbrecht & Berg (2013). 500 mg of mycelia were weighted and fractioned by forced stirring during 5 minutes in 150 mL of sterile distilled water. Aliquotes of 50 mL were transferred to sterilized glass flasks with 100 mL of sterile distilled water. The seedlings' roots were washed with sterile distilled water to eliminate substrate residues. Once cleaned, the plants were introduced inside the flasks containing the oomycetes solution, one plant per flask. The flasks were kept at 23 °C inside a bio-climatic chamber with 14/10 h of light and darkness periods, in order to conduct daily observations. At the end of the pathogenicity tests, the inoculated pathogens from necrotic roots were re-isolated, using hereinabove described methodology and replicates were made to confirm the isolates' identity.

RESULTS AND DISCUSSION

Derived from the purification of the strains, 40 isolates were obtained, highlighting the presence of two oomycetes (*P. cinnamomi* and a second oomycete). However, the second had a higher incidence rate with 72.2% and the rest (27.7%) for *P. cinnamomi*. Due to the above, the oomycete with greater incidence was morphologically identified, observing constrictive or lobulated elongated anteridia, and papillary non-globular circular sporangia, coinciding broadly (dimensions and structures) with that reported by de Cock *et al*. (2015) for *Phytopythium vexans*. This result is similar with that reported by Vawdrey et al. (2005) who used the selective medium P10ARP, isolating *P. vexans* more frequently than *Phytophthora palmivora*, noting that the latter was considered the main pathogen causing durian (*Durio zibethinus*) death in northern Queensland. The variant in our work was to add the active ingredient Hymexazole (50 µg / mL) to the selective medium. On the other hand, Kato et al. (1990) and later Davison & Ribeiro (1996) report resistance of some oomycetes (*Pythium*) to Hymexasol, without mentioning the pathogenicity of these species. In the molecular identification, similarity values of 99% were obtained with the sequences HQ85346 and HQ853459 of *Phytopythium* spp. already reported in the Mexican Republic; *Phythium vexans* sequences showed a similarity of 99% with the sequence LM651020, reclassified as Phytopythium vexans (Cock et al., 2015). While the isolate S4-39 showed a similarity of 99% with LM651019 (*Phytopythium vexans*),phytopathogen that causes the symptomatology of the avocado sadness disease reported by Rodríguez et al. (2014) in the Canary Islands, mentioning that some isolates were more aggressive than *P. cinnamomi*. Since there is literature confirming the pathogenicity of this oomycete, the pathogenicity test was performed. Pass by 24 h after inoculation, the plants showed apical decay; the leaves turned yellow after 120 h (post inoculation), and after 15 days the leaves and stems were dry and brittle. The isolate S4-39 caused the wilting of the inoculated plants, in the radicular system a necrosis was observed in some cases greater than the control inoculated with *P. cinnamomi*; while the control inoculated with sterile distilled water did not show any decay, yellowing or any other symptom related to the disease known as "Avocado sadness". Fulfilling the postulates of Koch, it gives us the certainty that *P. vexans* is the causal agent of the symptomatology of the sadness of the avocado tree. This phytopathogen agrees with the symptomatology described by Mora et al., (2007) for the oomycete *P. cinnamomi*. It is important to note that *P. vexans* has been reported since 2005, in where different authors obtain similar results to the present investigation, reporting its pathogenicity in vegetables and fruit trees, causing root rot in kiwi, apple and grapevine (Vawdrey et al., 2005; Spies et al., 2011; Polat et al., 2017).

Affectations have been reported in woody non-fruit species (family: fabaceae) in association with *Phytophthora palmivora* (Steinrucken et al., 2017). With the above, the first report of the oomycete *P. vexans* affecting avocado trees in the

most important producing region of the state of Michoacan in the Mexican Republic is confirmed.

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