

## ***Phytophthora heveae* causing basal rot of avocado fruit in Mexico.**

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### **Abstract**

During the summer of 2010 was observed in the avocado region of Michoacan and Nayarit, Mexico a high incidence of Hass avocado fruit affected by basal rot. Diseased fruits showed large necrotic lesions in the epidermis and flesh darkening, mainly in the distal region; fruits with apical and lateral lesions were also observed. The aim of this study was to determine the cause of the disease. From diseased fruits washed with tap water and disinfected with 3 % sodium hypochlorite, mycelial colonies with petaloid growth were obtained consistently in potato dextrose agar. From these colonies *Phytophthora heveae* was identified, based on morphological characteristics of the sexual structures developed in lima-bean agar and sporangia formed directly in the host. The identification was confirmed by amplification of 5.8S ribosomal DNA gene and internal transcribed spacers (ITS) with the ITS primers ITS5 and ITS4. The pathogenicity of isolates was confirmed in healthy fruits inoculated with LBA disks with mycelial growth of 48 h. Inoculated fruits were kept in a moist chamber at 25°C and they developed necrotic lesions 72 h after inoculation. To our knowledge, this is the first report of *P. heveae* causing fruit rot of avocado in Mexico.

### **Resumen**

Durante el verano del 2010 se presentó en la zona aguacatera de Michoacán y Nayarit, México, una alta incidencia de frutos de aguacate Hass afectados por pudrición basal; los frutos mostraron grandes lesiones necróticas en la epidermis y manchado de pulpa, principalmente en la región distal; frutos con lesiones apicales y laterales también fueron observados. El objetivo del presente estudio fue determinar la causa de la enfermedad. De frutos enfermos lavados con agua corriente y desinfectados con hipoclorito de sodio 3 %, se obtuvieron en papa dextrosa agar colonias miceliales blancas de crecimiento petaloide. A partir de estas colonias se identificó a *Phytophthora heveae* con base en las características morfológicas de las estructuras sexuales desarrolladas en frijol lima-agar y de los esporangios formados directamente en el hospedero. La identificación fue confirmada por la amplificación del gen 5.8S del rDNA y los espaciadores internos transcritos (ITS) con los iniciadores ITS5 e ITS4. La patogenicidad se verificó en frutos sanos inoculados con discos de LBA con crecimiento micelial de 48 h. Los frutos inoculados fueron mantenidos en cámara húmeda a 25°C y las lesiones necróticas se desarrollaron 72 h después de la inoculación. Este es el primer reporte de *P. heveae* causando pudrición del fruto de aguacate en México.

**Key words:** *Phytophthora*, avocado fruit, basal rot, ITS.

**Introduction.** In summer of 2010, a severe basal rot of Hass avocado fruit was observed in many commercial orchards in the Mexican states of Nayarit and Michoacan. Large black lesions in the epidermis and flesh darkening in the distal region characterize the disease. Apical and lateral lesions can occur. As the disease progress, the whole fruit is affected causing the fruit to fall. When humidity is high and temperature is over 25°C, affected fruits show reddish water-drops running down the fruit that carry *Phytophthora*-type sporangia. Young twigs located near the affected fruit, can show wilt and canker. Dropped fruits act as inoculum source for new infections. Low level fruits are more exposed to infection, but fruits located at second third of the canopy are infected to. The disease is more intense in warm climate regions and can increase to epidemic levels under constant rainfall. The disease occurs from July to September affecting fruits close to physiological maturity. Copper sprays are very effective for disease control.

It was postulated that this disease could be caused by a species of *Phytophthora*. Several species of *Phytophthora* have been associated with avocado fruit rot but only *P. cactorum* have

been formally reported in Spain (López-Herrera et al., 2005). The objective of this work was to identify the agent causing the basal rot of avocado fruit in México.

## Materials and methods

**Isolation.** Thirty samples of diseased fruits were obtained from six commercial avocado orchards in the Mexican states of Nayarit and Michoacan during the summer 2010. Fruit samples were washed under running tap water. Small segments (2 to 5 mm long) of affected tissue were selected at the margins of diseased tissue. Each segment was surface disinfested for 20 s with 3% sodium hypochlorite, washed with sterilized water, dried and immediately placed in Petri plates containing potato dextrose agar (PDA, DIFCO) amended with 0.2 mL of 92% lactic acid per liter of PDA. Cultures were incubated in darkness at 25°C for 7 days. Growth characteristics of mycelium on agar media were examined using a light microscope and tips from hyaline, coenocytic hypha from emerging colonies were sub cultured on PDA for further characterization.

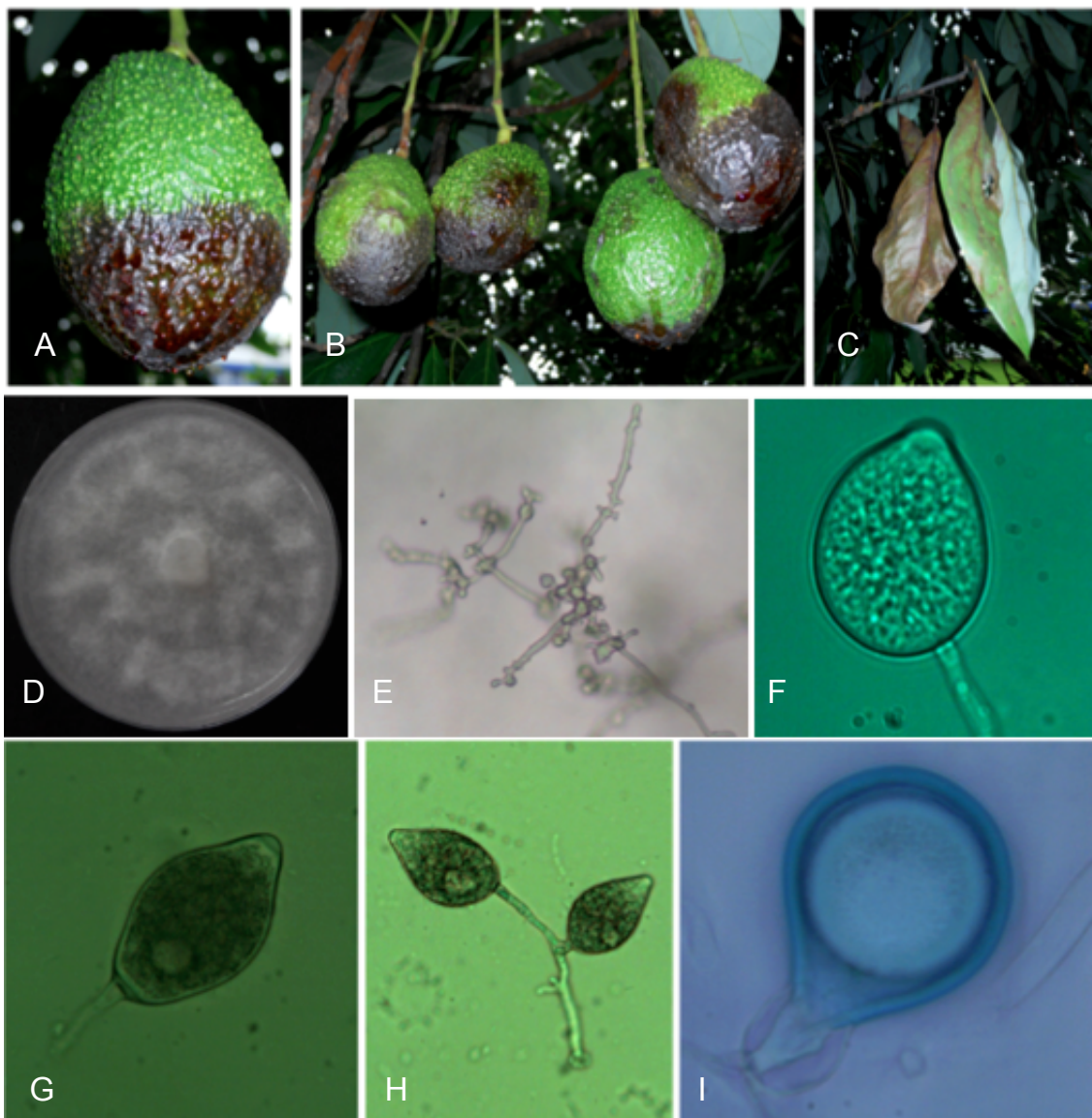
**Morphological characterization.** Isolates obtained were identified to species level with three basic morphological criteria and molecular characteristics. Colony morphology was characterized after 7 days at 25°C on lima bean agar (Gallegly and Hong, 2008). Mycelium growth habit, mycelium pattern and structures present in agar were determined. Sporangia morphology was determinate in 50 sporangia taken directly from diseased fruit and characterized under light microscope for shape, size, presence of papilla, proliferation and branching of the sporangiophores, and caducity. All the isolates produce abundant gametangia and oospore on standard media and were characterized for shape, size,

**Molecular characterization.** Four isolates (Ag16, Ag20, Ag25 and Ag28) were selected for DNA sequence analysis. Total genomic DNA was extracted from mycelium obtained from 7-day-old PDA cultures grown at 25°C, using the protocol described by Cenis (1992). A 1.5 ml Eppendorf tube was filled with 300  $\mu$ l of extraction buffer (200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) then 100 mg of fresh mycelium were added. The mycelium was crushed with a conical grinder, fitting exactly the diameter tube and actioned by electrical potter at 200 rpm for 3 minutes. After that, 150  $\mu$ l of 3 M sodium acetate, pH 5.2 was added, and tubes were incubated at -20°C for ten minutes. Tubes were centrifuged (11,300 x g, 5 min) and the resulting supernatant was transferred to another tube. Then, an equal volume of cold isopropanol (4°C) was added and incubated at room temperature by 5 min. The precipitated DNA was pelleted by centrifugation (11,300 x g, 5 min), washed in 70% ethanol, and re centrifuged (11,300 x g, 5 min). The pellet was collected and dried at room temperature for 20 min and re suspended in 50  $\mu$ l of TE buffer. The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified in a Multigene thermocycler using the primers ITS5 and ITS4 (White, Bruns, Lee, Taylor 1990). Each reaction mixture was 12.5  $\mu$ l, containing 1  $\mu$ l of template DNA (20 ng), 0.02 mM each dNTP, 0.2 pmol forward primer ITS5, 0.2 pmol reverse primer ITS4, 2.5 mM MgCl<sub>2</sub>, 0.02 units Taq DNA polymerase (Invitrogen), 1x PCR buffer (Invitrogen) and double distilled water. The reaction cycle consisted of initial denaturation at 96°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 80 s, and final extension at 72°C for 10 min. Following amplification, the products were checked on a 1% agarose gel stained with ethidium bromide and visualized under UV light (Transilluminator MiniBis Pro) to confirm successful amplification. A standard 100-bp molecular weight DNA marker (Invitrogen) was included. Products of PCR were purified and sequencing by Macrogen Corp (Rockville, MD, USA). Sequences obtained were subjected to a BLAST search (<http://www.phytophthoradb.org>) and aligned using the CLUSTAL W program (Thompson et al., 1994) prior to phylogenetic analyses to identify the closest related sequences. For phylogenetic inference, a neighbor-joining (NJ) tree was constructed with 1,000 bootstrap replicates. The NJ tree was performed using the computer program MEGA version 5 (Tamura et al., 2011).

**Pathogenicity tests.** Twenty-eight isolates were tested for pathogenicity on fruits of avocado cv. Hass (22% dry matter content), uniform in size and color. The fruits were washed in running tap water and surface disinfested for 1 min in 75% ethanol. Fruits were inoculated with a mycelial plug (5 mm in diameter) placed over the intact skin of basal area. Each isolate was inoculated in two fruits. Ten non-inoculated fruits, served as control. The fruits were placed in humid chamber at room temperature and evaluated at 24 h intervals.

## Results

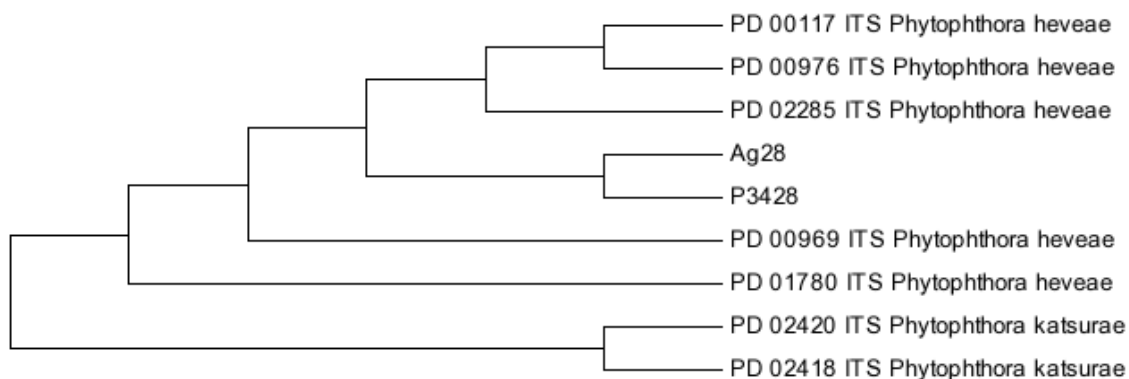
**Isolation and identification.** Twenty-eight isolates of *Phytophthora* spp were obtained from all samples collected. All isolates produced colonies with superficial mycelium in petaloid pattern; composed of hyaline, branched, coenocytic hyphae with abundant hyphal swellings small to large with no chlamydo spores presents. Sporangia papillate, with narrow pore; spherical, ovoid or ellipsoid; noncaduceous on sympodium simple sporangiophores; apical or lateral sometimes asymmetrically attached to sporangiophores. Homothallic, with abundant production of antheridia and oogonia on PDA and lima been agar. Antheridia were amphigynous, spherical or cylindrical; oogonia globose and smooth-walled with funnel shaped at the base. Sometime produced in groups. Oospore aplerotic, spherical, smooth and thick walled (Figure 1).



**Figure 1.** Basal rot of avocado fruit caused by *Phytophthora heveae*. **A**, Typical symptom of basal rot. **B**, Group of avocado fruits with basal and lateral black rot. **C**, Twigs wilt. **D**, Colony on PDA. **E**, Hyphal swellings. **F**, Sporangia asymmetrically attached to sporangiophore. **G**, Papillate sporangia. **H**, Sporangia on sympodium simple sporangiophore. **I**, Aplerotic oospore whit amphigynous antheridia.

**Molecular characterization.** The sequences of the ITS region were practically identical for isolates Ag16, Ag18, Ag20 and Ag28. BLAST searches indicated the relatedness of the isolates used in this study to *P. heveae* (>99%). Isolate Ag28 was 100% identical to Extype of *P. heveae*. NJ phylogenetic analysis of the sequence Ag28 confirmed the position of this isolate grouped in *Phytophthora* Clade 5 (Figure 2).

Homothallic production of oospores; presence of distinctive antheridium and oogonia morphology, sporangia and pedicel characteristics were consistent with the identification made based on ITS sequencing.



**Figure 2.** Neighbor joining tree of the ITS rDNA region of *Phytophthora* clade, showing the position of isolate Ag28 in relation to selected sequences from phytophthoradb.org and the Extype of *P. heveae* (P3428).

### Pathogenicity tests

All isolates tested for pathogenicity induced a 2 cm diameter black necrotic lesion on inoculated avocado fruits after 72 h of incubation. Inoculated fruits developed total rot after 7 days of inoculation, with white mycelium and sporangia covering the epidermal surface. No symptoms appeared on the control fruits. Reisolations made from disease tissue taken from inoculated avocado fruits were successful and the organism reisolated was confirmed as *P. heveae* by microscopic examination.

### Discussion

The first report of *P. heveae* attacking avocado comes from Zentmayer et al. (1978) who find the pathogen causing a new canker disease in Guatemala. Later *P. heveae* was reported in Mexico (Ceja et al., 2000) associated to avocado trunk canker but no reports exist of *P. heveae* affecting avocado fruit. López-Herrera et al. (2005) reports a fruit rot on avocado in Spain caused by *P. cactorum* and describes a symptomatology very similar to the caused by *P. heveae*. Basal rot of avocado fruit was observed by the first time in Michoacan, México in 1982, and the causative agent identified as *P. boehmeriae* (Solis, 1982), however there is no evidence that *P. boehmeriae* is actually present in México. The presence of *P. heveae* in avocado orchards represents a risk for avocado production, not only for the fruits damages, but also for its dispersal potential and its capacity to survival from one year to another in the canopy tree and debris soil.

### Conclusions

Morphological and molecular identification and results of pathogenicity test, confirmed that basal rot of avocado fruit in México is caused by *P. heveae*. To our knowledge, this is the first

report of *P. heveae* causing fruit rot of avocado in Mexico. Our finding is important because the presence of *P. heveae* in this area represents a risk for avocado production, due to its capacity to infect both fruits and trunk.

### Acknowledgments

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