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Armillaria gallica associated with avocado root rot in Michoacán

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ABSTRACT

Aims and background. Root rot and the death of avocado trees related to *Armillaria* is an emerging disease with a high economic impact in orchards established in previously forested areas of Michoacán. Nowadays, the species related with typical symptoms of wilting, yellowing, excessive fruit production and subcortical mycelia in the roots is unknown. The aim of this investigation was to molecularly characterize the species of *Armillaria* associated to avocado root rot.

Materials and methods. For the morphological and molecular characterization, 60 root simples from trees found in three commercial orchards with a putative presence of *Armillaria* were processed in a malt-agar extract. The DNA of purified isolations were amplified by PCR with genes RPB2 and TEF *α-1*. The sequences were aligned using MAFFT and the phylogenies were constructed using the maximum likelihood algorithm in IQ-TREE.

Results. Two species were consistently identified: *A. gallica* (20%) with a 100% homology, and *A. mexicana* (25%), with 98%. Another species that represented 55% of the isolations was not aligned with any group. Morphologically, the *A. gallica* basidiocarps coincide with the characteristics of this species.

Conclusions. This is the first report on *A. gallica* associated to avocado root rot in Michoacán.

Keywords: Basidiocarp, RPB2, TEF *α-1*, Wilting

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Introduction

In Mexico, 1.64 million tons of avocado were planted in 2023, accounting for 31% of the world's production with 179,914.30 ha planted. Out of this surface, Michoacán contributes with 992,816 tons for export to the USA, China, Korea and Chile (SIAP, 2023). The fungus *Armillaria* spp has been recorded in forest and fruit-producing ecosystems, as well as urban trees (Baumgartner, 2004). Trees under biotic or abiotic stresses are more susceptible to *Armillaria*, particularly in areas in which the forest surface has been replaced with commercial crops (Valdez *et al.* 2004). Recent studies on this pathogen describe some species as *A. mellea, A. ostoyae* and *A. mexicana* in peach (*Prunus* sp) and *A. mellea* in forests (Elías, 2018).

Armillaria has been recorded in Michoacán in some crops such as *Prunus pérsica* and *Persea americana*; in the latter, the species is unknown (Téliz and Mora 2015). In peach trees, there are reports of *A. mellea, A. gallica* and *A. mexicana* in Coatepec Harinas and Temascaltepec, State of Mexico (Elías, 2018). The species found in forest trees are *A. calvescens, A. gallica, A. mellea,* and *A. ostoyae*, reported in Chihuahua, Durango, State of Mexico, Guanajuato, Jalisco, Nuevo León, Puebla, Querétaro, Veracruz and Morelos (Alvarado *et al*., 2007). Among the different techniques to identify species with little morphological variation, somatic compatibility and molecular analysis stand out (Schulze *et al.*1997; Worrall, 1994; White *et al*. 1990). At a molecular level, elongation factor TEF-*1α* has been used for its phylogenetic usefulness and for being a preserved region (Hasegawa *et al.* 2010). Maphosa and collaborators (2006) used this gene for phylogenetic studies on *Armillaria* and they established the relationship between *Armillaria* species of the Northern and Southern Hemispheres. The aim of this investigation was to molecularly identify the *Armillaria* species related to root rot and wilting of avocado in the state of Michoacán.

Sampling and processing of roots and infected tissues. Roots with *Armillaria* spp. Mycelia were collected from 20 avocado trees in commercial orchards in the municipal areas of Charapan, Los Reyes and Nuevo Parangaricutiro, Michoacán. A total of 60 samples was transported to the laboratory to isolate the fungus. The mycelium was fragmented and disinfested in a 3% sodium hypochlorite solution and 70% ethanol for 10 and 5 min, respectively. Between solutions, it was rinsed with sterile distilled water to later dry the mycelium pieces in sterile wet wipes (Aguín *et al*. 2004).

Isolation and conservation of the strains. A total of 60 isolations were obtained from plantations in Petri dishes with a BDS medium (malt extract (AM) with 40 mg of benomyl, 20 mg of dicloran and 100 mg of streptomycin) (Aguín *et al.* 2004). In each dish, five fragments of mycelium were placed and kept at 24°C in the dark until the fungus began developing rhizomorphs (one month approximately). The strains were preserved in VCAM (Very Cold *Armillaria* Medium: 1.5% agar, 0.75% malt extract, 0.5% peptone and 0.75% dextrose) with five replications per strain.

DNA extraction and PCR. The DNA was extracted from three-week-old strains planted ina 1% malt extract (Figure 1), following the protocol by Cenis (2009).

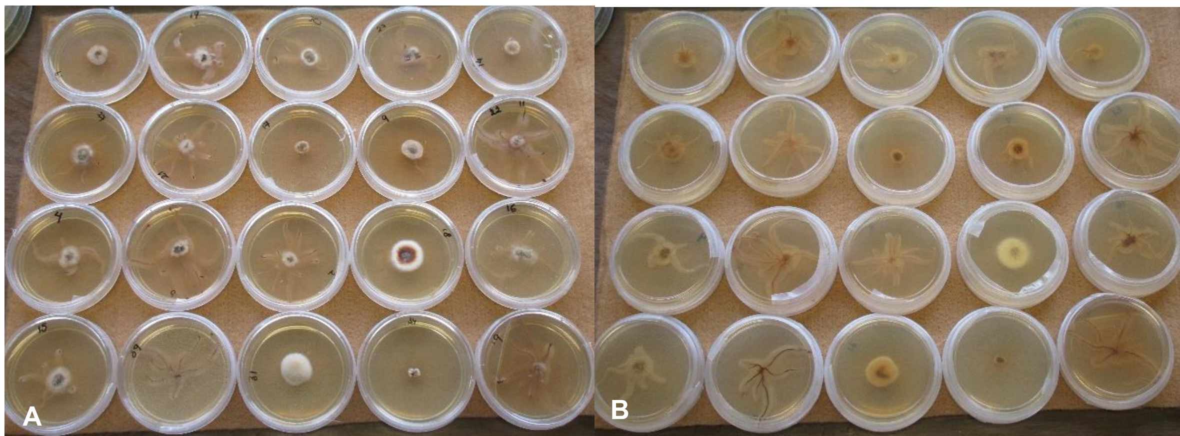

Figure 1. *Armillaria* morphotypes taken from avocado orchards in different municipal areas of the avocado belt in Michoacán. **A**. Front, **B**. Reverse.

TheconcentrationandabsorbanceoftheDNAwerequantifiedintheNanodrop2000c Spectrophotometer®. Regions of genes TEF *1-a* and RPB2 were amplified. For the TEF *1-α* elongation factor, primers EF1160R (5'CCGATCTTGTAGACGTCCTG3') and EFR595F (5´CGTGACTTCATCAAGAACATG3´) were used (Maphosa *et al*., 2006), along with RPB2-6F (5´ TGGGGKWTGGTYTGYCCTGC 3´) and RPB2-7R (5´ CCCATWGCYTGCTTMCCCAT 3´) for gene RPB2 (Brazee *et al.,* 2011). The PCR reactions were carried out in a total volume of 25 µL with 10 mM of Tris-HCl pH 8.0 Buffer, 50 mM KCl, 1.5 µM $MgCl_2$, 25 mM of each one of the dNTP's, 0.2 U of Taq polymerase DNA, 10 pM of each oligo (Invitrogen, USA) and 25 µg of genomic DNA. The PCR reactions were carried out in a Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA). The program consisted of an initial denaturalization cycle at 94°C for 2 min, 30 denaturalization cycles at

94°C for 30 s, an alignment at 56° for 30 s, an extension at 72° for 30 sec and a final elongation at 72° for 7 min for the elongation factor. For gene RPB2, the first cycle was at 95 °C for 2 min, followed by 30 cycles made up of a denaturalization at 94 °C for 30 s, an alignment temperature of 60 °C for 45 s, and an extension at 72 °C for 1 min and a final extension at 72 °C for 10 min (Brazee *et al.,* 2011). The amplicons generated were examined in a 2% agarose gel (p/v) dyed with SYBER Green (Invitrogen, USA). The amplification products obtained were sequenced by the company Elim Biopharmaceuticals Inc. (Hayward, CA. USA). The sequences obtained were edited by hand to eliminate the ambiguities in the ends and a *Blast* search was conducted to find those with a maximum identity with the Genebank NCBI sequences. The fasta files of *Armillaria* spp. sequences and those with higher homology were obtained for phylogenetic analysis, including a sequence of *Desarmillaria tabescens* (KY359140) for use as an outgroup. Sequence alignment was performed using MAFFT with default parameters (Katoh *et al.,* 2017). The resulting alignment was used to obtain an evolutionary model using the ModelFinder software (Kalyaanamoorthy *et al.,* 2017). Phylogenetic trees were generated using the TIM3e+G4 (elongation factor gene) and GTR+G (gen RPB2) models to calculate genetic distances. Dendrograms were generated using the ML criterion in IQ-TREE (Nguyen *et al.,* 2015). SH-aLRT (%), *aBayes support* and *ultrafast bootstrap (UFBoot) support* values were obtained for each bifurcation. Due to the congruence between the three support values, only SH-aLRT percentages greater than 50% were included in the tree. The trees were visualized and edited using the FigTree software, version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Amplification of genes RPB2, TEF *α-1* and phylogeny. The amplification of gene TEF α-1 yielded a band between 450 and 500 pb. Strain MICH29, gathered in Charapan, was identified as *A. gallica* according to BLAST analysis and the morphological characteristics of the basidiocarps (Figure 2).

Figure 2. *Armillaria gallica* basidiocarps gathered in Charapan, Michoacán. **A**. Mature *Armillaria gallica* basidiocarps (yellow cap with scales); **B**. Immature gray *A. gallica* basidiocarps with scales; **C**. Single basidiocarp with a single rhizomorph on the base.

The alignment with sequences from the GenBank displayed a similarity of 99% with sequence JF895870 corresponding to *A. gallica* (Brazee *et al*. 2011) (Figure 3).

This species has been reported in North America and it is broadly related to *A. calvescens*. *A. gallica* has been reported in forest species in Mexico (Alvarado *et al*., 2007) and in peach trees (Elías, 2018). However, this is the report on *A. gallica* associated to avocado root rot in Michoacán. Strain MICH32 and MICH32R was aligned with *A. mexicana*, although no sequences of gene RPB2 exist for this species. The strain 21 from San Juan Viejo was used independently between the clade of *A. mellea* and *A. mexicana*; the percentages of alignment with these species were 62% and 66%, respectively. Therefore, this species cannot be identified. There is probably a relation between these species, although for this particular strain, it is necessary to carry out a morphological description and multigene phylogeny that includes the amplification of at least three genes (e.g., ITS, TEF and RPB2). It cannot be ruled out that due to heterogenicity and little variation in this gene, it may be difficult to differentiate between some species (Hasegawa *et al*. 2010).

The sequences aligned with the elongation factor, TEF *1-α,* mentioned by Maphosa and collaborators (2006) help analyze the relation between species and identify them (Hasegawa *et al.* 2010). Isolate MICH29 is the only one that was grouped in the clade of *A. gallica* with an identity of 100% in regard to the GenBank data base and a fragment amplified between 600 and 700 pb, which is the fragment range obtained for *Armillaria*, using the elongation factor (Hasegawa *et al.* 2010; Maphosa *et al.* 2006). Using strain MICH21 the results were more consistent; *Armillaria* sp was grouped in a clade located between *A. mexicana* and *A. mellea*. The relationship between clades may be due to the possibility of the Southern Hemisphere being the origin of the ancestor of *Armillaria* spp (Maphosa *et al.,* 2006).

The results of the alignment and phylogenetic analysis of gene TEF *α-1* confirm that the relation between *Armillaria* species is very narrow, and that other parameters have to be considered to tell them apart, such as morphologic ones. Some species are significantly linked and ITS amplification is not enough to tell them apart (Coetzee *et al.* 2001). With this gene, the *Armillaria* species of the Northern Hemisphere were found to be widely related (Dunne *et al.* 2002). This fact is related to the interspecific hybridization, as in the case of *A. gallica* and *A. calvescens,* where both species are partially infertile, which is why there is thallus compatibility. Although the IGS and ITS are the most amplified regions for this genus, they are not reliable because there are cases in which the species cannot be differentiated, due to the high diversity of the IGS between isolations.

With this study, the geographic distribution of *A. gallica* broadens to Michoacán, in addition to the previous records in Chihuahua, Durango, State of Mexico, Guanajuato, Jalisco, Nuevo León, Puebla, Querétaro, Veracruz, Morelos (Elías, 2017). Regarding gene RPB2, there are not sequences of all the species in the

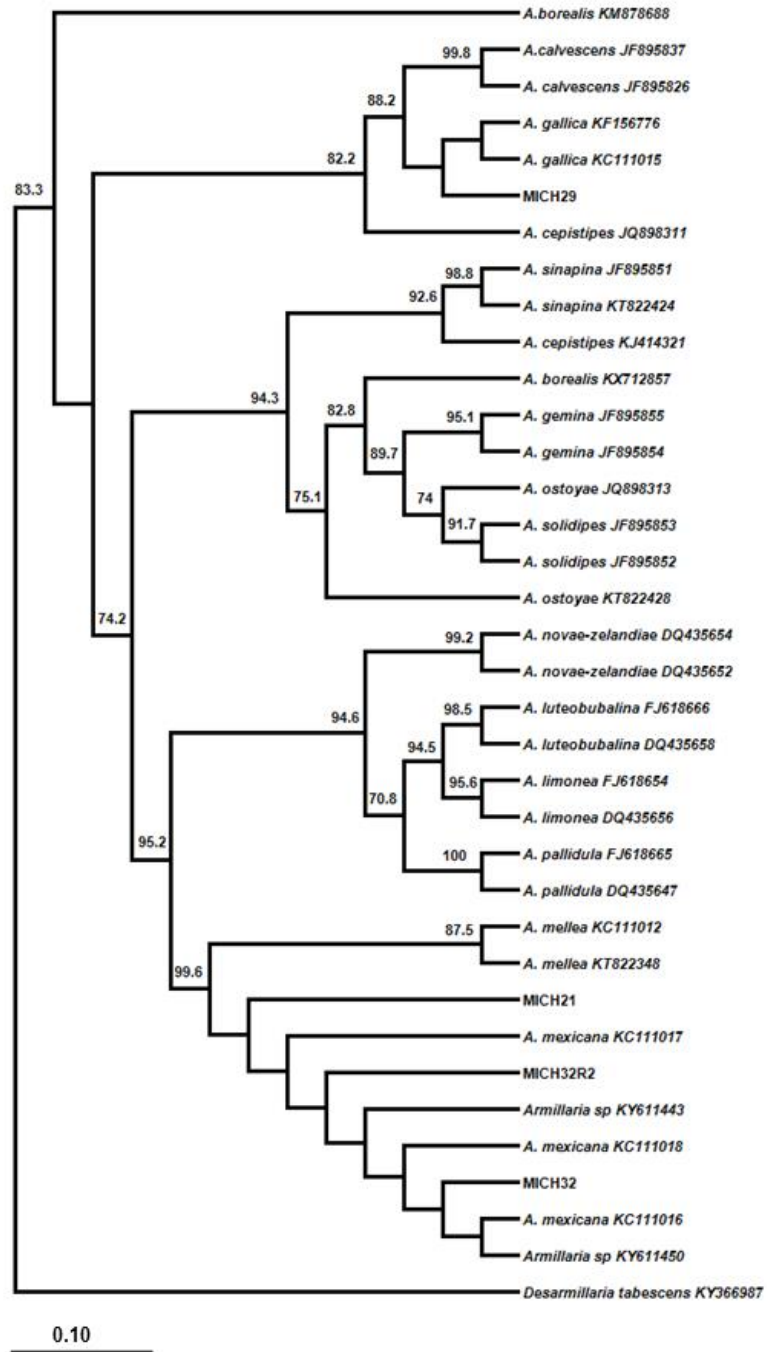

Figure 3. Phylogenetic analysis with *Armillaria* species sequences from avocado and from the GenBank amplified with the gene TEF *α-1*. Isolate MICH29 was directly grouped with the species *Armillaria gallica.* Strains MICH32 and MICH32R are broadly related to *Armillaria mexicana,* whereas MICH21 was grouped between the clade of *A. mellea* and *A. mexicana*

Genbank. However, the sequence of strain MICH29 was aligned 100% with *A. gallica*. Strain MICH21 was aligned with the clade of *A. mellea* (Figure 4).

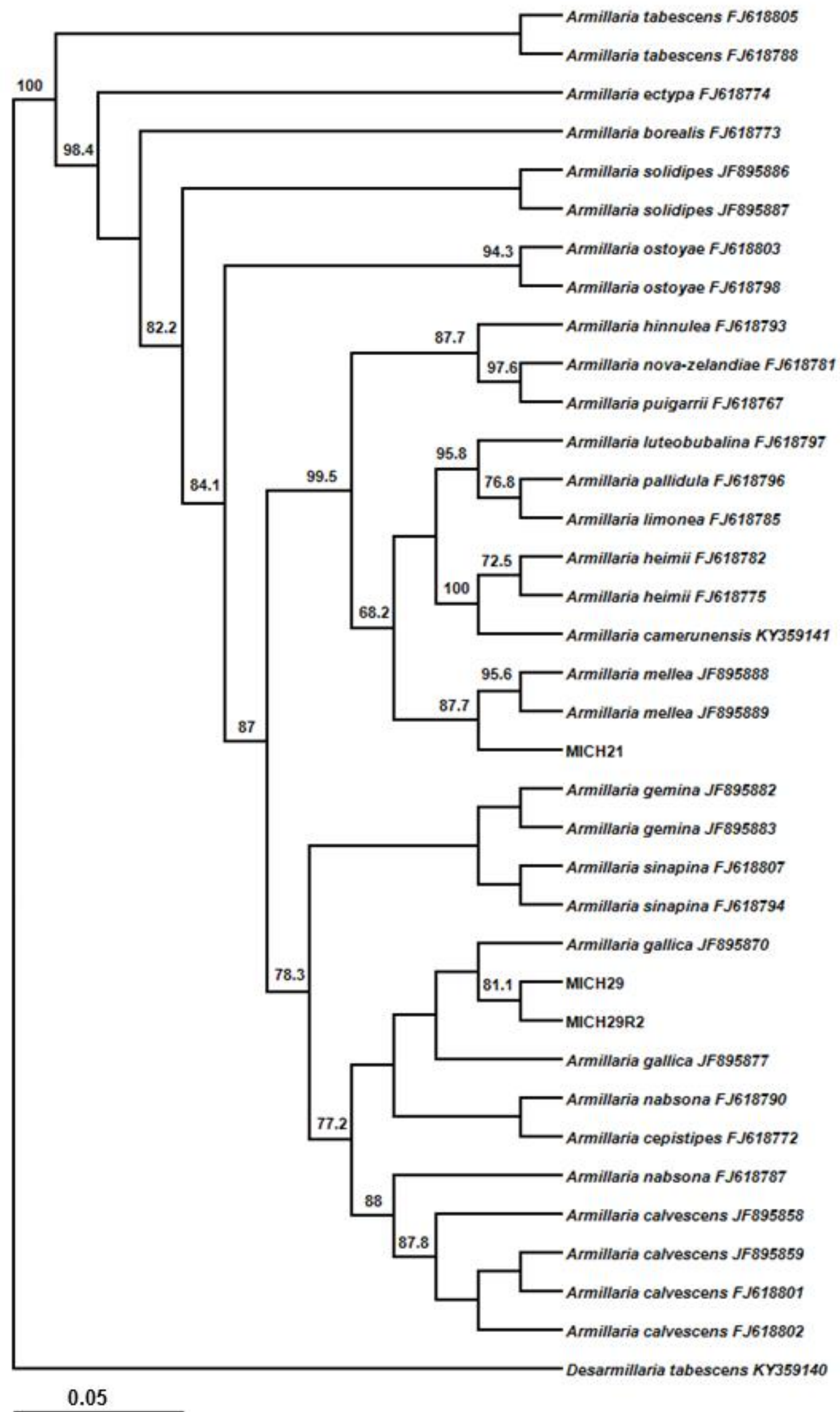

Figure 4. Phylogenetic analysis of sequences of *Armillaria* species from avocado and the GenBank amplified with gene RPB2. Strain MICH29 and MICH29R are grouped directly with the species *Armillaria gallica,* while MICH21 is related to *A. mellea.*

The amplification of gene RPB2 has been useful in the discrimination of species of closely related basidiomycetes. Nevertheless, this gene did not discriminate between the species of *A. calvescens* and *A. gallica* (Brazee *et al.,* 2011). To date, gene RPB2 has been scarcely used to examine closely related *Armillaria* species, but in comparison with the IGS and ITS sequences, they have been used in several phylogenetic studies. Regions of rDNA also provide a deficient resolution to differentiate closely related *Armillaria* species (Ota *et al.,* 2011; Kim *et al.,* 2006). In addition, the diploid mycelia often contain heterogenous sequences that may make analysis confusing (McLaughlin and Hsiang; 2010; Kim *et al.,* 2006).

Conclusions

Three *Armillaria* were found in avocado orchards established in soils with forest backgrounds in Michoacán. *A. gallica* was identified in seven out of 10 samples with more severe root rot symptoms in comparison with the other two species identified. *A. mexicana* was found in 25% and one unidentified species, *Armillaria* sp, in 55%. The elongation factor was the most adequate gene to identify species of *Armillaria.* The exploration of other genes is suggested to generate a concatenated phylogeny. The morphological identification of *Armillaria* species is complicated, due to the narrow characteristics between species. It is necessary to support molecular results with morphological data.

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