HISTOPATHOLOGY OF AVOCADO ROOTS (*Persea americana* MILL) INFECTED BY *Armillaria* SP. AND HISTOLOGICAL CHARACTERIZATION OF THE RHIZOMORPHS

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ABSTRACT

In the state of Michoacán, the substitution of forestry areas by avocado orchards has resulted in incidences of more than 50 avocado trees from two to 30 years old, attacked and killed by *Armillaria*, which has generated important economic losses. Structural alterations of the roots of avocado trees naturally infected by the fungus *Armillaria* sp. were characterized. Higher phenol content in the cortical tissue of healthy roots was observed compared with the vascular system; there was starch in both tissues. The medullar and cortical parenchyma, as well as the associated to the vascular tissue of roots, showed phenolic compounds, disintegration and cellular death, in addition to the occlusion of vessel elements by the presence of mycelium. The invasion of these tissues was intra and intercellular. No starch was present either in the cortex or in the vascular tissue of infected roots. Rhizomorphs were located between the phloem and the pericycle. Rhizomorphs of infected roods in the field, and those artificially developed showed the same structure, cortex, subcortex, primary medulla and hyphae with rounded to oblique cells, and without organelles; the most evident difference was the presence of mucilaginous tissue in rizhomorphs in greenhouse, with a yellow-creamy coloration at the beginning of the growth period, and reddish ochre to black at the end of the developmental period. This is the first record of the alterations in the morphology of roots of avocado trees caused by *Armillaria* and the description of the rhizomorphs in the roots of infected trees in orchards and seedlings in greenhouse.

Key words: *Armillaria*, avocado, rhizomorphs, root rot, histology.

INTRODUCTION

The forestry surface in the state of Michoacán is being partially replaced by avocado. These avocado orchards are exposed to the attack of plant pathogens that have evolved and coexist with forestry trees. *Armillaria* is a native pathogen in forestry fields with species such as *A. borealis*, *A. epistipes*, *A. ostoyae*, *A. gallica*, *A. tabescens* and *A. ectypa* (Aguín *et al*., 2004), which are responsible for root rot in several species of pines and oaks. The initial phase of the disease in forestry trees starts by yellowing and darkening of the leaves. Afterwards, resin secretion from roots and trunk starts (Mallet and Hiratsuka, 1987). Under the bark of the trunk and roots, fan-shaped mycelium develops, whereas rhizomorphs are formed exclusively in the roots (Van Der Kamp and Hood; 2002).

Armillaria is more common in avocado trees older than five years, whose death is accelerated under unfavorable and stressful environmental conditions (Ochoa, 2011). Histological alterations induced by *Armillaria* are unknown in avocado, which contrasts with several works that describe them in forestry trees. In *Larix occidentalis, Pseudotsuga menziessi* and *Picea sitchensis*, compartmentalization of tissue occurs, as well as the formation of callus and periderm (Robinson and Morrison, 2001; Solla *et al.,* 2002; Cleary *et al.,* 2012). Formation of resin was observed in *L. occidentalis, P. menziessi, Pinus radiata* (Van Der Kamp and Hood, 2002) and in *Thuja plicata* (Cleary and Holmes, 2011). These conditions induce the formation of a layer of suberized and lignified cells (Cleary *et al.,* 2012), as well as the differentiation of rhizomorphs (Solla *et al.,* 2002). The formation of cork layers occurs as a typical response due to resin release (Robinson and Morrison, 2001). Rhizomorphs penetrate the tissue by means of chemical and physical mechanisms; the invasion of these structures takes place on and below the bark, which exhibits cell death linked to the advance of the pathogen; finally, the rhizomorph becomes established between the cortex and the xylem (Leach, 1936). Rhizomorph hyphae invade vascular and parenchymatous cells of the xylem and the medulla. The presence of starch could be scarce in the cortex, whereas in the medulla it may be evident and occupy the tissue in its entirety (Leach, 1936; Solla *et. al.,* 2002). In *Glyricidia maculta*, compounds of phenolic origin and necrosis can be observed as responses towards the pathogen (Leach, 1936); cell death was also present in *P. menziessi (*Cleary and Holmes, 2011) and *P. radiata* (Van Der Kamp and Hood, 2002). These alterations can also be the result of the response towards abiotic factors, which is associated with defense mechanisms of the host (Cleary *et al.* 2012).

Regarding the rhizomorphs, their structure, development and functions have not been extensively studied (Snider, 1959). These structures are special morphological adaptations (Shaw and Kile, 1991), highly differentiated, autonomous, with apical growth, that can develop outside the food source or inside the substrate (Shaw and Kile, 1991, Issac, 1995). Rhizomorphs can reproduce vegetatively and spread from a host to another. Several works are limited to the study of rhizomorph development in *Armillaria mellea* (Snider, 1959; Harting, 1873). Townsend (1954) classified the rhizomorphs according to the arrangement of their tissues, whereas the mycelium was studied through electron microscopy (Berliner and Duff, 1965), and porous spaces in mycelial cells of the rhizomorph were observed (Issac, 1995; Moore, 1965). The first description of the apical growth of rhizomorphs was recorded by Brefeld in 1877. The importance of the study of the rhizomorph lies in its consideration as a structure in the process of somatic nuclear division in basidiomycetes (Motta, 1969), as well as its being a structure of protection and mobilization of nutrients of the pathogen (Issac, 1995).

To this date, there are no histological studies of avocado roots infected by *Armillaria*, or of the rhizomorphs, so the objective of this research was to describe the structural alterations in avocado roots infected by this pathogen, and histologically characterize the rhizomorphs from the field taken from avocado roots, and rhizomorphs developed in culture medium.

MATERIALS AND METHODS

Sampling. -Sampling was carried out in an orchard of avocado Hass, located in the municipality of Charapan, Michoacán (2352 meters above sea level, 19º 37’ 92’’ N and

102º 18’ 01.7’’ W). Avocado roots with signs and symptoms of *Armillaria* were collected and dissected in three 1cm-long segments to isolate the pathogen and for histological analysis. Root segments were disinfected with 3% sodium hypochlorite and 70% ethanol for 10 and 5 min, respectively. Samples were rinsed with sterile distilled water, and cultured in BDS (Benomyl – Dichloran - Streptomycin) and V8 (Agar – V8 juice) at room temperature in the dark.

Histological analysis. - Roots collected in the field and rhizomorphs collected in the field or grown in culture medium were cut in 1cm-long sections and fixed in a solution of formaldehyde – glacial acetic acid and 96% ethanol for 24 h (López *et al*., 2005; Cleary and Holmes, 2011). Samples were gradually dehydrated in 10, 20, 30, 40, 50 70, 85, 96 and100% ethanol for 5 h in each solution. Afterwards, they were placed in a mixture of absolute ethanol:xylene (1:1) for 5 h in each solution. The material was embedded in paraplast (SIGMA Chemical Co., USA) for 48 h at 60 °C. The samples were sectioned transversally and longitudinally in a rotary microtome (Spencer 820) with a thickness of 10 µm. The sections obtained were cleaned from paraffin and stained using the safranin-fast green differential technique, according to the protocol described by López *et al.* (2005). The sections were observed and described in an American Optical (Spencer) microscope. The photographic record was taken with the aid of a Carl Zeiss III photomicroscope coupled with a PAXcam digital microscope camera.

RESULTS

***Symptoms*. -** Infected trees exhibited yellowing, wilting, apical and partial leaf loss. In trees bearing fruits, flowers and fruits remained attached. Fan-shaped mycelium was found under the bark, and rhizomorphs were observed on roots and in a groove at the base of the trunk (Figure 1).

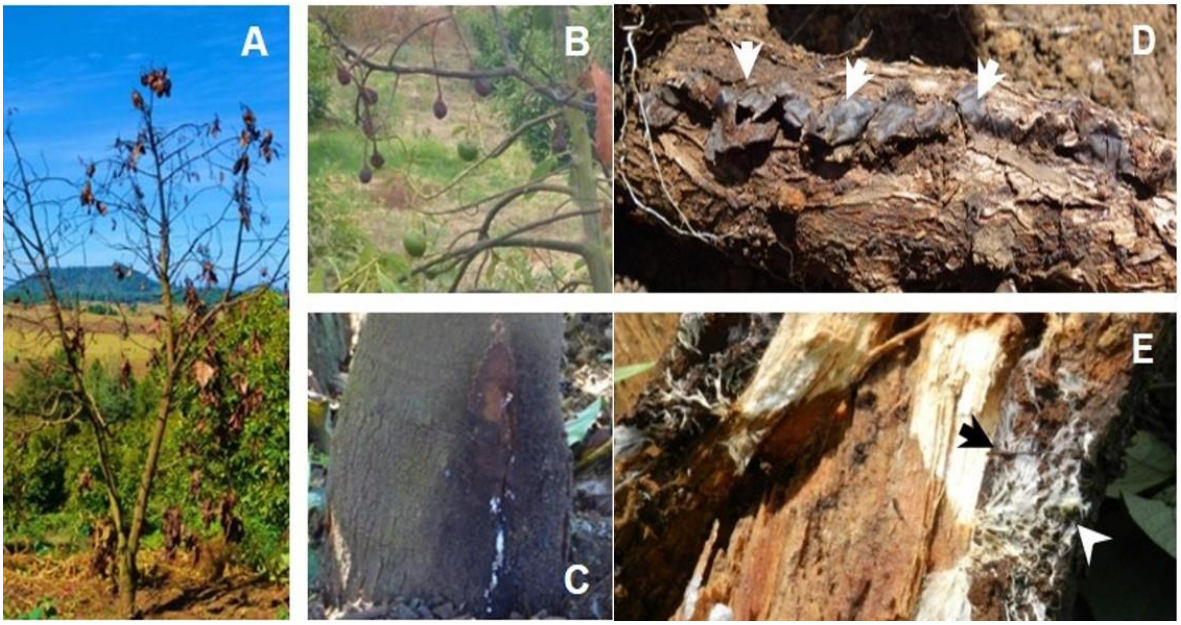

Figure 1. Symptoms induced by *Armillaria* in Hass avocado trees. Partial leaf loss (A), fruits attached to branches (B), Groove at the base of the trunk, observed in all symptomatic trees (C), Root with rhizomorphs (D), Root with subcortical fan-shaped mycelium (E).

***Histology of healthy plants*. -** The periderm in transverse sectioning was observed as a layer with six to seven strata of oblong, thin-walled cells. The cortex was formed by approximately 12 cell strata with reddish brown deposits, possibly of phenolic nature. The endoderm layer had three or four strata of cells that were smaller than cortical cells (Figure 2). The vascular cylinder showed the characteristics of roots with secondary growth. The phloem was located between the cortical parenchyma and the pericycle (from two to three layers of thin-walled cells); the xylem was observed as a continuous band of vessel elements, parenchyma and fibers (Figure 3). The medulla was formed by numerous isodiametric thin-walled cells and phenol deposits (Figure 3). All the tissues had starch, which was apparently more abundant in the xylem.

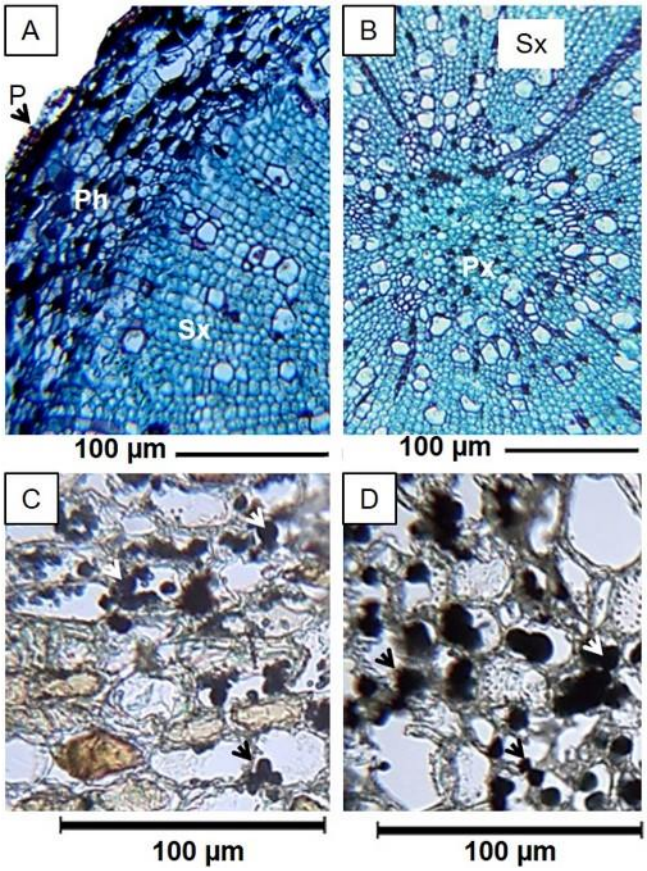

Figure 2. Transversal section of a healthy avocado root. P (Periderm), Ph (Phloem) (A), SX (Xylem) (B) C and D (starch granules *)

***Histology of infected plants.* -** In the roots, mycelium was observed in the periderm (Figure 4). Afterwards, the mycelium invaded intra and intercelularly the tissues of the cortex and endoderm, where some cells became necrotic and some others showed higher phenol content with respect to the healthy tissue (Figure 3A and 3C). Hyphae of *Armillaria* were longitudinally distributed in the transition zone between the cortex and the pericycle, where the mycelium started to differentiate into a more complex tissue (rhizomorph) (Figures 3B).

The mycelium that forms part of the rhizomorph had, in longitudinal sectioning, fibulae and a layer of schlerenchymatous cells (Figure 8). The vascular tissue and the medulla were also colonized by the fungus. The pathogen was observed intra and intercellularly in the parenchymatous cells of the vascular tissue, clogging the vessel elements (Figures 3D). In all the components of this tissue, phenols were observed. The histochemical test to determine presence of starch was negative in all root tissues of infected plants (Figure 4).

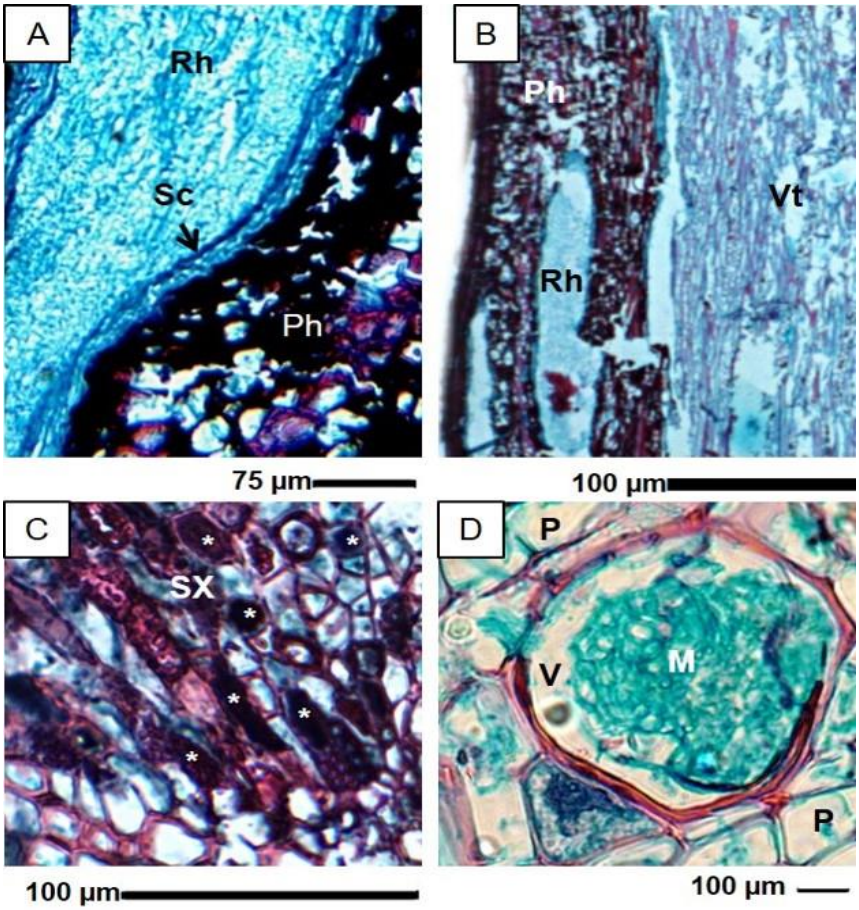

Figure 3. Longitudinal section A and B. Transversal section C and D of avocado roots infected by *Armillaria*. Ph (Phloem), Sc (Schlerenchymatous cells), Rh (Rhizomorph), P (Parenchyma), SX (Xylem), M (Mycelium), V (Xylem vessels), Vt (Vascular tissue).

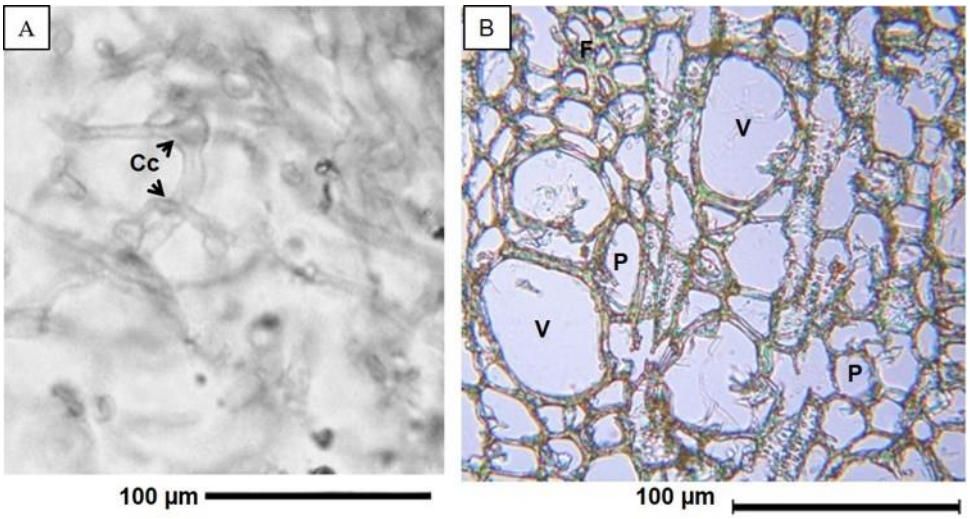

Figure 4. Sections of roots infected by *Armillaria*. A. Cc, clamp connections. B. Lugol's test; P parenchyma; V, xylem vessels.

***Rhizomorphs from the field*. -** The coloration of rhizomorphs varies from ochre to black; branching is dichotomous and the apical zone was not macroscopically observed with precision; there was no presence of mucilaginous tissue adhered to the cortex either.

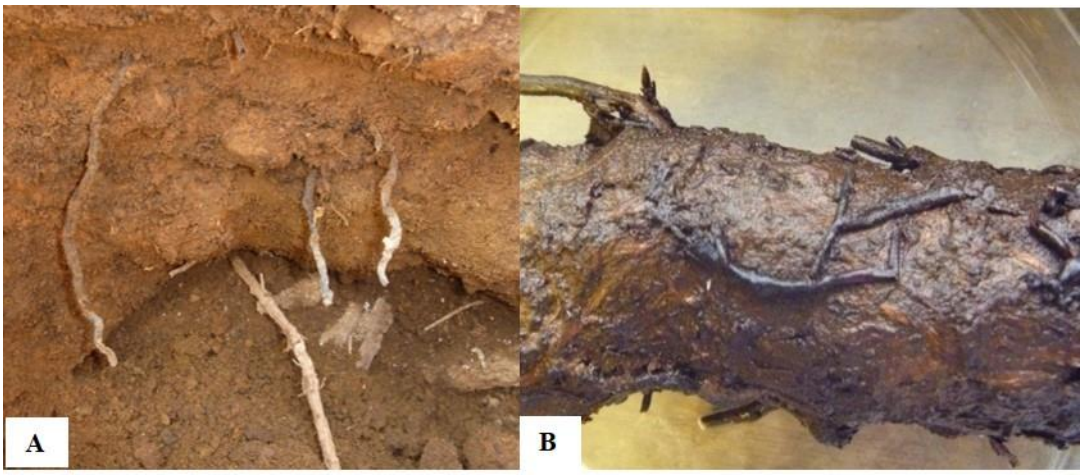

Figure 5. Rhizomorphs of Armillaria in natural conditions. Rhizomorphs developed in the soil (A); rhizomorphs on avocado roots (B).

Histological sections showed the characteristic tissues of the rhizomorph and its apical zone. Mature rhizomorphs are composed of three differentiated concentric layers. The apical center, or primary medulla, is differentiated into subcortex and cortex in longitudinal sectioning; in the primary medulla the mass of apical hyphae is located. Cells of the cortical and subcortical tissues are oblique, lacking organelles because it is a schlerenchymatous or protective tissue. Transversal sectioning shows the same tissues; however, the hyphae of

the fungus are dispersed and abundant compared with the longitudinal sectioning (Figure 6).

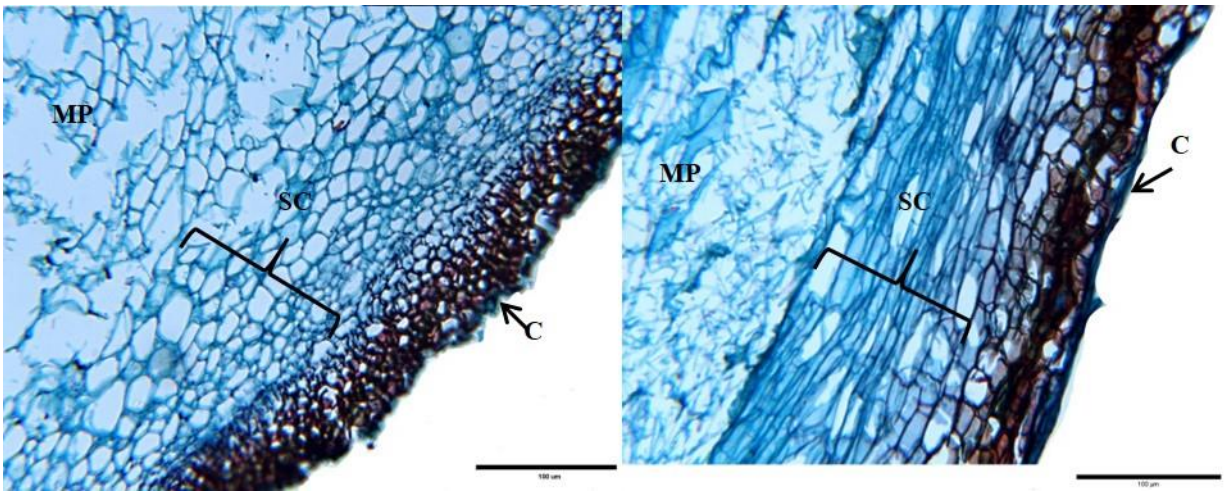

Figure 6. Histological sections of rhizomorphs from the field. Transverse section (A): MP, primary medulla; SC, subcortex; C, cortex; longitudinal section (B).

Laboratory rhizomorphs. - Rhizomorphs developed in culture medium in laboratory had a creamy-yellow coloration during the initial stage of development (14 days) and the presence of hyphae external to the tissue, represented by a mucilaginous tissue. Once they emerge from the medium and come into contact with oxygen, the tips of the rhizomorphs become black or ochre (Figure 7A). Histological sections showed the same structure of a rhizomorph from the field; however, cells of the cortex and subcortex had a paler coloration when compared with the rhizomorphs adhered to avocado roots in the field (Figure 7B).

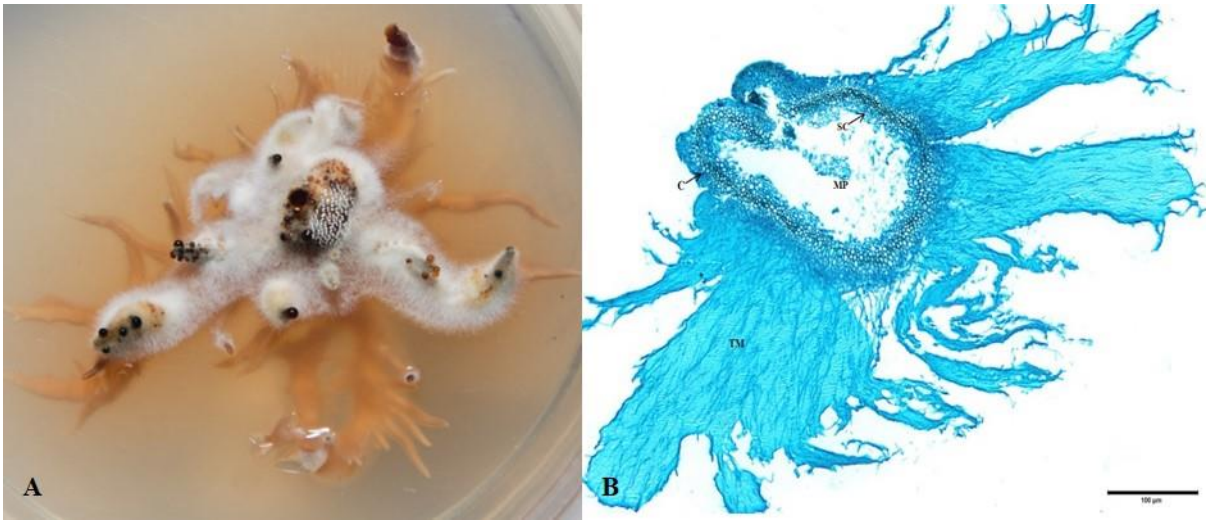

Figure 7. A. Rhizomorphs of *Armillaria* sp. developed *in vitro*. B. Transversal sections of the rhizomorph A: MP, primary medulla; SC, subcortex; C, cortex; TM, mucilaginous tissue.

DISCUSSION

The alterations observed in avocado were similar to those described in the association of *Armillaria* with *Larix occidentalis, Pseudotsuga menziessi, Pinus radiata* and *Thuja plicata* (Robinson and Morrison, 2001; Van Der Kamp and Hood, 2002; Cleary and Holmes, 2011), except that in avocado there was no formation of callus or resin. These results coincide with the studies carried out by Leach (1936) in seedlings of *Glyricidia maculata*.

The cells of the cortical and vascular tissue of avocado had deposits of phenols. The rhizomorph became established between the cortex and the xylem, and therefore, the phloem became completely disorganized; this stops nutriment supply, which coincides with the alterations described by Leach (1936) in *G. maculata*. Fungal hyphae invaded the elements of the xylem and some cells of the medulla. It is possible that such invasion takes place intra and intercellularly, as Solla *et al.,* (2002) reported in *Picea sitchensis*; however, we suggest that complementary studies becarried out using Transmission Electron Microscopy to corroborate the observations. In addition to the presence of phenols, there was necrosis of the bark, vascular and medullar tissue of the roots of avocado. It is possible that these alterations are due to the enzymatic activity of the lacasse, which is naturally produced by the rhizomorph (Worral and Hüttermann, 1986), although they could also be the result of structural and biochemical defense mechanisms, expressed by the plant in response to pathogen attack (Soustrade and Escarmant, 1997; Shaw and Kile 1991).

Starch was observed in the parenchymatous cells of the cortex, xylem and medulla of healthy roots, although it was not detected in infected roots, which could be due to the fact that the fungus used it as substrate to maintain its development during the invasion of the rest of the tissues of the root (Leach, 1936). The fungus induced the degradation and death of cells of the cortex and vascular tissue, as well as the accumulation of phenols. The rhizomorph became established between the cortex and the xylem, whereas vessel elements became obstructed by the presence of mycelium.

The morphology of the rhiozomorphs described coincides with that registered for *A. mellea* (Motta, 1982, 1969; Shaw y Kile, 1991; Harting 1870, 1874). The variation in coloration is due to melanin pigmentation; the tips of the rhizomorphs become black or ochre and have a higher growth, as Worral *et al.,* (1986), Smith and Griffin (1970) asseverate; these authors attribute the coloration of these cells to the enzyme lacasse or to the activity of p-diphenol.

Differences among the rhizomorphs coincide with Harting (1874), who described rhizomorph variation according to the environment where they develop. The growth and length vary depending on the amount of available oxygen, either in the soil or under controlled conditions (Smith and Griffin, 1970). The results of this study explain the activity of rhizomorphs after three weeks of having been isolated *in vitro*. The pigmentation or coloration may be due to the presence of three substances, *viz*. lacasse (Worral *et al*., 1986), melanin (Issac, 1995) or p-diphenol (Smith and Griffin, 1970).

The results of this study highlight the impact of changes in land use and the presence of emerging pathogens. The importance of *Armillaria* as pathogen of avocado trees in former forestry zones is increasing in Michoacán, Mexico. In some orchards, the incidence has reached 50 dead trees. Deforestation is also having a negative impact in the ecology of Michoacán, and in the future, it will affect health and productivity of avocado.

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