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Histopathology of *Peronospora tabacina* in tobacco

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ABSTRACT

Background/Objective. *Peronospora tabacina* is an oomycete that causes blue mold in tobacco. Little is currently known about the pathogenicity of this pathogen, as available studies have focused on general aspects of the infection, and detailed histological analyses are scarce. Therefore, the objective of this study was to describe the histological changes that occur during *P. tabacina* infection in artificially inoculated tobacco plants.

Materials and Methods. The infection process was analyzed by differential tissue staining with safranin-fast green in inoculated plants under greenhouse conditions. Tissue sections were made at 2, 4, 6, 9, 12, 18, 20, 24, 30, 36, 48, 60, 72, 96, 120, 148, 172, 196, and 220 hours after inoculation (hai).

Results. The presence of sporangiospores attached to the leaf cuticle was observed between 2 and 48 hai, which germinated from 96 hai. The pathogen penetrated the leaves directly through appressoria at 96 hai. The first infectious hyphae emerged from 96-120 hai, and between 168 and 192 hai, intracellular hyphae developed and colonized the interior of the tissues, causing cell collapse in the lacunar and chlorophyll parenchyma, as well as structural alterations in vascular bundles and epidermal cells. The pathogen completed its infection cycle by producing oospores and sporangia at 192 hai and is considered a source of inoculum for the infection of new tissues.

Conclusion. *Peronospora tabacina* is capable of directly penetrating tobacco leaf tissue through appressoria and developing a progressive infection both intercellularly and intracellularly, with severe alterations in leaf anatomy and completion of the cycle with the production of reproductive structures in a short period, reinforcing its potential as an aggressive pathogen under favorable conditions.

Keywords: Intracellular pathogen, Intercellular pathogen, Oospores, Pathogenesis, Oomycete, Biotroph

INTRODUCTION

Tobacco (*Nicotiana tabacum*) is grown in 124 countries (http://tobaccoatlas.org/topic/growing/) and is considered a commercial crop, important to the agricultural economies of these countries, particularly in developing nations in Asia and Africa (Nowicki *et al*., 2022). Due to its main use, which is in the production of cigarettes and cigars, among others, this crop represents economic and social benefits derived from both its exports and the creation of jobs in both rural and urban areas. Due to this, its production is a relevant activity in several parts of the world, such as Brazil, China, the U.S. and India (CONASAMA, 2023).

In Mexico, the tobacco-growing area has been stable for one decade. In 2008, 5,900 hectares were dedicated to the production of this crop, and by 2018, the figure increased to 6,600 (SIAP, 2023). In that year, tobacco production was 15,181 tons, increasing by 32% on 2008, and it became number seven among the main producers in the Americas, after Brazil, United States, Argentina, Cuba, Guatemala and Canada. In 2019, tobacco had an 11% increase in production, with the highest yield being in Nayarit, Mexico's leading tobacco-producing state, where the highest production levels are obtained in the month of May, providing around 43% of the annual production of the country. It is worth mentioning that the farmers in that state were paid more than 37,700 Mexican pesos per ton (CONASAMA, 2023).

As with other crops, tobacco is susceptible to damage caused by infectious pathogens belonging to the groups of fungi and oomycetes. In this regard, plant biotrophic oomycetes cause significant problems in the production and economic losses in modern agriculture, since they display high genetic variability and quick adaptation for their survival (Latijnhouwers *et al*., 2003).

Peronospora spp. normally limits itself to specific hosts or many related hosts and are capable of causing severe damages. They are biotrophic oomycetes that belong to the Oomycota phyllum, traditionally mistaken for fungi, but they are phylogenetically more similar to algae. They are characterized by being obligate pathogens that mainly infect dicotyledonous plants, showing a high degree of host specificity. These organisms can cause devastating diseases, such as blue mold, due to their ability to sporulate profusely and complete infectious cycles in short periods under favorable conditions (Lee *et al*., 2017). Economic losses have been reported in ornamental and food crops globally and the downy mildew disease caused by this phytopathogen is considered one of the most important diseases in tobacco crops, since it can cause severe economic losses (Thines and Choi, 2016). In the U.S., *P. tabacina* has even been classified as a potential biological weapon, since its presence can severely affect the country's economy in regard to the tobacco industry (Thines and Choi, 2016). Among the six most important biotrophic oomycetes, *Peronospora tabacina* is mentioned as a cause of the blue tobacco mold. This oomycete is an obligate biotrophe that requires the live tissue of the host to complete its life cycle (Perfect *et al*., 1999, Webster and Weber, 2007). The blue tobacco mold is a polycyclic disease and under favorable conditions to its development, the pathogen can complete its life cycle in 10 days or less (Heist *et al*., 2002). The primary and secondary inoculant of *P. tabacina* consists of asexual sporangia, which are typically produced in the leaves and stems of infected plants, with active sporulation observed in leaf tissue reaching up to one million sporangia per cm^2. This is an important factor that can contribute to the complete devastation of infected tobacco crops. The sporangia of this pathogen have the ability to travel long distances and cause diseases several hundred kilometers from their origin (Heist *et al*., 2004; Spring *et al*., 2018).

The symptoms that *P. tabacina* cause are commonly lesions or yellow spots in tobacco leaves that appear individually or in groups and are usually fused to form light-maroon necrotic areas. Some of the leaves display a downy bluish-gray mold on their upper side, which may come with upward deformations. When there is abundant sporulation, particularly under conditions of high relative humidity, the bluish color in the diseased plants becomes quite noteworthy, hence the name of the disease: the blue tobacco mold (Borrás-Hidalgo *et al*., 2010). Given the importance of preventing epidemics and economic losses caused by *P. tabacina*, implementing monitoring strategies that allow an early and reliable identification of possible virulence phenotypes is crucial. This may be achieved with the detailed characterization of the attributes of the pathogen that correlate with their infectious behavior (Blanco-Meneses and Ristiano, 2011).

Understanding the process and the strategies used by *P. tabacina* to infect tobacco plants is extremely important, since some strategies of certain pathogens are correlated with host specificity (Pring *et al*., 1995; Zhang *et al*., 2023). Therefore, identifying the infection process would provide useful tools for proper disease management by revealing the period during which the pathogen develops in the host. Currently, little is known about the *P. tabacina* pathogenesis process, as available studies have focused on general aspects of the infection, with histological analyses in *Nicotiana tabacum* being scarce (Borrás-Hidalgo *et al*., 2010; Ayliffe *et al*., 2008) are scarce. Therefore, the aim of this study was to describe the histological changes that take place during *P. tabacina* in artificially inoculated tobacco plants.

MATERIALS AND METHODS

Plant material and inoculum. Healthy, three-month-old tobacco plants were used from the Tlapacoyan cultivar, which were planted and kept under controlled conditions until their use. The experiment was carried out in the Phytopathology Laboratory of the Food and Development Research Center, Culiacán branch, and repeated on three independent occasions, during the months of January of 2022, 2023 and 2024, respectively. The Pt1SA isolate of *Peronospora tabacina*, from San Andrés, Tuxtla, Veracruz, Mexico, previously molecularly characterized using the specific oligonucleotides PTAB and ITS4 (Ramos-Barraza *et al*., 2023), was used as the inoculum. The sporangial suspension was prepared from leaves of previously inoculated tobacco plants with active sporulation, collecting the sporangia with a brush and adjusting the concentration to 1 x 10^6 sporangiospores mL^{-1}, using a Neubauer chamber for counting.

Inoculation of tobacco plants. The undersides of 15 tobacco plant leaves were disinfested with ethanol at 70% for 1 min, washed with distilled water and left to dry at room temperature. Ten 1 cm^2 sites were selected per leaf on each plant and inoculated using the point application technique with 10 µL of the sporangiospore suspension. As a control treatment, 10 µL aliquots of sterile distilled water were placed on five tobacco plants. Both the inoculated tobacco plants and the control tobacco plants were placed in a shade mesh-type greenhouse at ambient temperature, which ranged from 15 to 17 °C and a high relative humidity (85-100%), which was maintained naturally, due to the environmental conditions favorable for disease control.

From the tobacco plants, five tissue samples (1 cm^2 each) were taken from the inoculates sites 2, 4, 6, 9, 12, 18, 20, 24, 30, 36, 48, 60, 72, 96, 120, 148, 172, 196 and 220 hours after inoculation (hai), and placed in plastic cassettes for fixation. The proposed sampling scheme, with shorter intervals in the first hours after inoculation, and longer ones in later stages, was designed to capture the different phases of the *P. tabacina* infection process. This approach helps observe earlier events such as the germination and penetration of the pathogen, as well as later stages, such as the colonization and destruction of tissues. The samples were submerged in an FAA fixation solution FAA (10% formaldehyde, 5% glacial acetic acid, 50% ethanol at 96%) for at least 24 h. The tissues were washed three times in ethanol at 50% for 15 min each, then gradually dehydrated in an ethanol series (50, 70, 96 and 100 %) and later transferred to absolute alcohol-xylene (1:1), two xylene changes and two in Paraplast paraffin (3 hours for every step of dehydration). Tissue embedding consisted of immersing the samples in melted paraffin in metal molds, pointing them either longitudinally or transversally, to subsequently obtain sections using a microtome (Rojo-Báez *et al*., 2016; Cruz-Lachica *et al*., 2018).

Longitudinal and cross sections, 10 µm thick, were cut using a RM2125 RT rotary microtome (Leica Biosystems, Nussloch, Germany) and mounted on slides previously prepared with Haupt adhesive and water in a slide warmer (Thermo Fisher Scientific, U.S.A.), at a temperature de 40 °C for 24 h to dry. Subsequently, the paraffin was removed from the cuts with three xylene changes (3 min in each) and hydrated using a gradual series of ethylic (100, 96, 70 and 50%) for 3 min each. Staining in 1% safranin (prepared in 50% ethyl alcohol) was performed for 3 h. After the cuts, they were dehydrated in an ethyl alcohol series at 50, 70 and 96% (3 min in each) and stained with 1% fast green (Técnica Química®, Mexico)—prepared in 96% ethyl alcohol—for 30s. They were washed and dehydrated by passing them through 96 and 100% ethyl alcohol, for 3 min in each. Finally, the stained sections underwent three xylene changes (3 min in each), covered with Entellan resin and the coverslips were left to dry for 24 h (Fierro-Corrales *et al*., 2015). The histological preparations were observed under a Carl Zeiss Imager A2 optic microscope (Carl Zeis, Oberkochen, Germany) with a built-in camera to visualize tissue damage using 10, 20 and 40X lenses.

RESULTS AND DISCUSSION

At the beginning of the experiment, the anatomical characteristics of healthy tobacco leaves were observed, in order to establish a point of comparison with the infected structures. In the transverse and longitudinal sections of healthy leaves (Figure 1A and 1B), the adaxial and abaxial epidermis were identified, with no presence of the pathogen or signs of cell damage. Ungerminated *P. tabacina* were found attached to the surface of the leaf, between 2 and 48 hai (Figure 1C and 1D). The adhesion of spores to hostplants is essential for the onset of disease in pathogen-plant interactions (Perfect *et al*., 1999). The start of the germination of sporangia was observed to begin 96 hdi (Figure 2A) and the pathogen displayed the ability to form melanized appressoria at the apical end of the germ tubes of the sporangia, which were dark brown and ranged in shape from globose to irregular (Figure 2B). Through these structures, the pathogen attached to and penetrated the host via natural openings such as the leaf stomata (Figure 2A). The adhesion of the germinative tubes and the appressoria tends to be stronger than in the spores (Hardham, 2007).

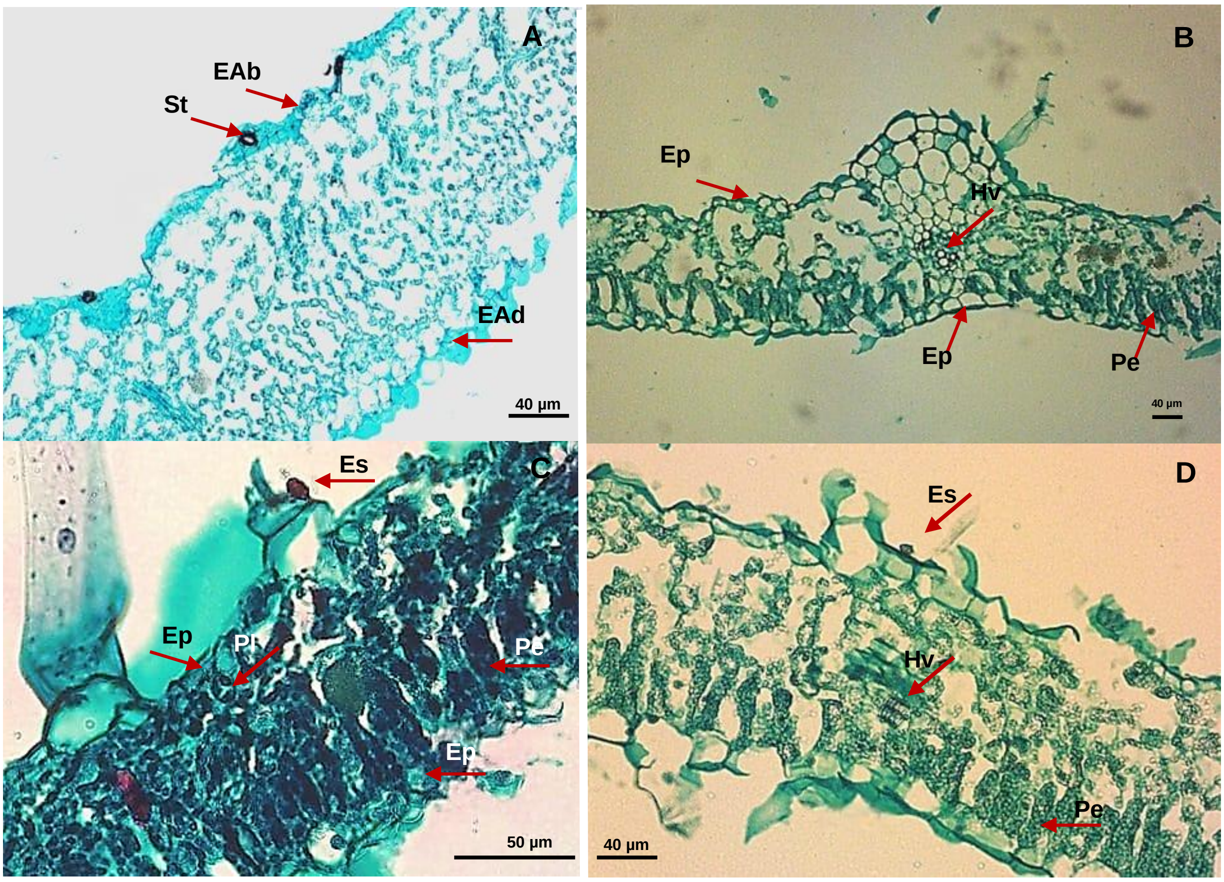

Figure 1. Stages of the *Peronospora tabacina* infection process in artificially inoculated tobacco leaves. A) Transversal section of a healthy leaf displaying the abaxial (EAb) and adaxial (EAd) epidermis and stomata (St). B) Longitudinal section of a healthy leaf displaying the epidermis (Ep), vascular bundles (Hv) and palisade parenchyma (Pe). C) Longitudinal section 24 hai with sporangia (Es) in the trichome base. D) Longitudinal section 48 hai with sporangia in the epidermis. Bar = 40 µm.

The adhesion of the appressoria is particularly strong to provide a firm base for penetration, as pointed out by Pain *et al*. (1996), who state that the fragments of the cell wall of the appressoria remain attached to the substrate after sonication or shaking. The direct germination of the sporangia has evolved independently in several genera (such as *Peronospora*). The way in which the pathogen penetrates is as diverse as the behavior of the sporangia. Meanwhile, root-parasiting root oomycetes tend to form appressoria to invade the internal tissues by directly penetrating the rhizodermic cells, or squeezing in between the middle lamella of the cells (for instance, *Pythium*, *Aphanomyces*, etc.). Other species that attack leaves in similar ways (such as, *Peronospora* and *Bremia*), use the stomata as points of entry (Spring *et al*., 2018a), although penetration has also been documented through other natural openings such as the trichomes or leaf hairs (Agrios, 2005; Judelson and Blanco, 2005; Hardham, 2007; Latijnhouwers *et al*., 2003).

After the formation of the appressoria, the formation of the first infection hyphae was observed, which appeared between 96 and 120 hai (Figure 2D). These hyphae emerged mainly through natural openings, such as the stomata (Spring *et al*., 2018a), and the penetration of a spore through a natural opening related to the base of a trichome was also observed (Figure 2A), suggesting that *P. tabacina* can use multiple natural entry forms into the foliar tissue (Agrios, 2005; Hardham, 2007). In this same interval, secondary hyphae were observed to invade epidermic cells intracellularly, mainly in the adaxial epidermis (Figure 2C). This finding coincides with reports by Milholland *et al.* (1981), who mention that, although *P. tabacina* is predominantly spread intercellularly through the vascular tissue, intracellular hyphae can be observed in the xylem vessels and the parenchyma cells.

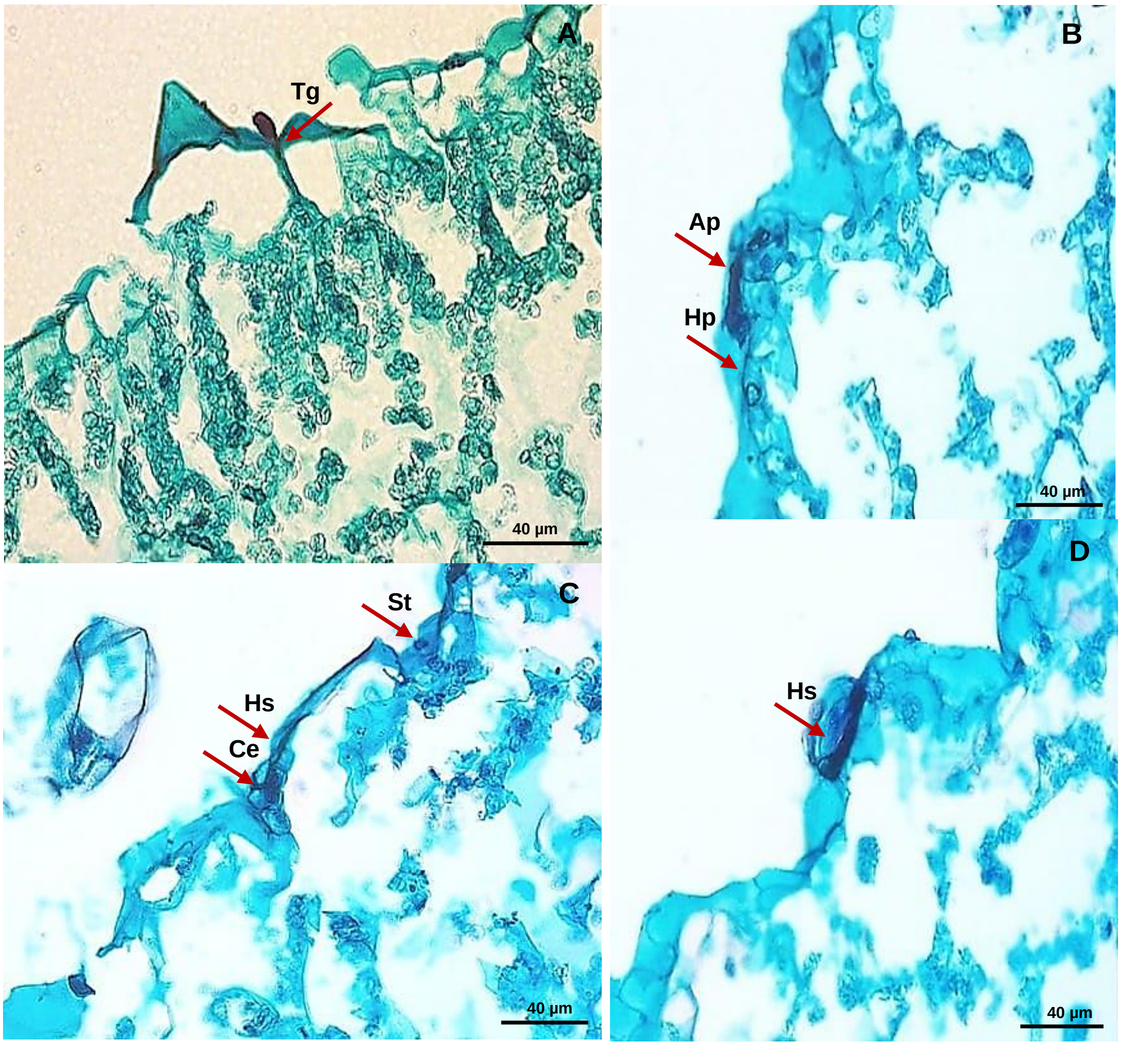

Figure 2. Development of infectious *Peronospora tabacina* structures in tobacco leaves. A) Germinative tube (Tg) emerging from the sporangium at 96 hai. B) Penetration hyphae (Hp) emerging from the appressorium (Ap) at 96 hai. C) Secondary hyphae (Hs) intracellularly invading epidermal cells (Ce) 96 hai. D) Infection hyphae (Hi) emerging from the appressorium and invading tissues at 120 hai. Barra = 40 µm.

These results show that *P. tabacina* is able to invade the tobacco leaf tissues, inter- and extracellularly, confirming the report by Milholland *et al.* (1981), on the presence of intercellular hyphae in the xylem of systemically infected plants. However, our findings broaden these observations showing an intracellular invasion in the plant tissues, such as the chlorenchyma and the vascular bundles, suggesting a more aggressive pathogenic strategy during the early stages of infection. This behavior has been backed by recent genomic and molecular studies, which highlight the advanced repertoire and effectors and colonization mechanisms of *P. tabacina*, facilitating its spread and establishment under specific environmental conditions (Derevnina *et al*., 2015; Borrás-Hidalgo *et al*., 2010).

Between 168 and 192 hai, the primary intracellular hyphae were observed to develop (Figure 3A), along with coenocytic secondary hyphae colonizing the insides of the tissues and causing cellular disorganization and destruction of the spongy parenchyma, chlorenchyma and chloroplasts, vascular bundles and epidermal cells of the leaf hoja (Figure 3B-5D). During the colonization of the host plants, the pathogenic oomycetes display two main forms of nutrition: biotrophy, in which nutrients are obtained from the living host cells, and necrotrophy, in which nutrients are obtained from dead host cells that have been attacked by the oomycete (Perfect *et al*., 1999). *P. tabacina*, the pathogen under study, belongs to the Peronosporaceae family, in which haustoria have been developed as a specialized cell structure that penetrates in the cell wall of the host, but that does not alter the plasmalemma (Agrios, 2005; Hardham, 2007). Thus, the affected plant cells stay alive, and their metabolism continuously supports the pathogen. This type of interaction is typical of the biotrophs such as mildews (Spring *et al*., 2018b).

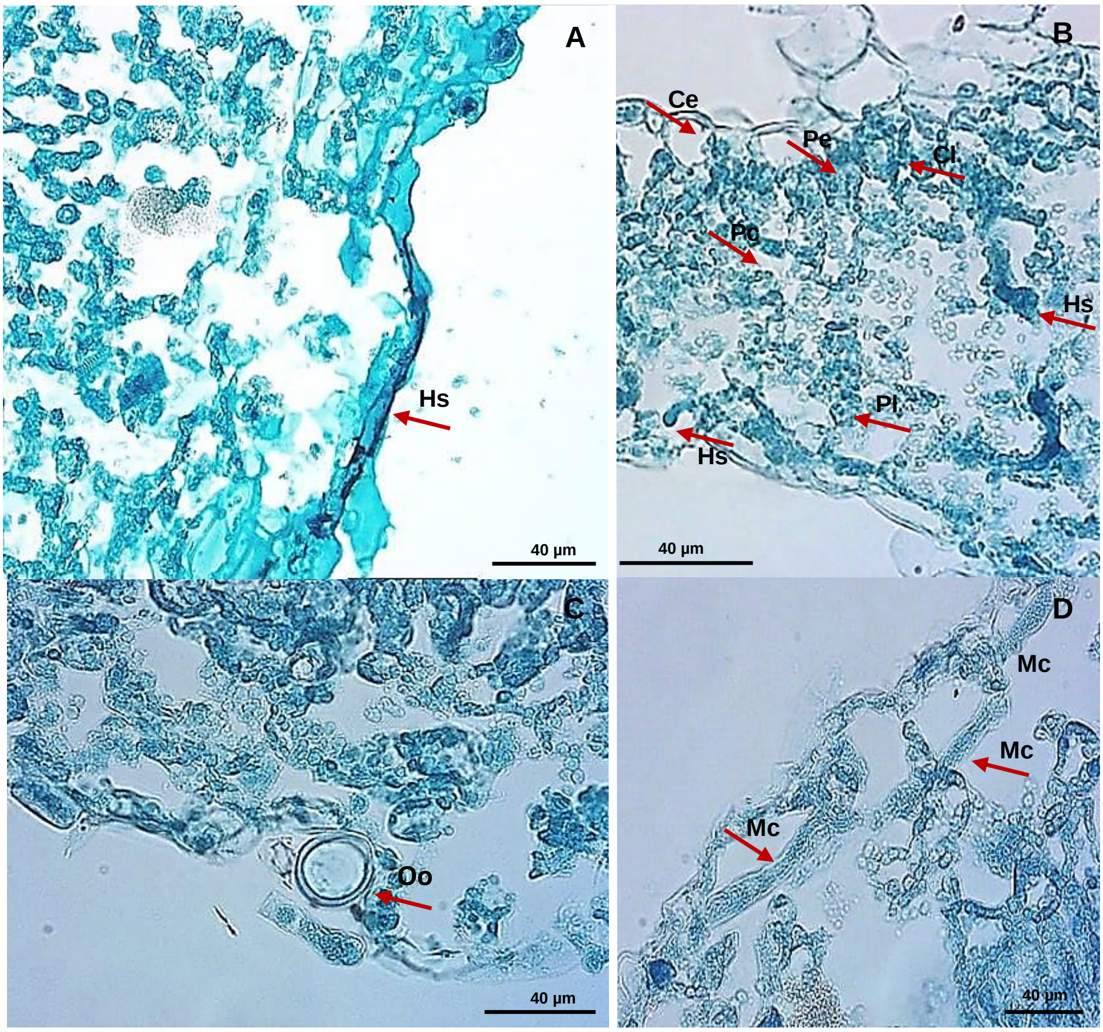

Figure 3. Advanced invasion of *Peronospora tabacina* on tobacco feal tissue. A) Secondary hyphae (Hs) invading intracellularly at 120 hai. B) Coenocytic hyphae growing intracellularly at 192 hai, causing cellular disorganization, and destroying spongy parenchyma (Pl), chlorenchyma (Pc) and chloroplasts (Cl). C) Formation of oospores (Oo) at 192 hai. D) Coenocytic mycelia (Mc) causing cell disorganization and destruction of tissues at 192 hai.

The healthy tobacco plants used for inoculation with the pathogen (Figure 4A) and the non-inoculated plants used as controls (Figure 4B) presented no symptoms during the assay. By contrast, the inoculated plants displayed the destruction of epidermal tissues (Figure 3A), associated to the appearance of yellow irregular spots on the surface of the leaves (Figure 4C) and with the presence of visible sporulation on the underside (Figure 4D). Between 192 and 216 hai, the pathogen completed its life cycle producing oospores and sporangia (Figure 3C), which are the source of inoculant for new infections. The oospores are formed inside the tissues of affected leaves when these make contact with the ground. When they mature, they do not germinate immediately, but after the leaf falls. These structures can remain visible on the ground and germinate under favorable conditions to infect young plants. The formation of oospores varies: in some cases, it is abundant and in others its presence is almost undetectable, probably due to adverse environmental conditions. Oospores have been proven to survive for long periods in the soil, which is a challenge for the management of diseases caused by oomycetes. For example, in *Plasmopara halstedii*, the causal agent of the sunflower mildew, oospores can stay alive for up to 10 years (Spring *et al*., 2018b).

Figure 4. Foliar symptoms caused by *Peronospora tabacina* (Pt1SA isolation) in tobacco. A) Healthy plants used for inoculations. B) Control plants, not inoculated. C) and D) Leaves with symptoms on the adaxial and abaxial surfaces from 168 hai.

In the case of *Phytophthora infestans*, responsible for the late blight in potato, its oospores have been reported to retain infectivity for up to 48 months, depending on the type of soil (Turkensteen *et al*., 2000). Likewise, in *Peronospora viciae* f.sp. *pisi*, the causal agent of the downy mildew in pea, oospores maintained a viability greater than 25% after 29 weeks at low temperatures (Inglis *et al*., 1997). These findings support the ability of oospores to persist in the environment, even in the absence of a host. In the specific case of *P. tabacina*, the production of oospores to as survival structures in the soil has also been documented, although their formation may vary significantly among isolates and under different environmental conditions (LaMondia, 2010).

The early detection of sporangia and oospores 96 hai is relevant, both from the biological and phytosanitary points of view, since it marks the start of the reproductive phase of *P. tabacina* before visible symptoms are manifested. Their ability to complete part of their life cycle in a relatively short period emphasizes the importance of implementing early monitoring strategies in tobacco crops. The timely detection of these reproductive structures may be key to applying preventive treatments that limit the spread of the pathogen and reduce the impact of the disease on the crop (Milholland *et al*., 1981; Borrás-Hidalgo *et al*., 2010; Derevnina *et al*., 2015).

In the histological sections made on tobacco leaves with visible symptoms of the disease caused by *P. tabacina*, a significant alteration was observed in the tissue architecture, with the presence of intercellular and intracellular coenocytic mycelia in the mesophyll, as well as the partial destruction of parenchyma cells and vascular tissues (Figure 5A-D). The hyphae were widely distributed between the intercellular spaces of the spongy parenchyma and in some cases, penetration structures were observed, which suggest invasive activity. These findings coincide with a report by Milholland *et al*. (1981), who described the colonization of *P. tabacina* in vascular tissues of systemically infected plants.

This generalized cell destruction not only compromises the integrity of the plant tissue but can also interfere with essential functions such as photosynthesis. The loss of cell organization observed in the epidermis, mesophyll and vascular bundles suggests a deterioration in the ability of the leaves to carry out key physiological processes. This phenomenon is consistent with the pathogenic mechanisms described for other oomycetes, such as *Phytophthora infestans*, whose invasion of the plant tissue directly affects the photosynthetic function (Latijnhouwers *et al*., 2003). In the case of *P. tabacina*, the induction of necrosis in the parenchyma cells could be the main cause of the foliar symptoms observed, such as the yellowing and the death of the tissue.

The histological alterations observed, such as the destruction of the epidermal cells, the invasion of the parenchyma and the formation of reproductive structures of the pathogen within the plant tissue have significant physiological implications in the tobacco plants. The disruption of the epidermis compromises the protective barrier of the leaf, facilitating the loss of water via transpiration and increasing the susceptibility to other pathogens. The colonization of the parenchyma, particularly in the palisade mesophyll, can interfere with photosynthesis, due to the reduction of functional cells, the loss of chloroplasts and cell collapse (Berger *et al.*, 2007; Rojas *et al.*, 2014). These modifications have a direct impact on the production of photoassimilates and, consequently, plant growth.

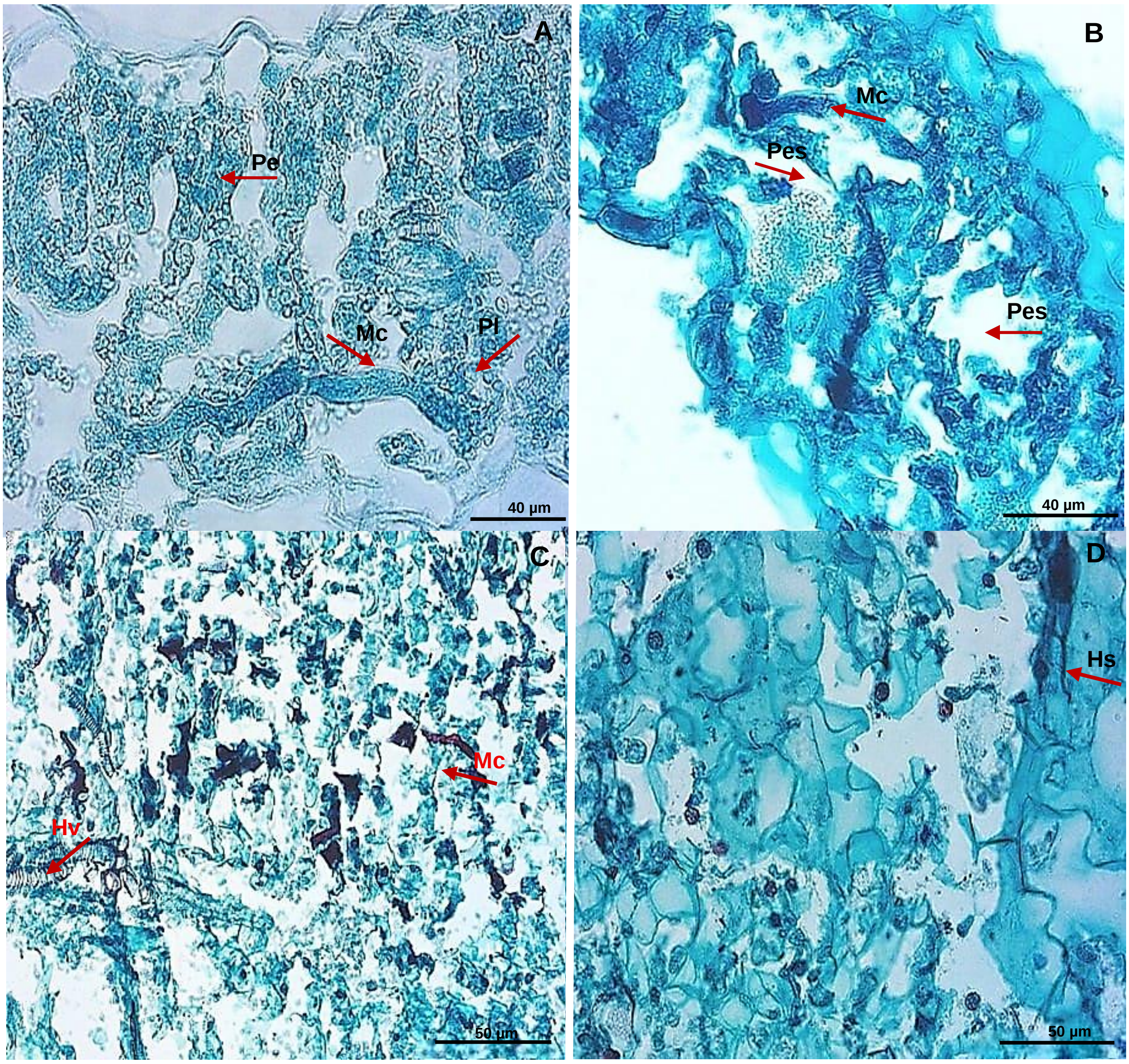

Figure 5. Longitudinal sections of the tobacco leaf at 192 hai and 216 hai displaying colonization of the foliar mesophyll by *Peronospora tabacina.* A) Coenocytic mycelium (Mc) invading the tissues of the palisade parenchyma (Pe) and the spongy parenchyma (Pl). B) Intercellular hyphae in the spongy parenchyma (Pes). C) Distribution of coenocytic mycelium in the leaf mesophyll. D) Accumulation of secondary hyphae (Hs) in intercellular spaces.

Likewise, the colonization of vascular tissues by *Peronospora* species interferes in the transportation of water and nutrients, which affects the cell turgor and the general metabolism of the plant (Latijnhouwers and Govers, 2003). These physiological alterations derive in visible symptoms such as chlorosis, necrosis and wilting, that can result in the functional loss of the affected organ (Ah-Fong *et al*., 2019).

In addition, pathotrophic pathogens, such as *P. tabacina* manipulate the metabolism of the host plant to favor its development, interfering with hormonal pathways and defense signals, which contributes to a greater functional deterioration of the infected tissue (Dodds and Rathjen, 2010; Lorrain *et al*., 2003). Altogether, this evidence reinforces the fact that the structural alterations observed in this study not only show the advance of the pathogen but also explain the physiological deterioration that jeopardizes the yield and survival of the plant. Given the significant impact of *P. tabacina* on the physiology of the plant and its ability to jeopardize essential functions, it is crucial to establish management strategies that reduce its propagation and adverse effects.

The detailed knowledge on the pathogenesis of *P. tabacina* obtained in this study may have significant implications on the integrated pest management (IPM) on tobacco crops. Given that the pathogen can invade the tissues intracellularly in the first 96 dai, an intensive monitoring is recommended in the first stages of cultivation, in order to detect spores and take timely preventative measures. In addition, the use of resistant varieties may play a key role in reducing the severity of the disease, which would also reduce the dependence on chemical treatments (Rivas, 2012).

This study also has implications on sustainable agriculture, since it provides a more accurate focus on how *P. tabacina* infects and affects the tobacco crops. The detailed understanding of the pathogenesis and the dynamics of the infection helps farmers implement mor efficient management practices, depending less on chemical products. Additionally, the use of biological management techniques and the selection of resistant varieties may contribute to a more sustainable control of the disease, reducing the environmental impact and improving the profitability of the crop in the long run (Blanco Marquizo *et al*., 2022; Singh *et al*., 2019).

CONCLUSIONS

This study helped describe the histological changes during the infection of *Peronospora tabacina* on artificially inoculated tobacco plants, providing evidence of its ability to infect its host intercellularly and intracellularly. Symptoms were observed on tobacco leaves 168 h after inoculation, whereas the production of sporangia and oospores began taking place after 96 h, completing its life cycle in this period. These findings contribute to the understanding of the de *P. tabacina* pathogenesis process, complementing the earlier studies on infection and offering relevant information for the development of disease management strategies.

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