Colegio de Postgraduados

Diagnosis of the causal agent of fruit necrosis in tabaquero pepper (*Capsicum annuum* L.)

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ABSTRACT

Objective: To identify the causal agent of fruit necrosis in tabaquero pepper (*Capsicum annuum*).

Design/methodology/approach: Since the planting and harvesting season of tabaquero pepper was suspended in 2022, a habanero pepper plantation exhibiting symptoms similar to those observed in tabaquero pepper was selected for pathogen isolation. Morphological and pathogenic characterization of the fungus associated with these lesions, as well as with foliar spots on the plants, was carried out. Additionally, molecular identification techniques using the 18S molecular marker were implemented to confirm the causal agent of anthracnose in this crop.

Results: The results indicate that the fungus belonging to the genus *Colletotrichum*, specifically the species *C. truncatum* (syn. *C. capsici*), may be the causal agent responsible for fruit and leaf necrosis in tabaquero pepper plantations in Macuspana, Tabasco, during the 2021-2022 season. Therefore, this research provides relevant information for the development of effective phytosanitary management and control strategies in current plantations.

Study limitations/implications: Greenhouse infrastructure conditions and climatic variables may have influenced the parameters obtained during the study. Additionally, the complete molecular identification of the selected fungi at the species level was limited due to the high costs associated with this process.

Findings/conclusions: Pathogenicity tests and the expression of symptoms in pepper fruits indicate that the fungus *Colletotrichum truncatum* may be the causal agent of fruit anthracnose and leaf spot in tabaquero pepper plantations in the municipality of Macuspana, Tabasco, Mexico. However, the need to implement more precise diagnostic techniques, such as microscopy and molecular analyses, is emphasized in order to detect the presence of the fungus at early stages of infection. This would not only improve phytosanitary control measures but also support the design of integrated management strategies aimed at reducing the spread of the pathogen in high-value commercial crops.

Keywords: *Colletotrichum*, Koch's postulates, pathogenicity, molecular, fungi.

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INTRODUCTION

Tabaquero pepper (*Capsicum annuum* L.) has been a key crop in the agriculture of Tabasco, Mexico, standing out as an economic driver for rural communities. For years, its production generated stable income and promoted both direct and indirect employment in activities such as planting, harvesting, and commercialization, thereby strengthening local

value chains. In 2014, the annual production of dried tabaquero pepper exceeded two tons, representing a value of approximately 19.5 million Mexican pesos, positioning the crop as a strategic element in the regional economy. However, this outlook was severely affected in 2022, when unfavorable environmental conditions led to the outbreak of a devastating disease in the crops. The high sensitivity of tabaquero pepper to humidity variations, as noted by Aceves-Navarro *et al.* (2008), increased the incidence of fungal diseases, severely impacting production. According to an institutional report by Ortiz (2022), the disease was characterized by brown lesions with sunken areas on the outer surface of the fruits, along with circular to ovoid spots. These symptoms were associated with anthracnose caused by the fungus *Colletotrichum*. This situation revealed the vulnerability of the agricultural sector to environmental challenges, the lack of infrastructure, and the absence of accurate diagnostics, all of which limited producers' ability to respond effectively. The consequences were devastating not only for farmers, who faced high phytosanitary control costs and economic losses, but also for the regional economy, as the abandonment of the crop disrupted the productive chains that had previously driven local development. In light of this scenario, there is an urgent need to identify and analyze the causal agent of necrosis in tabaquero pepper, particularly in the pepper-growing region of Macuspana, Tabasco, in order to develop strategies that can mitigate future impacts and help revitalize this high-value crop.

MATERIALS AND METHODS

Study area

The study was conducted in the municipality of Macuspana, Tabasco, in the Pantanos subregion, specifically in the ejido (communal farmland) Nueva Esperanza. Sample processing took place in the Biological Control Laboratory at the Colegio de Postgraduados, Tabasco Campus (17° 58' 34.92" N latitude and 93° 23' 14.10" W longitude).

Koch's Postulates

To confirm the causal agent of fruit and leaf sunken lesions and necrosis in tabaquero pepper, Koch's postulates were applied through the following steps:

Pathogen Collection and Isolation

In 2022, the economic losses derived from the production of tabaquero pepper led to the suspension of its cultivation in the main production areas, making it difficult to collect fruits for research purposes. For this reason, a habanero pepper (*Capsicum chinense* Jacq.) plantation located in the community of Arroyo Hondo Abejonal, municipality of Cárdenas, Tabasco, was selected. This plantation included four cultivated varieties. During field sampling, fruits displaying characteristic sunken lesions were collected, placed in plastic bags, and refrigerated for further analysis at the Biological Control Laboratory of the Colegio de Postgraduados (COLPOS).

The collected fruits were placed under humid chamber conditions at 30 °C for eight days to allow visible symptom development. For pathogen isolation, tissue fragments smaller than 1 cm^2 from affected areas were obtained and disinfected sequentially in distilled water, 1% sodium hypochlorite solution, and 70% ethanol. Subsequently, the fragments were plated onto Potato Dextrose Agar (PDA) medium and incubated in Petri dishes at 30 °C for 240 hours.

From the isolates obtained, four strains exhibiting morphological characteristics consistent with the genus *Colletotrichum* were identified. Based on mycelial growth and sporulation parameters, the strain used for the pathogenicity test was molecularly characterized.

Pathogenicity Test

A pathogenicity test was conducted using the microorganism isolated from habanero pepper fruits on a considerable number of healthy tabaquero pepper plants. For this, a conidial suspension was prepared following the methodology of Wang *et al.* (2021) with some modifications. Agar plugs with fungal mycelium were inoculated into Erlenmeyer flasks containing Potato Dextrose Broth (PDB) and shaken for 99 hours. Then, the conidial suspension was filtered, and a concentration of 1×10^8 spores mL^{-1} was prepared, which was placed into manual sprayers. Spraying was performed at a distance of 20 cm from the plants, while control plants were sprayed with water only. The plants were monitored daily for 30 days, documenting symptom development following the methodology of Lozano *et al.* (2015).

Morphological Identification

To identify the macroscopic characteristics of the isolated pathogen, the following descriptive parameters were considered: presence of mycelium, colony color on the front and reverse sides, texture, shape, growth on the plate, and edge margins (Benítez, 2007). For microscopic identification, descriptive parameters included the structures of hyphae, setae, acervuli, and conidia. Measurements of each structure were taken according to the methodology described by Smith (2004).

Molecular Identification

The selected strain was sent to the Potosino Institute of Scientific and Technological Research (IPICYT), LANBAMA Laboratory, where molecular analyses were performed using the 18S molecular marker (CGACGGGCGGTGTGTACAAGT). The consensus sequence obtained was compared with existing records in the NCBI database using the Basic Local Alignment Search Tool (BLAST) to identify regions of similarity. Sequence assembly was carried out using Chromas Pro software (version 2.6.6), followed by multiple sequence alignment with Clustal X software. Seaview software was used as a graphical editor for multiple sequence alignments (Perdomo, 2011). For the construction of the phylogenetic tree, the MegaAlign tool was employed. Maximum Likelihood analysis was performed using MEGA-X software with 1,000 bootstrap replicates. The model that best fit the sequences was the Tamura-Nei model (Tamura *et al.*, 2021).

RESULTS AND DISCUSSION

Pathogen Isolation

A strain of *Colletotrichum* was obtained from habanero pepper fruits exhibiting dark, sunken circular lesions, designated as "Colle-H". The colonies presented a circular shape with entire margins and a velvety/cottony texture, showing a dull gray pigmentation at the center and dark gray on the growth rays, along with concentric orange rings. These results are similar to those described by Pérez-Castro *et al.* (2003) in *Discorea* sp., where different morphotypes of the *Colletotrichum* genus exhibited mycelial growth of 1 to 2 cm per day. Likewise, colonies with velvety and cottony textures were observed, with pigmentation ranging from gray, black, to orange. This variability in sporulation and pigmentation among morphotypes is attributed to the different affected hosts and the specific agroclimatic conditions of each region. Microscopically, the strain exhibited typical structures of the genus *Colletotrichum*, such as pointed setae, short conidiophores, conidia, and the presence of acervuli. The conidia were fusiform, hyaline, with curved and pointed ends, measuring 20.2-22.4 μm in length and 3-3.01 μm in width. These results are similar to those reported by Rojo-Báez (2017), who highlighted the presence of acervuli, conidiophores, conidia, and abundant setae formed directly on the hyphae; the setae exhibited brownish coloration. The conidiophores can be hyaline, septate, and the conidia are unicellular, hyaline, smooth-walled, aseptate, with the central part generally slightly curved with parallel walls, measuring between 22.8-23.8 $\times$ 3-3.02 μm. These are typical descriptions of the species *C. truncatum*.

The phylogenetic analysis placed the "Colle-H" strain within the *Colletotrichum truncatum* clade (syn. *C. capsici*). These morphological and molecular results were fundamental for conducting pathogenicity tests on healthy tabaquero pepper plants.

Pathogenicity Test

The analysis conducted on the plants inoculated with the fungus allowed the observation of the sequence of events necessary for the establishment of the infection responsible for anthracnose. The pathogenicity tests confirmed that the fungal isolate (Colle-H) is pathogenic on tabaquero pepper fruits, as evidenced by the appearance of circular and ovoid necrotic lesions with sunken areas displaying light to dark brown tones. However, a relevant aspect of the process was the absence of apparent defense responses or immediate cell death during the initial days of infection, as characteristic symptoms only began to appear approximately eight days after inoculation. During this period, the affected fruits retained a vigorous appearance and showed no visible differences compared to the controls. Auyong (2012) attributes this characteristic to the pathogen's ability to colonize a wide range of hosts, which may be related to its capacity to adapt to different plant tissues and environmental conditions. As the days progressed after spraying, the symptoms did not appear at the same stage on the fruits as on the leaves, since the fruit cuticle remained intact and apparently undamaged. The infection period became visible during the second week. By day 12, the fruits began to exhibit the characteristic sunken appearance on the superficial epidermis; by day 15, damage caused by the fungus was observed, characterized by prominent, dark, sunken lesions with visible concentric rings. Between days 16 and 18, the presence of the fungus became more noticeable, with lesion pigmentation progressing to darker tones and a more necrotic appearance. Auyong *et al*. (2015) indicate that the rate of infection by *C. truncatum* may depend on the phenological stage of the host, environmental factors such as humidity and temperature, as well as biochemical changes associated with fruit ripening. In addition, Rojo-Báez (2017), in studies related to the infection process of *C. truncatum*, highlights that this pathogen has the ability to remain in an endophytic state until external conditions favor the manifestation of visible symptoms.

The initial infection events were similar in both green and mature fruit stages; however, on day 20, symptoms were observed in younger fruits, which showed limited development, early maturity, weakened attachment, and the presence of a water-soaked cuticle caused by the gradual degradation of the cell wall. The levels of fungal presence progressively increased and remained nearly constant; likewise, there was no reduction in the infection process, as anthracnose was observed in 90% of the inoculated plants until the end of the monitoring period. Mahasuk *et al*. (2009), in their studies on the pathogenicity of fungi of the genus *Colletotrichum*, specifically *C. truncatum*, during the seedling and fruiting stages of chili pepper, reported that young tissues are more susceptible to the development of anthracnose symptoms. However, the characteristic quiescent phase of the disease allows the fungus to remain latent and infect new host shoots, meaning the persistence of the pathogen can be prolonged in the crops. The symptomatology and appearance of symptoms have been variable between fruits and leaves. Than (2008), in his study on the presence of anthracnose in commercial plants, attributes these variations to the influence of external environmental factors such as temperature, humidity, precipitation, wind, light, among others. However, Rojo-Báez (2017) mentions that the infection process of *C. truncatum* in other hosts mainly involves intracellular and subcuticular colonization as an infection strategy, along with the production of enzymes (cutinase, polygalacturonase, pectate lyase, and pectin lyase) during the necrotrophic phase.

These results open an important space for discussion about the dynamics of fungal establishment in different hosts. The lack of an initial defense response by the infected plants could be related to the fungus's ability to go unnoticed by the plant's defense mechanisms during the early stages of infection.

Morphological Characterization

In this study, the growth rate of the fungus was classified as rapid, as by the tenth day of incubation the colony had already developed a complete circular shape. On the tenth day, the fungus showed invasive growth across the entire plate, reaching a diameter between 72 and 79 mm. This growth exhibited a velvety or cottony texture and was characterized by dark gray tones. In addition, a decrease in color intensity was observed in the radial and concentric rings, resulting in an overall grayish tone of the colony, with a slight white pigmentation at the margins. On the reverse side of the colony, the grayish tone intensified, becoming darker, and the concentric rings were less prominent and paler. Although the gray shades observed in the colony are a common characteristic of the *Colletotrichum* genus, variations in color intensity were noted as the culture developed. Over time, the colors became darker, and the concentric rings became more evident. These findings are consistent with those reported by Shin *et al.* (2024), who also observed that grayish tones are typical of *Colletotrichum*, although not exclusive to this genus. It is important to note that Adaskaved and Hartin (1997) and Casanova *et al.* (2016) indicated that morphological variations of colonies may be influenced by factors such as the culture medium and temperature fluctuations. According to Rojo-Báez (2017), *Colletotrichum* colonies can exhibit differences even within the same species, which may lead to difficulties in accurate species-level identification (Figure 1).

Microscopically, unicellular conidia measuring 20.2-22.4 μm in length and 3-3.01 μm in width were observed. These conidia had smooth walls, were aseptate, hyaline, and exhibited a slight curvature in the central region, with parallel walls ending abruptly in a rounded and truncated base, containing granular content. Likewise, the presence of acervuli, conidiophores, and abundant setae formed directly on the hyphae was observed. The setae were brown in color, and the conidiophores were hyaline, septate, branched, and densely clustered. The described features are fundamental in the infection process of *C. truncatum*, as they represent key elements for the development and dissemination of the disease in fruits and plant tissues. Rojo-Báez (2016) highlights that the pathogen's life cycle culminates with the production of acervuli loaded with conidia, which serve as the primary source of inoculum for new infections. These acervuli are associated with the appearance of dark, sunken lesions on fruits, characterized by a brown to black coloration.

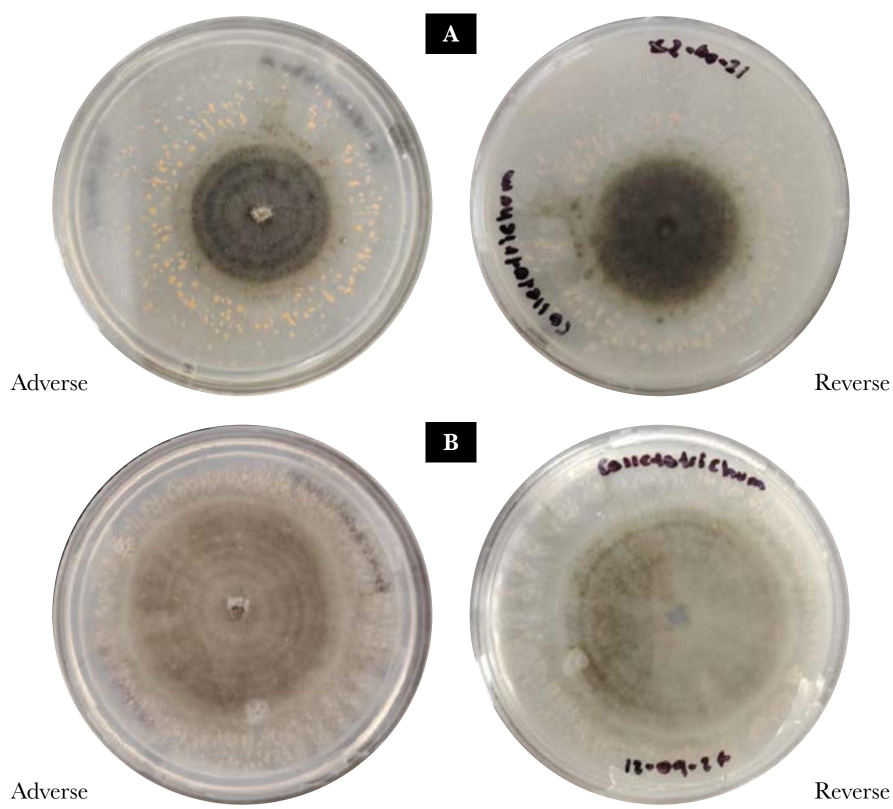

Figure 1. Strain Colle-H grown on potato dextrose agar (PDA) medium, incubated at 30 °C, after five (A) and ten (B) days of growth.

The cellular damage caused during the infection process increases as the acervuli proliferate in the affected areas, creating a favorable environment for the expansion of the fungus. The formation of appressoria —specialized structures that enable the fungus to penetrate the intact fruit cuticle— is crucial for the onset of infection. This mechanism is highly efficient, as it allows for the direct colonization of plant tissue without the need for prior wounding, which explains the pathogen's ability to infect healthy tissues under favorable environmental conditions (Figure 2).

Molecular Characterization

DNA amplification through PCR of the re-isolated fungal strain Colle-H using the 18S rDNA primer was carried out at the Instituto Potosino de Investigación Científica y Tecnológica (IPICYT), producing a PCR product of approximately 700 bp. The phylogenetic tree (Figure 3), reconstructed using MEGA 5 software, placed the sequence within the taxon of *Colletotrichum truncatum*, with a branch support value of 94%. These molecular results confirm that the isolates, based on the amplification of universal primers targeting the 18S rDNA gene, are considered reliable —since values below 50% are deemed unreliable. Furthermore, the bootstrap value of the Colle-H consensus sequence was greater than 99%, supporting the conclusion that the Colle-H strain corresponds

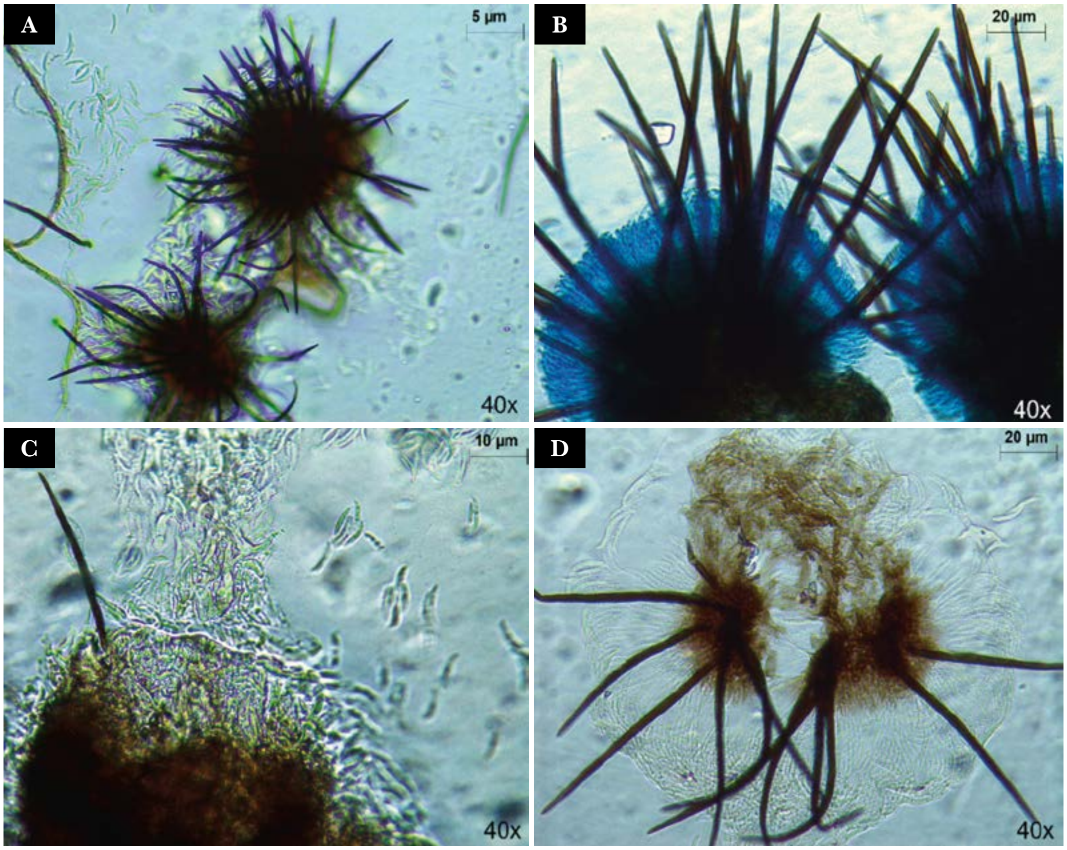

Figure 2. Acervuli (A, B, C, D), conidiophores (B), and setae of the fungus Colle-H (A, B, C), viewed at 40x magnification.

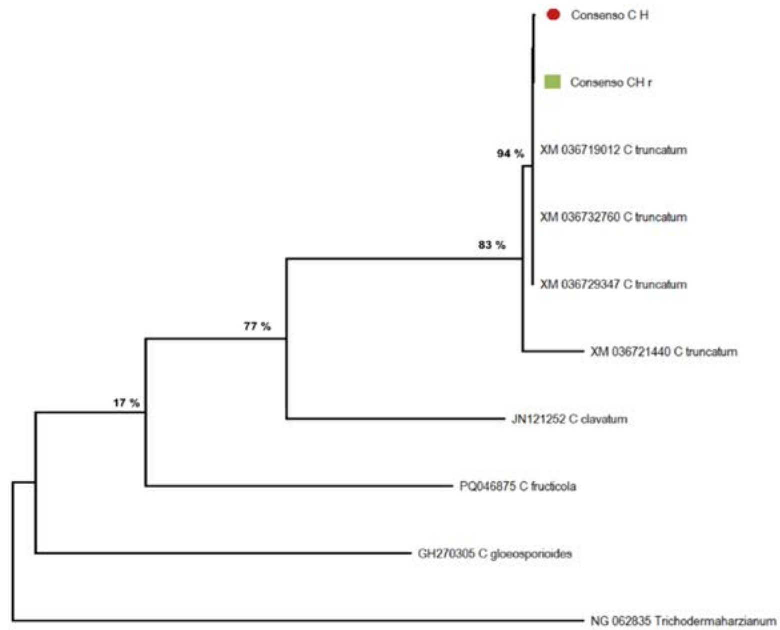

Figure 3. Phylogenetic relationship based on the 18S region. Tree constructed using MEGA 5 software, Maximum Likelihood (ML) method, Kimura 2-parameter model, and 1000 bootstrap replicates. Includes the sequence of the fungus Colle-H (Consensus C H) identified as *Colletotrichum truncatum* and three different species of *Colletotrichum*.

accurately to the species *C. truncatum*. These results are consistent with those reported by Rojo-Báez (2017), who molecularly identified *C. truncatum* isolates in papaya fruits using the 18S rDNA region. The use of this region facilitates amplification and makes it useful for the identification of fungi belonging to the species *C. truncatum*.

This comprehensive approach combining morphological and molecular techniques is essential, especially in the context of plant pathogen diagnosis, where accurate species identification can determine effective phytosanitary management strategies and contribute to the prevention of diseases in economically important crops. Moreover, precise identification not only allows for delimiting the impact of each species but also helps to better understand the ecological and evolutionary dynamics of the genus *Colletotrichum*.

These results open an important discussion regarding the dynamics of fungal establishment in different hosts. The lack of an initial defense response by the infected plants may be related to the pathogen's ability to evade the host's immune system during the early stages of infection. This phenomenon underscores the need to further investigate the interaction mechanisms between the pathogen and the host, taking into account factors such as environmental conditions and possible variations among cultivars. Furthermore, the similarities in symptomatic manifestations across different plant species suggest that certain infection mechanisms may be conserved within the *Colletotrichum* genus, which could provide valuable insights for developing integrated and effective management strategies to control this pathogen.

Finally, these findings highlight the importance of considering the physiological and biochemical characteristics of the host, as well as environmental factors that may influence symptom expression and disease severity. Further studies could explore the specific interactions between *Colletotrichum truncatum* (syn. *Capsici*) and different chili pepper varieties, aiming to identify potential differences in susceptibility and to develop cultivars more resistant to this type of infection.

CONCLUSIONS

Pathogenicity tests demonstrated that tchihe fungus *Colletotrichum truncatum* can be the causal agent of anthracnose in fruits and leaf spots in tabaquero pepper plantations in the study region. However, further tests and sampling in different tabaquero pepper growing areas where the causal agent is present are necessary to confirm the disease.

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