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RESEARCH ARTICLE

Morphological and multilocus molecular characterization of *Neocosmospora solani* and *Fusarium oxysporum* associated with gummosis in *Citrus sinensis* in Veracruz, Mexico

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Abstract

Citrus gummosis is a complex disease primarily associated with *Phytophthora* spp. in Mexico, particularly *P. nicotianae* (syn. *P. parasitica*) and *P. citrophthora*, which have historically been recognized as the main causal agents in the country's citrus-growing regions. However, several studies have reported additional fungal pathogens, such as *Lasiodiplodia* spp., involved in gummosis-like symptoms. The objective of this research was to identify phytopathogenic fungi associated with gummosis symptoms in orange orchards in Tuxpan, Veracruz. A total of 100 bark samples were collected from symptomatic trees across five representative zones of the municipality. Fungal isolation was performed on potato dextrose agar (PDA), yielding 25 isolates, from which *five* were selected based on morphological characteristics. Molecular identification was conducted through PCR amplification and concatenated sequencing of the internal transcribed spacer (ITS) region and the translation elongation factor 1-α (*tef1*) gene. The sequences were compared with reference data in the NCBI and Fusarium MLST databases. Phylogenetic analysis using Bayesian inference revealed that the isolates corresponded to *Neocosmospora solani* (2 isolates), *Fusarium oxysporum* (2 isolates), and an undescribed *Fusarium* species (1 isolate). The presence of *N. solani* and *F. oxysporum* as dominant species (99% sequence identity) suggests their potential role in the etiology of citrus gummosis. These findings expand the current understanding of citrus gummosis etiology in Mexico and underscore the importance of considering alternative fungal pathogens in diagnostic and management strategies.

Keywords: *Citrus* spp; Pathogenic fungi; Trunk canker; Phylogenetic analysis; Vascular discoloration.

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1. Introduction

Citrus gummosis is a destructive disease affecting citrus crops worldwide. In Mexico, gummosis has traditionally been attributed to species of the genus *Phytophthora*, particularly *P. nicotianae* (syn. *P. parasitica*) and *P. citrophthora*, which are considered the main causal agents of the disease in the country's major citrus-producing regions (Acosta-Pérez et al., 2012). These pathogens cause bark cankers, gum exudation, root rot, and progressive decline of affected trees, leading to significant economic losses. In many citrus-growing regions worldwide, species of *Phytophthora* have been reported as important causal agents of citrus gummosis. However, recent studies continue to identify additional species and new geographic records associated with this disease, reflecting its dynamic and evolving etiological landscape. For example, *Phytophthora parvispora* was identified as the causal agent of gummosis and root rot in pomelo (*Citrus grandis*), orange (*Citrus sinensis*), and lime (*Citrus aurantiifolia*) trees in northern Vietnam through morphological characterization, ITS and cox1 sequence analyses, and pathogenicity assays, which confirmed its ability to induce typical symptoms in different citrus hosts (Tran et al., 2023). This report highlights the continuing emergence and diversity of *Phytophthora* species associated with citrus diseases worldwide.

The etiology of citrus gummosis is more complex than previously recognized, involving a broader range of pathogens beyond the traditionally associated *Phytophthora* spp. New reports continue to identify emerging causal agents associated with gummosis and decline symptoms in citrus, highlighting the dynamic nature of this pathosystem (Wang et al, 2026). In this context, fusarioid fungi, including Fusarium and *Neocosmospora* spp., have been increasingly reported in association with gummosis, vascular discoloration, cankers, and dieback symptoms in citrus orchards, with confirmed pathogenicity in several cases (Sardooei et al., 2026). These findings support the hypothesis that citrus gummosis may result from a complex of pathogens rather than a single etiological agent. However, several studies conducted in different Mexican citrus-growing regions have shown that fungal pathogens other than Phytophthora may be associated with gummosis or gummosis-like symptoms. For example, *Lasiodiplodia citricola*, *L. pseudotheobromae*, and *L. theobromae* were identified in Persian lime (*Citrus latifolia*) trees in Morelos state exhibiting dieback and gum exudation (Guillén-Sánchez et al., 2019). More recently, Hernández-Castillo et al. (2024) isolated *Lasiodiplodia pseudotheobromae* from diseased citrus tissues in Yucatán and confirmed its pathogenic role through inoculation tests. These findings support a broader etiological spectrum for citrus gummosis in Mexico than previously recognized.

In this context, the current study investigates citrus gummosis cases in Tuxpan, Veracruz one of the key citrus-producing regions in the country. Veracruz is the leading state in citrus production in Mexico, both in planted area and production volume. Nevertheless, citrus growers in the region often operate under minimal-cost strategies, relying heavily on favorable environmental conditions. This situation, compounded by climate variability and soil degradation, increases crop vulnerability to pests and diseases. The persistence of gummosis and other trunk diseases, along with limited access to effective and affordable control methods, underscores the need to identify the full range of causal agents involved in citrus decline.

The objective of this study was to identify phytopathogenic fungi associated with gummosis symptoms in *Citrus sinensis* orchards in Tuxpan, Veracruz, through phylogenetic approach. The discovery of *Fusarium oxysporum* and *Neocosmospora solani* as potential contributors to this disease expands the current understanding of citrus gummosis etiology in Mexico and supports the need to reassess diagnostic and management strategies to reflect a more diverse fungal complex.

2. Methodology

2.1 Fungal isolation

A total of 100 bark samples were randomly collected from the trunks and branches of symptomatic orange trees located in five representative zones (central, north, south, east, and west) of the municipality of Tuxpan, Veracruz, Mexico. Sections of bark (5 to 10 mm wide) were excised from the margins of lesions exuding gum using a sterile scalpel. The samples were placed aseptically onto potato dextrose agar (PDA) plates and incubated at 28 °C for 7 days. A total of 25 fungal isolates were obtained (Figure 1). Selected isolates were subsequently used for pathogenicity testing, as well as morphological characterization and phylogenetic analysis.

2.2 Morphological characterization

Isolates were characterized at the genus level based on macroscopic and microscopic characteristics using taxonomic keys proposed by Barnett & Hunter (1998) and Leslie & Summerell (2006).

2.3 Molecular identification

Isolates were reactivated on PDA medium and incubated at 28 °C for 5 days. The mycelium was then harvested and transferred to 2 mL Eppendorf tubes for DNA extraction using a modified CTAB (2%) method (Doyle & Doyle, 1990). The mycelium was resuspended in 400 µL of HPLC-grade water, incubated at 55 °C for 15 min, followed by the addition of 34 µL of 3 M sodium acetate (CH_3COONa) and 1 mL of 95% ethanol. Samples were incubated at −20 °C for 60 min and then centrifuged at 11,500 × g for 10 min. The supernatant was discarded, and the pellet was washed with 600 µL of 70% isopropanol, centrifuged again, and air-dried in a laminar flow hood for 30 min. The DNA pellet was resuspended in 50 µL of HPLC-grade water. DNA quality and concentration were verified using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific, USA).

2.4 PCR amplification

Amplification of the internal transcribed spacer (ITS) region of rDNA was performed using primers ITS5

(5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The partial sequence of the translation elongation factor 1-α (*tef1*) gene was amplified using primers EF1 (5'-ATGGGTAAGGA(A/G)GACAAGAC-3') and EF2 (5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3') (Geiser et al., 2004). PCR reactions were performed in a 15 µL final volume containing 5× PCR buffer, dNTPs (20 µM each), primers (10 µM), 60 ng of genomic DNA, and 1.5 U of GoTaq DNA polymerase (Promega, USA).

PCR for ITS amplification was carried out with the following conditions: initial denaturation at 95 °C for 4 min; 35 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 10 min. Amplifications of the *tef1* included: initial denaturation at 94 °C for 15 min; 35 cycles of 94 °C for 30 s, 55 °C for 15 min, and 68 °C for 2 min; and a final extension at 68 °C for 5 min.

PCR products were verified by horizontal electrophoresis in a 1.5% agarose gel (Seakem, USA) stained with GelRed (Biotium, USA) and run at 88 V for 1.5 hours. Gels were visualized with an Infinity-3026 transilluminator (Vilber Lourmat, Germany) and digitized using Infinity-Capt software.

2.5 Sequencing

Amplicons were purified using the ExoSAP-IT enzymatic method (Affymetrix, USA) according to the manufacturer's instructions. Sequencing reactions were prepared using the BigDye Terminator v3.1 kit (Applied Biosystems, USA) in 96-well plates with a final volume of 20 µL containing 4 µL of Ready Reaction Premix (25×), 2 µL of BigDye sequencing buffer (5×), 4 pM of each primer, and 10 – 20 ng of template DNA. The thermocycling program included: initial denaturation at 96 °C for 1 min; 35 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Reactions were purified with ethanol/EDTA precipitation, air-dried, resuspended in Hi-Di Formamide (Applied Biosystems), and analyzed on a 3130 Genetic Analyzer with four capillaries.

2.6 Phylogenetic analysis

Consensus sequences were assembled and edited using BioEdit Sequence Alignment Editor v7.2.6 (Hall, 1999). These were compared to sequences in the NCBI and Fusarium MLST databases. Multiple alignments were performed with MAFFT version 7 (https://mafft.cbrc.jp/alignment/server/index.html) under default parameters. Sequences of *Fusarium* species complexes type strain deposited in the GenBank database were downloaded and included with the sequences obtained in this study. A concatenated alignment datasets was performed with Mesquite v4.01 modular system for evolutionary analysis (Maddison & Maddison, 2025). The best evolution model for each region or gene alignment was determined using JModeltest v.2.3 (Darriba et al., 2012). Phylogenetic reconstruction was conducted using Bayesian inference with MrBayes software (Ronquist & Huelsenbeck, 2003) with 1 million generations until the standard deviation of split frequencies dropped below 0.01. The first 25% of the generated trees were discarded as the burn-in phase, and posterior probabilities were determined for the remaining trees. The phylogenetic tree was rooted with *F. equiseti* accession number KX463032. Maximum-likelihood analysis was performed in raxmlGUI 1.5b2 (Silvestro & Micha-lak, 2012) using Rapid bootstrapping with 1000 iterations and the general time reversible model with a gamma distribution (GTR + G).

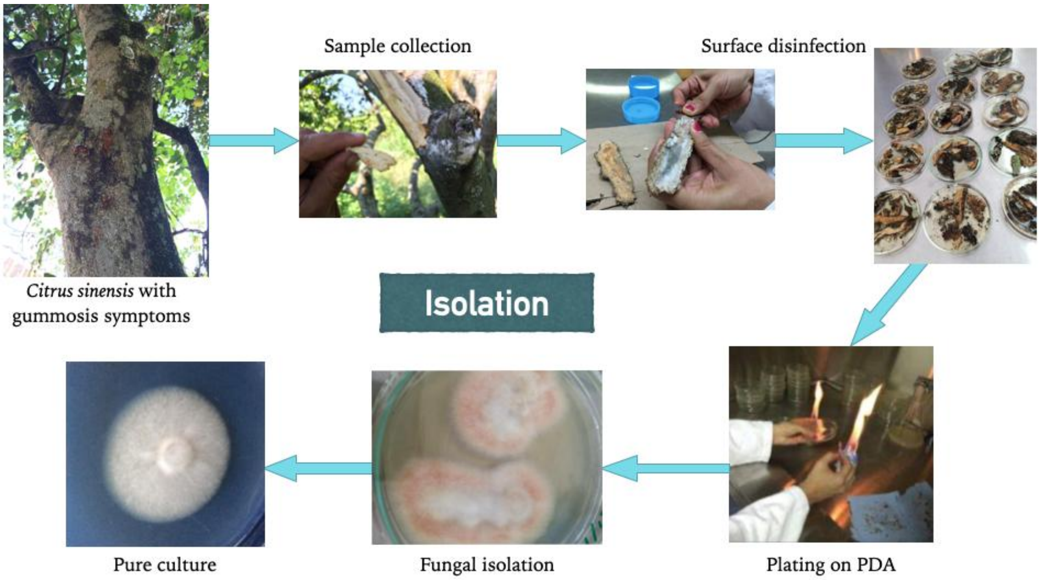

Figure 1. Graphical diagram of methodology of fungal isolation from gummosis-affected Citrus sinensis tissues.

The trees were edited in FigTree v1.4.4 software (http://tree.bio.ed.ac.uk/software/figtree/), and the resulted sequences were deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

2.7 Pathogenicity tests

Pathogenicity was assessed in 260-day-old *Citrus sinensis* seedlings. Cross sections were made on the stem near the root zone, and three additional wounds were made on the main roots. A 2-mm PDA disc containing fungal mycelium was placed on each wound site. The inoculated area was wrapped with transparent tape and covered with a plastic bag for 48 h to prevent desiccation, as described by Zhu et al. (2013). Control plants received PDA discs without mycelium. The experiment was conducted in triplicate and repeated twice for validation. Typical gummosis symptoms such as gum exudation, xylem discoloration, leaf chlorosis, and crown wilting were reproduced under greenhouse conditions in *C. sinensis* seedlings (Figure 2). These symptoms were absent from the control plants.

Figure 2. *Citrus sinensis* plant under laboratory conditions, used in pathogenicity tests, showing gummosis symptoms including gum exudation on the stem.

3. Results and discussion

3.1. Number of isolates obtained

A total of 25 fungal isolates were obtained from the 100 bark samples collected. Of these, 10 were purified, and five isolates were selected based on their pathogenicity for subsequent molecular identification. Typical symptoms of gummosis, including gum exudation, necrotic lesions, and bark cracking, were observed on the trunks and branches of infected trees (Figure 3).

Figure 3. Cross-section of *Citrus sinensis* trunk showing visible gummy exudation symptoms associated with gummosis.

3.2 Morphological characteristics

Colonies grown on PDA exhibited abundant white aerial mycelium with yellow to orange pigmentation. Microscopic observation revealed septate hyphae, abundant ovoid microconidia measuring 57-119 µm in length and 2 - 36 µm in width, produced on long conidiophores. Macroconidia were sickle-shaped, measuring 162 - 265 µm in length and 27 - 449 µm in width, with 2 - 3 septa. These morphological traits are consistent with descriptions reported by Barnett & Hunter (1999) and Montealegre et al. (2003). Colonies exhibited abundant white aerial mycelium with yellow to orange pigmentation, consistent with Fusarium-like fungi (Figure 4).

3.3 Molecular identification

Identification of the fungal isolates associated with gummosis symptoms in *Citrus sinensis* revealed a predominance of *Fusarium oxysporum* and *Neocosmospora solani*, which have 99% similarity in their *tef1* sequences. This finding is particularly significant given that *F. oxysporum* is not traditionally recognized as a primary causal agent of citrus gummosis, although it has been isolated from diseased roots and vascular tissues in other citrus decline syndromes (Xiao et al., 2025; Yaseen & D'Onghia, 2010). The presence of *N. solani* in two of the isolates aligns with recent reports of its pathogenic role in citrus dry root rot (Kurt, 2020) and root necrosis and wilting (Garzón-Nivia et al., 2025) and suggests that members of the *Neocosmospora solani* species complex (NSSC) may have a broader host range and symptomatology than previously documented. The confirmation of *Fusarium* spp. using both ITS and *tef1* markers supports the reliability of a multi-locus molecular approach in the identification of cryptic fungal pathogens. These results also highlight the limitations of relying solely on morphological traits, which may underestimate species diversity or lead to misidentification, especially among Fusarium-like taxa. The identification of one isolate as an undescribed *Fusarium* species further underscores the potential existence of novel or emerging fungal pathogens within Veracruz citrus orchards. Bayesian phylogenetic analysis based on ITS and *tef1* sequences confirmed the identity of the isolates (Figure 5). Considering that gummosis is typically attributed to *Phytophthora* spp., the consistent recovery of *Fusarium* and *Neocosmospora* from symptomatic bark tissues raises questions about the complexity of this disease's etiology. It is possible that these fungi act as secondary invaders or opportunistic pathogens facilitated by environmental stress, previous wounding, or chemical management practices such as the prolonged use of phosphonates, which may disrupt natural microbial antagonism and host resistance (Theron et al., 2025; Dann & McLeod, 2021).

Recent international investigations further support the involvement of multiple Fusarium and Fusarium-like species in citrus decline syndromes, including dry root rot (DRR) and gummosis. Similar trends have been reported in other horticultural crops affected by *Fusarium* spp., where integrated morphological and molecular approaches have proven essential for accurate species identification and for understanding pathogen diversity under different agroecological conditions (Ramos-Hernández et al., 2024; Zúñiga-Torres et al., 2025).

Figure 4. Colony morphology of a representative fungal isolate grown PDA at 28 °C for 7 days, showing abundant white aerial mycelium with concentric salmon-to-pink pigmentation toward the colony margin, characteristic of Fusarium-like fungi.

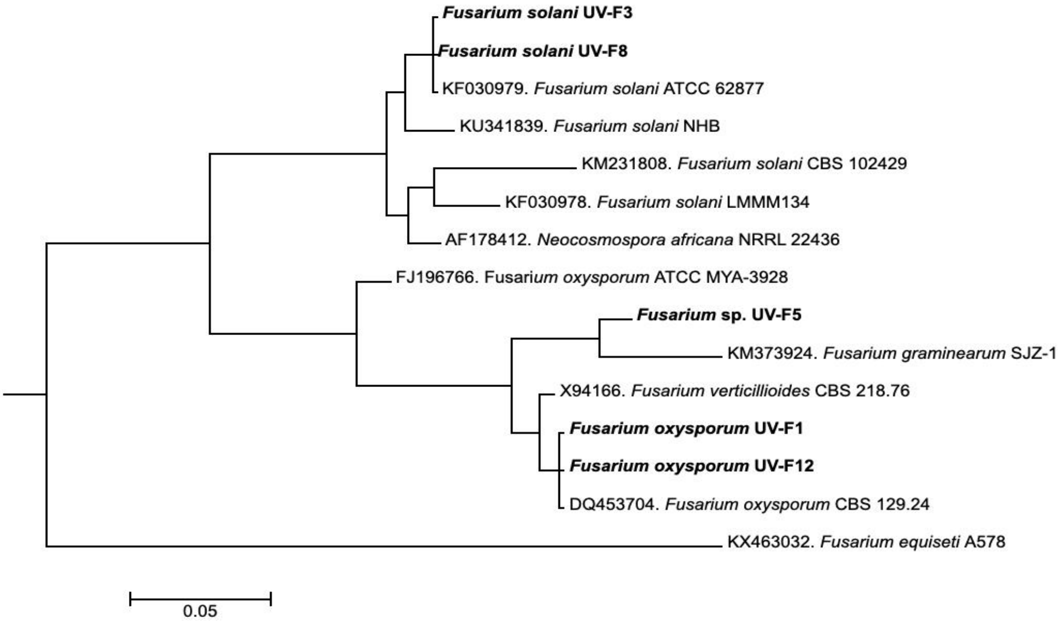

Figure 5. Bayesian phylogenetic analysis based on ITS and tef1 sequences confirming the identity of the fungal isolates.

These studies, carried out in Latin America, emphasize that the combination of ITS and tef1 sequencing increases diagnostic precision and supports the design of targeted management strategies adapted to local production systems. For instance, in Italy, a broad survey identified several species, *F. oxysporum*, *F. sarcochroum*, *F. ensiforme*, *F. citricola*, *F. salinense*, *F. siculi*, and *Neocosmospora* spp. associated with symptomatic tissues in citrus trees, including trunks, branches, and roots (Aloi et al., 2021). Similarly, studies in South Africa reported the presence of *N. solani* and at least ten other *Neocosmospora* species in DRR-affected groves, reinforcing their phytopathological relevance in citrus agroecosystems. These findings highlight the high species diversity and geographical variability of fungal pathogens affecting citrus, emphasizing the need for regional molecular surveillance. Considering these reports, the identification of *F. oxysporum* and *N. solani* in our study suggests a possible shift or expansion in the range of species associated with gummosis in Mexico.

In Mexico, the diversity of fungal pathogens associated with gummosis-like symptoms in citrus is increasingly evident. A study conducted in Morelos state identified *Lasiodiplodia citricola*, *L. pseudotheobromae*, and *L. theobromae* as causal agents of dieback and gummatous exudates in *Citrus latifolia* ('Persian 'lime) plantations. The isolates were confirmed through morphological traits, pathogenicity assays, and molecular analysis of the ITS region, with sequence similarities up to 99% and clear phylogenetic grouping into distinct clades (Guillén-Sánchez et al., 2019). This finding expands the spectrum of fungal pathogens involved in citrus decline in Mexico and highlights the geographic and host variability of gummosis-associated agents.

While these *Lasiodiplodia* species were identified in *C. latifolia* in central Mexico, the present study reports the occurrence of *F. oxysporum* and *N. solani* in *C. sinensis* in Veracruz, a region with distinct agroecological conditions. This contrast suggests that the etiology of gummosis may differ by citrus species, cultivar, environmental stressors, and regional management practices. Such diversity underscores the need for localized diagnostic approaches and region-specific disease management strategies, as pathogen composition may vary significantly across Mexico's citrus-producing areas. A similar study conducted in Tunisia provides additional support for the pathogenic role of *F. oxysporum* in citrus. In that report, unusual wilt symptoms were observed in *C. sinensis*' Washington Navel 'and *C. tangerina* cultivars grafted on sour orange (*C. aurantium*) rootstocks, with up to 67% disease incidence and no visible root rot. The isolated *F. oxysporum* strains were confirmed through molecular analysis of ITS region and *tef1*, showing 99% similarity to reference sequences. Pathogenicity tests reproduced wilt symptoms in greenhouse conditions, and *F. oxysporum* was successfully re-isolated, fulfilling Koch's postulates (Hannachi et al., 2014). This is consistent with our findings of *F. oxysporum* isolated from symptomatic bark tissues of sweet orange in Veracruz, suggesting that this species may be more widely associated with vascular and trunk diseases in citrus than previously recognized. Symptoms reported in both studies occurred in the absence of root necrosis, challenging the traditional association of *F. oxysporum* exclusively with root rot syndromes.

3.4 Pathogenicity assay

Inoculated *Citrus sinensis* seedlings developed characteristic gummosis-like symptoms, including vascular browning and gum exudation, at the inoculation sites, foliar yellowing, and eventual plant death. Lesions ranged from 1 to 3 cm in length and 0.5 to 2.5 cm in width. Control plants remained asymptomatic throughout the experiment. *Neocosmospora solani* and *Fusarium oxysporum* were consistently re-isolated from symptomatic tissues, confirming their pathogenicity and fulfilling Koch's postulates.

A comparison with previously reported studies on citrus gummosis pathogens is presented in Table 1.

Table 1
Summary of pathogenicity assays and comparison with recent literature on citrus gummosis-associated fungi

Pathogen	Host / System	Symptoms observed	Reference
Neocosmospora solani	*Citrus sinensis* seedlings (this study)	Xylem discoloration, gum exudation, lesions, leaf yellowing, plant death	This study
Fusarium oxysporum	*Citrus sinensis* seedlings (this study)	Vascular browning, wilting, gum exudation	This study; Garzón-Nivia et al. (2025); Zhu et al. (2013)
Phytophthora parasitica (syn. P. nicotianae)	*Citrus sinensis* and *C. latifolia* (Mexico)	Gummosis, stem lesions, fruit rot	Acosta-Pérez et al. (2012)
Fusarioid species (incl. *Neocosmospora spp.)*	*Citrus* spp. (China, field surveys 2021–2024)	Gummosis, necrosis, cankers, wood decay	Xiao et al. (2025)
Phytophthora parvispora	*Citrus spp.* (Vietnam)	Gummosis, root rot, fruit rot, leaf yellowing	Tran et al. (2023)
Neofusicoccum parvum	Citrus × limon (Italy)	Trunk and branch cankers, abundant gum exudation	Aloi et al. (2021)
Neoscytalidium dimidiatum	*Citrus* spp. (Italy)	Gummosis, canker, twig dieback	Polizzi et al. (2009)

These results, consistent with recent studies, support the increasing recognition of *Fusarium* and *Neocosmospora* species as important contributors to citrus gummosis and decline, highlighting the complexity of its etiology. In addition to well-documented gummosis pathogens, several studies have reported fusarioid species associated with vascular diseases, decline, and canker symptoms in citrus and other crops (Garzón-Nivia et al., 2025; Xiao et al., 2025), suggesting that these fungi may also contribute to gummosis-like syndromes under certain conditions.

4. Conclusions

This study identified five phytopathogenic fungal isolates associated with gummosis symptoms in *Citrus sinensis* in the municipality of Tuxpan, Veracruz, Mexico, were associated with the presence of *Neocosmospora solani* and *Fusarium oxysporum*, identified through morphological characterization and ITS and tef1-α sequence analyses. Pathogenicity tests confirmed the ability of both fungi to induce disease symptoms under controlled conditions, supporting their role in the development of gummosis.

These findings expand the current knowledge of the etiological agents associated with citrus gummosis in Mexico and highlight the need to consider fungal pathogens beyond traditionally recognized causal agents in disease diagnosis and management strategies.

Authors' contribution

Conceptualization: **S.M.K.L., A.M.R. and S.R.H.V.**; Data curation: **S.M.K.L.**; Formal Analysis: **A.M.R. and S.R.H.V.**; Acquisition of financing; **S.M.K.L.**; Investigation: **S.M.K.L. and G.C.J.C.**; Methodology: **S.M.K.L., G.C.J.C and A.M.R.**; Management of project: **S.M.K.L.**; Resources: **S.M.K.L.**; Software: **A.G.A.**; Supervision: **S.M.K.L. and A.M.R.**; Validation: **S.M.K.L., A.M.R., S.R.H.V. and G.C.J.C.**; Visualization: **A.G.A., S.R.H.V. and P.V.R.**; Write-Draft original: **S.M.K.L.**; Write- review and edition: **A.G.A., S.R.H.V., P.V.R. and A.M.R.**

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