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Fungal species characterization associated with vanilla root rot in the Totonacapan, Veracruz, Mexico

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Abstract

Vanilla (*Vanilla planifolia*) is a crop of great biocultural importance in the Totonacapan region in Mexico; however, producers face various phytosanitary problems that decrease production. The main one being crop loss caused by the fungus, *Fusarium oxysporum* f. sp. *vanillae*, a pathogen that causes a disease whose symptomatology is root rot that in severe cases causes the death of plants. Therefore, the study of the pathogen is crucial, due to the scarcity of genetic variability of vanilla, a condition that makes the crops equally susceptible to the pathogen. The present work aimed to generate and molecularly and pathogenically characterize a fu-ngal isolates collection obtained from *V. planifolia* with root rot. Roots with rot were collected in six municipalities of the Totonacapan region, for fungal isolation, a semiselective culture medium was used, later, the molecular characterization was carried out through the amplification and sequencing of the partial region of the translational elongation factor 1α gene (*TEF1*). The pathogenic characterization was carried out on vanilla leaves in a humid chamber, inoculating medium discs with mycelium, and making observations for 18 days every 48 h. As results, 94 strains were obtained, of which 81 belong to the genus *Fusarium* (42 *F. oxysporum* strains), eight to *Colletotrichum*, three to *Trichoderma* and two it was not possible to identify them, only 23 *Fusarium oxysporum* strains showed to be pathogenic on vanilla leaves with different degrees of virulence. The results of the molecular and pathogenic characterization will be crucial for further studies of comparative genomics of *Fusarium oxysporum*.

Keywords: Pathogenic and molecular characterization; *Fusarium oxysporum* f. sp. *vanillae*; *Vanilla planifolia*; Koch's postulates

1. Introduction

The *Fusarium* genus was first described by Link in 1809, it is a group of filamentous fungi that contains many agronomically important plant pathogens, mycotoxin producers, and opportunistic human pathogens [1]. The species of this genus, especially the pathogenic ones, have great economic importance because they are responsible for considerable annual crop losses, affecting a large number of species of agricultural importance [2]. Within the genus, the species *Fusarium oxysporum* and *Fusarium solani* stand out for being the only ones that affect a wide diversity of plants and for being present in almost the entire planet [3]. Unlike other species that are limited to certain geographic regions and crops, *F. oxysporum* and *F. solani* are widely distributed sharing morphological characteristics and ecological niches that make them very similar and difficult to differentiate [4]. Another particularity that these two species share are the hosts, for example, vanilla turns out to be within the wide range of hosts for these pathogens, since their presence has been reported in crops in the main vanilla-producing areas of the world, being *F. oxysporum* f. sp. *vanillae* the causal agent *V. planifolia* root rot [5]. Worldwide, root rot caused by *F. oxysporum* has been reported in

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vanilla in crops from the main producing countries in the world, such as Madagascar and Indonesia [5], which according to Food and Agriculture Organization (FAO) data, in 2020 ranked first and second place in production, respectively, followed by Mexico, Papua New Guinea and mainland China [6]. In Mexico, this disease has also been reported in vanilla fields in Nayarit [7], in the Huasteca Potosina regions [8] and in Totonacapan region [9] located in the Gulf-Central area of Mexico. The species *F. oxysporum* f. sp. *vanillae* is the causal agent of vanilla root rot, manifesting as black rot in roots, causing the wilting of the plants and in severe cases their death, resulting in the loss of plantations and reduced crop production [9]. The problem with *Fusarium* in the Totonacapan becomes even more relevant because, as indicated by Lubinsky [10], worldwide vanilla has its origins in this area and the fact that this is a clonal propagation crop means that there is little genetic variability, and in turn most crops become equally susceptible to said pathogen. Thus, the need arises to generate and characterize molecularly and pathogenically a collection of Fungal isolates and specifically *Fusarium* obtained from roots with vanilla rot from Totonacapan. With the development of this research, the bases are laid for the generation of knowledge that helps in the creation of more efficient proposals for the control and elimination of the disease, through strategies such as biocontrol, analysis of population structure, and comparative genomics of pathogenic and non-pathogenic isolates.

2. Material and methods

2.1. Collection of vanilla samples with root rot

The vanilla plant tissue consisted of roots and stems with symptoms of rotting and wilting, which was collected in the Totonacapan region, specifically in the municipalities of Papantla, Gutiérrez Zamora, Actopan, Vega de Alatorre, San Rafael and Tlapacoyan, in Veracruz. The tissue was stored in labeled bags and transported in a cooler until processing at the Phytosanitary Diagnostic Laboratory of the Ixtacuaco Experimental Field (CEIXTA) from National Institute of Forest, Agriculture and Livestock Research (INIFAP).

2.2. Fungal isolation from vanilla plant tissue

At CEIXTA, the collected tissue was processed using soap and chlorine to remove external contaminants, then ~5 mm^2 explants were sown in Peptone Chlorothalonil Agar culture medium, supplemented with Penicillin, Streptomycin and Dicloxacillin, to prevent bacterial growth [1]. After 48 h after sowing, the fungal isolates were transferred to 2% Water Agar medium, and then they were purified using the hyphal tip technique and stored at 16°C in the form of discs of SNA medium with mycelium (Spezieller Nährstoffarmer Agar).

2.3. Molecular characterization of fungal isolates

The 94 fungal isolates were grown in dextrose potato broth (PDB) for 7 days, then the mycelium was compacted, dried, and ground with liquid nitrogen for DNA extraction using the CTAB protocol [11]. The DNA extracted was used to amplify the partial region of the *TEF1* gene by PCR and the PCR products were resolved by electrophoresis in 1.5% agarose gel, later purified with Promega's Wizard SV Gel and PCR Clean-Up System kit and they ordered sequencing to the Potosí Institute for Scientific and Technological Research (San Luis Potosí, Mexico). The identity of the isolates was determined by analyzing the sequences in the BLAST program of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

2.4. Pathogenicity tests and Koch's postulates

Three healthy and physiologically mature *Vanilla planifolia* leaves were used for each isolate to be inoculated, and for the control. The leaves were disinfested with a 1% soap solution for 10 min, followed by 2% sodium hypochlorite for 2 min, and finally, they were rinsed with sterile distilled water, dried and placed in humid chambers. The inoculation was carried out following the protocol used by Adame-García [12]; each leaf was made a lesion with a sterile scalpel in the center of the leaf of ~2 mm where a 5 mm disk of PDA medium with mycelium was placed, the controls were inoculated with the disk of PDA medium without fungal growth. In order to determine the level of virulence through the percentage of damage on the leaves, considering the area of the damaged zone with respect to the total area of the leaf, four levels of pathogenicity were classified:

- Non-pathogenic, leaves without damage;
- Low virulence, lesion less than 25%;
- Medium virulence, lesion in the range of 25%-50% and
- High virulence, lesion greater than 50%. The cameras remained under observation for 18 days.

3. Results and discussion

3.1. Generation of the collection of isolates

It was possible to generate a collection of 94 fungal isolates with the collection and processing of plant tissue obtained from vanilla roots and stems, with symptoms of rot and wilting, from the Totonacapan region, which are sheltered in the microbiology area of the Phytosanitary Diagnostic Laboratory of the Ixtacuaco Experimental Field.

3.2. Molecular characterization of the isolates

The results after performing the BLAST analysis indicated that 86.17% (81) of the isolates belong to the *Fusarium* genus. 51.85% (42) correspond to the *F. oxysporum* species; 19.75% (16) are *F. solani*, which is not surprising, since in other works on vanilla, this species is highly represented in the tissue of *V. planifolia* and *V. pompona* [5]. 14.81% (11) belong to the *Fusarium incarnatum-equiseti* species complex (FIESC), a complex that is a common inhabitant of soils and found associated with plants as a saprophyte [11]. 2.47% (2) of the isolates are *F. fujikuroi*; 6.17% (5) *F. lateritium*; 2.47% (2) are *F. irregulare*; 1.23% (1) one isolate is *F. pseudocircinatum*; 1.23% (1) *F. sulawesiense* and finally 1.23% (1) is *F. culmorum* all these species have been reported as soil dwellers, saprophytes, or opportunistic pathogens [1]. On the other hand, 11.7% (13) of the isolates did not belong to the *Fusarium* genus, eight isolates were identified as *Colletotrichum gloesporioides*, a pathogen that causes anthracnose on vanilla leaves and fruits [13], three isolates are *Trichoderma* spp. and two isolates, no sequences were obtained (Figure 1).

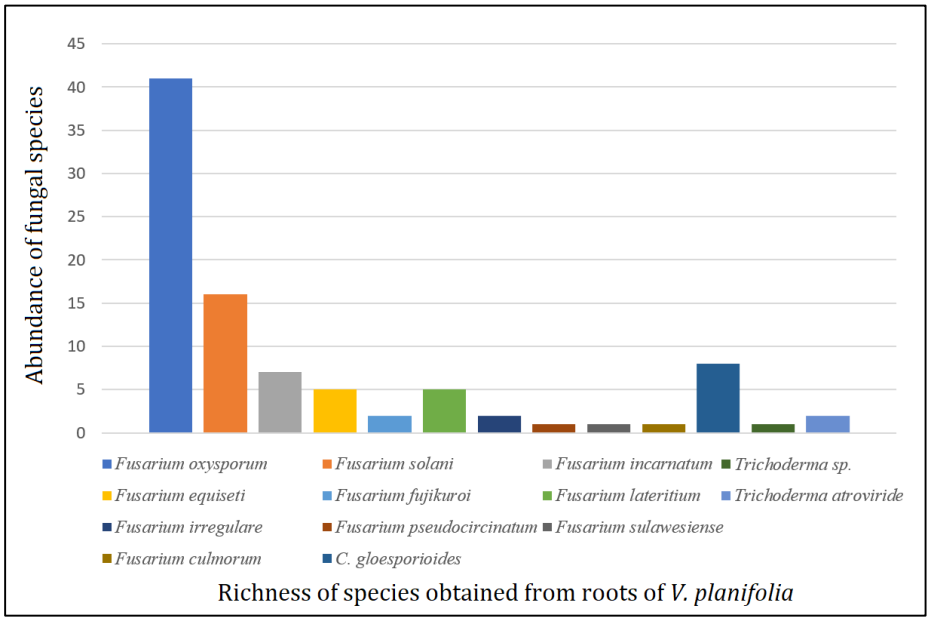

Figure 1 Richness and abundance of species obtained from root tissue of *V. planifolia* with symptoms of rot, by molecular identification of the strains using the amplification and sequencing of the *TEF1* gene

3.3. Pathogenicity tests and Koch's postulates

The symptoms on vanilla leaves that presented some level of damage, were observed to begin with moderate expansion of the mycelium outside of the agar disk on the leaf, later the color change began around the point of inoculation, from green to dark brown and in some cases to black, indicating the beginning of rot, which always progressed more rapidly towards the base or the apex compared to the sides, representative images are shown in Figure 2.

The results of the inoculation of the 94 fungal strains on healthy and mature *V. planifolia* leaves indicates that only twenty five of the forty two *Fusarium oxysporum* strains showed some degree of virulence on vanilla leaves, because these strains are *F. oxysporum* f. sp. *vanillae* [5].

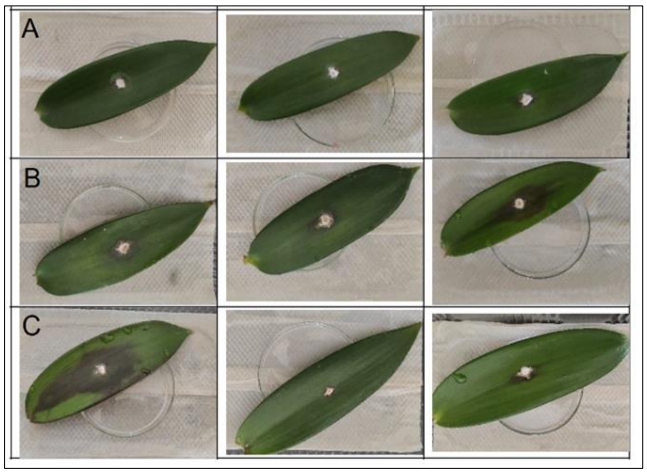

Figure 2 Representative images of pathogenicity tests on *V. planifolia* leaves, showing different degrees of virulence. A) IXF53 isolated from non-pathogenic endophytic *F. oxysporum* ; B) IXF41 isolated from moderately virulent *F. oxysporum* ; C) IXF50 isolated from moderately virulent *F. oxysporum*

4. Conclusion

At least nine species of the genus *Fusarium* are associated with vanilla roots with rot and wilting symptoms, however, only *F. oxysporum* f. sp. *vanillae* is capable of inducing symptoms on vanilla leaves under *in vitro* conditions. Subsequent studies using comparative genomics will help us understand the differences observed in the degree of virulence of *F. oxysporum*, as well as the possible molecular mechanisms involved during the interaction with vanilla.

Compliance with ethical standards

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Disclosure of conflict of interest

None of the authors have non-financial as well financial conflict of interest.

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