Pathogenicity of *Fusarium oxysporum* f. sp. *nicotinae* and *F. phyllophilum* in tobacco in the Granma region, Cuba

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Patogenicidade de *Fusarium oxysporum* f. sp. *nicotinae* e *F. phyllophilum* no tabaco na região de Granma, Cuba

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Abstract

Diseases caused by *Fusarium* spp. are considered cosmopolitan and affect a great diversity of crops worldwide. In the tobacco-growing areas of Granma province, located in eastern Cuba, these phytopathological problems cause significant losses in tobacco leaf yield and quality. To address this critical issue, a comprehensive study was conducted, focusing on disease monitoring and the identification of tobacco seeds and seedlings (cv. Corojo 2012) affected by the diseases. The study also examined the variables associated with the severity of the diseases. The results of the study indicated that the vascular wilt was caused by two distinct fungal pathogens: *F. oxysporum* f. sp. *nicotinae* and *F. phyllophilum*. The study revealed that the disease exhibited levels of recurrence in both space and time, reaching up to 62 % necrosis in seedlings with no chance of survival. This disease severity, measured on a maximum scale of five, attained a maximum score of 4.20. The findings indicated a substantial inhibition in the germination and growth variables of *Nicotiana tabacum*, which has a profound impact on the yield and commercial quality of *N. tabacum* leaves, particularly those intended for the Cuban cigar manufacturing industry. This underscores the necessity for expeditious diagnosis of the disease and the implementation of appropriate management strategies to mitigate the risks of its propagation in producing regions of Cuba and globally.

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Resumen

Las enfermedades causadas por *Fusarium* spp. son consideradas cosmopolitas y afectan a una gran diversidad de cultivos en todo el mundo. En las zonas tabacaleras de la provincia de Granma, ubicada en el oriente de Cuba, estos problemas fitopatológicos provocan pérdidas significativas en el rendimiento y la calidad de la hoja de tabaco. Por este motivo, se realizó un estudio basado en el monitoreo de la enfermedad, en el cual se determinaron las afectaciones en las semillas y plántulas de tabaco cv. Corojo 2012, así como las variables asociadas a la severidad de la enfermedad. Los resultados mostraron que el marchitamiento vascular fue causado por *F. oxysporum* f. sp. *nicotinae* y *F. phyllophilum*. Se encontró una recurrencia de la enfermedad en el espacio y el tiempo, con niveles máximos de un 62 % de necrosamiento en plántulas sin posibilidades de supervivencia, además de una severidad de la enfermedad de 4,20 en una escala máxima de cinco. Esto provocó una inhibición significativa en las variables de germinación y crecimiento de *Nicotiana tabacum*. Lo anterior posee incidencia en el rendimiento y calidad comercial de las hojas de *N. tabacum*, especialmente si se destinan a la industria de fabricación de puros cubanos. Esto pone de manifiesto la necesidad de realizar diagnósticos tempranos de la enfermedad y aplicar las alternativas de manejo correspondientes para minimizar los riesgos de diseminación en regiones productoras de Cuba y el mundo.

Palabras clave: distribución, enfermedad, hongos, intensidad.

Resumo

As doenças causadas por *Fusarium* spp. são cosmopolitas e afectam uma grande diversidade de culturas em todo o mundo. Nas zonas de cultivo de tabaco da província de Granma, localizada no leste de Cuba, estes problemas fitopatológicos causam perdas significativas no rendimento e na qualidade das folhas de tabaco. Por este motivo, foi realizado um estudo baseado na monitorização da doença, no qual se determinaram as afectações nas sementes e nas plântulas da variedade de tabaco Corojo 2012, bem como as variáveis associadas à severidade da doença. Os resultados mostraram que a murcha vascular foi causada *por F. oxysporum* f. sp. *nicotinae* e *F. phyllophilum*. Foi observada uma recorrência da doença no espaço e no tempo, com níveis máximos de 62 % de necrose em plântulas sem hipótese de sobrevivência, e uma severidade da doença de 4,20 numa escala máxima de cinco. Este facto provocou uma inibição significativa da germinação e das variáveis de crescimento da *Nicotiana tabacum*. Isto tem um impacto no rendimento e na qualidade comercial das folhas de *N. tabacum*, especialmente se forem destinadas à indústria cubana de fabrico de charutos. Este facto evidencia a necessidade de um diagnóstico precoce da doença e a aplicação das alternativas de gestão correspondentes para minimizar os riscos de disseminação nas regiões produtoras de Cuba e do resto do mundo.

Palavras chave: distribuição, doença, fungos, intensidade.

Introduction

Tobacco (*Nicotiana tabacum* L.) is one of the most commercially valuable solanaceae and the most widely cultivated non-edible agricultural product in the world, with a major impact on the culture, society and economy of tropical and subtropical countries. Its leaves, rich in organic compounds, are the basis of the tobacco industry for the production of cigarettes and cigars. Originally from the Andean region, its cultivation has spread to more than 125 countries thanks to its high adaptability to different agricultural ecosystems (Sosa-Sánchez *et al*., 2022; Gui *et al*., 2024).

Cuba is renowned for the production and export of dark tobacco, with growing demand in markets such as China, Spain and Germany. In 2022, exports of tobacco products reached US$ 275 million, representing more than 20 % of the national total. However, key tobacco-growing regions have experienced a 62 % drop in yields, attributed to lack of agricultural inputs, adverse weather conditions and fungal diseases (National Office of Statistics and Information [ONEI], 2022; Observatory of Economic Complexity [OEC], 2024).

In this context, several dark tobacco varieties have been studied with tolerance to infections of the fungi causing blue mould (*Peronospora hyocyami* de Bary), tobacco blight (*Phytophthora parasitica* Dast. var. *Nicotianae* Breda de Haan) and *Fusarium* (Ha-Thanh *et al*., 2022). Specifically, *F. oxysporum*, *F. solani* and *F. phyllophylum* are the most recurrent soil species associated with solanaceae and with the greatest impact on tobacco since, in addition to causing the collapse of xylem vessels causing wilting, they also affect the leaf of *N. tabacum* with the appearance of leaf spots and eventual necrosis (Sosa-Sánchez et al., 2022; González et al., 2023). *Fusarium oxysporum* is considered a complex of cryptic species, whose phylogenetic complexity makes species identification difficult (Ribeiro *et al*., 2022).

Specifically, vascular wilt in *Nicotiana tabacum* is caused by *Fusarium* f. sp. *nicotianae*, *batatas* and *vasinfectum*. Necrosis-producing variants complicate agronomic management by affecting leaves, which are key to premium cigar production. In addition, some variants can remain dormant in the soil in the form of chlamydospores, facilitating their spread on various hosts (Nikitin *et al*., 2023). However, for *Fusarium* infection to occur, processes such as degradation of host barriers, early signalling and interaction of key metabolites and molecules, including enzymes, toxins and membrane transporters, must be activated (Laraba *et al*., 2022). This suggests that some dark tobacco varieties in Cuba may be susceptible to *Fusarium* f. sp. associated with wilt.

Moreover, the symptoms are not only limited to the vascular bundles, but also affect leaf quality, precisely one of the most recognised characteristics of export tobacco (Sosa-Sánchez *et al*., 2022). In view of the above, this research was conducted to determine the phytopathogenicity of native isolates of *Fusarium oxysporum* and *F. phyllophylum* on *N. tabacum*.

Materials and methods

Isolation of *Fusarium* spp.

Samples of roots, stems and leaves with *Fusarium* symptoms were collected from commercial tobacco crops in Granma, Cuba, specifically in Bueycito (20°14’20. 5‘’N, 76°46’28.8‘’W), El Dorado (20°15’52.3‘’N, 76°44’43.8‘’W), Monjará (20°17’51.9‘’N, 76°36’08.7‘’W) and Los Cayos (20°18’20.2‘’N, 76°50’00.6‘’W). Symptoms observed included stunted growth, yellowing, leaf necrosis, vertical drying of leaves, browning of vascular tissues and chlorosis at the top of the plant (Gilardi *et al*., 2021). In addition, disease recurrence was recorded in two consecutive periods in 2019 and 2020. The isolation and morphological characterisation of the fungi was performed according to Sosa-Sánchez *et al*. (2022) and they were deposited in collections of the Universidad de Granma, Cuba, and the Centro de Investigaciones Biológicas del Noroeste (CIBNOR S. C., Mexico).

Primer-specific identification

DNA was amplified with the molecular marker for elongation factor 1α and primers EF1 [5'-ATGGGTAAGGA(A/G)GACAAGAC-3'] and EF2[5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3'] were used to differentiate *Fusarium* species (Nitschke et al., 2009; Retana et al., 2018). The procedure was performed at the Laboratory of Phytopathology, CIBNOR, La Paz, B.C.S., Mexico. DNA sequence comparisons with the GenBank® database (https://www.ncbi.nlm.nih.gov/genbank/) were used to determine species identity.

Seed and seedling conditioning

Seeds of the cultivar Corojo 2012 from the Unidad Empresarial de Base (UEB) de Acopio y Beneficio de Tabaco en Horno de Guisa, Bayamo, Cuba, with a germination power ≥85 % were used. The seeds, disinfected with 1 % sodium hypochlorite for 2 minutes, were sown in 3 L pots with a substrate of soil and organic sheep manure (3:1 v/v), sterilised at 150 ºC for 3 hours and rested for 12 hours before use. Management conditions followed the recommendations for cultivation (Espino *et al*., 2012).

Experimental inoculation of seeds and seedlings

At the Microbiology Laboratory of the Facultad de Ciencias Agrícolas, Universidad de Granma, Cuba, 25 previously disinfected seeds were placed in Petri dishes with sterilised filter paper. 600 µL of a spore suspension ($0.74.10^6$ macroconidia.mL^{-1}) of *Fusarium* spp. (isolates FO-B, FO-D, FP-E and FP-F) were added and the plates were incubated at 28 ± 2 °C, adjusting the humidity daily (≥85 %). After 7 days, *Fusarium* spp. colonies were counted and after 10 days the percentage of seed germination was assessed. After 28 days, whole seedling length (LP) and fresh mass per seedling (MFP) were measured to calculate the percentage of inhibition in the early developmental stages.

For inoculation, 10 homogeneous seedlings with the first four true leaves were used, a 1 cm cut was made at the apex of the main root. They were then immersed in a suspension with macroconidia ($0.74.10^6$ macroconidia.mL^{-1}) of the isolates for three hours. Control seedlings were immersed in sterile distilled water for the same time. After this process, they were transplanted into 250 mL plastic jars with sterilised substrate and watered every other day.

At 17 days post inoculation (dpi) disease intensity was measured using the formula of Townsend and Heuberger (1943), I (%) = [(∑ a x b) / N x K] x 100, where: a = number of plants or organs affected, b = grade of the scale, K = last grade of the scale used, N = total number of plants. Damage was recorded according to a scale of 0-5 degrees (table 1) and necrotic seedlings with no chance of survival were counted.

Experimental design and statistical processing

A completely randomised design with four treatments, a control without pathogen inoculation and 10 replicates was used for both trials. Percentage data were transformed prior to ANOVA using RAIZ^2 (x+1). A one-way ANOVA was performed and, when significant differences were found, Tukey's multiple comparison of means test ($p \leq 0.05$) was applied using InfoStat 2008 software (Balzarini *et al*., 2008).

Results and discussion

Distribution of *F. oxysporum* f. sp. *nicotianae* and *F. phyllophylum* in the tobacco-growing areas of Granma Province, Cuba

In the monitored areas, the genus *Fusarium* was found to be associated with vascular wilt. The species *F. oxysporum* f. sp. *nicotinae* occurred in Bueycito and El Dorado, while *F. phyllophylum* was found in Monjará and Los Cayos. Except for isolate FP-F (*F. phyllophilum*), which was not observed in the second monitoring cycle, the other isolates were recurrent in each locality. Molecular tests with specific primers confirmed the identity of the species. *Fusarium* recurrence is common and key to yield and quality losses (Kema *et al*., 2021).

The morphological characteristics of the isolates coincided with reference strains from the Microbiology Laboratory of the Universidad de Granma (Sosa-Sánchez *et al*., 2022). Field symptoms included wilting, stunting, yellowing, leaf and stem necrosis, interveinal chlorosis and browning of vascular tissue caused by *F. oxysporum* f. sp. *nicotianae* and *F. phyllophylum* (Pandey, 2023). Both species were associated with vascular wilt of tobacco in Granma province (table 2).

Native strains of *F. oxysporum* f. sp. *nicotianae* and *F. phyllophilum* may be related to others that share alternate hosts (tomato, vanilla, orchids). Recent studies suggest the possibility of forming lineages within the same species (Luna-Rodriguez *et al*., 2023). Vascular wilt disease is complex and multifactorial, with several species, variants and virulence factors interacting. *F. oxysporum* is considered a key pathogen because of its efficient seed and soil-borne transmission (Hudson *et al*., 2021).

Table 1. Damage scale from 0-5 to determine the severity of the disease in the different organs of the seedlings of *Nicotiana tabacum* cv. Corojo 2012.

Scale[1]	Síntomas[2]		
	Root	Stem	Leaves
0	Healthy plant	Healthy plant	Healthy plant
1	Slight root rot (< 10 %)	1-5 % vascular necrosis	Yellowing of basal leaves
2	Dark lesions on 25 % of the roots	5-15 % necrosis in vascular tissue	Yellowing of basal leaves and wilting of one or two leaves
3	50 % of the infected root. Severe necrosis on the main root	15-35 % necrosis in the vascular tissue.	Severe yellowing of leaves. 50 % of leaves wilted and growth inhibited.
4	75 % of the infected root. Crown lesions and wilting of old leaves.	35-67.5 % necrosis in the vascular tissue.	Widespread yellowing, vascular and root necrosis
5	100 % of the infected root. Wilt and death of basal leaves	67.5-100 % necrosis in vascular tissue	Necrotic plant

[1]Scale. [2]Raíz (Cakir *et al*., 2014), stem (Akhter *et al*., 2015), leaves (Rongai *et al*., 2017).

Table 2. Causal agent associated with vascular wilt and its spatio-temporal distribution in the tobacco growing region of Granma, Cuba.

Isolates	Key	Locality/Variety	Targeted detection[1]	Recurrence[2] (I cicle/II cicle)
F. oxysporum f. sp. *nicotina*e	FO-B	Bueycito/Habana 2000	+	+/+
F. oxysporum f. sp. *nicotinae*	FO-D	El Dorado/Habana 92	+	+/+
F. phyllophilum	FP-E	Monjará/Corojo 2012	+	+/+
F. phyllophilum	FP-F	Los Cayos/Habana 2000	+	+/-

[1]Specific detection for *F. oxysporum* y *F. phyllophilum* (Retana *et al*., 2018). [2]Recurrence in the field per cycle, (+) positive to the appearance of disease-associated symptoms under field conditions. (-) without apparent symptoms, monitoring performed in 2019 (I cycle) and 2020 (II cycle).

Effect of *F. oxysporum* f. sp. *nicotianae* and *F. phyllophylum* on seed germination and early seedling development of *N. tabacum* cv. Corojo 2012

Seed germination inhibition of tobacco cv. Corojo 2012 was observed with both *Fusarium* species, being *F. phyllophylum* FP-E the one that most affected this process (51.10 %). The other isolates showed values between 29.31 % and 33.11 %. The highest proliferation was recorded in isolates FP-E (5.25) and FP-F (3.25) compared to FON-B (2.25) and FON-D (2.75). The most significant (Tukey, p≤0.05) reduction in seedling mass and length was caused by isolates FP-E (56.31 %, 13.00 mm) and FON-B (51.58 %, 12.36 mm), while FP-F and FON-D reached lower values (table 3).

Table 3. Effect of *Fusarium* isolates on inhibition of germination, fresh mass and seedling length of *Nicotiana tabacum* cv. Corojo 2012.

Isolates[1]	Effect on seeds[2]		Fresh mass reduction[3]	Length reduction[4]
	G (%)	NC	(%)	(%)
FON-B	33.11^{b}	2.25^{b}	51.58^{a}	12.36^{a}
FON-D	31.02^{b}	2.75^{b}	33.34^{b}	10.56^{b}
FP-E	51.10^{a}	5.25^{a}	56.31^{a}	13.00^{a}
FP-F	29.31^{b}	3,25^{ab}	39.40^{b}	10.91^{b}
Control	0	0	0	0
CV *	5.70	6.96	11.10	8.75

[1]*Fusarium* isolates from the tobacco-growing region of Granma; *F. oxysporum* f. sp. *nicotinae* (FO-B), *F. oxysporum* f. sp. *nicotinae* (FO-D), *F. phyllophilum* (FP-E), *F. phyllophilum* (FP-F). [2]G = percentage of germination inhibition by the effect of the isolates of *Fusarium* spp. NC = Number of colonies (n=10). [3]Reduction of fresh mass due to the effect of the isolates of *Fusarium* spp. [4]Reduction of seedling length due to the effect of the isolates of *Fusarium* spp. CV: coefficient of variation. Different letters in the column show significant differences (Tukey, p≤0,05). Percentage values were transformed prior to ANOVA by $\sqrt{(x+1)}$.

Several studies documented the inhibitory effect of *Fusarium* on seed germination. Specifically, *F. oxysporum* f. sp. *vasinfectum* breed 4 (FOV4) reduces cotton germination by 5-20 % (Zhu *et al*., 2023), while *F. circinatum* decreases the viability of *Pinus greggii* seeds by 43 % (Garcia *et al*., 2017). In grasses, *F. graminearum*, *F. avenaceum*, *F. culmorum* and *F. poae* can inhibit germination by 65.5 % to 92.5 % (Browne and Cooke, 2005). This effect is associated with secondary metabolites, enzymes and toxins, as evidenced in *Orobanche ramosa*, where at least 18 toxins influenced germination inhibition at concentrations ≥10 µM (Zonno and Vurro, 2002).

The colony-forming ability may indicate the proliferation and possible pathogenicity of *Fusarium* on seed germination. Autochthonous isolates of *Fusarium* spp. from tobacco-growing areas in Granma, Cuba, showed high proliferation and infectivity at concentrations ≥1.10^{6} macroconidia.mL^{-1} (Sosa-Sánchez *et al*., 2022). In addition, *Fusarium* spp. can colonise the seminal embryo and prevent its germination (Pfenning *et al*., 2014).

This study confirmed that indigenous isolates of *F. oxysporum* f. sp. *nicotianae* and *F. phyllophilum* significantly reduce the fresh mass of seedlings of *N. tabacum* cv. Corojo 2012. Previous studies reported reductions of 86 % to 96 % in *Glycine max* and *Cicer arietinum* due to *Fusarium* spp. This decrease in growth can be attributed to damage to roots, stems and leaves, affecting water uptake, nutrients and photosynthesis (Zhang *et al*., 2021). Hu *et al*. (2023) reported that *F. oxysporum* f. sp. *fabae* caused vascular wilt in broad beans (*Vicia faba* L.), reducing growth by 6-25 %. Pastuszak *et al*. (2021) observed that *F. culmorum* infection of wheat (*Triticum* sp.) seedlings caused a 37 % loss in fresh mass of leaves and roots, as well as a reduction in chlorophyll a, b and carotenoid levels.

Severity of *F. oxysporum* f. sp. *nicotianae* and *F. phyllophylum* on *N. tabacum* cv. Corojo 2012

Root and leaf organs were more severely affected than the stem. Isolates FP-E and FON-B showed a higher disease severity on roots and leaves compared to FON-D and FP-F. In the stem, there were no significant differences between isolates. FP-E and FON-B also showed higher disease intensity and necrosis in plants compared to FON-D and FP-F, according to Tukey's test (p≤0.05) (table 4, figure 1).

Isolates FP-E (*F. phyllophilum*) and FO-B (*F. oxysporum* f. sp. *nicotinae*) showed higher virulence on the organs of *N. tabacum* cv. Corojo 2012, with clear symptoms of vascular wilting, chlorosis and necrosis in roots, stems and leaves 17 days after inoculation (dpi). These damages affect yield and marketable leaf quality, underlining the need for early diagnosis and management measures in the field (Qiu *et al*., 2023).

Results showed that inoculation of *Fusarium* by wounding the root system of seedlings was efficient in expressing disease symptoms. Retana *et al*. (2018) showed that *F. oxysporum* on *Apium graveolens* reached maximum disease expression at 35 days post inoculation (dpi). The pathogenicity of *Fusarium* spp. varies, with symptoms appearing as early as 5 days and reaching maximum expression at 17 dpi, causing up to 17 % plant mortality (Shen *et al*., 2023).

Espinoza-Ahumada *et al*. (2019) observed a severity of *Fusarium* spp. on melon cv. Top Mark with a damage gradology between 4 and 5 in seven days. Berruezo *et al*. (2021) noted severe *F. oxysporum* and *F. solani* damage on Virginia type tobacco in northwestern Argentina, attributable to pathogen virulence, inoculum concentration and variety tolerance. Carmona *et al*. (2020) recorded a maximum infection intensity of 94.5 % in tomato seedlings cv. Santa Cruz Kada at 30 days, slightly higher than the present investigation.

The highest averages of necrotic plants with no chance of survival were observed for *F. phyllophilum* (FP-E) and *F. oxysporum* f. sp. *nicotinae* (FO-B) at 12 days post inoculation (dpi). Khademi *et al*. (2020) found that both transgenic and natural seedlings of *N. tabacum* showed wilt, chlorosis and necrosis symptoms within seven days after inoculation with *F. solani* and *F. oxysporum*, with severe wilting and death in less than three weeks.

Table 4. Disease severity caused by *Fusarium oxysporum* f. sp. *nicotinae* and *Fusarium phyllophilum* on root, stem and leaf organs of *N. tabacum* cv. Corojo 2012.

Isolates	Root	Stem	Leaves	I[3]	Necrotic plants[4]
	DS[1] ± SD	DS ± SD[2]	DS ± SD	(%)	(%)
FON-B	4.17 ± 1.69 a	3.50 ± 1.72 b	4.31 ± 1.10 a	89.72 a	58.0 a
FON-D	3.40 ± 1.90 b	3.30 ± 1.83 b	3.70 ± 1.25 b	71.11 b	41.5 b
FP-E	4.26 ± 1.45 a	3.95 ± 1.45 b	4.40 ± 0.97 a	91.10 a	62.0 a
FP-F	3.80 ± 1.69 b	3.60 ± 1.90 b	3.80 ± 1.32 b	73.05 b	37.5 b
Control	0	0	0	0	0

[1]DS: damage scale, maximum value of 5. [2]SD: Standard deviation. [3]I. Disease intensity was determined using the Townsend and Heuberger formula. (1943), I (%) = [(∑ a x b) / N x K] x 100, where: a = number of plants or organs affected, b = scale grade, K = last scale grade used, N = total plants. Different letters in the columns indicate significant differences (Tukey, p≤0.05). [4]Percentage of necrotic seedlings with no chance of survival.

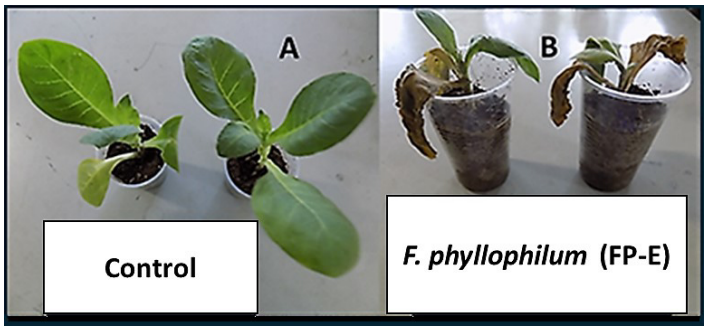

Figure 1. Severity of *Fusarium phyllophilum* (FP-E) on *Nicotiana tabacum* cv. Corojo 17 days after inoculation. A) control without inoculation, B) wilted and necrotic seedling with no chance of survival.

Conclusions

The vascular wilt disease caused by *F. oxysporum* f. sp. *nicotinae* and/or *F. phyllophilum* represents a limiting factor for tobacco production in Granma, Cuba. It was found a recurrence of 62 % with seedling necrosis and a severity of 4.20 in a scale of 5 grades, which affected the germination and seedling development variables. This has an impact on the yield and commercial quality of leaves, especially for the manufacture of Cuban cigars, underlining the importance of early diagnosis to establish management measures to prevent the spread of the pathogen in producing regions of Cuba and the world.

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