Basal rot in carnation (*Dianthus caryophyllus* L.) is caused by *Fusarium verticillioides* (Sacc.) Nirenberg

La pudrición basal en clavel (*Dianthus caryophyllus* L.) es causada por *Fusarium verticillioides* (Sacc.) Nirenberg

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ABSTRACT

The carnation is one of the most important products for export in the floriculture industry of Colombia. Fusariosis (a disease resulting from presence of *Fusarium*) appears on the crops in two forms: vascular wilt and basal rot. The first is caused by *Fusarium oxysporum* f. sp. *dianthi*, which is a well-characterized disease. The second, caused by *Fusarium verticillioides* (previously *Fusarium roseum*), is a non-characterized disease and its development in the plant has not been described in detail. The symptoms of basal rot were differentiated from vascular wilt, through infection of plants in the greenhouse, with isolates of *F. verticillioides* and *F. oxysporum* obtained from lesions of symptomatic plants. The fungi morphological characterization allowed differentiation of the isolates of these two species and their growth habits on different media. The sequencing of 8 different genes with more than 13 amplicons in the 2 species showed genetic differences that grouped the isolates into different taxa. Multilocus sequence typing analysis using DNA sequences of 8 different genetic regions confirmed the presence of *F. verticillioides*. In this study, the role of *F. verticillioides* was demonstrated in the stems of carnation in commercial crops that presented pathogenic lesions. According to the results of the study, *F. verticillioides* is the etiological agent that produces the basal rotting in carnation plants, alone or in association with *F. oxysporum*.

Key words: *Fusarium oxysporum, Fusarium roseum*, morphological characterization, vascular wilt.

RESUMEN

El clavel es uno de los productos más importantes para exportar en la industria floricultora de Colombia. La fusariosis (una enfermedad resultante de la presencia de *Fusarium*) aparece en el cultivo en dos formas: la marchitez vascular y la pudrición basal. La primera es causada por *F. oxysporum* f. sp. *dianthi* y es una enfermedad bien caracterizada. La segunda, causada por *Fusarium verticillioides* (anteriormente *Fusarium roseum*), es una enfermedad no caracterizada y su desarrollo en la planta no ha sido descrito en detalle. Los síntomas de la pudrición basal fueron diferenciados de los producidos por la marchitez vascular, a través de la infección de plantas en el invernadero, con aislados de *F. verticillioides* y *F. oxysporum* obtenidos de lesiones de plantas sintomáticas. La caracterización morfológica de los hongos permitió diferenciar las dos especies de *Fusarium* y se observaron sus hábitos de crecimiento en diferentes medios. Las secuencias de 8 diferentes genes con más de 13 diferentes amplicones en las 2 especies mostró diferencias genéticas que agruparon los aislados dentro de taxones diferentes. Análisis usando tipificación de secuencias múltiples de DNA confirmaron la presencia de *F. verticillioides*. En este estudio se estableció el papel de *F. verticillioides* presente en los tallos de clavel en cultivos comerciales que presentan lesiones patogénicas. De acuerdo con los resultados del estudio, *F. verticillioides* es el agente etiológico responsable de la pudrición basal en plantas de clavel solo o en asocio con *F. oxysporum*.

Palabras clave: *Fusarium oxysporum, Fusarium roseum*, caracterización morfológica, marchitez vascular.

Introduction

The global carnation market is projected to reach USD 3.266 billion by 2026, from USD 2.719 billion in 2020 (Global carnation sales market report, 2020). Fusariosis disease is a critical problem for carnation growers in Colombia as in most countries where carnations are produced. *Fusarium oxysporum* (FOX) f. sp. *dianthi* (Prill. and Delacr.) (Snyder & Hansen, 1940) has become the most significant pathogen in fusariosis disease in this crop (Arbeláez, 1987; Arbeláez, 1992; Borrero *et al.*, 2009). Large losses can occur due to the easy spread of the fungi across the infected cuttings, the fast dissemination by diverse means, the persistence of the pathogen in the soil, and the high cost of control

measures. All these causes confer special importance to this disease (Graces *et al.*, 2001).

Lopez and Fergus described the carnation basal rot for the first time in 1965. Since then, growers have related the disease to *F. roseum*. More recently, the species *F. roseum* has been replaced by other species of *Fusarium* (Burgess *et al.*, 1988). Arbeláez (1992) reported that both FOX and *F. roseum* could occur together in the same crop, with their masking symptoms making their identification difficult.

Fusarium verticillioides (Sacc.) (FVER) (Nirenberg, 1976) is an important phytopathogen in cereal crops that produces significant economic losses around the world. It attacks mainly corn, causing rotting in shoots, roots, and spikes (Bush *et al.*, 2004). Filgueira *et al.* (2007) first reported FVER presence on carnation stems with basal rot symptoms. The objective of our study was to identify the etiologic agent that produces basal rot on carnation and to describe the development of the disease in the plants.

Materials and methods

Biological samples

More than 1,500 plants with basal rot symptoms were used to isolate the fungus. These plants were collected between the 2012 and 2018 from 12 different carnation farms, located at the Bogotá plateau in the municipalities of Cajicá, Madrid, Siberia, Chía, Suesca, Sopó, Facatativá, and Tocancipá. All of them were in the Cundinamarca department of Colombia. The commercial carnation varieties sampled were: ´Nelson®', 'Tasman®', 'Delphi®', and 'Pink Nelson®', with basal rot symptoms as previously reported by Arbeláez (1987) and Filgueira *et al.* (2007).

Fungi isolation

All the stem fragments, sampled at the crown level of infected plants, were surface sterilized with 5% sodium hypochlorite for 1 min and then with 70% ethanol for 1 min and rinsed several times with sterile water. Once surface disinfection was completed, the stem was sectioned into discs of 2 mm thickness, which were then grown on Potato Dextrose Agar (PDA) (MERK) and Czapek Dox (OXOID) and incubated at 25°C for 7 d. Once the pathogen colony was visible, it was transferred to liquid Czapek Dox Broth Sigma with agitation for a week at 25ºC. Then, a single-spore culture was obtained from each isolation, three 1:10 serial dilutions of the spore suspension were made and 0.1 ml of the last dilution was taken and scattered on PDA. After incubation, a monosporic growth was selected and isolated on PDA. Finally, the fungi were conserved in

glycerol 50% at 4ºC. Colony characteristics such as color and appearance were observed in PDA cultures. The bright field in an Axioskop 2 Plus Carl Zeiss microscope was used to determine the presence, absence, and type of micro and macroconidia, the morphological characteristics of the mycelium, and possible formation of chlamydospores by the isolate. Asexual structures like macroconidia were measured using the Scion Image 4.0 free program.

Symptom characterization

On 53 carnation hybrid lines, the product of the crossing between varieties resistant and susceptible to FOX and one commercial variety 'Nelson®' hybrid lines and the commercial variety were established from seeds *in vitro* using MS basal media (Murashige & Skoog, 1962). One hundred microplants of each hybrid line and 'Nelson' variety were acclimated to outdoor conditions of the Bogotá plateau (maximum temperatures around 19 to 20°C [66 to 68°F] and minimum temperatures from 7 to 9°C [44.5 to 48°F]; the average annual relative humidity of 79%), with a vermiculite-like substrate. Four weeks later, plants of each hybrid line and commercial line were transplanted into pots. This evaluation used a quantitative measure of disease intensity with time (AUDPC) with analysis of variance by ANOVA in three repetitions that showed a normal distribution. The roots of each of the sixty plants were inoculated with 6 ml of a suspension of $1x10^6$ spores/ml (30 plants with FVER and 30 with FOX) using a syringe and injecting the fungus around the plant crown. Seven d after inoculation, the plants were re-inoculated with 200 μl of a suspension of $1x10^6$ spores/ml by puncturing next to the stem base (Filgueira, 2011). As a control, 20 plants of each hybrid line and the commercial variety were inoculated in the same way with distillate water. Finally, 20 plants were used as negative controls without inoculation.

Weekly for 4 months, the plants were observed and a detailed record of the appearance of symptoms was performed. The criterion used for the external symptom registration was yellowing and the discoloration or loss of green color was measured as presence/absence. The wilting of the foliage, stem, and buds was measured according to the following scale: absence 0, wilting of the stem base and cracking 1, wilting that affects the stem and leaves that can be observed in ¾ of plant 2, total wilting of the plant and death 3. Spots, such as stem discoloration, were measured as absence/presence. External necrosis, such as basal disintegration of the stem, was recorded as absence 0, moderate tissue damage 1, brown color in the stem base 2, and possible leaf necrosis, loss of turgor, stem decay, and dehydration 3. Finally, symptomatic plants were collected

at random, and the pathogen was isolated *in vitro* to corroborate the entrance of the pathogen to the plant. The incidence was estimated as the number of plants that are diseased, relative to the total number collected (Campbell & Neher, 1994).

In vitro evaluation of the plant response to *Fusarium* presence

In vitro evaluation consisted of an essay of "dual" cultures, in which undifferentiated cells were obtained from microplants leaf explants of 53 carnation hybrid lines, cultivated on MS basal media supplemented with 1 mg L^{-1} of 2,4-Dichlorophenoxyacetic acid (2,4-D) (Filgueira, 2011). One gram of *in vitro* cells was placed in a Petri dish (diameter 90 mm) border, with the same media as the callus induction, and cultured for a week at 26°C. Next, 10 μl of spore solution of the pathogen (FVER or FOX) at a concentration of 1x10^6 spores/ml was inoculated on a sterile circle of filter paper on a Petri dish located opposite to plant cells and then left for two weeks. Possible inhibitory (resistant) or not-inhibitory (susceptible) responses by plant cells towards pathogen growth were recorded measuring the growth of the fungi in the direction of the plant cells in centimeters (Filgueira, 2011).

PCR identification

For this purpose, four of the characterized isolates of both FOX and FVER that were placed by rapid growth in the media and did not present other biological contaminants were used. Fungal DNA was extracted, and PCR amplification was performed following the method described by Cenis (1992), with the modification by Abd-Elsalam *et al.* (2003). The fungal conidia were inoculated in 10 ml of liquid Czapek Dox Broth (Sigma®) for 72 h at 25°C; later, the fungal mycelia were centrifuged at 15000 g for 5 min. Next, the samples were washed with 2 ml of Tris Buffer (TE), (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and the mycelia were macerated in liquid nitrogen for 5 min. Three hundred μl of lysis buffer (200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added for 5 min, followed by the addition of sodium acetate 3 M (pH 5.2). The fungal samples were put at 20°C for 10 min and centrifuged at 15000 g for 5 min. The supernatant was transferred to a new tube and an equal volume of isopropanol was added and incubated at 4°C for 4 h. Finally, the suspension was centrifuged at 15000 g for 10 min. Ethanol 70% was added to the pellet to wash and then discarded. The DNA was suspended in 100 μl of TE.

The primers used to identify the fungi are summarized in Table 1. The PCR mixture contained 5 to 10 ng of template DNA, 1 μM of each primer, 3 mM of MgCl$_2$, 200 μM of dNTP, 2.5 U of Taq polymerase, and reaction buffer (50 mM KCl, 50 mM Tris-HCl; pH 8.3, 0.1 mg ml^{-1} BSA), in a final volume of 20 μl. Thirty PCR cycles with the following temperature regime were performed at 95°C for 2 min (only in the first circle), at 94°C for 1 min, at the annealing temperature for 0.5 min, and at 72°C for 1 min. The PCR products were analyzed by electrophoresis on 1% agarose gel stained with 0.5 μg ml^{-1} of ethidium bromide. As an external group, DNA of *Botrytis cinerea,* obtained under the same conditions, was used. The outgroup is important as a point of comparison for the in-group and allows the phylogeny to be rooted. Each amplicon was sequenced twice (forward and reverse) using the Sanger *et al.* (1977) method by Macrogen® (Korea). The DNA consensus sequence was compared with the GenBank database. Sequences that presented similar values higher

TABLE 1. Forward and reverse primers employed. Thirteen amplicons belonging to eight different genes or genetic regions were used to identify the fungi taxonomically.

Gene	Primer	Sequences	Sense	Size expected and size obtained	Author
β-Tubulin	Bt1α	5'TTCCCCCGTCTCCACTTCTTCATG3'	Forward	537bp 280bp	Glass and Donaldson, 1995
	Bt1b	5'GACGAGATGGTTCATGTTGAACTC3'	Reverse		
	Bt2a	5'GGTAACCAAATCGGTGCTGCTTTC3'	Forward	495pb 380bp	
	Bt2b	5'ACCCTCAGTGTAGTGACCCTTGGC3'	Reverse		
Histone 3	H31α	5'ACTAAGCAGACCGCCCGCAGG3'	Forward	447pb 540bp	
	H31β	5'GCGGGCGAGCTGGATGTCCTT3'	Reverse		
Calmodulin	Cmd-F	5´GATCAAGGAGGCCTTCTC3´	Forward	672pb 720bp	O'Donnell *et al.*, 2000
	Cmd-R	5´TTTTTGCATCATGAGTTGGAC3´	Reverse		
Elongation factor 1-α	EF1H	5'ATGGGTAAGGAAGACAAGAC3'	Forward	678pb 700bp	O'Donnell *et al.*, 1998
	EF2T	5'GGAAGTACCAGTGATCATTGTT3'	Reverse		

To be continued

Gene	Primer	Sequences	Sense	Size expected and size obtained	Author
Cytochrome C oxidase	AHyFuF	5'CTTAGTGGGCCAGGAGTTCAATA3'	Forward	568pb	Gilmore *et al.*, 2009
	AHyFuR	5'ACCTCAGGGTGTCCGAAGAAT3'	Reverse		
rDNA	ITS FuR	5' GCGACGATTACCAGTAACGA3'	Forward	416pb 398bp	White *et al.*, 1990
	ITS FuF	5'CAACTCCCAAACCCCTGTGA3'	Reverse		
	ITS1	5'TCCGTAGGTGAACCTGCGG3'	Forward	250pb 220bp	
	ITS2	5'GCTGCGTTCTTCATCGATGC3'	Reverse		
	ITS1	5'TCCGTAGGTGAACCTGCGG3'	Forward	570pb	
	ITS4	5'TCCTCCGCTTATTGATATGC3'	Reverse		
	ITS4	5' TCCTCCGCTTATTGATATGC3'	Forward	524bp 530pb	
	ITS5	5'GGAAGTAAAAGTCGTAACAAGG3'	Reverse		
Mitochondria small subunit	NMS1	5´CAGCAGTGAGGAATATTGGTCAATG3'	Forward	716pb 720bp	
	NMS2	5´GCGGATCATCGAATTAAATAACAT3'	Reverse		
Intergenic spacer rDNA	VERT1	5' GCGGGAATTCAAAAGTGGCC 3'	Forward	800bp	Patiño *et al.*, 2004
	VERT2	5' GAGGGCGCGAAACGGATCGG 3'	Reverse		
Intergenic spacer rDNA	VERTF1	5' GTCAGAATCCATGCCAGAACG 3'	Forward	400pb	
	VERTF2	5' CACCCGCAGCAATCCATCAG 3'	Reverse		

The DNA sequence data obtained in this study have been deposited in NCBI GenBank and the accession numbers are listed in https://www.ncbi.nlm.nih.gov/nuccore/?term=Fusarium%20filgueira.

than 97% with the morphologic species, using the BLAST tool, were used to generate a consensus using CLC DNA Workbench® software. The consensus sequences were compared by multiple alignments with CLUSTAL OMEGA in the MEGA7 software (Molecular Evolutionary Genetics Analysis), which was used to build the phylogenetic tree using the Neighbor-Joining distance method.

Processing of data

All biological materials in this study were randomly selected and the data were handled with descriptive statistics for qualitative dichotomous characteristics by presence or absence (P/A) of symptoms of necrosis, spots, curl, sporulation, stem chlorosis, turgidity loss, rachis, rot, and decay. The data were processed in the MINITAB Release 17 program using a model of logistic regression for the P/A of symptoms. The variance between assay groups with the ANOVA program was evaluated, and then the data adjustment in the model was tested. All the data were studied in time (d). Using the previous method, the tendency of the symptoms in time was observed, and finally, the Tukey's range test was applied.

The information for sequences of the different loci used in this study was processed using the Multi-Locus Sequence Analysis (MLST) tool of the CBS-KNAW Fungal Biodiversity Centre's Fusarium MLST website (https://fusarium.mycobank.org/) and the corresponding Fusarium-ID site hosted at the Pennsylvania State University

(http://isolate.fusariumdb.org). Dendrograms were generated by neighbour-joining and split decomposition with bootstrap analysis with 1,000 replications, using MEGAX.

Results

Symptoms in plants

In general, it is possible to distinguish fusariosis symptoms at the base of the plants in the crop field, *e.g.*, FVER produces a dry rot that ascends towards the medial part of the stem, forming caverns-like cracks with or without jumps. These lesions did not compromise the vascular bundle (Fig. 1A-C). In advanced stages, it was possible to detect a white to reddish cottony mycelium inside the lesion (Fig. 1G). On the other hand, the infection with FOX in the basal portion (crown) of the plant was characterized by a lesion in the form of a narrow ring limited to the vascular system that did not compromise the medullary tissue in the first stage of the infection (Fig. 1D-E). In some cases, it was possible to detect both fungi infecting the same plant (Fig. 1F). Other external symptoms of basal stem rot caused by FVER are: partial or complete chlorosis, generalized or partial wilting green, stem turgor loss, cracking in the crown, presence of conidiation structures, necrosis, and finally plant death (Fig. 1H).

The analysis of the fusariosis disease under field conditions showed that the Pink Nelson variety presented a higher FVER incidence of 7.1% (SD=1.2), followed by the Delphi

FIGURE 1. Symptoms of fusariosis in carnation plants. A-C) transversal and longitudinal cuts in the crown of carnation plants with the symptom of basal rot caused by FVER (C initial stage of invasion and B final stage); D-E) Transversal and longitudinal cuts in plants with symptoms of vascular wilt caused by FOX; F) Plant infected by both parasites with the symptom of basal rot (black arrow) and vascular wilt (red arrow); G-H) Carnation plants that present the final phase of the disease caused by FVER with cracking in the crown, the presence of fungal structures, and necrosis.

variety with 3.7% (SD=0.6) and the Nelson variety with 3% (SD=0.5) incidence. In the case of FVER, the maximum incidence observed was 10% (SD=1.6) as opposed to FOX, with a maximum incidence value of 68% (SD=5.7) in the farms.

Fungal isolation and preliminary characterization

The evaluation of 1,500 samples of monoconidial culture showed that, in 68.3% of the samples presenting symptoms of fusariosis, FOX was present, while in 8.2% of the samples, FVER was present, values close to that of the infected plants that were observed in the field. Ten percent of the samples presented both species simultaneously; 11.5% of the plants presented other *Fusarium* species (*F. graminearum, F. foetens, F. culmorum, F. avenaceum,* and *Fusarium* sp.), and, in 2% of the cases, it was not possible to identify

which of the parasites was present. Microscopically it was possible to identify septate mycelium in FVER isolates and production of long and slim macroconidia with thin walls, bent apical cells, and basal cells in the form of foot, with three to five septa. The morphological characterization of vegetative and reproductive structures of FOX and FVER is presented in Table 2. Macroconidia of FVER are shown in Figure 2A-B. Figure 2D shows observed simple branched FVER monophialides and abundant ovoid microconidia. The microconidia were only obtained on CLA (carnation leaf agar) media after 45 d of cultivation (Fig. 2B-C) and occurred separated from each other or in short and long chains (Fig. 2C). This last characteristic is important for distinguishing FVER from FOX. In this study, dark yellow *sporodochia* were observed in FVER *in vitro* media with more than 45 d of cultivation.

FIGURE 2. Isolation of FVER and FOX in nutrient media and microscopy recognition. A) Macroconidia of the FVER; B) FVER Macroconidia and microconidia. Bar is one μm; C) Production of FVER microconidia in a chain, 100X; D) FVER branched monophialide, 180X; E) Macroconidia, microconidia, and chlamydospores of FOX. Monophialides of FOX. A, C, D, and F, 1100X. B and E, 750X.

TABLE 2. Morphological characterization of FOX and FVER. The values are measurement averages. Range of the standard deviation in all the measures was between 0.001 and 0.15 μm.

		FOX	FVER
Macroconidia	**Form**	Straight or slightly curved	Long and thin
	Apical cell	Tapered and curved	Curved
	Foot cell	Definite	Definite
	Area (μm)	123	101
	Perimeter (μm)	779.5	911
	Major axis (μm)	290.5	363.5
	Minor axis (μm)	53.75	36
Microconidia	**Form**	Ellipsoidal, kidney-shaped	Oval
	Phialide	yes	no
	Area (μm)	0.11	0.07
	Perimeter (μm)	1.55	1.24
	Major axis (μm)	0.67	0.51
	Minor axis (μm)	0.22	0.17
Chlamydospores		yes	no

Tests of pathogenicity

In vitro interaction

Through the different tests, we study the processes of recognition and invasion of carnation plants by FVER. The first test was an *in vitro* assay, where the pathogen and undifferentiated cells of the plant can mutually recognize each other. In this case, it was possible to observe that the mycelia of the fungi grew mainly in the direction in which the plant cells were present (Fig. 3A, Fig. 4A). In the opposite case, where the cells present a resistant reaction to the presence of fungi, the fungi mycelia grew more slowly in the direction of the plant cells, as a type of growth inhibition reaction (Fig. 3B, Fig. 4A.). This evidences a host-parasite interaction between carnation cells and FVER. This behaviour was also observed in an *in vitro* assay with FOX (Fig. 3C-D., Fig. 4B).

To determine if the daily growth of the funguses in presence of resistant and susceptible carnation cells differs significantly with the "dual" culture in the Petri dish, variance analysis was done by d using ANOVA program with a *P*<0.05. The results show significant statistical differences between the fungi growth in the presence of resistant cells and susceptible cells (Fig. 4A-B).

Ex vitro interactions in the greenhouse

In the inoculation test of susceptible plants inoculated with FVER in germination trays, the first symptom of dry stem bark at the plant base was observed 15 d after the first
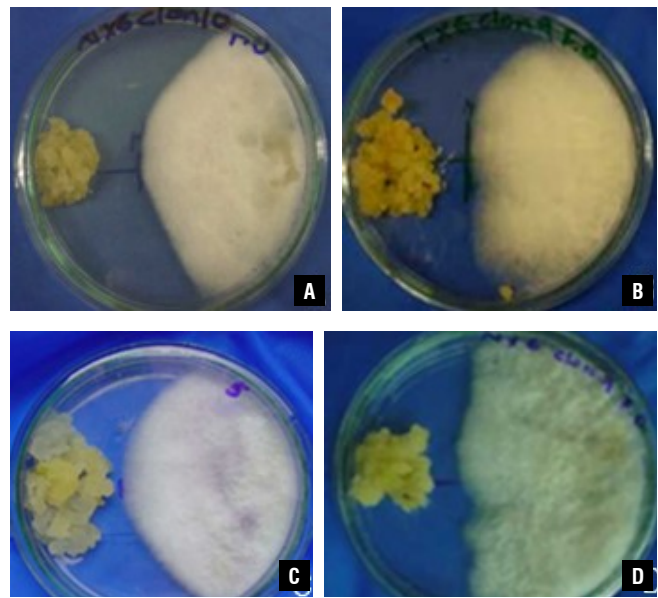
FIGURE 3. "Dual" culture assay *in vitro*. A) In the interaction between susceptible carnation cells and FVER, the fungus grew towards the carnation cells; B) Interaction between carnation-resistant cells and FVER indicating that the fungi stopped its growth by the presence of the resistant plant cells. An assay using FOX with carnation-susceptible cells C) and resistant cells D).

inoculation. This symptom became most prominent and ascended between 10 and 15 cm from the stem base by week 10 to 13. The Tukey´s test presented a value of 3.45 (α=0.05, K=3, df = 5) near week 7. It was possible to observe wilt with foliar yellowing on the leaves located near the plant base of the susceptible varieties. In this case, the Tukey's range test showed a value of 2.95 for this symptom by week 5. Additionally, internally in the plant base, a continuous or discontinuous pattern of rots at the same zone on the stem medulla was found, as shown in Figures 1A and C., which was near d 30 after the first inoculation. This symptom reached a value of 3.4 in the Tukey´s test near week 10. The rot spread on the stem base from the modular zone toward the vascular bundles and other stem tissues (Fig. 1B). The rots inside the stems sometimes appeared with red stains located in the modular zone, and wilting was evident from week 3. The Tukey´s test showed a value of 3.95 for this symptom after week 7.

Likewise, the spot symptom in the base stem indicated pathogen colonization, which was evident between weeks 3 and 4 after the inoculation. The Tukey´s test showed a value of 3.85 to 3.95 after week 10. The tissue necrosis symptoms were present posterior to the spot apparition, near week 4 (Fig. 1G). Plant behavior was similar in all the control groups. A comparative study with FOX (data not
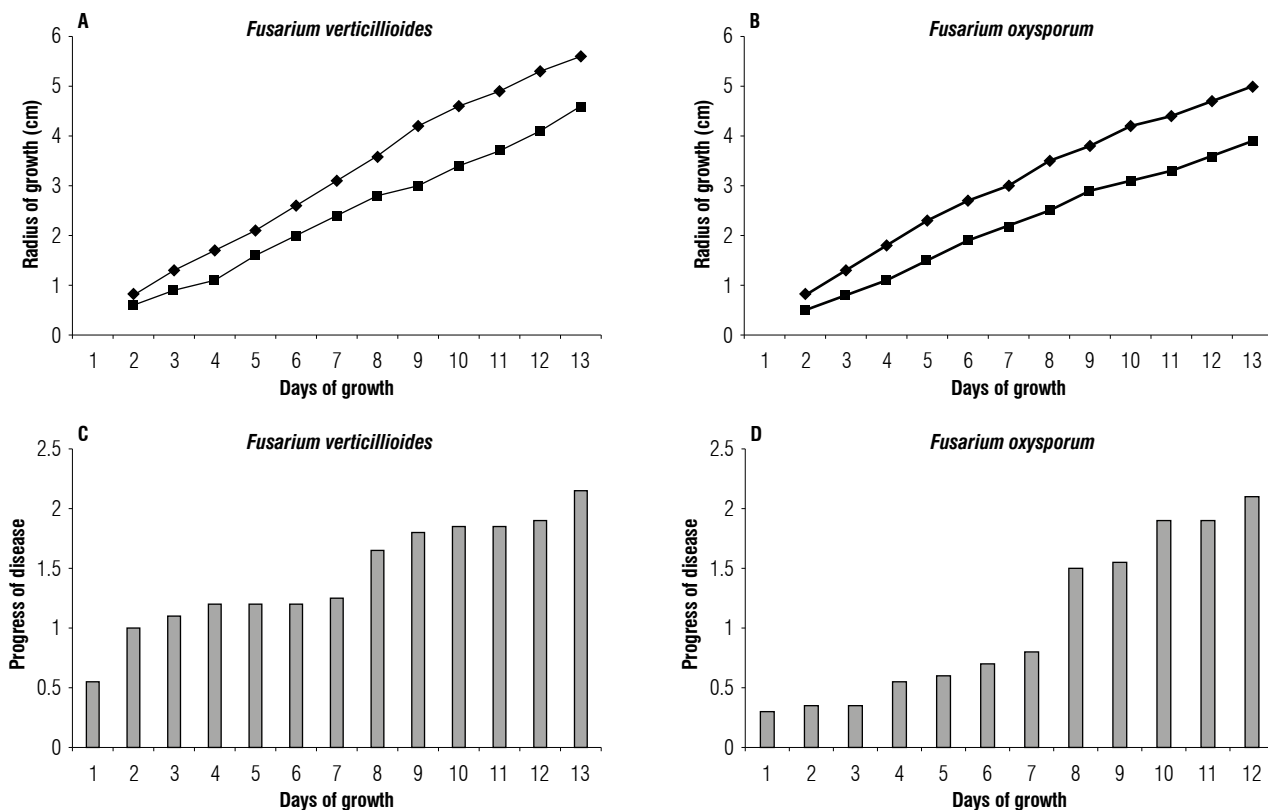
FIGURE 4. Fungi growth *in vitro* (A and B). Disease progress in the *ex vitro* test (C and D); A) FVER growth and B) FOX growth. The fungus growth is shown on *in vitro* assay ("dual" culture), in the carnation resistant cells interaction (■) with the susceptible cells (◆); C) Plants inoculated with FVER and D) plants inoculated with FOX. The plant wilting progress is recorded in days in the assay of plant inoculation in the greenhouse.

presented here) indicated a significant difference in the infection progress of the disease (Fig. 4C-D), such as the plant wilting progress over time. These data determine that FVER is more aggressive in the infection and colonization of the plant stem than FOX.

As mentioned above, we selected symptomatic plants randomly for each inoculation treatment and the re-isolation in vitro of the pathogen to confirm the presence or absence of the isolates, fulfilling Koch's postulates. Twenty plants of the susceptible commercial variety Nelson were re-inoculated with conidia of the pathogen obtained from infected plants from the first inoculation experiment. All these plants presented severe symptoms of basal rot with foliar yellowing, vascular chlorosis, medullar rot, and stem cupping. Fragments of this infected tissue were used to isolate the parasite and determine the presence of the fungus that was used to inoculate the initial plants like FVER.

Molecular identification

An adequate quantity of genetic regions was employed to appropriately identify the two species because of the

problems in using morphological characters in the identification of FOX and FVER. First, we used the ITS region for the identification of the genus to resolve the limitation that this genetic region presents for use as barcoding. Next, we used a group of primers to determine with the greater accuracy of the fungal species, especially when the study implicates phylogenetic analysis (Tab. 1). In this sense, the use of MLST is the best option to identify and obtain phylogenetic relationships between species of difficult identification (Matsumura, 2013).

Ten samples of each of the morphological species FVER and FOX were selected. To prevent slants or false positives, a preliminary molecular characterization by PCR using ITSs and EFs 1-α (Tab. 1) was performed to determine if the isolates previously classified as FOX or FVER belong to the *Fusarium* genus. Figure 5A-E shows the amplicons of the primers ITSFuF/ITSFuR, ITS1/ITS2, ITS1/ITS4, ITS4/ITS5, and EF-1H/EF-2T. The accession number (ID) to view the nucleotide sequence in the GenBank of NCBI of these amplicons is listed in Table 3. In most cases, such as amplicons ITS1/ITS2 and Elongation Factor 1-α, the

TABLE 3. GenBank (NCBI) IDs and comparisons with related sequences. The DNA sequences of FVER and FOX obtained in this study were compared using the BLAST tool, and the results show the identity percentage and the E value of the most related sequences.

Locus	Gene	FVER*	FOX*	Related specie	Percentage Identity	E value
β-Tubulin	Bt1	KF467337.1	KF467331	*F. verticillioides*	100	0.0
β-Tubulin	Bt2	KF467337	JF773354	*F. verticillioides*	97	0.0
Histone 3	H3-1	KF467389	KF467383	*F. verticillioides*	100	0.0
Calmodulin	Cmd	KF467350	KF467344	*G. moniliformis*	99	0.0
Elongation factor 1-α	EF	KF467376	KF467370	*F. verticillioides*	100	0.0
Cytochrome C oxidase	AHyFu	KF467363	KF467357	*G. moniliformis*	99	0.0
rDNA	ITS Fu	KF467402	KF467396	*F. verticillioides*	100	0.0
rDNA	ITS1-2	KF467415	KF467409	*F. verticillioides*	100	0.0
rDNA	ITS1-4	KF467428	KF467422	*F. verticillioides*	100	0.0
rDNA	ITS4-5	KF467441	KF467435	*F. verticillioides*	100	0.0
Mitochondria small subunit	NMS	KF467454	KF467448	*F. verticillioides*	100	0.0
Intergenic spacer rDNA	VERT	submitting	submitting	*G. moniliformis*	97	0.0
Intergenic spacer rDNA	VERTF	submitting	submitting	*G. moniliformis*	95	1 e-121

identity of these sequences with different sequences of *F. verticillioides* (*e.g.*, ID XR_001989346.1) in GenBank was 100% and the e-value 0.0.

Other genes used in this study permitted a more adjusted taxonomic classification. The accession numbers of Gen-Bank (NCBI) of the sequences obtained in this study with the different genes used are listed in Table 3. In the same way, Table 3 lists the identity percentages and the E values produced by the comparison between the sequences obtained in this study with the sequences of the closest taxonomic species, using the BLAST tool of NCBI.

Using the sequences obtained with the different amplicons, a MultiLocus Sequence-Typing (MLST) schema was built. The MLST schema used an agglomerative clustering of a complete linkage of eight different genes of FVER (EF1, Cmd, Bt1, VERT, VERTF, ITS1-2, ITS Fu, and NMS). The eight genes of FVERT were compared with thirty-one conserved housekeeping genes to derive a combination of alleles known as a sequence type (ST), using the *Fusarium*-ID site hosted at the Pennsylvania State University (Fig. 5). To obtain the MLST, we used the most representative FVER sequences, which obtained the highest percentage of identity and the lowest E value in the sequence alignment using the BLAST tool, as seen in Table 3. The MLST schema shows that the isolate that previously identified *F. verticillioides* is clustered with similar types like *Fusarium verticillioides* (*Fusarium fujikuroi* species complex), excluding *Fusarium oxysporum* (*Fusarium oxysporum* species complex) that are grouped in a different phylogenetic group (Fig. 5).

Discussion

Symptomatic and morphological recognition

This study showed that FVER is a pathogen of the carnation, which can lead to a confused diagnosis with carnation plants infected with FOX. Here, we demonstrated that FVER attacks carnation plants, producing some symptoms like those of carnation plants attacked by other *Fusarium* species. The morphological description of the species FVER agreed with those of Leslie and Summerell (2006) and other authors (Deepa *et al.*, 2018), with the presence of typical macroconidia, microconidia in a chain, and absence of chlamydospores.

In vitro tests showed resistance to FVER from cells of different hybrids and commercial varieties. This demonstrates that the plant can recognize FVER and elaborate a response to it. The pathogenicity test, the *in vitro* "dual" test is based on the chemical response, in which recognition of the pathogen activates the metabolism of defense in the plant cell wall (Bigeard *et al.*, 2015). In this assay, the undifferentiated cells of the hybrid lines, which have some type of resistance towards the pathogen, induced smaller displacements on the fungus on the nutrient media, supporting the hypothesis that antifungal molecules are produced by callus into the media (Buiatti *et al.*, 1987). This type of response has been already reported, characterizing lines of cells of tomato and evaluating them *in vitro* responses against FOX. In this case, a correlation between phytoalexin production and inhibition of fungal growth was reported (Storti *et al.*, 1989). In our case, in some "dual" tests, changes were
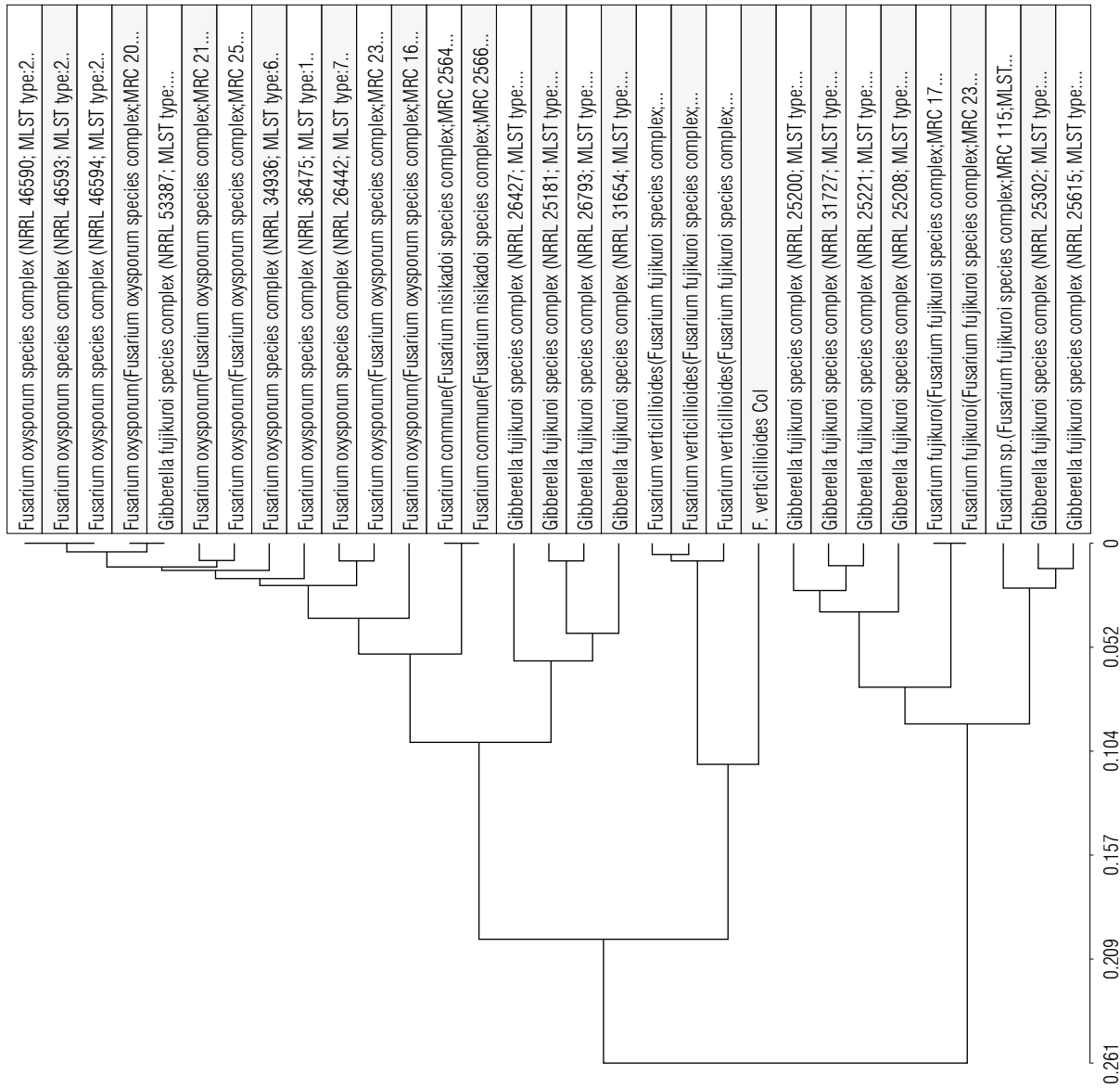
FIGURE 5. MLST scheme. FVER (*F. verticillioides* col) phylogeny inferred from an *EF1, Cmd, Bt1, VERT, VERTF, ITS1-2, ITS Fu,* and *NMS* dataset genes, in which six species complexes were resolved. The approximate number of phylogenetically distinct species within each species complex is based on an agglomerative clustering of complete linkage analysis.

presented in the form of the fungal growth front line. This clearly shows a response by the callus, which does not allow the fungi to continue its normal advance. Filgueira and Guardiola (1999) also reported this change in the growth direction of the fungus as a response to the resistance of the carnation callus to the pathogen presence. Several reports have shown similar results in their studies, such as the measured severity and disease incidence. They show correlations between the *in vitro* assays and those observed in the field (González *et al.*, 2004).

In the carnation, FVER produces basal rot, which is characterized as dry rot in the base of the stem, as previously observed in maize by Leyva-Madrigal *et al.* (2015). Sometimes the rot can be presented in phases as Filgueira reported in 2007 in a study of the carnation, and when the infection is advanced, cottony mycelium can be seen in the cave produced by the medullar rot. Also, posterior expansion of the infection is possible toward the vascular bundles and other stem tissues, producing stem rot disease. Externally, the stem in the base presented chlorosis, cortical necrosis, dry rot, and yellowing foliage.

Symptoms of the disease caused by FVER expand in the plant from bottom to top, similar to wilting caused by FOX. These two pathologies can be differentiated doing cross-section cuts to determine if necrosis is present in the medullary zone of the vessel bundles. In addition, it should be kept in mind that FVER does not produce an asymmetric yellowish color on the leaves. On the contrary, the leaves lose hydration, and the wilting shows a strong pink color in the stem. The FVER sporulation on the outer side of the stem is remarkably similar and consistent with rot caused by FVER in corn, where the rot is accompanied by sporulation (Rossi *et al.*, 2008).

In carnation plants, rot in the medullar zone can also be observed when attacked by species of *Fusarium* other than FOX, such as FVER, as reported by Filgueira *et al.* (2007). This suggests that the presence or proportion of these pathogenic species depends on the individual incidence of each pathogen. The greatest incidence of double infections was present with FOX and FVER together. These species colonizing the same plant evidenced low or non-existent ecological competition among the species. This result evidences a possible synergy between some of the species and stem colonization. Additionally, the presence of *Fusarium* in symptomatic plants was found in some carnation varieties.

Molecular identification

In all the cases analyzed using ITS primer, all the corresponding to the *Fusarium* genus were demonstrated in other cases by Glass and Donaldson (1995) and Tan and Niessen (2003). Furthermore, the amplicon obtained with the primers ITS 1/2 consistently showed that the species most related with the analyzed sequences was FVER. In general, the obtained size of the amplicons of this study did not differ significantly from the expected size, except for the case of β-tubulin which showed an appreciable difference (Tab. 1); the difference was not a problem when using the sequence as a tool to identify the related species in the GenBank. Most of the amplicons obtained with the different primers of the genes used in this study were identified as the species most related to FVER. This is the case of the β-tubulin, the Bt1a/Bt1b and Bt2a/Bt2b amplicons, the cytochrome C amplicon, histone-3, calmodulin, and mitochondrion small subunit amplicon, which presented identities above 97% and e-values in all cases near zero.

The product obtained by the primers VERT1/VERT2 and VERTF2/VERTF specific to identify FVER produced bands of the expected sizes of 800 and 400 bp, respectively, as reported by Patiño *et al.* (2004) in the isolates of FVER from the symptomatic flowers of farms. This is indicative of the species with Fumonisin gene presence. In addition, Fumonisin is a mycotoxin produced by some *Fusarium* species like FVER (Scott, 2012; Rosa Junior *et al.*, 2019).

On the other hand, the phylogeny analysis with the MLST schema using the genes of FVER *EF1*, *Cmd*, *Bt1*, *VERT*, *VERTF*, *ITS1-2*, *ITS Fu*, and *NMS* produced a tree with a clear aggregation of FOX isolates in a separate group of FVER species and with an important genetic distance (Fig. 5). All these results evidence that 1) FVER is present in carnation growing fields. This is notable because FVER is an important parasite of different crops of economic importance like cereals; 2) FVER has a type host-pathogen relationship with the carnation that had not been studied until now and produces notable symptoms in the plant leading to the die; 3) the disease known as *F. roseum* by carnation growers is a basal rot caused by FVER; 4) FVER and FOX can act synergistically to produce an infection of the plant that presents symptoms of both disease, vascular wilt, and basal rot; and 5) FVER is producing an emergent disease that could be related to climate change and must be acted upon.

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Conflict of interest statement

The authors declare that there is no conflict of interest regarding the publication of this article.

Author's contributions

All the authors of this article contributed in many forms to the final document. JJFD is the leader of the scientific group and compiled and wrote the manuscript. CMRS worked on the molecular identification of the species. CYQ discovered the parasite *F. verticillioides* in the carnation crop. JCS did the isolation and preliminary characterization of the fungi. IEM collected the samples on the farms and studied the incidence in the crop. JJFD formulated the overarching research goal and aims. CYQ and IEM applied statistical methods. JJFD obtained financial support. JJFD conducted the research and investigation process. JJFD and CMRS developed and designed the methodology. JJFD verified the overall reproducibility of results and other research outputs. JJFD, CMRS, CGQ and IEM prepared and presented the published work and oversaw its presentation. JJFD wrote and translated the initial draft. JJF carried out the critical review of the manuscript. All authors reviewed the manuscript.

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