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Fusarium oxysporum as the causal agent of vascular wilt in *Ruta graveolens L.* in Colombia

Fusarium oxysporum como agente causal del marchitamiento vascular en *Ruta graveolens* L. en Colombia

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ABSTRACT

The increasing demand for rue (*Ruta graveolens* L.), widely used for medicinal and aromatic purposes, has fostered the social development of farmers in the Village of San Raimundo Alto, in Granada, Cundinamarca, where small producers cultivate this plant. However, vascular wilt is a major phytosanitary problem, causing plant death, reduced production, and economic losses. The causal agent of this disease had not been previously identified in Colombia. This study aimed to identify the pathogen responsible for vascular wilt in rue. Symptomatic plants were collected from the Village of San Raimundo Alto and analyzed in the phytopathology laboratory of Universidad de Cundinamarca. For the isolation, twenty-four rue plants of two different ages (45 and 90 days after sowing) were processed. Morphological identification of the isolated fungi was performed considering macroscopic and microscopic characteristics. The *Fusarium*

genus was found in all isolates, and it was verified by the fulfillment of Koch's postulates that the fungus that causes the vascular wilt of rue plants was *Fusarium* sp. For molecular analysis of pathogenic isolates, DNA extraction was performed, and primers of internal transcribed spacers—ITS (ITS4 and ITS5) and elongation factor—TEF (EF1 and EF2) were used for polymerase chain reaction (PCR). The sequences obtained were compared with those deposited at the GenBank database (NCBI), identifying all isolates analyzed as *Fusarium oxysporum*, with identity percentages greater than 98%. To our knowledge, this is the first report in Colombia of this pathogen causing vascular wilting in rue.

Keywords: Aromatic plants; fungal pathogens; molecular identification; pathogenicity test; plant diseases; wilting.

RESUMEN

La creciente demanda de ruda (Ruta graveolens L.), ampliamente utilizada con fines medicinales y aromáticos, ha fomentado el desarrollo social de los agricultores de la Vereda San Raimundo Alto, en Granada, Cundinamarca, donde pequeños productores cultivan esta planta. Sin embargo, la marchitez vascular es un problema fitosanitario importante, que causa la muerte de las plantas, la reducción de la producción y pérdidas económicas. El agente causal de esta enfermedad no había sido identificado previamente en Colombia. Este estudio tuvo como objetivo identificar el patógeno responsable de la marchitez vascular en ruda. Se recolectaron plantas sintomáticas en la vereda San Raimundo Alto y se analizaron en el laboratorio de fitopatología de la Universidad de Cundinamarca. Para el aislamiento se procesaron veinticuatro plantas de ruda de dos edades diferentes (45 y 90 días después de la siembra). La identificación morfológica de los hongos aislados se realizó teniendo en cuenta características macroscópicas y microscópicas. En todos los aislamientos se encontró el género Fusarium, y se comprobó por el cumplimiento de los postulados de Koch que el hongo causante de la marchitez vascular de las plantas de ruda era Fusarium sp. Para el análisis molecular de los aislamientos patógenos se realizó la extracción de ADN, y se utilizaron los cebadores de los espaciadores transcritos internos-ITS (ITS4 e ITS5) y del factor de elongación-TEF (EF1 y EF2) para la reacción en cadena de la polimerasa (PCR). Las secuencias obtenidas se compararon con las depositadas en la base de datos GenBank (NCBI), identificándose todos los aislados analizados como Fusarium oxysporum, con porcentajes de identidad superiores al 98%. Hasta donde sabemos, este es el primer reporte en Colombia de este patógeno causante de marchitez vascular en ruda.

Palabras clave: Enfermedades de plantas; identificación molecular; marchitez; patógenos fúngicos, plantas aromáticas; pruebas de patogenicidad.

INTRODUCTION

Medicinal and aromatic plants are of economic and cultural importance, being close to 400 species, and their cultivation is mainly carried out by family workers (Ministerio de Agricultura y Desarrollo Rural, 2019). Rue (*R. graveolens* L.) is considered an aromatic herb of great utility for the pharmaceutical industry with the potential to combat infectious diseases of bacterial, fungal, and viral origin. Likewise, these plants have been shown to have anti-inflammatory and antioxidant properties (Pabón *et al*., 2017; Donadu *et al*., 2021; Ebrahimi *et al*., 2021; Mokhtar *et al*., 2022; Szewczyk *et al*., 2022; Dwivedi & Verma, 2024). Rue is also traditionally used to relieve joint pain, arthritis, and gout (Pa Badhusha *et al*., 2020).

According to Agronet (2021), the production of rue in the department of Cundinamarca was 144 tons. This crop is important for small producers in Granada, as it is easily marketed, has low production costs, and has a high profit margin, generating constant income. In Colombia, there are few reports of pathogens affecting rue, as is the case of ash (*Oidium* sp.), leaf blight (*Phoma* sp.), and scorch (*Cladosporium* sp.) (Alarcón, 2011). However, rue crops in this municipality are affected by vascular wilting, the causal agent of which is unknown, causing the death of the plants and generating up to 50% of losses in production (data provided by producers). Because there are no reports of pathogens causing the symptoms mentioned in this plant species in Colombia, this work aimed to identify the causal agent of vascular wilting in rue plants in Granada, Cundinamarca.

MATERIAL AND METHODS

Plant material. Samples of rue were taken on farms producing rue in the village of San Raimundo-Alto in the municipality of Granada, Cundinamarca. The village is 2,270 meters above sea level (masl), relative humidity is 70%, average annual rainfall is 1700 mm, and temperature ranges from 18 to 22°C.

Twenty-four rue plants (*R. graveolens* L.) were collected in the vegetative phase (45 and 90 days after sowing), showing visible symptoms of vascular wilt: yellowing of basal leaves, which lose turgor and dry but remain attached to the plant. This symptomatology advances upwards, causing a general wilt and death of the plant. Samples were wrapped in Kraft paper and transported in a fridge to the Phytopathology laboratory of the Universidad de Cundinamarca for processing.

Isolation. Four disease degrees were described based on visual characteristics present in the collected plants. Six plants in each degree were processed (three 45-day-old plants and three 90-day-old plants). For disinfection, the methodology of Hernández-Amasifuen *et al*. (2019) was followed with modifications: explants of the stem of 1 cm^2 were cut, disinfected superficially with 1% sodium hypochlorite for 30 seconds, 70% ethanol for 30 seconds, and three rinses with sterile distilled water for 30 seconds. Explants were cultivated in Sabouraud Oxoid agar supplemented with chloramphenicol (0.05 $g.L^{-1}$) and incubated at 25 °C for five days. Subsequently, we made monosporic cultures by preparing a spore suspension of 1 $x10^3$ $conidia.mL^{-1}$, which were plated on Sabouraud agar, and 24 hours later the individual colonies were transferred to a new culture medium to perform the pathogenicity tests (Fan *et al.*, 2024), and carnation leaves were sounded in agar to make observations under a microscope (Gibert *et al*., 2022).

Morphological identification. The observation and measurement of fungal structures was carried out with a microscope (Axiostar® dm 500), using the objectives 40X and

100X. The photographs were taken using the software program Motic® Images Plus 3.0 and the type of conidia and size of conidia were measured in micrometers (µm) Data were compared with the taxonomic key for imperfect fungi developed by Barnett and Hunter (1998). Also, the macroscopic characteristics were described (colony size, color, texture, presence, or absence of sclerotia, and mycelial diameter in PDA culture medium Oxoid agar) (Tilocca *et al*., 2020; Leslie & Summerell, 2006).

Molecular analysis. Molecular analysis was performed for six isolates of *Fusarium* sp. DNA extraction was carried out using the Quick-DNA kit Fungal/Bacterial Miniprep Kit (Zymo Research), and DNA from each isolate was quantified by spectrophotometry with a Nanodrop™ 2000 (Thermo ScientificTM). DNA-specific regions were amplified by polymerase chain reaction (PCR) using internal transcribed spacer primers (ITS) ITS 4 (5 - GGA AGT AAA AGT AAC G -3') and ITS 5 (5 - TCC TCC GCT TAT TGA GC -3) (White *et al*., 1990) and elongation factor (TEF) EF1 (5 - ATGGGTAAGGAAGACAAGACGAC - 3) and EF2 (5 - GGAAGTACCAGTGATCATGTT - 3) (O'Donnell *et al*., 1998).

The amplification mixture for each sample contained 1X Buffer, 2.5 mM $MgCl_2$, 0.2 µM of each primer, 1 unit of Taq polymerase, 0.2 mM dNTPs, and 2 µL DNA. Reactions were run under the following amplification conditions: 1 cycle of 94°C for 3 minutes, 35 cycles of 94°C/30 seconds, 52°C/30 seconds, and 72°C/30 seconds, and a final extension of 72°C for 7 minutes.

Amplified fragments were run by 1.5% agarose electrophoresis and checked using imaging gel documentation. PCR products were purified and sequenced in an ABI 3500 sequencer through the Sanger technique. Sequences were edited by BioEdit 7.2 software, and they were compared with the GenBank databases of the National Center for Biotechnology Information (NCBI) using the BLAST tool.

Pathogenicity tests. We adjusted concentrations of $1x10^6$ conidia.mL-1 in the Neubauer, and healthy rue plants in the vegetative stage were inoculated. Two inoculation methodologies were used: 1) root immersion for ten minutes Luo and Yu (2020) and 2) application of inoculum to sterilized substrate (soil with rice husk 2:1) (Zhu *et al*., 2020).

Three plants were inoculated with one isolate from each of the seven groups, and three untreated plants were left as absolute control. Each inoculation methodology was to plants aged 45 and 90 days, for a total of 48 plants per trial. The plants were kept in a screen house with an average temperature of 17°C and relative humidity of 80% in the village of San Raimundo Alto.

When plants showed obvious wilt symptoms at the end of the incidence and severity evaluation, they were taken to the laboratory for re-isolation.

Assessment of Incidence and Severity. Seven isolates of the genus *Fusarium* were evaluated under screen house conditions (average temperature 17°C and average relative humidity 80%). We conducted two trials as described in the previous section. Plants were arranged in a completely random design, and each treatment (isolate for a group) had three replicates. The percentage of incidence was determined considering the formulas established by Nofal *et al*. (2021): Incidence (%) = (number of diseased plants/total of plants observed) * 100. For severity percentage, the formula of El-Sersawy *et al*. (2021) was used: Severity (%) = (number of plants x scale degree/number of plants evaluated x scale number greater) * 100.

A percentage damage scale was designed for this work (Table 1).

Table 1. Severity scale in plants with symptoms of vascular wilting.

Degree	Percentage of wilted tissue (%)
0	0
1	1 – 25
2	26 – 50
3	51 – 75
4	76- 100

Statistical analysis. We evaluated incidence employing contingency tables and severity using an analysis of variance and a Tukey test using the free version of the Infostat statistical software.

RESULTS AND DISCUSSION

Description of symptoms observed in the field. The symptoms observed in rue crops at ''San Raimundo Alto'' village started with chlorosis in bottom leaves, loss of turgor, decrease in the growth rate, and wilt of the plant with the advance of the disease. Stems began a process of necrosis, which ended with the death of the plant (Figure 1). Many authors have described these symptoms in plants affected by vascular pathogens of the genera *Fusarium* (Srinivas *et al*., 2019; Rahman *et al*., 2021) and others like *Verticillium* sp. (Wang *et al*., 2021; Zhang *et al*., 2022).

Figure 1. Progress of symptoms observed in rue crops. Stage 0: no damage; stage 1: plant with wilting between 1 and 25%; stage 2: plant with wilting between 26 and 50%; stage 3: plant with wilting between 51 to 75%; stage 4: plant with wilting between 76 to 100%.

Macro characteristics and microscopes. Based on their macroscopic characteristics, we grouped 24 isolates obtained into 7 groups (Table 2). The isolates obtained presented a rapid growth rate (9 cm in diameter at 6 days of incubation) on Saboraud agar. Cottony air mycelium, beige, light ochre, light purple or light pink, and without the presence of sclerotia (Figure 2). At a microscopic level, all 24 isolates were observed as straight to slightly curved macroconidia with 2 to 3 septates ranging in size between 23-28 µm long and 3-4 µm wide, slightly falcated. Unicellular microconidia with an average length between 10-13 µm and 2-3 µm wide. Terminal, or intercalary, chlamydospores averaging 6 to 14 µm long by 3-4 µm diameter width and septate hyphae were also observed (figure 3). According to the macroscopic and microscopic characteristics, isolates correspond to *Fusarium* sp. (Ortega & Uribe, 2023; Hami *et al*., 2021; Meena & Roy, 2020).

Table 2. Macroscopic characteristics of seven groups of *Fusarium* sp.

Group	Growth rate	Color	Aerial mycelium
1	Rapid	Reverse fuchsia, front light purple	Cottony
2	Rapid	Reverse light purple, front light purple	Cottony
3	Rapid	Reverse Dark violet, front light purple	Cottony
4	Rapid	Reverse light yellow, front light ochre	Cottony
5	Rapid	Reverse Light orange, front beige	Cottony
6	Rapid	Reverse orange, front beige	Cottony
7	Rapid	Reverse beige, front light pink	Cottony

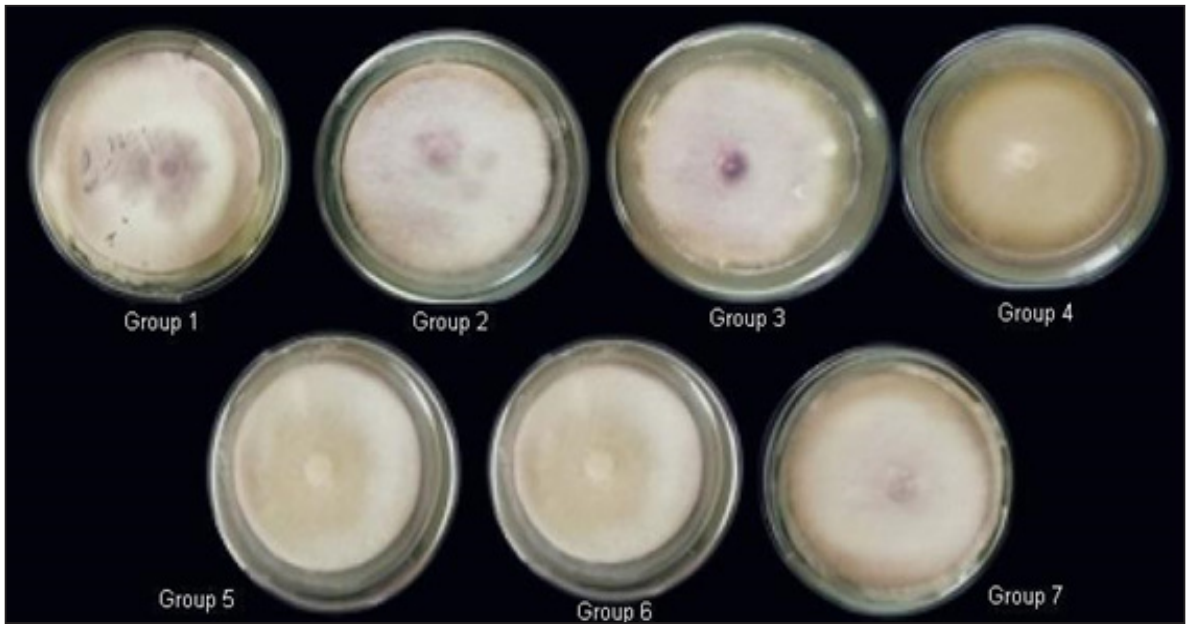

Figure 2. Macroscopic characteristics of seven isolates of *Fusarium* sp. All isolates had cottony aerial mycelium, some of which were front color: light purple (isolates 1, 2, and 3), light ochre (isolate 4), beige (isolates 5 and 6), and light pink (isolate 7).

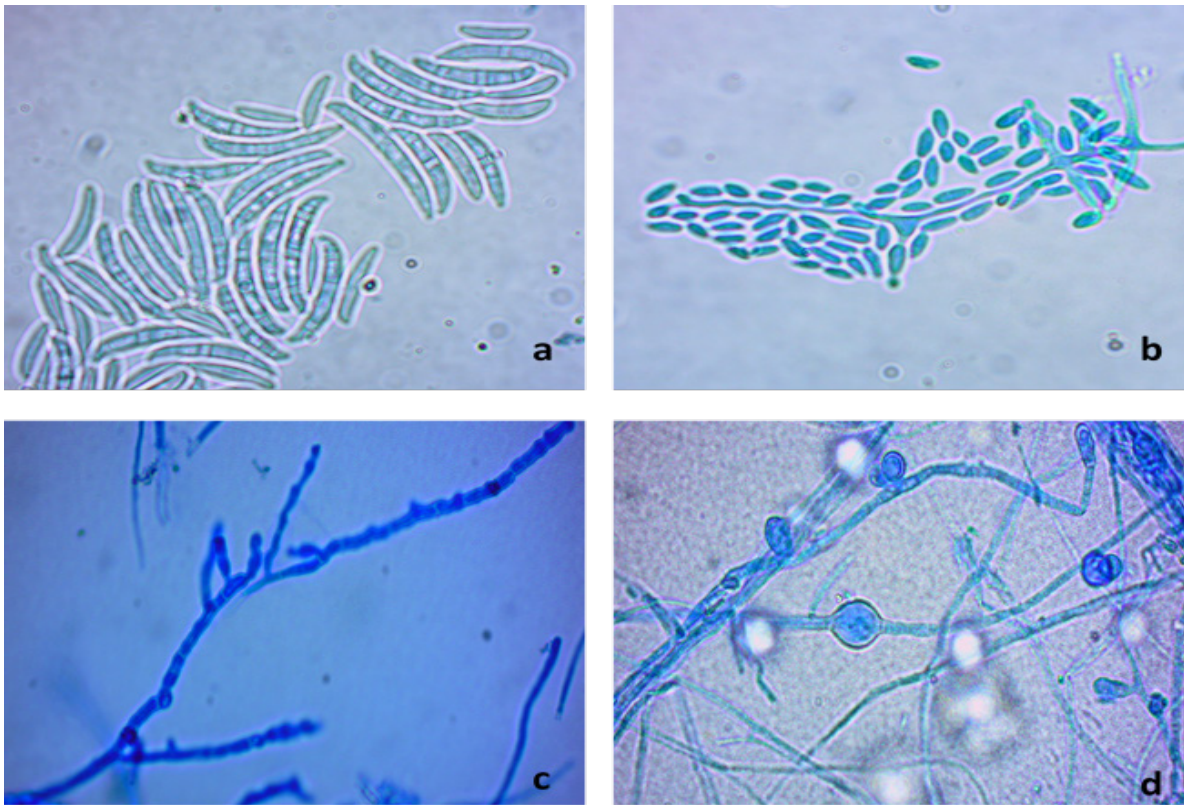

Figure 3. Microscopic structures from *Fusarium* sp. isolates: a) Macroconidia, b) Microconidia, c) Individual phialides, and d) Chlamydospores. Microscopic observations were made from 7-day-old colonies. Photographs taken with the 100X objective

Molecular identification. The sequences obtained from six isolates (two to seven groups) of *Fusarium* sp. were compared with the NCBI database, finding that these presented identity percentages greater than 98% with *Fusarium oxysporum* with both regions amplified. The coverage percentages were 100%, and the e-value was 0.0. (table 3). In rue, there are only two reports of plants with symptoms of vascular wilting, the first by Rathnamma *et al*. (1999) in Egypt, where the causal agent was identified as *Fusarium solani,* but they did not make a molecular identification but relied on the macroscopic and microscopic characteristics of the fungal isolates, and the second by Helmy *et al*. (2001) in India, where *Fusarium moniliforme, F. solani,* and *F. oxysporum, Rhizoctonia solani*, and *Pythium debaryanum,* among others, were identified. To date, there are no reports of vascular wilt in rue for Colombia or other countries in the Americas.

Table 3. Comparison of the sequences obtained with those deposited in the NCBI database.

Isolate	Amplified region	Species matched in the NCBI database	Accessions	Percentage of identity	Percentage of covering	e- value
2	TEF	*Fusarium oxysporum* isolates PG337, PG467, M9	MT630341.1 MT630343.1 KP964865.1	99,86%	100%	0.0
	ITS	*Fusarium oxysporum* clone 2014_1545	MN523129.1			
		Fusarium oxysporum f. sp. *cubense* isolate Chethali - Palakkad PKD 3	MN663157.1	99,81%	100%	0.0
3	TEF	*Fusarium oxysporum* isolates PG337, PG467, M9	MT630341.1 MT630343.1 KP964865.1	99,86%	100%	0.0
	ITS	*Fusarium oxysporum* isolate H.T S6	MN535091.1	100%	100%	0.0
		Fusarium oxysporum isolate IFOL6	OR345423.1	100%	100%	0.0
4	TEF	*Fusarium oxysporum* isolates PG337, PG467, M9	MT630341.1 MT630343.1 KP964865.1	99,72%	100%	0.0
	ITS	*Fusarium oxysporum* isolate LC1-5-1-8	PP838603.1	98,65%	100%	0.0
		Fusarium oxysporum isolate LA4	OR976033.1	98,84%	100%	0.0

Isolate	Amplified region	Species matched in the NCBI database	Accessions	Percentage of identity	Percentage of covering	e- value
5	TEF	*Fusarium oxysporum* isolates PG337, PG467, M9	MT630341.1 MT630343.1 KP964865.1	99,72%	100%	0.0
	ITS	*Fusarium oxysporum* strain GUCC2GF1	PP596543.1			
		Fusarium oxysporum isolate FOC_1	PP939721.1	99,62%	100%	0.0
6	TEF	*Fusarium oxysporum* isolates PG337, PG467, M9	MT630341.1 MT630343.1 KP964865.1	98,74%	100%	0.0
	ITS	*Fusarium oxysporum* isolate C632	KY910856.1			
		Fusarium oxysporum isolate AMU-FOL1	MN788643.1	99,25%	100%	0.0
7	TEF	*Fusarium oxysporum* isolates PG337, PG467, M9	MT630341.1 MT630343.1 KP964865.1	98,19%	100%	0.0
	ITS	*Fusarium oxysporum* voucher Zhaochanglin 1922	OQ996879.1	99,62%	100%	0.0
		Fusarium oxysporum isolate 36	EU839387.1	99,43%	100%	0.0

Pathogenicity tests, incidence, and severity of vascular wilt. The incidence and severity of vascular wilt were assessed for five weeks. No differences were observed between the inoculation methodologies evaluated, as symptoms appeared similarly over the time of evaluation.

The statistical analysis of the incidence was carried out using contingency tables since we have categorical data. In the first week, for the 45-day-old plants, differences between treatments were found, since the plants inoculated with the isolate of group 4 did not show symptoms, 4 of the 6 plants inoculated with isolates from groups 3 and 5 became sick (66%), and all of the plants inoculated with isolates from groups 6 and 7 showed symptoms (100%). Similarly, in week 2, plants inoculated with isolates from groups 1 to 4 showed 66% incidence, and all plants from groups 5 to 7 showed symptoms (100%). From week 3 to week 5, all plants inoculated with the different isolates showed wilting, and there were no significant differences between inoculated plants, but there were significant differences between inoculated and untreated plants. In 90-day-old plants, there were no significant differences in weeks 1, 2, and 3. In weeks 4 and 5, there were no significant differences between inoculated plants, but there were significant differences

between inoculated and untreated plants (figures 4 and 5). The data obtained from the incidence of the 7 groups were averaged, and it was found that 55% of plants 45 days old showed symptoms in the first week, and in the third week, all plants were wilted. The 90-day-old plants started to show symptoms in the third week. 100% incidence was reached in the fifth week. Untreated plants had no symptoms.

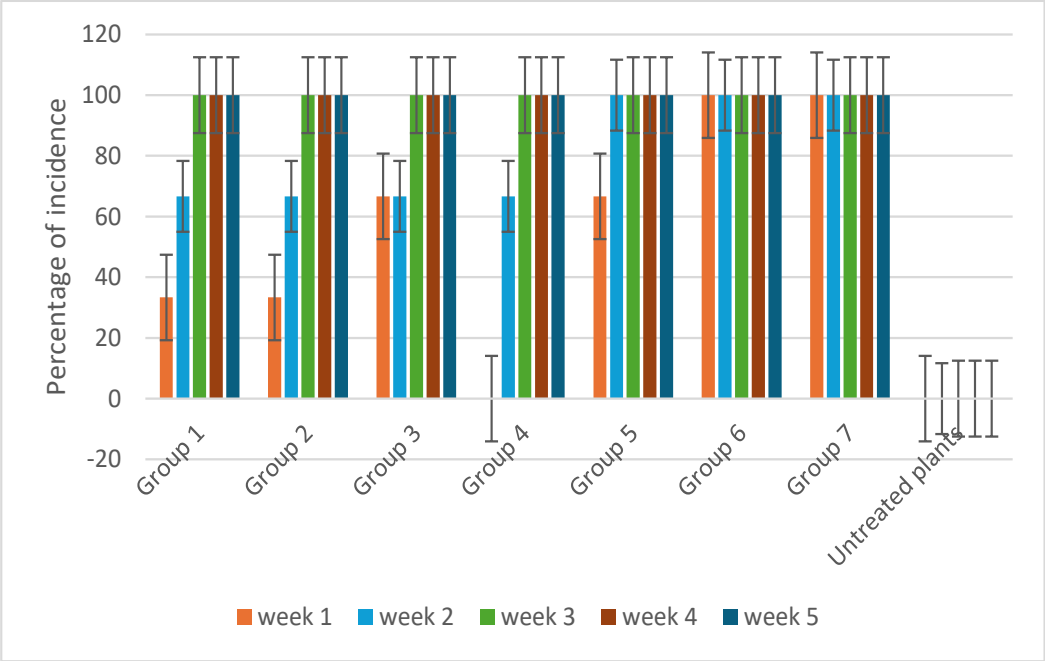

Figure 4. Incidence (%) of vascular wilt on 45-day-old plants

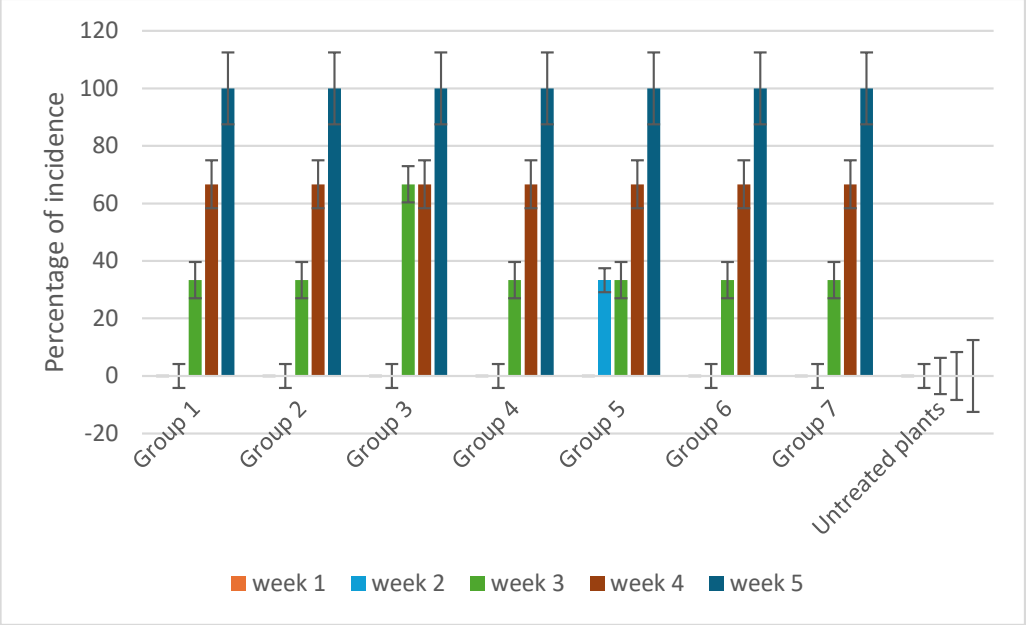

Figure 5. Incidence (%) of vascular wilt on 90-day-old plants

Similarly, we carried out an analysis of variance for the variable severity, finding that there are significant differences between the isolates evaluated. For the 45-day-old plants, in week 1, plants inoculated with isolate group 4 showed no symptoms. In weeks 2, 3, 4, and 5, there are no significant differences between inoculated plants, but there are significant differences between them and untreated plants. In 90-day-old plants, no symptoms were observed in week 1, and only a few plants inoculated with the group 5 isolate showed symptoms with a low severity percentage (5%). From week 3 to week 5, there are no significant differences between inoculated plants, but there are significant differences between inoculated and untreated plants (figures 6 and 7). In 90-day-old plants, the severity percentages are lower than those observed in 45-day-old plants. Rathnamma *et al*. (1999) inoculated rue plants with *F. solani* and found that symptoms appeared within 15 days and that young plants showed symptoms earlier than older ones. Helmy *et al*. (2001), report that rue seedlings inoculated with *F. oxysporum* showed severe disease. The results agree with those reported for other plants affected by *F. oxysporum*. Li *et al*. (2021) and Miao *et al*. (2021) found that young coriander (*Coriandrum sativum*) and boldo (*Coleus forskohlii* syn: *Plectranthus barbatus*) plants manifest symptoms rapidly within ten days after inoculation with F. *oxysporum* isolates. Lazreg *et al*. (2014) and Fernández-Herrera *et al*. (2020) were too determined that plants inoculated with different species of the genus *Fusarium* present symptoms more quickly among the youngest.

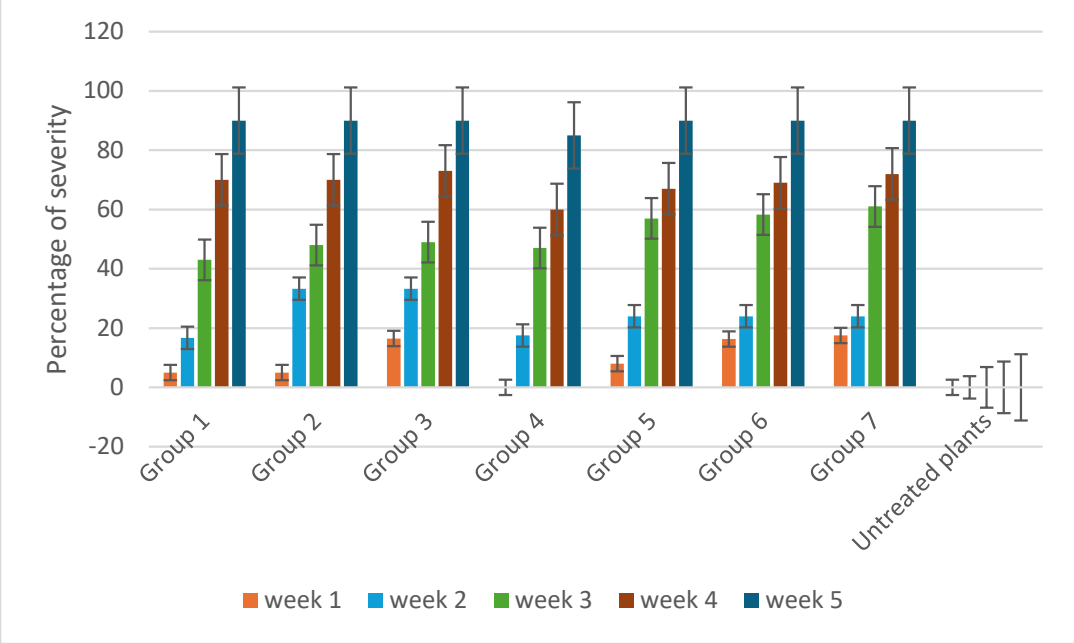

Figure 6. Severity (%) of vascular wilt on 45-day-old plants

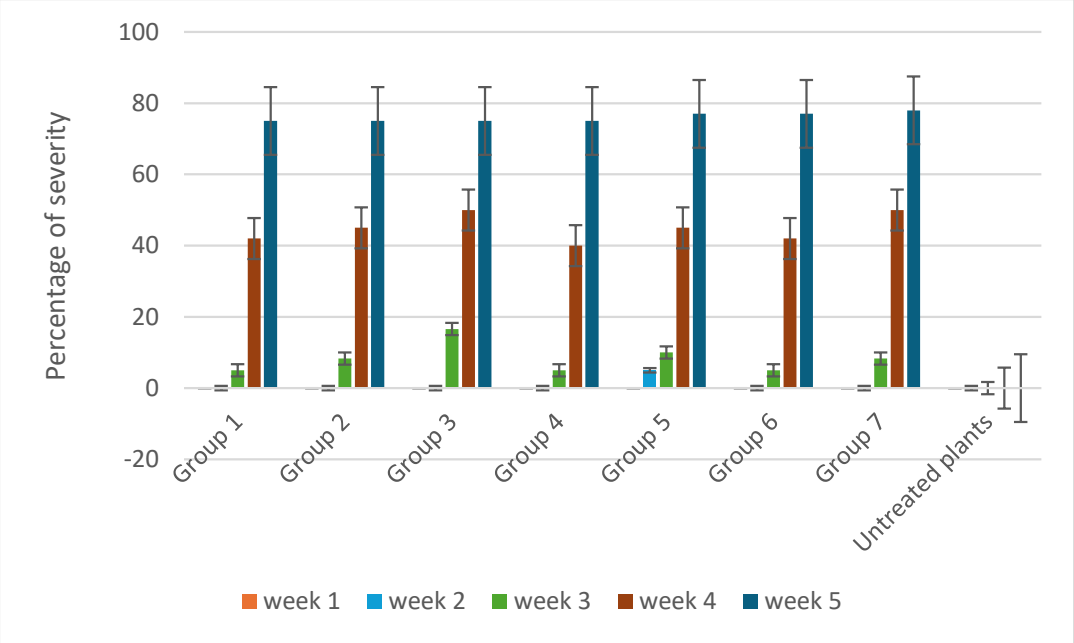

Figure 7. Severity (%) of vascular wilt on 90-day-old plants

CONCLUSIONS

The identification of *Fusarium oxysporum* as a causal agent of the vascular wilt of rue will allow farmers to implement preventive management practices focused on the plant pathogen. This approach intends to reduce the levels of incidence and severity of the disease, thereby minimizing economic losses. The description of the symptoms of vascular wilting in diseased plants in the field will serve as a reference to determining the progress of the disease in the field. Special care should be taken with plants during the early stages of cultivation, as young plants tend to manifest symptoms more quickly and more severely.

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