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Disease severity and relationship with the amount of fungal DNA and structural changes in resistant and susceptible bean cultivars infected with Fusarium wilt

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ABSTRACT. Fusarium wilt is an important soil-borne disease that affects the common bean. The disease is caused by *Fusarium oxysporum* f. sp. *phaseoli* (Fop), a fungus that invades the plant mainly through the roots and colonizes the xylem, causing wilting, vascular discoloration, chlorosis, stunting, and premature plant death. The objective of this study was to analyze Fop disease severity in the Mortiño (tolerant), BAT477 (resistant), and A211 (susceptible) differentiating cultivars of common bean. Another goal was to examine the relationship of the disease severity with the amount of DNA of the pathogen in the vascular system in the stem as well as structural changes in plants affected by Fop infection. Assessment of the level of xylem colonization by Fop isolated in these cultivars grown in an experimental field (plot size of 30 m^2) artificially infested with Fop showed in all the cultivars, a gradual increase in the fungus population over time, mainly during the maturation of the plants. The qPCR technique with the diseased genotypes collected at

five different times starting in V2 stage (second trifoliolate developed) in the biological replicates proved to be efficient in quantifying the relative amount of DNA of the fungus. In the scanning electronic microscopy and in the evaluation of the disease severity, the structures of the fungus and of the plant were different. In the A211 cultivar, formation of hyphae and microconidia of the fungus and tylose and amyloplasts of the plant were observed. In cv. Mortiño found hyphae of the fungus and tyloses of the plant. In BAT477, no pathogen structure was observed. The disease severity in the differentiating cultivars was correlated with the amount of fungal DNA and plant structures formed in reaction to the presence of the fungus detected mainly in the last collection at the R6 stage of the bean plants.

Key words: *Phaseolus vulgaris*; *Fusarium oxysporum* f. sp. *phaseoli*; qPCR; Scanning electron microscopy

INTRODUCTION

Fusarium wilt is an economically important soil-borne disease that affects common bean (*Phaseolus vulgaris*). Etiological agent of the disease is the fungus *Fusarium oxysporum* f. sp. *phaseoli* (Fop) (Alexopoulos et al., 1996). Fusarium wilt has been reported in all bean-producing regions of the world, and it is a serious problem in Latin America, Africa, and the northwestern United States (Pastor-Corrales and Abawi, 1987; Buruchara and Camacho, 2000). Temperatures in the range of 24-28ºC are ideal for development of the fungus; however, problems with this disease at higher temperatures has been observed in many bean-producing regions in the state of São Paulo in Brazil (Kimati, 1980). The types of spores produced by the fungus are macroconidia, microconidia, and chlamydospores. Macroconidia are fusoid and slightly curved and have three to five septa. Microconidia are elliptical, and chlamydospores are hyaline, intercalary, or terminal. Conidiogenous cells are short monophialides for *F. oxysporum* and long for *F. solani* (Nelson et al., 1993). The spores and the pathogen can be disseminated by contaminated seeds, wind, irrigation water that transports infested soil particles, and the conidia produced on the dead plant (Bianchini et al., 1997).

The occurrence and severity of this disease have increased due to a lack of necessary care in control methods. It is known that the damage caused by this disease is variable and may affect up to 80% of the crop, because the disease starts in small patches and, after a few years of cultivation, spreads throughout the crop (Sartorato and Rava, 1994). The fungus usually penetrates near the root tip, but it can also enter through wounds and natural openings, which are favored by the presence of nematodes in the area. Due to colonization of the vessels, the main reflex symptom is progressive yellowing from the lower to the upper leaves. As the disease progresses, the leaves acquire a light-yellow hue, entering senescence prematurely. When the disease affects young plants, reduced development and stunting are observed, which can lead to plant death. In the field, loss of turgidity is not always visible. The interior of the vascular tissues acquire a reddish-brown color (Bianchini et al., 1997).

Resistant genotypes are recommended as a convenient and effective strategy for disease control (Buruchara and Camacho, 2000). However, the development of new cultivars with a broad spectrum of resistance has been difficult, due to the high variability of the pathogen (Salgado and Schwartz, 1993). Pathogenic races have been described from various geographic regions. Race 1 was identified in South Carolina (USA) and in Portici (Italy) (Ribeiro and Hagedo, 1979), race 2 includes isolates from Brazil (Ribeiro and Hagedorn, 1979), race 3 occurs in Colombia (Salgado and Schwartz, 1995), race 4 includes an isolate from Colorado (USA) (Salgado and Schwartz, 1993), race 5 was identified in Greece (Kastoria) (Woo et al., 1996), race 6 in Spain (Alves-Santos et al., 2002), and race 7 in Greece (Alves-Santos et al., 2002).

Batista et al. (2017) inferred that resistance to Fop in beans is dominant, involving polygenes and with additive and dominance effects in the resistance genes. The objectives of our study were to analyze disease severity in differentiating cultivars of the common bean to Fusarium wilt and to exam the relationship of the disease severity with the amount of fungal DNA in the vascular system and to detect reaction structures of the plant as a consequence of Fop infection.

MATERIAL AND METHODS

Plant material

Mortiño (tolerant), BAT477 (resistant), and A211 (susceptible) are Fop-differentiating cultivars; that are used to examine the response of bean cultivars to Fop wilt. Sixty seeds of each of these cultivars were all sown in an experimental field area of 30 m^2 artificially infested with Fop races 1, 2, 3, 6, 7, and an undefined race of Fop. The experimental area belongs to the bean plant breeding program of the Instituto Agronômico (Fazenda Santa Elisa, IAC, Campinas, SP, Brazil) and has been inoculated over time with these races through the introduction of wheat seeds colonized by the pathogen. The control treatment without *Fusarium* consisted of seeds of the cultivars sown directly in 10 L vases containing autoclaved substrate kept in a greenhouse. The xylem vessel of the control plants was also analyzed for the presence of the fungus and detection of resistance structures.

The bean genotypes were collected at five different times for real-time PCR (qPCR), following the phenological stages of the plants (https://www.ag.ndsu.edu/crops/dry-bean-articles/stages-of-development). The 1st collection was carried out in the cultivars at the V_2 stage, when the primary leaves were already open, eight days after sowing. The 2nd collection was done at 18 days after sowing in the field, when the plants were in the V_3 and V_4 stages. At 28 days after sowing, the 3rd collection was performed, when the plants were between the V_4 and R_5 stages. The 4th collection was when the plants were in the R_6 stage, at the time the first flower opens, and the pods are formed. The 5th collection was carried out 10 days after flowering.

For evaluation of disease severity in plants in the field, the following rating scale was used: 1 – absence of symptoms; 2 – beginning of yellowing; 3 – stunting and 1 or 2 dead plants; 4 – stunting and 20 to 30 % dead plants; and 5 – more than 50% dead plants. Cultivars with score 1 were classified as resistant to Fop, cultivars with scores 2 and 3 were classified as tolerant, and cultivars with scores 4 and 5 were classified as susceptible.

Real-time PCR for cultivars colonized with *F. oxysporum* f. sp. *phaseoli*

After collecting the three cultivars (Mortiño, BAT477, and A211) colonized with Fop, DNA was extracted from the stem of the three biological replicates according to the Wizard Genomic DNA Purification kit (Promega). The full stem of each plant was ground in liquid nitrogen. Total DNA was quantified after electrophoresis in 1% agarose gel using lambda phage DNA (Fermentas, SP, Brazil).

The oligonucleotide used for the amplification of pathogen DNA was designed in the Primer3 program (http://frodo.wi.mit.edu/) (Rozen and Skaletsky, 2000). Alignment was performed in the BlastN of the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Amplification reactions were prepared using the DNA extracted from mycelium from a pure culture of Fop and *F. solani* as a template. Reactions were prepared for qPCR using 50 ng of DNA extracted from tissue colonized with the pathogen and 0.5 µL of the primer pair that was designed. The reaction consisted of 1 µL of DNA, 1 µL of each oligonucleotide, and 10 µL of the SYBR green Go*Taq* qPCR Master Mix (Promega), adjusting the final volume to 20 µL with Milli-Q water. The cycle used was 50°C for 2 minutes, 95°C for 10 minutes, 40 times 95°C for 15 seconds, and 60°C for 1 minute. The ABI PRISM 7000 SDS version 1.1 software was used (Applied Biosystems).

The *β*-actin gene was chosen as an endogenous control. The oligonucleotides used were F - 5' TACAACGAGCTTCGTGTTGC 3' and R - 5' CTTCTCTCTGTTGGCCTTGG 3'. Amplification efficiency was calculated in reactions containing serial dilutions of DNA at concentrations of 100, 10, 1, 0.1, and 0.01 ng/µL, which was extracted from tissue infected with Fop. Efficiency was determined by the linear relationship between the cycle in which fluorescence was detected above the minimum limit (Ct – cycle threshold) and the log of DNA concentration. Based on the slope value of the curve, amplification efficiency was calculated by the following formula: $E_{(\text{amplification efficiency})} = 10^{(-1/\text{slope})} - 1$

Three reactions were prepared for each DNA dilution, composed of 1 µL of DNA (from the serial dilutions), 0.5 µL of each oligonucleotide, and 10 µL of the SYBR green Go*Taq* qPCR Master Mix (Promega), adjusting the final volume to 20 µL with Milli-Q water. Healthy tissue, without the pathogen, was used as a negative control. The first collection of tissue colonized with the pathogen was used as a normalizer. The equation used for normalization was ΔCt = Ct (target gene) – Ct (endogenous control). The increase in target gene DNA concentration for each condition was calculated using the formula $\Delta\Delta Ct$ = ΔCt of the sample - ΔCt of the calibrator. The calibrator is the Ct value obtained for a specific sample; the increase in expression levels is always obtained in relation to the specific calibrator used. The relative amount was calculated using the formula $QR = 2^{-\Delta\Delta Ct}$. For all qPCR reactions, the dissociation curve was determined to verify nonspecific amplifications.

Quantification of the fungus was compared to a previously optimized standard curve and validated for amplification efficiency. This was performed using known concentrations of Fop DNA, obtained from the amplification of serial dilutions (10×) from 1×10^{-3} to 1×10^{-4} ng/µL. The mean Ct values (Y axis) were plotted against the log10 of the initial amount of template DNA (X axis) for each negative (no pathogen) and positive (fungal DNA) control included in all experiments to exclude or detect possible contaminations. The initial fungal DNA concentration was 100 ng. For the qPCR amplification reactions, 1 µL of DNA (from the serial dilutions), 0.5 µL of each

oligonucleotide, and 10 µL of the SYBR green Go*Taq* qPCR Master Mix (Promega) were used, adjusting the final volume to 20 µL with Milli-Q water. The same amplification conditions described above were applied. All Ct calculations, integration of the amplification efficiency data and statistical analyses were performed using StepOneTM Software V2.2.2.

Scanning electron microscopy (SEM)

The stem fragments from the Mortiño (tolerant to Fop), BAT477 (resistant to Fop), and A211 (susceptible to Fop) cultivars were collected. The material from the Mortiño and BAT477 cultivars was gathered 28, 42, and 55 days after sowing in the field infested with Fop. For the A211 cultivar, plant material was gathered at 28, 55, and 68 days after sowing, also from the field. The materials were fixed in 70% alcohol until preparation for observation in SEM. The freeze-fracture technique (Taylor, 2008) was used, in which the stem fragments were removed from the fixative, frozen, and sectioned under liquid nitrogen. The samples were then post-fixed in 70% ethanol for one hour and dehydrated in 96% ethanol for one hour, followed by three washes in absolute ethanol for 2 hours for each wash. Subsequently, the samples were dried in a critical point device (Blazers CPD/030) using liquid CO_2. The longitudinal and transverse sections of the stem were prepared on metallic supports (stubs) and plated with gold for 3 minutes in the Bal-Tec SCD 050 Sample Sputter Coater. The stem fragments were visualized in SEM (JSM-5800 LV) at the Electron Microscopy Laboratory of UNICAMP (Campinas, SP, Brazil).

RESULTS

Real-time PCR for *Fusarium oxysporum* f. sp. *phaseoli*

The oligonucleotide design resulted in four primers pairs: IAC-Fop 01, IAC-Fop 03, IAC-Fop 05 and IAC-Fop 06. They had 100% identity with *F. oxysporum* (Table 1). The primer pairs IAC-Fop 01, IAC-Fop 03, IAC-Fop 05 and IAC-Fop 06 amplified fragments of 221, 169, 187 and 178 bp, respectively. The total DNA extracted from the mycelium of the fungus *F. solani* f. sp. *phaseoli* was not amplified (Figure 1). For the qPCR reactions, the IAC-Fop 05 was selected among the other primer pairs and showed an amplification efficiency of 90% (Figures 1, 2, 3 and Supplementary 1).

Table 1. Results of analyses carried out in silico to confirm whether the fungal DNA fragment corresponded to the sequence of the *Fusarium oxysporum* pathogen.

Oligonucleotides	*e*-value	Maximum Identity	Number of the access	Identity of the genes and the region
IAC-Fop 01	0.89	100%	JQ265798	Pisatin demethylase
IAC-Fop 03	0.89	100%	AY337453	Alfa EF-1
IAC-Fop 05	0.89	100%	DQ831900	28S
IAC-Fop 06	0.90	100%	AB256871	PG5

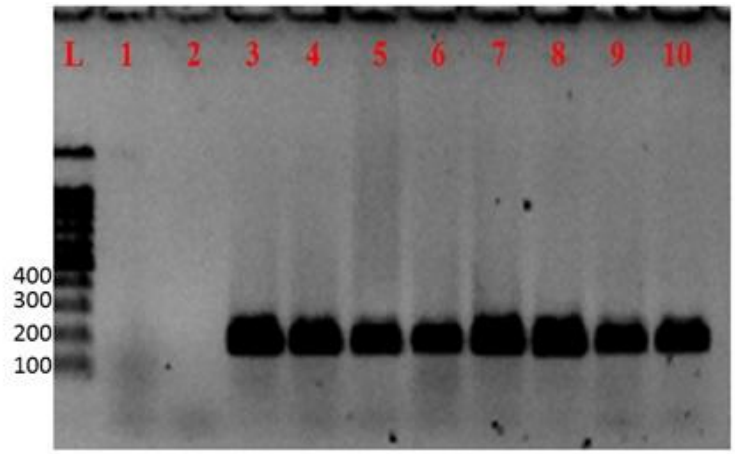

Figure 1. The IAC-Fop 05 primer pair in 1% agarose gel amplifying fungi DNA. 1 and 2 – DNA extracted from the mycelium of the fungus *Fusarium solani* f. sp. *phaseoli*, 3 and 4 – DNA extracted from mycelium of the American race of Fop (*Fusarium oxysporum* f. sp. *phaseoli*); 5 and 6 – DNA extracted from mycelium of the Brazilian race of Fop; 7 – DNA extracted from mycelium of the Colombian race of Fop; 8 – DNA extracted from mycelium of the European race of Fop; 9 – DNA extracted from mycelium of the Greek race of Fop; and 10 – DNA extracted from mycelium of the undefined race of Fop. L: Molecular weight marker of 100 bp.

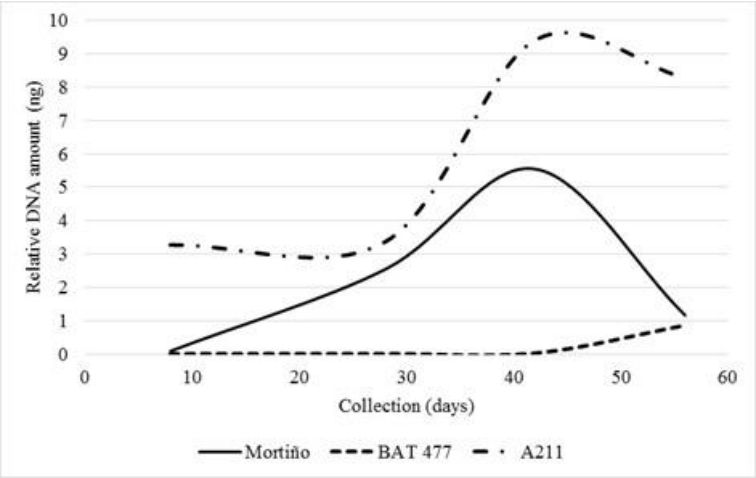

Figure 2. Relative amount of total Fop (*Fusarium oxysporum* f. sp. *phaseoli*) DNA measured inside Mortiño, BAT477, and A211 throughout infection with Fop using real-time PCR. The IAC-Fop 05 primer pair was used.

The standard curve for Fop was determined estimating the quantity of pathogen molecules in the colonized plants by comparing the average of Cts obtained in samples colonized with the fungus with the average of Cts obtained in the serial dilutions performed with the total DNA extracted from the pathogen mycelium. There was a linear range, with a correlation coefficient of 0.96 (Supplementary 2).

Comparison of the average of the Cts obtained for each collection date of the material colonized with the fungus and the average of the Cts obtained in the serial dilution carried out with the total Fop DNA can be associated with the relative amount of DNA in

the three cultivars (Supplementary 3). In all the cultivars analyzed, a gradual increase in the fungus population was observed over time (Supplementary 3).

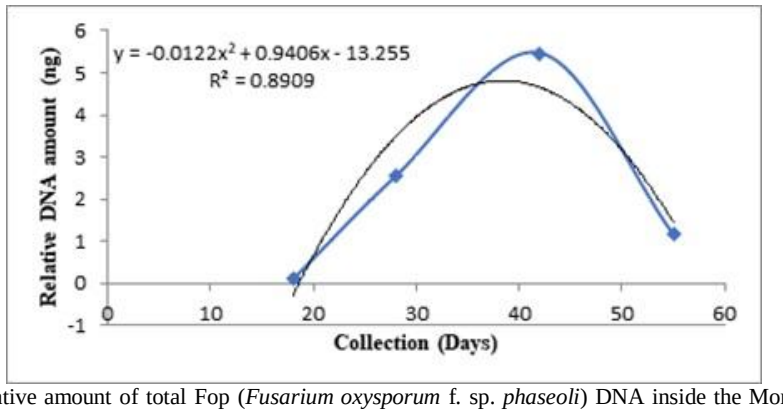

Figure 3. Relative amount of total Fop (*Fusarium oxysporum* f. sp. *phaseoli*) DNA inside the Mortiño cultivar throughout infection with Fop based on real-time PCR. The IAC-Fop 05 primer pair was used.

Relative amount of DNA and reaction of *F. oxysporum* f. sp. *phaseoli* under field conditions

The Mortiño cultivar was considered moderately tolerant (mean score 3.0, Table 2) to the fungus, with plants showing slight yellowing, and wilted plants at the end of the cycle. The value 5.55 ng was observed for the relative amount of DNA obtained with the qPCR (collection performed at 42 days after sowing) (Figure 2 and Table 3). Indeed, the highest relative amount of pathogen DNA found was observed in the collection performed at 42 days after sowing (Figures 2 and 3). After that collection, a reduction in the relative amount of DNA of the pathogen was observed in the plants. In the collection performed at 55 days, the value was 1.17 ng.

Table 2. Equation and R^2 of the relative amount of Fop (*Fusarium oxysporum* f. sp. *phaseoli*) DNA (ng) in the cultivars in the total of collections and disease severity average: Mortiño, BAT477, and A211 common bean cultivars.

Cultivars	Equation	R^2	DNA (ng)	Disease severity[1]
Mortiño	$y = -0.0122x^2 + 0.09406x - 13.255$	0.8909	2.34	3.0
BAT477	$y = -2E\text{-}05x^2 + 0.0022x - 0.019$	0.8660	0.02	1.0
A211	$y = -0.0023x^2 + 0.3264x - 2.6172$	0.8743	6.09	5.0

[1]Rating scale for evaluation of disease severity: 1: absence of symptoms; 2: beginning of yellowing; 3: stunting and one or two dead plants; 4: stunting and 20 or 30% dead plants; 5: above 50% dead plants. Cultivars with score 1 were classified as resistant to Fop, cultivars with scores 2 and 3 were classified as tolerant and cultivars with scores 4 and 5 were classified as susceptible. R^2 is the coefficient of correlation obtained for the standard curve.

The BAT477 cultivar showed resistance to the pathogen, and the plants did not show symptoms in the field (mean score 1.0; Table 2). The relative amount of DNA obtained for this variety was an average of 0.02 ng (Table 3), and a small amount of DNA was observed in all the collections (Figures 2 and 4).

Table 3. Relative amount of Fop (*Fusarium oxysporum* f. sp. *phaseoli*) DNA (ng) of the common bean cultivars at the four collection times (days): Mortiño, BAT477 and A211 cultivars.

Cultivars	Collection (Days)					
	8	28	42	49-68	R^2	Mean DNA amount (ng)
Mortiño	0.10	2.54	5.55	1.17	0.8909	2.34
BAT477	0.01	0.01	0.03	0.03	0.8660	0.02
A211	3.28	3.37	9.41	8.28	0.8743	6.09

R^2 is the coefficient of correlation obtained for the standard curve.

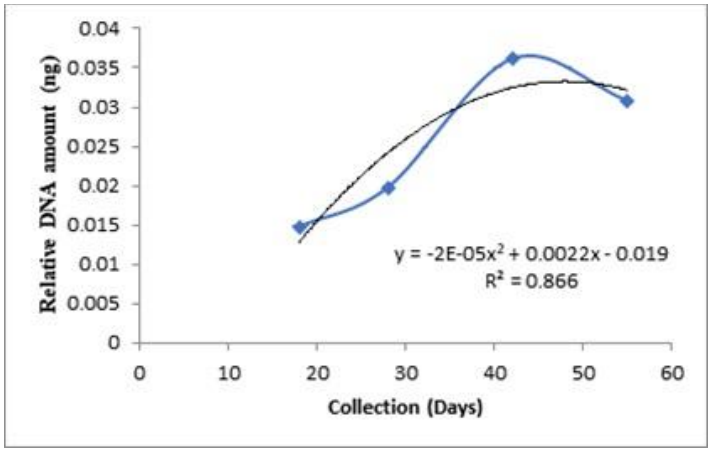

Figure 4. Relative amount of total Fop (*Fusarium oxysporum* f. sp. *phaseoli*) DNA inside BAT477 (common bean cultivar) throughout Fop infection based on real-time PCR. The IAC-Fop 05 primer pair was used.

The A211 cultivar was susceptible to the fungus when evaluated in the field, and the main symptoms observed were plant wilting (mean score 5.0; Table 2). For qPCR, the relative amount of DNA showed the highest peak in the collection period performed at 55 days after sowing, with a value of 9.41 ng (Figures 2 and 5). For the collection period performed at 68 days, the relative amount of DNA obtained was 8.28 ng (Table 3).

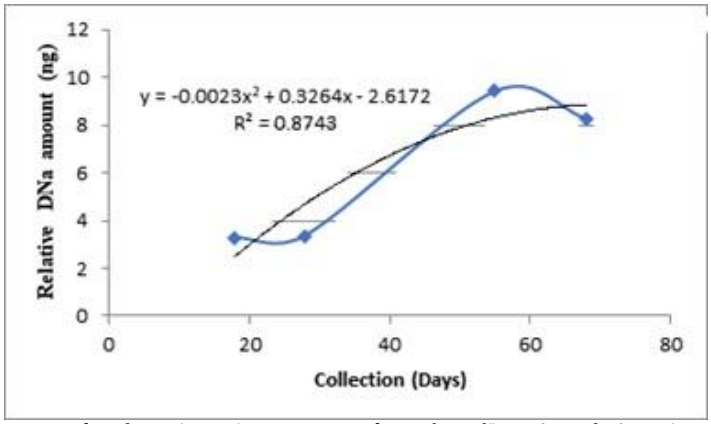

Figure 5. Relative amount of total Fop (*Fusarium oxysporum* f. sp. *phaseoli*) DNA inside A211 (common bean cultivar) throughout infection with Fop based on real-time PCR. The IAC-Fop 05 primer pair was used.

Scanning electron microscopy (SEM)

In scanning electron microscopy (SEM) for the Mortiño cultivar (classified as tolerant to Fop in field evaluations) in the collection at 28 days after sowing, the relative amount of DNA detected was 1.77 ng (Figure 2); formation of hyphae of the pathogen was not observed (Figure 6 A and B). In the collection at 55 days after sowing, the relative amount of DNA was 3.25 ng; formation of tyloses was observed (Figure 6 C). In the collection at 68 days after sowing, the relative amount of DNA detected was 1.17 ng (Table 3); formation of pathogen hyphae was perceived in intracellular spaces (Figure 6 D). In the field, the plants showed symptoms of dwarfism, and dead plants.

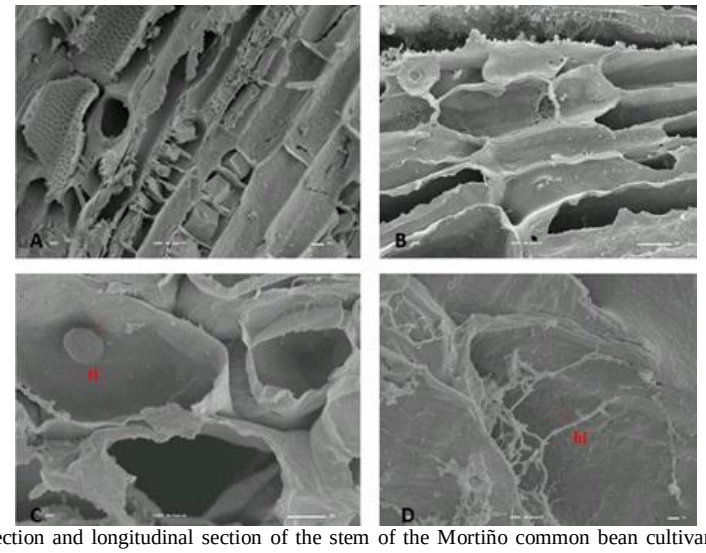

Figure 6. Cross section and longitudinal section of the stem of the Mortiño common bean cultivar colonized by Fop observed by scanning electron microscopy. A. collection at 28 days after sowing, B. and C. collection at 55 days after sowing, D. collection at 68 days after sowing. hi: hyphae; ti: tyloses. Bars: A = 1400 µm; B = 500 µm; C = 1500 µm; D = 4500 µm.

In the vascular system of the stem of BAT477, classified as resistant to Fop in the field evaluations, no structure (such as hyphae and spores) of the pathogen was observed (Figure 7). In Figure 7 A and B, referring to the collection at 28 days after sowing, there was no sign of colonization of the fungus; there was likewise no sign in the collections at 42 until 55 days after sowing (Figure 7 C and D). The relative amount of DNA for the three collection dates was very low or almost null, ranging from 0.01 ng for the third collection to 0.03 for the fourth and fifth collections (Table 3). Symptoms of fusarium wilt were not observed in the plants in the field.

For the A211 cultivar, the formation of hyphae was observed in the three collections, and this cultivar was classified as susceptible under field conditions. In Figure 8 A, referring to collection at 68 days after sowing, the relative amount of DNA was 3.37 ng (Table 3). In that collection, formation of pathogen hyphae appeared between the intracellular spaces. In Figure 8 B, referring to the collection at 55 days after sowing, the largest relative amount of DNA of the pathogen (9.41 ng) was observed. Degradation of the tissues and the formation of a mesh close to them caused by Fop could be seen. For the

collection at 68 days after sowing, the relative amount of DNA was 8.28 ng (Table 3), and image of tissues in SEM showed the formation of hyphae in intracellular spaces and the formation of microconidia (Figure 8 C). In the collection at 68 days after sowing, the presence of tyloses was also observed (Figure 8 D). In addition to the tyloses in that collection, many amyloplasts were detected (Figure 8 E).

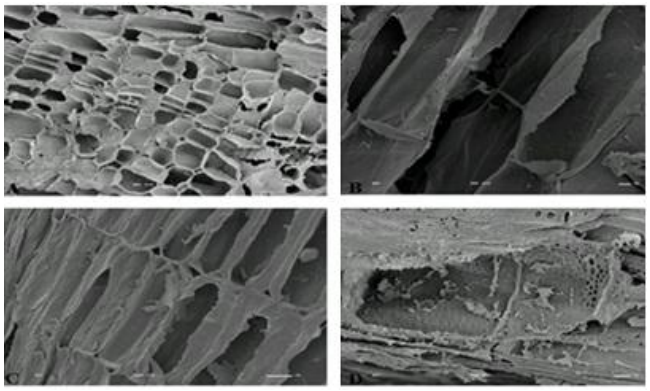

Figure 7. Cross section and longitudinal section of the stem of the BAT477 common bean cultivar colonized by Fop observed by scanning electron microscopy. A. and B. collection at 28 days after sowing, C. collection at 42 days after sowing, D. collection at 68 days after sowing. Bars: A and B = 170 µm; C = 1100 µm; D = 500 µm.

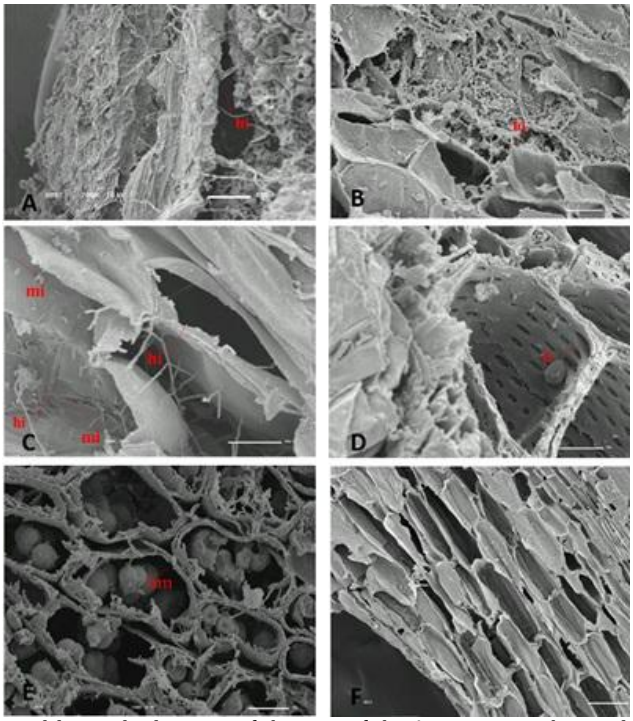

Figure 8. Cross section and longitudinal section of the stem of the A211 common bean cultivar colonized by Fop observed by scanning electron microscopy. A. collection at 28 days after sowing, B. collection at 55 days after sowing, C. and D. collection at 68 days after sowing, E. amyloplasts (collection at 68 days after sowing) F. control treatment. hi: hypha; ti: tyloses, mi: microconidia, am: amyloplast. Bars: A, B, D = 200 µm; C = 2000 µm; E = 400 µm; F = 1,700 µm.

In most of the cultivars, the highest peak of the relative amount of DNA was observed in the collections at 42 to 68 days after sowing, and the expression of symptoms was more evident. In the A211 cultivar, susceptible to Fop, from 28 to 68 days after sowing, the formation of hyphae and microconidia was observed; and in the cultivar Mortiño at 68 days after sowing, the formation of hyphae was also detected.

In the differentiating cultivars planted in autoclaved substrate in vases (control), fusarium wilt was not detected. Furthermore, no DNA of the fungus was detected and no structure of the plant reacting to the fungus was observed.

DISCUSSION

The sequence available in GenBank used for the design of the oligonucleotide IAC-Fop 01 (Table 1) corresponds to the gene of pisatin demethylase. This enzyme helps in the removal of phytoalexins. The host specificity of plant pathogens may be dictated by genes that allow pathogens to bypass host defenses. After recognizing a pathogen, plants initiate defense responses, which may include the production of antimicrobial compounds, such as phytoalexins (Ma et al., 2022). For the oligonucleotide IAC-Fop 03, the corresponding candidate gene was the protein responsible for the alpha elongation factor EF-1. This protein is the main transcriptional regulator of cellular response in Fop (de Carvalho Paulino et al., 2022). The oligonucleotide IAC-Fop 05 corresponds to the 28S region and this region is highly conserved intraspecifically, but variable between different species, which makes it possible to distinguish at the specific level (Sharma et al., 2018). This was the best oligonucleotide to identify *F. oxysporum* and was used in the qPCR analyses. The gene corresponding to the initiator IAC-Fop 06 is the PG5 gene, which encodes members of an important family of cell wall degradative enzymes (Hirano et al., 2009).

In the current study, the Mortiño cultivar was classified as tolerant to the fungus Fop. This finding is according to Paulino et al. (2020) that reported Mortiño with intermediate resistance to Fop. The BAT477 cultivar was described by Cross et al. (2000) as susceptible to Fop, while Henrique et al. (2015) showed that it was resistant to Fop race 2 and 3. This cultivar was classified in the present study as resistant to Fop. The A211 cultivar is a black bean reported as susceptible to Fop, according to France and Abawi (1994) and Paulino et al. (2020). The cultivar A211 was also susceptible to Fop in the current study.

In our study, it was observed that Fop infection in cultivars depends on the defense mechanism of each cultivar and on the possibility of the pathogen multiplying inside the host. The results presented in real-time PCR to evaluate the symptoms of Fusarium wilt in the field corroborated with observation of the tissues of the three different cultivars in methodology for SEM. Concerning pathogen multiplication in A211, susceptible to Fop, the mean relative amount of DNA was 6.09 ng, and observed pathogen hyphae and macroconidia in the tissues as well as formation of tylose and amyloplast. Degradation of the tissues and the formation of a mesh close due the presence of Fop could be seen in the A211 cultivar. For Mortiño, tolerant to Fop, the mean relative amount of DNA was 2.34 g, and presence of pathogen hyphae and tyloses were noted in intracellular spaces as well as dead plants and plants showing symptoms of dwarfism. Pereira et al. (2013) also studied the Fop infection process in bean cultivars resistant, intermediate, and susceptible to the pathogen. At 43 days after the inoculation, the stem was observed with a scanner electronic

microscope and resistant and intermediate cultivars presented an occluding material in the xylem vessels, which may have restricted tissue colonization by Fop.

BAT477 cultivar was classified as resistant to Fop and did not detect presence of the fungus and no plant resistance structure was observed in tissues, and no symptoms of Fusarium wilt occurred in plants in the field. Tyloses are stimulated by wounds, water stress, and by presence of pathogenic microorganisms, interrupting the flow of sap and, consequently, the advance of pathogens through the xylem (Kostea and Bart, 2013). Amyloplasts are large reservoirs of starch; in times of need (a lack of glucose), they can be reconverted into glucose. During an infection, they serve as a nutrient for the pathogen, and many amyloplasts in the host tissue is a sign of fungal colonization. This sign may explain the susceptibility of the A211 cultivar to the Fop. In addition, degradation of the tissues of the cultivar A211 can be explained by the reports of Bentes and Matsuoka (2002 and 2005) and Quadros et al. (2021), who state that the action of phytopathogens in plants causes disorganization in the cellular protoplasm and the death of the plant cells as well as the cells can be damaged and with the chloroplasts destroyed or without their content.

Cultivar BAT477, classified as resistant to Fop in the field evaluations, seems to have an effective mechanism of resistance to the pathogen. With scanning electron microscopy, it was not possible to detect structural changes. According to Pascholati and Leite (1995), the resistance mechanism of a plant against a fungus can be structural and/or biochemical. As for the biochemical, the phytoalexins is important, and these are antimicrobial compounds that are synthesized by plants and accumulate in cells in response to microbial infection (Paxton, 1981). Mendes et al. (2018) performed comparative analysis of the rhizosphere microbiome between the Fop-resistant (IAC Milenio) and susceptible bean (IAC Alvorada), and the results revealed a larger abundance of the genera *Pseudomonas* and *Bacillus* in the Fop-resistant cultivar. According to Mendes et al. (2013), these two rhizobacterial are known for biofertilization, stimulation of root growth, rhizo-remediation, control of abiotic stress and plant diseases. Therefore, for BAT477 cultivar the resistance mechanism needs to be better investigated to know if there are multiple factors involved or some specific against Fop.

In our study, the symptoms of fusariosis in the cultivars tested, as well as the highest and lowest peaks in the relative amount of DNA and the observation of fungal structures in plant tissues were more evident in the last collections (42 - 68 days). This collection period corresponded to the R_6 phenological phase of common bean, with the remobilization of photoassimilates and grain filling. According to reports of Wendland et al. (2018), the symptoms of plant yellowing, and wilting caused by the Fop pathogen occur from the flowering to the grain filling phase, and after this grain filling phase.

Results indicate that the qPCR assay is effective in detecting Fop in common bean and in assessing the level of fungus multiplication in plants. In the differentiating cultivar susceptible to Fop was observed the greater relative amount of pathogen DNA. In the Fop-tolerant differentiating cultivar, the relative amount of DNA was higher than in the Fop-resistant differentiator. The highest expression of symptoms was observed in the 5th collection, at the R6 stage of the bean plant, as well as the highest relative amount of DNA. qPCR to *Fusarium* is very used. Matthews et al. (2020) used qPCR with specific primers to *Fusarium oxysporum* f. sp. *cubense* isolates and were successful in detecting in Africa the pathogen in banana, soil, and water.

The disease severity in the differentiating cultivars was correlated with the amount of DNA of fusarium and plant structures formed in reaction to the presence of the fungus detected mainly in the last collection at the R6 stage of the bean plants.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Alexopoulos CJ, Mims CJ and Blackwell M (1996). Introductory Mycology. 4.ed. New York: J. Wiley, 880 p.

Alves-Santos FM, Cordeiro-Rodrigues L, Sayagués JM, Martin-Dominguez R, et al. (2002). Pathogenicity and race characterization of *Fusarium oxysporium* f. sp. *phaseoli* isolates from Spain and Greece. *Plant Pathol.* 51: 605-611.

Batista RO, Silva LC, Moura LM, Souza MH, et al. (2017). Inheritance of resistance to fusarium wilt in common bean. *Euphytica.* 213: 133. https://doi.org/10.1007/s10681-017-1925-1.

Bentes JLS and Matsuoka K (2002). Histologia da interação *Colletotrichum guaranicola* e *Paullinia cupana* var. *sorbilis* em clones resistente e suscetível. *Fitopatol Bras.* 27(1): 71-77. https://doi.org/10.1590/S0100-41582002000100011.

Bentes JLS and Matsuoka K (2005). Histologia da interação *Stemphylium solani* e tomateiro. *Fitopatol. Bras.* 30: 224-231.

Bianchini A, Maringoni AC and Carneiro SMTG (1997) Doenças do feijoeiro (*Phaseolus vulgaris* L.). In: Kimati H, et al. Manual de fitopatologia: doenças das plantas cultivadas. 3.ed. São Paulo: *Agronômica Ceres*. 2: 376-399.

Buruchara RA and Camacho L (2000). Common bean reaction to *Fusarium oxysporum* f. sp. *phaseoli*, the cause of severe vascular wilt in Central Africa. *J. Phytopathol.* 148(1): 39-45.

de Carvalho Paulino JF, de Almeida CP, Barbosa CCF, de Moraes GCG, et al. (2022). Molecular and pathogenicity characterization of *Fusarium oxysporum* species complex associated with Fusarium wilt of common bean in Brazil. *Trop. Plant Pathol.* 47: 485-494.

de Quadros FM, Garcés-Fiallos FR, de Freitas MB, Bouzon ZL, et al. (2021). Dual role of H_2O_2 in late stages of root colonization of resistant and susceptible bean plants by *Fusarium oxysporum* f. sp. *phaseoli*. *Physiol. Mol. Plant Pathol.* 115: 101686.

France RA and Abawi GS (1994). Interaction between *Meloidogyne incognita* and *Fusarium oxysporum* f. sp. *phaseoli* on Selected Bean Genotypes. *J. Nematol.* 26(4): 467-474.

Gonçalves JGR, Chiorato AF, da Silva DA, Esteves Jad, et al. (2015). Combining ability in common bean cultivars under drought stress. *Bragantia.* 74(2): 149-155.

Hirano Y and Arie T (2009). Variation and phylogeny of *Fusarium oxysporum* isolates based on nucleotide sequences of polygalacturonase genes. *Microbes Environ.* 24: 113-120.

Jakobek JL and Lindgren PB (2002). Expression of a bean acid phosphatase cDNA is correlated with disease resistance. *J. Exp. Bot.* 53(367): 387-389.

Kimati H (1980). Doença do feijoeiro (*Phaseolus vulgaris* L.). In: GALLI, F. (Coord.). Manual de Fitopatologia: doença das plantas cultivadas. São Paulo: *Agronômica Ceres*. 2(19): 297-318.

Koste AY and Bart PHJT (2013). The xylem as battleground for plant hosts and vascular wilt pathogens. *Plant Sci.* 4: 97-105.

Kouas S, Debez A, Slatni T, Labidi N, et al. (2009). Root Proliferation, Proton Efflux, and Acid Phosphatase Activity in Common Bean (*Phaseolus vulgaris*) Under Phosphorus Shortage. *J. Plant Biol.* 52: 395-402.

Ma L-J, Geiser DM, Proctor RH, Rooney AP, et al. (2013). Fusarium Pathogenomics. *Annu Rev. Microbiol.* 67(1): 399-416.

Ma T, Li Y, Lou Y, Shi J, et al. (2022). The Drug H+ Antiporter FgQdr2 is Essential for Multiple Drug Resistance, Ion Homeostasis, and Pathogenicity in *Fusarium graminearum*. *J. Fungi.* 8(10): 1009.

Matthews MC, Mostert D, Ndayihanzamaso P, Rose LJ, et al. (2020). Quantitative detection of economically important *Fusarium oxysporum* f. sp. *cubense* strains in Africa in plants, soil and water. PLoS ONE. 15(7): e0236110.

Mendes R, Garbeva P and Raaijmakers JM (2013). The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiol. Rev.* 37: 634-663.

Mendes LW, Raaijmakers JM, Hollander M, Mendes R, et al. (2018). Influence of resistance breeding in common bean on rhizosphere microbiome composition and function. *The ISME Journal.* 12: 212-224.

Nelson PE, Tousson TA and Marasas WF (1993). Fusarium species: an illustrated manual for identification Pennsylvania/London: The Pennsylvania State University Park and London, p. 193.

Pastor-Corrales MA and Abawi GS (1987) Reactions of selected bean germplasms to infection by *Fusarium oxysporum* f.sp. *phaseoli*. *Plant Dis.* 71(11): 990-993.

Pascholati SF and Leite B (1995). Hospedeiro: Mecanismos de resistência. In: Bergamin Filho A, Kimati H, Amorim L (Org.) Manual de Fitopatologia. In: São Paulo: *Agronômica Ceres*. p. 417-452.

Paulino JFd, Almeida CPd, Gonçalves GdMC, Bueno CJ, et al. (2020). Assessment of resistance in common bean to *Fusarium oxysporum* f. sp. *phaseoli* using different inoculation and evaluation methods. *Crop. Breed Appl. Biotechnol.* 20(3): e337620311. https://doi.org/10.1590/1984-70332020v20n3n45.

Paxton JD (1981). Phytoalexins- a working redefinition. *J. Phytopathol.* 101: 106-109.

Pereira AC, Cruz MFA, Júnior TJP, Rodrigues FA, et al. (2013). Infection process of *Fusarium oxysporum* f. sp. *phaseoli* on resistant, intermediate and susceptible bean cultivars. *Trop. Plant Pathol*. 38: 323-328.

Ribeiro RLD and Hagedo DJ (1979). Screening for resistance to and pathogenic specialization of *Fusarium oxyporium* f. sp. *phaseoli*, the causal agent of bean yellows. *Phytopathology*. 69(3): 272-276.

Ribeiro RLD and Hagedorn DJ (1979). Inheritance and nature of resistance in beans to *Fusarium oxysporum* f. sp. *phaseoli*. *Phytopathology*. 69(8): 859-861.

Rozen S and Skaletsky H (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol*. 132: 365-86.

Salgado MO and Schwartz HF (1993). Physiological specialization and effects of inoculum concentration on *Fusarium oxysporum* f. sp. *phaseoli* in common beans. *Plant Dis*. 77(5): 492-496.

Salgado MO and Schwartz HF (1995). Inheritance of resistance to a Colorado race of *Fusarium oxysporium* f. sp. *phaseoli* in common beans. *Plant Dis*. 79(3): 279-281.

Sasseron GR, Benchimol-Reis LL, Perseguini JMKC, Paulino JFC, et al. (2020). *Fusarium oxysporum* f. sp. *phaseoli* genetic variability assessed by new developed microsatellites. *Genet. Mol. Biol.* 43(2): e20190267.

Sartorato A and Rava CA (1994). Murcha ou amarelecimento de Fusarium. In. Sartorato, A, Rava, C.A (Eds). Principais doenças do feijoeiro comum e seu controle, p.175-190.

Sharma KD, Hemlata Rathour R, Kapila RK and Paul YS (2018). Detection of pea wilt pathogen *Fusarium oxysporum* f. sp. *pisi* using DNA-based markers. *J. Plant Biochem. Biotechnol*.27: 342-350.

Taylor J (2008). Cryo-Fracture or Freeze-Fracture, a Method to Expose Internal Tissue Surfaces and Cell Surfaces for Viewing in the Scanning Electron Microscope. *Micros. Today* 16(4): 56-59.

Vargas WA, Martín JM, Rech GE, Rivera LP, et al. (2012). Plant defense mechanisms are activated during biotrophic and necrotrophic development of *Colletotricum graminicola* in maize. *Plant Physiol.* 158(3): 1342-58.

Wendland A, Lobo Júnior M and Faria JC (2018). Manual de identificação das principais doenças do feijoeiro comum. Brasília: Embrapa 49 p.

Woo SL, Zoina A, Del Sorbo G, Lorito M, et al. (1996). Characterization of *Fusarium oxysporium* f. sp. *phaseoli* by pathogenic races, VCGs, RFLP, and RAPD. *Phytopathology*. 86 (9): 966-973.

Xue R, Wu J, Zhu Z, Wang L, et al. (2015). Differentially Expressed Genes in Resistant and Susceptible Common Bean (*Phaseolus vulgaris* L.) Genotypes in Response to *Fusarium oxysporum* f. sp. *phaseoli*. *PLOS ONE*. 10(6): e0127698.