GMR

The Original by FUNPEC-RP

Infection and colonization of common bean by EGFP transformants of *Fusarium oxysporum* f. sp. *phaseoli*

R.O. Batista[1], T.S. Leite[2], A. Nicoli[1], J.E.S. Carneiro[3], P.C.S. Carneiro[3], T.J. Paula Junior[4] and M.V. Queiroz[5]

[1] Instituto de Ciências Agrárias, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Unaí, MG, Brasil
[2] Instituto Federal do Sudeste de Minas Gerais, Campus Manhuaçu, Distrito Realeza, Manhuaçu, MG, Brasil
[3] Programa de Pós Graduação em Genética e Melhoramento, Universidade Federal de Viçosa, Viçosa, MG, Brasil
[4] Empresa de Pesquisa Agropecuária de Minas Gerais, Viçosa, MG, Brasil
[5] Departamento de Microbiologia, Universidade Federal de Viçosa, Viçosa, MG, Brasil

Corresponding author: R.O. Batista
E-mail: renata.batista@ufvjm.edu.br

Genet. Mol. Res. 18 (4): gmr18370
Received May 17, 2019
Accepted August 19, 2019
Published October 30, 2019
DOI http://dx.doi.org/10.4238/gmr18370

ABSTRACT. Fusarium wilt caused by the fungus *Fusarium oxysporum* f. sp. *phaseoli* (*Fop*) is one of the most important diseases of common bean and occurs in many regions of the world. However, there is little available information that could elucidate when and where the fungus attacks common bean plants. To study the colonization of common bean by this fungus, we transformed *Fop* Brazilian race 2 for *egfp* gene expression and used these fungal transformants to analyze the colonization process in common bean roots and stems. The combined use of driselase and lysing enzyme was sufficient for *Fop* mycelium protoplastization. The PEG-$CaCl_2$ transformation protocol allowed us to obtain stable transformed fungi, with colonies expressing the *egfp* gene. Regarding colonization by *Fop* transformants, the fungus grew intercellularly on the root hair epidermis at six days post-inoculation (DPI) and on parenchymal

cells at 11 DPI on plants of the susceptible line. At 19 DPI, the fungus reached xylem vessels, blocking water and mineral passage in shoots, resulting in wilt and plant death at 25 DPI. Using fluorescence to mark the fungus revealed details of *Fop* colonization of common bean tissues.

Key words: Fusarium wilt; *Phaseolus vulgaris*; Plant-pathogen interaction

INTRODUCTION

The common bean (*Phaseolus vulgaris*) is one of the most important food crops for human consumption, mainly because it is a rich source of protein, complex carbohydrates, and micronutrients (Nemli et al., 2015). In Brazil, the common bean is a principal source of daily protein consumption (Toledo et al., 2013). Unfortunately, its production is severely constrained by many pathogens, resulting in yield loss (Paula Junior and Wendland, 2012). Fusarium wilt of the common bean is caused by the soilborne fungus *F. oxysporum* f. sp. *phaseoli* (*Fop*). In Brazil it is the most important bean soil disease, occurring in many states and *Fop* isolates have high molecular diversity (Toledo‑Souza et al., 2012; Cruz et al. 2018). This disease results in severe losses that have gradually increased in Brazil, which is mainly in central pivot irrigation, due to intensive production practices, such as planting beans in succession in the same area (Ramalho et al., 2012; Paula Junior et al., 2015).

The fungus *Fop* infects the root system, moving through the epidermis, cortex and endodermal tissues and penetrating the xylem vessels through the pits. The main symptoms of fusarium wilt include foliar chlorosis, premature senescence of lower leaves, red-brown discoloration of vascular tissue and wilting, often lead to early maturity, reduced seed size, yield loss and plant death (Abawi and Pastor-Corrales, 1990; Brick et al., 2006). The most effective measure adopted by farmers to control economic losses from this pathogen is the use of resistant cultivars (Abawi and Pastor- Corrales, 1990; Brick et al., 2006; Paula Junior et al., 2015).

The *gfp* gene expressing **G**reen **F**luorescent **P**rotein (GFP) was amplified from the genome of the jellyfish *Aequorea victoria* (Chalfie and Kain, 1998) and has been widely used as a molecular marker to study the colonization of many different pathogens in plant tissues such as banana (Li et al., 2011), carnation (Sarrocco et al., 2007), tomato (Lagopodi et al., 2002), gladiolus (Lakshman et al., 2012), melon (Zvirin et al., 2010) and others crops. In the common bean, Niño-Sánchez et al. (2015) transformed two specific isolates of *Fop* from Spain to study the dynamics of colonization in roots and hypocotyl tissues.

We transformed *F. oxysporum* f. sp. *phaseoli* Brazilian race 2 for enhanced GFP (EGFP) expression to study infection and colonization in common bean tissues using *Fop*-transformed isolates.

MATERIAL AND METHODS

Fungal strain

Isolate *Fop* 46 denoted as Brazilian race 2 wildtype (WT) (Ribeiro and Hagedorn, 1979), was kindly provided by Centro Nacional de Pesquisa Arroz e Feijão (Embrapa Arroz

e Feijão). The isolate was grown at 25°C with 12 h under white light on Potato Dextrose Agar (PDA). Mycelial discs 8 mm in diameter were removed from the cultures and stored in sterile distilled water at 4°C for later use (Castellani, 1939).

Plasmid vector

In this study, we used the pSM1 plasmid vector (Pöggeler et al., 2003), which contains the *Escherichia coli* Hygromycin B phosphotransferase (*hph*) gene and the *Aequorea victoria egfp* gene. The *egfp* gene encodes EGFP, and the *hph* gene encodes resistance to Hygromycin B antibiotic, a common dominant selectable marker for fungal transformation.

The pSM1 plasmid was propagated according Sambrook et al. (1987) using the *E. coli* competent strain DH5α and purified using the PhoenIX™ Maxiprep Kit (MP Biomedicals, Santa Ana, CA, United States) according to the manufacturer's instructions.

Minimum inhibitory concentration (MIC)

To find the minimum concentration of Hygromycin B (from *Streptomyces hygroscopicus* - Sigma-Aldrich, Product number: H7772-1G) that inhibited the growth of *Fop* race 2 WT, the fungal culture was grown on PDA supplemented with different concentrations of Hygromycin B ($\mu g.mL^{-1}$): 25, 50, 100, 150, 200, and one negative control without antibiotic. For each concentration, we did three replications and incubated the plates at 25°C for 10 days.

Isolation of fungal protoplasts

Fop race 2 mycelia were collected from PDA plates, inoculated in Potato Dextrose Broth (PDB) and incubated at 25°C for 40 h with continuous shaking at 120 rpm. After incubation, the mycelium was collected on filter paper and washed three times with a saline solution (0.9% NaCl w/v). We weighed 480 mg of mycelium and transferred it to a 250 mL Erlenmeyer flask for protoplast isolation according to Pecchia and Anné (1989) with some modifications. Enzyme solutions were prepared with Driselase at 10 $mg.mL^{-1}$ (Driselase from *Basidiomycetes* sp. - Sigma-Aldrich, Product number: D9515-1G) dissolved in 10 mL of 0.7 M NaCl (pH 6.0) and filtered through a 0.22µm membrane to retain carbohydrates (mainly starch). Lysing Enzyme at 10 $mg.mL^{-1}$ (enzyme from *Trichoderma harzianum* - Sigma-Aldrich, Product number: L1412-25G) was added to the initial enzyme solution, and the solution of enzymes was added to the flask containing the mycelium. The suspension was incubated at gentle shaking (70 rpm) at 28°C, and protoplast release was monitored at every hour for 3 h by the microscopic observation of 10 µL in a hemocytometer. After 3 h, the solution was then passed through four layers of cheesecloth to retain mycelium and centrifuged at 1000 rpm for 10 min at 5°C, after which protoplasts were obtained in the supernatant fluid and the remaining mycelium was pelleted. The pellet was washed in 0.7 M NaCl (pH 6.0) and centrifuged again (1000 rpm, 10 min, 5°C) to collect any remaining protoplasts. Protoplast suspension was centrifuged (2500 rpm, 20 min, 5°C) and resuspended in 0.7 M NaCl (pH 6.0) to a final concentration of 10^6 protoplasts mL^{-1}. The solution was added to microtubes (1.5 mL) that was placed on ice for posterior use.

Fungal transformation

Fop race 2 WT was genetically transformed according to Sarrocco et al. (2007) with some modifications. The plasmid pSM1 (5 µg) was mixed with 200 µL of protoplast solution and 50 µL of PEG solution (25% polyethylene glycol 6000, 50 mM $CaCl_2$, 1 M Sorbitol, 50 mM Tris-HCl- pH 7.5) and incubated on ice for 20 min. Then, 500 µL of PEG Solution was added, and the suspensions were incubated at room temperature for 5 min. Aliquots of 250 µL were poured into Petri dishes containing 20 mL of regenerating medium (PDA supplemented with 1 M Sorbitol). Plates were incubated at 25°C and overlaid after 16 h with 5 mL of selective molten semisolid PDA medium [0.8% (w/v) agar, 45°C] supplemented with hygromycin B at MIC.

Protoplast regeneration and mitotic stability

Regeneration process of the protoplasts was observed at regular intervals beginning 72 h after antibiotic + PDA addition. Putative transformants that grew through the selective agar layer were transferred to fresh selective agar plates supplemented with Hygromycin B at MIC. These transformants were submitted to six consecutive subcultures on selective and non-selective PDA medium to confirm mitotic stability. Before each subculture, the fluorescence from the mycelium expressing the *egfp* gene was checked under a fluorescence microscope Olympus (Model IX 50) with a digital camera Q Color 3 (Olympus PM-C35DX) coupled to the program Q Capture Pro 6). UV fluorescent light with the filter U-MWU2 was used for EGFP detection.

Fungal colonies that maintained their growth and EGFP fluorescence were considered stable transformant strains. Single-spore colonies were obtained from fluorescent mycelia. Mycelial discs from fungal transformants were stored such as the fungal WT.

DNA extraction and PCR reaction

Fop WT strain and transformants were grown over cellophane on non-selective and selective PDA, respectively. With seven days, the mycelium was collected for DNA extraction in agreement with Specht et al. (1982). PCR was performed to detect the phosphotransferase gene (*hph*) in the putative transformants. An Eppendorf Mastercycler (Eppendorf, Germany) was programmed to carry out the initial denaturation at 95°C for 2 min, followed by 39 cycles at 95°C for 1 min, 60°C for 1 min and 72°C for 1 min and a final elongation at 72°C for 7 min. Amplification was performed on a final volume of 25 µL containing 5.0 µL of Go Taq® Flexi Colorless Buffer [5X] (Promega, Madison, Wisconsin, USA); 2.5 µL of $MgCl_2$ [25 mM] (Promega, Madison, USA); 1.0 µL of dNTPs [2.5 mM of each dNTP]; 1.0 µL of the primer hph1 (5’ CAGCGAGAGCCTGACCTATTGC 3’) [5 µM]; 1.0 µL of the primer hph2 (5’ GCCATCGGTCCAGACGGCCGCGC 3’) [5 µM]; 0.25 µL of Go Taq® DNA Polymerase [5 U/µL] (Promega, Madison, USA); 1.0 µL of genomic DNA [150 $ng.\mu L^{-1}$]; and 13.25 µL of autoclaved ultrapure water. After amplification, the PCR products of the *hph* gene (~690 bp) were separated by electrophoresis in a 1.2%

agarose gel (w/v) stained with ethidium bromide (0.2 µg.mL^{-1}). ϕX174/*HaeIII* (Promega, Madison, Wisconsin, USA) was used as a molecular size marker.

Southern Blotting

The integration of the *hph* gene in the genome of transformants was checked by Southern Blotting. The genomic DNA of *Fop* race 2 WT isolate and from putative transformants were digested with the restriction enzyme *Hind*III (Promega, Madison, Wisconsin, USA), which cut once within the integrated pSM1 plasmid, outside the *hph* gene. The digestion reaction was performed for a final volume of 50 µL with 8 µL of DNA (1 µg.µL^{-1}), 4 µL of *Hind*III (10 U.µL^{-1}), 10 µL of Buffer E (10X) (Promega, Madison, Wisconsin, USA), 1 µL of BSA (Bovine Serum Albumin Acetylated) (Promega, Madison, Wisconsin, USA) and 27 µL of ultrapure water to complete the volume. The pSM1 plasmid was the positive control. The reactions were placed in a water bath at 37°C overnight. After the cleavage, the digestion reactions were stopped by placing them in a water bath for 20 min at 80°C. The DNA digests were separated by 0.8% agarose electrophoresis run for 12 h at 40 V using the TAE 1X buffer (40mMTris-HCl, 20mM acetic acid, and 1mM EDTA).

Before blotting, the gel was treated with denaturation solution (1.0 M NaCl and 0.4 M NaOH for 1 h) and neutralization solution (1.0 M NaCl and 0.5 M Tris-HCl pH 7.2 for 1 h). Then, the DNA was transferred by capillary from the gel to a 0.45-µM nylon membrane (Amersham Hybond™-N^{+},GE Healthcare®). Pre-hybridization, hybridization, and post-hybridization washes were performed in agreement with Sambrook and Russell (2001). Hybridization was carried out using as a probe the ~690-bp PCR product of the *hph* gene present in pSM1. The probe was labelled using the PCR DIG Probe Synthesis Kit (Roche®, Germany), and the hybridizing bands were detected using the DIG DNA Labeling and Detection Starter Kit version 19 (Roche®, Germany) according to the manufacturer's instructions. DIG-labeled hybrids were detected using an anti-DIG-alkaline phosphatase conjugate and the substrates NBT (nitroblue tetrazolium salt) and BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt), which give a light blue precipitate. For each transformant, the number of integrated copies of pSM1 was counted using the *hph* gene as a probe.

Plant inoculation

Two susceptible and one resistant common bean genotypes were inoculated with two *Fop* race 2 *egfp* transformants. The genotypes were BRS Estilo and CNFC 11965 (both susceptible), as well as Pérola (resistant). The spore suspension of *Fop* WT, *Fop* race 2 *egfp* transformants and inoculation of common bean plants were prepared according to Pastor-Corrales and Abawi (1987) with the modifications cited by Batista et al. (2016). After the inoculation the seedlings were maintained in a greenhouse at Experimental Field Diogo Alves de Melo at the Federal University of Viçosa, Minas Gerais, Brazil (20°45' S and 42°51' W). For each plant genotype, 16 replications were obtained for each transformant, except the control plants that was

formed by four seedlings. Each pot was one repetition. Plants were grown at variable temperatures (20 - 25°C), irrigated every day or when necessary. All of the pots were fertilized 10 days post-inoculation (DPI) using urea as a nitrogen source. As soon as the symptoms appeared, the plants were analyzed.

Histopathological observation

Fluorescence Microscopy - Root hairs sampled from common bean were analyzed by fluorescence microscopy. Each of the examined root hairs was placed on a microscope slide with water and covered with a glass coverslip. UV fluorescent light with the filter U-MWU2 was used for EGFP detection. The fluorescence microscope that was used was Olympus (Model IX 50) with a digital camera Q Color3 coupled to the program Q Capture Pro 6.

Confocal Laser Scanning Microscopy - Stem and root sections were cut on a microtome (Model LPC, Rolemberg and Bhering), putted on microscope slide and visualized in Zeiss Laser LSM 510 Meta - Confocal Scanning Microscope. The specific excitation and emission of the EGFP protein were affected by excitation at 488 nm (first dichroic optical filter HFT 488/543) with an Argon laser (transmission 20%), collecting emitted light with a KP 545-nm filter, and using a plate for the second dichroic and no plate for the third channel. Images were acquired and observed using the Zeiss LSM Image Browser and processed using Adobe Photoshop (version 6.0).

RESULTS

Minimum inhibitory concentration

The MIC trial showed growth of *Fop* WT partially inhibited at 150 µg.mL^{-1} and totally inhibited at 200 µg.mL^{-1} hygromycin B (Figure 1). Thus, transformants were selected using 200 µg.mL^{-1} hygromycin B.

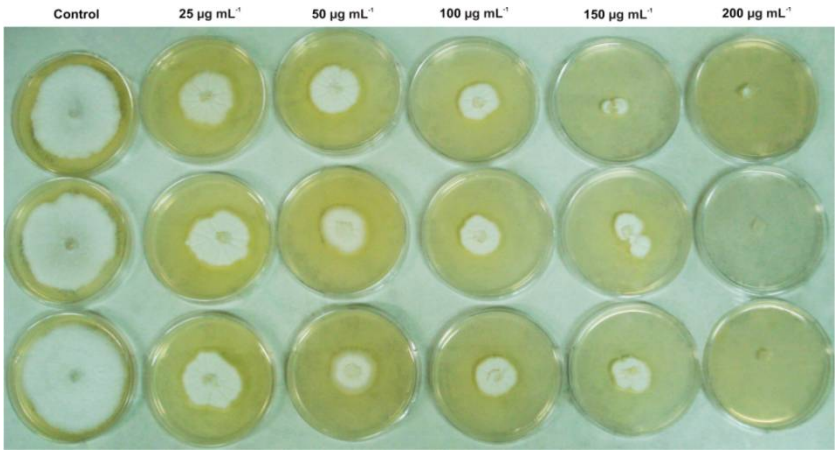

FIGURE 1. Assay of the resistance of *Fop* Brazilian race 2 to increasing concentrations of hygromycin B antibiotic to determine the minimum inhibitory concentration.

Isolation and regeneration of protoplasts

Uptake of protoplast was efficient with use of Driselase and Lysing Enzyme, obtained largest number of protoplasts after 3 h of digestion. Increasing this digestion time decreased the concentration of protoplasts. Approximately 90% of the mycelium of *Fop* race 2 formed a spherical shape (protoplast) as viewed under an optical microscope using a hemocytometer. The regenerative capacity of fungal protoplasts was efficient with 1 M Sorbitol (an osmotic stabilizer) and can be noted by the intense mycelium growth, identical to that of *Fop* WT.

The transformants and Southern Blotting

The transformant *Fop* colonies became visible four days after transformation, producing abundant mycelia. From a total of 200 hygromycin-resistant colonies that grew on the selective medium (PDA + 200 $\mu g.mL^{-1}$ hygromycin B), 60 colonies were able to express EGFP. The EGFP protein was visualized in the cytoplasm because of its fluorescence of mycelial cells and in the spores of pathogen (Figure 2 A-F).

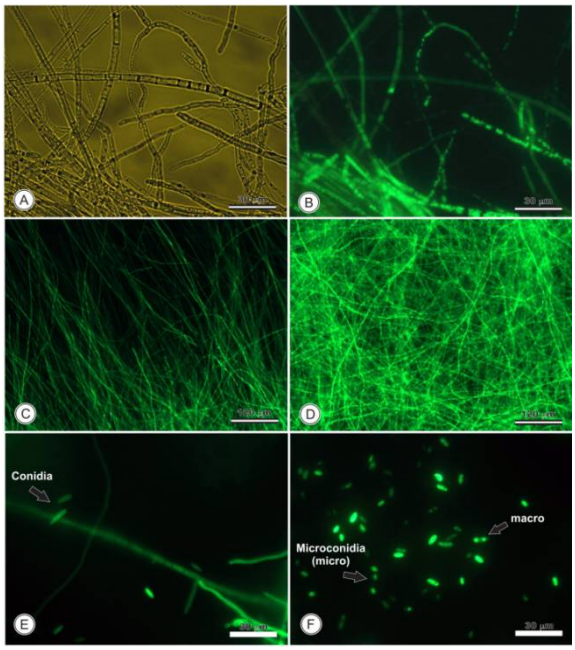

FIGURE 2. Microscopic images of the EGFP transformant mycelium of *Fop* race 2 observed under white light (A) and UV fluorescent light using the filter U-MWU2 (B). Fluorescence microscopy of *Fop* mycelium showing bright green fluorescence characteristic of EGFP expression. EGFP expression is distributed throughout the mycelial cytoplasm (C and D) and even in the spores: macro- and microconidia (E and F).

The stability of *egfp-Fop* transformants was analized by six consecutive transfers of a 9-mm-diameter plug (ten days old) of each putative transformant to selective medium alternated with non-selective medium. After all, from a total of 60 colonies, we selected five hygromycin-resistant colonies (EGFP1, EGFP2, EGFP3, EGFP4 and EGFP5) for later use. The growth, structures and morphology of the colonies were similar to *Fop* WT. They also showed a uniform and strong fluorescence EGFP, indicating presence of the *egfp* gene (Figure 2 C-F).

The integration of pSM1 containing *hph* into the genome of the transformants EGFP1, EGFP2, EGFP3, EGFP4, and EGFP5 was proved by PCR (Figure 3) with primers hph1 and hph2 amplified 690-bp DNA fragments that corresponded to *hph*.

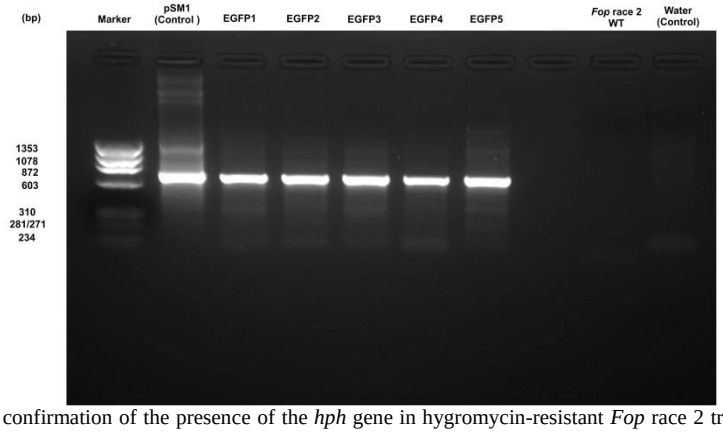

FIGURE 3. PCR confirmation of the presence of the *hph* gene in hygromycin-resistant *Fop* race 2 transformants. PCR was carried out using genomic DNA and the hph1 and hph2 primers, which amplified a 690-bp DNA fragment. Lane 1: DNA molecular size marker ϕX174/*Hae*III; Lane 2: positive control with the pSM1 plasmid vector; Lanes 3-7: genomic DNA from hygromycin-resistant *Fop* race 2 transformants (EGFP1 to EGFP5); Lane 9: genomic DNA from *Fop* race 2 WT (negative control); and Lane 10: ultrapure water (negative control).

Southern Blotting hybridization using as a probe the PCR product of the *hph* gene showed plasmid integrated into genomic DNA in one (EGFP4 and EGFP5) or more (EGFP1, EGFP2, and EGFP3) positions (Figure 4). Thus, single or multiple integrations of the plasmid were detected in the five transformants, and the hybridization pattern confirmed that all of the integrations in the fungal genome occurred randomly.

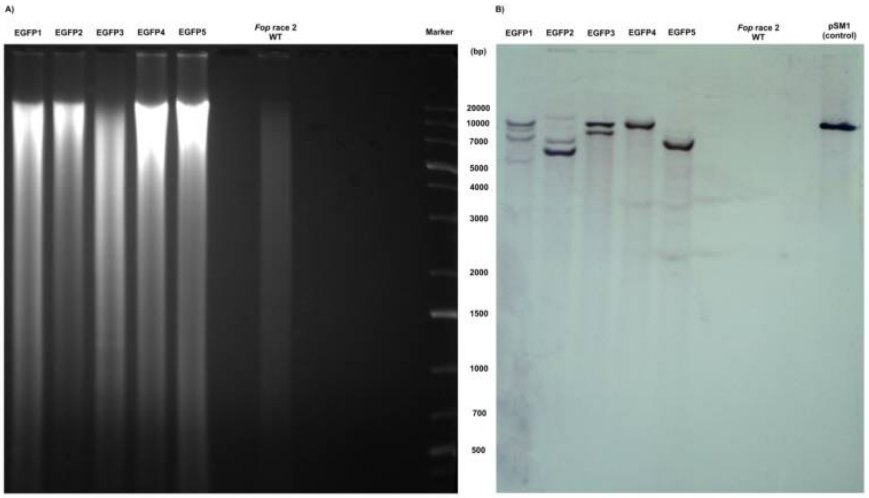

FIGURE 4. (A) Agarose gel electrophoresis (0.8%) of genomic DNA digested with the enzyme *Hind*III. Lanes 1-5: genomic DNA from *Fop* race 2 transformants (EGFP1 to EGFP5); Lane 7: genomic DNA from *Fop* race 2 WT; and Lane 11: DNA molecular size marker O'GeneRuler^{TM} 1 Kb Plus DNA Ladder (Thermo Scientific Fermentas, United States). (B) Southern blotting using the PCR product of the *hph* gene as a probe. Lanes 1-5: profile of bands of plasmid integrations and the corresponding sizes of the hybridized DNA fragments in the genomic DNA from *Fop* race 2 transformants (EGFP1 to EGFP5); Lane 7: genomic DNA from *Fop* race 2 WT (negative control for *hph* gene); and Lane 9: pSM1 plasmid vector (positive control).

Pathogenicity

No difference in the morphological, structural or growth characteristics was observed between *Fop* transformants and WT on PDA. The transformants EGFP4 and EGFP5 with just one copy of the plasmid in their genome were selected for inoculation in common bean. When inoculated in susceptible genotypes (BRS Estilo and CNFC 11965), these transformants caused fusarium wilt symptoms similar to WT. At 25 DPI, we observed wilting, leaf fall, followed by plant death (Figure 5A-B), with conidia in the stem (arrow in Figure 5C). There was no colonization or symptoms on the resistant cultivar (Pérola).

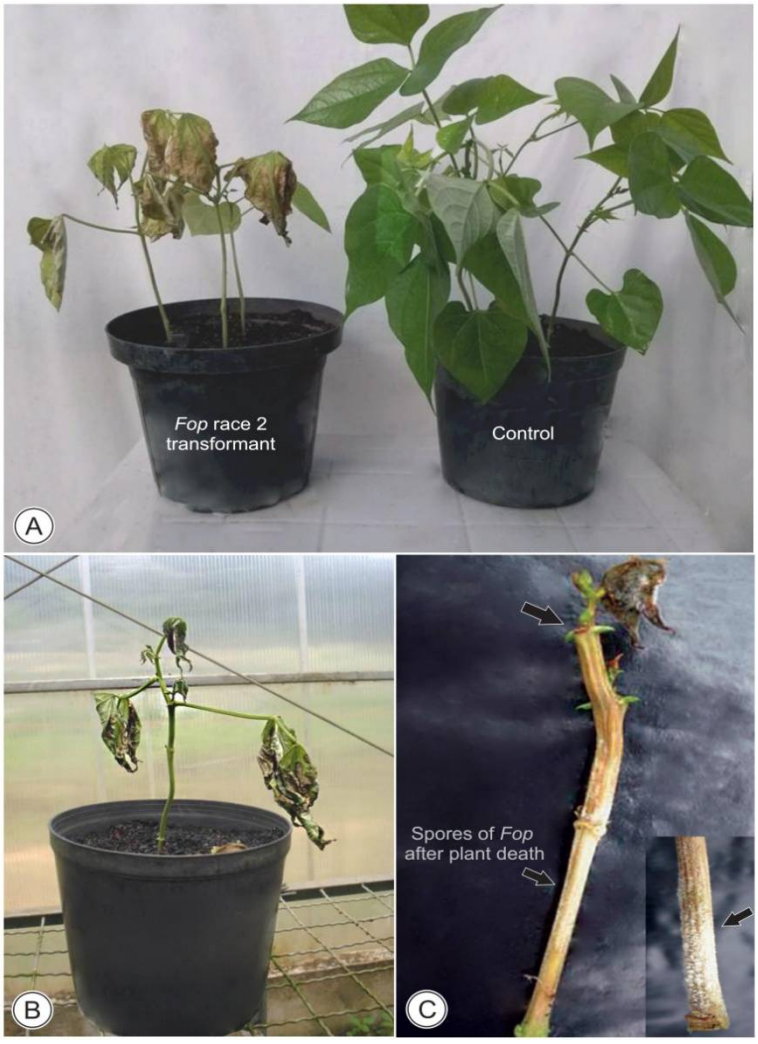

FIGURE 5. (A) Plants of the susceptible cultivar BRS Estilo inoculated with *Fop* race 2 transformants (EGFP4 or EGFP5) (left pot) and control plants (without pathogen inoculation - right pot) 20 days post-inoculation. (B) Wilted shoot and necrosis of leaves of the susceptible cultivar CNFC 11965 25 days post-inoculation. (C) Plant death with falling leaves, apex necrosis and pathogen sporulation in the susceptible cultivar BRS Estilo 25 days post-inoculation.

egfp-Fop colonization in common bean tissues

Using fluorescence microscopy, we found that the transformants EGFP4 and EGFP5 colonized roots systems from susceptible genotypes, first colonizing the root surface and penetrating the root hair epidermal cells (Figure 6 A-B). Approximately 6 DPI, *egfp-Fop* began colonization with hyphae growth throughout the intercellular spaces between root hair epidermal cells (Figure 6 A-B). The infection progressed to the main root, stem, and vascular cylinder. Unfortunately, using fluorescence microscopy, we could not perform internal root observation due the occurrence of common bean autofluorescence (Figure 6 E-H).

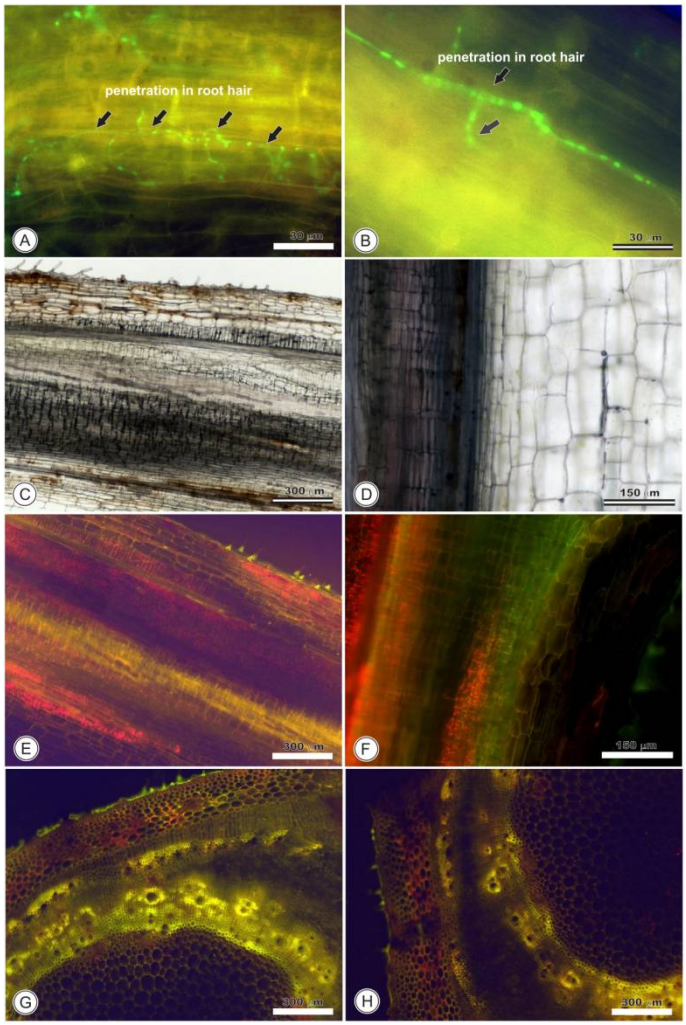

FIGURE 6. Fluorescence microscopy images of the penetration and colonization of *Fop* race 2 EGFP4 into the root hairs of a susceptible cultivar of common bean (A and B). Fluorescence images of longitudinal sections of common bean stems under white light (C and D) and UV fluorescent light (E and F) showing common bean tissue autofluorescence. Transverse section with intense autofluorescence (G and H).

In tissues of susceptible genotypes the root colonization to the stem was observed from 2 to 5 DPI under confocal microscopy. The colonization of the vascular cylinder was observed just at 19 DPI, while the colonization of parenchymal cells grow into the vascular cylinder at 21 DPI (Figure 7 A-F). At 25 DPI, the plant apex had severe necrosis and then plant death.

The detection of fungal transformants expressing EGFP occurred up to 11 DPI in the roots of susceptible genotypes. In the stem and parenchymal cells the transformants did not express EGFP (Figure 7 A-F). Finally, we could follow and confirm root colonization by the development of EGFP4 and EGFP5 in common bean tissues due to wilting, besides of mycelial and conidia during colonization (Figure 7 A-F).

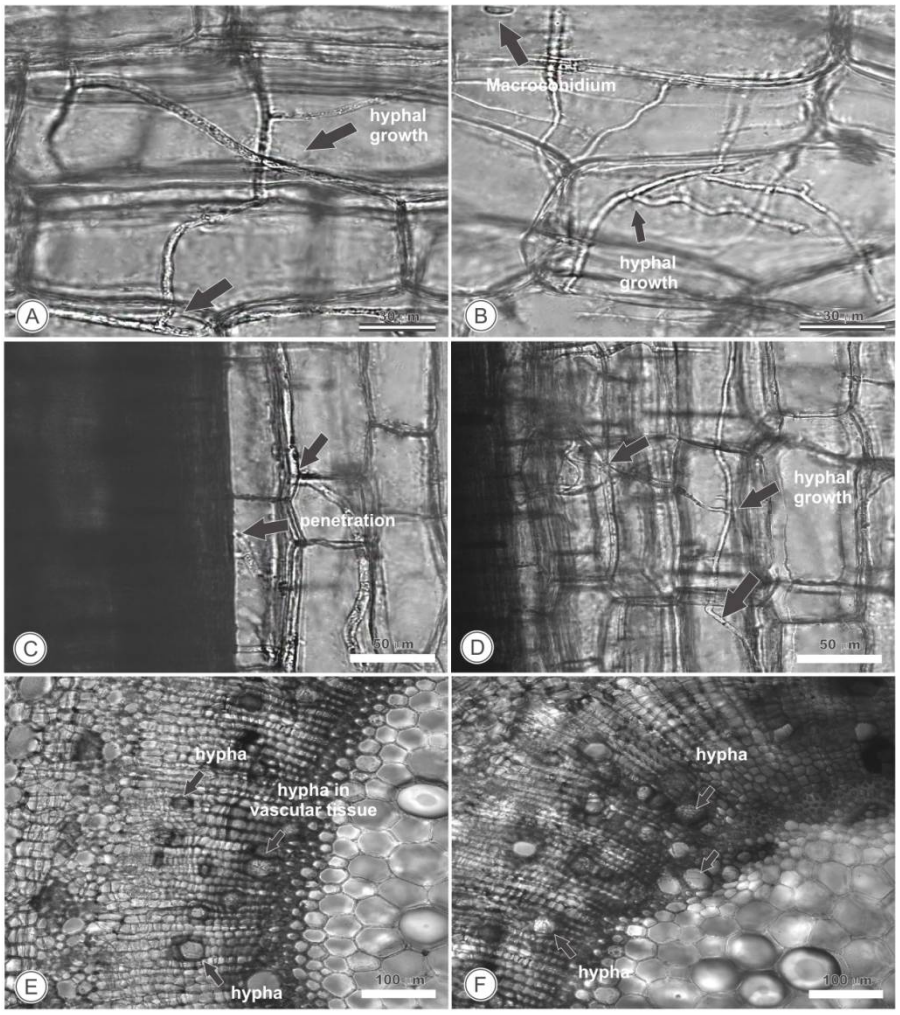

FIGURE 7. Confocal images of the longitudinal sections of common bean stem parenchymal cells colonized by *Fop* race 2 EGFP4 transformant (A and B). Colonization of parenchymal cells with mycelial growth in the direction of the vascular tissue (C and D). Hyphal growth in the vascular tissue (xylem vessels) of transverse sections of common bean stems (E and F).

DISCUSSION

Studies aiming to elucidate the infection process and dynamics of the colonization of common bean by *Fop* provide relevant information for understanding the pathosystem. Our work reports the production, isolation and analysis of *Fop* transformants expressing EGFP and their use to follow the common bean colonization process.

MIC

To select transformants of *Fop*, the MIC of the antibiotic Hygromycin B, a common dominant selectable marker, was determined. In this study, *Fop* race 2 WT was completely inhibited at 200 µg.mL^{-1} Hygromycin B (Sigma-Aldrich) in PDA. In contrast, 100 µg.mL^{-1} Hygromycin B was sufficient to inhibit WT colony growth in many studied *formae speciales* of *F. oxysporum* (Nonomura et al., 2001; Zvirin et al., 2010; Lakshman et al., 2012).

Isolation and regeneration of protoplasts

A total of 10^7 protoplasts mL^{-1} of *Fop* race 2 WT were obtained within 3 h of enzyme digestion. However, when we increased the digestion time, the number of protoplasts decreased. The bursting of protoplasts might be caused by membrane injury from the protease present in Lysing Enzyme. The proteolytic activity present in some enzymes can affect protoplastization efficiency and protoplast integrity (Liu and Zhu, 2000; Patil et al., 2015). The osmotic stabilizer used for protoplastization (NaCl 0.7 M pH 6.0) could maintain favorable conditions for enzymatic digestion and could maintain protoplast integrity until a new cell wall could regenerate (Singhvi et al., 2013; Zhao et al., 2014). Sorbitol benefited the protoplast regeneration in solid medium as demonstrated by the abundant mycelial growth in two days. This osmotic stabilizer has been successfully used for fungal protoplast regeneration (Sarrocco et al., 2007; Morocko-Bicevska and Fatehi, 2011; Feng et al., 2012). The protoplasts that were obtained were used for fungal transformation.

Transformation and Southern Blotting

EGFP detection was easily visualized using a fluorescence microscope, which made it possible to notice fungal structures expressing the fluorescent protein. The *egfp* gene encodes a protein that is produced in the cell cytoplasm, causing its visualization in all fungal structures, such as the mycelium, spores (macro- or microconidia), and cell wall. Microconidia with strong fluorescence demonstrated that fluorescence persisted throughout fungal reproduction.

After transformation, *egfp-Fop* race 2 transformants were tested for the genetic stability of the integrated plasmid. The transformants continued to express *egfp*, even after six transfers into new PDA medium with and without Hygromycin B. This result indicated that these are stable transformants. In addition, these transformants had similar growth characteristics, asexual structures and morphology to those of *Fop* race 2 WT and could be used for common bean inoculation.

Southern Blotting hybridization from the transformants showed single-plasmid integration into the genome of the transformants EGFP4 and EGFP5 while the other three transformants showed the insertion of two or three plasmid copies. In general, many copies of the plasmid should be integrated in the fungus genome mediated by PEG/$CaCl_2$ transformation using heterologous genes, but in this study, a single integration was obtained. Some studies that transformed *F. oxysporum* using the PEG/$CaCl_2$ system also obtained a single integration of the *egfp* gene and stable transformants (Pietro and Roncero, 1998, Nonomura et al., 2001, Visser et al., 2004, Sarrocco et al., 2007).

Common bean autofluorescence

On the fluorescence microscopy images, when we followed fungal colonization, we could observe the presence of common bean autofluorescence. Intense bright red and green colors of the main root and sections of stem tissues characterized this phenomenon. There is no information about common bean anatomy or autofluorescence in the literature. Using fluorescence microscopy, it was not possible eliminate common bean autofluorescence. However, using confocal microscope, filters that restrict the wavelengths of excitation and emission to nearly EGFP values (488 nm and 525 nm, respectively) could eliminate autofluorescence. Because of this, from this stage we decided to use only the confocal microscope.

Fluorescence of egfp-Fop transformants

In this study, *Fop* transformants isolates caused the common bean to die at 25 DPI. In the same way, using the disease severity scale of Pastor-Corrales and Abawi (1987), the authors found that *Fop* takes 21 days to kill susceptible genotypes. Niño-Sánchez et al. (2015) conducted assays to evaluate the patterns and dynamics of GFP transformants from *Fop* colonization in common bean plants grown in hydroponic culture. Plants inoculated with an aggressive strain were almost dead and showed a complete necrosis of the crown region at 21 DPI.

As seen in confocal microscopy images, *Fop* transformants intercellularly colonized the root parenchymal cells and were slow to reach the xylem vessels. Niño-Sánchez et al. (2015) reported the dynamics of highly and weakly aggressive *Fop* strains transformed to express the *gfp* gene by observing the colonization of common bean. The highly aggressive isolate presented a rapid initial colonization stage of parenchymal cells, after which it restricted its growth to the xylem vessels. On the other hand, the weakly aggressive isolate showed intense growth in the intercellular spaces of the parenchymal cells of the root but weak growth in the xylem vessels. This statement agreed with the growth observed in *Fop* race 2 transformants that had predominant parenchymal cells colonization and was a less efficient colonizer of the xylem vessels.

In confocal microscopy, the fluorescent fungal structures were not visualized in common bean tissues. The predominant intercellular colonization by *Fop* in the parenchymal cells of the root and crown and the weak colonization of the xylem vessels worsen the fungal observation. Thereat, we suggest that the long-term exposure of EGFP to laser excitation altered the fluorescent protein (Lippincott-Schwartz et al., 2003). This phenomenon, called photobleaching, has been previously reported (White and Stelzer,

1999; Heikal et al., 2001; Bogdanov et al., 2012). Photobleaching is a possible reason for the absence of EGFP visualization in the fungal structures in common bean tissues. In studies with fluorescence microscopy, brightness and photostability are desirable characteristics of fluorescent proteins for successful application (Bogdanov et al., 2012).

Because the hyphae and others structures of the fungus were observed in common bean tissues using confocal microscopy, the absence of fluorescence in *Fop* race 2-*egfp* transformants did not prevent fungal visualization and allowed us to follow the dynamic colonization process.

egfp-Fop race 2 colonization of common bean tissues

The infection process of EGFP4 and EGFP5 transformants consisted of spore germination and penetration through injuries made in bean roots. Hyphal growth in epidermal root hairs was observed at 6 DPI. Oren et al. (2003) and Lagopodi et al. (2002) also found that the first contact between *F. oxysporum* and their hosts begins predominantly by root hairs. From 6 to 11 DPI, the colonization expanded from root parenchymal cells to xylem vessels, where the pathogen remained in growth and sporulation. At this stage of disease development, the plant shoot was wilted, resulting in colonization. This wilt became severe with disease progress, which speeds up by upward mycelial growth in conducting vessels and the flow of crude sap that carries conidia, distributing them throughout the plant (Abawi, 1989; Pereira et al., 2013). The obstruction of conducting vessels contributed to plant death at 25 DPI. The spores on the surface of the dead plant under field conditions can infect neighboring plants and spread disease. Many factors, including natural injuries, nematodes, soil pests and pathogens, and compacted and soaked soil, can facilitate *Fop* infection under field conditions by benefitting pathogen development and disease incidence.

The infection of *egfp-Fop* race 2 transformants in root hairs begins with an intercellular colonization of the root system. Later, intercellular mycelial growth was predominant in the colonization of stem parenchymal cells. The preferential hyphal growth along the intercellular space during the stages of the disease resembles that other *formae speciales* of *F. oxysporum* as described by Lagopodi et al. (2002) and Li et al. (2011). On the other hand, similar intracellular growth during the late stages of disease has been reported as well (Lagopodi et al., 2002; Olivain et al., 2006). This study contributes to the knowledge the colonization of *Fop* in common bean tissues as well as the development stages exhibited by this pathogen.

CONCLUSIONS

Based on microscopic observation using fluorescent transformation of the Fusarium fungus, common bean infection and colonization by *Fop* race 2 was apparent at six days. The fungus grew intercellularly on the root hair epidermis and on parenchymal cells at 11 days. At 19 days, the pathogen reached the xylem and blocked water and mineral transport in the shoots, resulting in plant wilt and death at 25 days.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This research was supported by FAPEMIG – Fundação de Amparo à Pesquisa do Estado de Minas Gerais (CAG APQ-01073-13), as well through grants from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors also thank Embrapa Arroz e Feijão for providing the isolate and Núcleo de Microscopia e Microanálise of UFV for their support. This work is part of the doctoral thesis of the first author.

REFERENCES

Abawi GS (1989). Root rots. In: Schwartz HF, Pastor-Corrales MA (Eds.) Bean production problems in the tropics. 2nd Ed. Cali Colombia. CIAT. p. 105-157.

Abawi GS and Pastor-Corrales MA (1990). Root rots of beans in Latin America and Africa: Diagnosis, research methodologies, and management strategies. Cali Colombia, CIAT.

Batista RO, Oliveira AMC, Silva JLO, Nicoli A, et al. (2016). Resistance to Fusarium wilt in common bean. *Crop. Breed Appl. Biotechnol.* 16: 226-233.

Bogdanov AM, Kudryavtseva EI and Lukyanov KA (2012). Anti-fading media for live cell GFP imaging. *PLOS One.* 7: e53004.

Brick MA, Byrne PF, Schwartz HF, Ogg JB, et al. (2006). Reaction to three races of fusarium wilt in the *Phaseolus vulgaris* Core Collection. *Crop Sci.* 46: 1245-1252.

Castellani A (1939). Viability of some pathogenic fungi in distilled water. *J. Trop. Med. Hyg.* 42: 225-226.

Chalfie M and Kaine S (1998). GFP - Green Fluorescent Protein properties, applications and protocols. Wiley-Liss, New York, 385 p.

Cruz AF, Silva LF, Sousa TV, Nicoli A, et al. (2018). Molecular diversity in *Fusarium oxysporum* isolates from common bean fields in Brazil. *Eur. J. Plant. Pathol.* 152: 343-354.

Feng HT, Sun ZG, Li HJ, Qin O, et al. (2012). Preparation, purification and regeneration optimizing research of protoplasts from *Rhizoctonia solani*. *Afr. J. Microbiol. Res.* 6: 3222-3230.

Heikal AA, Hess ST and Webb WW (2001). Multiphoton molecular spectroscopy and excited-state dynamics of enhanced green fluorescent protein (EGFP): acid–base specificity. *Chem. Phys.* 274: 37-55.

Kendrick J and Snyder W (1942). Fusarium yellows of Beans. *Phytopathology*. 32: 1010-1014.

Lagopodi AL, Ram AF, Lamers GE, Punt PJ, et al. (2002). Novel aspects of tomato root colonization and infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as a marker. *Mol. Plant-Microbe.* 15: 172-179.

Lakshman DK, Pandey R, Kamo K, Bauchan G, et al. (2012). Genetic transformation of *Fusarium oxysporum* f. sp. *gladioli* with *Agrobacterium* to study pathogenesis in gladiolus. *Eur. J. Plant Pathol.* 133: 729-738.

Li C, Chen S, Zuo C, Sun Q, et al. (2011). The use of GFP-transformed isolates to study infection of banana with *Fusarium oxysporum* f. sp *cubense* race 4. *Eur. J. Plant Pathol.* 131: 327-340.

Lippincott-Schwartz J, Altan-Bonnet N and Patterson GH (2003). Photobleaching and photoactivation: following protein dynamics in living cells. *Nature Cell Biol. Suppl*. S7-14.

Liu W and Zhu WM (2000). Production and regeneration of *Trichosporon cutaneum* protoplasts. *Process Biochem*. 35: 659-664.

Moročko-Bičevska I and Fatehi J (2011). Infection and colonization of strawberry by *Gnomonia fragariae* strain expressing green fluorescent protein. *Eur. J. Plant Pathol.* 129: 567-577.

Nemli S, Kutlu B and Tanyolac B (2015). Determination of the population structure of common bean (*Phaseolus vulgaris* L.) accessions using lipoxygenase and resistance gene analog markers. *Biochem. Syst. Ecol.* 59: 107-115.

Niño-Sánchez J, Tello V, Castillo VC, Thon MR, et al. (2015). Gene expression patterns and dynamics of the colonization of common bean (*Phaseolus vulgaris* L.) by highly virulent and weakly virulent strains of *Fusarium oxysporum*. *Front. Microbiol.* 6: 234.

Nonomura T, Matsuda Y, Takasugi M, Ootani T, et al. (2001). A monitoring system for green fluorescent protein gene-transformed *Fusarium oxysporum* in melon seedlings. *J. Gen. Plant Pathol.* 67: 273-280.

Olivain C, Humbert C, Nahalkova J, Fatehi J, et al. (2006). Colonization of tomato root by pathogenic and nonpathogenic *Fusarium oxysporum* strains inoculated together and separately in the soil. *Appl. Environ. Microbiol.* 72: 1523-1531.

Oren L, Ezrati S, Cohen D and Sharon A (2003). Early events in the *Fusarium verticillioides*-maize interaction characterized by using a green fluorescent protein-expressing transgenic isolate. *Appl. Environ. Microbiol.* 69: 1695-1701.

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

Patil NS, Patil SM, Govidwar SP and Jadhav JP (2015). Molecular characterization of intergeneric hybrid between *Aspergillus oryzae* and *Trichoderma harzianum* by protoplast fusion. *J. Appl. Microbiol*. 118: 390-398.

Paula Junior TJ and Wendland A (Eds.). 2012. Melhoramento genético do feijoeiro-comum e prevenção de doenças. Epamig, Viçosa, Brazil.

Paula Júnior TJ, Vieira RF, Teixeira H, Lobo Junior M, et al. (2015). Doenças do feijoeiro: estratégias integradas de manejo. In: CARNEIRO JES, PAULA JÚNIOR TJ, BORÉM A. (Ed.). Feijão: do plantio à colheita. Viçosa, MG: Ed. UFV, p. 270-299.

Pecchia S and Anné J (1989). Fusion of protoplast from antagonistic *Trichoderma harzianum* strains. *Acta Hortic*. 255: 303-312.

Pereira AC, Cruz MFA, Paula-Júnior TJ, 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.

Pietro AD and Roncero IG (1998). Cloning, expression, and role in pathogenicity of pg1 encoding the major extracellular endopolygalacturonase of the vascular wilt pathogen *Fusarium oxysporum*. *Mol. Plant Microbe. Interact.* 1: 91-98.

Pöggeler S, Masloff S, Hoff B, Mayrhofer S, et al. (2003). Versatile egfp reporter plasmids for cellular localization of recombinant gene products in filamentous fungi. *Curr. Genet.* 43: 54-61.

Ramalho MAP, Abreu AFB, Carneiro JES, Wendland A, et al. (2012). Murcha-de-fusário. In: Paula Junior TJ, Wendland A. editors, Melhoramento genético do feijoeiro-comum e prevenção de doenças. Epamig, Viçosa, Brazil. p 127-138.

Ribeiro R and Hagedorn D (1979). Inheritance and nature of resistance in beans to *Fusarium oxysporum* f. sp. *phaseoli. Phytopathology*. 69: 859-61.

Sambrook J, Fritsch EF and Maniatis T (1987). Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory NY, USA.

Sambrook J and Russell D (2001). Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory NY, USA.

Sarrocco S, Falaschi N, Vergara M and Nicoletti F (2007). Use of *Fusarium oxysporum* f. sp. *dianthi* transformed with marker genes to follow colonization of carnation roots. *J. Plant Pathol.* 47-54.

Singhvi M, GAikaiwari S and Digambar G (2013). Protoplast formation and regeneration in *Acetobacter pasteurianus*. *Am. J. Bioeng. Biotechnol.* 21: 37-43.

Specht CA, Dirusso CC, Novotny CP and Ullrich RC (1982). A method for extracting high-molecular-weight deoxyribonucleic acid from fungi. *Analy. Biochem.* 119: 158-163.

TOledo-Souza EDD, Silveira PMD, Café-Filho AC and Lobo Junior M (2012). Fusarium wilt incidence and common bean yield according to the preceding crop and the soil tillage system. *Pesq. Agropec. Bras.* 47: 1031-1037.

Toledo NMV, Rocha LC, DA Silva AG and Canniatti-Brazaca SG (2013). Interaction and digestibility of phaseolin/polyphenol in the common bean. *Food Chem*. 138: 776-780.

VIsser M, Gordon TR, Wingfield BD, Wingfield MJ, et al. (2004). Transformation of *Fusarium oxysporum* f. sp. *cubense*, causal agent of Fusarium wilt of banana, with the green fluorescent protein (GFP) gene. *Australas Plant Pathol.* 33: 69-75.

White J and Stelzer E (1999). Photobleaching GFP reveals protein dynamics inside live cells. *Trends Cell. Biol.* 9: 61-65.

Zhao Y, Jiang C, Yu H, Fang F, et al. (2014). Genome shuffling of *Aspergillus glaucus* HGZ-2 for enhanced cellulase production. *Appl. Biochem. Biotechnol.* 174: 1246-1259.

Zvirin T, Herman R, Brotman Y, Denisov Y, et al. (2010). Differential colonization and defence responses of resistant and susceptible melon lines infected by *Fusarium oxysporum* race 1·2. *Plant Pathol.* 59: 576-585.

