MARCELLA VIANA DE SOUSA

TRANSMISSION, COLONIZATION AND MOLECULAR DETECTION OF *Fusarium oxysporum* f. sp. *phaseoli* IN COMMON BEAN SEEDS

LAVRAS – MG

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Orientador

Dr. José da Cruz Machado

Coorientador

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APROVADA em 27 de novembro de 2013.

Dr. José Otávio Machado Menten	USP/ESALQ
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*"**Progress** is impossible **without change**,*
and those who cannot change their minds
cannot change anything".

George Bernard Shaw

BIOGRAFIA

Marcella Viana de Sousa, filha de Luiza C. Viana de Sousa e de Octávio de Sousa Filho, nasceu em 21 de fevereiro de 1981, na cidade de Lavras (MG).

Concluiu o primeiro e segundo grau no Instituto Presbiteriano Gammon, em dezembro de 1998, na cidade de Lavras (MG).

Em agosto de 1999, ingressou no curso de Agronomia na Universidade Federal de Lavras (MG), obtendo o título de Engenheira Agrônoma em janeiro de 2004. Nesse período, desenvolveu trabalhos na Epamig e no Laboratório de Patologia de Sementes.

Iniciou o curso de Mestrado em Agronomia/Fitopatologia na Universidade Federal de Lavras, em Lavras (MG), em março de 2004, concluindo-o em fevereiro de 2006.

Trabalhou na Usina Bunge Itapagipe, em Itapagipe/MG como supervisora de produção industrial de açúcar e etanol no período de agosto de 2006 à setembro de 2008.

Em março de 2010, ingressou no curso de Doutorado em Agronomia/Fitopatologia, na Universidade Federal de Lavras (MG). Durante o doutoramento, no período de agosto de 2012 a agosto de 2013, realizou o doutorado sanduíche na Iowa State University. Em dezembro de 2013 defendeu sua tese, obtendo o título de Doutora em Ciências.

GENERAL ABSTRACT

The common bean (*Phaseolus vulgaris* L.) is a crop of great economic and social importance in Brazil and one of the basic diets of the Brazilian population. Several diseases occur in this crop, causing yield losses and/or decreases in seed quality, such as Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *phaseoli* (*Fop*). This organism can be spread by seeds and it is classified as a Regulated Non-Quarantine Pest in Brazil. The current known methods for its detection and identification in seeds are blotter tests and semi-selective agar medium, followed by a pathogenicity test. The goals in this work were to extend knowledge on seed transmission of *Fop* in common bean as well as to investigate the close interaction between that fungus in infected seeds through GFP technique associated to scanning electron microscopy and to establish a protocol for detection of this fungus in seeds by real time PCR (qPCR). In paper 1, transmission rates of the pathogen from artificially and naturally *Fop*-contaminated seeds to emerged plants were tested. Two strains of *Fop*, two genotypes of bean, two environment temperatures and four inoculum potentials were used in the experiments with artificially inoculated seeds, in order to assess the symptomatic and asymptomatic plants. The frequence of symptomatic plants was lower than 5% but the transmission rates of those plants were 100%. The transmission rates of asymptomatic plants were 57% and 49.7% for BRSMG Majestoso and Ouro Negro, respectively. In respect to comparison between temperatures, the rates were 54.4% at 20 ºC and 52.3% at 25 ºC. For *Fop* strains, the transmission rates were 83.6% and 94.2% for FOP005 and FOP014. The mean rate at P3 was 64.4% and 58% at P1. From the assays with naturally *Fop*-contaminated seeds, transmission rates were lower than those determined for inoculated seeds, ranging from 8.1% to 16.7%. In paper 2, *Fop* was transformed by green fluorescent proteins (GFP) containing the resistance gene of

hygromycin-B. Seed infection by the transformed *Fop* was visualized in the embryo, including the plumule, and in the endosperm. A large amount of fluorescent mycelium was observed externally on bean seedling roots, which presented vascular discoloration, which is the typical symptom of Fusarium wilt disease. In paper 3, the results of the experiments on molecular detection of *Fop* in common bean seed samples showed that the specific primers and probe used as part of the qPCR protocol in this study were viable to detect *Fop* in infected seeds with high sensitivity, at 0.25% of *Fop* incidence. TaqMan assays provided more reliable, sensitive, effective and quicker results than SYBR Green assays, which confirm previous reports for other pathosystems. Analysis of naturally *Fop*- contaminated seeds by qPCR correlated with results of the blotter test but further studies are needed to optimize sampling and subsampling of seed health testing using PCR-based assays.

Key-words: Fusarium wilt, green fluorescent protein, seed pathology, real-time PCR, *Phaseolus vulgaris*.

RESUMO GERAL

O feijoeiro (*Phaseolus vulgaris* L.) é uma cultura de grande importância econômica e social no Brasil por ser uma das dietas básicas da população brasileira. Diversas doenças ocorrem nessa cultura, causando perdas na produção e/ou reduzindo a qualidade das sementes, como é o caso da murcha de fusarium, cujo agente etiológico é *Fusarium oxysporum* f. sp. *phaseoli* (*Fop*). Esse organismo pode ser disseminado por sementes e é classificado como Praga Não Quarentenária Regulamentada no Brasil. Os métodos conhecidos para sua detecção e identificação em sementes são o *blotter test* e o meio semiseletivo, ambos seguidos pelo teste de patogenicidade. Objetivou-se, neste estudo, ampliar os conhecimentos na transmissão de *Fop* por sementes de feijão, assim como investigar a interação entre *Fop*, em sementes infectadas por meio da técnica de GFP, associada à microscopia eletrônica de varredura, além de estabelecer um protocolo para sua detecção em sementes de feijão por PCR, em tempo real (qPCR). No artigo 1, as taxas de transmissão do patógeno foram testadas, a partir de sementes artificialmente e naturalmente associadas ao *Fop* para plantas emergidas. Dois isolados de *Fop*, duas cultivares de feijoeiro, duas temperaturas e quatro potenciais de inóculo foram utilizados nos testes a partir de sementes artificialmente inoculadas, com a finalidade de avaliar as plantas sintomáticas e assintomáticas. A frequência de plantas sintomáticas foi menor do que 5%, com taxas de transmissão de 100%. As taxas de transmissão de plantas assintomáticas foram 57% e 49,7%, para BRSMG Majestoso e Ouro Negro, respectivamente. Em relação às temperaturas, as taxas foram 54,4% à 20 ºC e 52,3% à 25 ºC. Para isolados de *Fop*, as taxas de transmissão foram 83,6% e 94,2% para FOP005 e FOP014. A taxa média no P3 foi 64,4% e, 58% no P1. A partir dos ensaios com sementes naturalmente associadas ao *Fop*, as taxas de transmissão foram menores do que aquelas determinadas a partir de sementes

inoculadas, variando de 8,1% a 16,7%. No artigo 2, *Fop* foi transformado pela inserção do gene que expressa as proteínas fluorescentes verdes (GFP) e contém o gene de resistência à higromicina-B. A infecção das sementes pelo *Fop* transformado foi visualizada no embrião das sementes, na plúmula, e no endosperma. Grande quantidade de micélio fluorescente foi observado externamente nas raízes das plântulas de feijão, as quais apresentaram escurecimento vascular, sintoma típico da murcha de fusarium. No artigo 3, os resultados dos experimentos sobre detecção molecular de *Fop*, em lotes de sementes de feijão, mostraram que primers e sonda específicos, usados como parte do protocolo de qPCR neste estudo, foram viáveis para detectar *Fop* em sementes infectadas, com alta sensibilidade, a 0,25% de incidência do patógeno em sementes. Os ensaios com TaqMan forneceram resultados mais confiáveis, sensíveis, eficientes e rápidos do que aqueles com SYBR Green, confirmando os relatos anteriores para outros patossistemas. As análises de qPCR, a partir de sementes naturalmente associadas ao *Fop*, correlacionaram com os resultados obtidos em *blotter test*, mas estudos adicionais são necessários para otimizar a amostragem e subamostragem, nos testes de sanidade baseados em PCR.

Palavras-chave: murcha de fusarium, proteína fluorescente verde, patologia de sementes, PCR em tempo real, *Phaseolus vulgaris*.

SUMMARY

CHAPTER 1

1 GENERAL INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is a legume that provides dietary proteins and plays an important role in human nutrition. Brazil is the largest producer of this crop and has been affected by several diseases, such as Fusarium wilt, caused by the necrotrophic fungus *Fusarium oxysporum* f. sp. *phaseoli* (*Fop*). This disease has attracted special interest in the last years due to the increasing dissemination of the pathogen associated with the higher degree of mechanization in the fields, successive plantings in the same area and for producing more than one cycle per year (PEREIRA; RAMALHO; ABREU, 2009).

Fop can be spread over long distance by association with seeds (TOLEDO-SOUZA et al., 2012) and has been listed as a Regulated Non-Quarantine Pest in Brazil. As a risk pathogen the tolerance level of zero in 400 seed samples submitted to laboratories has been proposed for it in certification programs in order to avoid the inoculum spreading. Thus, studies of seed-to-plant transmission of the pathogen, influence of external biotic and abiotic factors on seed transmission, dynamic of seed infection and its colonization should be well known. Pathogen detection on seeds is performed by traditional incubation methods which involve plating seeds by blotter method, observation of morphological structures by microscopy and symptoms in plants by pathogenicity test, if necessary. These methods are labor-intensive and time-consuming. The incubation diagnostic procedure is rather questionable as to the occurrence of saprophytic strains of *F. oxysporum* on diseased common bean plants, which are morphologically identical to *F. oxysporum* f. sp. p*haseoli* (ALVES-SANTOS et al., 2002a). In order to establish a protocol for seed testing

to detect that pathogen, PCR-based methods which are faster, more reliable and accurate should be tested.

In this work, studies were performed with the objectives of elucidating the transmission rate, colonization and detection of *Fop* in common bean seeds.

The aim in the first study was to calculate the seed-to-emerged plant *Fop* transmission rate and its occurrence in different plant tissues in order to understand the seed-pathogen interaction, seed transmission mechanisms and the consequence of the fungus inoculum present on seeds.

In the second study, the aim was to closely follow the seed infection and colonization process through GFP markers and scanning electron microscopy.

The third work was proposed to establish a faster, efficient and reliable PCR-based protocol of seed health testing to be used in routine analysis for detection of such a pathogen.

2 LITERATURE REVIEW

2.1 Economic importance of *Phaseolus vulgaris* L.

Common bean is the third most important food legume crop worldwide (SCHWARTZ et al., 2005). It is widely consumed throughout the world and is considered a good source of protein (23%), complex carbohydrates, dietary fiber and some vitamins and minerals (CAMPOS-VEGA et al., 2013).

Common bean is the most widely grown of the four species belonging to the genus *Phaseolus*. It is widely cultivated in North, South, and Central America, Africa, Asia and throughout Europe (SCHWARTZ et al., 2005). Myanmar, India and Brazil are the largest *Phaseolus*-producing nations in the world; however, Myanmar and India produce large quantities of *Vigna* beans while Brazil is the largest producer of common bean (FOOD AND AGRICULTURAL ORGANIZATION OF THE UNITED NATIONS - FAO, 2012) with an annual production of 2,899,000 tons (ANUÁRIO…, 2013). Brazil also remains the most important country for consumption of beans in the world, followed by Mexico (FAO, 2012). These two countries are nearly self-sufficient in the crop, but bean imports can be essential to supplement periodic production shortfalls.

The largest producing state in Brazil is Paraná with 23.37% of the national production, followed by Minas Gerais with 22.17% (ANUÁRIO…, 2013), where the black and brown beans are the favorite among the consumers. For several communities in Brazil, the common bean stands for the major economical activity and alternative for many jobs.

The common bean crop (*Phaseolus vulgaris* L.) is cultivated in a large number of farms with variable sizes and farming systems in Brazil. The plant undergoes four distinct developmental stages during its life cycle that ranges from 65 to 100 days. The time period required to complete each stage varies

among cultivars and is influenced by environmental factors (SCHWARTZ et al., 2005).

The irrigation system used in some fields in Brazil as well as inadequate management practices are causes for the occurrence of many diseases in common bean, such as Fusarium wilt. According to literature, infected seeds are the major source for spreading the pathogen over long distances (SANTOS et al., 1996; SCHWARTZ et al., 2005).

2.2 General aspects of Fusarium wilt disease in common bean

Fusarium wilt was originally discovered in dry beans in California in 1928, and later found in large areas of the United States, Brazil, Colombia, Peru, Costa Rica, Italy, Spain, Greece, the Netherlands and Central Africa (ALVES-SANTOS et al., 1999; BURUCHARA; CAMACHO, 2000; CRAMER et al., 2003). The disease is considered important in Brazil due the lack of crop rotation, intensive cultivation of common bean per year and the intensive movement of machines and implements between fields (PEREIRA; RAMALHO; ABREU, 2009).

Fusarium wilt is caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *phaseoli* J. B. Kendrick & W. C. Snyder (*Fop*). Symptoms of the disease occur only on *Phaseolus* spp. but the pathogen is able to colonize the roots of other plants, particularly legumes, and produce chlamydospores without causing symptoms or disease. Infection of *Phaseolus* beans by *Fop* occurs through roots and hypocotyls, most commonly through wounds. Initial symptoms are yellowing and premature senescence of the lower leaves. The chlorotic symptoms progress up the plant until all leaves are bright yellow, followed by wilting and discoloration of foliage. If plants are infected when young, they remain stunted. The vascular tissues usually become reddish brown, often

extending beyond the second node (SCHWARTZ et al., 2005). Severe infections can kill the whole plant within a few weeks due the presence and activities of the pathogen in the xylem vessels of the plant. Only when the infected plant is killed by the disease do these fungi move into other tissues and sporulate at or near the surface of the dead plant (AGRIOS, 2005).

The optimum temperature for disease development is 20 °C. Extremes of soil moisture levels do not appear to be required for the disease to occur but can influence disease severity. Soil compaction and poor drainage also appear to aggravate disease severity (SCHWARTZ et al., 2005).

In general, little information is available on management of Fusarium wilt. Resistance to *Fop* is usually race specific (ALVES-SANTOS et al., 2002b), conferred by single to multiple genes from different races of beans, that have been incorporated with conventional breeding and molecular techniques into various bean cultivars (BRICK et al., 2006; CROSS et al., 2000; FALL et al., 2001; PEREIRA et al., 2013; PEREIRA; RAMALHO; ABREU, 2009; RIBEIRO; HAGEDORN, 1979a; RONQUILLO-LÓPEZ; GRAU; NIENHUIS, 2010; SALA et al., 2006; SALGADO; SCHWARTZ; BRICK, 1995). As a consequence, correct identification of the local race is essential for the choice of resistant cultivars (ALVES-SANTOS et al., 2002b). Crop rotations associated with the use of healthy seeds are important measures to reduce levels of inoculum in soil (TOLEDO-SOUZA et al., 2012). Chemical seed treatment (MACHADO, 1986) and reduction of soil compaction may also be useful in the control of Fusarium wilt (JENSEN; KURLE; PERCICH, 2004).

2.3 Morphological and genetic aspects of *Fusarium oxysporum* f. sp. *phaseoli*

According to Leslie and Summerell (2006), *Fusarium oxysporum* has been defined by morphology as an asexual reproductive structure and was placed in

the section Elegans by Wollenweber and Reinking in 1935. The fungus typically has hyaline, nonseptate chlamydospores (2-4 x 6-15 µm) and macroconidia formed in pale orange, usually abundant, sporodochia. The macroconidia are short to medium in length, falcate to almost straight, thin walled and usually 3-septate. The apical cell is short and is slightly hooked in some isolates. The basal cell is notched or foot-shaped. Macroconidia are formed from monophialides on branched conidiophores in sporodochia and to a lesser extent from monophialides on hyphae. Microconidia usually are 0-septate, may be oval, elliptical or reniform (kidney-shaped), and are formed abundantly in false heads on short monophialides. Maximum mycelial growth occurs on culture medium at 28 ºC (SCHWARTZ et al., 2005).

The pathogen is a common, widespread fungus found in soil, being spread over long distance by infected seeds (MBOFUNG; PRYOR, 2010; TOLEDO-SOUZA et al., 2012). The pathogen inhabits soil and can survive as chlamydospores in the absence of its hosts. Soil pH changes result in a transcription factor that activates alkaline-expressed genes and inhibits acid-expressed genes and thereby affect fungal cell growth, development, and possibly pathogenicity (AGRIOS, 2005).

Pathogenic variability has been analyzed in *Fop* by the specific pathogenic interaction of the fungus with a set of differential cultivars (WOO et al., 1996) and, so far, seven races have been described. The classification seems to be related to the geographic origin, as race 2 includes isolates from Brazil (RIBEIRO; HAGEDORN, 1979b), race 3 includes isolates from Colombia (SALGADO; SCHWARTZ; BRICK, 1995), race 4 includes one isolate from Colorado, USA (SALGADO; SCHWARTZ, 1993) and race 5 includes isolates from Greece (WOO et al., 1996). Races 6 and 7 were characterized in Spain (ALVES-SANTOS et al., 2002b). However, the results of pathogenicity and race characterization using the CIAT (Centro Internacional de Agricultura Tropical)

differential cultivars system of *Fop* isolates from Spain and Greece indicated that isolates classified in the same race were not homogeneous with respect to virulence (ALVES-SANTOS et al., 2002b; ZANOTTI et al., 2006).

Besides the pathogenic population, *F. oxysporum* is commonly isolated from asymptomatic roots of crop plants (GORDON; MARTIN, 1997). Saprophytic strains of *F. oxysporum* on diseased common bean plants are morphologically identical to *Fop*. Because of this, the use of molecular marker techniques, as the analysis of random amplified polymorphic DNA (RAPD), can facilitate the identification of the population (ZANOTTI et al., 2006).

Langin, Capy and Daboussi (1995) reported that the presence of transposable element *impala* in nonpathogenic isolates is able to genetically distinguish them from pathogenic ones.

Previous studies reported that highly virulent strains of *Fop* were able to kill the host in two weeks and weakly virulent strains caused only a lower degree of damage (ALVES-SANTOS et al., 2002a). These findings suggested a differential expression of the transcription factor genes involved in virulence, such as *Fusarium transcription factor 1*. The gene *ftf1*encodes a transcription factor containing a Zn(II)2-Cys6 binuclear cluster DNA-binding motif and it is present as a multiple copy gene in highly virulent strains of *Fop* (RAMOS et al., 2007). The corresponding gene was confirmed to possess the STE and C2H2 domains, characteristic of the fungal Ste12 transcription factor family of proteins (GARCÍA-SÁNCHEZ et al., 2010; VEGA-BARTOL et al., 2011). Homologs of Ste12 identified in several pathogenic fungi have been shown to be involved in pathogenicity (DENG; ALLEN; NUSS, 2007; PARK et al., 2004; TSUJI et al., 2003).

2.4 Interaction and transmission of *Fusarium oxysporum* f. sp. *phaseoli* in common bean seeds

Infected seed is one of the causes for the introduction of *Fop* into common bean fields or production areas. The presence of the pathogen does not assure however its transmission to plants due to interferences from several factors related with the host, environment and pathogen (MACHADO; POZZA, 2005). Once introduced, the pathogen can survive in soil over extended periods, on crop residues and nonhost crops, and by forming chlamydospores (HAEGI et al., 2013).

The pathogen transmission from seed to seedling/plant is influenced by several factors such as inoculum potential and position in seed, environment, time of infection, host resistance and soilborne microbiota (MACHADO; POZZA, 2005).

Velicheti and Sinclair (1991) observed that hyphae of *F. oxysporum* grew over the seed surface, in the hilar region, and seed coat of soybean. Sharma (1992 apud SINGH; MATHUR, 2004) observed that the hyphae of *F. oxysporum* were confined to the soybean seed coat and the hilar stellate parenchyma in asymptomatic seeds and in all layers of the seed coat, stellate parenchyma in symptomatic seeds with weak infection. In moderately infected seeds, inter and intracellular mycelium occurred in all components. Heavy colonization was found in the seed coat with mycelial mat in the parenchymatous region. In cotyledons the infection was more on the abaxial than the adaxial surface. Infection in the embryonal axis was rare. In heavily infected seeds, aggregation of mycelium occurs in different components including the embryonal axis.

In general, pathogen-seed interaction and transmission to plants can negatively affect the yield and seed quality, resulting in considerable economic

losses. For *F. oxysporum*-common bean seeds interaction results in reduced seed germination, reduced vigor (PRYOR; GILBERTSON, 2001), decreased pre-emergence and post-emergence (DUTHIE; HALL, 1987), stunted growth (SCHWARTZ et al., 2005) or a combination of symptoms.

The infection mechanisms involved in the interaction between seeds and plant pathogenic fungi vary according to seed structure and the nature of the pathogens. Seed with a large embryo, such as seeds of legumes, may be often expected to carry the infection of fungi in the embryo (NEERGAARD, 1977). Several methodologies are applied to analyze these aspects, making microscopic techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), and fluorescence microscopy, the tools that complement other methodologies, allowing the observation from bio-images of the changes produced.

The studies of transmission mechanisms of pathogenic fungi from seeds to plants have been developed by incubation of host tissues in sterile culture medium. After incubation, observations are performed in the microscopy to identify the typical structures of the pathogens (DUTHIE; HALL, 1987; GÖRE et al., 2011; SANTOS et al., 1996; SHAH; BERGSTROM, 2000). For example, 42.8% of *Fop* was transmitted from common bean seed to seedlings (SANTOS et al., 1996) but very little is known about the mechanisms involved.

Other techniques to monitor seed transmission of pathogens are structural analyses by using electron microscopy (WANG; MAULE, 1994), molecular markers as heavy phosphorus (BAHAR; KRITZMAN; BURDMAN, 2009) or through biochemical tests (MICHENER; PATAKY; WHITE, 2002).

Currently, green fluorescent protein (GFP) has shown to be useful for studying plant-fungus and fungus-fungus interaction *in vivo*. Lagopodi et al. (2002) visualized in details the tomato root colonization and infection processes by *gfp*-labeled *F. oxysporum* f. sp. *radices-lycopersici*. Sarroco et al. (2007)

followed the colonization of carnation roots by *F. oxysporum* f. sp. *dianthi* transformed with GFP and red fuorescent protein (DsRedFP), observing that the hyphae were confined within the vascular cylinder by the endodermal cells beginning from the zone of differentiation of vascular tissues, and were able to grow inside vessels. Vallad and Subbarao (2008) compared the infection and colonization steps of resistant and susceptible lettuce roots by *gfp*-tagged *Verticillium dahliae*. Other studies have demonstrated the transmission of the GFP-labeled *F. verticillioides* from inoculated maize seed to plants under different temperatures (MURILLO-WILLIAMS; MUNKVOLD, 2008; WILKE et al., 2007). The results of both studies indicated that, if maize seed is infected by *F. verticillioides*, seed transmission is common and symptomless systemic infection can be initiated under a broad range of temperatures.

2.5 Use of molecular techniques in seed health test for fungal detection

Plant pathogens that causes seed discoloration or are visibly evident as mycelium or as fruiting structures, have a greater chance to be detected and subsequently discarded (ELMER, 2001). However, most pathogens infest the seeds and use them as a vehicle for transportation of the inoculum over long distances (ELMER, 2001). There are many types of associations between seed pathogens and their hosts. Agarwal and Sinclair (1997) reported that pathogens associated with seeds are more localized on seed coats. This external contamination is also considered as infestation of seeds. The infection of the seeds is observed when the pathogen is internally localized on seeds, colonizing them.

Long distance movement and establishment of disease in foreign regions are favored by asymptomatic colonization of the seed by the pathogen (ELMER, 2001). So, accurate seed health testing for seedborne pathogens is an important

tool of disease management for reducing the chances of disease spread and for being helpful in decision making regarding the appropriate use of seed treatment, and other practical application in seed certification programs (MBOFUNG; PRYOR, 2010).

Seed health testing is an essential management tool for the control of seedborne and seed-transmitted pathogens and continues to be an important activity for their regulation and control through phytosanitary certification and quarantine programs in domestic and international seed trade (MORRISON, 1999).

F. oxysporum f. sp. *phaseoli* has been proposed in Brazil as 'Regulated Non-Quarantine Pest' and its tolerance level in common bean seed samples is zero from a total of 400 analyzed seeds in a routine test, according to National Program of Seed Health Quality Control. Conventional methods recommended for detection of *Fop* in common bean seeds are the blotter test and plating seeds in semi-selective medium supplemented with PCNB (ALBORCH; BRAGULAT; CABAÑES, 2010; BRASIL, 2009; SOUSA, 2006). These methods are not able to differenciate formae speciales, races or saprophytic isolates that are morphologically identical to pathogenic isolates. In this case, pathogenicity test should be performed to obtain reliable informations about the pathogen occurrence in each seed lot. In addition, those methods are time-consuming, labor intensive, require skilled personnel, and are not suited for the rapid and high-throughput type of testing, as demanded in screening commercial seed (ALVES-SANTOS et al., 2002a; MBOFUNG; PRYOR, 2010). The low level of sensitivity and specificity of the current biological methods are obstacles for their adoption in routine analysis (MBOFUNG; PRYOR, 2010).

Therefore the development of a reliable, rapid and sensitive diagnostic method that allows for the detection and quantification of *Fop* in common bean seeds is essential. The polymerase chain reaction (PCR) assay has been used

widely as a diagnostic method, as it allows for detection of extremely small quantities of specific target DNA (LEE; TEWARI; TURKINGTON, 2002).

Diagnostic methods based on polymerase chain reaction (PCR) have high analytical sensitivity to discriminate between different strains of fungi and have been used to detect a number of form speciales within the *F. oxysporum* complex (ALVES-SANTOS et al., 2002a; ATTITALLA et al., 2004; CHIOCCHETTI et al., 2001; DITA et al., 2010; MBOFUNG; PRYOR, 2010; SILVA; JULIATTI; JULIATTI, 2007; ZHANG et al., 2005). In addition to high sensitivity and specificity, PCR-based methods have the advantage to process a large number of samples within a short period of time and can be conveniently applied to commercial seed testing and certification. The method also detects all pathogen inoculum present both within the seed and on the seed surface (GLYNN; EDWARDS, 2010). However the high levels of polysaccharides and phenolic compounds frequently present in seed may affect the efficiency of DNA extraction, and the presence of PCR inhibitors can negatively impact successful amplification of the recovered DNA (DE BOER et al., 1995; MA; MICHAILIDES, 2007; MURILLO; CAVALLARIN; SAN SEGUNDO, 1998; ZOUWEN et al., 2002).

In literature several reports provide the potentiality of using molecular techiques in seed health testing. A successful example was the use of specific primers and PCR to identify *Tilletia indica* on wheat seed through the washing extraction method, separating *T. indica* from *T. barclayana* and other *Tilletia* spp (SMITH et al., 1996). Other reports of PCR detection and quantification were *Rhynchosporium secalis* in asymptomatic wheat seedlots (LEE; TEWARI; TURKINGTON, 2002) and *F. oxysporum* f.sp. *lactucae* in commercial lettuce seedlots (MBOFUNG; PRYOR, 2010). The results of both works demonstrated the potential of the PCR assays as an alternative seed health testing method.

Diagnostic methods based on polymerase chain reaction (PCR) have high analytical sensitivity (MBOFUNG; PRYOR, 2010) and have been developed along immunoassays and nondestructive seed health tests, such as ultrasound, optical and infrared analyses, and biopsis (the removal and analysis of tissue cores from seeds) (MUNKVOLD, 2009).

According to Munkvold (2009), the PCR-based methods for detecting pathogens in seeds have begun to be implemented in the vegetable seed industry and in some official seed testing laboratories for quality control, but this process has been slow in international seed testing programs. The author still described some reasons for the slow adoption: costs, technical expertise, poor quality DNA, PCR inhibitors from seed extracts (leading to false negatives), remnant DNA from nonviable pathogen propagules (potential for false positives) and sample sizes. Several strategies have been developed to overcome the technical impediments. For improving the DNA quality, commercial DNA extraction kits have been recommended but they are not able to remove all inhibitors in some specific cases (MA; MICHAILIDES, 2007). For attenuating the effects of PCR inhibitors, various techniques have been suggested by Ma and Michailides (2007), like the use of commercial DNA extraction kits combined with additions of amplification facilitators in DNA extraction and PCR reaction buffers. The magnetic capture hybridization (MCH) is another procedure that can concentrate target DNA and separate it from inhibitory compounds and nontarget DNA, increasing sensitivity of the PCR (HA et al., 2009; MUNKVOLD, 2009; WALCOTT; GITAITIS; LANGSTON JUNIOR, 2004). Some approaches to ensuring that PCR is detecting DNA from viable pathogen cells are the use of BIO-PCR coupled with nested-PCR, flow cytometry or propidium monoazide. BIO-PCR involves propagation of putative pathogen propagules on a culture medium and subsequent PCR (MUNKVOLD, 2009). Nested-PCR is also used to enhance the sensitivity and specificity of the detection (ROBÈNE-

SOUSTRADE et al., 2010). The use of propidium monoazide can selectively remove free DNA from dead cells (MUNKVOLD, 2009). Some conflicts exist about sample sizes and more studies need to be developed and refined about this topic.

The introduction of real-time PCR technology, which is characterized by the inclusion of a fluorescent reporter molecule in each reaction that yields increased fluorescence with an increasing amount of product DNA, has improved and simplified methods for PCR-based quantification. The quantification of a pathogen upon its detection and identification is an important aspect as it can estimate potential risks regarding disease development, spread of the inoculum, and economic losses. Apart from this potential, it provides the information required to make appropriate management decisions (LIEVENS; THOMMA, 2005). According to Mumford et al. (2006), there are ranges of alternative real-time detection chemistries which are used for the plant pathogens detection, including SYBR Green, TaqMan® and FRET. SYBR Green is a method based on DNA-intercalating dyes. Methods based on separation between the reporter and quencher dyes by cleaving of labeled-probe (TaqMan®) results in an increase of fluorescence, which is related to the amount of amplified product. Methods using fluorescent resonance energy transfer (FRET) probes, where spatial separation between the reporter and quencher dyes is achieved through a loss of complex secondary structure due to probe binding, as 'Molecular Beacons'and 'Scorpion primers'.

Several studies have been done to analyse the real-time PCR as a tool for seed health tests through detection and quantification of pathogenic fungi in different plants and seeds. Bilodeau et al. (2007) used three different reporter technologies, TaqMan, SYBR Green and Molecular Beacons to differentiate *Phytophthora ramorum* from 65 *Phytophthora* spp. and to detect the target DNA in infected plants of different hosts. The chemistries were *P. ramorum*-

detectable but TaqMan seemed to be more sensitive. Chilvers et al. (2007) used a real-time PCR with SYBR Green chemistry to quantify *Botrytis aclada*, *B. allii* and *B. byssoidea* associated with onion seeds, reporting the sensibility of the technique that was able to detect the pathogen in 5 of the 23 seedlots. Ioos et al. (2009) reported a new, sensitive and specific detection protocol combining an enrichment procedure with a TaqMan real-time PCR assay that allowed detection of 0.1% infestation level of *Fusarium circinatum* in artificially contaminated pine seeds as well as in seed obtained from naturally infected pine stands.

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CHAPTER 2 - PAPERS

Paper 1

Transmission of *Fusarium oxysporum* f. sp. *phaseoli* from seed to emerging plants of common bean

Transmissão de *Fusarium oxysporum* f. sp. *phaseoli* de sementes para plantas emergidas de feijão

Prepared according to Seed Science and Technology guidelines

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ABSTRACT

Fusarium oxysporum f. sp. *phaseoli* (*Fop*) causes severe losses in common bean fields and the pathogen can be seed-transmitted and disseminated over long distances. The objective in this work was to estimate the seed-to-emerged plant transmission rates of *Fop* in relation to different factors under controlled conditions. The work was conducted using artificially and naturally *Fop*-contaminated seeds. For seed inoculation by osmotic technique two cultivars (BRSMG Majestoso and Ouro Negro), two temperatures (20 and 25 ºC), two strains (FOP005 and FOP014) and four inoculum potentials (P0, P1, P2 and P3) were used. For naturally *Fop*-contaminated seed trials three common bean cultivars (Horizonte, Cometa and Valente) in three seed sizes (small, medium and large) at two temperatures (20 and 25 ºC) were used. The transmission rates from artificially and naturally contaminated seeds were 100% in symptomatic plants although the number of emerged symptomatic plants was lower than 5%. Asymptomatic plants contaminated with *Fop* were observed in high frequencies on different plant tissues, especially on hypocotyl and main root, confirmed by pathogenic *Fop*-positive bands in conventional PCR. A steep decline of the fungus recovery was observed in the cotyledons and first node tissues. From these results it was observed that the transmission rates of *Fop* from infected bean seeds to emerged symptomatic and asymptomatic plants is quite high and has to be considered as an important factor in the disease management.

Key-words: Fusarium wilt, *Phaseolus vulgaris*, seed infection, seed pathology.

RESUMO

Fusarium oxysporum f. sp. *phaseoli* (*Fop*) é responsável por perdas severas em campos de produção de feijoeiro, sendo o patógeno transmitido e disseminado por sementes a longas distâncias. Objetivou-se, neste estudo, estimar as taxas de transmissão de *Fop* das sementes para as plantas emergidas, em relação a diferentes fatores sob condições controladas. O trabalho foi conduzido utilizando-se sementes artificialmente e naturalmente associadas ao *Fop*. Para a inoculação das sementes através da técnica de condicionamento osmótico foram utilizadas duas cultivares (BRSMG Majestoso e Ouro Negro), duas temperaturas (20 e 25 ºC), dois isolados (FOP005 e FOP014) e quatro potenciais de inóculo (P0, P1, P2 e P3). Para ensaios com sementes naturalmente associadas ao *Fop* foram utilizadas três cultivares de feijão (Horizonte, Cometa e Valente), em três tamanhos de peneiras (pequeno, médio e grande) e em duas temperaturas (20 e 25 ºC). As taxas de transmissão de sementes artificialmente e naturalmente associadas ao *Fop* foram 100% para plantas sintomáticas, apesar do número de plantas sintomáticas emergidas ser menor do que 5%. Plantas assintomáticas e associadas ao *Fop* foram observadas em alta frequência, em diferentes tecidos da planta, especialmente no hipocótilo e raiz principal, confirmado por bandas positivas de *Fop* patogênico, em PCR convencional. Grande redução do crescimento fúngico foi observado nos cotilédones e inserção dos cotilédones. A partir destes resultados foi observado que as taxas de transmissão de *Fop* de sementes de feijão infectadas para plantas emergidas sintomáticas e assintomáticas foram elevadas e devem ser consideradas como um fator importante no manejo de doenças.

Palavras-chave: murcha de fusarium, *Phaseolus vulgaris*, infecção de sementes, patologia de sementes.

INTRODUCTION

Fusarium oxysporum Schlechtend:Fr. f. sp. *phaseoli* J. B. Kendrick & W. C. Snyder (*Fop*) is the causal agent of Fusarium wilt of common bean (*Phaseolus vulgaris* L.), which is distributed worldwide (Alves-Santos *et al.*, 2002a) as a soil inhabitant, being able to survive in the form of chlamydospores or in infected seeds (Schwartz *et al.*, 2005).

Symptoms of the disease are characterized by chlorosis of leaves, necrosis of the vascular system and general wilt and death of the colonized plant (Vega-Bartol *et al.*, 2011). Highly virulent strains are able to kill common bean plants in about two weeks (Alves-Santos *et al.*, 2002a). The optimum temperature for *Fop* development is 28 °C but for disease development is 20 °C (Schwartz *et al*., 2005).

The most efficient and viable management practices for Fusarium wilt control are the use of healthy seeds (Santos *et al.*, 1996) and use of resistant cultivars (Pereira et al., 2009). No information was found in literature about resistance related to seed transmission of *Fop* in common bean seeds. A study by Pereira *et al.* (2013) indicated that *Fop* structures were observed in the xylem vessels of resistant cultivar of *P. vulgaris,* although no disease symptom was observed in plants. These plants become an important inoculum source in the field but the question as to whether seed from resistant cultivars contaminated with *Fop* inoculum is able to transmit it to seedling continues to be misunderstood.

Santos *et al.* (1996) showed that infected common bean seeds with incidence of 14% by *F. oxysporum*, transmitted the pathogen to plants in a high percentage (42.8%). Some aspects such as genotype, environmental conditions and pathogenicity/ virulence of the fungus were not reported in their study.

Although Fusarium wilt is one of the most important diseases in common bean in Brazil (Paula Júnior *et al.*, 2006), little information is known about seed-*Fop* interaction as well as the plant-to-plant transmission rate in field conditions.

This work was proposed with the objective to determine the rates of seed transmission of *Fop* in common bean. This kind of information is essential for establishing health standars for that pathosystem which are of great interest in Seed Certification Programs.

MATERIALS AND METHODS

Origins of *Fop* strains, common bean cultivars and seed inoculation procedures. For this experiment two *Fop* strains, two cultivars of common bean, two temperatures and four inoculum potentials were used following the model proposed for other pathosystems (Botelho et al., 2013).

The two *Fop* strains, FOP005 and FOP014 were obtained from the Mycological Collection of Seed Pathology Laboratory, Lavras, MG, Brazil and from Agronomic Institute of Campinas, Campinas, SP, Brazil, respectively. These strains were identified as *F. oxysporum* on synthetic-nutrient-agar (SNA) and potato-dextrose-agar (PDA; Difco Laboratories, Plymouth, MN), according to Leslie & Summerell (2006). Single spore isolates were prepared and maintained on PDA. The pathogenicity was tested according to Alves-Santos *et al.* (2002a); FOP005 was identified as a highly virulent pathogen and FOP014 as a weakly virulent pathogen (data not shown). *Fop* was confirmed using specific primers to conventional PCR from Alves-Santos *et al.* (2002b).

The two *Phaseolus vulgaris* cultivars used, BRSMG Majestoso and Ouro Negro, are recommended for planting in the State of Minas Gerais (Paula Júnior *et al.* 2010). The absence of *F. oxysporum* in seeds was initially confirmed by blotter test (Brasil, 2009) and conventional PCR, using the primer set published by Alves-Santos *et al.* (2002b).

For inoculation of seeds, bulks of 1,000 seeds of each cultivar were inoculated through osmotic technique described by Sousa *et al.* (2008). Through that technique, seeds were surface disinfected by soaking for one minute in a 1% NaHClO solution followed by drying on a sterile filter paper in a laminar flow hood. Both isolates FOP005 and FOP014 were cultured for five days on potato-dextrose-agar (PDA) medium at 22 °C. Macro and microconidia were harvested by washing the surface of a culture with 10 mL of sterile distilled water. The

resulting suspensions were diluted with sterile water to obtain a final concentration of 10^6 spores mL^{-1} (counts adjusted with a hemacytometer). Inoculum suspensions were sprayed on seeds and on PDA supplemented by mannitol with osmotic potential adjusted to -1.0 MPa, according to software SPMM (Michel & Radcliffe, 1995). Seeds were kept at 20 °C, 12h photoperiod, for four incubation periods, 0, 36, 72 and 96h of the exposure seeds to *Fop* colonies. The different incubation periods were considered as different inoculum potentials (P0, P1, P2 and P3, respectively) of the pathogen. Artificially inoculated seeds were then removed and air dried overnight under a hood. Negative controls were prepared for each cultivar and incubation period using PDA supplemented by mannitol (-1.0 MPa) with absence of *Fop.*

Sowing artificially *Fop*-contaminated seeds to evaluate seed-to-plant transmission rate under controlled conditions.

To assess the incidence of seed infection by *Fop*, 100 inoculated seeds and 100 non-inoculated seeds, without surface disinfestation, were placed on blotter test moistened with PDB supplemented with 1 ppm of PCNB (Sousa, 2006). The seeds were kept in incubation room at 20 °C, 12 h photoperiod, for seven days. The incidence of seeds with *Fop* was recorded.

For estimating the potential rate of seed-to-plant transmission, the experiment was displayed in three blocks with 20 treatments (2 cultivars x 2 *Fop* strains x 4 inoculum potentials + 4 negative controls). Sixty seeds of each treatment were individually sowed in 300 mL-plastic cups, containing soil: sand: compost substrate (Tropstrato HA Hortaliças) in equal proportion (by volume). The cups were arranged in randomized blocks in two growth chambers with temperatures adjusted to 20 and 25 °C. Light was supplied in each shelf by three horizontally oriented cool white fluorescent bulbs (NSK T10 40W 6500K FL40T10-6 60Hz).

Final evaluations were made on emerged plants of 25 days-old by counting the stand and looking at typical symptoms of Fusarium wilt. Plants were considered symptomatic when they presented yellowing, wilt and/or vascular discoloration. Those plants were counted and expressed as a percentage of the total number of emerged plants in each treatment. The asymptomatic plants were also removed from the cups and 4-cm fragments of the main root, hypocotyl and first node were cut for examination. The cotyledons were collected from 10-15 day-old plants when they fell from the plants. Every tissue from each plant was surface disinfestated with 70% ethanol for 2 min, 1% sodium hypochlorite solution for 2 min, and sterile distilled water for 2 min and placed in PDA medium supplemented with 1 ppm PCNB. The materials were kept in an incubator with 12h photoperiod for seven days at 20 °C, and microscopically examined for morphological structures of *F. oxysporum*. Plant fragments colonized by *F. oxysporum* were scored as positive transmission, and conventional PCR were performed to confirm the identity of pathogenic *Fop*.

Symptomatic plants as well as each plant fragments of asymptomatic plants with or without presence of *F. oxysporum* growth were frozen in liquid nitrogen and ground into a fine powder. DNA extractions were performed using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's protocol.

Amplifications were performed in thermocycler (Multigene TC 9600-G; Labnet International Inc.; software V3.3.4C), primer set from Alves-Santos *et al.* (2002b) (B310: 5'-CAGCCATTCATGGATGACATAACGAATTTC-3' and A280: 5'-TATACCGGACGGGCGTAGTGACGATG-3'), and the components were added following the protocol of Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA). The amplification conditions were as follows: a denaturation step for 5 min at 94 °C, followed by 40 amplification cycles consisting of 1 min at 94 °C, 1 min at 65 °C, and 2 min at 72 °C. A final

extension step was performed for 5 min at 72 °C. Samples of the PCR products were run on 1.2% agarose gels with GelRed® (Biotium) in 1x Tris-borate-EDTA buffer, and DNA was visualized by L-Pix HE (Loccus Biotecnologia, Cotia, SP).

Sowing naturally *Fop*-contaminated seeds. Seeds from three cultivars of *Phaseolus vulgaris* L., Cometa, Horizonte and Valente, obtained in 2010 from *Fop*-infected fields of Embrapa (Rice and Bean) Arroz e Feijão, Santo Antônio de Goiás, GO, Brazil were used in this study. The *Fop*-susceptible cultivars were separated in sieve sizes during seed processing, and three different seed sizes, small, medium and large, were used. Seed infection incidence by *F. oxysporum* was determined by assaying 400 seeds without surface disinfestation on blotter test modified for addition of 1 ppm of PCNB.

Seeds were placed on PDA modified by addition of manittol -1.0 MPa, calculated by software SPMM (Michel & Radcliffe, 1995) for three days. Seeds with *Fusarium* sp. growth were collected and sown individually in 300 mL-plastic cups containing 1 part soil: 1 part sand: 1 part of organic compound (by volume). The experimental unit was 60 plastic cups, with three blocks arranged in randomized blocks in each of two growth rooms, at 20 and 25 °C. The plants developed until 25 days after sowing. The collecting period, types of fragments of plant with plating procedures on culture medium and analysis by conventional PCR were performed as the same for artificially *Fop*-contaminated seeds.

Symptomatic or asymptomatic plants that showed at least one fragment with *Fop*-mycelial growth, which was positive through conventional PCR, were counted as *Fop*-positive transmission in each treatment. The rates of transmission of *Fop* from seeds to plants were estimated by dividing the number of *Fop*-positive transmission by number of sowed seed and by multiplying by 100.

Data analysis. For both experiments, analyses of variance were made on transmission rate of symptomatic and asymptomatic plants from artificially and naturally *Fop*-contaminated seeds as well as the percentage of *Fop* occurrence in infected cotyledons, first node, hypocotyl and main root. A square-root transformation was used to equalize variances in the occurrence of the fungus in each plant fragment from naturally *Fop*-contaminated seeds. Analyses were conducted with the general linear model (GLM) procedure of SAS ver. 9.1 (SAS Institute Inc., Cary, NC, USA). Least significant difference (LSD) tests at the 0.05 level were calculated to compare means of cultivars and temperature, and Tukey test was used to compare means of isolates. Regression models were calculated to compare periods of exposition of seeds to *Fop* (inoculum potentials).

RESULTS

Artificially *Fop*-contaminated seeds. The initial germination percentages of common bean seeds of both cultivars used in this study, BRSMG Majestoso and Ouro Negro were 97% and 95%, respectively. The results of the botter test and conventional PCR showed that both common bean seed lots were *Fop*-free. After artificial seed infection by the osmotic technique, the incidence of *Fop* in inoculated seeds was 100%.

The influence of the biotic (cultivar, pathogen virulence, inoculum potential) and abiotic factors (temperature) were evaluated individually because no statistical differences were found by the interaction between them (Appendix 1).

Typical symptoms of Fusarium wilt in emerged plants of common bean at 25 °C were observed in 3.9% of the plants (Fig. 1A), 1.6% for BRSMG Majestoso and 2.3% for Ouro Negro (Fig. 1B) and 4.2% for highly-virulent strain (FOP005) compared to 2.3% of the weakly-virulent one (FOP014) (Fig. 1C). However, all symptomatic plants presented high *Fop* incidence in culture medium with a 100% transmission rate in these plants. Most incidence of the pathogen in emerged plants was found in asymptomatic plants (Fig. 1 A, B, C), with transmission rates ranging from 54.4% at 20 ºC to 52.3% at 25 ºC (Fig. 1A); 49.7% to 57% for Ouro Negro and BRSMG Majestoso (Fig. 1B); 83.6% to 94.2% for FOP005 and FOP014 (Fig. 1C), respectively.

Regression equations were obtained for potential transmission rates and seed-*Fop* contact period (inoculum potential). Linear equation was used relating that the potential transmission rate of symptomatic plants increased by 0.0273 for each 1 hour in the period of seed-*Fop* contact (Fig. 2A). The coefficient of determination was 0.78 and not significant. Polynomial equations showed maximum potential transmission rate for asymptomatic plants related to periods

of *Fop* exposure around 71 h, with significant coefficient of determination (0.97) (Fig. 2A).

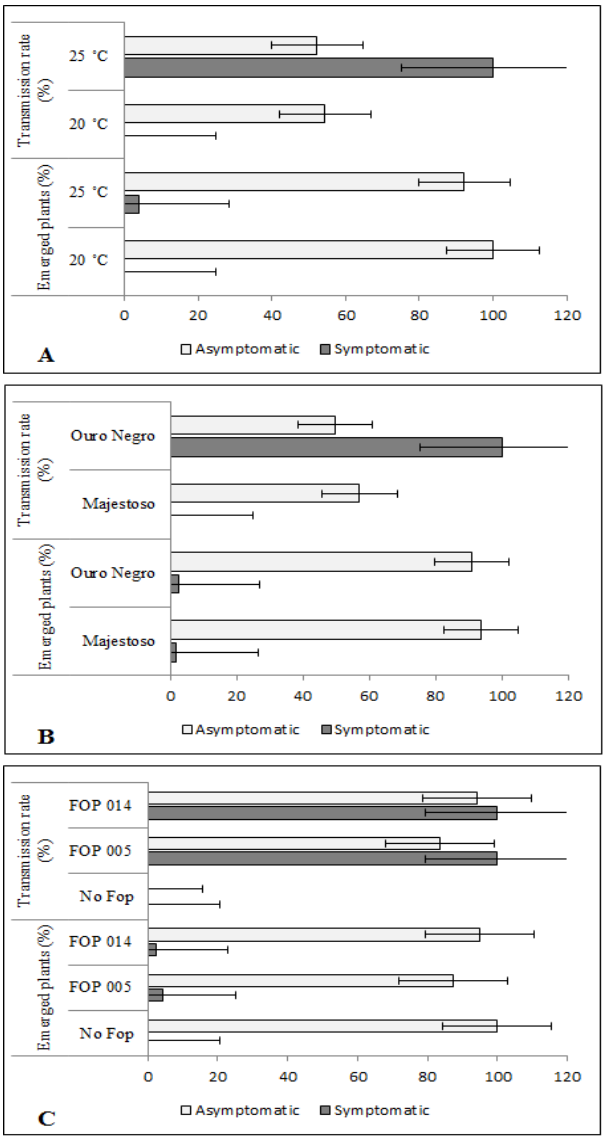

Figure 1. Seed-to-plant transmission rate (%) of *Fusarium oxysporum* f. sp. *phaseoli* and emerged plants (%) in inoculated common bean (*Phaseolus vulgaris* L.) seeds, with symptomatic and asymptomatic

plants: **A)** in two temperatures (20 and 25 °C); **B)** in two cultivars (BRSMG Majestoso and Ouro Negro); **C)** with two *Fop* isolates (FOP 005 and FOP 014) and no *Fop* (negative control)

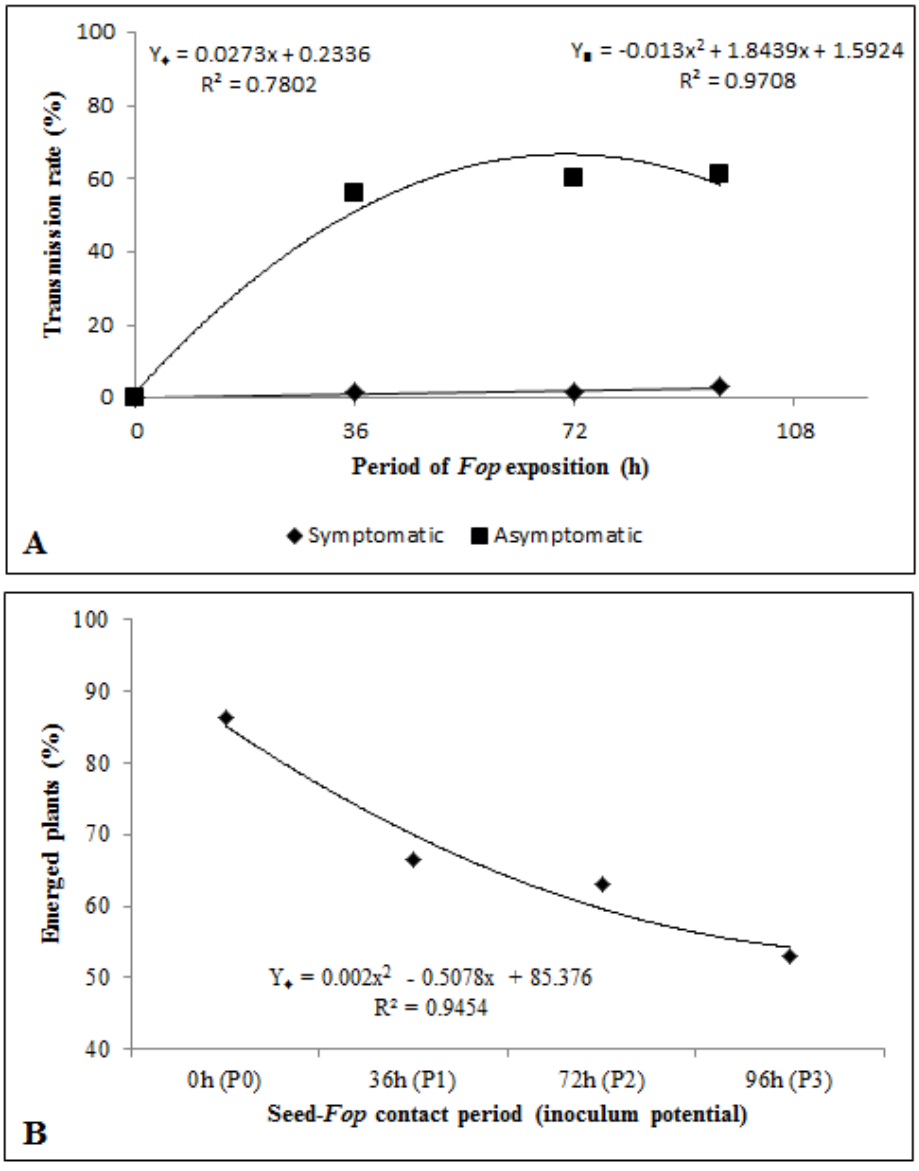

Figure 2. Inoculated common bean (*Phaseolus vulgaris* L.) seeds by *Fusarium oxysporum* f. sp. *phaseoli* in different seed-*Fop* contact periods

(inoculum potentials): 0h (P0), 36h (P1), 72h (P2) and 96h (P3). **A)** Seed-to-plant transmission rate (%) with symptomatic and asymptomatic plants; **B)** Emerged plants at 25 days after planting

The population of symptomatic plants ranged from 1.9% (P1) to 3.2% (P3) whereas asymptomatic plants with presence of pathogenic *Fop* ranged from 56.2% to 61.3% (Fig. 2A). The emerged plants from inoculated seeds showed variable decrease according to the inoculum potentials used in this study (Fig. 2B). The highest frequence of isolation of *Fop* occurred on hypocotyl followed by main root, cotyledons and first node of asymptomatic plants (Fig. 3). Recoveries of *Fop* from hypocotyl were 55.8, 57.6% for 20 and 25 °C (Fig. 3A), 56.7% for both cultivars (Fig. 3B), 0, 93, 96% for non-inoculated seeds, FOP005 and FOP014 (Fig. 3C), and 0, 62.4, 65.3, 61.4 % for P0, P1, P2 and P3 (Fig. 3D).

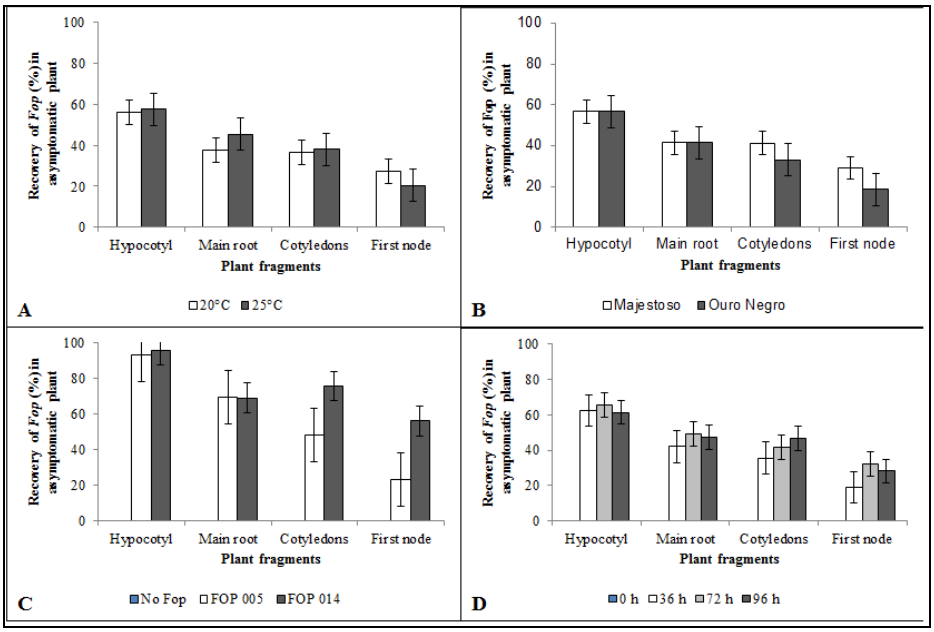

Figure 3. *Fusarium oxysporum* f. sp. *phaseoli* recovery frequency (%) in four different fragments (hypocotyl, main root, cotyledons and first node)

of 25-days old asymptomatic common bean plants (*Phaseolus vulgaris* L.) assessed, **A)** in two temperatures, 20 and 25 °C; **B)** in two cultivars, BRSMG Majestoso and Ouro Negro; **C)** with two *Fop* isolates, FOP 005, FOP 014 and no *Fop* (negative control); and **D)** in different seed-*Fop* contact period/inoculum potentials 0 h (P0), 36 h (P1), 72 h (P2) and 96 h (P3)

Naturally *Fop*-contaminated seeds. A low proportion of symptomatic plants, lower than 1.5%, were observed at 20 and 25 °C, in all cultivars and seed sizes (Fig. 4).

However, for the results of conventional PCR, all symptomatic plants were pathogenic *Fop*-positive corresponding to a100% transmission rate in these plants. The results of conventional PCR also showed that *Fop* as non-pathogenic *F. oxysporum* were present in asymptomatic plants. The colonies were similar in culture medium but they were genetically distinguishable. *Fop* was found in asymptomatic plants, having 13.0% and 10.4% transmission rate at 20 and 25 °C, respectively; for cultivars Valente, Cometa and Horizonte the transmission rates were 8.1%, 10.3% and 16.7%, respectively; 14.7%, 9.7% and 10.6% for small, medium and large sizes of common bean seeds, respectively (Fig. 4).

Fop was recovered from each plant fragment with great incidence in the hypocotyl followed by main root and a steep decline of the fungus recovery in the cotyledons and first node (Fig. 5).

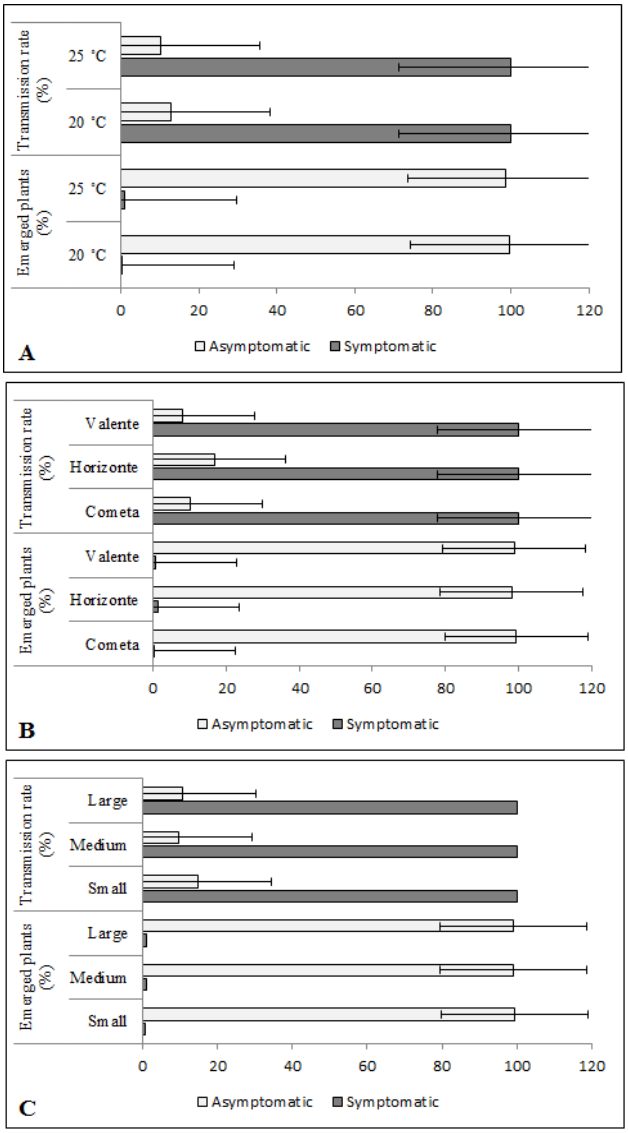

Figure 4. Transmission rate (%) of *Fusarium oxysporum* f. sp. *phaseoli* from common bean (*Phaseolus vulgaris* L.) seeds harvested in infected fields seeds to emerged symptomatic and asymptomatic plants: **A)** in two temperatures, 20 and 25 °C; **B)** in three cultivars, Valente, Cometa and Horizonte; **C)** with three seed sizes, small, medium and large, obtained by seed processing

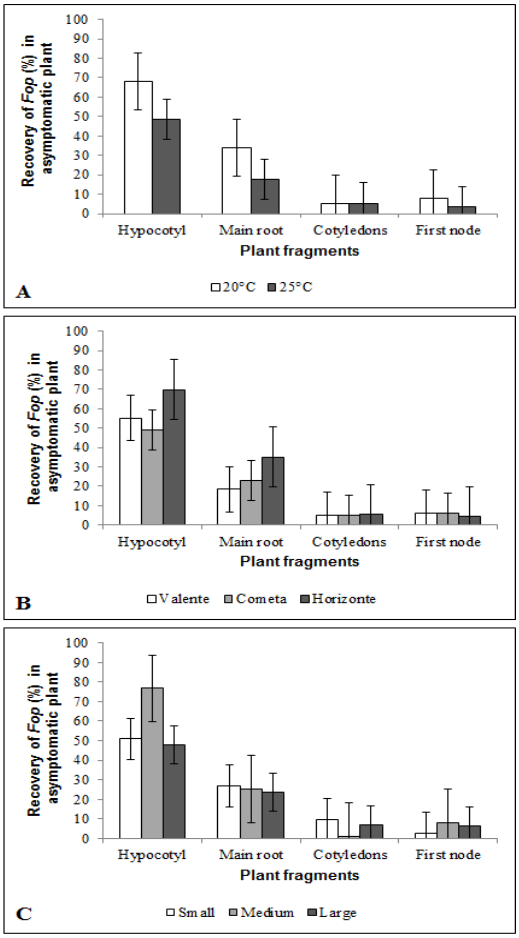

Figure 5. *Fusarium oxysporum* f. sp. *phaseoli* recovery frequency (%) in four different fragments (hypocotyl, main root, cotyledons and first node) of 25-days old asymptomatic common bean plants (*Phaseolus vulgaris* L.) assessed, **A)** in two temperatures, 20 and 25 °C; **B)** in three cultivars, Valente, Cometa and Horizonte; **C)** with three seed sizes, small, medium and large, obtained by seed processing

DISCUSSION

The seed-to-plant transmission rates, as calculated in this work, provided an estimate about the risk of using *Fop*-contaminated/infected seeds of common bean. The results indicate that by the individual analysis of the factors used in this study (Appendix 1), like temperature, host genotype, pathogen population and its biomass (inoculum potential) on seeds can affect the normal development of plants and the beginning of the disease process. In literature, information on that kind of interaction is found for some pathosystems such as those involving *F. verticillioides* in maize (Wilke *et al.,* 2007), *Verticillium dahliae* in cotton (Göre *et al.* 2011), *F. graminearum* in winter wheat (Duthie & Hall, 1987). In those pathosystems a close relationship was also seen between seed infection and effects of the pathogens on initial plant development.

The estimated rate of seed-to-plant transmission of *Fop* in artificially inoculated seeds was higher than rates that occurred for naturally *Fop*-contaminated seeds. The fungus biomass present on inoculated seeds may be higher which explains this difference. Plants incubated at 20 °C did not show Fusarium wilt symptoms at 25-days after sowing, and only a low percentage of symptomatic plants were observed at 25 °C (Fig. 1A). The influence of temperature on Fusarium wilt development in common bean has not been well characterized and understood. Ribeiro & Hagedorn (1979) reported that a low temperature, around 20 °C, is the optimum for this disease development. Other authors mentioned that high temperature associated to high moisture is the favorable environmental condition to cause Fusarium wilt disease in common bean (Pastor-Corrales & Abawi, 1987; Buruchara & Camacho, 2000; Pereira *et al.*, 2011). The results of this work indicate that, if seed is infected with *Fop*, seed transmission can be initiated under a broad range of temperature conditions.

The two genotypes exhibited asymptomatic plants with presence of the pathogenic *Fop* (Fig. 1B). The resistance of both cultivars used in the present work was not able to avoid the presence of pathogen biomass on seeds with their consequent infections, moving to plants showing or not showing typical symptoms.

In relation to virulence of *Fop* strains, a higher transmission rate was observed for a highly virulent isolate, FOP005 (Fig. 1C), causing death of the young plants with visible sporulation of the fungus at the surface of the dead plants in symptomatic plants. Weakly virulent isolate (FOP014) was able to cause some yellowing symptoms in leaves but not wilting and death during the experimental period. Both were seed-trasmitted at 83.55 and 94.24% to asymptomatic plants (Fig. 1C); more studies are required to understand if they can start an epidemy if some changes occur, like favorable environmental conditions.

The relation between transmission rate and inoculum potential (inoculum biomass) of *Fop* (Fig. 2A) was not explained by linear regression. The high inoculum potential in seeds (P3) probably caused the seed rot leading to the pre-emergence death of seeds. Thus, that migh be the cause of the low number of asymptomatic emerged plants (Fig. 2B) with infection by *Fop*.

The higher occurrence of *Fop* in the present work was observed in the hypocotyl fragments for all treatments followed by the main root (Fig. 3) differing from a previous study from Wilke *et al.* (2007) who observed recovery of transformant *F. verticillioides* at V2 stage ranging from 60 to 80% in stems of maize plants and more than 90% in roots. Some factors may explain these differences, such as the inoculation method used for both studies, the differences between species of *Fusarium* and the germination type for each family of plants (Poaceae and Fabaceae). The occurrence of *Fop* in the cotyledons and first nodes can be associated to infected cotyledons remain in Fabaceae plants until the first

true leaves are unfolded, facilitating the stem infection. After that, the cotyledons eventually fall off and continue on the soil, being an important source of inoculum dissemination.

Rates of seed-to-plant transmission of *Fop* observed in this study for naturally infected seeds are lower than the rate reported by Santos *et al.* (1996). The results in this work indicate that transmission rates of *Fop* in common bean, ranging from 10.6% to 18.1%, were temperature, genotype and seed-size dependents (Fig. 4). Santos *et al.* (1996) observed a 42.8% rate of transmission of *F. oxysporum* from 14% of contaminated seeds. Probably differences in methodology used in both studies may be the cause of those conflicting results.

The highest transmission rate in this study was observed for the cultivar Horizonte small-seeded size at 20 ºC. All symptomatic plants resulting from naturally *Fop*-contaminated seeds died and the fungus sporulated on their external surface. For asymptomatic plants, pathogenic *Fop* was recovered on all assessed plant fragments, confirmed by conventional PCR, with variable incidences (Fig. 5), according to the incidences observed for artificially infected seeds.

In this work the results indicated that *Fop*-contaminated common bean seeds are able to transmit the pathogen with observation of symptomatic and asymptomatic plants. Seed-transmitted *Fop* was shown to be dependent on the biomass and position of the inoculum in seeds, virulence of the pathogen as well as on some intrinsic characteristics of the host (resistance and seed size). Usually, the symptomatic emerged plants were lower than 4% with 100% of *Fop* transmission in these plants. Asymptomatic plants with the presence of *Fop* reached 94% at the highest values of inoculum biomass present on seeds. This information is of interest for establishing tolerance levels in seed certification programs and for improving the seed production and trade process. The establishment of phytosanitary standards is required, especially for Regulated

Non-Quarantine Pests due the great concern involving the seed movement and pathogen spreading. The results of this work confirm the huge importance of understanding the interaction between *Fop* and seeds of common bean while maintaining the management of this disease in practice in sight and the clear need of additional studies in this line of research.

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Paper 2

Studies of the association of *Fusarium oxysporum* f. sp. *phaseoli* in common bean seeds

Estudos da associação de *Fusarium oxysporum* f. sp. *phaseoli* em sementes de feijão

Prepared according to Scientia Agricola Journal guidelines

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ABSTRACT

Green fluorescent protein (GFP) has been used as a marker for studying the colonization of different crops by plant pathogens. In order to follow the colonization process by *Fusarium oxysporum* f. sp. *phaseoli* (*Fop*) in common bean seeds (*Phaseolus vulgaris*), that marker was tested using one pathogenic strain of *Fop* which was GFP-labeled following protocols described in literature. The transformant was tested in PDA containing hygromycin-B (300 µg mL^{-1}) and conventional PCR. The transformation of the pathogen can be considered successful by taking into account the high proportion of fungal colonies formed on PDA with hygromycin-B. Bean seeds, cv. Uirapuru, were surface-disinfected, dried in a hood overnight and inoculated with the transformed fungus using the osmotic technique. The pathogen was detectable in whole embryonic axis, including the plumule, and on endosperm of the common bean seeds, confirming the infection process. GFP-tagged mycelium was externally observed in the roots and hypocotyl of the plants. Vascular discolorations were well developed in advance of the *Fop*-infection. These results show that the pathogen was able to colonize both external and internal tissues of infected seeds as well as external and vascular tissues of the resulting plants.

Key-words: Fluorescence microscopy, Fusarium wilt, *Phaseolus vulgaris*, seed-pathogen interaction.

RESUMO

A proteína fluorescente verde (GFP) tem sido utilizada como um marcador nos estudos de colonização de fitopatógenos, em diferentes culturas. Com a finalidade de acompanhar o processo de colonização de *Fusarium oxysporum* f. sp. *phaseoli* (*Fop*), em sementes de feijão (*Phaseolus vulgaris*), aquela técnica foi testada usando um isolado patogênico de *Fop*, que foi marcado por GFP, seguindo protocolos descritos em literatura. O transformante foi testado em BDA, contendo higromicina-B (300 µg mL^{-1}) e PCR convencional. A transformação do patógeno foi considerada bem sucedida, tendo-se em vista a alta proporção de colônias fúngicas formadas em meio BDA, com higromicina-B. Sementes de feijão, cv. Uirapuru, foram desinfestadas superficialmente, secas em câmara de fluxo overnight e inoculadas com o transformante pela técnica de condicionamento osmótico, descrita em literatura. O patógeno foi detectado em todo o eixo embrionário, incluindo a plúmula, e no endosperma de sementes de feijão, confirmando o processo de infecção nessa interação. O micélio marcado por GFP foi observado externamente nas raízes e hipocótilo das plantas. Escurecimento vascular foi bem desenvolvido com o avanço da infecção por *Fop*. Esses resultados indicaram que o patógeno foi capaz de colonizar ambos os tecidos internos e externos de sementes infectada, assim como tecidos externos e vasos das plantas resultantes.

Palavras-chave: microscopia de fluorescência, murcha de fusarium, *Phaseolus vulgaris*, interação semente-patógeno.

INTRODUCTION

Fusarium wilt is one of the most important diseases in common bean crop, caused by the fungus *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *phaseoli* J. B. Kendrick & W. C. Snyder (*Fop*). The pathogen inhabits soil in the form of chlamydospores and may also infest seeds (Schwartz et al., 2005). Seeds can carry the inoculum with no external symptoms of infection, one of the most important sources of the pathogen dissemination over long distances, and still transmit the pathogen, even in low levels of inoculum.

The mechanism of *Fop*-seed colonization and transmission are not well understood. For some fungal-plant interactions those processes have been investigated by means of the scanning electron microscope (Alves et al., 2003; Pereira et al., 2013) and/or green fluorescent protein (GFP) expression *in planta* (Nonomura et al., 2001; Lagopodi et al., 2002; Sarrocco et al., 2007). The main difference between both techniques is that through GFP it is possible to follow the pathogen and to understand the infection process in real time. This marker has been used for studying the colonization of the host plant by pathogenic fungi, such as different formae speciales of *Fusarium oxysporum*, in many economically important crops (Nonomura et al., 2001; Lagopodi et al., 2002; Sarrocco et al., 2007). Vallad and Subbarao (2008) studied the infection and colonization of GFP-tagged *Verticillium dahliae* in lettuce. The authors reported the seedborne infection was limited to the maternal tissues of the achene, including the pappus, pericarp, integument and endosperm; but the embryo was never reached. However, there are few studies monitoring the pathogen on the seed colonization process. In order to understand the common bean seed association processes by *Fop* a virulent strain of *Fop* was transformed and seeds were inoculated with the transformed fungus with the objective to follow the seed and seedling/plant infection and colonization.

MATERIALS AND METHODS

Strain selection and origin. The *Fusarium oxysporum* f. sp. *phaseoli* (*Fop*) strain was obtained from Mycological Collection of Seed Pathology Laboratory at Federal University of Lavras, MG, Brazil. The strain LAPS 156 was identified as *Fop* based on morphological characteristics (Leslie and Summerell, 2006) and pathogenicity test (Alves-Santos et al., 2002a). Using the diagrammatic scale from CIAT (Pastor-Corrales and Abawi, 1987), the strain was identified as a highly virulent pathogen, causing death or severely infected common bean plants with 100% of the foliage showing wilt, yellowing, chlorosis, necrosis or premature defoliation.

Fungal transformation. The selected *Fop* strain, LAPS-156, was transformed with genes for green fluorescent protein (GFP) and hygromycin-B resistance using plasmid pSC001, provided by T. van der Lee (Plant Research International, The Netherlands).

Plasmid pSC001 contains the gene *GFP* attached to a *PtoxA* promoter from *Aspergillus nidulans*. It also includes a hygromycin-B phosphotransferase gene (*hygB*) for selection in fungi.

Protocols for preparation of protoplasts were adapted from *F. graminearum* by Maier et al. (2005) with some modifications. The strain was grown on potato-dextrose-agar (PDA) for six days. Fifty mL of the conidial suspension with concentration adjusted to 10^6 conidia mL^{-1}, were added to 250 mL of potato-dextrose-broth (PDB) and incubated at 28 ºC for 18 h on a rotary shaker at 125 rpm. The resulting mycelial mass was filtered and rinsed once with 0.7 M NaCl solution. Then, 100 mg of the dried mycelia were incubated with 3 mL of the lyzing enzymes (Sigma – Aldrich, St. Louis) solution, obtained with 0.7 M NaCl solution and 10 mg mL^{-1} of the enzyme at 28 ºC for 3h in constant agitation (75

rpm) to digest the cell walls. Protoplasts were harvested by filtering through two layers of sterile cheesecloth. The filtrate was centrifuged for 5 min at 4 ºC and 2,000 rpm and the pellet washed by adding 3 mL of 0.7 M NaCl cold solution. The pellet was resuspended in four parts of STC (0.8 M sorbitol, 50 mM Tris [pH 8.0], and 50 mM $CaCl_2$) and one part of SPTC (0.8 M sorbitol, 40% PEG4000, 50 mM Tris [pH 8.0], and 50 mM $CaCl_2$). The protoplasts were adjusted to 10^7 protoplasts mL^{-1} in 100 µL of suspension and 10 µL of transforming plasmid DNA (0.35-1.66 µg $µL^{-1}$) were added to the tube and incubated on ice for 30 min. One ml SPTC was mixed with the suspension and incubated at room temperature for 20 min. Protoplasts were mixed gently into 200 mL of regeneration medium (yeast extract [0.1%], caseinhydrolysate [0.1%], sucrose [1.0 M], granulated agar [1.6%]; at 43 ºC), poured in 94 mm Petri dishes (20 mL per plate) and incubated at 25 ºC for 72 h. Upon fungal growth, 10 mL of selective agar (1.2% granulated agar in water containing hygromycin-B [300 µg mL^{-1}]) were overlaid and further incubated. Transformants were obtained after 4 - 15 days. They were transferred to fresh plates of CM medium with 300 µg mL^{-1} of hygromycin-B and incubated at 25 ºC. The transformed isolates appeared bright green fluorescence under fluorescence stereomicroscope Leica DFC 310 FX with 480 nm excitation/500-to-550 emission filter block. The transformed *Fop* isolates were purified by single-spore isolation, and tested for their pathogenicity, according to Alves-Santos et al. (2002a). One of these transformed isolates was randomly selected for use in further experiments.

The stability test was applied by transferring eight consecutive times of a mycelium disk of the transformant to PDA without the addition of hygromycin-B. After that, a mycelium disk was transferred to PDA with addition of hygromycin-B [300 µg mL^{-1}] and whether hyphae grew on it, the transformants

were stable. The fluorescence of the transformed isolates was observed under microscope to confirm if they remained stable.

Determining *gfp* presence. For DNA and protein extraction, stable transformed *Fop* isolates and the progenitor WT isolates were grown on sterile potato-dextrose-agar (PDA) at 25 ºC for seven days. Mycelial mats were harvested and ground into a fine powder under liquid nitrogen. Forty mg of the powder were used for DNA extraction using the commercial kit Wizard® Genomic DNA Purification Kit (Promega, Madison, WI), according to manufacturer's protocol. The quality and quantity of DNA were ascertained via a NanoDrop-1000 spectrophotometer (Thermo Scientific) at 260 nm, and adjusted to a final concentration of 20 ng μL^{-1} with ultrapure water. To confirm the presence of the *gfp* gene, conventional PCR was performed on transformed and WT isolates using forward 5'-GCGACGTAAACGGCCACAAG-3' and reverse 5'-CCAGCAGGACCATGTGTGATCG-3' primers which amplified a 606-bp fragment of the *gfp* sequence (de Silva et al., 2009). A 25 µL-PCR mix was prepared, containing 1x Platinum® PCR buffer (Invitrogen, Carlsbad, CA); 25 mM $MgCl_2$ solution; 1 mM dNTP mix; 0.25 µM forward primer; 0.25 µM reverse primer; 1U Platinum® Taq DNA polymerase (Invitrogen); 2.0 µL template DNA. PCR reactions were carried out in a Multigene TC 9600-G (Labnet International Inc.; software V3.3.4C) cycler programmed for the following parameters: 94°C for 5 min, 35 cycles at 94°C for 1 min, 53°C for 2 min, and 72°C for 1.5 min, and a final extension period of 5 min at 72°C (de Silva et al., 2009). PCR-amplified DNA fragments were fractionated in 1.2% of agarose gel in 1x Tris-borate-EDTA buffer and visualized in GelRed™ (Biotium).

Morphological and cultural characteristics. For the set-up of this experiment, the selected transformed isolate was transferred to PDA and PDA amended with hygromycin-B (300 mg μL^{-1}) to examine the morphology and cultural characteristics of *Fop*. The WT isolate was used as the control. For the evaluation of mycelial growth index, mycelium disks of 4 mm in diameter were placed in the center of the 94 mm Petri dish containing the culture media and incubated at 25 °C and 12-hour photoperiod. The diameter of the mycelium growth was evaluated daily up to the seventh day. Four treatments were obtained from the combination of two isolates on two culture media. Five replicates were used per each treatment and the experiment was designed in completely randomized treatments. The data were estimated in mycelial growth index by using the formula of Maguire (1962). The data were submitted to analyses of variance and Tukey's test.

MGI = C1/N1 + C2/N2 +…+ Cn/Nn , which:
MGI: mycelial growth index
C1, C2, Cn: mycelium growth measure (cm) day-by-day
N1, N2, Nn: number of days.

Seed inoculation. A commercial common bean seed lot (*Phaseolus vulgaris*) cv. Uirapuru was obtained from Federal University of Lavras / Department of Biology (Lavras, MG, Brazil). The absence of *F. oxysporum* was initially confirmed by blotter test (Brasil, 2009) and conventional PCR, using the primer set published by Alves-Santos et al. (2002b). For that, seeds were surface disinfected in 1% NaHClO solution for 2 minutes followed by drying on a sterile filter paper in a laminar flow hood.

To investigate the penetration step of *Fop* in common bean seeds, five seeds were inoculated by the osmotic technique by exposing them to wild-type *Fop*

culture for 36, 72 and 96 h. The negative control was *Fop*-free seeds. All samples were prepared for observation in SEM. Seeds of each treatment were immersed in Karnovsky solution (Karnovsky, 1965) for 24 h and soaked three times for 10 min with sodium cacodilate buffer and transferred to an aqueous solution containing 30% glycerol for 30 min. Seeds were cross-sectioned in liquid nitrogen using a scalpel (Alves et al., 2003). Sections were transferred to 1% aqueous solution of osmium tetroxide for one hour at room temperature and subsequently dehydrated in an increasing acetone concentration series (25, 50, 75, 90 and 100%) for 10 min each and after critical point dried (Balzers CPD 030). Processed materials were fixed on aluminum stubs with a double faced tape, sputter coated with gold (Balzers SCD 050) and observed under a LEO EVO 40 XVP scanning electron microscope (SEM). Images were obtained at lower and higher magnifications and were processed using the Software Corel Draw 12.

Cultures of wild-type and transformed isolates were grown on PDA medium for seven days at 25°C and 12 h photoperiod. Fifty seeds were exposed on the colonies for 72 h; ten seeds were directly observed on fluorescence stereomicroscope Leica DFC 310 FX. Histopathological sections from inoculated seeds were done in order to observe the infection of the seed tissues. Four replications containing 10 inoculated seeds were sowed on paper-towel and incubated at 25 ºC with 100% humidity. Twenty seedlings were taken at three and five days after sowing on paper-towel for observation in fluorescence stereomicroscope Leica DFC 310 FX. After external observation, seedling sections were prepared to visualize the internal tissues in an Epi-fluorescence microscope with Apotome system (Carl Zeiss) in order to analyze the *Fop* infection and colonization in seeds and seedlings.

RESULTS AND DISCUSSION

The transformants produced a bright green color in whole cytoplasm of the mycelia, macro and microconidia but not in the nucleus (data not shown). The bands obtained with transformed isolates by polymerase chain reaction (PCR) confirmed the presence of *gfp* gene in those populations. PCR detected a 606-bp gene sequence from the *gfp* gene for all transformed isolates and no bands were detected in wild-type isolates. The pathogenicity of the GFP-tagged *Fop* was maintained and the transformant was considered as a highly virulent strain. These results showed that *gfp* plasmid had integrated the *Fop* genome in 21 from 38 (55%) hygromycin-B-resistant transformants expressing the green fluorescent protein. Nineteen of them had reached high mitotic stability with seven successive transfers on PDA and they were demonstrated as pathogenic as wild-type *Fop*, causing very severe symptoms in common bean plants, which 60-100% of the foliage showing wilting, chlorosis, necrosis, premature defoliation or dead plants (data not shown).

On the basis of brightness and stability of the fluorescence and hygromycin-B resistance, similarity of the transformed colony morphology to the wild-type was confirmed. The measurements of the mycelium diameter of both colonies showed that the mycelial growth index was higher on PDA than on PDA supplemented with hygromycin-B (Fig. 1). Transformed and wild-type colonies showed the pale violet color of the typical mycelia of *Fop* on PDA medium. Shape and size of the transformed colonies in PDA and the morphological structures were similar to the wild-type colonies. The addition of the antibiotic to the medium had selected the resistants. Some differences such as mycelial growth index and mycelium color were found in PDA supplemented with hygromycin-B, but only GFP-tagged *Fop* grew up on PDA supplemented with hygromycin-B (Fig. 1), wherein they showed a different mycelial color ranging

from white to orange. The same behavior with different pathogenic fungi was found in previous studies (Lorang et al., 2001; Visser et al., 2004; de Silva et al., 2009).

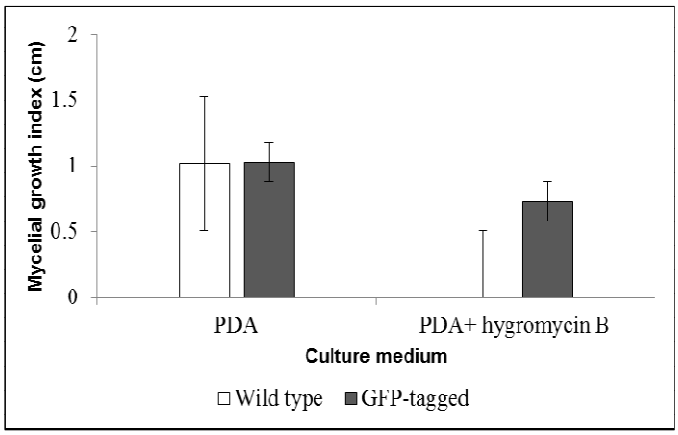

Fig. 1. Mycelial growth index (MGI) from wild type and green fluorescent protein-tagged *Fusarium oxysporum* f. sp. *phaseoli* isolates. The isolates were cultured for seven days in potato-dextrose-agar (PDA) or PDA supplemented with hygromycin-B and the diameter of the colonies were measured daily

From the inoculated common bean seeds with *Fop*, which was carried out to know the most appropriate inoculum potential for studying the interaction in this pathosystem with observation in SEM revealed interesting findings. For example, no mycelium was observed on *Fop*-free seed coat (Fig. 2A) but hyphae had colonized the seed coat after 36 h of contact with *Fop* colony and initiated the seed infection process (Fig. 2B). Small conidiophores, the main morphological structure of *F. oxysporum*, were observed on seeds which were exposed to fungal colony for 72 h (Fig. 2C) as well as a large amount of the hyphal penetration into the seeds directly through the cuticle (Fig. 2D, E) and with appressorium formation (Fig. 2F). After 96 h of contact of the seeds with

the *Fop* colony a large amount of hyphae was developed on the seed surface making difficult the observation of the penetration process (data not shown).

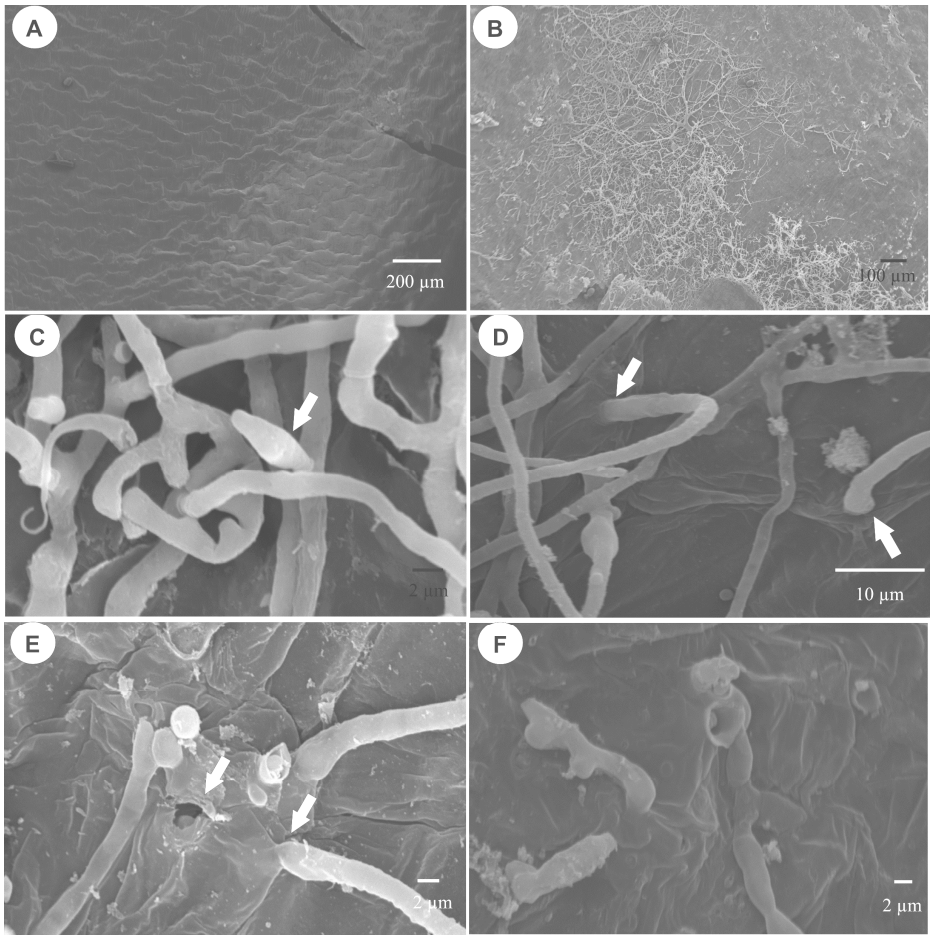

Fig. 2. Scanning electron micrographs of common bean (*Phaseolus vulgaris*) seed surface. **A)** *Fusarium oxysporum* f. sp. *phaseoli*-free seed coat; **B)** Hyphae of *F. oxysporum* f. sp. *phaseoli* colonizing and covering the seed coat after 36 h of inoculation; **C)** Hyphae with small conidiophore on seed coat after 72 h of inoculation; **D)** Direct penetration of hyphae into the seeds after 72 h of inoculation; **E)** Hyphae adhered to seed coat and a hole found on seed surface after the complete penetration process; **F)** Hyphal penetration with formation of appressorium

The observation on SEM is very useful but caution has to be taken concerning the presence of other fungi which may be present in the tissues of the seeds. A similar infection process was reported by Rahman et al. (2010) who observed direct penetration of hyphae of *F. moniliforme* into soybean seeds after 48 h of inoculation, with or without appressorium formation. Murillo et al. (1999) also showed the direct penetration of the maize seed pericarp by *F. verticillioides* three days after sowing the inoculated seeds.

From the Epi-fluorescence microscope observations of 72h-*Fop* inoculated seeds, the development of *Fop* mycelium on embryo was confirmed under favorable conditions, infecting the seeds. The mycelium had colonized the tegument, cotyledons and embryonic axis of the seeds (Fig. 3). The GFP marker has proved to be a useful tool to investigate the interaction of *Fop* and seeds of common bean.

GFP-expressing transgenic isolates can be visualized in living tissues without any processing or manipulation of the samples (Oren et al., 2003). In the present study, hyphae eventually grew into cotyledons of the seeds (Fig. 3B), a common source of starch, and was able to attack the embryonic axis, where a large amount of mycelium on plumule or first leaves was visible (Fig. 3A) when compared with the radicle. These results differed from Vallad and Subbarao (2008) who reported that *Verticillium dahliae* never colonized the embryo of susceptible lettuce cultivar in naturally infected seeds. In the present study, the seeds were artificially inoculated for 72-h and then the inoculum load might be higher than in naturally infected seeds. In the seedling post-emergence step, the present *Fop* in contaminated cotyledons can be transmitted to seedlings where it can move to vascular tissue. When the two primary leaves are unfolded in the vegetative growth stage, the cotyledons eventually fall off and continue on the soil, being an important source of inoculum in the field. The same behavior may occur for contaminated seed coats that remain on the soil (Fig. 3C, D).

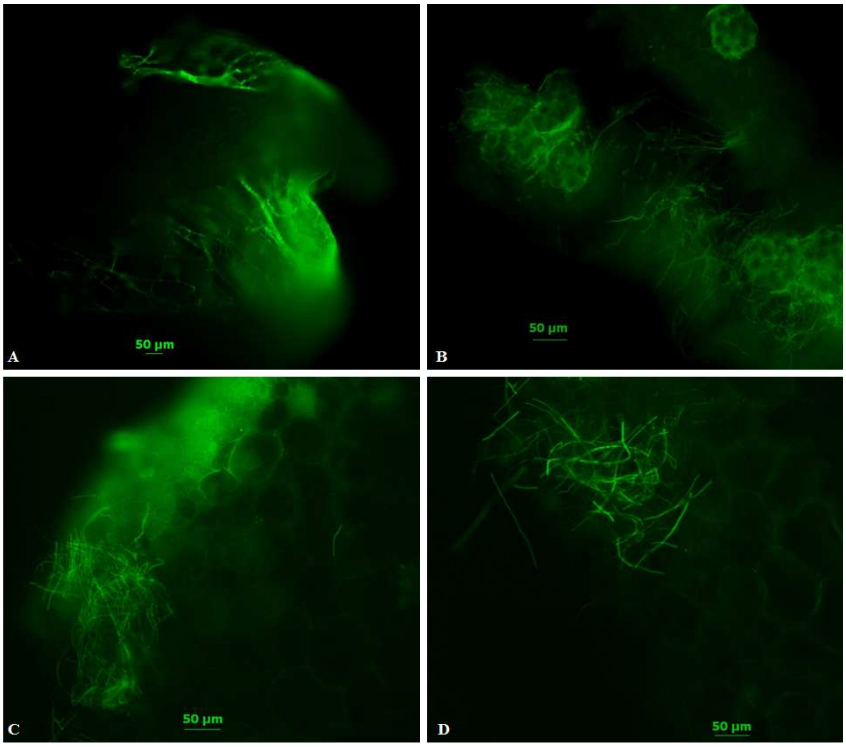

Fig. 3. Epi-fluorescence microscope images of the sections of the infected common bean seeds inoculated with *Fusarium oxysporum* f. sp. *phaseoli* isolate expressing green fluorescent protein (GFP). (**A)** Infected plumule from embryo; **(B)** Infected cotyledons; **(C and D)** Seed coat (tegument) and cotyledons of the seeds

For the rolled-towel assay, *Fop* mycelia start to grow up on roots three days after sowing and a few symptoms were observed. After five days, the roots of the seedlings/plants were externally covered with *Fop*-mycelium (Fig. 4), and necrotic lesions and collapse of infected secondary roots were observed. The green fluorescence was intense when a large amount of the transformed-*Fop* mycelium colonized the common bean roots five days after sowing (Fig. 4A) compared to three days after sowing (Fig. 4B). These results are consistent with those from Dowd et al. (2004) who observed that more plant genes were induced in *F. oxysporum* f. sp. *vasinfectum*-infected hypocotyl tissues of cotton,

compared with infected root tissues, suggesting the fungus may be suppressing plant defense responses in the root tissue and presenting more disease symptoms.

Fig. 4. Fluorescence stereomicroscope images of the colonized common bean root by *Fusarium oxysporum* f. sp. *phaseoli* isolate expressing green fluorescent protein (GFP). Seeds were incubated on GFP-tagged isolate for 72-h and incubated on rolled-towel under favorable conditions **A)** for three days; **B)** for five days; **C)** Negative control

Even though the main root and crown region exhibited severe vascular discoloration, the fungus was absent upon closer examination. The infected tissues were not detected by fluorescence microscopy examination, but only by culturing on a semi-selective medium.

Some previous microscopic studies had examined the root interactions of many economically important crops with different *formae speciales* of transformed-*Fusarium oxysporum* (Lagopodi et al., 2002; Nonomura et al., 2001; Sarrocco et al., 2007). Nevertheless, studies about pathogen-seed interactions are still poorly understood. In this paper the transformed *Fop* was visualized during the infection and colonization process from common bean seeds, confirming the presence of *Fop* inoculum on embryo under favorable conditions of environment and pathogen virulence.

CONCLUSIONS

By GFP marker it was possible to observe that under favorable conditions, *Fop*-hyphae present on common bean seed surface may start the infection process by direct penetration using mechanical force with or without forming appressorium. The fungus colonizes the cotyledons and embryo of common bean seeds, becoming an important source of dissemination of *Fop*. The pathogen moves with the seedling development, colonizing external and internal roots, with observation of rotted roots and vascular discoloration symptoms in young plants.

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Paper 3

Detection of *Fusarium oxysporum* f. sp. *phaseoli* in common bean seeds by real-time PCR assays

Detecção de *Fusarium oxysporum* f. sp. *phaseoli* em sementes de feijão através de testes de PCR em tempo real

Prepared according to Plant Pathology guidelines

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ABSTRACT

Fusarium oxysporum f. sp. *phaseoli* (*Fop*) can be introduced into *Phaseolus vulgaris* fields through infested seed, persisting in soil as chlamydospores. PCR-based seed health testing methods can aid in prevention of the long-distance spread of this pathogen by contaminated seeds. The objective of this study was to develop a rapid, accurate and sensitive protocol for the reliable detection of *Fop* in common bean seeds through real-time PCR assays (qPCR). Seed lots with infection incidence ranging from 0·25 to 20% were prepared by mixing known amounts of *Fop*-infected seeds with *Fop*-free seeds of seven cultivars. Direct comparisons between SYBR Green and TaqMan qPCR methods were performed using the primers based on *Fop* virulence factor *ftf1*. The primers produce a 63-bp product for highly virulent strains of *Fop* but do not produce a positive reaction with non-pathogenic *F. oxysporum* from *P. vulgaris* or other sources. Under optimized conditions, both qPCR assays detected 0·25% of *Fop* infection (one infected seed mixed with 399 *Fop*-free seeds), but in the SYBR Green assay PCR inhibitors interfered with the quantification of target DNA. Significant linear regression models describing the relationship between results of the qPCR assays and the artificial infection levels were found for all cultivars although the TaqMan assay yielded more reliable quantification. Using a TaqMan assay, naturally infected seeds of three cultivars and three seed sizes were tested, and *Fop* was detectable in the small-seeded lots of two cultivars only. Our results suggest that the TaqMan assay developed in this study is a useful tool for detection and quantification of *Fop* in seeds.

Key-words: DNA quantification, Fusarium wilt, *Phaseolus vulgaris*

RESUMO

Fusarium oxysporum f. sp. *phaseoli* (*Fop*) pode ser introduzido em campos de *Phaseolus vulgaris* através de sementes contaminadas, sobrevivendo no solo por meio de clamidósporos. Os testes de sanidade baseados em PCR podem ajudar a prevenir a dispersão do patógeno a longas distâncias por meio de sementes contaminadas. Objetivou-se, neste estudo, desenvolver um protocolo rápido, eficiente e sensível para detecção confiável de *Fop* em sementes de feijão por meio de PCR em tempo real (qPCR). Lotes de sementes com incidência de infecção variando de 0,25 à 20% foram preparados através de misturas de sementes infectadas e não infectadas de sete cultivares. Comparações entre SYBR Green e TaqMan foram realizadas usando-se os primers que amplificam o gene *ftf1* relacionado ao fator de virulência de *Fop*. Eles produzem um amplicon de 63 pb para isolados de *Fop* altamente virulentos, mas não produzem uma reação positiva para os isolados não patogênicos de *F. oxysporum* de *P. vulgaris* e outras culturas. Sob condições otimizadas, ambos os ensaios de qPCR detectaram 0,25% de infecção por *Fop* (uma semente infectada em mistura com 399 sementes sadias) mas alguns inibidores de PCR interferiram na quantificação do DNA-alvo em ensaios com SYBR Green. Modelos de regressão lineares significativos descreveram a relação entre os resultados dos ensaios de qPCR e os níveis de infecção artificiais para as sete cultivares embora TaqMan apresentou os resultados mais confiáveis. Utilizando-se TaqMan, sementes naturalmente infectadas de três cultivares e tamanhos foram testadas e *Fop* foi detectável em lotes de sementes pequenas de duas cultivares. Os resultados deste trabalho sugerem que o método de TaqMan pode ser útil para a detecção preventiva e quantificação do DNA de *Fop* em sementes de feijão.

Palavras-chave: quantificação de DNA, murcha de fusarium, *Phaseolus vulgaris*

INTRODUCTION

The nutritional value of common bean, *Phaseolus vulgaris* L., is high, with significant concentrations of protein and minerals, and as a result, this crop is gaining increased international attention in agriculture research for its critical role in enhancing the nutritional quality of diets (Schwartz *et al.*, 2005). According to the Food and Agricultural Organization (FAO), in 2011 India, Myanmar and Brazil were the leading bean producers; India and Myanmar producing large quantities of *Vigna* beans and Brazil the largest producer of common bean with an annual production of 3,434,370 metric tons.

Common bean production is severely constrained by a number of crop pathogens including *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *phaseoli* J. B. Kendrick & W. C. Snyder (*Fop*), the causal agent of Fusarium wilt, a disease that can reduce bean yields by as much as 30% (Abawi & Pastor-Corrales, 1990; Cramer *et al.*, 2003). Fusarium wilt symptoms include foliar chlorosis, premature senescence of lower leaves, red-brown discoloration of vascular tissue, and wilting, which often lead to early maturity, reduction in seed size, yield loss, and plant death (Abawi & Pastor-Corrales, 1990; Brick *et al.*, 2006; Ronquillo-Lopez *et al.*, 2010). The pathogen is considered to be a soil inhabitant that is most likely introduced into bean fields through the use of contaminated farm implements or infested seed (Toledo-Souza *et al.* 2012). It is believed that the greatest potential for long distance dissemination of *Fop* is through seed (Santos *et al.*, 1996; Vilela *et al.*, 2010), with seed-to-plant transmission rates as high as 42·8% from naturally infested common bean seeds (Santos *et al.*, 1996).

An important preventive management tool for reducing the spread of pathogens into new regions is accurate seed health testing (Mbofung & Pryor, 2010). Most current phytosanitary seed-health testing is based on incubation methods or microscopy-based methods (Munkvold, 2009), which often require

confirmatory pathogenicity tests. For determining the incidence of *Fop* infection in bean seeds, the routine testing currently used is a blotter test followed by a grow-out test (MAPA, 2009). A semi-selective media, Nash & Snyder medium + PCNB (Nishimura, 2007), followed by identification of pathogens via microscopy, is also traditionally recommended. These methods are labor intensive, require skilled personnel, and are time consuming. The occurrence of saprophythic strains of *F. oxysporum* on bean seeds and other related species which are morphologically similar to *F. oxysporum* f. sp. *phaseoli* are important limitations of these biological methods (Alves-Santos *et al.*, 2002a). Many of these limitations can be overcome by utilizing PCR-based methods for seed health testing (Munkvold, 2009).

PCR-based detection methods are useful tools that have been developed and applied to many seedborne pathogens (Munkvold, 2009) and have been proven to be reliable alternative methods for making accurate decisions regarding the acceptability of seeds for sale, appropriate use of seed treatment, for seed certification programs (Mbofung & Pryor, 2010) and for differentiating between nonpathogenic and pathogenic forms of *F.* o*xysporum* (Alves-Santos *et al.*, 2002a). Previous studies showed that a *Fusarium* transcription factor, *ftf1*, contains a Zn(II)2-Cys6 binuclear cluster DNA-binding motif that is related to a virulence factor, and is characteristic of virulent *Fop* strains (Ramos *et al.*, 2007; Garcia-Sanchez et al, 2010; Vega-Bartol *et al.*, 2011). This gene makes an excellent candidate for the development of a molecular diagnostic method for the detection of *Fop* in bean seeds.

The majority of studies that have used PCR for specific detection of pathogens in seeds are qualitative tests to detect the presence of the pathogen, and not to quantify the level of pathogen contamination (Gracia-Garza *et al.*, 1999; Chiocchetti *et al.*, 2001; Pryor & Gilbertson, 2001; Kulik, 2008; Mbofung & Pryor, 2010). An assay to quantify the amount of pathogen DNA in seed

health testing is desirable to measure the actual infection level of the seed lot and this can be achieved through real-time PCR (Glynn & Edwards, 2010).

Real-time PCR has great potential to replace or complement current seed health assays for several reasons, such as speed, specificity, sensitivity and quantification of the target DNA (Ganchon *et al.*, 2004; Munkvold, 2009). Additionally, a large number of samples can be processed within a short period of time and it can be conveniently applied to commercial seed testing and certification (Mbofung & Pryor, 2010). The advantages of real-time PCR for seed health testing are well documented but data comparing two of the most frequently used chemistries, SYBR Green and TaqMan, is still limited. Thus in addition to establishing a real-time PCR assay for the detection of *Fop* in bean seed, we also compared the relative efficacy of these two approaches.

MATERIALS AND METHODS

Fungal isolates and DNA extraction. Isolates of *Fop* and other fungal isolates used in this study are listed in Table 1 with their geographical origin and range of virulence. Cultures were grown on wheat-germ medium and potato-dextrose-agar (PDA; Difco Laboratories, Plymouth, MN) at 22°C. After seven days, each isolate was identified based on standard morphological criteria (Leslie & Summerell, 2006) followed by pathogenicity testing (Alves-Santos *et al.*, 2002b).

For DNA extraction, liquid cultures were prepared by flooding PDA plates containing 5-day-old fungal colonies with 10 mL of sterile distilled water and dislodging mycelia and conidia with a sterile stick. The final concentration of the inoculum suspension was adjusted to 10^6 conidia ml^{-1} and an aliquot of 2 mL was added to 50 mL of sterile liquid DNA medium (3 g yeast extract, 3 g malt extract, 5 g peptone, 20 g dextrose, 2 g NH_4SO_4 per liter of distilled water). Cultures were incubated on a rotary shaker at 120 rpm for two days at 22 °C. Mycelia were harvested by filtration through Miracloth (EMD Biosciences, Inc., La Jolla, CA), lyophilized and then individually ground with a sterilized pestle and mortar. The fungal DNA was extracted as described by Zelaya-Molina *et al.* (2011). The quality and quantity of DNA was ascertained via a NanoDrop-1000 spectrophotometer (Thermo Scientific) at 260 nm, and adjusted to a final concentration of 20 ng μL^{-1} with TE buffer.

Table 1. Fungal isolates used in this study and characterized by pathogenicity, virulence and qPCR amplification

Species[a]	Isolate	Host	Geographic origin	Source[b]	Patho-gen[c]	qPCR[d]
Fop	ATCC 18131	*P. vulgaris*	California	ATCC	HVP	+
Fop	FOP 8	*P. vulgaris*	Colorado	HFS	HVP	+
Fop	FOP 16	*P. vulgaris*	Colorado	HFS	HVP	+
Fop	FOP 31	*P. vulgaris*	Netherlands	HFS	HVP	+
Fop	FOP 48	*P. vulgaris*	Colorado	HFS	WVP	-
Fop	FOP 52	*P. vulgaris*	Colorado	HFS	HVP	+
Fop	FOP 58	*P. vulgaris*	Spain	HFS	HVP	+
Fop	LAPS 152	*P. vulgaris*	Brazil	MHDM	HVP	+
Fop	LAPS 153	*P. vulgaris*	Brazil	MHDM	HVP	+
Fop	LAPS 154	*P. vulgaris*	Brazil	MHDM	HVP	+
Fop	LAPS 155	*P. vulgaris*	Brazil	LAPS	HVP	+
Fop	LAPS 156	*P. vulgaris*	Brazil	LAPS	HVP	+
Fop	LAPS 157	*P. vulgaris*	Brazil	LAPS	WVP	-
Fop	LAPS 164	*P. vulgaris*	Brazil	LAPS	HVP	+
Fop	LAPS 165	*P. vulgaris*	Brazil	LAPS	WVP	-
Fop	LAPS 168	*P. vulgaris*	Brazil	LAPS	WVP	-
Fop	CML 144	*P. vulgaris*	Brazil	LHP	WVP	-
Fop	2556-1	*P. vulgaris*	Brazil	MFI	HVP	+
Fop	6825-1	*P. vulgaris*	Brazil	MFI	WVP	-
Fop	9455	*P. vulgaris*	Brazil	MFI	HVP	+
Fop	9840	*P. vulgaris*	Brazil	MFI	WVP	-
Fop	FOPS 018	*P. vulgaris*	Brazil	LAPS	HVP	+
Fop	FOPS 019	*P. vulgaris*	Brazil	LAPS	HVP	+
Fop	FOPS 020	*P. vulgaris*	Brazil	LAPS	HVP	+
Fop	FOPS 021	*P. vulgaris*	Brazil	LAPS	HVP	+
Fop	FOPS 022	*P. vulgaris*	Brazil	LAPS	HVP	+
Fop	FOPS 023	*P. vulgaris*	Brazil	LAPS	HVP	+
Fop	FOPS 024	*P. vulgaris*	Brazil	LAPS	HVP	+
Fop	FOPS 025	*P. vulgaris*	Brazil	LAPS	HVP	+
Fop	FOPS 026	*P. vulgaris*	Brazil	LAPS	HVP	+
Fov	CML 1098	*Gossypium hirsutum*	Brazil	LHP	NP	-
F. oxysp.	20L6	*Glycine max*	Benton, IA	ME	NP	-
F. oxysp.	258L1	*G. max*	Blackhawk, IA	ME	NP	-
F. oxysp.	258L3	*G. max*	Blackhawk, IA	ME	NP	-
F. oxysp.	5L7	*G. max*	Carrol, IA	ME	NP	-
F. oxysp.	42L9	*G. max*	Floyd, IA	ME	NP	-

...Continue...

Table 1. *continuation…*

Species[a]	Isolate	Host	Geographic origin	Source[b]	Patho-gen[c]	qPCR[d]
F. oxysp.	20L6	*G. max*	Benton, IA	ME	NP	-
F. oxysp.	34L2	*G. max*	Hamilton, IA	ME	NP	-
F. oxysp.	34T3	*G. max*	Hamilton, IA	ME	NP	-
F. oxysp.	4T6	*G. max*	Sioux, IA	ME	NP	-
F. oxysp.	258L8	*G. max*	Blackhawk, IA	ME	NP	-
F. oxysp.	11L8	*G. max*	Lyon, IA	ME	NP	-
F. oxysp.	34L4	*G. max*	Hamilton, IA	ME	NP	-
F. oxysp.	61L5	*G. max*	Taylor, IA	ME	NP	-
F. oxysp.	55L8	*G. max*	Johnson, IA	ME	NP	-
F. oxysp.	36L8	*G. max*	Marshall, IA	ME	NP	-
F. oxysp.	68T10	*G. max*	Polk, IA	ME	NP	-
F. oxysp.	372T7	*G. max*	Harrison, IA	ME	NP	-
F. oxysp.	219L6	*G. max*	Cass, IA	ME	NP	-
F. oxysp.	CML755	*G. max*	Brazil	LHP	NP	-
F.comm.	258T7	*G. max*	Blackhawk, IA	ME	NP	-
F.comm.	48T1	*G. max*	Carroll, IA	ME	NP	-
F.comm.	311T7	*G. max*	Carroll, IA	ME	NP	-
F.semit.	009	*P. vulgaris*	Brazil	LAPS		-
F.sol.	008	*P. vulgaris*	Brazil	LAPS	HVP	-
F.vert.	010	*Zea mays*	Brazil	LAPS		-
M. phaseol.	2964	*G. max*	Guthrie Co.	ESR		-
S. sclerot.	Ss1	*G. max*	Nashua, IA	ESR		-

[a] Abbreviations for especies of fungal isolates are as follows: *Fop* = *Fusarium oxysporum* f. sp. *phaseoli*; *Fov* = *Fusarium oxysporum* f. sp. *vasinfectum*; *F. oxysp*. = *Fusarium oxysporum*; *F.comm. = Fusarium commune*; *F. sol. = Fusarium solani*; *F. vert. = Fusarium verticillioides*; *M. phaseol. = Macrophomina phaseolina*; *S. sclerot. = Sclerotinia sclerotiorum*.

[b]Abbreviations for sources of fungal isolates are as follows: ATCC = American type Culture Collection, Rockville, MD, USA; HFS = H.F. Schwartz, Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO, USA; MHDM = M.H.D MORAES, Departamento de Fitopatologia e Nematologia, Escola Superior de Agricultura "Luiz de Queiroz", Piracicaba, SP, Brazil; LAPS = Laboratorio de Patologia de Sementes, Universidade Federal de Lavras, Lavras, MG, Brazil; LHP = L.H. Pfenning, Departamento de Fitopatologia, Laboratorio de Micologia, Lavras, MG, Brazil; MFI = M.F. Itto, Instituto Agronomico de Campinas, Campinas, SP, Brazil. ME = M. Ellis, Department of Plant Pathology and Seed Science Center, Iowa State University, Ames, IA, USA; ESR = E.Saalau-Rojas, Plant and Insect Diagnostic Clinic, Iowa State University, Ames, IA, USA.

[c]Abbreviations for virulence of the strain: HVP = highly virulent pathogen; WVP = weakly virulent pathogen; NP = non-pathogenic.

[d]qPCR assay was carried out using the primer set QFOP2A/QFOP2B. +: positive amplification, -: negative amplification.

PCR protocol optimization. *Fusarium* transcription factor 1(*ftf1*) sequences generated from Ramos *et al.* (2007) were used to design the primers and probe (Table 2), using the program Primer Express® Software version 3·0 (Applied Biosystems). The primer pairs used in previous studies by Ramos *et al.* (2007) and Ren-Feng *et al.* (2012) (Table 2) also were validated with a small sample of *Fop* isolates used in this study (data not shown). A temperature gradient was performed to test the primer sets, using a highly virulent strain of *Fop* (ATCC 18131). A 25 µL-PCR mix was prepared, containing 1x Green GoTaq® Flexi buffer (Promega, Madison, WI); 2·5 mM $MgCl_2$ solution; 0·39 mM dNTP mix; 2·5 µM forward primer; 2·5 µM reverse primer; 5U GoTaq®DNA polymerase (Promega); 2·0 µL template DNA. PCR reactions were carried out in a Bio-Rad thermal cycler programmed for the following parameters: 94 °C for 5 min, 30 cycles at 94 °C for 30 sec, 52-60 °C for 30 sec, and 72 °C for 1 min, and a final extension period of 10 min at 72 °C. Based on the gradient PCR the optimum annealing temperature was determined to be 60 °C and the primers selected for use were QFOP2A/QFOP2B as they did not undergo primer dimerization at low temperatures (data not shown).

In order to determine the specificity of the primers QFOP2A/QFOP2B, all isolates listed in Table 1 were tested. The qPCR assays and data analysis were performed on a StepOnePlus™ Real-time PCR system (Applied Biosystems). For the SYBR Green assay, the PCR mix was as follows: 2 µL of total genomic DNA, 25 nM of each primer, 2x SYBR Green, for a total reaction mixture of 20 µL. The qPCR cycling conditions were as follows: 95 °C for 10 min, followed by 38 cycles of 95 °C for 15 sec and 58 °C for 1 min, and the melt curve phase of one cycle at 95 °C for 15 sec, 58 °C for 30 sec and 95 °C for 15 sec (ramp speed 0·5 °C s^{-1}). To determine the sensitivity of the primers, 10-fold serial

dilutions of *Fop*-total genomic DNA from highly virulent isolates (Table 1) were prepared in TE buffer (concentrations ranged from 10 ng μL^{-1} to 10 fg μL^{-1}). The dilution series of genomic DNA from a pure culture of *Fop* (FOP 58) was used to create a standard curve. The qPCR assay was carried out as per the protocol described above. Samples were run in triplicate including the negative control, in which the DNA template was replaced with sterile water.

Table 2. Oligonucleotides and probe designed for quantification of *Fusarium* transcription factor 1 (*ftf1*) gene of *Fusarium oxysporum* f. sp. *phaseoli* in common bean (*Phaseolus vulgaris* L.)

Primer/probe	Sense	Sequence (5' – 3')	Ampl. size (bp)	Ref.
QFopta700A	Forward	CCTGCAAGCACTGCCTACAC	121	Ramos *et al.* (2007)
QFopta700B	Reverse	TCGCTTATCGAGCGTAACCA		
QFopA	Forward	ACATAGCGGTCTACCGTTCG	149	Ren-Feng *et al.* (2012)
QFopB	Reverse	GGTTACAGGAAGCCAAACCA		
QFOP2A	Forward	GCCTCGGACAAATTACATGGTT	63	This study
QFOP2P	Probe	**VIC**-TGCTCGATAAGCGACCGA		
QFOP2B	Reverse	ATGATGCGTTCTTCCATGCA		

Seed inoculation with *F. oxysporum* f. sp. *phaseoli*. The commercial bean (*Phaseolus vulgaris*) seed lots (Tiger's Eye, Boston Favorite, Ireland Creek Annie, Arikara Yellow, Bountiful, Carioca and Dart) were obtained from Seed Savers Exchange (Decorah, IA, USA), Harris Moran Seed Company (Modesto, CA, USA) and Grupo Farroupilha (Patos de Minas, MG, Brazil) for use in this study. Seed size was characterized for each cultivar by weighing four subsamples of 100 randomly selected seeds of each cultivar. Cultivars were categorized as "large-seeded" (≥ 40 g / 100 seeds) or "small-seeded" (< 40 g / 100 seeds) in large and small seed sizes (Table 3). The absence of *F. oxysporum*

was initially confirmed by blotter test (MAPA, 2009) and conventional PCR, using the primer set published by Alves-Santos *et al.* (2002b). One hundred seeds of each cultivar were artificially inoculated with a conidial suspension of *Fop* using the water restriction technique described by Sousa *et al.* (2008). Briefly, seeds were surface disinfested by soaking for 2 minutes in a solution containing 1% NaHClO, followed by complete drying on a sterile filter paper under a laminar flow hood. The isolate FOP58 (Table 1) was cultured for five days on potato-dextrose-agar (PDA) medium at 22 °C. Macro and microconidia were harvested by washing the surface of a culture with 10 mL of sterile distilled water. The resulting suspension was diluted with sterile water to obtain a final concentration of 10^6 spores mL^{-1} (determined with a hemacytometer). The inoculum suspension was sprayed on seeds, which were then incubated in a growth chamber in the dark for three days at 25 °C. This was then followed by air drying overnight under a hood.

Artificial infection levels, ranging from 0·25% to 20·00% seed infection incidence, were created by mixing *Fop*-inoculated seeds with non-infected seeds (Table 4). Negative control seed lots consisted of 400 *Fop*-free seeds (Table 4).

In order to test the sensitivity of *Fop* detection by qPCR even at low levels of fungal DNA per seed, 100 inoculated seeds of cultivar Carioca were surface disinfested with 1% NaHClO for two minutes, dried in a hood overnight, and blends of seeds with different artificial infection levels (Table 4) were prepared for both disinfested and non-disinfested seeds and results compared for the TaqMan PCR assay.

Table 3. Commercial common bean (*Phaseolus vulgaris* L.) seed lots used in this study. One hundred seeds of cultivars from different companies were taken randomly, weighed and compared for sorting the seed size

Seed infection	Company	Cultivar	Seed size	100-seed weight (g)[1]
Artificially infected seeds	Seed Savers Exchange (Decorah, IA, USA)	Tiger's Eye	Large	53·20
		Boston Favorite	Large	51·84
		Ireland Creek Annie	Large	52·56
		Arikara Yellow	Small	35·28
		Bountiful	Small	34·73
	Grupo Farroupilha (Patos de Minas, MG, Brazil)	Carioca	Small	27·80
	Harris Moran Seed Company (Modesto, CA, USA)	Dart	Small	25·28
Naturally infected seeds	EMBRAPA Arroz e Feijão (Santo Antônio de Goiás, GO, Brazil)	Cometa	Large	24·80
		Cometa	Medium	16·73
		Cometa	Small	9·64
		Horizonte	Large	28·19
		Horizonte	Medium	22·39
		Horizonte	Small	15·46
		Valente	Large	26·92
		Valente	Medium	21·72
		Valente	Small	15·89

[1]The 100-seed weight is an average of four replications.

Naturally infected seeds. Common bean seeds naturally infected with *Fop* were harvested from an infested area at EMBRAPA Arroz e Feijão, a Brazilian Agricultural Research Corporation. The seeds of three cultivars of common bean (Cometa, Valente and Horizonte) were separated by sieve into three different sizes (small, medium and large). The seed sizes were characterized by weighing four subsamples of 100 randomly selected seeds of each category within each cultivar (Table 3). The presence of *Fop* in these seeds was confirmed by blotter test (MAPA, 2009) and conventional PCR, using specific primers from Alves-Santos *et al.* (2002b).

Table 4. Artificial infection level of seeds with *F. oxysporum* f. sp. *phaseoli* in seven bean cultivars

Level	Infected seed/seed tested	# replications/# seeds in each rep	Seed infected with *F. oxysporum* f. sp. *phaseoli* (%)
1	0/400	4/100	0·00
2	1/400	1/400	0·25
3	2/400	2/200	0·50
4	4/400	4/100	1·00
5	80/400	4/100	20·00

DNA extraction from seed samples. Seed samples with differing artificial infection levels (Table 4) as well as naturally infected seeds (Table 3) (400 seeds per treatment) were ground into a fine powder using a Fresh Grind™ Coffee Grinder (Hamilton Beach Brands, Inc.) prior to DNA extraction. To ensure that cross contamination did not occur the stainless steel grinding chamber of the grinder was treated with 70% ethanol and 10% NaHClO between samples. Eight subsamples (50mg each) per contamination level were used for DNA extraction.

In order to determine the most effective method to eliminate PCR inhibitors, four different DNA extraction protocols were tested: a conventional CTAB chloroform: isoamyl alcohol protocol (Graham *et al.*, 1994); a protocol that combined three detergents with proteinase K (Zelaya-Molina *et al.*, 2011); DNeasy® Plant Mini Kit (QIAGEN); and Extract-N-Amp™Seed PCR kit (Sigma-Aldrich). The genomic DNA was subsequently used as the template for the qPCR assays.

qPCR assay. Quantitative PCR was carried out on the StepOnePlus™ Real-time PCR system (Applied Biosystems). Each DNA sample was loaded in triplicate in a total reaction volume of 20 µL per sample with each reaction mix containing 10 µL of 2x SYBR Green Mastermix (Applied Biosystems), 0·15µL of each primer (concentrations 2·5 pmol μL^{-1}), 2 µL of template DNA, and 7·7 µL of nuclease-free water. The following cycling conditions were used: 95 °C for a 10-min denaturation step; followed by 38 cycles of amplification at 95 °C for 15 sec and 60 °C for 1 min; and melt curve analysis of heating to 95 °C, cooling to 60 °C for 1 min, and heating to 95 °C at a rate of 0·5 °C/5 sec. For the TaqMan assay, each qPCR reaction contained 1x TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 900 nM of each primer QFOP2A and QFOP2B, 250 nM TaqMan MGB probe QFOP2P and 2 µL template DNA in a total volume of 20 µL. The qPCR cycling conditions for the TaqMan assay included a holding stage at 50 °C for 2 min and 95 °C for 20 sec; followed by 38 cycles of amplification at 95 °C for 1 sec and 60 °C for 20 sec.

Data analysis. Any DNA extract for which the real-time PCR assay produced a cycle threshold (Ct) greater than that of the 1-pg standard was regarded as undetermined, and a value of 0 was noted for DNA quantify. For the artificially infected seeds, a 2x4 (qPCR assays x artificial infection levels) factorial was

used in the ANOVA analysis for each cultivar. Linear regression analysis was performed using SAS version 9·3 (Statistical Analysis Software, Cary, NC) to plot the quantity of *Fop* DNA as well as the Ct value against the artificial infection levels for seven cultivars of common bean.

RESULTS

Primer screening and qPCR assay parameters. The primer set QFOP2A/QFOP2B amplified a 63-bp product and showed a high specificity for highly virulent strains of *Fop*. The real-time PCR assay did not amplify DNA from weakly virulent and non-pathogenic strains of *F. oxysporum* and other bean pathogens listed in Table 1. In the SYBR Green assay, the primers successfully amplified target DNA from all of the highly virulent strains of *Fop* that were evaluated, with an efficiency of 97·517%, slope of -3·383 and R^2 of 0·94 (Fig. 1). In sensitivity tests with serial dilutions of *Fop* total genomic DNA, the limit of quantification was 2 pg of target DNA with a Ct value of 36·3 (Fig. 1). The melting curve for PCR products at the end of the cycling reactions revealed a single dissociation peak at 77·26 ± 1°C, indicating the specificity of the primers. For the TaqMan MGB probe assay, the limit of quantification of target DNA was 1pg. The standard curve between log of DNA concentration vs. Ct value generated a linear fit with a slope of -3·46 and linear regression coefficient (R^2) of 0·997 and the PCR efficiency was 94·544% (Fig. 1).

DNA extraction and real time PCR tests on common bean seeds. Use of the DNeasy® Plant Mini Kit resulted in less inhibition of the PCR reaction than the conventional CTAB chloroform: isoamyl alcohol protocol or the protocol combining three detergents with proteinase K.

Statistically significant differences were observed between artificial infection levels for each cultivar. Significant linear regression models describing the relationship between results of the qPCR seed assays and the artificial infection levels were found ($P < 0·0001$) for the SYBR Green as well as the TaqMan assays.

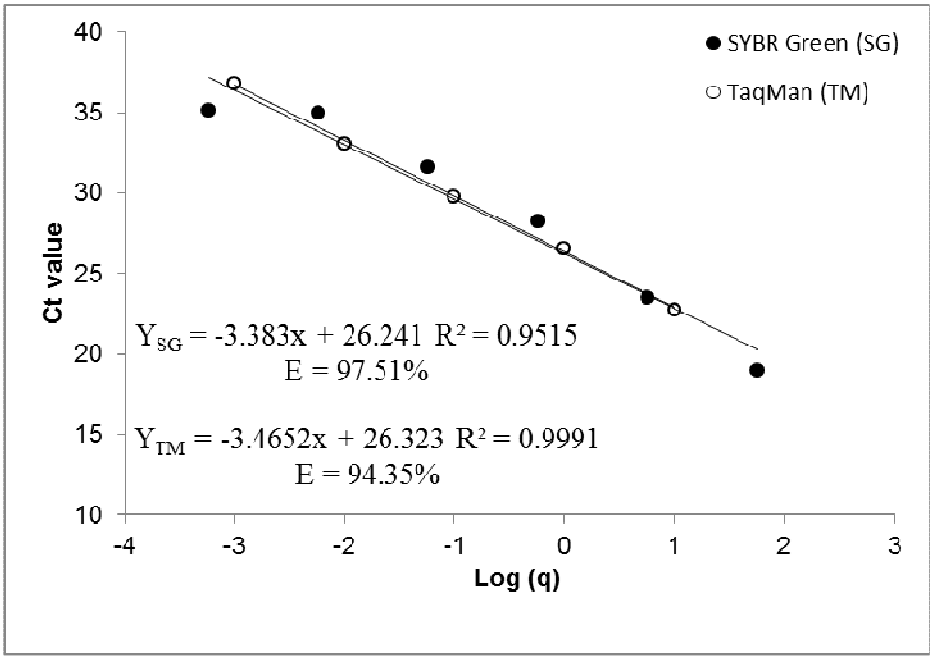

Figure 1. Standard regression lines of five-point 10-fold serial dilution of *Fusarium oxysporum* f. sp. *phaseoli* DNA comparing SYBR Green and TaqMan assays. Threshold cycles (Ct) were plotted against the log of genomic DNA standard curves of known concentrations. Data are an average of three separate runs of the qPCR assay

Pathogen DNA quantity decreased as the percent of seed infection decreased (Fig. 2), increasing the Ct value (Fig. 3). In the SYBR Green assay, the mean Ct value ranged from 23·961 to 37·462 according to the infection level and the amount of *Fop* genomic DNA present, which was estimated based on the known

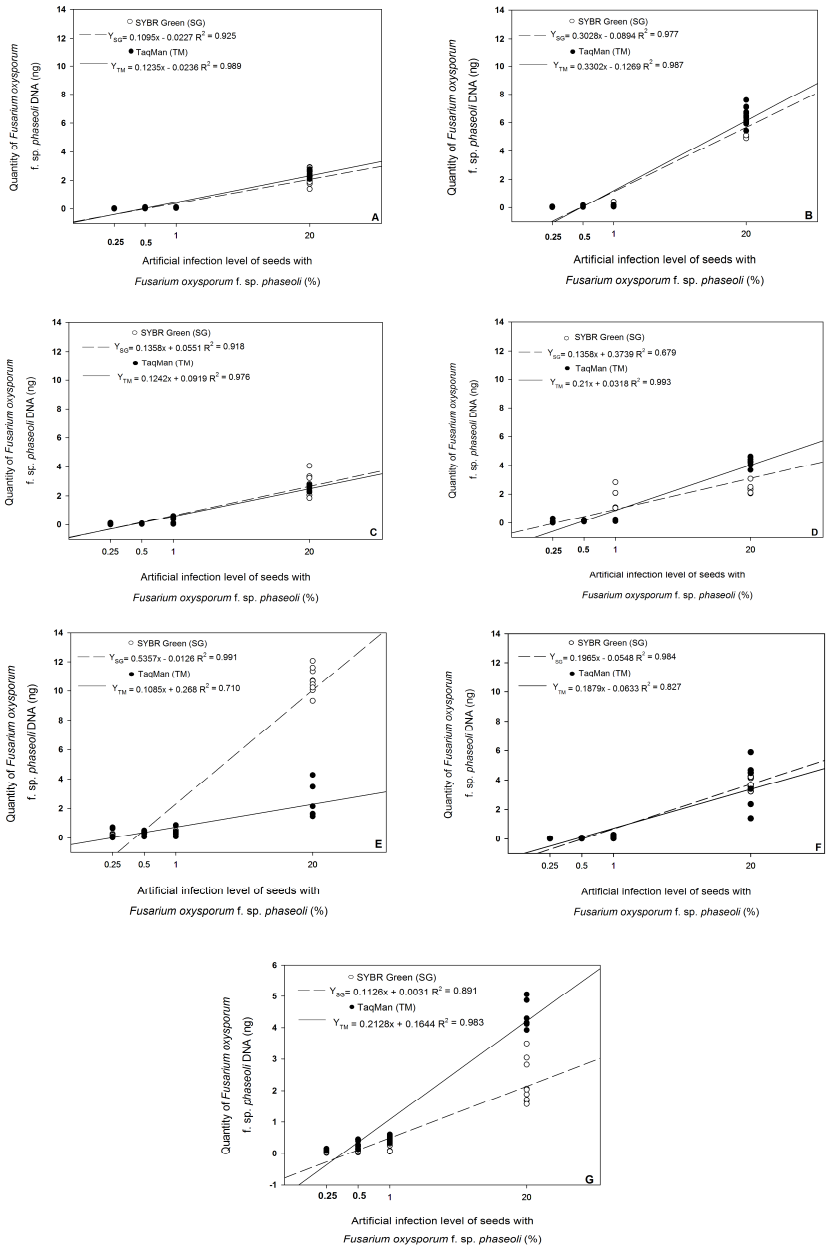

Figure 2. Regression lines of detection and quantities of *Fusarium oxysporum* f. sp. *phaseoli* DNA (ng) plotted against the artificial infection levels of seven common bean cultivars, comparing SYBR Green and TaqMan

assays by real-time PCR. A) Tiger's Eye; B) Boston Favorite; C) Ireland Creek Annie; D) Arikara Yellow; E) Bountiful; F) Carioca; G) Dart. Data are an average of nine replications in three separate runs of the qPCR assay

standard samples, and ranged from 10·70 ng to 9·2 pg. However, two dissociation peaks were observed from the melting curve analysis, one at 77·26 ± 1 °C and the other around 62 ºC, indicating that secondary nonspecific products were present in the samples. The second peak was lower when the DNeasy® Plant Mini Kit was used in comparison with the conventional CTAB chloroform: isoamyl alcohol protocol (data not shown).

The mean quantity of the target DNA detectable in blended seed samples varied with the artificial infection level, qPCR assays, bean seed genotype, pathogen biomass present in the artificially inoculated seeds and seed size (Fig. 2).

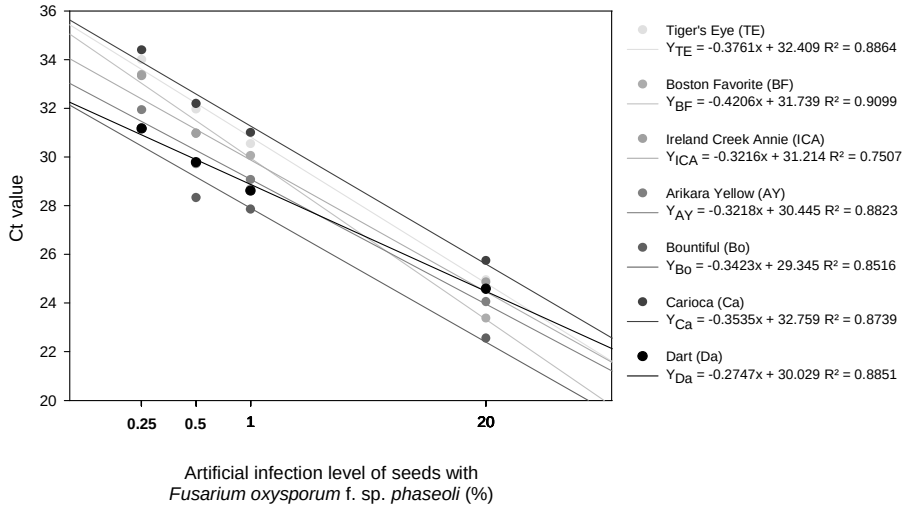

Figure 3. Regression lines of threshold cycles (Ct) for seven cultivars plotted against the artificial infection levels of common bean seeds with

Fusarium oxysporum f. sp. *phaseoli*, detected by TaqMan qPCR assay. Each dot represents an average of 24 data points

The slopes generated from the TaqMan and SYBR Green assays were similar in all cultivars, except for Dart and Bountiful. The TaqMan efficiencies ranged from 91 to 95% for larger-seeded beans and 75 to 99% for the smaller-seeded beans (Fig. 2). The efficiencies ranged from 71 to 91% for SYBR Green assays (Fig. 2).

The amount of *Fop* DNA analyzed by the TaqMan assay was greatly reduced by the disinfestation step after seed inoculation but infected seeds were still detectable even in a seedlot with 0.25% infection (data not shown).

Naturally infected seeds were positive in the smaller-seed size for two cultivars, Cometa and Valente, in both qPCR assays. In the SYBR Green assays, the Ct values were 37·18 and 37·28, which correlates to approximately 0·0160 ng of *Fop* DNA. In the TaqMan assays, the Ct values were 37·39 and 37·46, which correlates to approximately 0.0010 ng of DNA.

DISCUSSION

A rapid, sensitive and accurate seed health testing method to detect *Fop* in seeds would be a useful tool to prevent the spread of this pathogen and to ensure that seed quality and phytosanitary requirements are met. Real-time PCR-based *Fop* detection and quantification assays were developed in this study. Specific oligonucleotides allowed for the detection of highly virulent strains of *Fop* in bean seed samples without the need to perform a pathogenicity test. PCR primers to discriminate highly virulent strains of *Fop* have been previously developed using random amplified polymorphic DNA (RAPD) based on a sequence characterized amplified region (SCAR) and dispensable chromosome (Alves-Santos *et al.*, 2002a; Ramos *et al.*, 2007; Garcia-Sanchez *et al.*, 2010; Vega-Bartol *et al.*, 2011). In this study, the primer set and probe were re-designed using the sequence of *Fusarium oxysporum* zinc finger transcription factor 1 (*ftf1*), (GenBank accession number ABB97391), and adapted for detection of *Fop* in bean seeds. The primer set QFOP2A/QFOP2B amplified a 63-bp DNA fragment from *Fop* and highly virulent strains were detectable at 1 pg of DNA. The assay was highly specific when evaluated against template DNA from a range of closely related and unrelated fungal species, including common fungal beans seed microbiota. Additionally, the assay did not amplify common bean DNA. These results support the hypothesis by Alves-Santos *et al.* (2002a) and Ramos *et al.* (2007) that *ftf1* is associated with the virulence phenotype in strains of *Fop*. The use of reliable oligonucleotides that allows for the distinction of highly virulent strains and weakly/non-virulent strains of *F. oxysporum* in bean seeds is of great diagnostic importance, particularly as they are morphologically indistinguishable. In addition the test outlined in this study is less laborious and time consuming than the typical pathogenicity test that is currently used for routine analysis.

A coffee grinder was used in this study to grind the bean seeds into a fine powder, which we determined is an essential step in obtaining *Fop* DNA for the qPCR assays. However, PCR inhibitors are commonly found in DNA extracts from seeds (Pryor & Gilbertson, 2001; Walcott *et al.*, 2004; Ma & Michailides, 2007) and often interfere with DNA amplification in PCR assays. In this study, the use of the DNeasy® Plant Mini Kit decreased the PCR inhibitors, improving the sensitivity of the SYBR Green as well as TaqMan qPCR assays. However, some secondary nonspecific products were still present in the DNA harvested from seeds, as evidenced by the second peak 10 °C below the main peak, interfering with target DNA quantification when SYBR Green was used. New methodologies, such as magnetic-capture-hybridization-PCR (MCH-PCR), have been developed that significantly increase the sensitivity of pathogen detection using qPCR (Walcott *et al.*, 2004; Ha *et al.*, 2009), and adapting this technique for the detection of *Fop* in bean seeds should be explored. We determined that the TaqMan assay was more reliable and more sensitive than the SYBR Green. This is the result of the specific nature of the probe such that a fluorescent signal is only generated in the presence of the specific PCR product, in contrast to the SYBR Green dye which intercalates with any double stranded DNA. Although no significant differences could be found in the amount of target DNA between the two assays, TaqMan assay results were more reliable with higher slopes and linear regression coefficients for the different cultivars (Fig. 2). These results are consistent with those reported by Chen *et al.* (2013) who observed that the TaqMan MGB probe and SYBR Green dye assays were quite similar.

Seed sampling is key to obtaining accurate seed health test results due the low transmission rates of many important pathogens in seeds (Morrison, 1999). In both qPCR assays *Fop* DNA was detectable in 0·25% of seed infection incidence, corresponding to one infected seed mixed with 399 *Fop*-free seeds. At present, the common seed health testing method for detecting *F. oxysporum* in

bean seeds is a blotter test (MAPA, 2009). Usually a sample of 400 bean seeds is taken from the representative seed lot and is then used for the blotter test without surface disinfestation. In this study, although 400 seeds were used, the seeds were divided into subsamples of 400, 200 or 100 seeds, respectively (Table 4). We ascertained that using 100-seed subsamples produced less variation in the quantities of target DNA obtained for each cultivar (Fig. 2). This is likely due to the fact that the fungal spore contamination is more homogeneous in the lower volume of powder, than it would be in a composite sample.

Another important consideration is that the inoculum load in individual infected seeds may vary, and this could influence the amount of pathogen DNA quantified by PCR (Glynn & Edwards, 2010). For this reason, qPCR results can vary widely in samples according to seed size and infection incidence (Fig. 2, 3). We observed this in our study, and chose two cultivars (one small- and one large- seeded) to further assess the relationship between visual infection severity of individual seeds and the corresponding quantities of fungal DNA. Severely infected seeds of cv. Bountiful (small-seeded) generated a template DNA quantity around 75 ng. The quantities of DNA for the weakly infected seeds were close to 8 ng. For the severely and weakly infected bigger-seeds, cv. Ireland Creek Annie, the DNA quantities ranged between 2 and 27 ng (Fig. 4). For seed size, in the same inoculation period, the smaller seeds had increased mycelium biomass on the seed surface compared to the larger ones. As a result we compared the biomass of the pathogen to seed size, and determined that quantity of target DNA varied more in the smaller-seeds than in the bigger-seeds (Fig. 4). The four smaller-seeded cultivars used in this study generated smaller slopes, thus decreasing the qPCR efficiencies for both techniques (Fig. 2). Clearly the number of seeds as well as the number of subsamples required needs to be optimized to improve the sensitivity, cost-efficiency and accuracy of the sampling procedure.

We compared non-disinfested artificially infected seeds with seed that were surface disinfested and found that surface disinfestation significantly decreased the amount of external pathogen biomass on seeds. Given the high specificity of the primer set and TaqMan qPCR assay we were still able to detect *Fop* infection 0·25% even when seed surface disinfestation was performed (data not shown). The reduction in fungal biomass associated with surface disinfestation also was evident when surface-disinfested and non-disinfested seeds were planted in the greenhouse. Emergence was 90% for *Fop*–free seeds, 56·7% for surface disinfested seeds, and 25% for non-surface disinfested ones (data not shown). Our results indicate that greater biomass of *Fop* on seed surface facilitates detection by the qPCR assay.

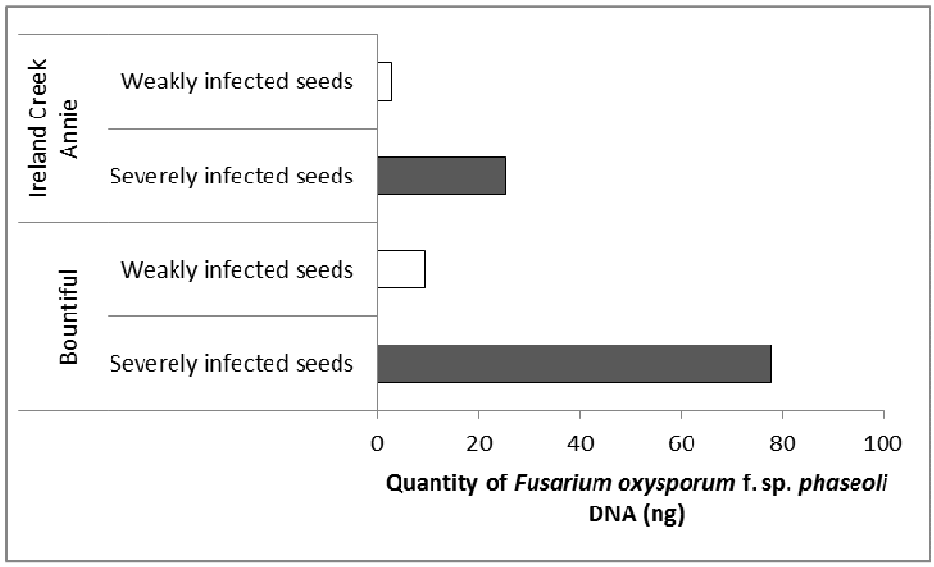

Figure 4. Quantification of *Fusarium oxysporum* f. sp. *phaseoli* DNA in seeds of a large-seeded (Ireland Creek Annie) and a small-seeded (Bountiful) bean cultivar using the TaqMan qPCR assay. Seeds were separated into weakly or severely infected categories according to visual signs of the pathogen. Each data bar represents an average of

nine replications of infected seeds that were individually analyzed by TaqMan qPCR assay

The objective of this study was to improve the reliability and increase the speed of the detection method for *Fop* on seeds using the same conditions as the standard seed health test (i.e. using 400 seeds without surface disinfestation). The SYBR Green assay was effective for detection but not for quantification of *Fop* in common bean seeds, presumably due to the presence of PCR inhibitors, as evidenced by the peak around 62 °C for *Fop*-free seeds (negative control) (data not shown). Highly-virulent *Fop* was successfully detected and quantified, even at low levels, in naturally infected samples using the DNeasy® Plant Mini Kit, specific primer set and TaqMan qPCR assay. Demonstrating that this protocol is a viable alternative to the current seed health testing method, providing accurate, sensitive, and reliable detection and quantification of *Fop* in common bean samples. Speed is particularly enhanced from one day to completion compared with 7-30 days for the blotter test and grow-out test. For laboratories that have real-time PCR equipment and technical expertise, the cost for a routine analysis using this protocol for *Fop* detection in common bean seeds is moderate.

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FINAL COMMENTS

The seed quality in crops such as common bean is of fundamental importance based on the fact that seeds may carry on infective inoculum of several pathogens as *Fusarium oxysporum* f. sp *phaseoli* (*Fop*) which causes one of the most damaging diseases to that crop, besides being a soilborne pathogen. By the intensive cultivation of beans the inoculum of *Fop* accumulates in the soil and in a short time these areas become useless for this type of culture for long periods of time. In this way the control of common bean seed health against pathogens as *Fop* and other equally pathogenic nature, becomes essential. For the successful of *Fop* management is needed that reliable and faster detection methods of this pathogen in seeds are ready and available for using by the certification programs in addition to seed treatment whose aim is avoid the seeds as the vehicle of the pathogen inoculum, spreading it over long distances between fields.

Although information is found in the literature on various aspects related to the life cycle of the pathogen and the disease cycle in Brazil conditions, it is clear that the more solid knowledge about this pathosystem, especially regarding the interaction between the pathogen and bean seeds, is still low and this has created difficulties in the development of more effective strategies to control this disease in Brazil. Importantly, the Fusarium wilt in bean is widespread in regions of this crop and none diagnosis test of this pathogen inoculum in commercial seed lots in the country has been performed in organized and systematic way. One of the arguments against adoption of seed health testing for *Fop* has been the lack of reliable and faster methods for its detection.

One of the purpose of the studies in this thesis was to determine the transmission rate of *Fop* in bean seeds by using artificially and naturally *Fop*-contaminated seeds and having as reference some factors that interfere more

directly on the cycle of this biological interaction, as in the case of temperature, pathogen virulence, genotypes of host, and the inoculum potential that can be associated with seeds used by producers of this species. In Paper 1, the aim was to establish the transmission rate of the fungus in question from seeds. By the inoculation technique used, different levels of pathogen infection were obtained, and thereafter making evaluation on the transmission rates in both symptomatic and asymptomatic plants under controlled conditions of cultivation. From the results, rates of the pathogen transmission by seeds of bean cultivars ranged from 49.7 to 100%. Noteworthy is the high number of non-germinated seeds resulting from the action of the pathogen in this work. This inoculum plays certainly an important role as inoculum source for the disease development in field, being responsible for the survival of the pathogen in these circumstances. This research line should be pursued with the focus now on *Fop* transmission from infected plants to seeds in the field.

The observation of the infection and colonization processes and movement of *Fop* from infected bean seeds to resulting plants is of great interest in order to make decisions on the appropriate use of those seeds in practice. For this type of study, the GFP technique proves extremely effective for providing conditions to make observations in real time on the various factors interfering in this biological interaction. The results of the studies described in Paper 2, make clear that the use of the GFP technique coupled with observations in the scanning electron microscope are important tools for better understanding the models of colonization and parasitism of plant tissues from infected seeds. By GFP markers and scanning electron microscopy was possible to verify and to register the colonization of different tissues of seeds, including the embryo and thus understanding one cause of death of infected seeds in pre-emergence. In lower inoculum potential the pathogen tends to colonize the tissues of the seed coat, and at higher inoculum potential the pathogen parasites the embryo, thus

compromising the performance of seeds. By means of this tool it is possible to quantify and to monitor the dynamics of parasitism of the fungus in question in the tissues of plants grown from inoculated seeds. The next step in this line of research should be the use of the GFP technique to follow the movement and colonization of tissues of plants resulting from infected seeds by the pathogen in question.

The need to provide methods for increased accuracy and faster execution for detection of pathogens in seeds is in general a constraint on agricultural systems with high demand for seed health analysis. For identification of pathogens with morphological similarities as in the case of *Fusarium* species, the development of specific and reliable methods for their detection in seeds is a goal of current research programs in the country. In addition to biological methods with some selectivity properties for the species *Fusarium oxysporum*, use of PCR-based techniques are needed not only to differentiate those species with high accuracy but to provide conditions to make the seed health testing viable for application in the certification programs. By means of qPCR assay, the object of the study in Paper 3 was shown that *Fop* even in low incidence, 0.25%, and with lower inoculum potential was reliably detected in common bean seeds. For the adoption of this technology in seed health quality programs in the country further steps have to be followed such as the protocol validation by the accreditated authorities.

APPENDIX

Appendix 1. Summary table of analysis of variance regarding to the percentage of stand, potential transmission rates of symptomatic plants, asymptomatic plants and total.

Source[1]	DF	Mean Square and Pr > F			
		Stand	Symptomatic	Asymptomatic	Total
T	1	10.7573	445.2865*	127.4321	96.2995
Cv	1	5516.6537*	14.5675	1678.2792*	1380.1275*
S	2	7216.8982*	160.3750*	95851.1530*	102568.4686*
Ip	3	1444.5083*	18.2978	177.7598*	246.0402*
t*cv	1	1611.9246*	14.5675	0.9830	23.1189
t*s	2	147.4537	60.3750	27.2395	63.0475
t*ip	3	862.0424*	18.2978	11.3019	3.9061
cv*s	2	2432.1759*	29.5115	68.8262	70.3149
cv*ip	3	173.8830	0.0184	56.5654	57.2761
s*ip	4	686.3426*	15.8257	49.8161	77.7450
t*cv*s	2	248.8426	29.5115	6.5487	63.4621
t*cv*ip	3	893.1607*	0.0184	30.2921	31.7082
cv*s*ip	4	904.3982*	6.1000	70.7170	26.2019
t*cv*s*ip	8	246.7593	10.9629	62.0922	37.4094
Block	1	30.8196	0.3011	73.1239	82.8089
CV		22.856	197.46	11.44	9.24

[1] Abbreviations for: t = temperature; cv = cultivar; s = *Fop* strain; ip = inoculum potential.