SHORT COMMUNICATION

MOLECULAR DETECTION OF *THECAPHORA FREZII* IN PEANUT (*ARACHIS HYPOGAEA* L.) SEEDS

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SUMMARY

Thecaphora frezii is the causal agent of peanut smut, important disease in the Argentine production area. The pathogen can be dispersed by kernels, through surface contamination or small lesions. The aim of this work was to design specific primers to develop a Polymerase Chain Reaction assay for the detection of *Thecaphora frezii* in peanuts kernels. The ITS region of four *T. frezii* isolates was amplified and sequenced. A consensus sequence was obtained and a pair of primers specific for the pathogen, named TF-2F and TF-2R, was synthesized. The primers were used for specificity and sensitivity tests. The former test involved a PCR using DNA from *T. frezii* and other seed-transmitted pathogens (*S. rolfsi*, *S. minor*, *Fusarium solani* and *S. sclerotiorum*). A 190 bp fragment was obtained for the *T. frezii* isolates, whereas no amplification was observed for the remaining pathogens. For the sensitivity test, positive results were observed at concentrations up to 3×10^{-4} pg of the target DNA. The specificity of this method, along with its sensitivity, makes it an important tool for epidemiological studies of peanut smut.

Keywords: PCR, peanut, smut, teliospora.

Argentina is one of the main peanut producing countries worldwide. Each year, 350 to 400 thousand hectares are cultivated, producing about one million tons (Pedelini, 2014). The first record of the presence of peanut smut was reported by Marinelli *et al.* (1995). Affected pods exhibited hypertrophy and spongy consistency, whereas the kernels were replaced, partially or totally, by a reddish-brown mass of teliospores. *Thecaphora frezii* was identified as the causal agent of the disease. Peanut smut was first reported in wild peanut samples from Aquidauma, Mato Grosso, Brazil (Carranza and Lindquist, 1962). Since it was first reported, the disease has increased in intensity, reaching 100% prevalence in the 2011/2012 crop season (Bonessi *et al*., 2011; Cazzola *et al*., 2012), producing yield losses of 51% in some plots (Oddino *et al*., 2010). Once the teliospores are on the soil, their germination is stimulated by the presence of the peg that penetrates the contaminated soil, producing a local infection and colonization of the plant tissue (Marinelli *et al*., 2008; Marraro Acuña, 2012) (Fig. 1). Conforto *et al.* (2013) confirmed that the pathogen belongs to a different species from the ones reported from the genus *Thecaphora*, using molecular tools.

The pathogen is dispersed through different means. Wind dissemination occurs via the dust generated during harvesting of infested peanut plots, which carries teliospores that are deposited at different distances from the source of inoculum. Agricultural machinery is another dispersal agent, since teliospores can adhere to different surfaces (Marraro Acuña and Haro, 2011). Kernels are the most efficient long-distance dispersal agent. Surface seed contamination by teliospores occurs mainly during shelling, when smutted pods release millions of teliospores that adhere to the healthy seed surface (Marinelli *et al*., 2008). Teliospores can also occur in small lesions of kernels that are not detected in controls (Marinelli *et al*., 2008).

To date, teliospores of *T. frezii* present in peanut kernels have been detected using light microscopy (Marraro Acuña *et al*., 2012). Molecular techniques based on DNA analysis are an alternative method for teliospore detection. These techniques have the advantage of being highly sensitive, specific, and independent of the environmental effect (Narayanasamy, 2011). They are also time-effective, because a high number of samples can be processed at the same time. The aim of this work was to design specific primers to develop a Polymerase Chain Reaction (PCR) assay for the detection of *Thecaphora frezii* in peanuts kernels.

Samples of infected kernels were collected by Conforto *et al.* (2013) from four peanut-growing regions in Argentina. Two isolates (Cba-GD1 and Cba-GD2) were obtained from General Deheza, Córdoba (32° 45′ 20.53″ S, 63° 46′ 56.5″ W), one isolate (Cba-CH1) from Charras, Córdoba (33° 1′ 33.03″ S, 64° 2′ 49.44″ W) and one isolate (Sa-EM1) from Embarcación, Salta (23° 12′ 37.16″ S,

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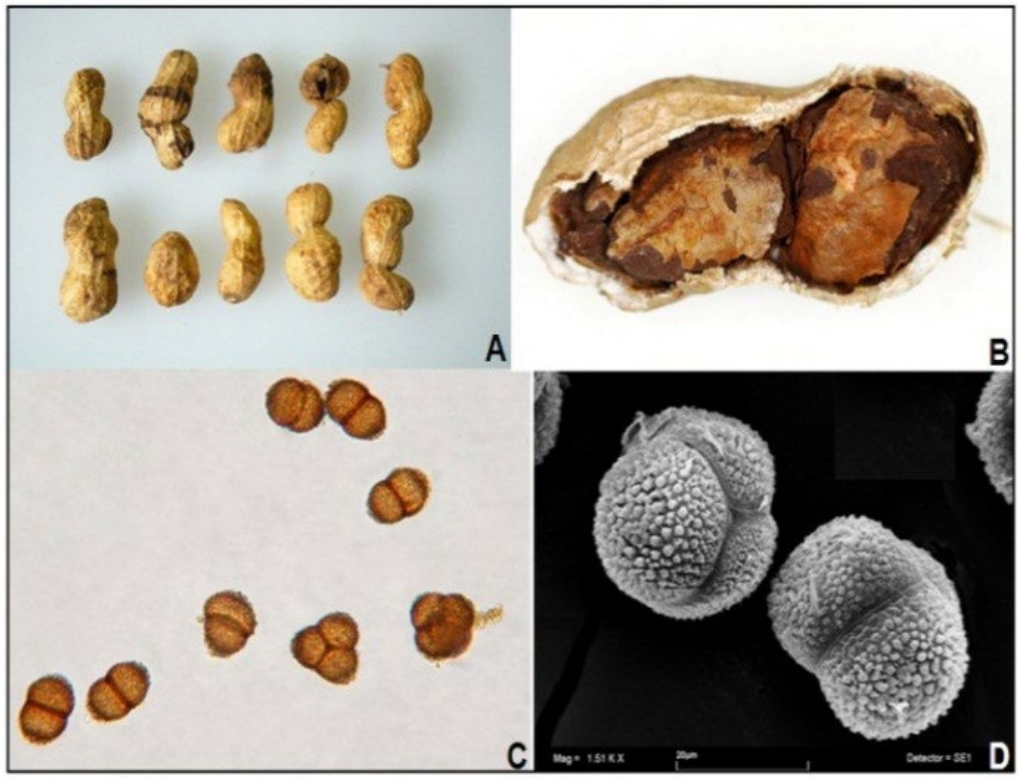

Fig. 1. A: Peanut pods showing symptoms of hypertrophy caused by smut. B: Pod with completely smutted kernels. C: Teliospores of *T. frezii* observed under light microscope (20×). D: Teliospores of *T. frezii* observed under scanning electron microscope (Núcleo de apoio à pesquisa em microscopia eletrônica aplicada à pesquisa agropecuária. Piracicaba, Brazil).

64° 5′ 50.3″ W). DNA was extracted following the CTAB protocol, with modifications (Doyle and Doyle, 1990; Conforto *et al*., 2013). Kernels (300 g) surface-contaminated with teliospores were washed in 100 ml sterile distilled water. The resulting suspension was placed in two 50-ml Falcon tubes and centrifuged at 3,500 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 ml sterile distilled water. Then, 700 µl of suspension from each tube was put in a 1.5-ml Eppendorf tube and centrifuged at 3500 rpm for 5 min. The supernatant was discarded and the pellet was crushed with a sterile PP EPPI micropestle plus 200 µl CTAB buffer. After crushing, other 300 µl of CTAB buffer was added; the remaining process followed the method of Doyle and Doyle (1990). Quality of the extracted DNA was verified in 1% agarose gel electrophoresis stained with ethidium bromide and visualized under UV light. PCR of the DNA samples was performed using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') – ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes and Bruns, 1993). PCR reaction mixture was prepared in a total volume of 25 µl, containing 0.5 U GoTaq® DNA polymerase and 5× buffer (Promega, USA), 0.25 µM of each primer, 0.25 µM dNTP, and 1 µl DNA (300 pg). PCR conditions were as follows: initial denaturation at 94°C for 5, followed by 32 cycles at 94°C for 45 s, 58°C for 45 s and 72°C for 45 s, with a final extension at 72°C for 10 min. PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide and observed under UV light. The obtained fragments were purified via Wizard® columns (Promega, USA) and automatically sequenced (Macrogen, Korea). The obtained sequences were assembled using the SeqMan software (Lasergene software, DNASTAR ver. 8.0.2, 2008). A consensus sequence was obtained and used to design a set of specific primers for *T. frezii* with the program Primer3 version 0.5 available online (Koressaar and Remm, 2007). The specific primers were synthesized by Invitrogen™.

Specificity was determined via a PCR using the DNA extracted from the different *T. frezii* isolates (Cba-GD1, Cba-GD2, Cba-CH1 and Sa-EM1) and from four isolates of fungal species transmitted by peanut kernels (*Sclerotium rolfsii*, *Sclerotinia minor*, *Fusarium solani* and *Sclerotinia sclerotiorum*). The DNA of the last four pathogens was extracted using the protocol described by Andrade *et al.* (2004). The primers used were those designed to detect *T. frezii*; universal primers ITS1 and ITS4 were used as positive control. Sensitivity was determined using 3×10^{-6} to 3×10^{4} pg of serially diluted DNA of *T. frezii* as DNA templates for PCR amplification. PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, 56°C for 1 min and 72°C for 1 min, with a final extension at 72°C for 5 min.

The DNA fragment of approximately 700 bp was amplified for the four *T. frezii* isolates using the ITS1/ITS4 universal primers. The sequences obtained from the different isolates were deposited in GenBank (NCBI)

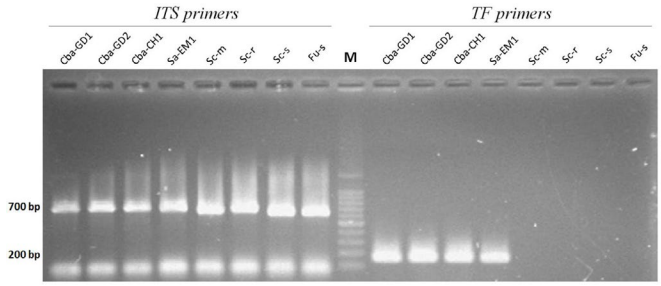

Fig. 2. Agarose gels with the PCR reaction products using universal primers ITS1/ITS4 (as a positive control) and the specific primers designed for *T. frezii* TF-2F/TF-2R. 300 pg of DNA from each fungal species were used in PCR reactions. M: Molecular marker (100 bp). Cba-GD1, Cba-GD2, Cba-CH1, Sa-EM1: Isolates of *T. frezii*. Sc-m: *Sclerotinia minor*. Sc-s: *Sclerotinia sclerotiorum*. Sc-r: *Sclerotium rolfsii*. Fu-s: *Fusarium solani.*

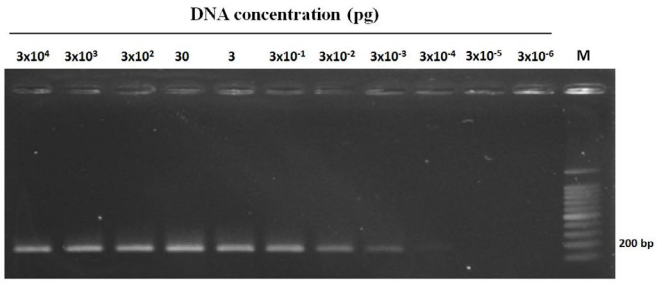

Fig. 3. Agarose gels with the PCR reaction products using a series of DNA dilutions (ranging from 3×10^{-6} to 3×10^{4} pg) from *T. frezii* as templates. M: Molecular marker (100 bp).

with accession numbers: Cba-GD1 isolate (KP994417), Cba-GD2 (KP994418), Cba-CH1 (KP994419), and Sa-EM1 (KP994420). Specifics primers TF-2F (5'ATGTCAAAGAGTGCGAAGAC3') and TF-2R (5'TATCTTGCTGGTAGGCTGTT3') were designed. The amplified product was 190 bp in size and corresponds to a segment specific for *T. frezii*.

In the specificity test of PCR using primers TF-2F/TF-2R, positive results were obtained for the four *T. frezii* isolates (Cba-GD1, Cba-GD2, Cba-CH1 and Sa-EM1). Negative results were observed for the isolates Sc-m (*S. minor*), Sc-s (*S. sclerotiorum*), Fu-s (*Fusarium solani*) and Sc-r (*S. rolfsii*) (Fig. 1). To determine the sensitivity, amplification products were obtained using up to 3×10^{-4} pg of DNA from *T. frezii*. As the signal generated by the latter concentration was very faint, the PCR detection limit is between 3×10^{-4} and 3×10^{-3} pg (Fig. 2).

Here, the method for the detection of *T. frezii* in peanut kernels was adjusted using PCR. This technique is specific, sensitive and rapid, providing advantages over other detection techniques, such as light microscopy (Guillemette *et al*., 2004). For this purpose, DNA extraction from contaminated kernels was optimized by washing them and recovering the teliospores from the resulting water. This proved to be a simple procedure to obtain the teliospores present on the seed surface, which is in agreement with results reported by Marraro Acuña *et al*. (2012).

Specific primers for *T. frezii* detection were synthesized using the ITS region. This region in fungi is characterized by its relatively short sequence (between 600 and 800 bp); it is easy to amplify using universal primers and can be amplified from degraded or low-concentration DNA samples (Lorena *et al*., 1999; Villalobos *et al*., 2005; Magalhães de Abreu and Pfenning, 2012). In addition, the divergent sequence of rDNA ITS region facilitates differentiation of species within a genus (Zambino *et al*., 1992; Ward and Bateman, 1999; Conforto *et al*., 2013). These differences allowed us to design specific primers for the detection of *T. frezii* in symptomatic kernels, the main long-distance dispersal agent of the pathogen (Lee *et al*., 2002; Marinelli *et al*., 2008). The negative results obtained by *S. minor*, *S. sclerotiorum*, *F. solani and S. rolfsii* emphasize the specificity of the primers designed; this is very important since these pathogens can also be transmitted by seed (Zhao *et al*., 2007).

The availability of a method to detect *T. frezii* spores in peanut seed provides an important tool for the study of peanut smut epidemiology. PCR allows for detection of teliospores in low concentrations of 10 (3×10^{-4} pg of DNA) to 100 (3×10^{-3} pg of DNA) teliospores in 400 kernels, whereas the detection by microscopy is used for samples with high levels of infestation (10^{6} teliospores) (Marraro Acuña *et al*., 2012). While conventional PCR provides information on the absence or presence of the pathogen, real time PCR are being fine-tuned for quantification of the pathogen load in kernels and soil samples. This would facilitate the correlation between disease incidence index and the pathogen load already present in the field or transported to other plots (Oddino *et al*., 2010).

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