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Detection of *Fusarium verticillioides* by PCR-ELISA based on *FUM21* gene

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ABSTRACT

Fusarium verticillioides is a primary corn pathogen and fumonisin producer which is associated with toxic effects in humans and animals. The traditional methods for detection of fungal contamination based on morphological characteristics are time-consuming and show low sensitivity and specificity. Therefore, the objective of this study was to develop a PCR-ELISA based on the *FUM21* gene for *F. verticillioides* detection. The DNA of the *F. verticillioides*, *Fusarium* sp., *Aspergillus* sp. and *Penicillium* sp. isolates was analyzed by conventional PCR and PCR-ELISA to determine the specificity. The PCR-ELISA was specific to *F. verticillioides* isolates, showed a 2.5 pg detection limit and was 100-fold more sensitive than conventional PCR. In corn samples inoculated with *F. verticillioides* conidia, the detection limit of the PCR-ELISA was 1×10^4 conidia/g and was also 100-fold more sensitive than conventional PCR. Naturally contaminated corn samples were analyzed by PCR-ELISA based on the *FUM21* gene and PCR-ELISA absorbance values correlated positively ($p < 0.05$) with *Fusarium* sp. counts (CFU/g). These results suggest that the PCR-ELISA developed in this study can be useful for *F. verticillioides* detection in corn samples.

1. Introduction

Corn (*Zea mays* L.) is the most cultivated cereal in the world and presents a broad spectrum of applications (USDA, 2017). The total world production of corn was 1 billion and 38 million in 2014 (FAO, 2015). Brazil is the third largest corn producer in the world and accounted for 88 million tons, and exported 35 million tons in 2016 (CONAB, 2017).

Corn is one of the major components of the animal feed and is the staple food in many countries (Nuss and Tanumihardjo, 2010). Nevertheless, it is susceptible to fungal contamination which affects grain and seed productivity and quality. *Fusarium verticillioides* (Sacc.) Nirenberg (synonym, *F. moniliforme* (J.) Sheldon; teleomorph, *Gibberella moniliformis* (synonym *G. fujikuroi* mating population A) is a primary corn pathogen and can cause from asymptomatic infection to rotting of all parts of the plant (CAST, 2003). Furthermore, this fungus produces fumonisins, a group of mycotoxins which can cause toxic effects in humans (Chuturgoon et al., 2014; Marshall et al., 2017; Wang et al., 2014) and animals (Szabó et al., 2016; Vendruscolo et al., 2016).

Fumonisins are mainly produced by *F. verticillioides* and *F. proliferatum* (T. Matsushima) Nirenberg (CAST, 2003) and are associated with leukoencephalomalacia in equines (Vendruscolo et al., 2016), pulmonary edema in pigs (Pósa et al., 2016), and have hepatotoxic and hepatocarcinogenic effect in rats (Riedel et al., 2015; Szabó et al., 2016). In humans, fumonisins are related to neural tube defects (Marshall et al., 2017; Missmer et al., 2006), esophageal and liver cancer (Chuturgoon et al., 2014; Wang et al., 2014). The International Agency for Research on Cancer (IARC, 2002) classified fumonisins as Group 2B, i. e. possibly carcinogenic to humans.

Studies carried out in Africa, Asia, Europe and South America have shown that the incidence of fumonisins in corn samples can reach 100% and in some cases with mean levels above the maximum limit recommended by the European Union for corn intended for direct human consumption (1000 μg/kg) (European Commission Regulation, 2007; Fu et al., 2015; Leggieri et al., 2015; Okeke et al., 2015; Oliveira et al., 2017; Phuong et al., 2015;

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Scussel et al., 2014).

Because of high contamination of corn by *F. verticillioides* and fumonisins and the health problems caused by these mycotoxins, it is essential to develop sensitive and specific methods to detect *F. verticillioides* contamination. Conventional methods for identification and detection of fungal contamination include culture in several media, microscopic examination and chemical analysis of chitin, ergosterol and secondary metabolites (Yeni et al., 2014). These methods have low specificity and sensitivity and are time-consuming, except for the identification of secondary metabolites by chromatography and mass spectrometry. In spite of the high sensitivity and specificity, they are laborious, use toxic reagents and require an extensive sample clean-up.

Capillary electrophoresis, biosensors and immunological methods varying from simple lateral flow immunoassay and ELISA (Enzyme Linked Immunosorbent Assay) to highly sophisticated immunosensors have been used for fungi and mycotoxin detection (Turner et al., 2015; Vallejo-Cordoba and González-Córdova, 2010; Zhao et al., 2014). Foodborne pathogens can also be identified by nucleic acid-based assays, including polymerase chain reaction (PCR), randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and quantitative PCR (qPCR) which give reliable, high-throughput, reproducible and specific results (Yeni et al., 2014).

The PCR based on genes involved in fumonisin production can be used to detect *F. verticillioides* in corn. The amplified PCR product is commonly detected by electrophoresis and is qualitative (Sue et al., 2014). An attractive approach is the detection of DNA using ELISA, also called ELOSA (Enzyme Linked Oligosorbent Assay) (Bernard et al., 2006), that allows semi-quantitative analysis of several samples in a single test with high sensitivity and specificity (Grimm and Geisen, 1998).

The genes directly involved in the fumonisin biosynthesis are organized into a gene cluster, known as the fumonisin biosynthetic (*FUM*) gene cluster (Proctor et al., 2003). In *F. verticillioides* the cluster consists of 17 coordinately regulated genes, designated *FUM1*, *FUM2*, *FUM3*, *FUM6*, *FUM7*, *FUM8*, *FUM10*, *FUM11*, *FUM13*, *FUM14*, *FUM15*, *FUM16*, *FUM17*, *FUM18*, *FUM19*, *FUM20* and *FUM21* (Brown et al., 2005, 2007; Butchko et al., 2003, 2006; Proctor et al., 2003).

FUM21, the last *FUM* gene described so far, probably codes a transcription factor that positively regulates the *FUM* genes expression (Brown et al., 2007). The presence of a Zn (II) 2Cys6 DNA-binding domain in the predicted protein suggests that this gene is involved in transcriptional regulation (MacPherson et al., 2006).

In addition to *Fusarium* species, some *Aspergillus niger* strains have also been reported as fumonisin B_2 and B_4 producers (Mogensen et al., 2010) and the presence of 11 genes similar to the *F. verticillioides FUM* genes, including the *FUM21* gene was reported in this species (Frisvad et al., 2007).

Divakara et al. (2014) developed a multiplex PCR to differentiate toxigenic and non-toxigenic *Fusarium* sp. using primer for the *FUM21* gene along with the *FUM1* and *FUM8* genes and observed positivity to the *FUM21* gene only in fumonisin producing *F. verticillioides* isolates, indicating that the *FUM21* gene shows greater potential to differentiate fumonisin producers from fumonisin non-producer isolates.

Therefore, the objective of this study was to develop a PCR-ELISA based on the *FUM21* gene to detect *F. verticillioides* in corn.

2. Material and methods

2.1. Fungal isolates and growth conditions

F. verticillioides isolates (97K, 103F, 103G, 113F and 119Br) and *Penicillium variabile* belong to the Mycological Culture Collection of the Department of Food Science and Technology at the State University of Londrina.; *F. graminearum* FSP27 and FRS26 were provided by the Mycological Culture Collection of Laboratory of Toxigenic Fungi and Mycotoxins of the Department of Microbiology of Biomedical Sciences Institute, University of São Paulo (São Paulo-Brazil); isolates of *F. verticillioides* (578, 636, 637, 638, 639, 642, 643, 644, 645, 646), *F. graminearum* 102, *F. proliferatum* 559, *F. subglutinans* 332, *Aspergillus niger* (911, 104CF, 219CF, 444CF, 642AN), *A. carbonarius* (168, 180) and *A. ochraceus* (4363, 4368) were provided by the Mycological Culture Collection of Department of General Biology, State University of Londrina, Paraná, Brazil. All isolates were routinely grown in potato dextrose agar at 25 °C.

Fumonisin ($FB_1 + FB_2$) production by *F. verticillioides* strains was as follows: 103 F (5119 μg/g), 97K (4051 μg/g), 119BR (4048 μg/g), 113F (724 μg/g), 103G (224 μg/g), 578 (105 μg/g), 636 (1.07 μg/g), 637 (1.23 μg/g), 638 (2069 μg/g), 639 (14,449 μg/g), 642 (0.33 μg/g), 643 (5292 μg/g), 644 (18,244 μg/g), 645 (8300 μg/g), 646 (14,777 μg/g).

2.2. DNA extraction from fungal isolates

The DNA of fungal isolates was extracted according to the method described by Van Burik et al. (1998) with some modifications. Briefly, mycelium of the *F. verticillioides* isolates grown on potato dextrose agar in Petri dishes were removed with inoculating loop and conidial suspension was prepared with sterile distilled water containing 0.1% Tween 80 (v/v). Conidia counts were determined with a hemocytometer and the inoculum concentration was adjusted to 10^6 conidia/ml. An aliquot of 1 ml conidia suspension (10^6 conidia/ml) was inoculated in potato dextrose broth and the culture was incubated for 3 days at 28 °C. Then, the mycelium was harvested by filtration and approximately 500 mg were transferred to a mortar followed by the addition of 500 μl lysis buffer (10 mmol/l Tris HCl pH 7.5; 20 ml/l Triton X-100; 10 mmol/l sodium acetate; 1 mmol/l EDTA pH 8.0; 10 g/l SDS). The mycelium was macerated and transferred to a microtube with 3 glass beads (3–4 mm) and phenol, agitated at 50 Hz for 3 min in the TissueLyser (Qiagen, Hilden, Germany) and centrifuged for 10 min at 9000 x *g*. A mixture of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant followed by agitation at 30 Hz for 3 min and centrifugation. A mixture of chloroform: isoamyl alcohol (24:1) was added to the supernatant followed by agitation at 30 Hz for 3 min and centrifugation (this process was repeated until the supernatant was clear). The supernatant was then treated with RNAse (20 mg/ml) diluted 1:100 for 30 min at 37 °C. Then the DNA was precipitated with absolute ethanol and the pellet was washed with 70% ethanol and dried at room temperature. The DNA was then re-suspended in ultrapure water and stored at −20 °C until use.

The DNA concentration was measured at A260 nm and the DNA quality was determined by calculating the ratio between A260 nm and A280 nm using a NanoDrop Lite spectrophotometer. The DNA was considered suitable when the ratio was approximately 1.80.

2.3. Conventional PCR

The PCR was carried out in a final reaction volume of 25 μl. The amplification mixture consisted of 2.5 μl of DNA template at 10 ng/μl for DNA of pure fungal isolates and at 20 ng/μl for DNA of corn samples, 1.5 mmol/l $MgCl_2$, 1× PCR buffer (Invitrogen, Carlsbad, USA), 200 μmol/l dNTP mix (Invitrogen, Carlsbad, USA), 1.25 U of Taq polymerase (Invitrogen, Carlsbad, USA) and primers at a concentration of 0.2 μmol/l (Invitrogen, Carlsbad, USA). The set of primers designed by Divakara et al. (2014) from the *FUM21* gene (*FUM21F*: 5′ GCAACATACAAGGGGGAGTT 3′, *FUM21R*: 5′