A multiplex real-time PCR method using hybridization probes for the detection and the quantification of *Fusarium proliferatum*, *F. subglutinans*, *F. temperatum*, and *F. verticillioides*

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ABSTRACT

Maize contamination with *Fusarium* species is one of the major sources of mycotoxins in food and feed derivates. In the present study, a LightCycler® real-time PCR method using hybridization probes was developed for the specific identification, detection, and quantification of *Fusarium proliferatum*, *Fusarium subglutinans*, *Fusarium temperatum*, and *Fusarium verticillioides*, four mycotoxin-producing pathogens of maize. Primers and hybridization probes were designed to target the translation elongation factor 1α (EF-1α) gene of *F. subglutinans* and *F. temperatum* or the calmodulin (*Cal*) gene of *F. proliferatum* and *F. verticillioides*. The specificity of the real-time PCR assays was confirmed for the four *Fusarium* species, giving no amplification with DNA from other fungal species commonly recovered from maize. The assays were found to be sensitive, detecting down to 5 pg and 50 pg of *Fusarium* DNA in simplex and multiplex conditions respectively, and were able to quantify pg-amounts of *Fusarium* DNA in artificially *Fusarium*-contaminated maize samples. The real-time PCR method developed provides a useful tool for routine identification, detection, and quantification of toxigenic *Fusarium* species in maize.

Introduction

The species *Fusarium proliferatum* (Matsushima) Nirenberg, *Fusarium subglutinans* (Wollenw. & Reinking) P.E. Nelson, Toussoun & Marasas, *Fusarium temperatum* Scauflaire & Munaut and *Fusarium verticillioides* (Saccardo) Nirenberg are pathogens of maize, causing ear rot and stalk rot in tropical and temperate regions (Bottalico 1998; Leslie & Summerell 2006; Scauflaire *et al.* 2011b). In addition to crop losses and reduction of seed quality, these *Fusarium* species produce mycotoxins that accumulate in plant tissues and were proven to pose serious problems for both human and animal health

(Logrieco *et al.* 2002; Desjardins 2006). Each species present a specific toxin profile, including fumonisins, fusaric acid, moniliformin, beauvericin, fusaproliferin, fusarins and enniatins (Leslie & Summerell 2006; Scauflaire *et al.* 2012). These four species belong to the *Gibberella fujikuroi* species complex (GFSC) which was divided into three phylogeographic clades: the African clade, which includes *F. verticillioides*; the American clade, which includes *F. subglutinans* and *F. temperatum*; and the Asian clade, which includes *F. proliferatum* (O'Donnell *et al.* 1998).

Due to morphological similarities of species in the GFSC, identification is usually based on various approaches combining morphological species recognition (MSR), biological species recognition (BSR) with diagnostic sexual crosses, and phylogenetic species recognition (PSR) using DNA sequence polymorphisms (Taylor *et al.* 2000; Kvas *et al.* 2009). Nevertheless, most protocols used for MSR and BSR are time-consuming, labour-intensive and require considerable expertise in *Fusarium* taxonomy and physiology, because of morphological similarities and interspecific sexual crosses that may occur under laboratory conditions (Leslie & Summerell 2006). As the rapid identification of these *Fusarium* species is critical to evaluate quality of feed samples or to assess their occurrence in fields during the growing season, there is a need to develop a complementary tool using PCR techniques that allow for rapid, specific, sensitive, and reliable diagnosis of *Fusarium* species. Among the different PCR techniques (Munaut *et al.* 2011), the real-time PCR using hybridization probes shows high specificity and sensitivity and combines the fast *in vitro* amplification of DNA with immediate fluorescence detection of the amplicon, allowing DNA quantification (Caplin *et al.* 1999; Poitras & Houde 2002). In this method, the acceptor probe is labelled with a fluorescent dye at the 5′ end and the donor probe with a fluorescein at the 3′ end. When donor and acceptor probes hybridize to adjacent regions on the target DNA in close proximity, Fluorescence Resonance Energy Transfer (FRET) interaction can occur. The fluorescent donor molecule is excited by an external light source which subsequently transfers its energy to the acceptor fluorophore. The excited acceptor emits light of a different wavelength which can be detected and measured by the LightCycler® instrument.

To achieve a rapid specific and sensitive method for the detection and the quantification of *F. proliferatum*, *F. subglutinans*, *F. temperatum*, and *F. verticillioides* in maize samples, the following steps of this work were (i) to develop species specific primers and hybridization probes designed from the translation elongation factor 1α (*EF-1α*) or the calmodulin (*Cal*) gene sequences, (ii) to evaluate the specificity and the sensitivity of the real-time PCR assays in simplex and multiplex conditions, and (iii) to validate the method by detection and quantification of the four *Fusarium* species in artificially *Fusarium*-contaminated maize samples.

Materials and methods

Fungal strains

In order to develop the real-time PCR assays, 29 strains of 13 *Fusarium* species and eight strains of eight other fungal genera commonly recovered in maize fields were used in this study (Table 1). All *Fusarium* strains were taxonomically well identified by morphological and phylogenetic characterization performed in previous studies (Britz *et al.* 1999; Zeller *et al.* 2003; Scauflaire *et al.* 2011a, b). The identification of each other fungal species was checked by morphological characteristics and by comparing the internal transcribed spacer (ITS) sequences of ribosomal DNA with data from GenBank.

Maize samples

Artificially *Fusarium*-contaminated maize samples were analyzed for the detection of *Fusarium proliferatum*, *Fusarium subglutinans*, *Fusarium temperatum*, and *Fusarium verticillioides* in the validation assays. The artificial contamination of stalks was performed as described by Danielsen *et al.* (1998) by insertion of a *Fusarium*-inoculated toothpick in the second internode of a 7-week-old maize plant. *Fusarium proliferatum* MUCL 53606, *F. subglutinans* MUCL 52467, *F. temperatum* MUCL 52463, and *F. verticillioides* MUCL 53471 were individually cultured in 10-mL tubes containing autoclaved toothpicks and 5 mL malt extract 2 % broth medium (20 g of malt extract L^{-1}, Duchefa, Haarlem, The Netherlands), and were incubated for 2 weeks at 25 °C in the dark. In addition, *Fusarium*-coinoculated toothpicks were prepared with all possible combinations of the two, three or four *Fusarium* strains. The test was duplicated and included control plants with insertion of a sterile toothpick. After 11 d, maize plants were collected and *Fusarium*-contaminated stalks were stored at −30 °C.

For kernel contamination, the four *Fusarium* strains as well as all their combinations were cultured in duplicate on 40 g of doubly autoclaved maize kernels that were adjusted to approximately 50 % moisture in 100 mL plastic flasks and inoculated with one plug of 25 mm² of 7-day-old mycelium grown on potato dextrose agar (PDA; Sharlau, Spain). Cultures were incubated at 25 °C for 3 weeks in the dark. Harvested culture material was lyophilized and stored at −30 °C until use. Control was similarly treated, except that it was not inoculated.

DNA extraction

To produce biomass for DNA extraction, fungal strains were grown in the dark at 25 °C for 5 d in malt extract 2 % broth medium (20 g of malt extract L^{-1}, Duchefa, Haarlem, The Netherlands) on a rotary shaker (100 rpm). Mycelium was harvested by centrifugation and the pellets were lyophilized and stored at −20 °C. To obtain genomic DNA from *Fusarium*-contaminated maize samples, 100 mg of infected tissue were sampled and were placed in a sterile tube. Both lyophilized mycelia and maize tissues were disrupted in a sterile tube with silica beads (Biospec Products, Bartlesville, USA) in the MagNA Lyser cell disrupter (Roche Diagnostics, Mannheim, Germany). Fungal DNA was extracted and purified using the Invisorb Spin Plant MiniKit (Invitek, Berlin, Germany) according to the manufacturer's recommendations. Purified DNA was quantified by the NanoDrop ND-1000 Spectrophotometer (Saveen Werner, Malmö, Sweden) and stored at −80 °C.