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Development of a qPCR assay for the detection of heat-resistant *Talaromyces flavus*

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ABSTRACT

Heat-resistant fungi of the species *Talaromyces flavus*, which inhabits soil and can contaminate fruits, constitutes significant impact on spoilage of heat-processed food. *T. flavus* possess the ability to produce numerous mycotoxins and is able to survive the process of pasteurization what makes it a treat to food industry. Up to date there is no rapid and reliable method to detect and identify *T. flavus*. Therefore in this study, a sensitive method for detecting *T. flavus* was developed. The primers (Tf1_F/R) specific to detection of *DNA replication licensing factor* gene of *T. flavus* were designed. With this set of primers, a qPCR reaction with SybrGreen detection was developed. The specificity of assay with use of 5 *T. flavus* strains and 35 other fungal isolates was tested. The detection threshold was 200 fg of *T. flavus* genomic DNA. The developed method was able to detect 640 ascospores in 1 g of strawberry fruits and soil samples.

1. Introduction

Fungal spoilage of heat-processed food origins from raw material that is contaminated with organisms belonging to heat-resistant fungi (HRF) group. The contamination of raw material begins from its contact with soil, which is the main residue of heat-resistant fungi such as *Talaromyces flavus* (Amaeze et al., 2010; Dethoup, 2007; Frąc et al., 2015). HRF, including *T. flavus* are reported to possess worldwide distribution and have been isolated from soil and agricultural products in several countries around the world, like Iran, Thailand, Nigeria or Finland (Amaeze et al., 2010; Beuchat and Pitt, 2001; Dethoup, 2007; Dijksterhuis, 2007; Fravel and Adams, 1986; Naraghi et al., 2010). Moreover, despite the fact that exact values of losses are not known, HRF are estimated to cause millions of dollars damage in fruit-juice production solely in United States of America (Dijksterhuis, 2007; Tournas, 1994).

T. flavus is a filamentous fungus of the *Trichocomacae* family. It develops in anamorphic *(Penicillum vermiculatum*) and teleomorphic (*T. flavus*) stadiums (Dethoup, 2007; Stolk and Samson, 1972).

T. flavus is one of the most common fungi belonging to this HRF group. The HRF are known for their ability to withstand high temperature treatment, such as pasteurization process (Jesenská et al., 1993; Samson et al., 2011; Sokołowska, 2010). *T. flavus* is able to survive heating to 90 °C for 6 min and to 95 °C for 1 min in glucose tartrate heating medium of pH 5.0 and 16° Brix (King, 1997). Moreover, certain isolates of *T. flavus* are able to produce numerous mycotoxins, such as talaromycin or mitrorubrin (Ayer and Racok, 1990; Proksa, 2010). *T. flavus* is able to grow under both aerobic and microaerobic conditions. These features affect significantly on spoilage of heat-processed food, especially fruits, causing danger of economic losses in food industry and may pose a risk for consumers health (Frąc et al., 2015; Pieckova and Jesenska, 1997; Proksa, 2010). Moreover, the HRF are noted to resist other growth control factors such as antibiotics or fungicides, for example amphotericin B, cycloheximide and 3-amino-1,2,4-triazole (Panek et al., 2016). Therefore, the best solution to provide safety of heat-processed products is early detection of the HRF occurrence. During last years there emerged many methods to detect fungal contamination in food products. Successful PCR methods for detection of HRF of genus *Byssochlamys* and *Neosartorya* were proposed (Nakayama et al., 2010; Yaguchi et al., 2012). Moreover, Loop-Mediated Isothermal Amplification method (LAMP) (Notomi et al., 2000; Nagamine et al., 2002) recently found application in detection of fungi, such as *Fusarium*, in food products (Abd-Elsalam et al., 2011; Niessen, 2013; Ghosh et al., 2015; Ayukawa et al., 2016). However, there is no published quick nor specific method addressing detection on HRF *T. flavus*.

Therefore, the aim of this study was development of sensitive and rapid method for detection of *T. flavus*. To fulfil this task we developed polymerase chain reaction based method with real-time amplicon detection by SybrGreen fluorescence assay. The qPCR method (Higuchi et al., 1992; Higuchi et al., 1993) may be used as a tool to detect the lowest quantities of target DNA. The sensitivity and specificity of reaction are the most essential features of *T. flavus* detection assay, so in

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this work both features of developed assay were tested. More than that, an impact of addition of *N*,*N*,*N*-trimethylglycine (betaine) on sensitivity of reaction was evaluated. Betaine is reagent that is reported for its role in improving of PCR specificity and yield (Frackman et al., 1998; Henke et al., 1997; Zhang et al., 2009).

2. Material and methods

2.1. Development of the qCR assay

Five nucleotide sequences of *T. flavus* (accession number GU396448.1.- GU396452.1) were retrieved from GenBank and were aligned using MEGA6 software (Tamura et al., 2013). The primers were designed to be highly specific to the *DNA replication licensing factor gene* (RLF) of *T. flavus* using software Primer3Plus 2.4.0 (Untergasser et al., 2012) (Table 1). The specificity of primers was confirmed by BLAST searches performing in GenBank database indicating no cross-reacting sequences with other fungi. The alignment of sequences and mapping of primers on *DNA replication licensing factor gene* sequence can be seen in supplementary materials (S1 and S2)·Therefore, it was hypothesized that qPCR reactions with DNA isolated from *T. flavus* would give a positive results, and that reactions with DNA isolated from other species would be negative.

T. flavus were cultured on Potato Dextrose Agar (PDA) at 27 °C for 7 days. The mycelium was sterilely transferred into 2 ml-tubes containing 500 and 250 mg of sterile glass beads with 3.15 mm and 1.45 mm diameter respectively, 400 µl of Lyse F buffer (EURx), 10 µl of Proteinase K (EURx) and 3 µl of RNase A (EURx). The mycelium was homogenized in FastPrep-24 instrument (MP Biomedicals) at 6 m/s for 40 s, and after sonicated in 240 W ultrasonic cleaner (Polsonic SONIC-6) for 10 min. Then genomic DNA from fungal mycelium was isolated using Plant & Fungi DNA Purification Kit (EURx) according to manufacturer protocol and suspended in 200 µl of Tris-HCl buffer (10 mM Tris-HCl, pH 8.5).

The quality of isolated DNA was assessed by measurements of the absorbance spectrum with 220–350 nm wavelength using spectrophotometer NanoDrop (Thermo Fisher Scientific). Then the DNA quantity and quality was estimated on the basis of 260 nm absorbance measurement and calculated absorbance ratios for 260 nm/230 nm and 260 nm/280 nm.

The qPCR was performed using 7500 Fast thermocycler (Applied Biosystems). The reaction was conducted in 25 µl volume containing: 8 µl deionized nucleases free water, 12.5 µl SYBR Green qPCR mastermix (EURx, SG qPCR Master Mix 2X), 0.4 µM each primer, 30 nM ROX (EURx), 8 mM UNG (EURx) and 2 µl of template DNA. The qPCR conditions were: one cycle in 50 °C for 2 min for UNG activation, one cycle in 96 °C for 10 min, 40 cycles: 96 °C for 15 s, 58 °C for 5 s, 72 °C for 15 s, 80 °C for 1 min. A melting curve was prepared from 65 °C to 95 °C with an increment of 0.016 °C per second. All reactions were performed in biological triplicates. All reactions were followed by melting curve analysis.

Positive reactions in this study were defined as an appearance of amplification plot and peak of appropriate melting temperature on derivative melting curve. The results were visualized as amplification plots (ΔRn) expressed as raw SybrGreen fluorescence units divided by reference dye ROX fluorescence diminished by baseline value in function of reaction cycles. The threshold cycle (C_t), expressing an intensification between an amplification curve and a threshold line, was also calculated allowing to evaluate the differences in amplification rate of various environmental samples and pure strains.

2.2. Assessment of specificity using fungal isolates

DNA extracted from pure culture of fungal strains (Table 2) was used to test the specificity of the designed qPCR assay. The fungal strains used in this study were identified by sequencing the internal transcribed spacer 1 and/or D2 region of large subunit of rRNA gene and comparing them to MicroSeq, GenBank and Mycobank databases. The assessment of specificity was performed using five *T. flavus* strains from international culture collections and thirty five various fungal isolates. Those included heat-resistant fungi, common cosmopolitan fungi and isolates derived from strawberry fruits or soil samples collected from strawberry plantations. The fungal isolates were cultured on PDA at 27 °C for 7 days. Samples of purified genomic DNA diluted to concentration of 2.5 ng/µl were used as templates for qPCR assay. Non-*T. flavus* strains were discriminated from *T. flavus* by evaluation of the amplification plots (ΔRn) appearance.

To confirm the specificity of amplification, the agarose electrophoresis of reaction products (2% TAE, 7 V/cm, 60 min) was performed and comparison of obtained electropherogram and melting curves was done. It allowed determining the melting temperature of specific reaction product.

2.3. Assessment of sensitivity using T. flavus

The sensitivity assessment was performed by the reaction with DNA serial dilutions of *T. flavus* DSM 63536. To test lowest amount of DNA detectable by qPCR assay, the DNA solution (100 ng/µl) was used as template for PCR and serially diluted 10-fold to obtain the following concentrations: 10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl, 100 fg/µl, 10 fg/µl and 1 fg/µl, which were used as a template for qPCR reactions in volume of 2 µl. Moreover, same sensitivity assay in presence of DNA (1 ng of DNA per reaction) isolated from other studied species in single reaction was performed. Sterilized DirectQ water was used as non-template control (NTC).

2.4. Assessment of specificity using environmental samples

The specificity assessment using environmental samples was performed by the reaction with DNA isolated from strawberry fruits and soil samples contaminated by fungal strains (Table 2). Three variants of assessment were performed. First, contamination of strawberry fruits and soil samples with ascospores from all tested *T. flavus* strain in single reaction. Second, contamination of strawberry fruits and soil samples with cells from all tested non-*T. flavus* isolate in single reaction. Third, contamination of strawberry fruits and soil samples with cells from each tested isolate in single reaction. Moreover, the negative controls were prepared with use of sterile DirectQ water instead of fungal cells. Samples were contaminated by addition of about 64,000 fungal cells suspended in 1 ml of sterile water to 1 g of strawberry fruits and soil samples. Next, contaminated samples were vortexed for 15 s and 200 µl of suspension was transferred to sterile tubes to perform DNA isolation. DNA extraction from contaminated strawberries was performed using procedure described above while DNA from contaminated soil samples was isolated with Soil DNA Purification Kit (EURx) according to manufacturer protocol. Sterile DirectQ water was used as non-template

Table 1
Primers for the PCR and the qPCR assay.

Species	Targeted sequence	PCR product (bp)	qPCR product T_m (°C)	Primer name	Primer sequences 5′ → 3′	Primer T_m (°C)
Talaromyces flavus	DNA replication licensing factor gene	346	83.34	Tf1_F	GTCTCACGGCTGCTGTTATG	59.7
				Tf1_R	TCCTTGGAGGGGGTATCGAG	60.9