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Differentiation and identification of grape-associated black aspergilli using Fourier transform infrared (FT-IR) spectroscopic analysis of mycelia

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ABSTRACT

The purpose of this study was to evaluate the potential of FT-IR spectroscopy as a high-throughput method for rapid differentiation among the ochratoxigenic species of *Aspergillus carbonarius* and the non-ochratoxigenic or low toxigenic species of *Aspergillus niger* aggregate, namely *A. tubingensis* and *A. niger* isolated previously from grapes of Greek vineyards. A total of 182 isolates of *A. carbonarius*, *A. tubingensis*, and *A. niger* were analyzed using FT-IR spectroscopy. The first derivative of specific spectral regions (3002–2801 cm^{-1}, 1773–1550 cm^{-1}, and 1286–952 cm^{-1}) were chosen and evaluated with respect to absorbance values. The average spectra of 130 fungal isolates were used for model calibration based on Discriminant analysis and the remaining 52 spectra were used for external model validation. This methodology was able to differentiate correctly 98.8% in total accuracy in both model calibration and validation. The per class accuracy for *A. carbonarius* was 95.3% and 100% for model calibration and validation, respectively, whereas for *A. niger* aggregate the per class accuracy amounted to 100% in both cases. The obtained results indicated that FT-IR could become a promising, fast, reliable and low-cost tool for the discrimination and differentiation of closely related fungal species.

1. Introduction

Fungal members of *Aspergillus* section *Nigri* group (black aspergilli), saprophytes generally considered ubiquitous in nature, are responsible for food spoilage and ochratoxin A (OTA) production in several food commodities (Ostry et al., 2002; Palumbo et al., 2010; Copetti et al., 2010; Hayrettin et al., 2012; Perrone et al., 2013). Black-spored species belonging to this group, particularly the biseriate species *A. carbonarius* and species of *Aspergillus niger* aggregate (*A. niger* and *A. tubingensis*), are the most frequently isolated species from grapes, raisins and wine (Chulze et al., 2006; Gómez et al., 2006; Moss, 2008; Chiotta et al., 2009). Several studies have clarified that the main OTA producer from this group is *A. carbonarius* which produces this mycotoxin very consistently and to a lesser extent the so-called *Aspergillus niger* aggregate, particularly *A. niger* and *A. tubingensis* (Magnoli et al., 2003; Bellí et al., 2005; Perrone et al., 2006; Samson et al., 2007; Lasram et al., 2012). OTA is a secondary metabolite with nephrotoxic, immunosuppressive, teratogenic, and carcinogenic properties which has been classified as group 2B carcinogen by the International Agency for Research on Cancer (1993). It has been reported as the first mycotoxin commonly found in grape products and therefore the European Union has set permissible limits of OTA intake from dried vine fruit to 10 μg kg^{-1}, and from grape juice and wine to 2 μg kg^{-1} (European Commission, 2005).

In food mycology, identification schemes are based on phenotypic and/or genotypic characterization. The former methods are mainly focused on fungal growth on different agar media, followed by carefully scrutinizing the morphological characteristics of the mycelium, and on microscopic observations. Additionally, physico-chemical reaction patterns can be also used as phenotypic methods for fungal identification (Samson et al., 2007; Meijer et al., 2011). Genotypic methods concern the polymerase chain reaction (PCR) of amplified DNA, using conserved sequences of mitochondrial and ribosomal genes and internal transcribed spacer regions (ITS) (Martínez-Culebras et al., 2009; Schoch et al., 2012).

The characterization of black aspergilli is considered one of the most confusing and difficult task due to indistinguishable differences among the species. Thus, fungal taxonomy of *Aspergillus* section *Nigri* group is unclear and many attempts have been undertaken to find appropriate taxonomic criteria (Dachoupakan et al., 2009; Meijer et al.,

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2011; Silva et al., 2011; Kizis et al., 2014). Although some species, such as *A. carbonarius* and uniseriate species, namely *A. aculeatus* and *A. japonicus*, can be easily recognized based on morphological criteria and microscopic observations, identification of other species remains problematic and insufficient (Abarca et al., 2004; Samson et al., 2007). Molecular characterization and phylogenetic analysis of representative black aspergilli based on Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) has been proposed as the most accurate method to identify fungal species (Martínez-Culebras and Ramón, 2007; Spadaro et al., 2012). Even though the RFLP-based studies for black aspergilli species are precise, molecular techniques remain fastidious, expensive and demand special laboratory skills and facilities (Rozynek et al., 2004).

Fourier Transform Infrared Spectroscopy (FT-IR) is a rapid method with high sensitivity, robustness and ease of use for the identification and differentiation of microorganisms causing food spoilage (Garon et al., 2010; Kaya-Celiker et al., 2015). The mid-FT-IR spectroscopy is a simple instrumental technique that can give evidence for the presence of various functional groups whereby vibrational motions of molecules either absorb or reflect radiation at wavenumbers of 4000–400 cm^{-1}. The absorption changes at specific frequencies allow the determination of which molecular groups are present, and how they are arranged or interact by means of an FT-IR spectrum. The spectral profile provides information about important macromolecules like proteins, lipids, phosphate-containing compounds, phospholipids, and carbohydrates present in cells. Classification is based on the analysis of the "molecular fingerprint" which is obtained by this spectrum (Naumann, 2000).

FT-IR spectroscopy has been successfully applied for the identification of filamentous fungi. Specifically, Garon et al. (2010) used this technique for the discrimination of closely related *Aspergillus* species (*A. flavus*, *A. fumigatus*, and *A. parasiticus*) which have been previously collected from feed and bioaerosols in agricultural environment, and concurrently for the differentiation among aflatoxigenic and non-aflatoxigenic isolates. Tralamazza et al. (2013) have also examined the ability of FT-IR as an alternative technique to molecular procedures for fungal identification. Particularly, they investigated the potential of FT-IR to discriminate and classify three environmental *Aspergillus* species (*A. niger*, *A. ochraceus*, and *A. westerdijkiae*) isolated from coffee beans and highlighted the ability of FT-IR to distinguish *Aspergillus* species as an alternative to molecular procedures. In another study, FT-IR spectroscopy was successfully employed for the differentiation of 16 isolates belonging to five *Fusarium* species (Nie et al., 2007). Other researchers also reported that FT-IR can be used for the identification and characterization of filamentous fungi and yeasts (Fischer et al., 2006; Santos et al., 2010; Shapaval et al., 2012; Zervakis et al., 2012; Lecellier et al., 2014, 2015; Kaya-Celiker et al., 2015). All these studies indicated that FT-IR spectroscopy can be effectively used as a rapid routine method of high specificity, reliability, and with minimal sample preparation for fungal taxonomy. The Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectroscopy, employed in this study for the analysis of fungal mycelia, is an alternative FT-IR spectroscopy method that was developed by Goodacre et al. (1996) for the spectroscopic analysis of powders and materials with rough surfaces and offers the advantage of simple sample preparation and the potential to analyze nontransparent materials.

The purpose of this study was to evaluate the potential of FT-IR spectroscopy as a high-throughput method for rapid differentiation among the ochratoxigenic species of *A. carbonarius* and the non-ochratoxigenic or low toxigenic species of *A. niger* aggregate, namely *A. niger* and *A. tubingensis*. This method was used to establish a comprehensive database of taxonomically well-defined black aspergilli species to aid in their taxonomy. Compared to previous studies the importance of this work is based on the differentiation of closely related species of *Aspergillus* section *Nigri* group using a high-performing technique. To our knowledge this is the first attempt to discriminate black aspergilli species using FT-IR spectroscopy.

2. Materials and methods

2.1. Fungal isolates

A total of 182 fungal isolates were analyzed in this study from which 91 isolates have been identified as *A. carbonarius* and 91 as *A. niger* aggregate (of which 80 were identified as *A. tubingensis* and 11 as *A. niger*). All of them have been previously isolated from grapes of Greek vineyards. Specifically, 115 fungi were taken from the culture collection of the Laboratory of Food Microbiology and Biotechnology (LFMB) of the Agricultural University of Athens. These fungi have been isolated during the 2012 harvesting period (from August to September) and identified at species level by molecular techniques as reported in a previous work (Kizis et al., 2014). The remaining 67 fungi were isolated during the 2014 harvesting period (in September) and identified at species level by PCR-RFLP of 5.8S-ITS gene region as detailed elsewhere (Bisbal et al., 2009; Kizis et al., 2014). Reference strains were obtained from the fungal collection of the LFMB for *A. carbonarius* (F26, F40), *A. niger* (F7, F88), and *A. tubingensis* (F31, F65).

2.2. DNA extraction and amplification of fungal isolates

2.2.1. Culture conditions and DNA extraction

Unidentified isolates from the fungal collection of the harvest year 2014 and reference strains were grown in Yeast Extract Sucrose broth (YES; yeast extract 20 g; sucrose 150 g; distilled water, *c.* 1000 ml) at 30 °C for 48 h. Then, mycelia were aseptically collected, washed thoroughly with ethanol (96%, *v/v*) and dried using Whatman No. 1 filter paper. Finally, 100–200 mg of each sample were lyophilized for 24 h and ground into a fine powder. DNA extractions were performed using the innuPREP Plant DNA kit (Analytik Jena, Germany) according to the manufacturer's instructions.

2.2.2. PCR amplification

The isolates were amplified using PCR primers ITS1 and ITS4. PCR reactions were performed in a 50 μl final volume, containing 1 × standard reaction buffer (Kapa Biosystems, Japan), 2.0 mM $MgCl_2$, 300 μM dNTPs (each), 300 nM primers (each), 100 ng DNA template and 1.25 U of Taq polymerase (Kapa Biosystems, Japan). The reaction mixtures for PCR primers ITS1/ITS4 were performed in a thermal cycler (Bio-Rad Laboratories, USA), starting with an initial denaturation step at 95 °C for 5 min, followed by 39 cycles consisting of 30 s at 95 °C, 30 s at 52 °C and 40 s at 72 °C, and a final extension step at 72 °C for 10 min. Then, PCR products were digested at 37 °C for 3 h with the restriction enzymes *Hha*I, *Hinf*I and *Rsa*I (New England Biolabs, UK). Digestions were performed in a 20 μl reaction volume containing 2 μl of 10 × reaction buffer, 10 μl of each amplicon and 1.5 U restriction endonuclease and 0.2 μl BSA (10 μg/l) for *Hha*I digestions at 37 °C for 3 h. All PCR amplicons and their restriction digestion fragments were separated by electrophoresis at 100 *V*, 1 × TAE buffer, in 1% and 3% agarose gels, respectively. Finally, gels were stained in ethidium bromide solution (0.5 mg/ml) and photographed under Ultra Violet (UV) light using a Gel Doc XR + system (Bio-Rad Laboratories, USA). Molecular sizes of the DNA were estimated by comparison with the DNA standard GeneRuler 100 and 50 bp DNA ladders (Thermo Scientific, USA).

2.3. FT-IR analysis

2.3.1. Fungal culture and sample preparation

All fungal species were cultivated on Malt Extract Agar (MEA; malt extract, 20 g; peptone, 1 g; glucose, 20 g; bacteriological agar, 20 g; distilled water, *c.* 1000 ml) medium into 9 cm Petri dishes for 7 days at 25 °C. Then, spores of each culture were extracted with the aid of a loop, transferred into a Petri dish containing 20 ml of YES broth medium (composition as mentioned previously) and incubated at 30 °C