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CHARACTERIZATION AND IDENTIFICATION OF *ASPERGILLUS* SECTION *FLAVI* ISOLATES FROM PORTUGUESE ALMONDS USING A POLYPHASIC APPROACH INCLUDING MALDI-TOF ICMS

Paula Rodrigues[a,b], Cledir Santos[a,], Armando Venâncio[a] and Nelson Lima[a]

[a] *Department of Biological Engineering, University of Minho*
[b]*CIMO – School of Agriculture, Polytechnic Institute of Bragança*
E-mail: prodrigues@ipb.pt

INTRODUCTION

Aspergillus is a large genus, with a complex and ever evolving taxonomy. Section *Flavi* is one of the most significant sections in the genus, and is one of the best studied among fungi, for the numerous industrial applications as well as for food safety issues.

Section *Flavi* is composed of a large number of very closely related species. While these species are difficult to differentiate morphologically and even genetically, they differ in a characteristic that is of paramount importance for food safety, as some are responsible for the production of the highly toxigenic aflatoxins. Taxonomy and species identification are therefore subject of great interest for scientists aiming to clarify the species concept and limits within the section. In this sense, the establishment of schemes for species and for aflatoxigenic strains identification that are simultaneously accurate, sensitive, robust and expedite is mandatory.

At present, reliable identification schemes typically imply the analysis of a wide variety of morphological, biochemical and molecular traits. Recently, Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Intact Cell Mass Spectrometry (MALDI-TOF ICMS) has been used to generate spectra of protein masses in a range of 2,000 to 20,000 Da, which result in a *taxa* specific fingerprint. This technique has already shown high potentialities to discriminate very closely related *taxa*, but has rarely been used in fungal species identification, either on its one or as part of a polyphasic scheme of identification.

AIMS

This work aimed to: i) characterize the population of *Aspergillus* section *Flavi* collected from Portuguese almonds in relation to their aflatoxigenic potential; ii) identify the isolates by applying a set of morphological, biochemical, molecular and spectral analyses (polyphasic approach); iii) compare the data obtained from the various approaches in terms of sensitivity, reliability and user-friendliness; and iii) determine the validity of MALDI-TOF technique for the identification of closely related field isolates of section *Flavi*.

METHODS

Samples of almonds originating from Trás-os-Montes (Moncorvo) and Algarve (Faro) were collected at different stages of production: field, storage and processing. All fungi belonging to genus *Aspergillus* section *Flavi* were isolated from almonds in Malt Extract Agar (MEA) supplemented with 10% NaCl and were, on a preliminary phase, differentiated from other sections based on their colony color on Czapek Yeast Autolysate (CYA) and on Aspergillus flavus and parasiticus Agar (AFPA).

These fungi were then further characterized by morphological, biochemical, molecular and spectral analyses. For the morphological analysis, fungi were

cultured on three different media (MEA, CYA and CYA supplemented with 20% sucrose) and were characterized for several macro and micro morphological features. Morphological analysis was complemented with biochemical analyses, which consisted of determining, by HPLC, the extrolite profiles relative to aflatoxins and cyclopiazonic acid. The molecular analysis of the isolates, which was applied to a restricted group of 24 isolates, was based on the sequencing of partial calmodulin gene. These sequences were then compared with sequences from reference strains obtained from GenBank. Spectral analysis of the same 24 isolates was made by MALDI-TOF ICMS to obtain spectra of protein masses in a range of 2,000 to 20,000 Da, using 2,5-dihydroxybenzoic acid (DHB) as matrix. Spectra were obtained using Shimadzu Axima-LNR equipment and treated for fungal identification using SARAMIS™ Package. Each set of data was used to generate a dendrogram of relatedness.

RESULTS

In our survey, 352 fungi belonging to section *Flavi* were isolated. From the preliminary morphological analysis, three groups (morphotypes) were identified: 29 isolates (8.2%) were morphologically identified as "*A. tamarii* morphotype", 128 isolates (36.4%) as "*A. flavus* morphotype", and 195 isolates (55.4%) as "*A. parasiticus* morphotype". In total, 231 isolates (66%) were aflatoxigenic: 28% of the *A. flavus* (*lato sensu*) isolates and 100% of the *A. parasiticus* (*lato sensu*) isolates were found to produce aflatoxins. No aflatoxin production was detected for any of the *A. tamarii* (*lato sensu*) isolates.

As for species identification, the genotypic and spectral analyses clustered the selected 24 isolates into the same 3 groups created by morphological analysis. Furthermore, all sets of data, including the morphological complemented with extrolite profile, were able to further resolve the isolates into more restrictive clusters. They all positioned 5 of the 24 isolates in two unidentified terminal clades closely related to *A. parasiticus*, and divided the *A. flavus* morphotype in two terminal clades.

CONCLUSIONS AND FUTURE WORK

The majority of the isolates obtained from Portuguese almonds were found to be aflatoxigenic, which may constitute a problem in terms of food safety.

At the species identification level, good agreement was obtained between the 3 methods of analysis since all generated similar dendrograms with concordant strain clustering.

Morphological analysis has shown sensitive and reliable as a preliminary method for species identification only when complemented with the extrolite profile. The calmodulin gene showed to be a robust and reliable genomic marker for this group of fungi, providing good DNA barcoding potential. MALDI-TOF ICMS results confirmed that this technique is as good as the calmodulin gene analysis for fungal identification. MALDI-TOF ICMS technique is rapid, reliable and inexpensive in terms of labour and consumables when compared with other biological techniques. At present, it adds a step to the polyphasic scheme of identification, which is essential whenever there is a paucity of characters for defining many fungal species.

Finally, the problematic strains are currently under further study using multilocus sequence typing (MLST) approach with 5 extra genes.

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