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Characterization of *Aspergillus* section *Flavi* isolated from fresh chestnuts and along the chestnut flour process

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ABSTRACT

An extensive sampling of *Aspergillus* section *Flavi* considered to be the main agent responsible for aflatoxin contamination, was carried out in the field and along the processing phases of chestnut flour production in 2015. Fifty-eight isolates were characterized by means of biological, molecular and chemical assays. The highest incidence of *Aspergillus* section *Flavi* was found in the field. The identification of the isolates was based on β-tubulin and calmodulin gene sequences. *A. flavus* was found to be the dominant species, and this was followed by *A. oryzae* var *effusus, A. tamarii, A. parasiticus* and *A. toxicarius*. Nineteen percent of the strains produced aflatoxins *in vitro* and forty percent *in vivo*. The pathogenicity assay on chestnut showed 56 virulent strains out of 58. The molecular, morphological, chemical and biological analyses of *A. flavus* strains showed an intraspecific variability. These results confirm that a polyphasic approach is necessary to discriminate the species inside the *Aspergillus* section *Flavi*. The present research is the first monitoring and characterization of aflatoxigenic fungi from fresh chestnut and the chestnut flour process, and it highlights the risk of a potential contamination along the whole chestnut production chain.

1. Introduction

European sweet chestnut (*Castanea sativa* Mill.) is mainly grown in the temperate regions of continental Europe, and it has a considerable economic value, with an annual production of about 170,000 tons in Europe. According to FAO, Italy is the main European producing country, with a cultivated area of 46,000 ha and a production of 43,000 tons/year, followed by Portugal and Spain (20,000 tons), Greece and France (10,000 tons) (Livre Blanc Chataigne, 2014). Almost 20% of the total production is used to make chestnut flour, dried chestnuts and the marrons glacé sweet.

Several studies have reported the presence of spoilage fungi, such as *Penicillium* spp. and *Aspergillus* spp., and the contamination of chestnuts by mycotoxins, particularly aflatoxins (AFs) (Bertuzzi et al., 2015; Overy et al., 2003; Pietri et al., 2012; Rodrigues et al., 2013). *A. flavus* is the main contaminant and the most frequent species of *Aspergillus* in chestnut (Klich, 2007; Wells and Payne, 1975).

AFs are secondary metabolites, which have toxic effects, including mutagenic, teratogenic and immunosuppressive activities. These molecules are mainly produced by *Aspergillus* section *Flavi,* and the most frequently studied species are *A. flavus*, which is able to produce aflatoxins B1 (AFB_1) and B2 (AFB_2), and *A. parasiticus*, which can also produce aflatoxins G1 (AFG_1) and G2 (AFG_2) (Bennett and Klich, 2003). The incidence of toxigenic *A. flavus* strains is about 40%, while most strains of *A. parasiticus* are aflatoxigenic (Frisvad et al., 2006). The maximum permissible levels in Europe of AFB_1 in food and the maximum sum of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) in nuts are specified in European Commission Regulation (EU) No 165/2010. Exceeding the AFB_1 and total AF thresholds in chestnuts and the related products, which have been established as 2 and 4 μg/kg, respectively, leads to product withdrawal from the market and is subjected to criminal procedures. The European Rapid Alert System for Food and Feed (RASFF, 2011) has in fact reported several cases of aflatoxin

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contamination in chestnut products in Italy. The above reported limits, which are lower than those of other nuts, are leading to a reduction in the commercialization of chestnut products. Therefore, national institutions in Europe have proposed modifying the thresholds. Moreover, they are funding research with the aim of understanding how and when these mycotoxins are produced in chestnuts, and at preventing and controlling their occurrence.

Aflatoxigenic fungi have been reported as frequent contaminants of food and feed, and their population tends to increase during storage, with *A. flavus* being the main species that has been found, while *A. parasiticus* seems to be less prevalent (Essono et al., 2009).

As reported by the US Council for Agricultural Science and Technology (CAST, 2003), the environmental conditions where tree nuts are grown, stored and processed, are known to promote the growth of fungi and AF production, and expose nuts to a moderate risk of aflatoxin contamination.

The members of the *Aspergillus* section *Flavi* show a high level of genetic variability, and this makes it difficult to use traditional identification methods, based on the analysis of micro and macromorphological parameters, such as the colony diameter, the color and the texture of the conidia, and the growth rate (Klich and Pitt, 1988; Kumeda and Asao, 1996). Rokas et al. (2007) highlighted the problem of species identification, due to the high degree of DNA similarity of some species of the section *Flavi*, such as *A. flavus/A. oryzae* and *A. parasiticus/A. sojae*. The use of a single molecular approach for sequencing, combined with a comparison with unverified sequences deposited in public databases, contributes to creating an ambiguous identification of the species (Samson et al., 2014).

Furthermore, intraspecific variability has also been shown for chemical characterizations, as not all isolates belonging to the same species produce the expected metabolites, particularly AFs (Giorni et al., 2007; Rodrigues et al., 2009; Vaamonde et al., 2003). As reported by Samson et al. (2014), a polyphasic approach is required, in which morphology, molecular traits, and extrolite analysis are combined. A molecular method, based on multi locus sequence analysis with at least two conserved regions, and the analysis of extrolites, such as AFBs and AFGs, cyclopiazonic acid and aspergillic acid, are necessary (Rodrigues et al., 2011).

Knowledge about the presence of aflatoxigenic fungi and their aflatoxigenic potential is crucial to guarantee consumer safety, and to elaborate standard operational procedures for the production of chestnut flour and dried chestnut. This present study has been aimed at characterizing the biological, molecular and chemical properties of several strains of *Aspergillus* section *Flavi* isolated in the field or along the chestnut flour production chain.

2. Material and methods

2.1. Fungal strain

Aspergillus section *Flavi* strains were collected from field chestnuts and during chestnut flour production. The field strains were isolated from chestnuts harvested in October 2015, in the Piedmont region (Ormea, Perlo and Viola, in the Cuneo province - Italy). Moreover, the study considered three chestnuts lots of different origin to isolate the strains along the chestnut production chain: one from Italy (Parenti, Calabria), one from Albania (Tropojë, Scutari) and one from Spain (Ourense, Galicia). For each chestnut lot, the following phases were considered: dried chestnuts (phase 1); sorted dried chestnuts (phase 2); chestnut granulate (phase 3); roasted chestnut granulate (phase 4); roasted chestnut flour (phase 5). An additional sample of commercial chestnut flour from Italy (Lisio, Piedmont), which had been stored for 6 months, was also included.

Fifty-eight isolates, selected on the basis of the source of isolation and morphotype (Table 1), were maintained, as monoconidial cultures, in tubes of Potato Dextrose Agar (PDA, Merck, Germany) and characterized throughout the experiments.

2.2. Sampling and aspergillic acid production

Aspergillus Flavus and Parasiticus Agar (AFPA) (Fluka, Germany), supplied with Chloramphenicol Selective Supplement (Fluka) and PDA (Merck), were used for the sampling.

Sampling was carried out randomly on 30 fresh chestnuts harvested in field per replicate, with five replicates. The samples were surface-disinfected with 1% sodium hypochlorite and washed in sterile deionized water. Four fragments of each fruit were cut and plated onto the two media. The Petri dishes were regularly inspected, every 2 days, for 10 days of incubation, and all the colonies of *Aspergillus* section *Flavi* were collected and transferred to AFPA medium. The material from the processing phases (from 1 to 4) was surface-disinfected and plated, as previously described. Each sample was composed by 3 replicates of 10 chestnuts (phases 1 and 2) or 4 replicates of 10 granulate pieces (phases 3 and 4). Chestnut flour (phase 5 or commercial sample; 20 g each replicate and 3 replicates) was previously homogenized with a Stomacher in sterile deionized water, and homogenates were taken and plated, after serial dilution (from 1:10 to 1:10,000), onto AFPA medium (Corry et al., 2003). Sampling was performed according to Pitt et al. (1983). Briefly, after 2 days of incubation at 30 °C in the dark, the re-isolates were controlled for the presence of orange-yellow pigmentation on the reverse side of the colony, formed as a result of the reaction between ferric citrate and aspergillic acid. *Aspergillus* section *Flavi* incidence was calculated, as described by Lione et al. (2015), as the percentage of contaminated samples over the total samples. The experiment was repeated twice.

2.3. Molecular analysis

The isolates for the molecular studies were grown on Yeast Extract Sucrose Broth (20 g yeast extract, Merck; 150 g glucose, Sigma Aldrich, Germany; 1 L H_2O), incubated at 28 °C in the dark. The mycelium was collected after 7 days, and DNA was extracted using Omega E.Z.N.A. Fungal DNA Mini Kit (VWR, USA), according to manufacturer's instructions. Partial amplification of the β-tubulin and calmodulin genes was obtained using the bt2a (5′-GGTAACCAAATCGGTGCTGCTTTC - 3′) and bt2b (5′ - ACCCTCAG-TGTAGTGACCCTTGGC - 3′) primer pair and the cmd5 (5′ - CCGAG-TACAAGGARGCCTTC - 3′) and cmd6 (5′ - CCGATRGAGGTCA-TRACGTGG - 3′) primer pair, respectively. PCR was carried out in a total volume of 25 μL containing: 2.5 μL of Buffer 10 X, 0.5 μL of MgCl2, 0.75 μL of dNTPs (10 mM), 1 μL of each primer (10 mM), 0.2 μL of Taq DNA polymerase (Qiagen, Germany) and 40 ng of template DNA. Thermal cycling programs were performed according to Samson et al. (2014). The obtained amplicons were run on a 1% agarose gel with 1 μL of GelRed™ (VWR) at 100 V/cm for 45 min, and compared with positive controls. Gel Pilot Wide range Ladder (Qiagen) was used to compare the expected size of the bands. The amplified DNA fragments of both genes were purified using QIAquick© PCR purification Kit (Qiagen), and sequenced in both directions by Macrogen, Inc. (The Netherlands).

Forward and reverse sequences of each gene were used to create a consensus sequence, using a DNA Baser program (Heracle Biosoft S.R.L., Romania), and alignment was performed using CLUSTALW through Molecular Evolutionary Genetics Analysis (MEGA6) software, version 6.0. After cutting the trimmed regions and manual correction, a dataset was obtained of 484 bp for the β-tubulin genes