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Electrochemical identification of toxigenic fungal species using solid-state voltammetry strategies

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ABSTRACT

An electrochemical methodology for the characterization of mycotoxin-producing fungal species from the genera *Aspergillus* and *Fusarium* using solid-state voltammetry is described. Upon attachment of fungal colony microsamples to glassy carbon electrodes in contact with aqueous acetate buffer, characteristic voltammetric signals mainly associated to the oxidation of polyphenolic metabolites are recorded. The possibility of fungi-localized electrochemical processes was assessed by means of electron microscopy and field emission scanning electrochemical microscopy coupled to the application of oxidative potential inputs. Using pattern recognition methods, the determined voltammetric profiles were able to discriminate between mycotoxin-producing fungi from different sections and to identify selected toxigenic species of the *Aspergillus* and *Fusarium* genera isolated from grapes and cereals.

1. Introduction

Mycotoxins are a group of secondary metabolites produced by fungi, especially of the genera *Aspergillus* and *Fusarium*, in agricultural commodities. Mycotoxin-producing species are habitual saprophythes or parasites of agricultural crops world wide, affecting mainly to cereals, fruits and nuts. Toxigenic isolates from these especies can be found throughout the food chain, including pre- and post-harvest, storage, food processing and transportation (Giorni, Bertuzzi, & Battilani, 2016; Magan & Aldred, 2007).

The European Commission recommends the application of good agricultural practices to prevent contamination of food by mycotoxins and early detection and control of toxigenic fungi because chemical, physical, and biological detoxification strategies for mycotoxins, have proven to be too expensive, inefficient and some of them can produce toxic residues and undesirable changes in foods and feeds. Accordingly, efficient tools available for the early detection of mycotoxigenic species are helpful to prevent the entry of these toxins into the food chain.

An important problem appearing in the study of fungal contamination on foods is the identification of toxigenic fungal species. Traditional methods used to detect these species in foods are based on culturing in different media and morphological studies. This approach, however, is time-consuming, laborious and often difficult even for expert taxonomists and has been complemented by methods based on DNA detection assays, mainly polymerase chain reaction (PCR). Several PCR protocols have been developed and applied successfully to detect mycotoxigenic species in food products. Although PCR methods, in particular those based on multicopy target sequences (Jurado, Vázquez, Marín, Sanchis, & González-Jaén, 2006; Mulè, Susca, Stea, & Moretti, 2004), are specific and sensitive (Niessen, 2007), they require a relatively complicated sequence of processes, namely, DNA extraction, amplification reactions and species-specific PCR assays. Accordingly, the implementation of rapid and efficient methods for species identification in all the stages of food production is necessary.

One plausible way for identifying fungal species is the determination of their secondary metabolite profiles, in particular, the mycotoxin profile. This route is facilitated by the recent advances on mycotoxin detection using affinity biosensors based on antibodies and aptamers (Castillo et al., 2015; Evtugyn et al., 2013; Vidal et al., 2013; Yang, Lates, Prieto-Simón, Marty, & Yang, 2012).

In this work, we propose an alternative route for the identification of fungal species based on the record of the voltammetric response due to metabolites such as anthraquinones or naphthopyrones containing phenolic units, melanin and other compounds existing in relatively large concentration in the fungi body and having 'direct' electrochemical activity. To increase sensitivity, the voltammetry of immobilized particles (VIMP)

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methodology was applied using fungal colony samples directly attached to glassy carbon electrodes in contact with aqueous acetate buffer. The VIMP, a technique developed by Scholz, Schröder, Gulaboski, and Doménech-Carbó (2014), involves the record of the electrochemical response of sparingly soluble solids attached to inert electrodes that come into contact with suitable electrolytes. Among other materials (Doménech-Carbó, Labuda, & Scholz, 2013), this methodology has been applied to vegetal tissues (Doménech-Carbó, Domínguez, Hernández-Muñoz, & Gavara, 2015; Domenech-Carbó, Ibars, et al., 2015) and plant extracts (Ortiz-Miranda et al., 2016) for obtaining chemotaxonomic information able to be correlated with phylogenetic trees (Domenech-Carbó, Ibars, et al., 2015). It is pertinent to emphasize that the proposed methodology, to the best of our knowledge has not been applied previously to the identification of toxigenic fungi, yields species discrimination without the need for identifying individual electroactive compounds nor determining mycotoxins.

Keeping in mind the interest of rapid procedures for routine characterization of toxigenic fungi, the aim of this study was to develop an easy and rapid electrochemical method for discriminating the most important toxigenic fungal species from cereals and grapes. Cereals are considered the first source of mycotoxins in the diet. The most important mycotoxins, such as aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FBs), type B trichothecenes, especially deoxynivalenol (DON), zearalenone (ZEA) and type A trichothecenes, mainly T-2 and HT-2 toxins (T-2 and HT-2) have been reported to occur in cereals (Serrano, Font, Ruiz, & Ferre, 2012). The International Agency for Research on Cancer (IARC) has classified them into groups 1B, 2B, 2B, 3, 3 and 3, respectively. AFs have been recognized as natural compounds having the highest teratogenic and carcinogenic potential for humans known (IARC, 2012). All these toxins, are regulated in cereals and by-products in the European Union (European Commission, 2006; 2007), although only recommendations about permissible levels of T-2+HT-2 in cereals have been reported (European Commission, 2013). Others important mycotoxins found in cereals, such as beauvericin (BEA) and enniatins (ENs) considered "emerging" mycotoxins (Sifou et al., 2011), are not currently regulated. The most frequent toxigenic fungi related with these toxins in cereals are: *Aspergillus flavus* and *A. parasiticus,* from section *Flavi* (AFs) (Giorni et al., 2016), *A. niger* and *A. carbonarius,* from section *Nigri* and *A. ochraceus, A. steynii* and *A. westerdijkiae* from section *Circumdati* (OTA) (Mateo, Gil-Serna, Patiño, & Jiménez, 2011), *Fusarium verticillioides*, *F. proliferatum* and *F. subglutinans* from section *Liseola* (FBs), *F. graminearum* and *F. culmorum* from section *Discolor* (type B trichothecenes and ZEA) (Gil-Serna et al., 2013; Piacentini, Savi, Pereira, Vildes, & Scusse, 2015), *F. sporotrichioides*, *F. langsethiae* and *F. poae* from section *Sporotrichiella* (type A trichothecenes) (Edwards, Imathiu, Ray, Back, & Hare, 2012), and *F. oxysporum* from section *Elegans* (BEA and ENs) (Song, Lee, Lee, Ha, & Lee, 2009) among others.

After cereals, wine is considered the second source of OTA in the diet. The relevance of this mycotoxin in wine and others grape products has been reported (Mateo, Medina, Mateo, Mateo, & Jiménez, 2007) and the native ochratoxigenic mycobiota of grapes has been studied by conventional and PCR techniques. The most frequent ochratoxigenic species in vineyards are *A. carbonarius*, *A. tubingensis* and *A. niger* (the two last from the *A. niger* aggregate). Whereas *A. carbonarius* can be microscopically distinguished by conidial size and ornamentation, all the taxa in the *A. niger* aggregate are morphologically indistinguishable and additional PCR techniques are required for their identification (Medina, Mateo, López-Ocaña, Valle-Algarra, & Jiménez, 2005; Niessen, 2007; Perrone et al., 2006).

In order to assess the possibility of a localized electrochemistry eventually involving trans-cellular membrane electrochemistry, as described for other biological systems (Domenech-Carbó et al., 2016; Yu, Wang, Zhu, Bao, & Gu, 2014), field emission scanning electron microscopy (FESEM) and scanning electrochemical microscopy (SECM) techniques were also used.

2. Experimental

2.1. Toxigenic fungal species

Isolates of the target species assayed (see Table 1) were previously isolated from grapes and cereals grown in Spain. Identification of fungi was carried out by conventional and PCR protocols following the methodology previously described by Medina et al. (2005) and Mateo et al. (2011) and associated references for *Aspergillus* spp., and by Gil-Serna et al. (2013) and associated references for *Fusarium* spp. isolates are held lyophilized in 15% glicerol at −20 °C at the fungal collection of the Mycology and Mycotoxins Group in the Department of Microbiology and Ecology (University of Valencia).

2.2. Reagents and standards

Standards of mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, DON, 3AcDON, ZEA, T-2, HT-2, BEA, ENs), 2-mercaptoethanol, pentafluoropropionic anhydride (PFPA) and 4-dimethylaminopyridine (DMAP) were purchased from Sigma (Sigma-Aldrich, Alcobendas, Spain). Acetonitrile, chloroform, acetic acid, methanol (all LC grade) and phosphoric acid (85%, A.R.) were from J.T. Baker (Deventer, the Netherlands). Yeast extract, sodium acetate, ethanol and toluene were from Panreac (Montcada i Reixac, Barcelona, Spain). Pure water was obtained from a Milli-Q Plus apparatus (Millipore, Billerica, MA, USA). Tween 80 and standardized 70–230 mesh aluminium oxide 90 were from Merck (Darmstadt, Germany). Glass microfibre filters and filter papers (Whatman No. 4) were from Whatman (Maidstone, UK). Ortophthaldialdehyde (OPA) was purchased from Fluka (Alcobendas, Spain). Sep-Pak Plus C18 cartridges were supplied by Waters Co. (Milford, MA, USA).

2.3. Mycotoxin producing potential of the isolates

2.3.1. Culture media and incubation conditions

Assays for toxin production by *Aspergillus* spp. were carried out in Yeast Extract Sucrose (YES) medium (20 g agar, 20 g yeast extract, 150 g sucrose, 1 g $MgSO_4 \cdot 7H_2O$, 1000 mL water). The medium was autoclaved at 115 °C for 30 min, the water activity (a_w) was adjusted to 0.98 by addition of exact glycerol weights using specific curves for YES and then it was poured into sterile 9 cm Petri dishes. 3 μL of a spore suspension of 1×10^6 spores/mL of each isolate were inoculated on the center of Petri plates and incubated at 25 °C for two weeks. Assays for toxin production by *Fusarium* spp. isolates were carried out using cereal grains as culture media. The most appropriate cereal grains for each toxin production were used (Medina et al., 2006) (Table 1). To prepare the media, 100 g of cereal grains were autoclaved for 20 min at 121 °C. Then, a_w of grains was adjusted to 0.98 by addition of sterile pure water. Grains were inoculated with 100 μL of a spore suspension of 1×10^6 spores/mL and incubated at 25 °C for two weeks with periodic shaking to facilitate their homogenization. A RTD-502 unit (Novasina GmbH, Pfäffikon, Switzerland) was used to perform all a_w measurements. During incubation time, inoculated flasks and Petri dishes were enclosed in sealed plastic containers together with beakers of a glycerol-water solution matching the same a_w as the cultures to maintain a constant equilibrium relative humidity inside the boxes and to promote toxin production. All experiments in flasks and Petri dishes were carried out in triplicate.

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