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.

Keywords: mycotoxin-producing fungi; voltammetry; species identification.

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, afecting 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, 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 (Yang, Lates, Prieto-Simón, Marty & Yang, 2012; Evtugyn et al., 2013; Vidal et al., 2013; Castillo, Spinella, Poturnayova, Snejdarkova, Mosiello & Hianik, 2015).

In this work, we propose an alternative route for the identification of fungal species based on the record of the voltammetric response due to other than mycotoxin 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) 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 et al. (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, this methodology has been applied to vegetal tissues (Doménech-Carbó et al., 2015a; b) and plant extracts (Ortiz-Miranda et al., 2016) for obtaining chemotaxonomic information able to be correlated with phylogenetic trees (Doménech-Carbó et al., 2015b). It is pertinent to emphasize that the proposed methodology that, 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 et al., 2015), *F. sporotrichioides*, *F. langsethiae* and *F. poae* from section *Sporotrichiella* (type A trichothecenes) (Edwards, 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; 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 (Doménech-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, Gil-Serna, Patiño and Jiménez (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 -20ºC 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). Orto-phthaldialdehyde (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 ºC for 30 minutes, 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. Three 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 ºC 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 ºC. 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 ºC 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. Cultures performed in cereal grains were dried at 40 ºC and finely ground before analysis for mycotoxins.

2.3.2. Mycotoxin extraction, clean-up, and analysis

AFB_1, AFB_2, AFG_1 and AFG_2: twelve agar culture plugs of biomass were cut out of the agar across the colony of *A. flavus* and *A. parasiticus* using a cork borer. These plugs were placed into a small glass beaker and weighed. AFs were extracted by adding 5 mL chloroform, capping, and shaking in orbital shaker (Infors-HT Aerotron, Bottminghen, Switzerland) for 1 hour. Four mL were filtered through a 0.5 g alumina cartridge using a vacuum elution manifold. The cartridge was washed with 2 mL chloroform, which were joined together with the previous filtrate. The solvent was evaporated to dryness under a slight stream of N_2 and the residue was suspended in 250 µL of water:methanol (80:20, v/v). After centrifugation for 10 min at 14000 rpm, 50 µL of the supernatant was injected into the LC-fluorescence detection (LC-FLD) system. Excitation and emission wavelengths were 362 nm and 435 nm, respectively.

OTA: toxin extraction and LC analysis was made according to Medina et al. (2006). Briefly, 2 g of culture was mixed with 200 µL of a 0.1 M solution of H_3PO_4, extracted with chloroform (3×3 mL) and filtered. After evaporation of the solvent, the residue was dissolved in 0.5 mL of acetonitrile:water:acetic acid, 49.5:49.5:1 (v/v/v) and analyzed using a LC-FLD system. Excitation and emission wavelengths were 330 and 460 nm, respectively.

FB_1 and FB_2: extraction and analysis was performed as described by Medina et al. (2006). Two g of ground culture was extracted with 10 mL acetonitrile:water (50:50,

v/v) and the extract was cleaned-up using Sep-Pak C18 cartridges. Derivatization of purified FBs was carried out with OPA. The FB OPA-derivatives were analyzed using a LC-FLD system. Excitation and emission wavelengths were 335 and 440 nm, respectively.

DON and 3AcDON: extraction and analysis were performed as described by Valle-Algarra et al. (2005). Two g of ground dried culture was extracted with acetonitrile:water (84:16, v/v). After filtering through Whatman No. 4 filter paper, the filtrate was cleaned-up by solid-phase extraction, derivatized with DMAP and PFPA and analyzed by GC-ECD using splitless injection.

ZEA: extraction and analysis was carried out following the methodology described by Medina et al. (2006) slightly modified. Two g of dried ground culture was extracted with methanol:1% aqueous NaCl (80:20, v/v) (2×10 mL) and cleaned-up using Sep-Pak Florisil cartridge. The final solution was analyzed by LC-FLD-DAD (arranged in tandem). For FLD, excitation and emission wavelengths were 274 nm and 440 nm, respectively.

T-2 and HT-2: toxin extraction and analysis was performed according to Mateo et al. (2013). Briefly, 2 g of ground dried culture was extracted with acetonitrile/water (84/16, v/v) in an orbital shaker (Infors-HT Aerotron, Bottminghen, Switzerland). The extract was filtered through Whatman No. 4 filter paper. Three mL of filtrate extract was passed through solid-phase extraction cartridge prepared as described by Valle-Algarra et al. (2005). The cartridge was washed with 2 mL of extraction solvent and pooled eluate was dried at 45 ºC, under a gentle stream of N_2, solved in 250 µL of pure water and injected into the LC-DAD system.

BEA and ENs: extraction and analysis was made following basically the methodology described by Sifou et al. (2011). Two g of dried ground culture was extracted with 15 mL of acetonitrile:water (85:15, v/v). The extract was centrifuged and, after evaporating the supernatant to dryness, the residue was solved in 2 mL of extraction solvent and analyzed by LC-DAD.

2.3.3. Chromatographic equipment

The LC system consisted of a Waters 600E system controller, a Waters 717 Plus autosampler, a Waters 474 scanning fluorescence detector and a Waters 996 diode array detector (Waters) set after the fluorescence detector to provide identity confirmation by comparison of UV spectra with that of the standard. Millennium32 software version

3.01.05 (Waters Co., Milford, MA, USA) was used to control the chromatograph and to process the signals. Separation was performed using a C18 Zorbax Eclipse Plus® (150 x 4.6 mm, 3.5 μm) (Agilent Technologies, Waldbronn, Germany), with a guard column of the same material. The GC system consisted of a HP-6890 Plus gas chromatograph, equipped with a ^{63}Ni ECD (Hewlett-Packard, Avondale, PA, USA) and an Agilent 7683 Series injector (Agilent Technologies). A fused-silica capillary column HP-5 (Agilent Technologies) was used.

2.4. Electrochemical measurements

Aqueous acetic acid/sodium acetate (HAc/NaAc) buffer solutions, concentration 0.25 M and pH 4.75, were used as supporting electrolytes for the electrochemical measurements. These were performed at 298±1 K in a CH cell using a laboratory CH I660 potentiostat (Cambria Scientific, Llwynhendy, Llanelli, Wales, UK). A BAS MF2012 glassy carbon working electrode (GCE, geometrical area 0.071 cm^2), a platinum wire auxiliary electrode and an Ag/AgCl (3M NaCl) reference electrode were used in a conventional three-electrode arrangement. Voltammetric measurements were carried out after transferring a ca. 0.1 mg amount of fungal sample on the surface of a GCE. Additional experiments were performed by means of the extraction method (Doménech-Carbó, Domínguez, Hernández-Muñoz & Gavara, 2015a). Here, *ca*. 1 mg of fungal sample was powdered with an agate mortar and pestle by adding 0.5 mL of ethanol for 2 min. Then, 50 μL of the resulting suspension were dropped onto the GCE surface. After solvent evaporation in air, the electrode was inserted into the electrochemical cell and electrochemical runs were performed. Linear potential scan (LSV), cyclic (CV) and square wave voltammetry (SWV) were used as the detection modes.

SECM experiments were performed with CH 920c equipment using a microdisk platinum electrode tip (CH 49, diameter 20 μm), a Pt substrate electrode (geometrical area 0.018 cm^2) and a Ag/AgCl reference electrode. The bipotentiostat mode was used to apply potentials to the tip (E_T) and the electrode substrate (E_S). The rate of scanning of the tip over the substrate was 20 μm/s for all experiments, the distance between tip and substrate being of the order of the tip electrode radius. A *ca*. 0.5 mg fungi fragment was fixed onto a carbon paste bed (50% w/w graphite powder plus nujol oil) forming a fine layer on a Pt substrate electrode. 2.0 mM $K_4Fe(CN)_6$ solution in 0.25 M HAc/NaAc aqueous solution at pH 4.75 was used as a redox probe.

2.5. Field emission scanning electron microscopy (FESEM) data

The microstructure of different fungal species was observed by using field emission scanning electron microscope (Model S-4800, Hitachi Ltd., Tokyo, Japan) operating at 20 kV. Samples were first fixed with Karnosky method 0.5% (v/v) glutaraldehyde and 2.5 % (v/v) paraformaldehyde in 50 mM phosphate buffer (pH 6.8) for 8-10 h at 4 °C, then rinsed with the same buffer for 3 h. Afterwards, samples were post-fixed with 1% (w/v) osmium tetroxide in the same buffer for 2 h at 4 °C. After dehydration for 24 h by a graded ethanol series with double steps of 10 min (30, 50, 70, 90 and 100) at room temperature, the samples were critical-point dried, mounted on stubs, and sputter-coated with palladium-gold.

3**. Results and discussion**

3.1. Mycotoxin production by the different isolates

Table 1 shows the mycotoxin levels found in the cultures *in vitro* of the isolates of the different species studied. All selected isolates were characterized as mid-to-high mycotoxin-producing fungi and one or more mycotoxins were detected in the different cultures.

3.2. General voltammetric pattern

Application of previously reported methodology for electrode conditioning, based on the formation of microparticulate films of ethanolic extracts of fungi produced well-defined voltammetric responses but exhibiting relatively high dispersion in the relative peak current values of the different signals (see Figure 1 in Mateo et al., submitted). Accordingly, the voltammetry of fungi microsamples directly attached to the GCE substrate was tested in contact with aqueous acetate buffer at pH 4.75. As can be seen in Fig. 1 for a) *A. carbonarius*, b) *A. niger* (An27g), c) *A. ochraceus* and d) *F. langshetiae*, upon scanning the potential from –0.85 V *vs* Ag/AgCl in the positive direction, a series of anodic peaks at potentials between –0.4 and +1.1 V were recorded. The voltammetric profiles exhibited significant differences between the different fungal species thus denoting that there is possibility of defining different voltammetric profiles for each of the studied species, as detailed below (see Figs. 2 and 3 in Mateo et al., submitted).

In all cases, repeatability tests (see Fig. 1a) performed on three independent measurements on GCE with renewed samples of the same fungi provided peak potential variations lower than ±20 mV and variations in the relative peak current for the different pairs of signals lower than 15%. It is pertinent to note that the amount of sample transferred to the electrode cannot be controlled accurately so that the absolute value of the peak currents can vary significantly from one modified electrode to another; accordingly, only current ratios are usable for defining shape-dependent parameters.

The observed voltammetry can mainly be attributed to the electrochemical oxidation of polyphenolic compounds existing in the fungi, as judged upon comparison with the voltammetry of different polyphenolic compounds (rutin, gallic acid, ellagic acid, quercetin, morin, maclurin, chlorogenic acid, among others) previously described (Doménech-Carbó et al., 2015a,b; Martini, De Carvalho, Blasco, & Doménech-Carbó, 2015; Ortiz-Miranda et al., 2016). Accordingly, the voltammetric peaks can correspond to the oxidation of several compounds containing similar functional units (for instance, *o*-catechol oxidation to *o*-quinones), so that the recorded voltammetric peaks correspond to multi-product signals. For this reason, and in order to compare the voltammograms of different species, the signals will be grouped in the following on the basis of peak potentials differing in values of at least between 50 and 100 mV (*vide infra*).

3.3. Electrochemical pathway

In the case of fungal samples directly attached onto the electrode surface, the observed voltammetric response can either be due to compounds released from the cellular content as the result of the moderately abrasive conditioning of the electrode, but also to possible transmembrane electrochemistry processes as described for several biological systems (Doménech-Carbó et al., 2016; Yu et al., 2014).

Among the variety of reported fungal secondary metabolites (Vishwanath, Sulyok, Labuda, Bicker & Krska, 2009), there is a variety of compounds containing electrochemically oxidizable functional groups. These include, among others, anthraquinonic and naphtapyrone compounds containing *o*- and *p*-diphenol units (fonsecinone A, nigerone). Other possible electroactive components are aspergillin, the green pigment of *A. niger*, and melanin, a polymeric substance constituted by dihydroxyindole units that is sparingly soluble in water. The electrochemical oxidation

of melanin can proceed via the phenolic and indolic motifs. The *o*-phenol units are oxidized to the corresponding *o*-quinones whereas the indolic unit yields the corresponding imine (a scheme was depicted in Mateo et al. submitted, Fig. 4). Given the variety of existing electroactive compounds and their presence in variable amounts depending on the fungal species, the voltammetric response of fungal samples would consist, in agreement with experimental data, on a series of overlapping peaks whose relative intensity will be sensitive to the relative amounts of the different electroactive species. Accordingly, the obtained voltammograms define an 'electrochemolomic profile' characterizing the vegetal species (Ortiz-Miranda et al., 2016).

Both the inner and/or outer layers of the cell wall contain melanin, a polyfunctional pigment, which contributes to the ability of fungi to survive in harsh environments (Eisenman & Casadevall, 2012). The melanin coating of the spores contains ionizable groups and influences the zeta-potential of the spores (Grimm, Kelly, Völkerding, Krull, & Hempel, 2005). Studies on *A. niger* have shown that the surface coating on top of the spore wall plays a crucial part in the electrostatic nature of the spores and that deprotonation of melanin-bound carboxyl groups is most probably responsible for pigment release under acidic conditions (Wargenau et al., 2011).

In order to test the possibility of spore wall permeation during electrochemical turnovers, SECM experiments on microsamples of fungi fixed onto a carbon paste substrate bed in contact with 2.0 mM $K_4Fe(CN)_6$ plus 0.25 M HAc/NaAc solution at pH 4.75 were carried out. SECM images were recorded by fixing the tip potential (E_T) at a value (+0.45 V) where $Fe(CN)_6^{4-}$ oxidation takes place under diffusion-controlled conditions, as ensured by voltammograms previously performed in the solution. When no potential was applied to the substrate (Fig. 2a,c), the exposed regions of the base electrode display a positive feedback response whereas the regions covered by the fungi body produce negative feedback, consistently with its insulating nature. Under these conditions, the tip current essentially reflects the topography of the fungal body, which appears as a series of irregular crusts accompanied by a flat region corresponding to the surrounding carbon paste bed. When a potential sufficiently positive (+0.80 V) to promote the oxidation of the fungal compounds was applied to the substrate (Fig. 2b,d), both the color map and the topographic image show significant changes, those being particularly abrupt in the boundary region between the fungal body, the base electrode and the electrolyte. These features can be interpreted in terms of the existence of

localized regions where the cellular damage facilitates the release of electroactive compounds without discarding the possibility of a certain degree of transmembrane electrochemistry.

FESEM examination of spores during electrochemical turnovers was consistent with the above considerations. Fig. 2e,f depicts FESEM images of spores of *A. flavus* e) before and f) after application of an oxidative potential input of +0.80 V during 5 min to fungal samples attached to graphite electrodes in contact with aqueous acetate buffer. In both species, the conidia were globular or subglobular, with delicately spiny ornamentation. After application of a potential sufficiently high to promote electrochemical oxidation processes (+0.80 V) such as in Fig. 1 subtle but significant morphological changes were detected in the FESEM images of the conidia. In the case of *A. flavus*, the warty ornamentation was retained or lightly smoothed whereas the size was apparently reduced and the shape moved to rounded cuboid. Such changes can be interpreted in terms of the weakening of the spore wall architecture favored by the deprotonation of melanin-bound carboxyl groups being accompanied by the release of inner components. In the case of *A. carbonarius* (see Mateo et al., submitted, Fig. 5), the spiny ornamentation of the parent conidia was considerably smoothed after oxidative potential inputs and the size was clearly decreased. Possibly, there is a loss of external wall regions associated to the electrochemical runs. For our purposes, the relevant point to emphasize is that both SECM and FESEM examination of conidia after oxidative stages supported the idea that there is a localized electrochemical response associated to voltammetric experiments at fungi-modified electrodes with involvement of conidia wall permeation and release of electroactive components.

3.4. Species discrimination

As showed in Fig. 1, where the SWVs of microsamples of the species *A. carbonarius*, *A. niger* (An27g), *A. ochraceus* and *F. langsethiae* were depicted, the voltammetric profiles differed significantly from one species to another. In order to characterize the different types of fungi, two successive approaches were used. The first classification scheme was based on the appearance or not of well-defined voltammetric peaks at selected potentials. Fig. 3 depicts a diagram summarizing the voltammetric peaks appearing at well-defined intervals of potentials (minimal separation of 100 mV) for fungal isolates in this study. One can see that there is a clear discrimination between

two genera, *Fusarium*, characterized by the presence of a signal at +0.90 V under our experimental conditions, and *Aspergillus*, where this signal is absent. Within this last genus, the sections *Flavi* and *Circumdati* were characterized by the common presence of a signal at –0.60 V, absent in the species of the section *Nigri*. In turn, sections *Flavi* and *Circumdati* can be discriminated based on the presence in the former of a signal at +0.50 V.

Using the same criterion of presence/absence of voltammetric peaks, *Fusarium* species of the different studied sections were characterized using voltammetric signals at +0.6 V, –0.1 and +0.4 V. Fig. 4 shows a cascade scheme (Doménech-Carbó, Doménech-Carbó, Calisti, & Maiolo, 2010) for the sequential discrimination between the studied fungi at the level of genera and sections. By using the presence/absence of voltammetric peaks criterion, it is possible to discern, in several cases, between different species within the same section. For instance, *A. steynii* yields a main oxidation signal composed by overlapping peaks at +0.80 and +1.0 V preceding weak anodic waves at +0.2, 0.0, –0.1, –0.4 and –0.6 V, whereas *A. ochraceus* and *A. westerdijkiae* provide a staircase-type profile, with peaks at 0.0, +0.2, +0.6, +0.7 and +1.0 V accompanied by an ill-defined shoulder near to –0.6 V.

In order to refine electrochemical discrimination at the species level, a second approach, based on pattern recognition strategies, can be used. In this approach, normalized voltammograms, obtained as the ratio between the current at a given potential, $I(E)$, and the maximum current in the voltammogram, $I(\max)$, as described in the literature (Doménech-Carbó et al., 2015a; b) were used. Representative examples of normalized voltammograms are shown in Fig. 6 in Mateo et al., submitted.

This can be seen in Fig. 5a, where the two-dimensional diagram plotting $I(-450)/I(\max)$ vs. $I(+700)/I(\max)$ averaged ratios for *Aspergillus* species is shown. One can see that, accounting the standard deviations of the current ratios from replicate experiments, the species of the different sections (*Flavi*, *Nigri* and *Circumdati*) fall in separated regions of the diagram and that, with the exception of *A. ochraceus* and *A. westerdijkiae*, each species is placed in a region clearly separated from the other species of the same section. Similar results were obtained for *Fusarium* species. Fig. 5b shows the representation of $I(-450)I(+900)$ vs. $I(+400)/I(+900)$ were one can see that again the different sections (*Liseola*, *Sporotrichiella*, *Elegans* and *Discolor*) are clearly separated and that the different species can also be discriminated.

Consistently, in cases where two isolates of the same species, were assayed (*A. niger*, *A. tubingensis* and *A. steynii*), the corresponding representative points in the diagram were closely located. Only in the case of *A. steynii*, the two studied isolates were distinguishable in the diagram, thus suggesting that, in favorable cases, an electrochemical access to the study of strain variability within the same species might be possible.

The obtained electrochemical data were used, taking the normalized voltammograms (I (E)/I (max) data), for determining similarity relationships by means of hierarchical cluster analysis, as already described (Doménech-Carbó et al., 2015a; b) It is pertinent to note that taxonomy of *Aspergillus* species has changed regularly in time. Since 1985 (Gams et al., 1985) the groups have had status in the taxonomic hierarchy as sections, but the resolution of species and sections is often problematic (Rokas & Carroll, 2005). Voltammetric data provided a dendrogram reflecting the similarity of the normalized SWVs (in terms of $I(E)/I$(max) *vs* applied potential data) of the studied *Aspergillus* species in close agreement with the phylogenetic tree (see Fig. 7 in Mateo et al., submitted) derived from DNA sequences determined for beta tubulin (BT2), calmodulin (CF), ITS and lsu rDNA (ID) and RNA polymerase II (RPB2) in *Aspergillu*s isolates (Peterson, 2008), thus illustrating the capabilities of the voltammetric methodology to yield information able to be correlated with phylogenetic data.

4. Conclusions

Upon attachment to glassy carbon electrodes, colony deposits of *Aspergillus* spp. and *Fusarium* spp. isolated from grapes and cereals provided well-defined voltammetric responses in contact with aqueous acetate buffer at pH 4.75. This voltammetry was dominated by anodic processes associated to the oxidation of metabolites containing phenolic and indolic units. SECM and FESEM examination of colony deposits before and after application of oxidative potential inputs evidenced the appearance of subtle but significant textural and morphological variations in the conidia, which were consistent with the assumption that weakening of the spore wall architecture and release of electroactive components occurred possibly accompanied by some transmembrane electrochemistry.

The obtained voltammetric profiles defined an electrochemical fingerprint able to be used for the rapid characterization of toxigenic fungal species from different sections of the above-mentioned genera. Peak potential data permitted to discriminate unambiguously between the genera *Aspergillus* and *Fusarium*, and between the different sections within each one of these genera. A second level of discrimination, between different species can be obtained from current measurements at different potentials. Application of hierarchical cluster analysis to voltammetric data provided an electrochemical tree for *Aspergillus* species that was entirely consistent with the corresponding phylogenetic tree, thus illustrating the possibilities of electrochemical methods in the field of food mycology.

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Declaration of interests

The authors declare no conflict of interests with other people or organizations.

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611

Figure captions

Fig. 1. SWVs of: a) *A. carbonarius* (three replicate experiments are superimposed), b) *A. niger* (An27g), c) *A. ochraceus* and d) *F. langsethiae* microsamples attached to GCE in contact with 0.25 M HAc/NaAc aqueous solution at pH 4.75. Potential scan initiated at –0.85 V in the positive direction; potential step increment 4 mV; square wave amplitude 25 mV; frequency 5 Hz. Criteria for current measurements are depicted in c).

Fig. 2. SECM a,b) map colors and c,d) topographic images of a *A. carbonarius* microsample colony deposited onto a carbon paste substrate immersed into 2.0 mM $K_4Fe(CN)_6$ plus 0.25 M HAc/NaAc, pH 4.75. E_T = +0.45 V; a,c) E_S = 0.00 V; b,d) E_S = +0.80 V. FESEM images of a spore of *A. flavus* e) before and f) after application of an oxidative potential input of +0.80 V during 5 min to fungal samples attached to graphite electrodes in contact with 0.25 M HAc/NaAc aqueous buffer at pH 4.75.

Fig. 3. Voltammetric peaks appearing at well-defined intervals of potentials (minimal separation of 100 mV) in SWVs of microsamples of fungal colonies of the assayed species. Conditions such as in Fig. 1.

Fig. 4. Cascade scheme for the electrochemical discrimination of fungi of different sections of *Fusarium* spp. and *Aspergillus* spp. based on the presence/absence of voltammetric peaks in data such as in Fig. 1.

Fig. 5. Two-dimensional diagram from current ratios at selected pairs of potentials recorded in square wave voltammograms such as in Fig. 1. a) Plots of I(–450)/I(max) vs. I(+700)/I(max) for *Aspergillus* species; b) plots of I(–450)I(+900) vs. (I+400)/I(+900) for *Fusarium* species. Error bars correspond to extreme values determined in three replicate experiments for each specimen.