Vol.52, Special Number: pp.1-9, November 2009
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

An Overview on Molecular Markers for Detection of Ochratoxigenic Fungi in Coffee Beans

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ABSTRACT

Ochratoxin A (OTA) is one of the most abundant food-contaminating mycotoxins. Its occurrence in several agricultural commodities has been considered a worldwide problem. This toxin is mainly produced by Aspergillus species. OTA has nephrotoxic, immunosuppressive, and carcinogenic effects and consequently the contamination with this toxin represents a high risk for human and animal health. In the last 5 years, several investigators have applied molecular methods in order to develop PCR assays for identifying and quantifying OTA-producing fungi in coffee beans samples. The main objective is to allow the detection of microorganisms capable of producing OTA, preferentially prior to ochratoxin production and accumulation. In this contribution several of these attempts will be reviewed and discussed.

Key words: ochratoxigenic fungi, molecular marker, PCR detection, molecular identification

INTRODUCTION

Mycotoxins are small organic molecules with grat diversity in chemical structure and biological activity. They are toxic compounds, occasionally very hazardous to animals and human. The main source of mycotoxins is plant food, which are contaminated during harvesting, transport, storage, and manufacture, or even in the field (Smith and Henderson, 1991). Their influences on human and animal health range from neurotoxic, teratogenic, immunosuppressive, nephrotoxic, hepatotoxic to carcinogenic effects (Geisen, 1998). About 20 different mycotoxins are significant to human health (Geisen, 1998; Bennett and Klich, 2003). Demand still exists for rapid and reliable techniques to detect mycotoxins and mycotoxin-producers (Russell and Paterson, 2006).

Ochratoxin A (OTA) is a mycotoxin that is receiving increasing attention worldwide because of its severe nephrotoxicity. Moreover, the International Agency for Research on Cancer has classified OTA as a Group B2, i.e. the metabolite is a probable human carcinogen. As reviewed by Petzinger and Weidenbach (2002), OTA-contaminated foods are abundantly found. Examples are grains, coffee beans, spices, nuts, grapes, and figs (Bayman et al., 2002; Jorgensen and Jacobsen, 2002; Battilani et al., 2003; Taniwaki et al., 2003). OTA is not totally decomposed during most food processing stages such as cooking, washing, and fermenting. Because OTA is not totally decomposed during

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most processing stages it has been also detected in manufactured food products, such as bread, beers, wine, coffee, and chocolate (Jorgensen et al., 1998; Visconti et al., 2001; (Fujii et al., 2007). Based on its possible effects on animal and human health, most countries have imposed statutory limits for OTA in some foods. For both roasted coffee beans and ground coffee beans the maximum tolerable level of OTA in the European Community is 5μg kg^{-1} (Official Journal of The European Union, 2005). Until 2004, the major fungal source of OTA in coffee was *Aspergillus ochraceus* and *A. carbonarius*, with minor contribution from *A. niger*. After advances in molecular and fungal metabolite techniques, new species were described and distinguished in the section *Circumdati* and *Nigri*. Nowadays, *A. westerdijkiae* is being recognized as the main OTA producer especially in arabica coffee, while *A. carbonarius* occurs most frequently in robusta coffee (Taniwaki et al., 2008).

The severe consequences of OTA contamination argue for methodologies that can be efficiently use to detect OTA producers in food. In this review some of the relevant molecular approaches, which have been carried out in order to detect and quantify the main OTA producers in coffee beans will be present.

Molecular Detection of Ochratoxigenic Fungi

The conventional scheme of isolation and identification of ochratoxigenic fungi from food samples are time-consuming and require high knowledge on fungal taxonomy. Even with taxonomical expertise, identification is commonly difficult regarding some fungi genus that contains a large number of closely related species. The application of molecular biology techniques can help to overcome these problems, because it can reduce the time for from several days to some hours and it allows precise species identification.

The polymerase chain reaction (PCR) assay has allowed precise identification and fast detection of ochratoxigenic species without the need for isolating pure cultures.

In contrast to others mycotoxin, the OTA biosynthetic pathway was not well characterized in any of the OTA-producing species and consequently the genes that encode enzymes involved in the biosynthesis of this secondary metabolite are poor known. Because of this, several PCR-based assays developed during the last five years have been used genes which were not associated to mycotoxin biosynthesis, as those based on ribosomal RNA, β-tubulin, and calmodulin genes (Perrone et al., 2004; Patiño et al., 2005; Morello et al., 2007) or have been based on anonymous DNA sequences. Anonymous DNA sequences are obtained from an unbiased sampling of genomic DNA and these may or may not contain functional genes. Developing markers from anonymous sequences requires comparative analyses among related species of DNA profiles generated from randomly amplified fragments by using RAPD (Random Amplified Polymorphic DNA) or AFLP (Amplified Fragment Length Polymorphism) (Williams et al., 1990; Vos et al., 1995).

Ochratoxin A was discovered as a secondary metabolite of *A. ochraceus* strains, which belongs to *Aspergillus* section *Circumdati*. Until recently, this species was considered the main fungal source of OTA in coffee. However, based on polyphasic taxonomy, Frisvad et al. (2004) proposed the division of formal *A. ochraceus* species into two species, *A. ochraceus* and *A. westerdijkiae*. These species are very similar and several isolates previously identified as *A. ochraceus* are now recognized as *A. westerdijkiae*, including the original OTA-producing strain (NRRL 3174). The first report of a diagnostic PCR assay for ochratoxigenic fungi dates from 2003 (Schmidt et al., 2003). The authors investigated the genetic relatedness among 70 *A. ochraceus* strains by using AFLP markers. A certain number of AFLP bands distinctive for *A. ochraceus* were detected. Three of these bands were cloned and sequenced and after that the sequences were used to design three primer-pairs specific for *A. ochraceus*. The specificity of the primer-pair OCA-V/OCA-R was tested with DNA of several different target strains as well as closely related *Aspergillus* and *Penicillium* spp. and DNA isolated from non-infected green coffee. Currently we know that this primer-pair is able to amplify DNA sequence from *A. ochraceus* and *A. westerdijkiae*, because it was developed previous to the division of formal *A. ochraceus* species into the two species above mentioned. Research in our group found that *A. westerdijkiae* is the main OTA producing species found in Brazilian coffee beans (*Coffea arabica*) (Fungaro et al., 2004b; Morello et al., 2007). Amplification and sequencing of the ITS1-5.8S-ITS2 region from several Brazilian strains of both species showed specific nucleotide variations (ITS

and β-tubulin genes) characterizing *A. westerdijkiae* and *A. ochraceus* (Fungaro et al., 2004a; Fungaro et al., 2004b; Morello et al., 2007). The nucleotide variations found between the β-tubulin gene sequences obtained from *A. ochraceus* and *A. westerdijkiae* were exploited by Morello et al. (2007) for designing species-specific primers for *A. westerdijkiae* detection in coffee beans (Bt2Aw-F/Bt2Aw-R). By using this primer-pair a 347 bp-amplicon was visualized in all *A. westerdijkiae* isolates but no PCR assay product was observed from *A. ochraceus* isolates. The Bt2Aw primers were successfully applied for detecting the 347 bp-amplicon when using DNA collected from coffee beans inoculated with *A. westerdijkiae.*

Although conventional PCR assay is a valuable tool for detection and monitoring mycotoxigenic fungi, it is not appropriate to quantify a given fungus species in a food sample. Small difference in reaction efficiency per cycle can result in a substantial difference in the final product quantity and so the extrapolation to the initial concentration of the template in the sample is very difficult to do (Hill, 1996). Fortunately, the introduction of the Real-Time PCR technology has increased the reliability of PCR results compared to those obtained by conventional methods, thus opening new avenues for quantifying ochratoxigenic fungi in food. Real-Time PCR is more sensitive than classical PCR and does not require gel electrophoresis. The analysis can be concluded with less than 5h. This attribute of Real-Time PCR significantly reduces time and manual labor, making it appropriate for large-scale analyses. The use of fluorophores is common to most of these methods and is described in detail by Boysen et al. (2000). By using Real-Time PCR it is possible to detect an increase in fluorescence emission during the reaction that is proportional to the initial copy number of the target sequence. The initial amount of template DNA is inversely proportional to a parameter measured for each reaction, which is denoted the threshold cycle (C_t). The C_t value is the PCR cycle when the fluorescence signal increases above the background threshold. Application of this method to natural samples provided an estimate of infection by a give species. Morello et al. (2007) evaluated the potential of the Real-Time PCR approach for quantification of this species in coffee beans. Green coffee beans were inoculated with 10^6 *A. westerdijkiae* conidia and incubated for 192 h. DNA extraction and colony-forming unit (cfu) assay were performed each 48h. It was observed high correlation between the cfu data and the fungal DNA content in coffee beans. The authors also assessed the sensitivity of this method in order to detect *A. westerdijkiae* in coffee beans. According to their data positive signal may be obtained with less than 10 copy number of *A. westerdijkiae* haploid genome. This value also means less than 10 haploid genomes per 0.1 g of coffee beans. Real-Time PCR sensitivity level was more than 100 times higher than the cfu technique.

Patiño et al. (2005) developed specific PCR assay for detection of *A. ochraceus* species based on ITS sequences. The primer-pair denoted OCRA1/OCRA2 was designed on the basis of ITS sequence comparison of several strains of *Aspergillus* species. The specificity of the primer-pair was tested on a number of *Aspergillus*, *Penicillium*, *Cladosporium*, *Botrytis*, and *Alternaria* strains commonly associated with grapes, cereals, and coffee. A single fragment of about 400 bp was only amplified when genomic DNA from *A. ochraceus* strains was used. No product was observed with genomic DNA from the *Aspergillus* isolates other than *A. ochraceus* nor in the case of other genera. According to the authors the sensitivity of PCR assay based on ITS sequences was higher (1 and 10 pg of DNA template per reaction) than primers based on single copy gene (0.1 and 1 ng of DNA template per reaction). The authors do not mentioned about the new species *A. westerdijkiae*. Probably the primer-pair proposed does not distinguish between *A. westerdijkiae* and *A. ochraceus.*

The ochratoxigenic species *A. carbonarius* and *A. niger* belong to section *Nigri*, which is an important group of species in food mycology. As commented by Samson et al. (2007), black aspergilli are one of the more complex groups concerning their classification and identification, and numerous taxonomic schemes have been proposed. The differences between some species belonging to section *Nigri* are very slight and their identifications require molecular analysis.

Specific PCR assay for detection of *A. carbonarius* species was developed by Patiño et al. (2005) based on ITS sequences. The primer-pair denoted CAR1/CAR2 was able to provide an amplicon of 420 bp only when genomic DNA from *A. carbonarius* strains was used. Schmidt et al. (2004) used AFLP to detect specific markers

for *A. carbonarius*. A certain number of amplified fragments were found to be specific to this species. The marker fragments were cloned and sequenced and used to design a primer-pair specific to detect this species. The primer-pairs A1B-fw/A1B-rv and C1B-fw/C1B-rv provided amplicons of 189 bp and 351 bp, respectively, in all *A. carbonarius* isolates tested. Based on alignment of calmodulin (*cmdA*) gene sequences, Perrone et al. (2004) identified regions suitable for design specific PCR primers for the detection of *A. carbonarius* strains. The primer-pair (CARBO1/2) produced a PCR product of 371 bp and the sensitivity was about 12 pg when using pure total genomic DNA. Although the PCR assay was useful in screening isolates of black aspergilli from grapes, the authors did not use it to detect *A. carbonarius* strains directly from grape samples.

Several strains representing closely related black aspergilli, i.e. *A. carbonarius*, *A. niger*, *A. foetidus*, and *A. tubingensis* were analyzed by RAPD with the aim of developing species-specific primers for the detection of *A. carbonarius* in coffee beans (Fungaro et al., 2004a). An example of a typical RAPD pattern is given in Figure 1. Some DNA bands were present in all *A. carbonarius* strains and absent in all strains of *A. niger* and *A. tubingensis*. One of these bands was cloned and sequenced, and then used to design a primer-pair specific to *A. carbonarius* ($OPX7_{809}$-F/$OPX7_{809}$-R). By using this primer-pair the authors successfully detected an amplicon of 809 bp when DNA from coffee beans infected with *A. carbonarius* strains was used. No cross-reaction was observed using DNA from coffee beans infected with closed related black aspergilli. Similarly, based on RAPD markers, Sartori et al. (2006) developed specific primers for *A. niger* detection. The primer-pair denoted $OPX7_{372F}$/ $OPX7_{372R}$ provided an amplicon of 372 pb in all *A. niger* stricto sensu isolates, and no amplification product was observed in reactions using DNA from related species. This PCR assay was successfully applied for detecting *A. niger* in coffee beans.

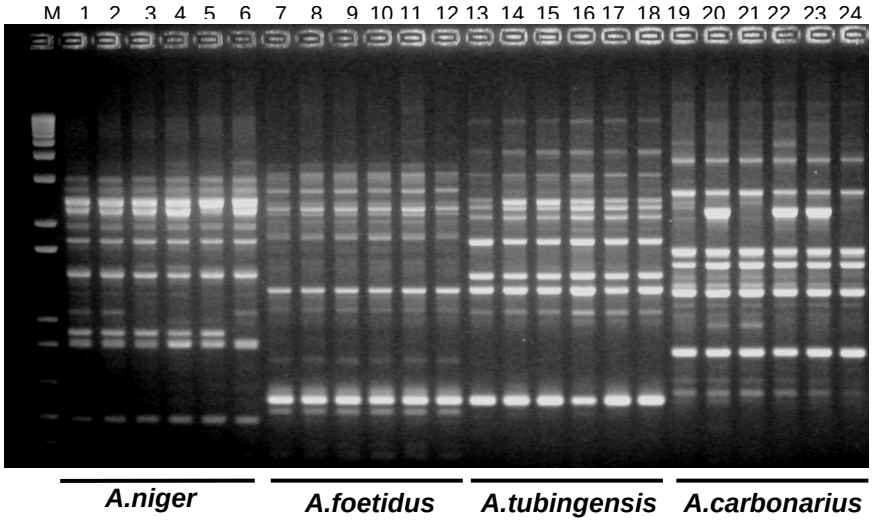

Figure 1 - Amplification of polymorphic DNA from *Aspergillus niger* strains (lanes 1-6), *A. foetidus* strains (lanes 7-12), *A.tubingensis* strains (lanes 13-18) and *A. carbonarius* strains (lanes 19-24), using the OPX7 random primer. The molecular weight standard (lane M) is a 1Kb plus DNA ladder (Invitrogen Life Technologies).

Because *A. ochraceus* (now *A. westerdijkiae*), *A. carbonarius* and *A. niger* are the major species for colonizing Brazilian coffee beans and producing OTA our research group developed a multiplex PCR assay (m-PCR) useful to detect the three target fungi species directly from sample of this commodity (Sartori et al., 2006). The m-PCR is a procedure that allows the simultaneous amplification of more than one target sequence in a single PCR reaction, decreasing the number of

reaction to be performed to assess the possible presence of different species in a food sample. The authors firstly analyzed the value of the m-PCR assay with DNA obtained from coffee beans inoculated with these three species. The Figure 2, shows the amplification profiles using simultaneously the primer-pairs designed for *A. ochraceus*, *A. carbonarius*, and *A. niger*. Amplification products of 260 bp, 809 bp and 372 bp, in a single PCR reaction confirmed the presence of *A. ochraceus*, *A. carbonarius*, and *A. niger*, respectively. The usefulness of the m-PCR assay was also analyzed using coffee bean samples collected in farm.

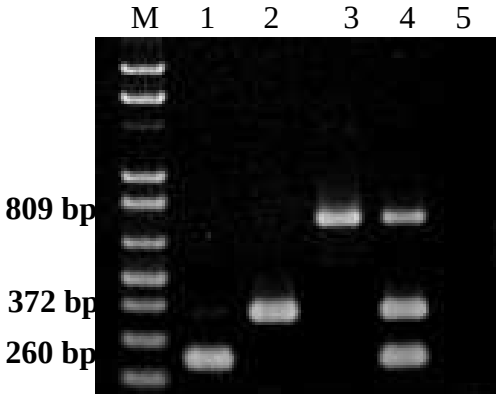

Figure 2 - Amplification products obtained by using DNA from inoculated coffee beans. Lane 1: DNA from coffee beans inoculated with *Aspergillus ochraceus* by using OCA V and OCA R primers; lane 2: DNA from coffee beans inoculated with *A. niger* by using $OPX7F_{372}$ and $OPX7R_{372}$ primers; lane 3: DNA from coffee beans inoculated with *A. carbonarius* by using $OPX7F_{809}$ and $OPX7R_{809}$ primers; lane 4: multiplex PCR using DNA from coffee beans inoculated with *A. ochraceus*, *A. niger*, and *A. carbonarius* and the 3 sets of primer pairs; lane 5: negative control (DNA from coffee beans without fungal inoculation). The molecular weight standard (lane M) is a 1Kb DNA ladder (Invitrogen Life Technologies).

As above mentioned, sequences of functional genes may also be used as target for designing PCR primers to detect mycotoxigenic fungi. However, in contrast to others mycotoxin, the OTA biosynthetic pathway was not well characterized in any of the OTA-producing species and consequently the genes that encode enzymes involved in the biosynthesis of this secondary metabolite are poor known. Based on OTA structure the necessity of various enzymes catalyzing key reactions in the formation of OTA can be expected and some teams of researchers are looking for the genes related to OTA biosynthesis (Lebrihi et al., 2003; Geisen et al., 2004; Atoui et al., 2006; O'Callaghan et al., 2006; Bogs et al., 2006). A polyketide synthase can be postulated as involved in OTA biosynthesis, because isocoumarin group of the OTA is probably a pentaketide formed from acetate and malonate via a polyketide synthesis pathway (O'Callaghan et al., 2003). The diversity of polyketide synthase genes has been investigated (Atoui et al., 2006) in *A. carbonarius*. Two non conserved sequences in the acyltransferase domain of a polyketide synthase gene, denoted *Ac12RL3*, were used as a target sequence to detect specifically *A. carbonarius* by PCR assay. The primer-pair, Ac12RL_OTAF/ Ac12RL_OTAR generated a 141 bp PCR product in all *A. carbonarius* isolates studied and none of the other species gave a positive result with this PCR primer set (Atoui et al., 2007). This specific primer-pair was successfully employed for the direct quantification of *A. carbonarius* in grape samples but not in coffee beans. With the same objective, i.e. to quantify *A. carbonarius* in grape samples, Atoui et al. (2006) used a specific primer-pair (Ac12RL_OTAF/Ac12RL_OTAR) that was designed from the acyltransferase (AT) domain of the polyketide synthase sequence (Ac12RL3) to

amplify 141 bp PCR products. By using Real-Time PCR conjugated with SYBR® Green I dye, the authors found a positive correlation (r^2=0.81) between *A. carbonarius* DNA content and OTA concentration in 72 grape samples. Table 1 summarizes the primer-pairs sequences designed for detection OTA-producing fungi in coffee beans.

Table 1 - Primer sequences used for detection of ochratoxin-A producing fungi in coffee beans.

Species	Utility	Target region	Primer pair	Reference
A. ochraceus	Species-specific detection	AFLP Marker	F5' ATACCACCGGGTCTAATGCA R5' TGCCGACAGACCGAGTGGATT	Schmidt et al., 2003
A. ochraceus	Species-specific detection	rRNA gene	F5' CTTCCTTAGGGGTGGCACAGC R5' GTTGCTTTTCAGCGTCGGCC	Patiño et al., 2005
A. westerdijkiae	Species-specific detection and quantification	β-tubulin gene	F5' TGATACCTTGGCGCTTGTGACG R5' CGGAAGCCTAAAAAATGAAGAG	Morello et al., 2007.
A. niger	Species-specific detection	RAPD marker	F5' CAGTCGTCCAGTACCCTAAC R5' GAGCGAGGCTGATCTAAGTG	Sartori et al., 2006.
A. carbonarius	Species-specific detection	rRNA gene	F5' GCATCTCTGCCCCTCGG R5' GGTTGGAGTTGTCGGCAG	Patiño et al., 2005
A. carbonarius	Species-specific detection	AFLP marker	F5' GAATTCACCACACATCATAGC R5' TTA ACTAGGATTTGGCATTGA AC	Schmidt et al., 2004
A. carbonarius	Specie-specific detection and quantification	*pks* gene	F5' AATATATCGACTATCTGGACGAGCG R5' CCCTCTAGCGTCTCCCGAAG	Atoui et al., 2007
A. carbonarius	Species-specific detection	RAPD marker	F5' AGGCTAATGTTGATAACGGATGAT R5' GCTGTCAGTATTGGACCTTAGAG	Fungaro et al., 2004a
A. carbonarius	Species-specific detection	*cmdA* gene	F5'AAGCGAATCGATAGTCCACAAGAATAC R5' TCTGGCAGAAGTTAATATCCGGTT	Perrone et al., 2004

Several analytical methods for the detection of OTA exist and the level of this mycotoxin can readily be measured very accurately in food, but this kind of analysis only returns a positive result once the toxins have been formed. Similarly several methods for the detection of ochratoxigenic species have been described, but the presence of an ochratoxigenic fungal species in a sample does not ultimately mean that the toxin itself is present. As occurs with other mycotoxins, the substrate on which the fungus grows, as well as the moisture content and temperature can determine if the mycotoxin will be produced. Moreover, there are great differences between strains of an ochratoxigenic species concerning their ability for toxin production. We have previously shown that 30% of *A. westerdijkiae* strains isolated from coffee beans are unable to produce detectable amounts of OTA (Morello et al., 2007). Based on these points, more important for a meaningful monitoring of OTA in food would be the measurement of the rate of expression of mycotoxin genes, because these genes are frequently expressed some days prior to the mycotoxins production and thus would allow an early warning (Schmidt-Heydt and Geisen, 2007).

The first relevant report of the cloning and characterization of putative polyketide synthase gene (*pks*) from *Aspergillus* involved in OTA biosynthesis was done by O'Callaghan et al. (2003). The authors used a molecular strategy denoted suppression subtractive hybridization PCR-based and discovered that this *pks* gene is expressed only under OTA permissive conditions and only during the early stages of the mycotoxin synthesis. A mutant in which the *pks* gene has been interrupted was not able to synthesize OTA. Afterward the authors examined OTA production by *A. ochraceus* grown under different nutritional and environmental conditions. Quantifications of transcript accumulation of the *pks* gene showed that *pks* transcription is tightly linked to OTA production (O'Callaghan et al., 2006). However it is important to state that although this gene is really more expressed by a positive strain under OTA permissive conditions, no information was available about the expression of this gene by OTA-non-producing strains.

Preliminary investigation carried out by our group

showed that the *pks* gene, described by O'Callaghan et al. (2003), is in fact significantly more expressed by *A. westerdijkiae* when grown in permissive conditions to the production of OTA than when grown in restrictive conditions to the production of this toxin. However, when a negative strain is cultivated in permissive conditions to the production of OTA, the *pks* gene is similarly express as the positive strain; though there is no OTA production (not published data). This fact is probably because other secondary metabolites can share this *pks* gene. This observation means that elucidation of differentially expressed genes between OTA-producing strains and OTA-non-producing strains is still required.

Representational Difference Analysis (RDA), as firstly described by Lisitsyn et al. (1993) is a powerful differential hybridization method to identify unique DNA sequences out of two complex and highly related genomes. With equal success, this approach was adapted by Hubank and Schatz, (1994) aiming at the identification of specific genes differentially expressed (cDNA RDA), and it has been employed for expression profiling in fungi species. We are using RDA approach in order to detect differences in gene expression between *A. westerdijkiae*

OTA-producing and non-producing strains grown under permissive conditions for OTA production. Until now, we identified two genes encoding oxidoreductases (P450-AL and OXI-1) that are markedly differentially expressed between OTA producing and non-producing strains of *A. westerdijkiae* (unpublished data). Comparative proteomics analysis are also in progress in our laboratory in order to detect differences in protein expression between *A. westerdijkiae* OTA-producing and non-producing strains grown under permissive conditions for OTA production. Some successful was already obtained as illustrated in Figure 3.

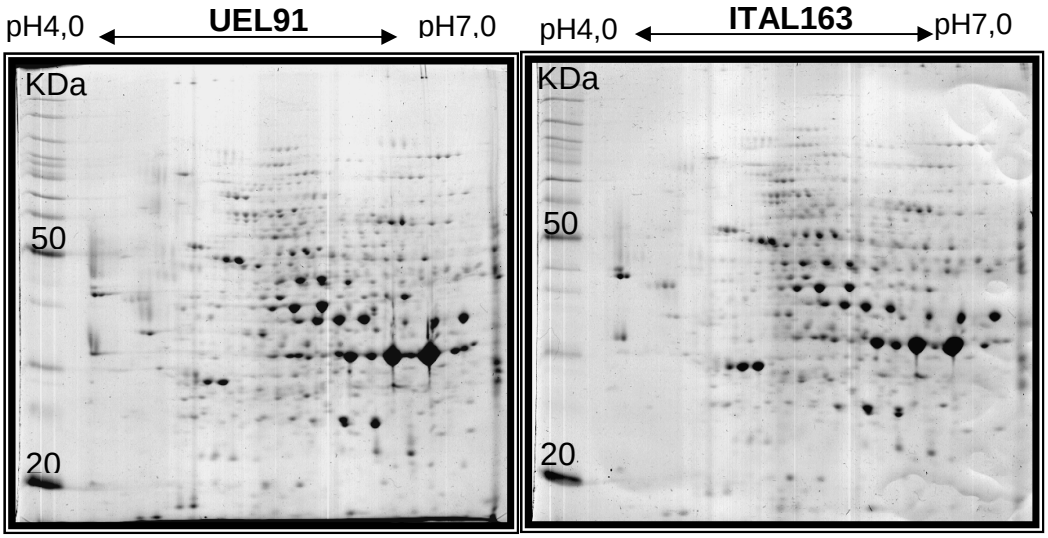

Figure 3 - Comparative proteomics analysis between *Aspergillus westerdijkiae* OTA-producing (UEL91) and non-producing (ITAL 163) strains grown under permissive conditions for OTA production.

Finally, our group has been engaged in an effort to find genome differences that could be useful for distinguishing toxigenic from non-toxigenic *A. niger* strains. Very recently we described that one *pks* gene is specific to OTA-producer strains. As this species is frequently applied for several industrial purposes and it is often found in a number of agricultural products, we are supposing that this *pks* gene will be very useful as a molecular marker for discriminating *A. niger* strains concerning their ability to produce OTA (unpublished data). This approach will guarantee that products of fungal fermentation or maturation are ochratoxin-free.

Concluding, over the last 5 years, several molecular assays for identification and fast

detection of ochratoxigenic species without the need of isolating pure cultures were published. These assays include conventional PCR, multiplex PCR, Real Time PCR and RT-Real Time PCR.
We are optimistic that in the near future the molecular technologies will be useful as a preventive approach to minimize the entry of ochratoxin into the food chain.

ACKNOWLEDGMENTS

Our study was supported (grants and fellowships) by the following Brazilian institutions: Fundação Araucária, Conselho Nacional de Pesquisa (CNPq), Financiadora de Estudos e Projetos (FINEP), Coordenadoria de Aperfeiçoamento de Pessoal do Ensino Superior (CAPES), Rede Integrada de Biotecnologia Aplicada ao Desenvolvimento Sustentável da Agricultura Familiar no contexto do Agro-negócio Paranaense (BIOAGROPAR) and Laboratório de Apoio à Pesquisa Agropecuária (LAPA).

RESUMO

Dentre as micotoxinas que contaminam produtos destinados à alimentação humana e animal, a ocratoxina A (OTA) é uma das mais freqüentemente encontrada. A sua ocorrência em vários produtos agrícolas tem sido considerada um problema de amplitude mundial. Esta toxina é produzida principalmente por fungos do gênero Aspergillus. A OTA tem efeitos nefrotóxico, imunossupressor e carcinogênico. A contaminação de alimentos com esta toxina representa risco para a saúde animal e humana. Nos últimos cinco anos, vários investigadores têm desenvolvido métodos moleculares para identificação e quantificação de fungos produtores de OTA em amostras de grãos de café. O objetivo desta revisão é apresentar e discutir as várias estratégias desenvolvidas recentemente para a detecção de fungos potencialmente produtores de OTA.

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