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Characterization of Molds from Dry-Cured Meat Products and Their Metabolites by Micellar Electrokinetic Capillary Electrophoresis and Random Amplified Polymorphic DNA PCR

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ABSTRACT

Molds are common contaminants of dry-cured meat products in which mycotoxins could be synthesized if stored under favorable conditions. Thus, efficient and accurate characterization of the toxigenic molds from dry-cured meat products is necessary. A micellar electrokinetic capillary chromatography (MECC) method was tested to analyze secondary metabolites produced by 20 mold strains commonly found in dry-cured meat products. In addition, their random amplified polymorphic DNA (RAPD) genotypes were determined by using a PCR method. Although peak profiles of the secondary metabolites differed among mold strains of different species, they were similar in the same species. MECC analysis showed that 10 of the 20 molds tested produced mycotoxins, including patulin, penicillic acid, cyclopiazonic acid, mycophenolic acid, aflatoxin B1, sterigmatocystin, and griseofulvin. The RAPD analysis yielded a different pattern for each of the mold species tested. However, strains of the same species showed similar RAPD profiles. A high correlation between RAPD analysis and MECC was observed, since strains of the same species that showed similar RAPD patterns had similar profiles of secondary metabolites. RAPD patterns with primer GO2 and MECC profiles, either singly or combined, could be of great interest to distinguish toxigenic from nontoxigenic molds in dry-cured meat products.

Molds are common contaminants of dry-cured meat products, where they find the appropriate conditions to outgrow bacteria, particularly in those of intermediate water activity that are kept at room temperature. Most species found in these products are able to synthesize mycotoxins in a meat substrate when grown under favorable conditions *(11, 15)*. These secondary metabolites are highly mutagenic and are considered potent human carcinogens *(13)*.

Appropriate characterization of toxigenic molds from dry-cured meat products is necessary to prevent health hazards. The conventional way to characterize them requires time-consuming approaches, including culture of molds, extraction of secondary metabolites with organic solvents, and detection with a sensitive method such as high-performance liquid chromatography coupled to mass spectrometry. This method is unfavorable because of the labor-intensive steps, such as extensive extraction, cleanup procedures for good chromatographic separation, the use of complex gradient mobile phases, and large quantities of organic solvents. The capillary electrophoresis (CE) technique may be used as an alternative, since it offers many advantages over the conventional chromatographic techniques, including rapid analysis, reduction in the use of organic solvents, small sample volume, and increased efficiency and resolution *(2, 18)*. A mode of CE termed micellar electrokinetic capillary electrophoresis or micellar electrokinetic capillary chromatography (MECC) has recently been applied to the determination of fungal metabolites, including aflatoxins and ochratoxins *(10, 13, 16, 17)*. MECC is performed by adding micelle-forming compounds such as sodium dodecyl sulfate (SDS) in the running buffer at amounts above their critical micelle concentrations *(18, 20)*. This method could be useful to detect mycotoxins during the characterization of toxigenic molds. In addition, peak profiles analysis of secondary metabolites obtained by MECC could be useful for rapid differentiation of toxigenic molds.

On the other hand, the phenotypic trait production of secondary metabolites may be coupled to the genotype. For example, there is a high correlation between the production of the secondary metabolites and genotype patterns of the *Penicillium roqueforti* strains *(5)*. If genotype patterns are coupled to production of secondary metabolites, they could be used as a genetic marker to rapidly identify the toxigenic molds.

The random amplified polymorphic DNA (RAPD) technique is a powerful tool for determining genetic differences of various microorganisms *(19)*. Depending on the choice of random primer, the resulting RAPD pattern can be species specific *(6)* or can differentiate between various RAPD genotypes within a species *(7)* or even among strains *(1)*.

In this work, a MECC method was assayed for secondary metabolites detection to characterize toxigenic molds. In addition, RAPD analysis was used to characterize the genotype patterns of the mold strains.

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TABLE 1. *Mycotoxins detected by MECC in the mold strains tested*

Strain[a]	Mycotoxin detected
Aspergillus flavus CECT 2686	Aflatoxin B1
A. flavus CECT 2687	Aflatoxin B1, ciclopiazonic acid
A. niger CECT 2089	ND[b]
A. oryzae CECT 2094 T	ND
A. parasiticus CECT 2682	Aflatoxin B1, penicillic acid
A. parasiticus CECT 2688	Aflatoxin B1, penicillic acid
A. versicolor CECT 2664	Sterigmatocystin
A. versicolor CECT 2903	Sterigmatocystin
Aspergillus spp. A161	ND
Aspergillus spp. A261	ND
Penicillium griseofulvum CECT 2919	Patulin, griseofulvin
P. aurantiogriseum CECT 2918	Penicillic acid
P. brevicompactum CECT 2316	Mycophenolic acid
P. chyrsogenum Pg222	ND
P. chrysogenum Pg223	ND
P. restrictum Pr341	Penicillic acid, patulin
P. commune Pc222	ND
P. expansum Px121	ND
P. expansum Px341	ND
P. viridicatum Pv221	ND

[a] Strains obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain) and strains isolated from dry-cured ham *(3, 12)*.
[b] ND, no mycotoxins detected.

MATERIALS AND METHODS

Strains and culture conditions. Twenty representative strains of *Aspergillus* and *Penicillium* species commonly found in dry-cured meat products *(3, 12)* were used in this study (Table 1). Ten strains were obtained from the Spanish Type Culture Collection (CECT). The remaining mold species were collected from dry-cured ham throughout the ripening process *(3, 12)*. Except for *Penicillium chrysogenum* and *Penicillium viridicatum* Pv221, most of the strains isolated from dry-cured ham were characterized as toxigenic using the brine shrimp, cytotoxicity, and Ames tests *(12)*. All of the molds used were purified on repeated cultivation on malt extract agar (2% malt extract, 2% glucose, 0.1% peptone, 2% agar) before analysis.

Production and extraction of secondary metabolites. Inoculation of mold strains was done in three points per plate in malt extract agar. For each mold tested, three plates were incubated for 7 days at 25°C.

The content of three petri dishes was transferred to a plastic bag and macerated with 150 ml of chloroform in a Stomacher Lab Blender 400 (Seward Medical, London, UK) for 4 min. After 1 h, the slurry was filtered twice through anhydrous sodium sulfate with Whatman no. 1 filter paper (Whatman International, Maidstone, UK). Then the filtrate was evaporated in a rotatory evaporator (model VV2000, Heidolph, Kelheim, Germany) at 40°C. The residue was resuspended in 5 ml of chloroform, filtered through a 0.45-μm pore size nylon membrane (MSI, Westboro, Mass.), and evaporated to dryness under a gentle stream of nitrogen. The extracts were stored at 4°C in the dark and were resuspended in 1 ml of 50% acetonitrile before the CE analysis.

Analysis of secondary metabolites by MECC. Secondary metabolites were analyzed by MECC according to the method of Tsao and Zhou *(16)*. The CE instrument used in this study was a Beckman P/ACE 5500 model with a photodiode array detector and a cartridge interface with cooling option (Beckman Instruments, Inc., Fullerton, Calif.). A fused silica capillary of 57 cm length and 75 μm inside diameter was used for separation. The capillary was conditioned between analyses by flushing at high pressure (20 psi) with sodium hydroxide (0.1 mM, 3 min) and purified water (3 min) before flushing with the running buffer of 25 mM sodium tetraborate and 50 mM SDS (pH 9). Conditions of analysis included a constant potential of 15 kV, maximum current at 200 μA, and a capillary cassette temperature of 23°C. Sample injection was at low pressure (0.5 psi) for 3 s. The total running time of the analysis was 30 min. The absorbance was recorded at 200- and 280-nm wavelengths. For each peak, a spectrum of absorbance between 190 and 600 nm was obtained in the photodiode array detector. Mycotoxins produced by molds commonly found in dry-cured meat products, including citrinin, penicillic acid, ochratoxin A, cyclopiazonic acid, mycophenolic acid, zearalenone, aflatoxin B1, sterigmatocystin, griseofulvin, and patulin (Sigma Chemical Co., St. Louis, Mo.), were used as standards.

DNA isolation. Two grams of frozen mycelium was ground to a fine powder in liquid nitrogen and homogenized in 4 ml of TES buffer (0.05 M Tris-Cl, pH 8; 0.005 M EDTA; 0.05 M NaCl), and SDS was added to a final concentration of 1% (wt/vol). Then 200 μl of proteinase K (10 μg/μl) was added, incubated at 60°C for 35 min, cooled on ice, and extracted with phenol-chloroform-isoamyl alcohol (25:24:1). The suspension was centrifuged at 3,000 × *g* for 2 min. The upper phase containing DNA was transferred to another centrifuge tube and precipitated by adding 3 M sodium acetate to a final concentration of 10% (wt/vol) and two volumes of cold ethanol. After centrifugation, the pellet was resuspended with sterile water and treated with 50 μl of RNase (10 μg/μl). Finally, DNA was precipitated again as indicated above.

RAPD PCR. The isolated chromosomal DNA was diluted to 100 ng/μl and used as a template for RAPD PCR. The primer GO2 (5′-AAGGCGGCAG-3′) was used *(4)*. Reaction was performed in a total volume of 50 μl, containing 100 ng of DNA, 10 mM Tris-HCl, 100 μM each of dATP, dCTP, dGTP, and dTTP,

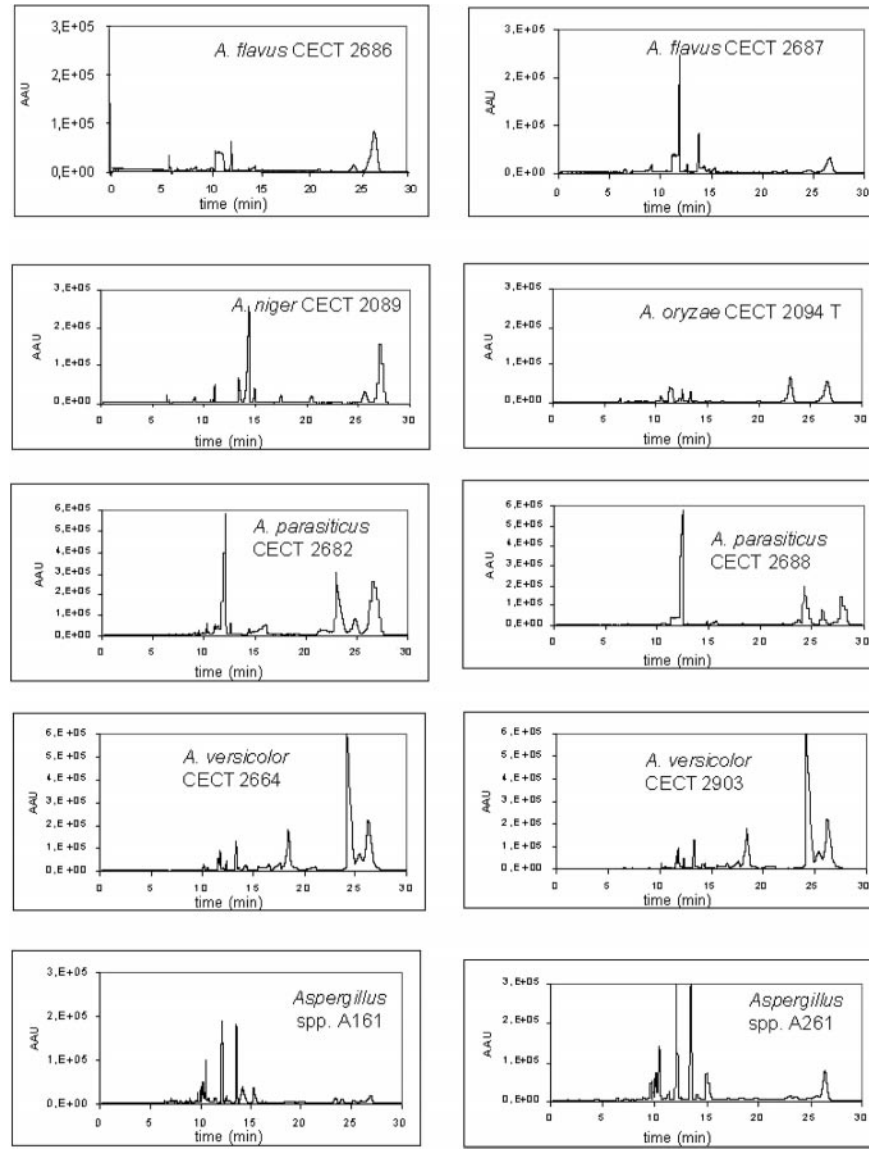

FIGURE 1. *Micellar electrokinetic capillary chromatography electropherograms at 200 nm of the* Aspergillus *strains tested.*

3 mM $MgCl_2$, 1 U of *Taq* polymerase (Finnzyme, Espoo, Finland), and 1 μM primer GO2. The reactions were performed in a thermal cycler (model iCycler 170–8731, Bio Rad, Hercules, Calif.) for 30 cycles using the following conditions: 1 min at 94°C, 1 min at 35°C, and 5 min at 54°C. A final step of 5 min at 72°C was performed. The amplification products were analyzed by submerged gel electrophoresis in 1% agarose gels using Tris–acetic acid–EDTA buffer at 70 V for 1.5 h. The gels were stained with ethidium bromide (0.5 μg/ml), and the products were visualized by UV transillumination and photographed. DNA molecular size markers of 0.5 to 10 kbp from Amersham Biosciences (Uppsala, Sweden) were used to determine the size of the PCR products. Electrophoretic patterns were compared using ID Image Analysis Software (Kodak Digital Science, Rochester, N.Y.).

RESULTS

Secondary metabolites. Figures 1 and 2 show the electropherograms of secondary metabolites from the different molds tested. Detected peaks were eluted between 6.5 and 30 min. Although it was not possible to identify all the detected peaks, very different peak profiles were observed among mold strains of different species (Figs. 1 and 2). However, very similar patterns were found among strains of the same species (*Aspergillus flavus* CECT 2686 and CECT 2687; *Aspergillus parasiticus* CECT 2682 and CECT 2688; *Aspergillus versicolor* CECT 2664 and CECT 2903; *P. chrysogenum* Pg222 and Pg223; and *Penicillium expansum* Px121 and Px341). The two strains of *Aspergillus* spp. (A161 and A261) also showed similar MECC patterns. Peaks from strains of the same species with the same retention time showed identical spectra in the diode array analysis (data not shown).

The electropherogram of the standard mycotoxins is presented in Figure 3. Purity of the peaks was tested by analysis of the UV spectra obtained in the diode array detector. At a 200-nm wavelength, peaks eluted at 10.8, 11.6, 13.4, 13.8, 14.1, 21.9, and 24.1 min were identified as citrinin, penicillic acid, ochratoxin A, cyclopiazonic acid, mycophenolic acid, zearalenone, and aflatoxin B1, respectively. Sterigmatocystin and griseofulvin were eluted at the same retention time (25.6 min), but they were distinguished by their own spectra. Thus, sterigmatocystin showed maximum absorbance at 251 and 335 nm, whereas griseofulvin showed maximum absorbance at 213, 221, and 291 nm.

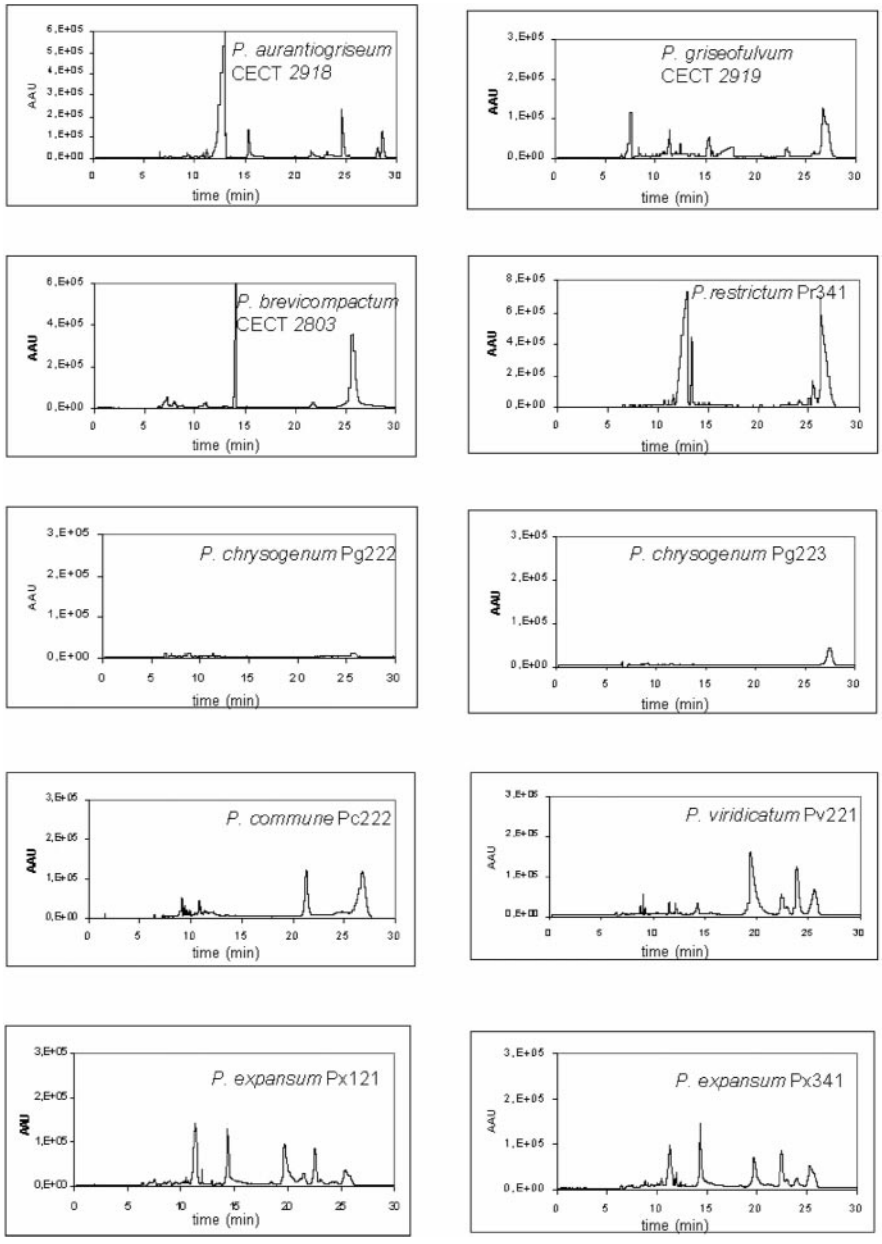

FIGURE 2. *Micellar electrokinetic capillary chromatography electropherograms at 200 nm of the* Penicillium *strains tested.*

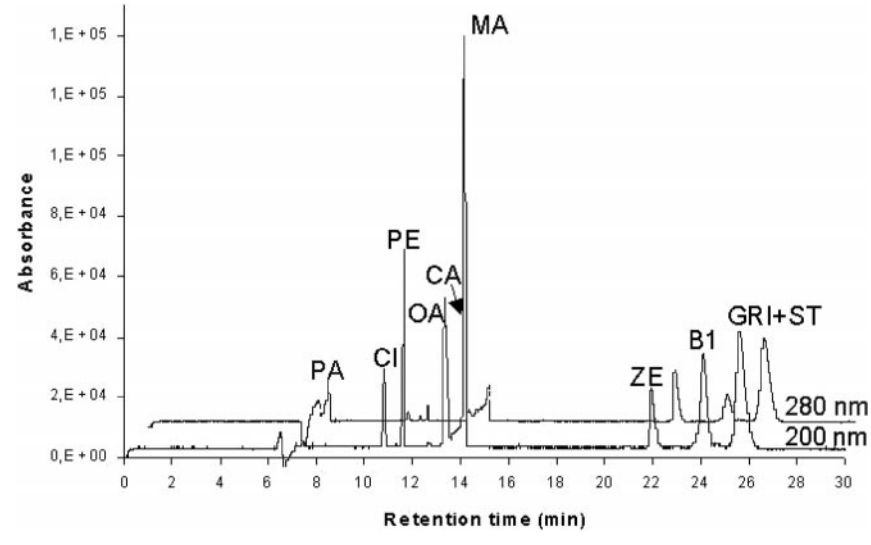

FIGURE 3. *Micellar electrokinetic capillary chromatography electropherogram at 200 and 280 nm of the standard mycotoxins: patulin (PA), citrinin (CI), penicillic acid (PEN), ochratoxin A (OA), ciclopiazonic acid (CA), mycophenolic acid (MA), zearalenone (ZA), aflatoxin B1 (A1), griseofulvin (GRI), and sterigmatocystin (ST).*

Patulin was detected at the 280-nm wavelength at the 7.5-min retention time (Fig. 3).

Most of the previously mentioned mycotoxins were detected among the electropherograms of the molds tested (Table 1). Thus, aflatoxin B1 was produced by *A. flavus* CECT 2686 and CECT 2687 and *A. parasiticus* CECT 2682 and CECT 2688, cyclopiazonic acid by *A. flavus* CECT 2687, patulin by *Penicillium griseofulvum* CECT 2919 and *Penicillium restrictum* Pr341, and sterigmatocystin by the strains of *A. versicolor* CECT 2664 and CECT 2903. Penicillic acid was detected in the electropherograms of the strains of *A. parasiticus* CECT 2682 and CECT 2688, *Penicillium aurantiogriseum* CECT 2918, and *P. restrictum* Pr341. Mycophenolic acid was produced by *Penicillium brevicompactum* CECT 2316.

RAPD analysis. The GO2 primer generated reproducible RAPD patterns for the different mold strains, each containing four to six major prominent bands with fragment lengths between 100 and 3,000 bp (Fig. 4). By comparison of the RAPD patterns, many differences at species level could be found. However, strains of the same species showed similar RAPD patterns. Thus, *A. flavus* CECT 2686 and CECT 2687 showed the same bands of approximately 932, 779, 469, and 412 bp; *A. parasiticus* CECT 2682 and CECT 2688 showed bands of 1280, 1048, 905, 631, 540, and 412 bp; *A. versicolor* CECT 2664 and CECT 2903 showed bands of 1122, 854, 670, and 412 bp; *Aspergillus* spp. A161 and A261 showed bands of 1048, 905, 854, and 670 bp; *P. expansum* Px121 and Px341 showed bands of 1280, 932, 752, and 505; and finally, *P. chrysogenum* Pg222 and Pg223 showed identical RAPD patterns.

DISCUSSION

The CE method can be used to obtain a specific profile of secondary metabolites. These profiles allow the tested molds to be distinguished at species level, since most of the obtained secondary metabolites are different among species. In accordance with data registered in the Spanish Type Culture Collection *(8)*, most of the mycotoxins synthesized by the strains tested are different at a species level. However, similar peak profiles were obtained for those tested strains of the same species, given that production of secondary metabolites is similar within a species.

Mycotoxins citrinin, penicillic acid, ochratoxin A, cyclopiazonic acid, mycophenolic acid, zearalenone, and aflatoxin B1 were properly separated in a 30-min MECC analysis. The exceptions were sterigmatocystin and griseofulvin, which were eluted at the same retention time, although they could be distinguished by UV spectra of the peak. Several CE methods have been reported to analyze separately cyclopiazonic acid *(14)*, patulin *(16)*, aflatoxins B1, B2, G1, and G2, and ochratoxin A *(13)*. By using the MECC method developed in the present work, at least 10 mycotoxins can be detected in a single analysis.

Ten of 20 molds tested produced at least one of the mycotoxins included in the analysis. Most of the mycotoxins detected in the strains of the Spanish Type Culture Collection confirmed previous results *(8)*. In addition, mycotoxins not reported in some these strains were detected by MECC analysis, such as aflatoxin B1 in *A. flavus* CECT 2686 and *A. parasiticus* CECT 2686 and sterigmatocystin in *A. parasiticus* CECT 2664. From the strains isolated from dry-cured ham, only *P. restrictum* Pr341 was detected in any of the mycotoxins analyzed. This strain was reported as toxigenic in brine shrimp, cytotoxicity, and Ames tests *(12)*. Although none of the analyzed mycotoxins were found in the electropherograms of the strains *Penicillium commune* Pc222, *P. expansum* Px121 and Px221, or *As-*

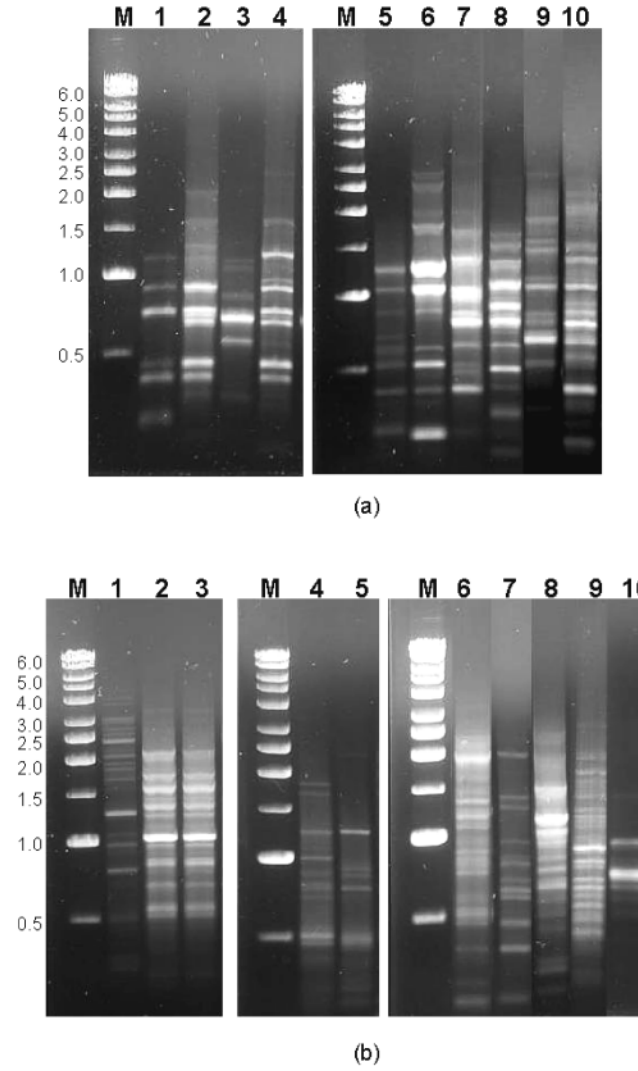

FIGURE 4. *Agarose gel of RAPD PCR products with primer GO2 of the different mold species tested. (a)* Aspergillus *strains: lane 1,* A. flavus *CECT 2686; lane 2,* A. flavus *CECT 2687; lane 3,* A. niger *CECT 2089; lane 4,* A. oryzae *CECT 2094 T; lane 5,* A. parasiticus *CECT 2682; lane 6,* A. parasiticus *CECT 2688; lane 7,* A. versicolor *CECT 2664; lane 8,* A. versicolor *CECT 2903; lane 9,* Aspergillus *spp. A161; and lane 10,* Aspergillus *spp. A261*. (b) Penicillium *strains: lane 1,* P. viridicatum *Pv221; lane 2,* P. chrysogenum *Pg222; lane 3,* P. chrysogenum *Pg223; lane 4,* P. expansum *Px121; lane 5,* P. expansum *Px341; lane 6,* P. commune *Pc222; lane 7,* P. restrictum *Pr341; lane 8,* P. aurantiogriseum *CECT 2918; lane 9,* P. griseofulvum *CECT 2919; and lane 10,* P. brevicompactum *2316. (M) DNA markers consisting of 10 double-stranded DNA fragment with sizes of 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, and 10 kbp.*

pergillus spp. A161 and A261, they were reported as toxigenic by the above tests *(12)*. Most likely, others mycotoxins, different from those analyzed, are produced by them, which may be on the nonidentified peaks of their electropherograms. The strains *P. chrysogenum* Pg222 and Pg223 and *P. viridicatum* Pv221, reported as nontoxigenic *(12)*, did not produce any of the mycotoxins. In addition, the electropherograms of the nontoxigenic *P. chrysogenum* were nearly void of peaks, indicating secondary metabolites. Thus, the analysis of secondary metabolite profiles by MECC is useful for mold characterization. Furthermore, analysis of the individual peaks could provide information related to mycotoxin production.

RAPD analysis with primer GO2 yielded different band patterns for each of the mold species tested. RAPD with primer GO2 has been reported as an effective means to differentiate phenotypically near-identical species of industrial *P. chrysogenum* and *Penicillium nalgiovense (4)*. Nevertheless, by using the described primer, similar RAPD patterns were observed for strains of the same species, which indicated that these strains have a high degree of similarity at the genetic level. A high correlation between RAPD analysis and MECC is expected, since strains of the same species that show similar RAPD patterns have a similar profile of secondary metabolites. In addition, when greater differences in the RAPD patterns are observed, as happened with mold strains from different species, greater differences were detected in the profile of secondary metabolites. This means that the RAPD pattern could be coupled to the phenotypic trait of the production of secondary metabolites. A high correlation between RAPD patterns and the production of mycophenolic acid has been reported for *P. roqueforti* strains tested as cheese starter cultures *(5)*. Also, some secondary metabolites (cyclopiazonic acid, rugulovasine, and cyclopaldic acid) produced by *P. commune* correlated with RAPD grouping *(9)*. The relationship between RAPD patterns and production of secondary metabolites could be of great interest in food safety because a simple analysis of RAPD patterns, in association with the typical profile of secondary metabolites, could be sufficient to differentiate toxigenic from nontoxigenic molds.

In conclusion, the MECC method allows for obtaining profiles of secondary metabolites that can be used to characterize molds at the species level. In addition, RAPD patterns obtained by using the GO2 primer may be useful for differentiating mold strains at the species level. Although more strains need to be tested, RAPD patterns with primer GO2 may be coupled to the phenotypic trait of the production of secondary metabolites. Thus, MECC and RAPD profiles as a single or combined analysis could be useful for distinguishing toxigenic from nontoxigenic molds in dry-cured meat products. These methods can be used to select safe molds as starter cultures for the meat industry and also for monitoring the occurrence of toxigenic molds in dry-cured meat processing as part of hazard analysis critical control point programs.

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